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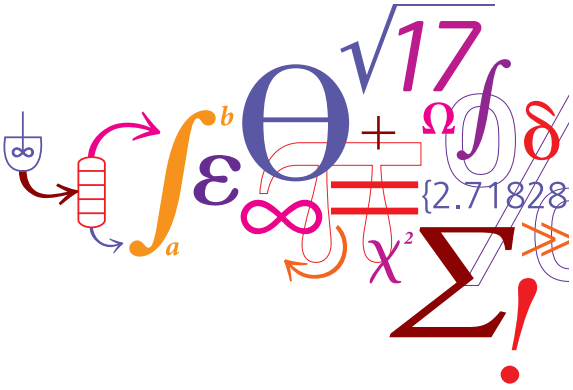

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Ph.D. Thesis
May 2013

DTU Chemical Engineering
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A methodology for development of biocatalytic processes

PhD Thesis

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Abstract

The potential advantages displayed by biocatalytic processes for organic synthesis (such as exquisite selectivity under mild operating conditions), have prompted the increasing number of processes running on a commercial scale. However, biocatalysis is still a fairly underutilised technology. As a relatively new technology biocatalytic processes often do not immediately fulfil the required process metrics that are key for an economically and/or environmentally competitive process at an industrial scale (high concentration, high reaction yield, high space-time-yield and high biocatalyst yield). These process metrics can often be attained by improvements in the reaction chemistry, the biocatalyst, and/or by process engineering, which often requires a complex process development strategy. Interestingly this complexity, which arises from the need for integration of biological and process technologies, is also the source of the greatest opportunities. Indeed, recombinant DNA technology offers a superb complement to process technologies. Potentially this is one of the biggest advantages of biocatalysis when compared with conventional chemical catalysis, where all the reaction boundaries are fixed by the physical and thermodynamic properties of the reaction compounds. Therefore, the main avenue that still remains to be explored by process engineers is how to promote process development in a systematic way rather than on a case-by-case basis, as is frequently the case today.

One of the main challenges in process development is selecting between different process alternatives. The development effort for a novel process is considerable and thus, an increasing number of conceptual process design methods are now applied in chemical industries. Since the natural environment of the biocatalyst is often very different from the operating conditions suitable for a viable process (high substrate and product concentrations, unnatural substrates, presence of organic solvents, etc.), process development strategies are particularly relevant for biocatalytic processes. However, state-of-the-art methodologies for process development applied to biocatalysis often prove to be unsuccessful. At the early development stage the biocatalysts are usually still under development and many of the reactions have not yet achieved their full potential, many of the process technologies are not yet well described and their relationship with the overall process is not clear.

The work described in this thesis presents a methodological approach for early stage development of biocatalytic processes, understanding and dealing with the reaction, biocatalyst and process constraints. When applied, this methodology has a decisive role in helping to identify many of the process bottlenecks up-front and in a straightforward way, whilst indicating development targets, allowing a better use of resources and shortening development time. The methodology is illustrated through three different case studies: ε-caprolactam production by a multi-enzymatic process, chiral amine production using ω-transaminase and finally long-chain chiral aliphatic

alcohol production by a bi-enzymatic system. For each case study presented, a different tool is used to guide development and evaluate the process when different levels of underlying process knowledge are available.

The first case study presents a rational approach for defining a development strategy for multi-enzymatic processes. The proposed methodology requires a profound and structured knowledge of the multi-enzyme systems, integrating chemistry, biological and process engineering. In order to suggest a reduced number of feasible process design options, cofactor and interaction matrices are used, identifying the challenges and addressing them by selecting appropriate process configurations. Based on this information, feasible flowsheets and mass and energy balances are identified. By applying evaluation tools, the number of options can be much reduced and the current process bottlenecks identified. By applying *a priori* this methodology, the laboratory experts are better able to understand the most favourable operating conditions at full-scale and thus be able to collect information at these relevant conditions.

In the second case study, windows of operation are used to quantify and visualise process performance and feasibility when interactions between process technologies and biocatalyst performance (or reaction) are significant. The methodology constitutes a useful tool that provides easy interpretable results to enable rational design choices of different available process technologies. In the particular case of the asymmetric synthesis of chiral amines, the reaction constraints (thermodynamic equilibrium) must be solved prior to implementation and these fix the hard boundaries of the operating space. Improvements in the biocatalyst specific activity are also required for a successful full-scale implementation.

In the third case study a methodology for bottleneck analysis is presented, incorporating process modelling and engineering evaluation tools. The benefit of such models, when integrated with evaluation tools, is that they can be used to predict the process performance and identify bottlenecks, without requiring experimental examination thereby reducing the resources and time for process development. The use of this methodology in the context of reaction engineering is to propose new operating conditions at which the process performance is improved, while identifying the remaining bottlenecks and suggesting further research efforts.

Although the proposed methodology is still in its infancy when compared with other established process development methodologies, it provides a good overview of the whole reaction system and process. The proposed methodological approach establishes a systematic evaluation of different process options and indicates required fundamental data collection and development efforts for further development stages. This methodology could be greatly enhanced by the implementation and integration of *in-silico* tools for property and thermodynamic data as well as process mechanistic models to assist in the selection of process technologies.

Resumé

De potentielle fordele ved brug af biokatalytiske processer af organisk syntese (såsom høj selektivitet under milde omstændigheder), har igangsat en øget mængde af processer på kommerciel skala. Til trods for dette er biokatalytiske processer stadig ikke en udbredt teknologi. Som en relativ ny teknologi er det ofte, at biokatalytiske processer ikke opfylder de proces krav, der er essentielle for en økonomisk og/eller miljømæssig konkurrencedygtig proces på industriel skala (høj koncentration, højt reaktionsudbytte, højt volumetrisk produktivitet og højt katalyseudbytte). Disse proces indikatorer kan dog oftest opnås ved at forbedre den kemiske reaktion, biokatalysatoren og/eller ved brug af procesteknik, der dog ofte kræver en kompleks proces udviklingsstrategi. Interessant er det, at den kompleksitet, der opstår ved nødvendigheden af at integrere biologiske- og proces-teknologier, også er årsagen til de største muligheder. Netop rekombinant DNA teknologi er et fremragende supplement til procesteknologier. Dette er potentielt en af de største fordele ved biokatalyse sammenlignet med konventionelt kemisk katalyse, der er begrænset af de fysiske og termodynamiske egenskaber af reagenser. Derfor er den største udfordring for procesingeniører inden for biokatalytiske procesudvikling, er at promovere procesudvikling på en systematisk måde, i modsætning til "case-by-case" som er tilfældet i dag.

En af de største udfordringer i procesudvikling er at vælge mellem forskellige proces alternativer. Omkostninger ved at udvikle en ny proces er betydelige og derfor bruges en øget mængde af konceptuelle procesdesign metoder inden for den kemiske industri. Eftersom de naturlige forhold for en biokatalysator oftest er meget forskellige fra de forhold der er gældende for en levedygtig proces (højt substrat og produkt koncentration, unaturlige substrater, tilstedeværelsen af organiske solventer osv.), er proces udviklingsstrategier især relevant for biokatalytiske processer. Ikke desto mindre har "state-of-the-art" metodikker for proces udvikling, anvendt på biokatalyse, ofte vist sig ikke at være succesfulde. På et tidligt udviklingsstadie er biokatalysatoren som regel stadig under udvikling og de katalytiske reaktioner har stadig ikke opnået deres fulde potentiale. Yderligere er mange af de anvendte procesteknologier ikke velbeskrevet og deres forhold til den overordnede proces er stadig ikke klart.

Arbejdet i denne afhandling præsenterer en metodisk strategi til udvikling af biokatalytiske processer på et tidligt stadie ved at forstå og håndtere reaktionen, biokatalysatoren samt proces begrænsningerne. Ved anvendelse, har denne metodik en afgørende rolle i at hjælpe med at identificere flaskehalsen i processen på en direkte måde og samtidigt indikere udviklingsmål, hvilket muliggør bedre udnyttelse af ressourcer og nedsætter udviklingstiden. Metodikken er eksemplificeret gennem 3 forskellige "case studies": produktionen af ε-caprolactam ved en multienzymatisk proces, chiral amin produktion ved brug af ω-transaminase og sluttelig produktionen af langkædet alifatiske alkohol i et bi-enzymatisk system. For hver "case study" er der

brugt et forskelligt værktøj til at guide udviklingen og evaluere processen når forskellige niveauer af underliggende procesviden er tilgængelige.

Det første case study præsenterer en rationel tilgang til at definere en udviklingsstrategi for multienzymatiske processer. Den forslåede metodik kræver en omfattende og struktureret viden omkring multienzymatiske systemer, der integrerer kemi, biologi og procesteknik. For at kunne foreslå et antal af mulige procesdesign løsninger, er cofaktor- og interaktionmatrixer brugt til at identificere udfordringerne og adressere disse ved at vælge passende proceskonfigurationer. Baseret på denne information blev mulige proces diagrammer, samt masse og energi balancer, identificeret. Ved at anvende evalueringsværktøjer (økonomisk og miljømæssig evaluering) kan antallet af muligheder reduceres og de nuværende flaskehalse identificeres. Ved at anvende denne metodik a priori er laboratorieeksperterne i stand til at bedre forstå de mest favorable fuld skala operationelle betingelse og dermed i stand til at indsamle information ved disse betingelser.

I det andet case study er ”window operations” brugt til at kvantificere og visualiserer proces præstationen og gennemførlighed når vekselvirkninger af procesteknologier og ydedygtighed af biokatalysatore (eller reaktionen) er signifikante. Metodikken udgør et brugbart værktøj der gør det let at fortolke resultater som gør det muligt at foretage rationelle valg mellem forskellige tilgængelige procesteknologier. I det specifikke tilfælde af asymmetrisk syntese af chirale aminer skal begrænsningerne ved reaktionen (termodynamisk ligevægt) løses før implementeringen og dette udgør de hårde grænser for det operationelle rum. Yderligere forbedringer i den specifikke aktivitet af biokatalysatoren er også nødvendige for en succesfuld fuld skala implementering.

I det tredje case study præsenteres en metodik for flaskehalsanalyse som indeholder proces modellering og tekniske evaluerings værktøjer. Fordele ved integreringen af sådanne modeller med evaluerings værktøjer er at de kan blive brugt til at forudsige proces præstationen og identificere flaskehalser, uden at det kræver eksperimentelle undersøgelser og dermed reduceres ressourcerne og tiden for proces udviklingen. Brugen af denne metodik i forbindelse med reaktionsteknik foreslår nye operationsbetingelser hvor præstationen af processen forbedres samtidig med at resten af flaskehalsene identificeres og yderligere undersøgelser foreslås.

Selvom den foreslået metodik stadig er i et fosterstadie sammenlignet med andre mere etableret proces udviklingsværktøjer og metoder, tilbyder den et overblik over hele reaktionssystemet og processen. Desuden etablerer den foreslået metodiske tilgang en systematisk evaluering af forskellige proces muligheder (f.eks. proces diagrammer), og indikerer nødvendige fundamentale dataindsamlinger og anstrengelser for at opnå yderligere udviklingsstadie. Dog kunne den metodik forbedres betydeligt ved implementeringen af *in-silico* værktøjer for egenskaber og termodynamiske data forudsigelse og proces mekanistiske modeller, til at hjælpe med udvælgelsen af procesteknologier.

Preface

This thesis was prepared at the Department of Chemical and Biochemical Engineering (KT), at the Technical University of Denmark (DTU) in partial fulfilment of the requirements for acquiring the Ph.D. degree in engineering.

The work here presented was developed at the Centre for Process Engineering and Technology (Process, DTU Chemical Engineering) in the period from March 2010 until early May 2013. Professor John M. Woodley (DTU Chemical Engineering) was the principal supervisor for the project, with Dr. Pär Tufvesson (DTU Chemical Engineering).

The project was funded by BIOTRAINS Marie Curie ITN, financed by the European Union through the 7th Framework people Programme (grant agreement no.: 238531). Furthermore, collaborations with Evonik Industries AG and DECHEMA Society for Chemical Engineering and Biotechnology were established under the framework of the AMBIOCAS project financed through the European Union 7th Framework Programme (grant agreement no.: 245144).

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Joana Lima Ramos

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List of abbreviations

Abbreviation	Description
6AHA	6-aminohexanoic acid
ADA	N-(2-acetamido)iminodiacetic acid
ADH	Alcohol dehydrogenase
Ala	Alanine
AlaDH	Alanine dehydrogenase
AM-101	AMMONENG^{TM} 101
APH	Acetophenone
API	Active pharmaceutical ingredient
ATP	Adenosine-5'-triphosphate
BVMO	Baeyer-Villiger monooxygenase
CDW	Cell dry weight
CFC	Trichlorofluoromethane (Freon-11)
CF_{ox}	Oxidised electron transfer cofactor
CF_{red}	Reduced electron transfer cofactor
CHMO	Cyclohexanol monooxygenase
CR	Carbonyl reductases
CSTR	Continuous stirred tank reactor
CYP450	Cytochrome P450
DSP	Downstream process
EBR	Expanded bed reactor
EC	Enzyme class
EHS	Environmental, health and safety
EMR	Enzyme membrane reactors
FBR	Fluidized bed reactor
GCM	Green chemistry metrics
GDH	Glucose dehydrogenase
GRAS	Generally regarded as safe
HR-P	Highly porous polystyrene divinylbenzene copolymer
IL	Ionic liquid
IPA	Propan-2-amine
IS(c)PR	*In-situ* (co-)product removal
ISCPR	*In-situ* co-product removal
ISPR	*In-situ* product removal
ISSS	*In-situ* substrate supply
KRED	Ketoreductase
***Lb*ADH**	Alcohol dehydrogenase from *Lactobacillus brevis*
LCA	Life cycle assessment
MINLP	Mixed integer nonlinear programming
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCE	New chemical entities
PAF	Potentially affected fraction of species
PBR	Packed bed reactor

Abbreviation	Description
scCO2	Supercritical carbon dioxide
SFPR	Substrate feeding and product recovery
SPE	Solid-phase extraction
STR	Stirred tank reactor
TAm	ω-Transaminase
ThDP	Thiamine diphosphate
TK	Transketolase
VOC	Volatile organic compounds
wt.	Weight
PEA	(S)-1-phenyl-ethylamine
PES	Polyethersulfone
PLP	Pyridoxal-phosphate
PSE	Process systems engineering
RM	Raw materials

List of nomenclature

Nomenclature	Description	Unit
AP	Acidification potential	gSO_2-eq
ADXs	Amine donor excess	$\frac{mol_{amino\ donor}}{mol_{substrate}} - mol_{substrate}$
AE	Atom economy	$MW_{product}/MW_{substrate}$
BWP	Bulk waste	g
CAPEX	Capital cost	€
CME	Carbon mass efficiency	$g_{C\text{-}product}/g_{C\text{-}reagents}$
ΔG_r	Free Gibbs energy	J/mol
ee	Enantiomeric excess	$\frac{mol_{enantiomer\ S/R}}{mol_{enantiomer\ S} + mol_{enantiomer\ R}}$
E-factor	Environmental factor	$g_{waste}/g_{product}$
EMY	Effective mass yield	$g_{product}/g_{non\text{-}benign\ reagents}$
EP	Nutrient enrichment potential	gPO_4^{3-}-eq
ETP	Eco-toxicity potential	$PAF.m^3.day$
GWP	Global warming potential	gCO_2-eq
HTP	Human-toxicity potential	cases
HWP	Hazardous waste	g
i	capital charge factor	No units
k	Annuity factor	No units
K_{eq}	Thermodynamic equilibrium constant	No units
LogP	Partition coefficient	No units
MW	Molecular weight	g/mol
ν	Volumetric flow	L/h
OPEX	Operational cost	€
pK_a	Acid disassociation constant	No units
PMI	Process mass intensity	$g_{reagents}/g_{product}$
POP	Photochemical ozone formation potential	gC_2H_4-eq
P_{vap}	Vapour pressure	mbar
ρ	Density	kg/m^3
RME (or ME)	Reaction mass efficiency	gproduct/greagents
S_{aq}	Aqueous solubility	g/L_{water}
SI	Solvent intensity	$g_{solvent(s)}/g_{product}$
SOP	Stratospheric ozone depletion potential	$gCFC_{11}$-eq
STY	Space-time yield	$g_{product}/(L_{reactor}.h)$

Nomenclature	Description	Unit
SXs	Substrate excess	$\frac{mol_{co\text{-}substrate}}{mol_{substrate}} - mol_{substrate}$
T	Temperature	^{o}C
τ	Residence time	h
t	Equipment economic life time	years
T_b	Boiling point	^{o}C
T_m	Melting point	^{o}C
TTN_i	Reagent *i* total turnover number	$mol_{product}/mol_{reagent\ i}$
V	Volume	m^3
WI	Water intensity	$g_{water}/g_{product}$
Y_{biocat}	Biocatalyst yield	$g_{product}/g_{biocatalyst}$
$Y_{reaction}$	Reaction yield	$mol_{product}/mol_{substrate}$ (%)
Y_{sp}	Yield coefficient of product from substrate	$g_{product}/g_{substrate}$
Y_{sRE}	Yield coefficient of recombinant enzyme from substrate	$g_{recombinat\ enzyme}/g_{substrate}$
Y_{sx}	Yield coefficient of biomass from substrate	$g_{biomass}/g_{substrate}$

Table of contents

1 Introduction

1.1 Biocatalysis for industrial production of chemicals

The chemical industry has a central role in modern society, since it provides society with a large number of value-added products and is one of the biggest economic sectors worldwide [1]. More recently, the conventional chemical industry has been forced to innovate in order to maintain a competitive position and to successfully penetrate already saturated markets [2]. This has resulted in increasing focus on production of chemicals from renewable sources, promoting greener synthetic routes and generating less toxic by-products and waste, without compromising the product quality. Green Chemistry, defined as "the design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances" [3], has been promoting the design of next generation processes and products, by providing guidelines for environmentally friendly and economically competitive processes (compiled in the 12 Principles of Green Chemistry [4] and engineering [5]).

Using alternative substrates, energy sources and innovative synthetic routes, bioprocesses have brought many innovations to the polymer, biofuel, textile, food, health care and pharmaceutical industries, amongst others [6]. Bioprocesses can be classified into fermentation and biocatalysis. Fermentation refers to the use of growing cells to make the product of interest; Biocatalysis may broadly be defined as the use of biocatalysts, which can be crude extracts, purified enzymes, or whole-cells (i.e. resting cells) and these can be in their free or immobilised form. Fermentation technologies can be applied to produce both large molecules, such as enzymes, peptides, therapeutic proteins (e.g. antibodies and insulin) and proteins used in the food industry (e.g. feed additives [7]), but also small molecules, such as metabolites of fermentation processes, e.g. ethanol, 1,3-propanediol, succinic acid and butanol [8]. Biocatalytic processes deal exclusively with the organic synthesis of chemicals (small molecules) such as building blocks for added-value chemicals, amino acids, agrochemicals and active pharmaceutical ingredients (APIs). [1,9]. The scope of this thesis is confined to biocatalytic processes.

In the past decades, enzyme and whole-cell biocatalysis has been applied in the production of various chemicals, mainly optically active intermediates such as fine chemicals and pharmaceutical intermediates [9,10]. Contributing to such a fast growth is the fact that the number of commercially available enzymes isolated from different biological sources has increased significantly [11]. The exquisite selectivity of enzyme-catalysed reactions yielding single stereoisomers, with few side reactions and easier separation of products [11,12] under mild reaction conditions (pH, temperature, often aqueous conditions, etc.) make biocatalytic processes well positioned to contribute to greener industrial processes, in line with the 12 Principles of Green Chemistry [4].

Joint efforts between biologists, chemists and, more recently, process engineers have also created new opportunities for using biocatalysis in the production of lower value chemicals and biofuels [13], where the cost structure puts even higher demands on process intensity and productivity to achieve a competitive process.

Despite the many potential advantages of biocatalytic processes and the increasing number of processes running on a commercial scale [14], when compared with conventional chemical industrial catalysis the use of biocatalytic processes is a younger technology [15] and, as with any emerging technology, many start-up difficulties are encountered [11]. Enzymes found in nature operate at the host conditions, which are typically mismatched with the conditions required for an industrial chemical processes, such as high substrate and product concentrations, unnatural substrates, presence of reaction additives (e.g. solvents), among others. Hence, often biocatalytic processes per se do not meet the required process metrics that are key for an economically feasible process at an industrial scale (high concentration, high yield, high space-time yield and high biocatalyst yield) [13,16].

In spite of some pioneering examples where biocatalytic processes (e.g. lipase catalysed) are already operating at full-scale for production of added-value chemicals, the true expansion of the enzyme toolbox[1] for organic synthesis is yet to come, as the next generation of enzymatic synthesis involves more challenging enzyme systems and reactions (e.g. amination, oxidation, carbon-carbon formation reactions) both in single and multi-step reaction systems. Many of these promising biocatalytic reactions and the corresponding biocatalysts are still under development and, as a result, many of the reactions are neither well developed nor optimised. Hence, the process technologies are not yet fixed and many alternative process configurations are possible.

The prospects of biocatalytic process lie on its multidisciplinary character: chemistry, biology and process engineering. This multidisciplinary character provides certain flexibility during the process synthesis and design since many of the identified process challenges can be overcome by efforts in one or more of these disciplines. This is probably one of the biggest advantages when compared with conventional chemical catalysis, where all the reaction hurdles are, to some degree, more constrained by the physical and thermodynamic properties of the reaction compounds and catalyst(s).

Due to the multidisciplinary character that brings new opportunities for biocatalytic processes, a systematic approach for process synthesis and design should indicate the research efforts required in each development area. Therefore, the main avenue that remains to be explored by process engineers is how to promote and conduct process development in a systematic way (and not on a case-by-case basis, as it is currently done). That is to say, a truly rational and systematic approach for process synthesis and design, leading the research focus for each area (chemistry, biology and process

[1] Enzyme toolbox: available enzymes for organic synthesis

engineering) from the early stage of process development. Such a structured approach would result in a better use of resources, a reduced development time and an increased understanding of the fundamental system constraints.

Even though this type of systematic approach is quite common in the conventional chemical industry (see section 2.1), the multidisciplinary nature of biocatalytic processes raises many different questions that need to be addressed simultaneously. This makes the application of a systematic methodology for conventional chemical compounds difficult to apply in biocatalytic processes, especially during the early stage when the biocatalyst performance needs to be strongly improved.

1.2 Scope of the work and specific research goals

This PhD project aimed at developing a systematic approach able to assist process synthesis and design for biocatalytic systems during early development stage, which integrates economic and environmental analysis and other well-established engineering tools (such as kinetic modelling).

This methodology has been developed for different industrial sectors (bulk, fine and pharmaceutical chemicals) and diverse understanding levels of the process and intrinsic constraints. Hence, the first goal of this methodology is to understand the information required for development and decision-making. Further, it is aimed at identify suitable process techniques (eliminating some less favourable options) and to identify the issues on which future research efforts should be focused.

The following specific objectives were addressed:

- To propose guidelines for threshold values for process metrics;
- To develop fast and accurate methodologies for cost and environmental analysis;
- To understand the process and the reaction constraints, identifying the important challenges when developing a new process, such as setting threshold values and guidelines for the process metrics;
- To identify suitable process techniques (eliminating some less favourable options);
- To assist in decision-making, regarding process flowsheet design, biocatalyst formulation (crude extract, purified enzyme or whole-cell and soluble or immobilised);
- To guide research during catalyst development, providing guidelines for biocatalyst stability and activity at relevant working conditions.

1.3 Thesis Outline

This thesis is divided into three parts: background, methodology for process development and case studies.

- Part I (Chapter 2) provides an introduction and background to the next generation of biocatalytic processes, indicating the major challenges and process technologies currently available for industrial application of biocatalytic processes.
- Part II (Chapters 3 - 5) focuses on methodologies for process design in biocatalysis:
    - Chapter 3 includes the current state-of-the-art in process synthesis and design in the conventional chemical industry and the tools currently available for bioprocess development (including biocatalytic processes);
    - Chapter 4 introduces feasibility and evaluation tools that have been developed as a part of the proposed methodology;
    - Chapter 5 is dedicated to explaining the proposed methodological approach for guiding development effort and the design of biocatalytic processes during early development stage.
- Part III (Chapters 6 - 9), exemplifies the application of the methodology for process development to three case studies and explains how different tools are applied for different levels of information (i.e. *a priori* available knowledge). The case studies (and corresponding tools) are presented in a crescent order of pre-available knowledge.
    - Chapter 6 gives an overview of the case studies, including a summary of the information available and the tools applied in each case study.
    - Chapter 7 presents how the application of 'cofactor and interaction matrices' can assist during process design of a multi-enzymatic process in the production of ε-caprolactam (bulk chemical).
    - Chapter 8 analyses the underlying constraints of a ω-transaminase-catalysed reaction using a 'window of operation' in order to identify suitable process strategies for the production of an optically pure chiral amine (pharmaceutical).

- Chapter 9 presents the application of a 'bottleneck analysis' for reaction optimisation of hardly water-soluble chiral aliphatic alcohols (fine chemical) towards a cost effective production.

- Part IV (Chapters 10 and 11) presents a general discussion of the methodology and case studies, the most significant conclusions and proposes possible directions for further research that may lead to a more efficient system process design and data collection.

1.4 Publications included in the thesis

The following submitted publications have resulted from the work presented in this thesis and submitted manuscripts are provided in the Appendix 1.

Tufvesson, P., J. Lima-Ramos, M. Nordblad and J. M. Woodley (2011) Guidelines and cost analysis for catalyst production in biocatalytic processes. Org. Process Res. Dev. 15:266-274.

Parts of this publication were included in Chapter 5 to explain the routine applied in simplified economic analysis. Furthermore, guidelines for biocatalytic yield presented in the Chapter 6 correspond to an adaptation of the above publication.

Tufvesson, P., J. Lima-Ramos, J. S. Jensen, N. Al-Haque, W. Neto and J. M. Woodley (2011) Process considerations for the asymmetric synthesis of chiral amines using transaminases. Biotechnol. Bioeng. 108:1479-1493.

Parts of this publication were reproduced in Chapter 2 and Chapter 9. References to more recent studies were included when suitable.

Part I

Background

2 Overview of biocatalytic processes

2.1 Introduction

Despite the large portfolio of added-value products in the chemical industry there is an on-going tendency to innovate the conventional chemical industry towards process improvements, cost reductions and increased quality, safety, health and environment profile of production processes[17,18]. White biotechnology, also known as industrial biotechnology [19], has been emerging not only as a suitable replacement technology to the conventional chemical synthesis of these products [1], but also as a route to new products (Figure 2.1). During the last few decades, considerable progress has been made in biotechnology research, which is ultimately reflected in the increasing number of bioprocesses that have been implemented at industrial scale [20]. Bioprocesses have provided many innovative routes for the chemical industry, by fulfilling many of the fundamental Principles of Green Chemistry [4,5,21,22].

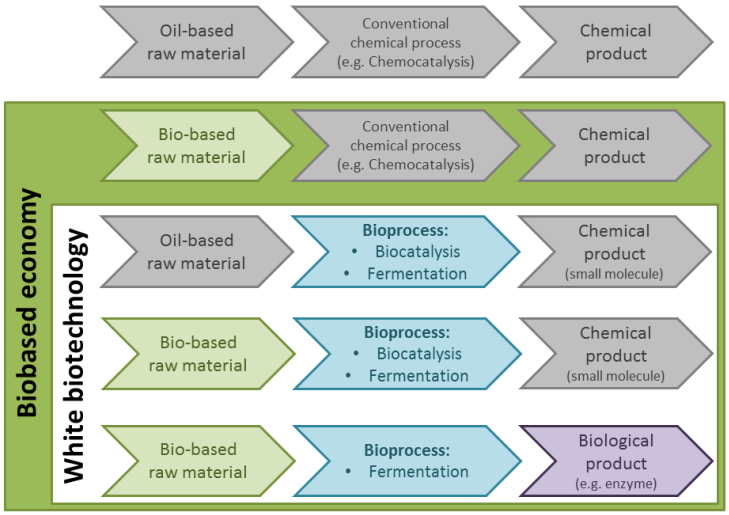


Figure 2.1. Production of chemical products and the White Biotechnology perspective

Among the bioprocesses, biocatalytic processes are especially attractive for the organic synthesis of industrial relevant products, since: 1) enzymes are natural catalysts produced by fermentation, possibly from renewable feedstocks; 2) enzymes are usually non-toxic catalysts and prevent large consumption of metals (unlike many of the metal and organometallic catalysts); 3) the processes are generally operated at moderate reaction conditions (temperature, pressure and pH levels) leading to lower energy consumption; 4) enzyme-catalysed reactions are usually very selective leading to high product purity, decreased waste production, facilitating the downstream process; 5) biocatalytic processes are commonly run in aqueous reaction media, preventing large

consumption of organic solvents; and 6) the possibility of improving the biocatalyst by recombinant DNA technology [17,23].

These advantages put forward a number of potential economic and environmental benefits, in line with the 12 Principles of Green Chemistry [4]. These benefits often meet the chemical industry's requirements in response to the market demands to find "greener" routes and processes, preserving or improving product quality [24]. Hence, in the past decades, enzyme and whole-cell biocatalysis has been applied in the production of different chemicals, mainly optically active intermediates such as fine chemicals and pharmaceutical intermediates [9,10]. Contributing to such a fast growth is the fact that the number of commercially available enzymes isolated from different biological sources has expanded rapidly [11]. More recently, joint efforts between biologists, chemists and engineers has produced new opportunities for biocatalysis also in lower value chemicals and biofuels [13].

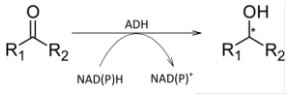
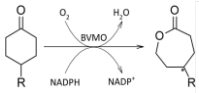
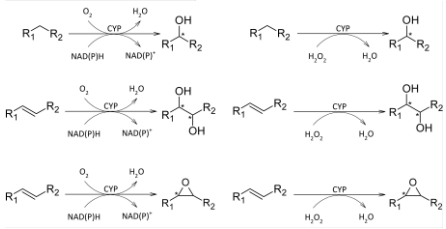
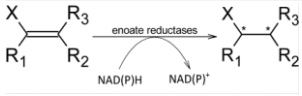
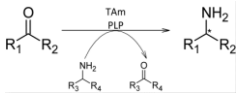
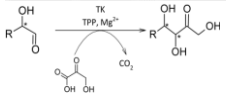
Currently the focus in biocatalytic processes is mostly on single-step reactions with one or two substrates [18]. However, multi-step reactions and multi-component reactions (such as reactions catalysed by cytochrome P450) have been considered as an alternative to chemical-catalysis [18,25] by combining different enzyme catalytic activities in a sequential manner. However, many challenges remain in the effective scale-up of processes using enantioselective enzymes in organic synthesis.

2.2 Next-generation biocatalytic processes

Some areas in biocatalysis, such as the use of hydrolases (EC 3 like acylases, amidases, esterases, lipases, proteases), have become well established in organic synthesis [18] and these biocatalytic reactions represent most of the enzyme-catalysed reactions in industry (such as in resolutions, deracemizations and desymmetrizations) [24]. However, recent reports [17,18,26] have identified other relevant enzyme classes suitable for organic synthesis. Oxidoreductase enzyme class (EC 1), including NAD(P)H-dependent alcohol dehydrogenases (ADH, EC 1.1.1.X), Baeyer-Villiger monooxygenases (BVMO, EC 1.14.13.X), cytochrome P450 (CYP450, EC 1.14.X.X), and enoate reductases (EC 1.3.1.31), have been identified as a relevant enzyme class for the synthesis of many industrial organic products [18,23,24,26] (Table 2.1). In addition, interesting enzyme-catalysed reactions are chiral amine synthesis applying ω-transaminases (aminotransferases, Tam, EC 2.6.1.X) [26] (Table 2.1) and asymmetric C–C bond formation using lyases (e.g. transketolase, TK, EC 2.2.1.X, and ThDP-dependent lyase, EC 4.1.X.X) (Table 2.1).

Enzymes within the oxidoreductase enzyme class (EC 1) catalyse oxidation and reduction reactions. The industrial application of oxidoreductases covers the synthesis of (non-natural) amino acids, chiral alcohols, amines and amides [14].

Table 2.1. Emerging relevant enzymes for organic synthesis of industrially relevant products

Enzyme Class	Enzyme	Example of the catalysed reaction	Main challenges
Oxidoreductase (EC 1)	Alcohol dehydrogenase (EC 1.1.1.X)		Cofactor recycling Enzyme stability [27]
	Baeyer-Villiger monooxygenase (EC 1.14.13.X)		Cofactor recycling Substrate/ Product inhibition O_2 transfer rate
	Cytochrome P450 (EC 1.14.X.X)		H_2O_2 inhibition and stability [28] Membrane integration [28] Cofactor recycling [29] Substrate inhibition [29] Electron transfer [29]
	Enoate reductases (EC 1.3.1.31)		Cofactor recycling
Transferases (EC 2)	ω-Transaminase (EC 2.6.1.X)		Thermodynamic equilibrium [30] Substrate/ Product inhibition
	Transketolase and Transaldolase (EC 2.2.1.X)		Thermodynamic equilibrium Substrate/ Product inhibition Product stability

* Chiral centre; X electron-withdrawing group

Enzyme catalysed oxidations display several advantages when compared with traditional organic chemistry, since they circumvent the use of flammable and halogenated solvents, high-valent metals and the use of stoichiometric oxidants in excess [24]. Despite the importance of oxidoreductases in organic synthesis, many of the subclasses of this family are still not fully explored [14,26]. Redox biocatalytic processes, normally require two oxidoreductases — one for the biotransformation and one for cofactor regeneration. Hence, the majority of enzyme-catalysed redox processes make use of metabolising cells, with enzymes from all classes being active at the same time as oxidoreductases, in order to promote effective *in-situ* cofactor recycling [12]. Interestingly, even in the absence of cell growth, cofactors can be effectively recycled, as shown for reductions mediated by baker's yeast cells [31].

Perhaps the most established biocatalytic redox reaction is the ketone reduction yielding a chiral alcohol, using nicotinamide nucleotide-dependent dehydrogenases (also known as alcohol dehydrogenases (ADH), ketoreductases (KRED) and carbonyl reductases (CR)) [27], due to the enantiospecificity in reduction of prochiral ketones (Table 2.1).

Regardless of the great progress in the application of nicotinamide-dependent dehydrogenases in the organic synthesis of industrially relevant compounds, other enzyme subclasses catalysing oxidation and reduction reactions are yet to be explored. Among the potentially interesting enzyme catalysed redox reactions, Baeyer-Villiger monooxygenases (BVMO) are notable for their ability to catalyse the selective insertion of an oxygen atom into a cyclic ketone to create a lactone [32], an important reaction for the synthesis of multi-cyclic lactones for potential use as pharmaceutical intermediates with high regio- and stereo-selectivity [24] (Table 2.1).

Further, the heme-containing cytochrome P450 (CYP450) oxygenases are generally recognised as highly relevant biocatalysts for the fine and pharmaceutical industries, as these enzymes are involved in the biosynthesis of several pharmaceuticals and steroids [28,29] due to their ability in catalyse the regio- and stereospecific oxidation of non-activated hydrocarbons [33] (Table 2.1).

Finally, there is an increasing interest in enoate reductase enzymes that catalyse the selective reduction of carbon–carbon double bonds that are substituted with electron-withdrawing groups [34] (such as a ketone, aldehyde, carboxylic acid, ester, anhydride, lactone, imide or nitro group [35]) yielding the synthesis of (up to) two chiral centres and thereby, of particular interest for the production of chiral materials.

Among the industrially relevant biocatalytic aminations, transaminases have received increased interest for the asymmetric synthesis of amines from prochiral ketones [36-38]. Transaminases can be applied for either kinetic resolution of racemic compounds or asymmetric synthesis starting from a prochiral substrate. However, despite being a potentially attractive technology for the industrial production of optically pure chiral amines due to the favourable reaction thermodynamics, asymmetric resolution is hampered by a maximum yield of 50% [36]. Therefore, the transaminase-catalysed asymmetric synthesis of chiral amines is more attractive for emerging biocatalytic processes, despite the frequently observed thermodynamically challenged reaction.

Carbon–carbon bond formation reactions are significant in organic synthesis to set up the carbon backbone of organic molecules [39]. Currently, most C-C forming enzymes have been applied to the synthesis of building blocks containing hydroxylated chiral centres [40]. C-C bond formation reaction involves nucleophilic attack and thus often poses additional challenges, as it requires an exact stereochemical control over separate fragments [40]. Due to the exquisite selectivity of enzyme-catalysed reactions, the application of a biocatalytic process for the synthesis of new compounds through C-C bond formation is especially relevant. These enzyme-catalysed reactions can be performed by the transketolases and transaldolases family and by enzymes belonging to the lyases enzyme class (such as ThDP-dependent enzymes, aldolases, among others). Transketolases (EC 2.2.1.X) catalyse the reversible transfer of a two-carbon ketol moiety from a ketose to an aldose. However, some studies have reported substrate inhibition

at relevant industrial concentrations as well product degradation throughout the reaction [41]. Further, in the lyases enzyme class, ThDP-dependent enzymes are also capable of catalysing a broad range of lyase and ligase reactions. Excellent reviews have been published covering C–C bond formation by enzymes [39,42,43]. However, despite the few examples where C-C forming examples were successfully scaled-up [43], most of the studies are currently focused in enlarging the substrate scope and, thus, the underlying challenges of these reactions at industrially relevant conditions are not yet fully identified [42].

Even more interesting than to explore the unique catalytic ability of these emerging enzymes, is to explore multistep chemistry, since synthetic processes to generate a desired product often require sequential synthetic reactions. Thus, promising future applications are envisaged for these processes [44,45]. However, multi-enzyme processes feature a high degree of complexity due to the interaction of the different components in the reaction mixture and the often encountered mismatch between optimum reaction conditions of each individual catalytic step.

2.2.1 Challenges and process development technologies

In general, the promising enzyme-catalysed reactions are now well launched due to the continued expansion of commercially available enzyme libraries and the increasing number of enzymes being expressed in GRAS (generally regarded as safe) organisms. Although advances in recombinant DNA technology offer huge potential to improve the biocatalyst performance, increasing tolerance to solvents, increasing the activity under operating conditions (e.g. high substrate or product concentrations and unnatural substrates), this is a time and resource consuming activity [46]. Further, for some small-scale products (less than 5 tons/year) and high market value, it might not be worthwhile to develop a biocatalyst based upon on the selling price of the product [14], as the biocatalyst cost contribution for high value products are often large. Developing a biocatalyst for a small process can lead to great investment of resources in the catalyst development that might not be translated in a significant decrease of the operating costs and thus, the investment made might not always be recovered.

Hence, it is also necessary to keep in mind that the integration of complementary technology platforms in manufacturing processes, such as fermentation (being increasingly implemented due to significant developments in pathway engineering and synthetic biology) and homogenous and heterogeneous catalysis is required. This integration implies that the implementation of biocatalytic steps should be matching the conditions of both synthetic and recovery steps. Many of the emerging biocatalytic reactions are still finding their position in the chemical industry, due to the mismatch

between the bench- and large-scale requirements. As the scale of biocatalytic step increases, more emphasis is required in promoting more cost-effective processes.

2.2.1.1 Process intensification

Among the requirements for a process to be economically viable, threshold values for given metrics must be attained (see Chapter 5, Section 5.2.1). Product concentration is a particularly challenging process metric to be successfully targeted and achieved, especially when moving from laboratory to full-scale implementation. Depending on the market sector, the usual requirement is to achieve product concentrations above 50 g/L [13]. In their natural conditions (i.e. inside the host organism) enzymes operate at maximum substrate and product concentrations within the range of millimolar. Hence, at large-scale, enzymes operate well outside from their natural conditions, with obvious consequences on the biocatalyst activity and stability. Further, the high concentrations required in industrial processes may also lead to multiphase reactions since the substrate and/or product of interest in the chemical industry often displays a low aqueous solubility [14].

In biocatalytic processes, efficient increase in concentration can be achieved in two complementary ways: protein engineering and process engineering. Protein engineering can be used to improve the biocatalyst tolerance to high concentrations of substrate and/or product [47,48]. Interestingly, process engineering solutions can potentially not only tackle the problem of the inhibitory effects of high concentrations of substrate and product, but also overcome substrate solubility problems: substrate can be added to the reaction by the addition of a second phase to the reaction medium (organic solvent or solid resin) or by operating the reactor in a fed-batch mode. The use of organic solvents might bring some extra consideration during scale-up, since the list of suitable solvents is limited, as they are required to be GRAS approved. Further, the addition of a second phase (organic solvent or solid resin) requires good mixing in order to avoid mass transfer limitations.

The use of *in-situ* product removal (ISPR) where the product is removed during the course of the reaction can lead to significantly higher productivity [49] by avoiding the build-up of inhibitory product concentrations in the reactor. Furthermore, the use of polymeric resins or organic solvents, may constitute an opportunity to implement ISPR with a controlled substrate feeding (*in-situ* substrate feeding, ISSS) [50] also known as substrate feeding and product recovery (SFPR). Further, for thermodynamically challenged reactions (such as ω-transaminase-catalysed reactions) the application of a highly selective ISPR technique is of particular relevance to displace the reaction equilibrium towards the product, enabling a higher conversion and thus an economically feasible process. The most suitable ISPR techniques are those involving partitioning of the product into a second liquid phase (extraction with organic solvents) or the adsorption onto or generation of a solid phase (resin adsorption), although other

examples can be found where extractive distillation, crystallisation, membrane separation, among others [51] are used. However, most of the ISPR techniques reported to date are not selective enough for a successful and significant equilibrium displacement.

2.2.1.2 Biocatalyst formulation

Using whole-cells may bring several benefits to the process, such as improved enzyme stability. It can also enable *in-situ* regeneration of expensive cofactors. However, due to the possibility of side reactions, the use of whole-cells requires additional downstream process cost for product recovery. Isolated enzymes are particularly interesting for synthetic routes, due to their selectivity and purity of product stream. Additionally, the use of isolated enzymes brings simplicity to the process (avoiding undesired side reactions) but the trade-off is higher upstream costs (for enzyme recovery and purification) [16] and therefore re-use of the enzyme is often required to design an economically competitive process.

As a balance between these trade-offs (and as a rule of thumb), the crudest possible form of the enzyme (e.g. whole-cell or lysate) should be used, without compromising the product quality [13]. For economic reasons whole-cell biocatalysts are typically preferred over isolated enzymes [26] (see Section 7.2.2).

Further, biocatalytic processes require a clean product stream [52], not only in terms of undesired by-products, but also avoiding protein contamination in the product. For this reason, large-scale biocatalytic processes require the use of immobilised biocatalyst, enabling the recycling and reuse of the biocatalyst [53]. Such recycling is also required for the process economic viability, in order to compensate for a costly upstream, comprising not only enzyme recovery and purification but also the immobilisation step. Moreover, operating with immobilised enzymes offers more options for alternative reactor design [54].

Immobilisation is particularly relevant when using organic solvents since under these conditions enzymes are prone to aggregation, affecting their activity and stability [15] (see Section 7.2.2). To date, there is not a general routine to select an immobilisation technique, which encompasses an analysis of stability, activity, handling and cost of the immobilised enzyme and the physicochemical properties of the immobilisation matrix, enzyme surface interactions and the reaction media [55-57].

2.2.1.3 Operating mode

The operating mode (batch, fed-batch and continuous) has a major influence on the liquid reactive phase of the process and thus, must be identified to correctly assess the mass (and energy) balances. For biocatalytic processes, the operating mode is mostly decided based upon on the reaction characteristics such as inhibitory effects, unfavourable thermodynamic equilibrium and low compound solubility. When the

process is operated in batch mode, all the reagents are fed into the reactor and the product is then recovered after the reaction is completed. Further, the conversion that can be obtained from the reactor is a function of the batch duration. However, this type of operating mode is not suitable for cases where a strong substrate inhibition is observed. In such cases, the process can be operated either in a fed-batch or in continuous mode. In fed-batch mode there is a periodic (or continuous) dosing of substrate into the reactor, while the product concentration builds up until the end of the reaction. In continuous operation mode, the reagents are continuously fed into the reactor and product is continuously recovered. This implies that the reactor is operating at the effluent substrate feed-rate; hence reaching complete conversion is impossible.

2.2.1.4 Reactor design

Reactor selection for biocatalytic reactions is usually based on cost, space, mass transfer, kinetics, heating and cooling, easiness of operation, operation mode and reusability of the catalyst [58].

A stirred tank reactor (STR) is the most common reactor set-up, due to its simple setup, flexibility, well-mixed behaviour and ease of operation. It can be applied to reactions catalysed by whole-cells as well as soluble or immobilised enzymes [15,25]. However, this type of reactor might cause mechanical shear stress (due to stirring and reactive oxygen species) on the biocatalyst, with consequent decrease in activity [52,59], which implies an adjustment in the operating conditions (e.g. increase the residence time) or dose more biocatalyst to the reactor [15]. In particular, continuous stirred tank reactor (CSTR) is appropriate for the enzymatic reactions with substrate inhibition [60] since it operates at low substrate concentrations where the reaction rate is higher.

The packed-bed reactor (PBR, plug-flow behaviour) is an alternative reactor design when operating with immobilised enzymes. The main advantages of the PBR compared to a STR are: lower investment cost; high volumetric productivity; low voidage; low mechanical stress; suitable for reactions with high product inhibition [60]; simplified separation of the enzyme from the product stream [15] and; when run at shorter residence times, may lead to fewer side reactions [25]. On the other hand, drawbacks of using a PBR include internal and external mass-transfer limitations, channelling effects over the bed and PBR are therefore, not suitable when operating in multi-phase reactions. In addition, pH control, ISSS and ISPR is difficult during a single pass through a packed bed. However, these limitations can be overcome when running several PBRs sequentially (or in a loop), with pH adjustment, substrate feeding or product removal in between [15].

A fluidised bed reactor (FBR) is particularly advantageous when using smaller particle-size in order to improve the contact and/or reaction of multi-phases (liquid–gas, liquid–liquid and liquid–solid) [52], as the solid particles are held in suspension within the reactor by means of fluids passing through the system and is therefore, of relevance

when using polymeric resins for ISPR or immobilised enzymes. When compared with PBR, FBR display several advantages: well-mixed behaviour, lower pressure drop, flow field is uniform and the formation of preferential channels is minimised [61] while keeping low mechanical shear stress. A variant of FBR is expanded bed reactor (EBR, plug-flow behaviour) where the reaction mixture includes suspended solids (such as polymeric resins for ISPR). The particles in the liquid may pass freely through the spaces in the bed without becoming trapped (unlike observed for PBR and FBR).

Some of the advantages and disadvantages of the most common reactors used in biocatalytic reactions are listed in Table 2.2.

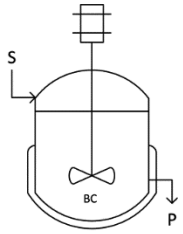
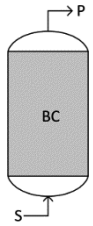
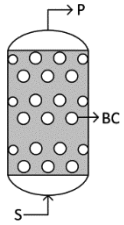
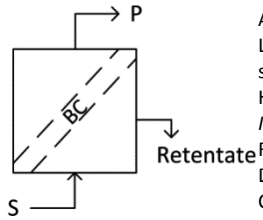
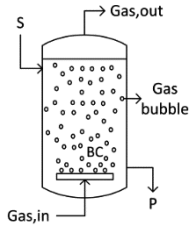
2.3 Concluding remarks

In the most prominent biocatalytic processes (such as those designated in this chapter as the next generation of biocatalytic processes), chemists have been putting great effort into finding new catalytic activities, to synthesise new compounds and to find new chemical routes for the synthesis of chemical compounds [62,63]. On the other end, biologists have been focusing their efforts on broadening the substrate range, improving specific biocatalyst activity and stability at operating conditions, or changing the enzyme selectivity [64-66]. Process engineers have been exploring new avenues to overcome many of the process limitations in areas that cannot be fully tackled by the other disciplines: shifting the thermodynamic equilibrium (by ISPR); and overcoming substrate and product solubility limits. However, process engineers have also been trying to reduce the burden on the biology side by developing new technologies to overcome substrate and product inhibition (by ISSS, ISPR and reactor design), or to improve enzyme stability (by immobilisation).

For instance, in a biocatalytic process, biologists can assist enzyme expression enhancement during the biocatalyst production by metabolic engineering or improved biocatalyst stability and activity by protein engineering. Process engineers can tackle the same challenges by opting for a different catalyst formulation (immobilised), altering the reactor configuration, applying a substrate feeding strategy (fed-batch mode, ISSS) and/or substrate removal (ISPR). As seen in conventional chemical catalysis, in biocatalytic processes, in theory, the only boundaries are the physical and thermodynamic properties of the system and even these can be eventually overcome by introducing a second liquid phase to increase compounds solubility, choosing a slightly different chemical route (e.g. different electron acceptor or donor) or optimising reaction conditions (pH, temperature etc.).

The multidisciplinary character of biocatalytic processes brings new opportunities within the field. Hence, a suitable structured approach for conceptual process synthesis should indicate the efforts required in each research area, where the different disciplines (i.e.

Table 2.2. Different reactors for multi-enzymatic processes

Reactor type	Reactor representation	Advantages	Disadvantages
Stirred tank reactor (STR)		Allows soluble and immobilised enzymes and whole-cell Multiphase media Good operational control Simple modelling Simple construction Low downtime (easy to clean)	Possible inactivation of biocatalyst Difficult soluble enzyme recycling
Packed bed reactor (PBR)		Allows soluble and immobilised enzymes and whole-cell Low enzyme damage due to shear stress High conversions achieved and volumetric productivities Shorter residence times Easy enzyme recycling, separation and exchange Low investment cost	Thermal gradient Difficult to control Complex modelling Mass transfer limitations Not suitable for multi-phase reactions
Fluidized bed reactor (FBR)		Allows soluble and immobilised enzymes and whole-cell Good enzyme mixing Suitable for multi-phase reactions Lower pressure drop Uniform flow field Good operational control Easy enzyme recycling, separation and exchange	Possible inactivation of biocatalyst Constrained by the particle size and density
Membrane bioreactor (EMR)		Allows soluble enzymes Low enzyme damage due to shear stress High conversions achieved *In-situ* separation Retention of enzymes and cofactors Dosing of a reagent Compartmentalisation	Complex modelling Poor control Membrane fouling Restrictions on the volumetric flow rate High energy utilisation
Bubble column reactor (BCR)		Simple design High heat transfer area Low enzyme damage due to shear stress Low maintenance costs	High sparging rates are required for a turbulent flow Bubble coalescence

chemistry, biology and process engineering) are aware of each other and of the cost of developing a technology. For instance, product inhibition can often be overcome by ISPR, which is often a less laborious and extensive task than developing the catalyst by enzyme engineering. This PhD project proposes a methodological approach to promote process development in a systematic way (and not by a case-by-case as it is currently done for the many of the aforementioned challenging biocatalytic processes). The proposed methodology can direct the research focus for each area (chemistry, biology and process engineering) from the early stage of process development, allowing a better use of resources and a reduced the development time.

Part II

Process development for biocatalytic processes

3 State-of-the-art in methodological approaches for conceptual process development

One of the main challenges in process development at early stage is selecting between different process alternatives (i.e. flowsheets, technologies, catalysts, among others). This challenge becomes even more crucial when developing biocatalytic processes, as the environment in which biocatalytic reactions naturally occur (i.e. inside the cell) is often very different from the operating conditions that ensure the economic and environmental viability of the process (high substrate and product concentrations, presence of organic solvents, etc.). Furthermore, reaction, biocatalyst and process constraints in biocatalysis are often correlated [36,46,67,68]. Thus, development strategies for biocatalytic processes require a deep understanding of the underlying constraints and should include a synergy between chemists, biologists and process engineers in order to overcome these.

In general, chemical processes must be operated effectively and efficiently [69,70]. Indeed, one of the main challenges of an engineer is to select a process route among different process options. Conceptual process synthesis has proven to be of high relevance in chemical process industries [71] as process development and design typically constitutes a small proportion (about 10 to 15%) of the total resources during the development process of a new product [72]. However, the decisions made in this step account for a large fraction (between 70 to 80%) of the total production costs [72]. The importance of a correct assessment is especially significant for bioprocesses, a less mature technology in the chemical industry, where most of the biocatalysts and host organisms are still under development and many of the reactions have not yet achieved their full potential.

An increasing number of conceptual process design methods are now applied in chemical industries in order to: design innovative processes by considering new raw materials, feedstock or routes [73,74]; re-design a process or; retrofit the process equipment in an existing process [70].

The following section is dedicated to the systematic methodologies developed for conventional chemical industries, followed by the state-of-the-art in methodologies for bioprocess development. Later, tools and the methodology proposed in this thesis are presented (Chapters 4 and 5, respectively).

3.1 Methodologies used in conventional process development

In the last five decades, there have been a large number of methodologies that propose an expedited approach to design new or optimised processes [75]. Regardless the methodology followed, the conceptual process synthesis includes: problem definition; goal definition; synthesis of solutions; analysis of solutions; evaluation and reporting [73].

Indeed, process synthesis methodologies and tools have reached such a level of maturity that they are successfully applied in industry, providing several advantages in process selection, in particular when aiming for energy, capital and operating costs savings [73-75].

There are three main conceptual process synthesis methods: optimisation-based methods, knowledge-based methods and hybrid approaches. The main idea of the optimisation-based approach is to formulate a new flowsheet in the form of an optimisation problem (e.g. improving the economic or environmental profile of the process) [71]. Whilst a knowledge-based method is focused on the representation and knowledge organisation of the design problem, meaning that the limiting steps are first identified and the conceptual process synthesis aims at overcoming the identified limitations [71]. The hybrid (or combined) approach combines simultaneous adoption of mathematical algorithms (as in optimised-based methods) and the hierarchical design procedure (used in the knowledge-based methods) [76].

3.1.1 Optimisation-based method

The optimisation-based method solves an optimisation problem where a mathematical representation reflects the requirements for an optimal solution [77-79]. For this purpose, an objective function is defined, including conceivable unit operations and a mathematical model that connects the different units of operation, mass and energy flows as well as capital and operating costs. The cost optimisation function is defined together with all constraints and variables. The output of an optimisation-based method for conceptual process synthesis is an optimal value for a set of variables calculated using algorithms (such as mixed integer nonlinear programming, MINLP and genetic algorithms, GA) [71]. This method has been applied in industry both for optimising an existing process for the production of bulk chemicals [80] and optimising a design derived from a knowledge-based method [73,81]. The main drawbacks of this methodology are: lack of an ability to automatically generate a flowsheet superstructure; considerable computational effort [71]; the optimum solution found can only be guaranteed for the alternatives considered earlier [81]. Hence, this conceptual process synthesis approach encounters great difficulties when dealing with poorly

defined processes, such as is often the case for bioprocesses during early development stage.

3.1.2 Knowledge-based approach

Knowledge-based or heuristic approaches rely on the long-term experience and expertise of the engineers and researchers. These methodologies are used to narrow down the list of possible operating units based on the experience of the process engineer. A systematic heuristic methodology, also called hierarchical heuristic method, proposes a procedure where the heuristic rules used for decision-making are applied at different design levels to generate the flowsheet alternatives [82,83]. During the conceptual process design the level of detail increases (as well as the information available) and therefore the alternative process flowsheet evolves. The hierarchical heuristic method defines: 1) operating mode (batch, fed-batch, continuous); 2) flowsheet input and outputs; 3) recycle structure; 4) separation system; 5) heat recovery and/or integration [71]. This methodology has been applied in the synthesis of new process flowsheets [84-86], separation processes [84,87,88], and for waste minimisation [89]. Even though this approach allows a rapid identification of flowsheets that are "near" optimal solutions, it is sequential and hierarchical structure does not allow interaction between the different levels and is thus, not suitable for biocatalytic processes, where improvements in the biocatalyst performance influence the flowsheet selection.

3.1.2.1 Conflict-based approach

A conflict-based approach is a knowledge-based method and it rest on the identification of system conflicts and contradictions for the solution of a given problem [2,90]. New process syntheses are attained by modification of a certain system aiming to overcome the internal contradiction. This methodology looks at the design problem as sub-problems, overcoming the aforementioned limitation of the hierarchical heuristic methodology regarding the lack of interconnection between the hierarchical design level and the limitations of insufficient problem representation. Conflict-based approach has been successfully applied to distillation column design and waste management [90,91] and biomass gasification [2]. However, this methodology implies an *a priori* synthesis of processes, as it only explores conflicts in synthesised solutions and is therefore not suitable for very early development stage.

3.1.2.2 Case-based reasoning

Case-based reasoning is a methodology that reuses solutions that were successfully applied before to similar problems. This systematic approach implies that the new synthesised problem is matched against previous cases by computing the degree of similarity in order to find the most similar problem and its solution. This methodology has been applied in design of distillation systems [92]. However, case-based reasoning

deals with very specific data and relies on reusing results and experience to solve new problems. In addition, the outcome solutions from this methodology are inevitably strongly influenced by previous designs, thus not allowing truly innovative breakthroughs.

3.1.2.3 Means-ends analysis approach

Means-ends analysis methodology sees a chemical process as sequence of different units of operation aiming at the sequential elimination of differences in physicochemical properties between raw material and final product(s) [84,93]. The means-ends analysis starts with an initial state and successively applies transformation operators to create new intermediary states with fewer property differences. This methodology has been applied in early systematic process synthesis for overall process flowsheets [93,94] where there were enough detailed specifications for the starting material and the product. One of the major drawbacks of this approach is that the means-ends analysis can only consider a limited number of properties simultaneously, while others are temporarily ignored and their impacts disregarded. Due to the correlation often observed between the different biocatalyst properties and the reaction components physicochemical properties this methodology is not suitable for biocatalytic processes. Further, the search method does not guarantee a feasible approach.

3.1.2.4 Phenomena-driven design

Phenomena-driven design is focused not on the unit of operation level itself but at the phenomena that occur within this unit of operation, exploring the relationship between physicochemical properties, the operation conditions and the operating unit. This methodology has been applied in separation process design [95] where the number of alternatives for each separation task is reduced by acknowledging the relationship between the thermodynamic data of the mixture at different operating conditions for each separation technique [96]. However, this methodology is based on opportunistic task identification (due to the predefined hierarchical levels, action and influence of the process phenomena) and it does not meet the general process needs [71].

3.1.3 Hybrid approaches

Hybrid approaches combine physicochemical property insights with mathematical algorithms [97]. These methods are usually implemented in a systematic procedure, where the output of a step is the sequential input of the following step. The final step of the hybrid methodology involves a simulation in order to verify the generated process solution and corresponding flowsheet [98]. Even though this methodology has been successfully applied in the chemical industry [99], it requires a large computational effort and large amount of data along with *a priori* knowledge of the process.

3.2 Tools applied to assist process development in bioprocesses

If for conventional chemical industries, where the process design options are well known and described, it is difficult to find a single suitable systematic approach for conceptual process synthesis. In bioprocesses, this is an even more ambitious task since many of the process technologies are not yet well described and their relationship with the overall process is not clear. Nevertheless, Grossmann and Westerberg [69] have identified bioprocesses as one of the areas that will receive increasing attention in the chemical industry and thus it is expected that the suitable methodologies for conceptual process synthesis and design for these processes will be developed and established.

In comparison with other conventional chemical processes, bioprocesses (including biocatalytic processes) have a wider multidisciplinary character (such as microbiology, molecular biology, chemistry and process engineering) which brings new opportunities for the production of new chemicals. However, the multidisciplinary nature of biocatalytic processes can also be seen as a weakness for implementation of a new process if the efforts in each discipline are not well targeted and defined. The use of the concept of process systems engineering (PSE) as a guide for bioprocess development is still in its infancy and many of the industrially successful biocatalysed processes are the result of case-by-case approaches [15]. Hence, an early stage systematic process design would likely assist the process engineer to achieve the full potential of the bioprocess leading to reductions in development time and savings in R&D.

Although a systematic design framework has not yet been proposed, solving a wide range of design problems based on a large range of potential solutions, there are a few methodological approaches that are able to assist in bioprocess development, such as process and kinetic modelling, sensitivity and uncertainty analysis, regime analysis and windows of operation.

3.2.1 Process and kinetic modelling

Process and kinetic modelling allows an efficient evaluation of different process options by providing a dynamic and quantitative description of the process. The design and optimisation of a certain process is strongly dependent on a reliable mathematical model that accurately describes the biocatalytic reaction(s) as well as the required engineering principles. Moreover, modelling saves time and manpower in experimental investigation [44] by predicting the system behaviour [100] and assisting in formulation of experimental design [101] where specific highly relevant variables can be investigated and thus experimental work can be focused on the information required to improve the process and model [101]. In order to model the process, a variety of experimental data is required, including thermodynamic, physicochemical and kinetic data [102]. Once this

data has been put together, process design software (such as ASPEN, SuperPro Designer or ProII) can be used to simulate different process configurations. Although these are fairly common tools in both conventional chemical processes and in bioprocesses, many compounds involved in biocatalytic processes are newer than those found in conventional chemical processes and therefore data is often not available [15] (e.g. physicochemical properties, reaction thermodynamic equilibrium constant, Michaelis constant and inhibition constant), or at least not available at relevant process conditions. Hence, it is necessary to develop a methodology that is able to identify and define, in early stage of the process design, the optimal process conditions.

3.2.2 Sensitivity and uncertainty analysis

Sensitivity and uncertainty analysis are methodologies that can be applied to study the process model's robustness, to quantify the likely variation in the process outcome and to identify the source of variation in the process performance [103]. The ability to identify the source of variation in the process can assist in the conceptual process synthesis by relating the different variation sources with different process scenarios [15]. Uncertainty analysis quantifies the overall uncertainty of the process model predictions (i.e. outputs) by studying the propagation of various sources of uncertainty [104]. Further, this analysis also provides information about the operating variables that need to be controlled, and is thus relevant when designing a process control strategy. These tools provide a better understanding of process variations and therefore they are extremely useful for a correct scale-up, since it is necessary to ensure a reproducible process where the product has a consistent quality [105]. In addition, this analysis also provides a certain adaptive character to the process and kinetic modelling by indicating the reliable variables that require close monitoring during the model recalibration once the process conditions are changed [106]. Finally, this analysis provides a quantifiable evaluation of the parameters that are most relevant in the process or kinetic model, allowing model simplification [107]. However, sensitivity and uncertainty analysis require a fairly good understanding and definition of the process design.

3.2.3 Regime analysis

Regime analysis is a useful tool for identifying constraints in the process and analysing the potential benefits of relaxing these [108,109]. To set a regime analysis it is necessary to choose a set of process metrics (such as product concentration, reaction yield, space-time yield and biocatalyst concentration) or different mechanisms involved in the process (mixing, mass transfer, reaction rate) [108] that can illustrate the effect of limiting regimes and simultaneously allow for sensitivity analyses of the different

process conditions [109]. Regime analysis based on process characteristic parameters (e.g. power input per volume, residency time, maximum, oxygen mass transfer coefficient) can give a quick estimation of the performance of bioreactors at large-scale [110]. Hence, for rate-limiting mechanisms (mixing, oxygen transfer, among others) the results of regime analysis can assist in solving scale-up problems [110]. This tool gives an insight how the process metric can change with alteration of one process parameter (e.g. biocatalyst concentration). For instance, in a whole-cell process, when the oxygen supply is limited, the catalyst concentration can be used to identify oxygen-limited regimes. An increased cell concentration leads to a reaction rate limited process due to competition for oxygen from metabolism. While decreasing the cell concentration implies an increase of the space-time yield due to a lower biocatalytic activity [111]. Nevertheless, this methodology does not explain the effect of altering the process design, such as adding an auxiliary phase, ISPR or improving the biocatalyst.

3.2.4 Windows of operation

Windows of operation are two-dimensional process maps displaying regions of feasibility (or operating ranges for input operating variables) where the process can be operated within user-defined constraints [15,112]. These constraints should reflect the required performance level, equipment limitations or physicochemical boundaries of the system [113]. Thus, this tool facilitates the prompt evaluation of the designed process [114]. Moreover, windows of operation allow a rapid definition of the operating spaces and a greater understanding of the influence that key operating variables have on the overall system behaviour [115].

Since engineering design problems are often complex and involve many interdependent variables, the selection of the operating variables is an essential first step when creating a window of operation.

During process design, several process options are tested, aiming at matching a specific objective for the output variable (measuring the process performance). Throughout this process, some of the process option combinations will not attain the desired performance. Those where the process performance is achieved define the operating region [112].

Hence, the selected axes must be two input operating variables that can be controlled and that have a strong influence on the process performance. Further, these input variables must be able to reflect the process options analysed, preferentially through precise relationships (e.g. mathematical models) between the two selected variables.

To create a window of operation, the collected process data is represented for a single output variable (Process metric 1 in Figure 3.1) as a function of two input variables

(Variable 1 and 2 in Figure 3.1) in a 3D plot, or in a contour plot [113]. The output variable is thus represented as a series of contour lines for combinations of input independent variables [113]. The relationship between two input variables and the process metric defines a window of operation [112]. By specifying required performance limits for the output variables of interest, one single contour line can be selected. Overlaying multiple contour plots for different dependent output variables enables the identification of windows of operation, where both independent control variables (Variable 1 and 2 in Figure 3.1) are feasible and the output variables reach a defined performance level (Figure 3.1) [113]. This approach allows the determination of the operating space, where all the performance criteria can be achieved simultaneously, leading to one single window of operation. These features allow the examination of multiple operating strategies and the ability to compare alternative processes whilst assessing their relative performance levels.

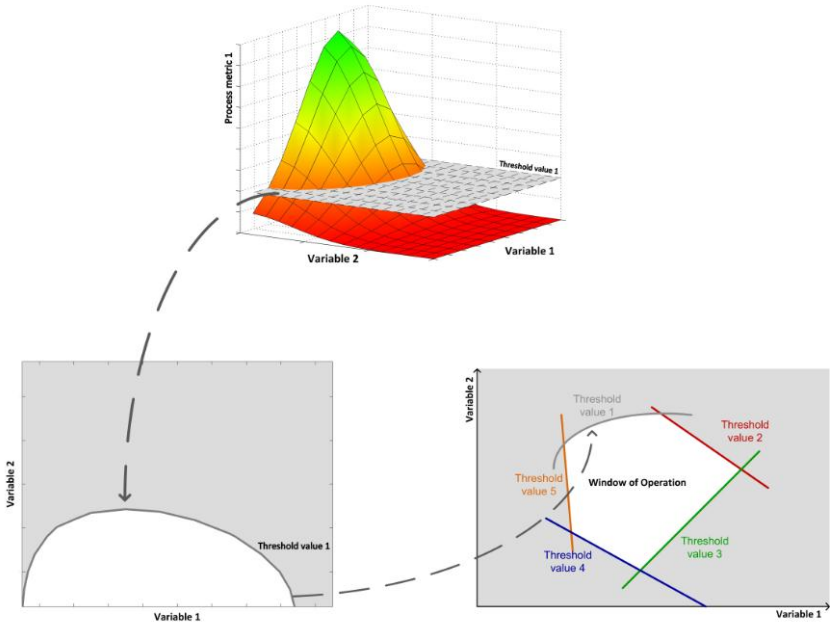


Figure 3.1. Generic window of operation; the area confined by the constraints is the resulting window of operation

Furthermore, the shape, size and position of the window of operation are dependent on the level of relaxation of each of the user-defined constraints [113]. Constraints have different origins such as performance-related constraints (e.g. biocatalyst specific reaction rate, minimum reaction yield or final product concentration), physical constraints (e.g. water-solubility of the reaction components), thermodynamic constraints (e.g. reaction equilibrium) and/or biological constraints (e.g. enzyme inhibition, stability). However, some of the defined constraints cannot be modified.

Hence, constraint relaxation aiming to enlarge or enable an operating space might not always have a physical meaning. It is thus essential to distinguish between hard constraints and soft constraints. Hard constraints are typically physical constraints (such as water-solubility and reaction thermodynamic equilibrium) in which constraint borders do not change when there is a slight variation of the operating conditions. On the other hand, common soft constraints in biocatalysis are those related to the biocatalyst (e.g. specific biocatalyst activity). Adjusting these soft constraints is, in part, dependent upon the amount of effort placed on biocatalyst development [112], since a more demanding soft constraint (e.g. specific activity) requires an extended development effort [65]. Soft constraints can be modified in two ways: 1) by relaxing the threshold of the process performance metric; 2) by putting in place a different process technology (e.g. soluble enzyme replaced by an immobilised enzyme).

In addition, when several dependent variables are included in the analysis, it is likely that relaxation of user-defined performance metrics will be necessary in order to obtain a feasible operating region where all the dependent variables can be accommodated (Figure 3.2).

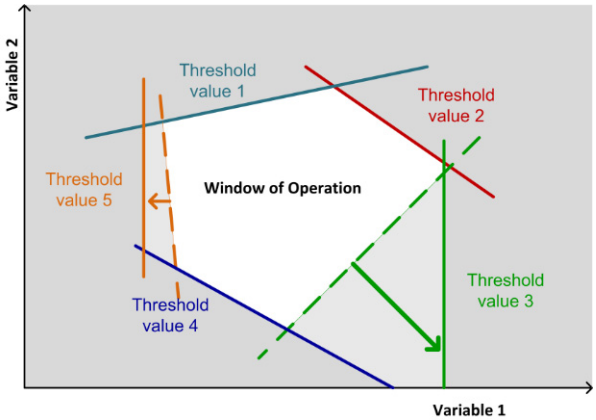


Figure 3.2. Enlarged generic window of operation; the area confined by the constraints is the resulting window of operation. Constraint 3 and 5 are soft constraints. Constraints 1, 2 and 4 are hard constraints.

Studying the size of the window of operation might be enlightening for a more complete process understanding. The size of the window of operation is related to its robustness. If the window of operation is large or there are multiple windows, it is easier to achieve the desired performance. Furthermore, a combination of windows of operation and Pareto optimal point approach[2] [116] might be beneficial in examining the impact of the

[2] Pareto optimal point approach: "constraint-oriented method" where a multiple-objective optimisation problem is converted into a scalar optimisation problem by minimising or maximising one objective-function while the other objective-functions remain constrained [320]. The set of Pareto optimal solutions is then generated by replacing each objective-function sequentially for different constraints. Pareto optimal point is

process operating input variables on the dependent variables, leading to a maximized performance. On the other hand, if the feasible space is small and tightly constrained, there is a limited range for the operation input variables (i.e. reduced window of operation), implying working at a more rigorously controlled process [117,118].

The window of operation methodology allows visualisation of data to aid decision-making by providing a process map for the feasible window of operation where a user-defined level of performance is achieved [112]. Further, this methodology also provides a graphical understanding and management tool for the several trade-offs between the input operating variables and the performance variables [113], as well as between different process options available in the process design.

To date, windows of operation have been used mainly for process control and development of suitable process control strategies by defining suitable operation spaces [117,119]. Further, windows of operation were also used as a tool to identify key process constraints, for process debottlenecking and assessing process feasibility at different process development stages (e.g. process design, scale-up and optimisation) [115,118].

Windows of operation to assess process control are probably the most common 'windows' in the scientific literature. Examples can be found in many areas in chemical engineering such as: distillation columns [120,121], syngas production [122], hydrogen production by iodine-sulphur cycle [119], but also within bioprocesses during production process of intracellular protein production [114,116,117,123], refolding of recombinant protein [124], design of chromatographic steps [125-127], to optimise cultivation conditions of mammalian cells [113,128] and to improve reaction conditions in multi-enzymatic systems [25,129]. One example of the use of the windows of operation is the selection of optimum operating conditions (e.g. substrate, product or biocatalyst concentration, temperature, pH, pressure, etc. [112]) and identifying suitable trade-offs between them [117] in order to achieved defined performance metrics (e.g. reaction rate, biocatalyst yield, operational cost, etc.). Figure 3.3 shows the window of operation for the process of a generic bi-enzymatic reaction system. The window of operation for a bi-enzymatic one-pot process is built by overlapping the individual window of operation for each enzyme. The resulting window of operation marks the operating range for the assessed control variables (in Figure 3.3, Variable 1 and Variable 2) where the performance metric is achieved.

a point in the feasible space, where no other point in this space exists yielding an improvement in one objective function without causing deterioration in the other criteria(on) [320].

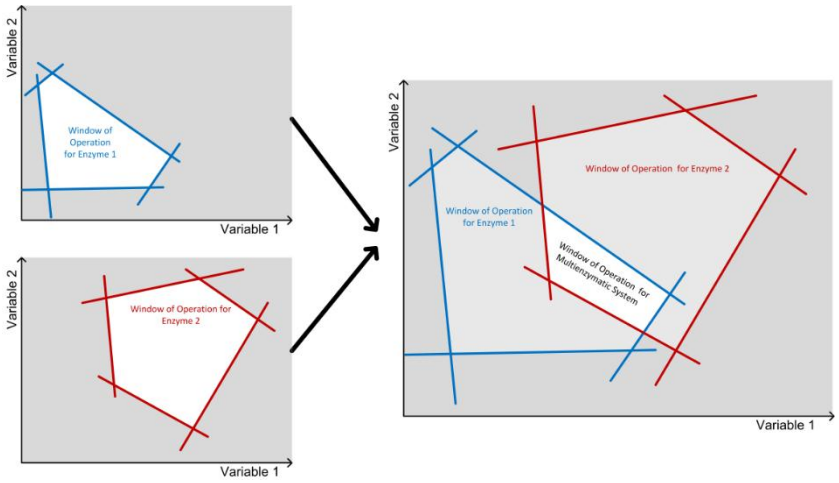


Figure 3.3. Schematic illustration of a window of operation for a bi-enzymatic reaction system for a generic performance metric

4 Tools developed in this thesis

In this chapter, 'cofactor and interaction matrices' and 'windows of operation' are described as engineering tools for assessing process feasibility. These feasibility tools endeavour to establish feasible flowsheets where reaction, biocatalyst and process constraints are all fulfilled. Further, performance evaluation tools assessing the economic viability of the process and its environmental impact are also discussed.

The engineering feasibility tools integrate the concepts introduced in the previous chapter (in particular those developed for bioprocesses, Section 3.2) and goes beyond these by coupling them with evaluation tools. Hence, the procedure applied when using these feasibility and evaluation tools takes into account the biocatalyst limitation(s) (such as inhibition and stability), reaction stoichiometry, mass and energy balances and process flowsheets, for specific raw material(s) and product(s). These tools are extremely useful in evaluating the effects of process improvement efforts (including reaction, biocatalyst and process development) on the process feasibility and performance at a very early stage of development. Further, when different routes and/or flowsheets are under consideration, a comparison by applying the presented feasibility and performance evaluation tools to the various options allow the elimination less favourable process configurations, thereby focusing the development efforts on those most likely to be successful at industrial scale.

4.1 Process feasibility tools

4.1.1 Cofactor and interaction matrices

Many of the biocatalytic reactions of interest for organic synthesis are oxidative and reductive reactions (redox reactions), which require the use of so-called "free coenzymes" (e.g. NADP(H), NAD(H), FAD(H2)) [130]. However, these cofactors are complex, unstable [131] and quite expensive [130]. Therefore, an efficient regeneration system is required in order to ensure process economic viability.

For a redox biocatalytic process, running with isolated enzymes the reaction system must be designed in such way that it includes *in-situ* cofactor regeneration routes. This may be achieved by implementing a network multi-enzymatic system. If such a multi-enzyme system is applied, it is convenient to analyse the flow of the electron donor and acceptor in order to decide which reactions should proceed in the same vessel. Alternatively, whole-cells where these cofactors are synthesised and regenerated as a part of cellular metabolism may be used [130]. The selection of whole-cell biocatalysts can offer a continuous source of cofactors, which, in some cases, could simplify the reaction structure, as no extra enzymes would be required for cofactor recycling [130].

An interaction matrix is a process design tool to assess feasibility. This is a particularly important evaluation tool when dealing with multi-enzyme systems, as it provides a better understanding of the overall system. This tool has proven to be extremely useful in gathering information related to reaction and process characteristics that must be considered for kinetic modelling [44]. However, within the framework of process development, this feasibility tool assists process design by narrowing down the number of process options.

The tool identifies the different interactions that may occur between the different compounds (e.g. substrate(s), products, by-product(s), intermediate(s), cofactors, etc.) and enzymes catalysing a specific biocatalytic step. In order to build an interaction matrix the different components are arranged in rows and the enzymes arranged in columns [44]. The matrix is then filled by defining the relationship between each compound and enzyme, e.g. substrate (S), product (P), inhibitor (I), activator (A), or non-interactive (x) (see also Chapter 7). This information can be gathered directly from the reaction structure, from scientific literature and/or experimental data from the laboratory.

For kinetic modelling of multi-enzyme processes, the interaction matrix can be used to identify an inhibitory compound and indicate that a new parameter should be added to the reaction rate kinetic model.

However, in the context of process design, the interaction matrix indicates the key reaction considerations that will affect or modify the flowsheet and/or mass and energy balances by indicating if the consecutive reaction can be carried out in the same vessel, the requirements for ISPR, inhibitory intermediates, etc. Hence, this tool indicates a limited number of viable (and preliminary) process options to be evaluated later using economic and environmental analysis. Detailed description of this tool can be found in Chapter 7.

4.1.2 Windows of operation

The ultimate role for windows of operation is to be used as a tool for process improvement. However, to date this methodology has only been applied for process debottlenecking or to assist during control design (see Section 3.2.4). Therefore, it was proposed that a methodology based on windows of operation principles to be applied during the early stage of process design and development, assisting during selection of process technologies and possible process flowsheets.

This rational approach highlights the most critical constraints during process selection, which are used in the methodology to plot the process performance (i.e. axes for the windows of operation). Further, the methodology is able to represent graphically the

effect of the design choices. In this methodology, it is clear that different process alternatives lead to different constraint values (e.g. modifying the ISPR method put in place). Thus, the application of windows of operation in this context serves as a feasibility analysis of the different process technologies available and the potential effects of future improvements.

This methodology provides a systematic approach for data collection and allows the exploration of conceptual scenarios to find the conditions under which a given process would be both feasible and competitive, while providing guidelines for directing future research in the performance of process options adopted throughout process development (see Chapter 5).

Moreover, this tool aims at supporting decision-making actions, not only for the process technologies available, but also for the synthetic route itself. For instance, when a new pharmaceutical compound is desired, being able to make accurate decisions regarding its synthetic route in a very short time is of great value to the process success as the speed of development is crucial for the economic feasibility of the process [132]. This approach provides a short-cut methodology to quickly and approximately assess the feasibility of a given synthetic route. On the other hand, for large-volume and low-price compounds (such as bulk chemicals) and also for generic pharmaceutical compounds, there is an increasing pressure to provide quality and efficiency, while improving safety, reducing production costs and pursuing greener processes [133]. For these processes, windows of operation are a useful tool to direct future research efforts and assist during selection of process design options. Detailed description of this tool and the systematic methodology to build a window of operation can be found in Chapter 8.

4.2 Performance evaluation tools

4.2.1 Economic evaluation

Economic evaluation is a decision-making tool to quantitatively estimate the expected profitability of a process, often in comparison with other choices [134]. The four essentials of an economic study are: problem definition; cost estimation; revenue estimation and profitability analysis as well as; a characterisation of the uncertainty and risk (Figure 4.1).

Cost estimates should be made throughout all the early stage of a project even when complete specifications (or other data) are not available [135]. Cost estimation is extremely useful during the development of a chemical process since it allows cost control and debottlenecking. At a research level, it plays an important and useful role in research guidance by pinpointing the process weakness [136].

Cost estimation can be divided into two categories: capital investment (CAPEX) and operating cost (OPEX) (Figure 4.1).

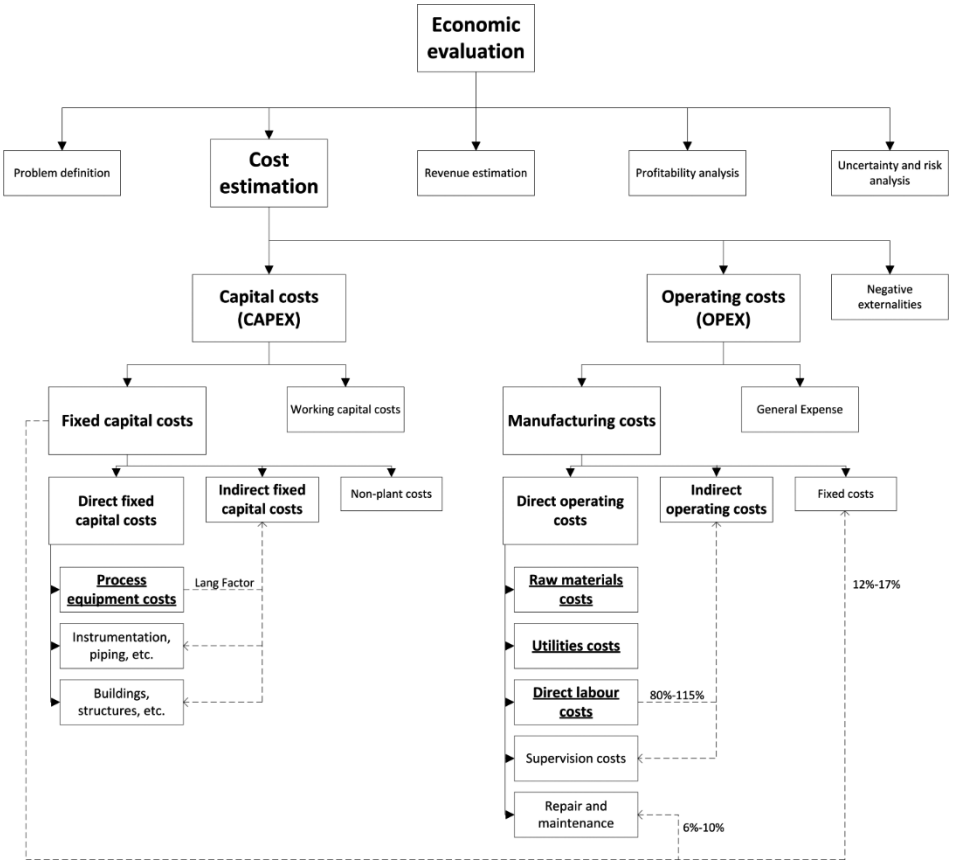


Figure 4.1. Cost estimation categories and sub-categories that are important for cost analysis.
Underlined costs are calculated separately, while other costs are estimated using rules of thumb and factors (_____) based on the cost estimation of the underline costs

4.2.1.1 Capital costs (CAPEX)

Capital investment corresponds to the sum needed to get the project started, for the machinery and equipment installation and can be classified in fixed capital and working capital [135]. Fixed capital stands for the capital necessary for all installed equipment and accessories required in the process operation and start-up [134,137]. Fixed capital comprises the price of purchasing, delivery and installation of equipment, piping, automated control, buildings and structures, site preparation, land (direct plant costs), engineering and construction (indirect plant costs) and contractor fees and contingency allowances (non-plant costs) [134,135]. Working capital is the sum required for the day-to-day operation and includes the cost of inventories, supplies and some of the start-up

expenses. The working capital cost was not accounted for in the cost evaluation methodology presented here.

The accuracy of a fixed capital estimate is a function of the design effort involved [134]. As the project design is refined, the estimate evolves from the various preliminary phases into more detailed construction estimates [134]. Evaluation of costs in the preliminary design phases involves guesses and the application of rules-of thumb [137].

Equipment cost

In simpler approaches, the calculation of CAPEX is focused on the process itself, excluding site-wide auxiliaries, off-site and land-related items [134,135,137,138]. The basis of a fixed capital estimate is equipment cost data. From this information, and by application of factors, the fixed capital investment can be calculated [135,137,139]. To obtain current equipment cost data, one should ideally solicit bids from vendors [137]. Other useful tools available to estimate the equipment cost are the available databases and process design software (such as ASPEN or SuperPro Designer). Unless stated otherwise, in this thesis, equipment cost data was obtained consulting the MatChe Inc. website[3].

The cost-capacity plot (or six-tenths rule) was applied when the effect of equipment scale was desired (Equation 4.1). However, one must be aware of the limited extrapolation capacity of this estimation [134,135].

$$\text{Cost of equipment B} = \text{Cost of equipment A} \times \left(\frac{\text{Capacity of equipment B}}{\text{Capacity of equipment A}}\right)^{n} \quad \textbf{Equation 4.1}$$

In the equation, n may vary between 0.4 and 0.9, depending on the type of the equipment and the operation conditions (pressure and temperature), 0.6 is the average value for all the equipment [134,137].

Other capital investment costs

In the early stage of process development, the level of detail does not usually allow for an accurate and reliable calculation of these expenses. Hence, in order to obtain the total investment cost, the equipment cost is multiplied by a factor to cover the costs for all supporting equipment and services [135]. In order to obtain the other costs that constitute the fixed capital (other direct capital costs - excluding equipment – and indirect capital cost), a multiplying factor (Lang, Hand, Wroth, Garrett and Guthrie factors) can be applied to the cost of the equipment delivered. These factors include the cost related with piping, automatic controls, insulation, painting, electrical work, engineering costs, etc. [137] (Figure 4.1). The selection of the most suitable factor to be applied depends on the level of detail in the cost estimation. Detailed information

[3] www.matche.com

concerning the aforementioned factors can be found in several process design handbooks [134,135,137].

Total capital cost/Annuity

A total capital investment, or a fixed capital investment, can be converted to an equivalent annual capital investment cost using an approximate capital charge factor (amortisation or annuity factor, k, Equation 4.2), which multiplies the capital investment to give a yearly capital cost, providing a convenient short-cut approach to use in annual cost estimation [134].

$$k = \frac{i}{1-(1+i)^{-t}} \qquad \textbf{Equation 4.2}$$

The capital charge factor (or interest factor, i in Equation 4.2), which is provided by finance groups, is typically around 7% for the chemical industry [134]. The typical equipment economic lifetime (t in Equation 4.2) is 10 to 15 years [137].

4.2.1.2 Operating costs (OPEX)

The operating (or manufacturing) cost is an important part of the economic evaluation. It consists of direct, indirect and fixed costs. At the early development stage, the operating costs that need to be determined are raw materials, utilities (including waste management) and operating labour. Other direct production costs (such as supervision, repair and maintenance), indirect and fixed operating costs might be calculated through direct labour cost and/or annual capital investment cost (Figure 4.1).

Raw materials

Estimates for raw material consumed are obtained directly from the mass balances. The prices of many raw materials can be obtained from the suppliers, by consulting trade journals (European Chemical News or Chemical Marketing Report) [134,137], through personal contact with the chemical companies, or using a chemical market information provider, such as ICIS[4]. A list of the raw materials prices used in this thesis is compiled in Appendix 2.

Utilities

Utility requirements, including the cost of heating and energy (for agitation), can be obtained from mass and energy balances. The energy necessary for heating can be calculated directly from the physicochemical properties of the materials used. While the energy necessary for mixing and aeration can be calculated using rule of thumb values [140]. Suppliers or purchasing agents should be contacted for the latest prices.

Although waste treatment is not usually part of the process design and cost model, waste disposal is an important process cost that should not be disregarded [137,141].

[4] www.icis.com

Typically wastewater treatment costs are 0.5-2 €/m^3 (dependent on location), and non-hazardous solid waste disposal has a cost of around 25 €/ton [141].

Labour

Labour needs are highly dependent on the plant scale. However for processes within the same capacity range, the labour needs do not increase in a direct correlation with the process volume. Therefore, in this study it was assumed that labour needs did not increase with scale. Direct (or operating) labour costs are normally estimated from the flowsheet, typical labour needs (personnel per unit of operation) [134], by applying the Wessel method, using the Ulrich table [137], using design software such as SuperPro Designer, or by experience about labour requirements for the whole process. Typically, the operating labour costs account for up to 15% of the total operating cost [135]. The direct labour needs are determined through typical labour requirements and in discussion with industry. Labour rates can be obtained from the union contract, from company labour relation supervision or from local statistical institutes (e.g. Eurostat, US Bureau of Labour Statistics).

Other operating costs

Other production costs can be calculated from direct labour costs or from annual capital investment estimates. Supervision costs (direct operating costs) and indirect operating costs (including payroll overhead, quality control, royalties and plant overheads) correspond normally to 80% to 115% of the total direct labour costs (Figure 4.1). Annual maintenance (direct operating costs) including labour and material make up for 6% to 10% of the fixed capital investment [134,137] (Figure 4.1). Fixed operating costs are insensitive to the production level and include depreciation, taxes, property rents, insurance, etc. corresponding to 12% to 17% of the annual capital investment cost Figure 4.1. Annual maintenance and fixed costs were not considered when determining the production cost.

4.2.1.3 Assumptions in simplified cost estimation

Within the scope of this thesis, one objective was to develop a fast and accurate method for cost analysis. Since many data are not widely available, in particular where the process design is not fixed, assumptions have to be made (see also Appendix 2). Table 4.1 summarises the main considerations in constructing the proposed economic assessment methodology.

When difficulties in obtaining raw material prices from the suppliers were experienced, the prices were estimated from laboratory chemical suppliers, and subsequently divided the original price by 10 to 30 depending on the original package size. Although, uncertainty of this approach is high it is still considered a good starting point for cost estimations. In any case, raw materials, utilities and equipment costs have been confirmed with industry (see Appendix 2).

Table 4.1. Summary of the considerations and sources of information used in the economic model

Cost	Contribution to Cost	Consideration
CAPEX	Equipment cost	MatChe Inc., process design software (ASPEN or SuperPro Designer)
	Other capital investment costs	Lang factor [140]: 5.0 (typical for fluid processing units [143])
	Annuity	From equation (Equation 4.2) k= 0.142, based on i=7% and t=10 years
	Equipment scale-up	n=0.6
OPEX	Raw materials	Market quotations, laboratory chemical suppliers
	Utilities	0.1 €/kWh (European Energy Portal[5])
	Waste handling	1 €/m^3 [142]
	Labour	30€/h (Eurostat[6])
	Supervision cost and indirect OPEX	100% of the direct labour
	Annual maintenance	10% of the annual capital investment cost.
	Fixed OPEX	15% of the annual capital investment cost

k - annuity factor; i - capital charge factor (or interest factor); t - the equipment economic lifetime

Evaluation of the costs in the preliminary design stage involves guesses and applications of rules-of thumb, therefore the quality and accuracy of these estimations are dependent on the skill and experience of the engineer [134]. For the methodology proposed the accuracy is considered to be about ±30%. Regardless of the level of detail and complexity in an economic study and in the project design, a certain degree of uncertainty will always remain [135]. This makes it is necessary to evaluate the effect of certain modifications to the original project on the total project cost by performing a sensitivity analysis to the cost used to calculate the process economic performance.

4.2.2 Environmental evaluation

In the last decade, an awareness of the impact that chemical production processes can potentially have on the environment has become a rising factor of concern. White biotechnology has been developing new processes that can potentially replace many of the conventional chemical processes, with a consequent possible reduction of chemical effluent and energy demand. Despite the great environmental advantages that biotechnology can bring to the chemical industry, being “bio” does not necessarily mean that a process is sustainable or more environmentally friendly than a conventional synthetic route and often questions regarding the benefits of these new routes against the traditional chemical processes are raised. Therefore, a fair comparison between the different synthetic routes is required and it is imperative to evaluate different process

[5] www.energy.eu
[6] ec.europa.eu/eurostat

options using economic assessments and environmental metrics side by side to find the most efficient and sustainable process configuration.

There are two well-established environmental metrics used to assess the environmental impact of a process: green chemistry metrics and life cycle assessment impact factors.

4.2.2.1 Green chemistry metrics (GCM)

Experts often find it difficult to assess bioprocesses, by virtue of the limited data available [143]. Indeed, the published number of complete environmental assessments of biocatalytic process is very limited [143]. However, when a comparison between synthetic routes is made, there are several simpler approaches to quantify the process environmental performance [144-146]. Among those, green chemistry metrics have been developed to measure the greenness of a given process according to the Green Principles [3-5]. Green chemistry metrics can be divided into reaction-related metrics and process-related metrics.

Reaction-related metrics

The green chemistry metrics that are included in the reaction-related metrics group intend to quantify exclusively the greenness of the reaction chemistry. Some of the reaction-related metrics include atom efficiency (AE), reaction mass efficiency (RME) and carbon mass efficiency (CME).

Atom Efficiency (AE)

Atom efficiency (AE, or atom economy) measures how much of the starting material ends in the desired product [147] (Equation 4.3). Hence, this metric assesses the reaction chemistry. The driver behind this metric is to fulfil the 12 principles of Green Chemistry, aiming at a product synthesis with high reaction yield and low waste [22,147]. This principle proposes that the chemists design a reaction where all the atoms of the substrates are included in the structure of the final product. This is often observed for biocatalytic reactions, with the exception of transferases (EC 3, where a donor is required). AE is an easy to use metric, based on the reaction stoichiometry and mechanism [148]. However, it does not consider the by-products produced, or co-substrates used and it is based only on the reaction chemistry, not taking into account the overall process.

$$AE = \frac{MW_{Product}}{MW_{Substrate(s)}} \qquad \textbf{Equation 4.3}$$

Reaction Mass Efficiency (RME)

Reaction mass efficiency (RME or just mass efficiency) is a metric developed by GlaxoSmithKline (GSK). This metric takes into account the reaction yield, the actual molar quantities of reagents and atom economy [144]. RME can be calculated by computing the quotient of the mass of the product by the mass of all the reagents in the

process (Equation 4.4) [144]. However, this metric does not account for the waste generated.

$$RME = \frac{m_{Product}}{\sum_i m_{Reagent_i}} \qquad \textbf{Equation 4.4}$$

Carbon Mass Efficiency (CME)

Like the mass efficiency, carbon mass efficiency (CME, or just carbon efficiency) is also a green chemistry metric developed by GSK to measure the sustainability of the processes within the framework of the 12 Principles of Green Chemistry [145]. CME is defined as the percentage of carbon in the reagents that remains in the final product (Equation 4.5) and takes into account the reaction yield, stoichiometry and the amount of carbon in the reagents that is incorporated into the final product [145]. Similarly, this metric takes into account not only the reaction chemistry, but also the reaction related issues of the process. However, as noted for the previous metric, CME does not account for the waste generated during the process.

$$CME = \frac{m_{C\text{-}Product}}{\sum_i m_{C\text{-}Reagent_i}} \qquad \textbf{Equation 4.5}$$

Process-related metrics

Process-related metrics intend to quantify the overall process (including the reaction chemistry). Some of the reaction-related metrics include process mass intensity (PMI), effective mass yield (EMY), E-factor, water and solvent intensity (WI and SI) and C-factor.

Process Mass Intensity (PMI)

Process Mass Intensity is the metric chosen by the American Chemical Society Green Chemistry Institute's Pharmaceutical Roundtable as a high-level metric to evaluate the sustainable manufacturing of a given process [149]. PMI is defined as the total mass of materials used to produce a specified mass of product [149] (Equation 4.6). When calculating PMI the starting point is the commonly available materials [149]. Hence, the metric accounts for all the steps in the chemical synthesis including the catalyst production and all materials (water is also considered), that are used directly in the process [150]. Further, PMI also includes the downstream process steps required for isolating and purifying the final product at the required quality [150]. However, PMI does not include specific concerns regarding the environment, health and safety of the raw materials used or the waste produced.

$$PMI = \frac{\sum_i m_i}{m_{Product}}$$ **Equation 4.6**

Effective Mass Yield (EMY)

Effective mass yield is defined as the percentage of desired product relative to the mass of all non-benign materials used in its synthesis (Equation 4.7) [151]. Unlike the above described metrics, this metric highlights the reagent(s) and reaction additive(s) toxicity. Despite the fact that this metric has been developed to assess only the reaction step, the same concept can be extrapolated to assess the full process (i.e. all steps of a synthetic path from commonly available materials to the final purified product). However, EMY lacks the definition of non-benign reagents, currently defined as "those by-products, reagents or solvents that have no environmental risk associated with them" [151]). Nevertheless, this definition cannot specify nor quantify the environmental risks. Furthermore, EMY only highlights one potential environmental impact (toxicity), disregarding others, such as global warming potential or waste generated. For example, when assessing a biofuel production process, this metric would give a favourable score to the process, since typically the reagents are renewable feedstocks and would be considered benign reagents.

$$EMY = \frac{m_{Product}}{\sum_i m_{\text{non-benign Reagent}_i}}$$ **Equation 4.7**

E-factor

The E (environmental) factor analyses the amount of waste formed in the synthesis of chemical compounds [152], estimating the amount of waste created per kg of product produced. Therefore, this green chemistry metric has been recognised as a valuable measure to provide information about the environmental performance and waste footprint of a given synthetic route [153]. In the E-factor, the waste is defined as everything leaving the process boundaries except the desired product. Therefore, E-factor takes into account the reaction yield, including solvent and reagent losses, process aids, etc. [154]. E-factor includes not only the reaction chemistry and the process options related with the reaction, but it can also include all the steps in a chemical synthesis, upstream or downstream of the reaction step. However, this green chemistry metric does not consider what type of by-product or waste is generated.

E-factor can also be used for multi-step reactions, though the result only provides a benchmark guide for different sectors and markets of the chemical industry [155]. In Table 4.2 the E-factor of different categories of chemicals are shown [155].

Table 4.2. Typical E-Factor values (without water) in the chemical industry

Industrial Sector	Volume (ton/year)	E factor ($kg_{waste}/kg_{product}$)
Bulk chemicals	10^4-10^6	<1 – 5
Fine Chemicals	10^2-10^4	5 - >50
Pharmaceutical Chemicals	10-10^3	21 - >100

Water is generally excluded from the E-factor as the inclusion of water could lead to excessive E-factors for some processes (such as biocatalytic processes), making a meaningful comparison of the results difficult [153]. Since water is usually benign, the solvent of choice for green chemistry is water, while in the conventional chemical synthesis organic solvents are often preferred [21]. There is a historical perception that water by itself does not have a significant environmental impact. However, one must remember that many chemical processes require highly purified water and there are life cycle impacts related to the water purification step. In addition, in many chemical synthetic routes, a mixed aqueous-organic waste stream is generated and therefore additional units of operation are required to further separate the phases prior to the wastewater treatment plant. Furthermore, nowadays assessing the water footprint of a given process is also a measure of sustainability, since in many parts of the world competition for water is becoming more of a concern and seems certain to become a greater issue in the future [156]. Therefore, two types of E factor were considered including water (Equation 4.8) and excluding water (Equation 4.9) consumption.

$$\text{E factor} = \frac{\sum_i m_{waste_i}}{m_{Product}} \qquad \textbf{Equation 4.8}$$

$$\text{E factor} = \frac{\sum_i m_{waste_i} - m_{water}}{m_{Product}} \qquad \textbf{Equation 4.9}$$

Solvent intensity (SI) and Water intensity (WI)

From a careful assessment of many of the synthetic routes to chemical products, solvents have been found to be one of the biggest mass contributors [157]. This is especially true for the pharmaceutical industry where the solvents typically contribute 80% to 90% of the mass intensity of a process [158]. Hence, solvent intensity (SI) was developed to tackle the problems raised when applying the E-factor, by analysing and quantifying the amount of all solvents used in the processes for the synthesis of a chemical (Equation 4.10). A particular version of solvent intensity is water intensity (WI) where the focus is on analysing the amount of water used throughout the whole process (Equation 4.11). However, the type of solvent or the quality of the water used is not specified and a methodology for measuring the relative greenness of a given solvent is

still required. Furthermore, the environmental issues related with the solvent recovery are not addressed by this metric.

$$SI = \frac{\sum_i m_{solvent_i}}{m_{Product}} \quad \textbf{Equation 4.10}$$

$$WI = \frac{\sum_i m_{H_2O_i}}{m_{Product}} \quad \textbf{Equation 4.11}$$

C-factor

The C-factor expresses the amount of CO2 produced per mass of product formed [159] (Equation 4.12). The innovation in this green chemistry metric is the life cycle thinking, as it includes all the CO2 produced from the raw material production, preparation, conversion and purification of the chemical. However, this metric focuses only on one environmental concern (global warming potential) [160]. Nevertheless, the production of chemical products (mainly fine chemicals and pharmaceuticals) entails other environmental concerns, such as emission of volatile organic compounds (VOCs), toxicity or nutrient enrichment. Thus, the exclusive use of the C-factor for environmental assessment leads to a risk of these issues being neglected. Furthermore, C-factor only accounts for the emission of CO2, giving a misleading result, especially for renewable resources where emissions of methane (CH4) and nitrous oxide (N2O) are equally (if not more) worrying in their potential contribution to global warming [161].

$$C\ factor = \frac{\sum_i m_{CO_{2_{emitted_i}}}}{m_{Product}} \quad \textbf{Equation 4.12}$$

4.2.2.2 Simplified Life cycle assessment (LCA)

The use of simple metrics is an attempt to measure the process chemistry and efficiency in a straightforward way. It does not require many process details and is therefore attractive for initial process design decisions. However, with simplicity might come several drawbacks, such as the fact that most of the metrics do not distinguish between waste types and emissions [158]. Moreover, many of these metrics do not consider the waste generated upstream or downstream of the investigated process step. At the other end of the spectrum, a more elaborate and comprehensive tool to quantify environmental effects is Life Cycle Assessment (LCA). Unlike green chemistry metrics, LCA is not specific for (bio)chemicals, chemical or bioprocesses. Indeed LCA was developed to be a suitable environmental assessment tool for all kinds of products and processes and there are LCAs published for different products from food [162] to television sets [163]. LCA is a standardized (and regulated) tool (ISO 14040) that provides detailed information about the type of emissions and the environmental impact over

the life cycle of a product or functional unit. LCA provides a framework for reporting applicable green metrics reflecting the whole life cycle of a given product (i.e. from raw material to the disposal stage) [164]. LCA metrics can be reported as inventory data (energy consumption, raw material consumption or emissions), measure of individual potential environmental impacts (such as global warming, acidification and nutrient enrichment potential), or as an aggregated score or index (such as EDIP, CML; Eco-Indicator, etc.) [161]. However, LCA is often a laborious task as it requires a large amount of data from a variety of sources [158] and therefore is not yet a widespread practice. Further, many of the raw materials common in bioprocesses were not yet correctly modelled and assessed in this methodology.

One of the most relevant steps of the LCA is to calculate the environmental impacts. These are classified in impact categories (e.g. global warming, photochemical ozone formation, human and eco toxicity potential). The environmental impacts are classified according to their radius of action into global, regional or local impacts (Table 4.3). The substance's impact potential is expressed in an equivalency factor (gCO_2-eq, gC_2H_4-eq, etc.). The equivalency factor expresses the emission measured relative to a reference substance. Substances contributing to more than one type of environmental impact require an equivalency factor for each type of impact. For example, emission of methane contributes for both global warming potential and photochemical ozone formation. Hence, emission of 1 g methane is translated on the impact assessment as 25 gCO_2-eq and 0.007 gC_2H_4-eq [161]. Table 4.3 shows the environmental impact potential and the equivalent unit (or equivalency factor).

Table 4.3. Environmental impact potentials

Type of Environmental Impact	Environmental Impact	Abbreviation	Equivalency Factor
Global	Global warming potential	GWP	gCO_2-eq
	Stratospheric ozone depletion potential	SOP	$gCFC_{11}$-eq
Regional	Photochemical ozone formation potential	POP	gC_2H_4-eq
	Acidification potential	AP	gSO_2-eq
	Nutrient enrichment potential	EP	gPO_4^{3-}-eq
Local	Eco toxicity potential	ETP	PAF*.m^3.day
	Human toxicity potential	HTP	Cases
	Hazardous waste	HWP	Kg
	Bulk waste	BWP	Kg

*PAF - Potentially affected fraction of species

The importance of a given impact category varies depending on the type of chemical assessed [165]. Several companies are reporting the environmental profile of their processes and products by describing emission of greenhouse gases or energy savings. Nevertheless, when performing an environmental assessment on a given chemical, one should choose the most relevant impact potential. For example, solvents are one of the biggest mass contributors in the production of fine or pharmaceutical chemicals due to

the low water solubility of many substrates and products of interest [158] and thus, VOCs emissions are mainly due to solvent use [161]. Hence, when assessing this type of chemical processes, it would be more relevant to study the impact assessment of regional and local impacts, such as photochemical ozone formation potential (instigated by high concentrations of VOCs) and eco and human toxicity potential (due to emission of toxic particles during the chemical production process [166]), since a reduction of these impacts might represent improvements in the process. On the other hand, when fuel production from renewable resources is evaluated, it is would be more beneficial to analyse the impact on greenhouse gas emissions, nutrient enrichment potential and land use (due to the crop growth). When comparing process options for the manufacture of biofuels, large volume (bulk) (bio)chemicals, or in fermentation processes (such as for the biocatalyst production), the impact of the cultivation of the raw materials (for the carbon-source) on global warming and nutrient enrichment potential is influenced mainly by the choice of crop, but also by the process yield. Therefore, the yield coefficient of biomass on substrate (Y_{sx}) could be a very useful assessment metric. Primary energy demand is also a suitable metric to measure the process efficiency, mostly for the fermentation and downstream processing steps. In a similar way, acidification potential can also measure the process efficiency, as the most significant man-made sources of acidification (e.g. SO_2) are combustion processes for electricity and heat production [161]. However, this relationship is very dependent on the type of fuel used for energy production and its sulphur content.

5 Description of a systematic methodology for biocatalytic process development

5.1 Introduction

In Chapter 3, it was emphasised that the approaches for process synthesis commonly applied to classical chemical processes are usually not suitable for biocatalytic processes. Furthermore, the current tools for development of bioprocesses (in particular for biocatalytic processes) do not incorporate the comprehensive perspective required for the process design (i.e. do not consider all the different alternatives). Current tools are often focused on only one process step and this relies heavily on experimental evaluation and pilot plant tests.

The significant potential for improvement of biocatalytic processes (in particular biocatalyst improvements by protein engineering) and the implications that these might have in the selection of process options and in the process performance, hinder the successful application of the conventional approaches. Hence, there is a current lack of a systematic approach to guide the development and design of biocatalytic processes, which can promptly evaluate the feasibility of a large number of alternative processes (typical during early development stage), while identifying and targeting improvements required for a feasible process. Additionally, a suitable methodology for the development of biocatalytic processes should also define beforehand the conditions required for a feasible process (i.e. defining the basic lines, operating conditions of the full-scale process, rather than providing all the process details) and thus, it provides guidelines for experimentation by correlation with the underlying knowledge [167].

Applying a systematic methodology in the early development stage brings many advantages: evaluating the process feasibility; forecasting the major process challenges; understanding the trade-offs when applying a given process technology option (i.e. the advantages and limitations of a given process consideration) and; identifying and ranking the most suitable strategies.

The methodology developed in this thesis uses several engineering tools, such as widely used economic and environmental evaluation (by applying a simplified version of the life cycle assessment and calculating green chemistry metrics, see Section 4.2), process modelling and bottleneck analysis. Moreover, specific tools used in bioprocesses, such as cofactor and interaction matrices analysis and operating windows (see section 4.1.1 and 4.1.2, respectively).

5.2 Methodological framework

A process development methodology should reflect the considered reaction, biocatalyst and process conditions. A methodological approach for process development should be an iterative process, as a decision taken in one step will affect the subsequent step and consequently the final outcome. Therefore, reformulations of the process are required in order to attain the most suitable process design.

The workflow and the tools included in the methodology are outlined in Figure 5.1. The whole evaluation methodology contains five steps. A detailed description of each step is given in the following sections.

5.2.1 Step 1: Define threshold values for process metrics

The integration of the objective function for cost optimisation is incorporated from the very first step of the proposed methodology. Based on the industrial sector targeted (i.e. bulk chemical, fine chemical or pharmaceutical) the engineer can estimate the annual production, the expected market value and revenue. The market value and annual production (defining which market segment the product of interest falls into) have a great influence on the scale, mass and energy balances. Further, the threshold values for the process metrics (biocatalyst yield, reaction yield, space-time yield and final product concentration) are also greatly influenced by the market segment (see Section 5.2.1.1). Additionally, when this information is not available it is necessary to use rules of thumb or analogies with other industrial processes.

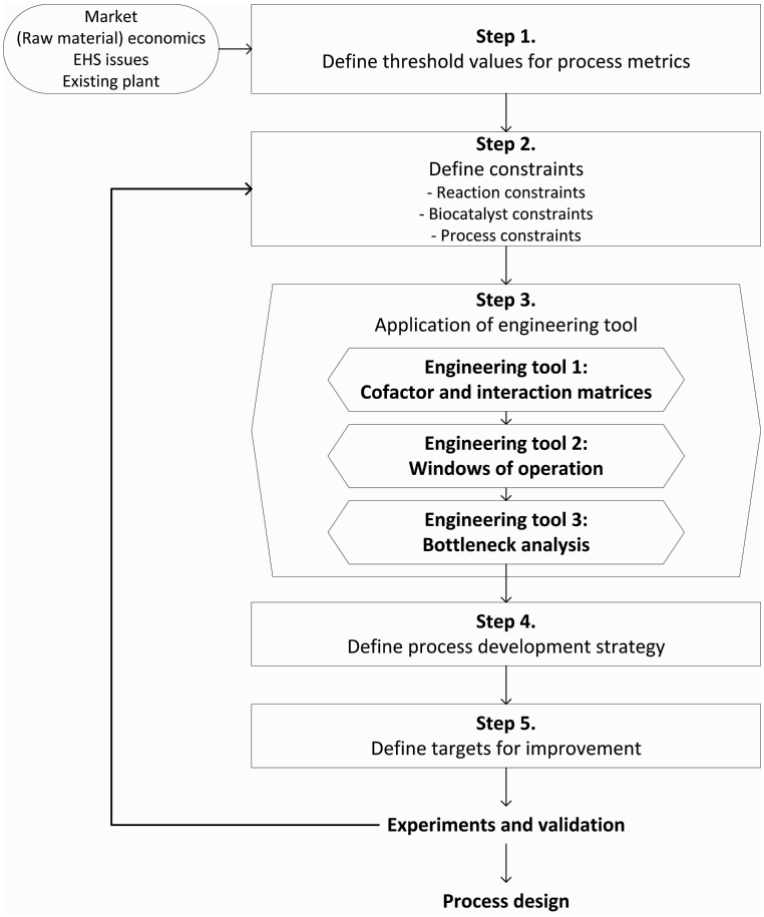


Figure 5.1. An overview of the proposed methodology for process development in biocatalysis EHS – Environmental, health and safety

5.2.1.1 Methodological constraints: guidelines for process metrics

Process scale-up is one of the fundamental steps in process development [168]. Even though this stage of the process development is often carried out only in industry, there is increasing interest in academia to develop and prove relevant scalable technologies. Process scale-up requires not only an increase in volume (of flow rates), but first and foremost an increase in the process mass metrics (product concentration, reaction yield, biocatalyst yield and space-time yield, Figure 5.2). Within the framework of the methodology suggested the establishment of the threshold values for these process metrics is of particular relevance since the success of the process development at large-scale requires a good understanding of the biocatalyst and the physicochemical environment at this scale [52]. Many constraints will only be observed under the operating conditions at large-scale (biocatalyst inhibition and solubility at operating

concentrations, mass transfer limitations, among others) thereby, influencing the selection and adoption of different process technologies. This section compiles previously suggested guidelines [14,16,24,49] for the process metrics for different industrial sectors (Table 5.1 and Table 5.2) .

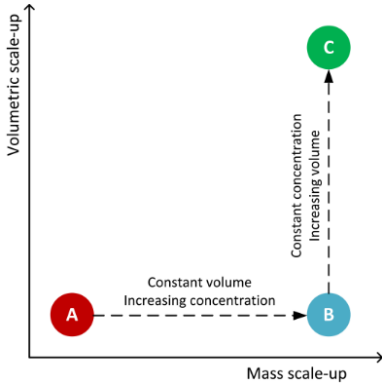


Figure 5.2. Methodology for process scale-up; **A** common starting point when developing a biocatalytic process (low volume and process mass metrics); **B** developed biocatalytic process (low volume and high process mass metrics); **C** common ending point for an implemented biocatalytic process (high volume and process mass metrics)

Catalyst production

The biocatalyst cost is dependent on the efficiency of its production. Originally, commercial enzymes were recovered from “simple” fermentation broths of their naturally occurring microorganisms, with a relatively low enzyme concentration of about 10 $g_{enzyme}/L_{fermentation}$ [169]. In the last decade, several methods have been developed enabling efficient expression and production of recombinant proteins (enhancement of secretion efficiency, prevention of inclusion bodies formation, co-expression of chaperones among others) [170]. Consequently, nowadays, industrial production of enzymes is performed in fungal (yeast) or bacterial hosts exploiting the expression of heterologous genes, applying recombinant DNA technology to maximize product purity and economy of production, resulting in higher enzyme concentrations (often above 30 g_{enzyme}/L) [169].

Enzyme concentration (g_{enzyme}/L) as a fermentation metric is of particular interest when operating with free enzymes and immobilised enzymes. When operating with free enzymes the biocatalyst should be preferentially used in its crudest form, since the purification steps significantly increase the biocatalyst cost (up to 10-fold [16]). Furthermore, these purification steps often affect the enzyme activity negatively. Nevertheless, operating with crude lysates might lead to side activities complicating the final product recovery. Enzyme concentration is ultimately related to enzyme yield, host-cell concentration (g_{CDW}/L) and total protein concentration ($g_{total\ protein}/g_{CDW}$).

When operating with "resting" microbial cells (i.e. whole-cells) where the enzyme of interest is (are) overexpressed, it is required to quantify the overexpressed protein(s) inside the cell. Hence, it is also required to set a threshold value for the protein expression level ($g_{recombinant\ protein}/g_{total\ protein}$), measuring how easy it is to express the desired enzyme(s). In recent improvements of host expression systems, recombinant enzymes reached up to 30% of the total cellular proteins under the control of an inducible promoter without the formation of inclusion bodies [171]. When targeting the overexpression of more than one enzyme in a whole-cell, it is perhaps more relevant to know how much of the total protein corresponds to the overexpressed enzyme(s) ($g_{recombinant\ protein}/g_{total\ protein}$), so that the relative activities can be balanced (see Section 7.4.2).

In recent years, the possibility of obtaining the desired biocatalyst at reasonable cost has become a reality, in particular for an increasing number of enzymes (such as lipases). However, for other enzymes (such as ω-transaminases and monooxygenases) the fermentation development still represents a challenge [172]. Table 5.1 summarises the fermentation metrics for different levels of development in the biocatalyst production. Unless stated otherwise, the metrics for "average" were used.

Table 5.1. Guidelines for fermentation metrics [16]
Note: Higher values might be achieved for secreted enzymes at optimised conditions

	Units	Low	Average	High
Cell density	g_{CDW}/L	10	50	100
Total protein concentration	$g_{total\ protein}/g_{CDW}$	0.40	0.50	0.60
Protein overexpression level	$g_{recombinant\ protein}/g_{total\ protein}$	0.10	0.20	0.30
Enzyme concentration	g_{enzyme}/L	0.40	5	18

Threshold values for process metrics

When developing a biocatalytic process, the engineer should consider a holistic design approach. Hence, the allowable costs for the different steps of a biocatalytic process (fermentation, catalyst formulation, reaction and downstream processing) should be integrated, setting the boundaries for the design exercise. By setting threshold values to the so-called 'process metrics' for each industrial sector (Table 5.2), the engineer can identify the efforts required and select between the different process alternatives available, in order to ensure a feasible process (and thus, generating feasible flowsheets). Hence, five process metrics are proposed in order to evaluate the required development efforts and to assess the contribution of each individual step towards process feasibility: biocatalyst yield (assessing the cost of the biocatalyst); reaction yield (as a measure of the raw materials cost); space-time yield (accounting for CAPEX, utilities and labour); product concentration and enantiomeric excess (assessing the downstream processing contribution).

Even enzymes for which the optimisation of the expression system is not fully realised can be applied in industrially relevant processes [14], as the catalyst cost does not mean much in itself. The relevant question to analyse is how much the cost of the catalyst (including fermentation and catalyst formulation) contributes to the final production cost (when compared with competing synthetic processes). Furthermore, the environmental impact of the biocatalyst should also be included in the overall process assessment, since the fermentation step can have a large impact on land use footprint, nutrient enrichment potential (due to growing of the carbon source crop) and acidification potential (due to energy demand) [173]. Therefore, processes where the catalyst contribution is significant lead to a less sustainable process. It is therefore necessary to set a threshold value for the efficiency of the biocatalyst (biocatalyst yield, $g_{Products}/g_{Biocatalyst}$). This metric sets targets for the biocatalyst activity and stability.

Efficient conversion of the raw material (RM) is also a requirement for the process success, since high reaction yield (% $mol_{Product}/mol_{Substrate}$) simplifies the downstream separation and leads to a more cost-effective process, lowering the economic and environmental contribution of the raw material to the final process performance. As raw materials costs are often in the range of 40% to 90% of the total processing costs [83], dependent on the industrial sector (Table 5.2), there are different threshold values for the suggested reaction yield for each market. When aiming for the production of a low value chemical (bulk chemical), there is a small gap between the cost of the raw materials and the profitable product cost. Therefore, the production costs are often dominated by the cost of the raw materials and very high reaction yields are required. Furthermore, achieving a high conversion of the oil-based raw material leads to lower cumulative energy requirements (and consequently lower global warming, acidification potentials low human and eco toxicity environmental impacts), while for bio-based raw materials a high reaction yield might lead to lower nutrient enrichment potential (due to the use of fertilisers during the growing of crops) and land use. For thermodynamically challenged reactions, the allowable cost contribution of the raw material determines the efforts required for displacing the equilibrium (excess of co-substrate, ISPR, etc.). However, for low value chemicals, the reaction thermodynamics might influence the process viability, since these types of chemicals have a small allowable cost for downstream process (for co-substrate recovery) and often cannot afford the implementation of expensive process technologies to shift the equilibrium (e.g. membrane technology or resins for ISPR).

When developing a new process, the business drivers are two-fold: economic (CAPEX and OPEX) and environmental (greenhouse gas emissions) and thus, energy requirement reductions are often targeted [80]. High space-time yield (STY or volumetric productivity, $g_{product}/L_{reactor}/h$) is required to lower the capital costs, energy requirements for stirring and heating during the reaction (lowering utility costs, emissions and environmental impacts related with energy production) and labour related costs. This

process metric assesses the speed at which the reaction occurs, the equipment occupancy time and the maximum annual production. When performing cost evaluation there is a trade-off between the STY and biocatalyst yield (Table 5.3), since operating at higher biocatalyst loading increases the STY (reducing the time that reaction takes to reach completion and consequently CAPEX, utilities and labour related costs) at the expense of increasing the biocatalyst cost contribution.

Recovery of the final product from the reaction phase is a critical step and it is often left out when developing and assessing a biocatalytic processes. Clearly the extent (and consequently the allowable cost) of the DSP is dependent on the product's subsequent use. Despite being beneficial for biocatalyst activity and stability (Table 5.3), operating at low product concentrations ($g_{Products}/L_{reactor}$) might compromise the DSP cost due to the high volume of water (or organic solvent) that it is necessary to evaporate. Hence, operating at low product concentration increases the energy requirements of the process (and consequently the energy costs and the emissions related with energy production), the solvent intensity (leading to VOCs emissions), the process water footprint and volumetric capacity of the DSP units of operation (with subsequent increase of the capital costs). Hence, unless the product is removed during the course of the reaction by ISPR, there is a trade-off between the biocatalyst activity and stability (and consequently the biocatalyst yield) and the final product concentration, that determines the process viability. Furthermore, chirality is often a requirement for many products, such as chiral drugs, agrochemicals, food additives and fragrances [174] and thus the enantiomeric excess (*ee* %, $\frac{mol_{enantiomer\ S/R}}{mol_{enantiomer\ S} + mol_{enantiomer\ R}}$) is also an important process metric, since further enantio-separations can increase the DSP costs and determine the process viability [174].

Table 5.2. Assumed threshold values for process metrics

Process Metrics	Cost	Annual Production	Biocatalyst yield	Reaction yield	Space-time Yield	Product concentration	*ee*
Units	(€/kg)	(ton/year)	$g_{product}/g_{biocatalyst}$	%	$g_{product}/L_{reactor}/h$	$g_{product}/L_{reactor}$	%
Bulk chemical	0.5 - 10	10^4 - 10^6	10^3 - 10^5	>95	>20	>300	>90%
Fine chemical	10.0 - 50	10^2 - 10^4	10^2 - 10^3	>90	>2.5	>150	>95%
Pharmaceutical chemical	>100	10 - 10^3	10 - 10^2	>90	>1	>60	>95%

Table 5.3. Trade-offs between process metrics reflecting the process economic and environmental performance

Process Metric	**Biocatalyst yield**	**Reaction yield**	**Space-time Yield**	**Product concentration**	***ee***
Units	g $_{product}$/g $_{biocatalyst}$	%	g $_{product}$/L $_{reactor}$/h	g $_{product}$/L $_{reactor}$	%
Process Step	Fermentation	Raw materials production	Equipment	Downstream process	
Economic performance	Biocatalyst allowable cost	Raw materials allowable cost	Equipment, labour and utilities allowable cost	DSP allowable cost	
Environmental performance	Growing crops (Land use and EP) Energy (ACP)	PMI, EMY and E-factor <u>Oil-based RM:</u> Energy, GWP, ACP, Toxicity ETP, HTP <u>Bio-based RM:</u> Land use and EP	Energy (GWP, ACP)	Water footprint (WI) Solvent intensity (SI): (POP) Energy (GWP, ACP)	
↑ [Substrate]	↓ [a)]	↓ [a)]	↑	↑	n.a.
↑ [Biocatalyst]	↓	n.a.	↑	n.a.	n.a.
↑ τ (residence time) or batch-time	↑	↑ [b)]	↓	↑ [b)]	n.a.

[a)] If substrate and product inhibition
[b)] If not thermodynamically limited
n.a. not affected

5.2.2 Step 2: Define constraints

Information about the process is highlighted in this step. The quality of the design is very much dependent on the quality of the information gathered at this point. Therefore, a deep search in the scientific literature and discussion with experts in the field is required in order to avoid proposing solutions that are in reality impractical. Indeed, for someone unfamiliar with the process or case study this is probably the most laborious and time-consuming step of the whole methodology. Hence, resources spent on development need to be used in the most efficient manner, collecting relevant information for process design. The information required should cover reaction, biocatalyst and process constraints.

Other constraints can also be related with the intellectual property rights, so-called 'freedom to operate' (FTO). FTO is an evaluation of whether the designed process can infringe on the patent, design or trademark rights of another entity. Hence, a FTO analysis should be always performed in order to avoid developing a process where the chosen technologies are covered by intellectual property rights. Nevertheless, it can also be possible to develop and design a process that is protected by intellectual property rights. Hence, it is required to evaluate the cost of the royalties and licensing fees and clear performance benefit by putting in place the technology.

5.2.2.1 Reaction constraints

A graphical identification of the reaction(s) in the process is required. The main, secondary, competitive, reversible and undesirable reactions should be included here, since these affect the reaction yield and consequently, the process performance. It is also important to mention if these reactions occur in cascade, parallel or in a network structure [101].

Biocatalysts, like organocatalysts, have no impact on the position of the thermodynamic equilibrium [175]. The reaction yield in thermodynamically constrained reactions (such as reversible reactions) is determined by the reaction thermodynamic constant (Keq). Hence, in order to select the appropriate process option to displace the equilibrium, it is necessary to characterise the reaction in terms of its Keq under operating conditions (pH, temperature and pressure). This information can be obtained either experimentally [30] or in Thermodynamics of Enzyme-catalysed Reactions Database (TECRDB, [176]).

Finally, it is also necessary to compile knowledge about the compound involved in the reaction, such as the physical and chemical properties. When developing a process the most relevant properties are aqueous solubility (S_{aq}), density (ρ), vapour pressure (P_{vap}), partition coefficient (LogP), boiling point (T_b), melting point (T_m), acid dissociation constant (pK_a), among others. There are several databases where such information can be obtained [178-181]).

5.2.2.2 Biocatalyst constraints

Kinetic information describing the effects of the operating conditions on the biocatalyst activity and stability is also required. This information should comprise of specific biocatalyst activity ($mol_{Product}/(g_{biocatalyst} \cdot min)$), substrate and/or product inhibition effects (if observed) and biocatalyst stability in half-life time ($t_{½,\ biocatalyst}$). This information might not always be readily available, but at least basic information on how these parameters are affected under operating conditions is required. Furthermore, for cofactor dependent enzymes, it is also necessary to report the cofactor specificity (e.g. NADP(H)- or NAD(H)-dependent enzymes), as different cofactors imply different a different cofactor stability and cost and therefore, threshold values for total turnover number (TTN) should also be attained for an economically feasible process.

5.2.2.3 Process constraints

Selected process options should be in accordance with the previously defined reaction and biocatalyst constraints. The process constraints include the boundaries of the process options selected. These can have different natures, depending on the category selected: reactor selection, ISPR technique selected, selection of the immobilisation technique, among others (Table 5.4). For later stages of the process development (see example in Chapter 9), these constraints include operating mode, reactor type (and thus,

maximum catalyst loading), product recovery technique (including selectivity and capacity), etc.

Table 5.4. Examples of possible process constraints, for three distinct groups of process options

Process option group	Examples of options	Design variable
Reactor	Stirred tank reactor (STRs)	Catalyst loading
	Packed bed reactor (PBR)	Catalyst loading Mass flow velocity
	Membrane bed reactor (MBR)	Catalyst loading Volumetric flow (depending on the membrane)
Immobilisation technique	Carrier-free	Enzyme loading Stability Cofactor leaching
	Carrier-bound	Protein loading Cofactor leaching
	Entrapment	Catalyst loading Mass transfer limitation
	Membrane entrapment	Catalyst loading Volumetric flow (depending on the membrane)
In-situ product removal (including co-product)	Adsorption Resins	Selectivity Capacity
	Membrane	Membrane Cut-off
	Bi-phasic System (organic solvent)	Selectivity Solubility of the reaction compounds
	Enzyme degradation or recycling	Specific activity
	Evaporation	Bi-phasic diagram (vapour-liquid diagram)

5.2.3 Step 3. Application of engineering tool

This thesis proposes three different engineering tools to be applied at different stages of the design: cofactor and interaction matrices, windows of operation and bottleneck analysis. As the process development progresses to later stages, the reaction, biocatalyst and process constraints become delineated with increasing detail. Therefore, different tools are required in order to narrow down the development space toward a suitable and feasible process design at different levels of process understanding.

Cofactor and interaction matrices (Table 5.5) aim at understanding the major reaction and biocatalyst constraints, in order to propose the most suitable process options. Normally, at this early stage, there are no major process constraints to take into account, as many of the process options are not yet selected. The outcome of this tool is a limited number of possible biocatalyst formulations and reactor configurations to operate the process (see Chapters 6 and 7). Later in the development procedure, other constraints

(at the previously sketched process design) are often brought to light (e.g. thermodynamic equilibrium, kinetics and stability at operating conditions, among others). To overcome these limitations, there is a number of process options that canbe put in place (also with constraints in their applicability). The section of the most suitable process options(s) can be assisted by applying windows of operation. **Windows of operation** (Table 5.5) are used as a tool to understand the benefits and limitations of applying a given combination of process technologies, ultimately providing an operating map indicating areas where different process technologies might be successfully applied. Moreover, windows of operation indicate the development areas where efforts should be focused on in order to achieve the threshold values for process metrics (see also Chapters 6 and 8). The selection of these areas can be ultimately done by applying a more detailed process evaluation (such as bottleneck analysis). **Bottleneck analysis** (Table 5.5) provides the final tuning for process optimisation, before proceeding to pilot plant tests. The outcome of this tool is a revamped design and the benefits of undergoing reaction, biocatalyst and process optimisation are evaluated (see also Chapters 6 and 9).

By applying the proposed methodology, promising strategies to improve the process performance are identified and further experimental evaluation and validation is carried out (Table 5.5 and Figure 6.1). The rational approach for each applied tool is given in the corresponding chapters (Chapter 7, 8 and 9, respectively).

Table 5.5. Tools for process development and required information

Constraint Group	Engineering tool		
	Cofactor and interaction matrices	**Windows of operation**	**Bottleneck analysis**
Reaction	Interaction between components	Interaction between components Thermodynamic equilibrium	Interaction between components Thermodynamic equilibrium Reaction conditions
Biocatalyst	Substrate, intermediates and production inhibition Cofactor requirements Activity (simple kinetics)	Substrate, intermediates and production inhibition Cofactor requirements Kinetics	Substrate, intermediates and production inhibition Catalyst formulation Cofactor requirements Kinetics
Process		IS(c)PR removal capacity	Reactor design IS(c)PR removal capacity Solubility limit

5.2.4 Step 4: Process development strategy

The outcome of the aforementioned tools is a development strategy where the most promising process configurations are identified. In this step of the methodology, a scenario analysis (what-if analysis) to the operating space is performed. Since

biocatalytic processes often need a great development effort to meet competitive operating conditions, it is necessary to revaluate the effect of modifying soft constraints (i.e. resilient parameters in the process design). Nevertheless, even when a feasible flowsheet is attained the engineer should revaluate the operating space and understand the benefits of relaxing one or more soft constraints by improving the biocatalyst or process technology(ies) associated with this constraint. Furthermore, information should be acquired at the conditions as close as possible to the defined development strategy.

5.2.5 Step 5: Define targets for improvement

Performance evaluation tools (such as economic and environmental assessment) are applied in conventional chemical process design and synthesis as an objective function to attain an optimal design. Together with kinetic and process modelling tools, the process feasibility can be proven conceptually before entering the laboratory [104,181] while experimental resources can be used to collect relevant information for decision-making and/or improve the process models. This procedure will allow the assessment of the impact of modification or improvements in the process metrics, while setting targets for the research efforts in the process technology(ies) and/or in improving the biocatalytic performance.

Besides, some knowledge about the development time of each targeted area is important. For instance, improving the biocatalyst activity at operating conditions might be a laborious task (up to 18 months [65]), while screening for a suitable IS(c)PR technology that is able to keep the product concentration below the inhibitory limit might be facilitated by high throughput experimentation in micro-reactors.

5.3 Concluding Remarks

This chapter has presented a general methodology for process development in biocatalytic processes, by applying three tools for different stages of process development (see also Chapter 6). The correct application of this methodology is highly dependent on the information known beforehand. The outcome of this methodology is: 1) a limited number of experiments for achieving the process development targets; 2) a reduced number of experiments for decision-making during the process design stage and; 3) defined operating conditions where the process achieves the threshold values for the process metrics. In the following chapters, the methodology will be illustrated with three different case studies, applying the proposed tools to the diverse industrial sectors.

Part III

Case studies

6 Introduction to the case studies

In this thesis, a systematic methodology for early stage development of biocatalytic processes is proposed, aiming for more efficient and competitive process design. The multidisciplinary nature of biocatalytic processes implies that several considerations (reaction, biocatalyst and process) need to be appraised simultaneously, making the application of a traditional systematic methodology for conventional chemical processes difficult (see Section 3.1), particularly during the early stage of development, when little detail about the process is known. In general, for biocatalytic processes, the success of the scale-up is based upon on the effort put into developing the process. Improvements in biocatalytic processes can be obtained by careful choice and optimisation of the reaction conditions, biocatalyst and/or the process technology(ies). Hence, it is crucial to identify where the research efforts should be focused in order to attain a viable process and/or ascertain when such technology has been sufficiently developed. A summary of the procedure is depicted in Figure 6.1.

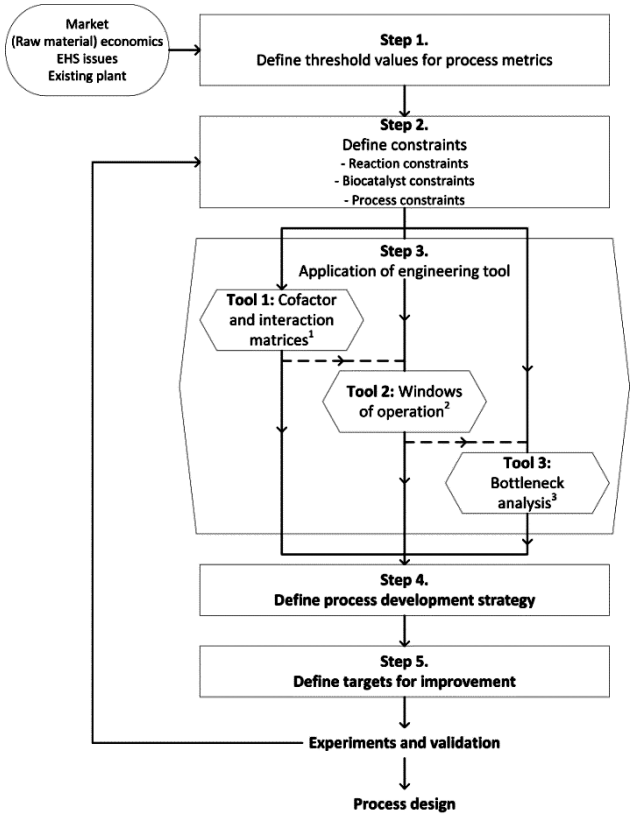


Figure 6.1. Overview of the proposed methodology for process development during early stage; Number 1, 2 and 3 refers to the case studies of the same number (Chapters 7, 8 and 9)

The synthesis and design of processes is a complex and multidisciplinary problem and several operational constraints (market competition, profitability, environmental regulations and existing facilities) can set threshold values for process metrics (e.g. biocatalyst yield, reaction yield, space-time yield and final product concentration and enantiometic excess, Step 1. in Figure 6.1).

In the following step of the proposed methodology (Step 2. in Figure 6.1), constraints of different origins (reaction, biocatalyst and process constraints) are used in order to rule out undesired or infeasible solutions. This information will be fed into the tools developed in this thesis (windows of operation, cofactor and interaction matrices and/or bottleneck analysis). The outcome of these tools is a collection of feasible development options assuming further reaction, biocatalyst and process optimisation (Step 3. in Figure 6.1). By performing a scenario analysis, targets for development can be set (Step 4. in Figure 6.1).

Three case studies have been selected in order to develop the methodology presented in this thesis. Valuable contributions were obtained from each case study, not only in developing and in demonstrating each tool required in this methodology, but also in compiling the information required for process development. The three case studies are:

- Case Study 1: Multi-enzyme system for biocatalytic production of caprolactam
- Case Study 2: Chiral amine production using ω-transaminase
- Case Study 3: Chiral aliphatic alcohol production using alcohol dehydrogenase

The first case study proposes a multi-enzyme system for synthesis of a commodity chemical, where the process success is greatly determined by the market and the competing technologies. In this case study, **cofactor and interaction matrices** are used as a development tool to guide process design (see Section 5.2.3). This tool assesses the reaction constraints, how these can affect the choice of the biocatalyst formulation and the process synthesis. In this case study different catalyst formulations (free-enzyme, whole-cell, immobilised) and consequently, different flowsheets are compared in terms of their impact on the process economic and environmental performance. The outcome of this case study is a limited number of flowsheets where the biocatalytic process presents a competing alternative to the conventional chemical route. For this purpose, guidelines for recombinant DNA technology (both in protein and in genetic engineering) are given (see Chapter 7).

The second case study explains the use of **windows of operation** (see Section 5.2.3) as a tool to assist during process synthesis of high value chemicals (chiral amines) through asymmetric synthesis using transaminase, where the speed of process development has great impact. Many of the proposed transaminase syntheses do not yet fulfil the required economic metrics necessary for process scale-up. Frequently encountered

challenges for the biocatalytic synthesis of chiral amines using ω-transaminases include potentially unfavourable thermodynamic equilibrium, low biocatalyst activity and stability, as well as substrate and product inhibition. To overcome these limitations there are several possible process solutions as well as solutions via biocatalyst engineering. Potential process solutions include operating with an excess of substrate (e.g. addition of an excess of amine donor), application of *in-situ* product removal and *in-situ* co-product removal (IScPR) or a combination of these. By applying the window of operation, a systematic approach is provided for data collection and option selection, exploring conceptual scenarios where the process would be feasible and competitive and defining an operating space, i.e. a set of operating conditions that define the process flowsheet (see Chapter 8).

In the third case study, flowsheet, mass and energy balances are established based on an already running process for the biocatalytic synthesis of long-chain chiral alcohols using a bi-enzymatic system. However, the process does not comply with the threshold process metrics and the current design is not economically viable. By applying the **bottleneck analysis** (see Section 5.2.3), comprising both economic and environmental assessment of the process, the main limitations are identified. The outcome of the methodology is a revamped design and evaluation of the benefits of undergoing reaction and process optimisation. By applying the proposed methodology, promising strategies to improve the process performance are identified and further experimental evaluation and validation is carried out. By iteration, this tool is able to identify the required process improvements in order to attain a competitive process (see Chapter 9).

In order to give a general idea of the third part of this thesis Table 6.1 summarises the three case studies including the previously available data in the scientific literature, tools applied and the steps that were performed following the described methodology.

Table 6.1. General overview of the case studies

	Case Study		
	Caprolactam (Chapter 7)	**Chiral amine (Chapter 8)**	**Chiral aliphatic alcohol (Chapter 9)**
Reaction System	9 compounds	4 compounds	4 compounds
	6 enzymes	1 enzyme	2 enzymes
	2 redox cofactors (require regeneration)	1 cofactor	1 redox cofactor (requires regeneration)
Step 1. Define threshold values for process metrics			
Market considerations	Commodity chemical	Pharmaceutical intermediate	Fine chemical
	Competing technology	Speed of development	
Economic considerations	$C_{Substrate}$ (€/kg) ≈ $C_{Product}$ (€/kg)	$C_{Substrate}$ (€/kg) <<< $C_{Product}$ (€/kg)	$C_{Substrate}$ (€/kg) < $C_{Product}$ (€/kg)
Environmental considerations	Improve EHS profile	Avoid protection and de-protection steps Improve atom economy Improve reaction efficiency	Avoid protection and de-protection steps Improve atom economy Improve reaction efficiency
Existing process	No	No	Yes
Step 2. Define process constraints			
Reaction constraints	Cofactor recycling	Thermodynamic equilibrium	Cofactor recycling
	Interaction between components (Probably) thermodynamic equilibrium		
Biocatalyst constraints	Protein overexpression	Activity Substrate and product inhibition	NADP(H)-dependent enzyme
Process constraints	One pot, multi-pot processes Batch	Co-substrate excess ISPR capacity and selectivity IScPR capacity and selectivity	Membrane reactor (biocatalyst loading) Substrate solubility limit (ionic liquid, solubiliser) Downstream process selectivity
Operating mode	Batch	Fed-batch	Continuous
Step 3. Application of engineering tool			
Tools for process development	Cofactor and interaction matrices	Windows of operation	Bottleneck analysis

Table 6.1. General overview of the case studies (Continuation)

	Case Study		
	Caprolactam (Chapter 7)	**Chiral amine (Chapter 8)**	**Chiral aliphatic alcohol (Chapter 9)**
Step 4. Define process development strategy			
Options selected	Biocatalyst formulation: Whole-cell	Technologies for shifting the thermodynamic equilibrium: simultaneous removal of product and co-product, with an excess of amine donor	Reaction conditions: partial recycling of the reaction media, glucose and cofactor concentration, enzyme:cofactor ratio and enzyme ratio
Reaction optimisation	Improve TTN_{Ala}:enzyme ratio	Enzymatic cascade for degradation or recycling of the co-product	Improve $TTN_{NADP(H)}$ (reaction engineering) Optimise glucose concentration
Biocatalyst optimisation	Overexpression level of each recombinant protein Biocatalyst formulation	Improve activity and stability Biocatalyst formulation	Not applicable Biocatalyst formulation
Process optimisation	Multi-pot strategy	Implement ISPR and IScPR	Improve conversion: increase enzyme concentration; increase residence time, increase enzyme concentration and volumetric flow rate Increase substrate solubility: modify solubiliser
Step 5. Define targets for improvement			
Reaction optimisation	Minimum TTN_{Ala}	Not applicable	$[NADP(H)]_{optimum}$; pH; Enzyme ratio; $[Glucose]_{optimum}$
Biocatalyst optimisation	Not applicable	Minimum biocatalyst activity	Not applicable
Process optimisation	Not applicable	Required ISPR and IScPR capacity and selectivity	Solubiliser capacity

7 Cofactor and interaction matrices for process development of multi-enzyme systems

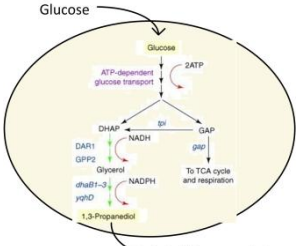
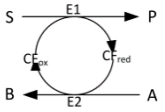
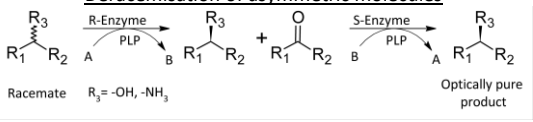
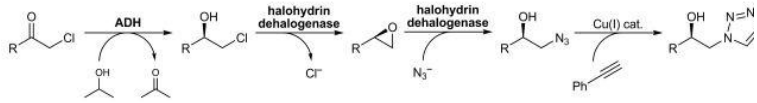
7.1 Introduction

Increasing pressure to operate greener and cheaper processes has stimulated the chemical industry into developing alternative routes to fulfil market demands without compromising product quality [182], opening new opportunities for the application of biocatalysis in organic synthesis [25]. Synthetic routes often require sequential reactions in order to obtain the desired product, implying the use of more than one enzyme or organocatalyst in consecutive reaction steps. From this perspective, the expansion of the enzyme toolbox opens new opportunities for biocatalysis [63], where by mimicking the metabolic networks inside the cell, biocatalysis can stand as a suitable and greener alternative to the conventional chemical routes. These processes are called multi-enzymatic processes. Multi-enzymatic processes are defined by using two or more enzymes catalysing a group of reactions in a defined pathway [183,184]. In these types of processes, the catalytic activity of all the enzymes involved in the synthetic route can be explored. The substrate is converted to the first intermediate, which is then converted by the following enzyme and so on, until the desired product is obtained. In principle, this procedure can simplify the downstream steps since the intermediates are consumed and thus, often eliminated from the reaction medium. Hence, several promising applications are envisaged for multi-enzymatic processes, making these a promising next-generation of biocatalytic processes [44,184,185].

To date, several studies have reported the use of multi-enzyme systems for direct fermentation of the carbon source to the product (in so-called de-novo pathways) [184,186], *in-situ* cofactor regeneration [130,187-190], deracemisation of asymmetric molecules [191-193] and cascades [45,184,194-196] (Table 7.1), among others. Nevertheless, multi-enzymatic processes are often very complex systems, with many interactions between enzymes, substrate(s), intermediates and product(s), affecting the catalyst activities [45]. Furthermore, as observed for more conventional biocatalytic processes (i.e. single enzyme), the required final product concentrations for an economically viable process are often much higher than those observed in the biocatalyst's natural environment, often compromising the performance of the biocatalyst.

Despite the increasing interest of the chemical industry in implementing multi-enzymatic processes, very few cases have been successfully scaled-up. Santacoloma [101] and Xue [25] with their co-workers have contributed with an overview of process engineering insights to the field of multi-enzyme processes. The first, by developing a

Table 7.1. Examples of *in-vivo* and *ex-vivo* multi-enzyme systems performed in single or multiple reactors

Catalyst	Reactor	Examples
Living microorganisms (*in-vivo* processes)	Single reactor	*De-novo* pathway  Adapted from [186]
Resting cells (whole-cell) or isolated enzymes (*ex-vivo* processes)	Single reactor	*In-situ* cofactor regeneration  Deracemisation of asymmetric molecules 
	Multiple reactors	Cascades  Adapted from [195]

methodological framework for process modelling and therefore contributing for the understanding of multi-enzymatic processes [101]; and the second, by highlighting the technology options and tools available for the development of multi-enzymatic processes [25]. However, to date process option selection for implementation of these processes has been performed on a case-by-case basis. Hence, a methodology that integrates the chemistry, biological and process engineering challenges is required for the full implementation of multi-enzymatic processes. In this chapter, the methodology developed in this thesis is proposed to assist in the selection of possible process options (such as biocatalyst formulation) aiming for the development of a sustainable process. For this purpose, cofactor and interaction matrices (see Section 4.1.1) were employed as a tool to identify feasible process configurations. The outcome of the proposed tool is a list of possible flowsheets where the reaction, biocatalyst and process constraints are overcome. These flowsheets are then evaluated in terms of their economic and environmental performance. In doing so, the

most favourable flowsheets and operating conditions can be identified. Consequently, the conditions for biocatalyst screening and development as well as the main process bottlenecks can be identified. Furthermore, research resources can be directed to collect relevant data for decision-making (e.g. biocatalyst specific activity, enzyme expression level and required co-substrate excess).

7.2 Process considerations for development of multi-enzymatic process and tools

The establishment of a multi-enzyme cascade process is still a major challenge in white biotechnology [25,44] as these processes are characterised by a high degree of complexity due to the combination of several catalytic activities [101], often with a mismatch of operating conditions. There are a wide range of process options that must be considered simultaneously, such as biocatalyst formulation options (whole-cell or isolated enzyme in their free or immobilised form) and reactor design (e.g. STR or PBR) (see Section 2.2.1). The correct choice of each of these process options constitutes a major challenge in multi-enzymatic processes and it has a strong influence on the process performance and viability.

7.2.1 Reaction constraints

When developing a multi-enzyme system there are three main chemistry constraints that the process engineer should have in mind during process design: 1) the cofactor balance (i.e. a cofactor consumed in one step must be regenerated in a later or parallel step of the multi-enzyme reaction), since a viable process requires an *in-situ* regeneration of the electron transporting cofactors, such as NAD(P)H; 2) interactions between the different compounds involved in the multi-enzyme system (e.g. inhibitory effects or side reactions), so the concentration of inhibitory substrate(s), intermediate(s), or product(s) are controlled by balancing the catalytic activity of the enzymes involved in the reaction system and/or by implementing process technologies (*in-situ* substrate supply, product removal, etc.) and; 3) reaction thermodynamics, since this often determines the reaction yield and the required efforts in process engineering (e.g. by applying selective IS(c)PR) to displace the reaction yield to values where the process is competitive.

In order to guide the reader through the following sections, consider the following reaction system (Figure 7.1), including 5 sequential enzyme-catalysed reactions transforming a substrate A to a product F, where components B to E are reaction intermediates and CF is an electron transfer cofactor required in the first and third

reaction steps (catalysed by E1 and E3). For simplicity, the presented general reaction scheme is similar to the multi-enzyme reaction for the synthesis of ε-caprolactam (see Section 7.4).

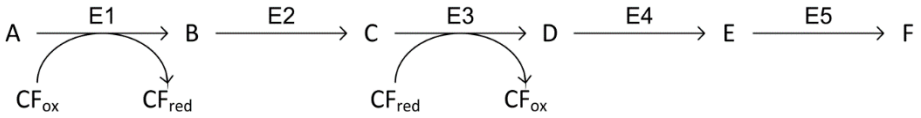


Figure 7.1. General reaction scheme for a multi-enzyme cascade process from substrate A to product F involving enzymes E1 to E5, cofactor CF and intermediates B to E.

7.2.1.1 Cofactor matrix

Many of the enzymes of interest for organic synthesis, such as those that catalyse oxidative and reductive reactions, require the presence of the so-called "free coenzymes" (e.g. NADP(H), NAD(H), and FAD(H2)) [130]. These play a role as hydrogen, oxygen or electron transporters between coexisting reactions (either sequential, parallel or network reactions). An efficient cofactor regeneration system to balance the cofactor use is indispensable for the multi-enzyme reactions involving oxidative and/or reductive reactions, since cofactors are complex, unstable [131] and quite expensive [130] and thus, for process success cannot be provided in stoichiometric amounts.

The cofactor matrix is a tool to gather information about the cofactor shuffling between its oxidized and reduced forms across the different reaction steps of a multi-enzymatic process (see Section4.1.1). In order to build a cofactor matrix, the different cofactors in their oxidized and reduced form are arranged in rows and the enzymes involved in the multi-enzyme system are arranged in columns. The matrix is then filled by defining the relationship between each cofactor form and each enzyme, i.e. substrate (S), product (P), or x when no interaction is observed. Table 7.2 shows the cofactor matrix of the cascade reaction displayed in Figure 7.1.

Table 7.2. Cofactor matrix for the general enzymatic cascade;
S substrate; **P** product; **x** no reaction

Cofactor	Enzyme E1	Enzyme E2	Enzyme E3	Enzyme E4	Enzyme E5
ox: CF_{ox}	S	x	P	x	x
red: CF_{red}	P	x	S	x	x

The process must be designed in such way that it includes the cofactor regeneration. The first and third catalytic steps (E1 and E3) require the use of cofactors as electron donor and acceptor respectively (i.e. they consumed the oxidized and the reduced

forms of the cofactor, respectively). Since the cofactor consumed by enzyme E1 is regenerated in the third step of the enzymatic cascade, the logical process design, for economic reasons, is that these two reactions exist in the same reactor.

When using whole-cells, cofactors are synthesised and regenerated as a part of cellular metabolism [130] (see also Section 7.2.2). The pyridine nucleotide cofactors NAD(H) and NADP(H) are indispensable cofactors of the cell, since they are the main electron carriers in reduction and oxidation reactions [189]. Hence, the selection of a whole-cell as the biocatalyst can offer a continuous source of cofactors, which could, in some cases, simplify the reaction structure, since no extra enzymes would be required for cofactor recycling [130]. Despite the fact that whole-cells have some reserves of cofactors, cofactor depletion can be a problem in particular when the enzyme using these cofactors is overexpressed on the host cell. Therefore, it is often necessary to co-express the enzyme(s) involved in the cofactor regenerating systems, in order to promote a continuous source of cofactor, avoiding that the cofactor supply becomes reaction rate-limiting.

7.2.1.2 Reaction thermodynamics

The design of natural pathways in living organisms typically implies that the first and the last reactions of a given pathway are thermodynamically favourable (i.e. have a large and negative value for the Gibbs free energy, ΔG_r) [140]. The reaction design in native pathways allows feasible metabolic pathways at low substrate and/or high product concentration, respectively [140], so the reaction can, theoretically, achieve full conversion.

However, for organic synthesis (i.e. non-natural pathways) in most of the cases this does not always hold true. Several enzyme catalysed reactions of interest are reversible [197] and the maximum reaction yield is thus determined by the thermodynamic equilibrium constant. The knowledge of the reaction thermodynamics is crucial during process design since it will determine which process solution(s) are feasible at industrial scale and where the process performance requirements (such as reaction yield and product concentration) are achieved [36]. The different strategies adopted for displacing the reaction equilibrium towards the product(s) side have different cost structures and different implications for the process design. In general, the easiest option for shifting the equilibrium towards a high yield of the product would be, in principle, to use an excess of co-substrate. However, the use of this strategy is restricted to limited number of cases where the equilibrium is only slightly unfavourable, since at industrial scale the substrate concentration needs to be kept at high levels and there will be an upper limitation of how large an excess of co-substrate can be used, due to the solubility of the co-substrate (see Chapter 8). Perhaps a straightforward strategy in multi-enzyme process is to couple thermodynamically favourable steps with more challenging reactions in order to drive the overall reaction

to completion. Other methods to shift the equilibrium towards the synthesis of the desired product include to selectively remove the product or co-product from the reaction phase during course of the reaction (i.e. apply ISPR [49-51,198]). Another alternative to overcome the challenging thermodynamic reaction equilibrium is to couple the challenged reaction with one side reaction (i.e. a parallel reaction) that convert the co-product into a nonreactive species or back to the original substrate [38,199].

7.2.1.3 Interaction Matrix

An interaction matrix identifies the different interactions (or relationships, e.g. inhibitory effects) that might happen between the different compounds (substrate(s), products, by-product(s), intermediate(s), cofactors, etc.) and the enzymes catalysing a specific biocatalytic step.

In order to build an interaction matrix, the different components are arranged in rows and the enzymes are arranged in columns [44]. The matrix is then filled by defining the relationship between each compound and each enzyme, i.e. substrate (S), product (P), inhibitor (I), activator (A), or non-interactive (x). Some of this information can be drawn directly from the reaction structure and complemented through experimental procedures (or reported results in scientific literature). During kinetic modelling, an interaction matrix can be used to identify an inhibitory compound indicating that a new parameter (inhibition constant) should be added to the reaction rate kinetic modelling formulation [101]. However, in the context of process design, the interaction matrix indicates the key process considerations that will affect or modify the flowsheet, mass and energy balances. Hence, this tool indicates a limited number of viable (and preliminary) process options to be later evaluated using economic and environmental analysis.

This is a particularly important evaluation tool when dealing with multi-enzyme systems, as it provides a visual understanding of the overall system. Table 7.3 shows an example of the interactions that exist between the compounds in the cascade reaction displayed in Figure 7.1.

In this example, the substrate A is inhibiting the enzyme catalysing the third reaction step (E3). The cofactor matrix determined that for the process viability the reactions catalysed by E1 and E3 need to occur in the same place. Hence, a substrate feed strategy (*in-situ* substrate supply or fed-batch) needs to be put in place, in order to maintain the concentration of substrate below the inhibitory levels.

The intermediate E, produced in the reaction catalysed by E4, is also an inhibitory compound to this enzyme. Thus, it is required to keep the concentration of this intermediate at a low level. This can be attained by levelling (i.e. balancing) the enzyme activity of E4 and E5, in such way that the rate of production of the

intermediate E (r_{E4}) is balanced with the rate of consumption of this intermediate (r_{E5}), keeping the intermediate E concentration below E4 inhibitory level.

Table 7.3. Interaction matrix for the general enzymatic cascade; **S** substrate; **P** product; **I** inhibitor; **A** activator; **x** non-interacting compound (no reaction)

	Enzymes				
Compounds	**E1**	**E2**	**E3**	**E4**	**E5**
Compound A	S	x	I	x	x
Compound B	P	S	x	x	x
Compound C	x	P	S	x	x
Compound D	x	x	P	S	x
Compound E	x	x	x	P I	S
Compound F	x	x	x	x	P I
	Cofactors				
CF_{ox}	S	x	P	x	x
CF_{red}	P	x	S	x	x

Furthermore, the interaction matrix also shows that product inhibition in E5 is observed. ISPR techniques can be a suitable strategy to reduce the product (F) concentration below its inhibitory level without compromising the process performance metrics (namely the final product concentration).

In summary, the analysis of the reaction constraints tell the process designer that the first three reactions (catalysed by E1, E2 and E3) need to take place in the same reactor, independently of the type of catalyst formulation adopted. Moreover, a strategy for product recovery in order to operate at high specific biocatalyst activity is also required.

7.2.2 Biocatalyst options

7.2.2.1 Whole-cell and isolated enzyme options

Multi-enzymatic processes can be carried out in intracellular (i.e. *in-vivo*) processes, where the enzymatic reactions are carried out inside the cell (resting or growing), or extracellular (i.e. *ex-vivo*) processes, where the reactions are taking place outside the cell using free-enzymes (either isolated, in crude extract, or immobilised) [44]. The choice of the biocatalyst can largely determine the design of biocatalytic processes [200].

Despite the several advantages of operating with *in-vivo* (such as the natural recycling systems of the cofactors and lower production costs [201]), the use of cells (growing or resting) in organic synthesis is not a simple task, as it requires the manipulation of metabolic pathways and gene regulation in order to control the sequential enzymatic reactions inside the cell (for resting whole-cells). While for growing cells (i.e. fermentation processes), the control of the metabolic flux is even more challenging. Aside from shifting the carbon flow to the desired synthetic pathway, the cells must also keep their metabolic functions for growth, with consequent production of metabolites (leading to higher DSP efforts) [186]. Moreover, the transport of the substrate over the cell membrane is often limited [202]. In addition, the regulation of the catalytic activity of the different enzymes involved in the enzyme cascade is dependent on the concentration of each individual enzyme inside the cell. In other words, when a tight regulation of the concentration of each enzyme involved in the cascade is not achieved, the reaction rate for the target reaction is as fast as the speed of the slowest enzyme. Hence, it is necessary to control the protein overexpression level in the host. For resting cells (whole-cell biocatalysts) maintenance, replication and function of the recombinant DNA in a host organism requires energy [203,204]. The maintenance energy of a recombinant organism increases due to promoter induction causing high-level expression of the target gene cloned in the host bacteria [205].

Many of the aforementioned drawbacks encountered for whole-cell processes can be overcome by putting in place a multi-enzymatic system of isolated enzymes or cell-free systems (generally based on crude extracts [206]). For isolated enzymes, when the process requires cofactor regeneration, it is necessary to dose the cofactor, which can drastically raise the production cost, even when a cofactor recycling system is put in place, as these compounds are unstable and thus hinder their reuse and recyclability [187]. Moreover, there are a limited number of enzymes and enzyme functions commercially available [207,208]. Further, the cost of isolated enzymes and their operational stability at industrially relevant conditions is still a major challenge for the successful implementation of biocatalytic processes. However, this later issue can be potentially overcome by enzyme immobilisation (see Section 7.2.2.2).

Recent studies of cell-free systems suggested that many of the drawbacks of operating with whole-cells or isolated enzymes have been overcome, expanding the capabilities of natural biological systems [206,209]. In this approach, inspired by the *in-vivo* options (fermentation and resting whole-cells), the complex biological system is released by cell lysis [209]. The main advantage in relation to *in-vivo* processes is that there is no mass transfer barrier (such as cell membrane) allowing a direct access of the substrate to the enzymes [209]. Further, the multi-enzyme components (natural and unnatural) can be added or synthesised and can be maintained at precise ratios [206]. Thereby, the chemical environment can be controlled and sampled [206]. The

activity of the different enzymes involved in the cascade reaction might be controlled by inducing overexpression of the desired pathway during cell growth, whilst side or competing pathways can be knocked-out by the action of cell-native proteases [209]. The application of cell-free biology in organic synthesis also allows the combination of pathways and enzymes from different hosts [209]. However, to put in place this technology, a comprehensive understanding of the regulatory mechanisms for the metabolic networks is required.

The characteristics related to the different types of catalyst that can be used in a multi-enzymatic process are listed in Table 7.4.

Table 7.4. Comparison of intra and extracellular catalyst for multi-enzymatic process

	Intracellular (*in-vivo*) catalysis		**Extracellular (*ex-vivo*) catalysis**	
	Growing whole-cell (Fermentation)	**Resting whole-cell**	**Isolated enzyme**	**Cell-free system**
Complexity	Very complex	Complex	Less complex	Complex
Process control (T, pH)	Online monitoring	Possible	Possible	Possible
Process control (substrate, intermediates and products concentrations)	Unlikely	Difficult	Easy	Possible
Metabolic control (regulation of enzyme activity)	Difficult	Possible	Easy	Possible
Process robustness (reaction reproducibility)	Variable	Reproducible	Reproducible	Reproducible
Reaction yield (substrate utilisation for product formation)	Low	High	High	High
Toxic intermediates or substrates	Problematic	Relevant	Less relevant	Less relevant
Formation of by-product	Very possible	Possible	Less possible	Possible
Cofactor regeneration	Easy	Easy	Possible	Possible
Stability	Low	High (if immobilised)	High (if immobilised)	Depending on the cell state when harvested
Mass transfer of substrates and products	Limiting	Limiting	Not relevant	Not relevant
Biocatalyst cost	Low	Low	High	Low
Downstream cost	High	High	Low (depends on inhibitory effects)	High
Research required for development	High	High	Lower	High

7.2.2.2 Soluble and immobilised options

Many of the reported multi-enzymatic reactions are carried out at laboratory scale using soluble enzymes. Nevertheless, this approach is perfectly adequate to achieve a good understanding of the reaction mechanism and the interaction between the different compounds in the reaction media. The use of soluble enzymes when scaling-up constitutes a problem for the economic viability of the process, due to the difficulty in separating the enzymes at the end of the reaction and the low operational stability of these. Since the cost of the biocatalyst can constitute a considerable portion of the

process operational costs [16], it is necessary to reuse the enzymes, in order to attain an economically competitive biocatalyst allowable cost.

The separation of soluble enzymes from solution requires ultrafiltration membranes [209]. Nevertheless, the use of these membranes implies an increase in utilities costs (due to the high energy requirements) and the operating costs (due to membrane fouling and thus reduction in lifetime). However, there are a few examples where the use of this technology has proven to be an economically competitive process option [209,210]. Hence, a comparative evaluation of the applicability of membrane technology is required.

Enzyme immobilisation can constitute a suitable alternative, since this strategy is able to improve the enzyme stability, enabling the use of alternative reactors, simplifying the downstream process and preventing carry-through of protein activity to the subsequent operating unit [57,211]. Enzymes can be immobilised on different types of supports, such as polymeric matrices (including resins, cellulose or hydrogel) [55], magnetic particles [212], encapsulation (e.g. polyethylenimine microspheres) [213], carrier materials (e.g. dendrispheres) [57], or through methods of enzyme self-immobilisation (e.g. CLEC, CLEA, Spherezyme) [57]. For many of the aforementioned enzyme immobilisation techniques, co-immobilisation of two or more enzymes (co-localization) has been reported [25,57]. Impressively, some of these studies have successfully co-immobilised systems requiring cofactor regeneration [214,215]. However, it has been reported that cofactor leaching might occur [216]. The immobilisation procedure is still a rather costly process and the immobilisation of the enzyme and cofactor may only be cost effective if there is a significant increase of their operational stability. In addition, during the immobilisation process, the enzyme may lose its activity, some of the optimal operational conditions might be affected, the apparent Michaelis constant might change [217] (due to the partition effect inside and outside the carrier [217]) and new mass transfer limitations might arise, negatively affecting the reaction rate.

For the aforementioned reasons the use of immobilised catalyst(s) in multi-enzyme systems might not be so simple, especially when cofactors are involved in the reaction (such as the reactions catalysed by E1 and E3 at the cascade reaction displayed in Figure 7.1).

7.2.3 Process technology options

The process considerations are essential to formulate mass and energy balances and to achieve a feasible process. Therefore, these should be consistent with the reaction and biocatalyst considerations listed above. Moreover, it is necessary to document with a

certain level of detail and accuracy the process considerations envisaged here, as they can be submitted to a scenario analysis during process debottlenecking. Process options include multi-step or one-pot processes, reactor design, operating mode, process control, process intensification options, among others. Considerations about operating mode, process control and process intensification options have been summarised in Chapter 2, and more detailed information can be found in the scientific literature (e.g. [25,101]).

7.2.3.1 Multi-step and one-pot processes

Multi-enzymatic processes can be operated either in a single reactor or in a battery of reactors. In theory, for an *n*-step multi-enzymatic reaction, the number of possible processes is 2^{n-1}. For the 5-step cascade (Figure 7.1), there are 16 possible flowsheets. However, by putting in place a cofactor matrix and interaction matrix (summarising reaction constraints) and the information gathered about the reaction thermodynamics, the number of flowsheets is narrowed down to 4 possibilities (Figure 7.2).

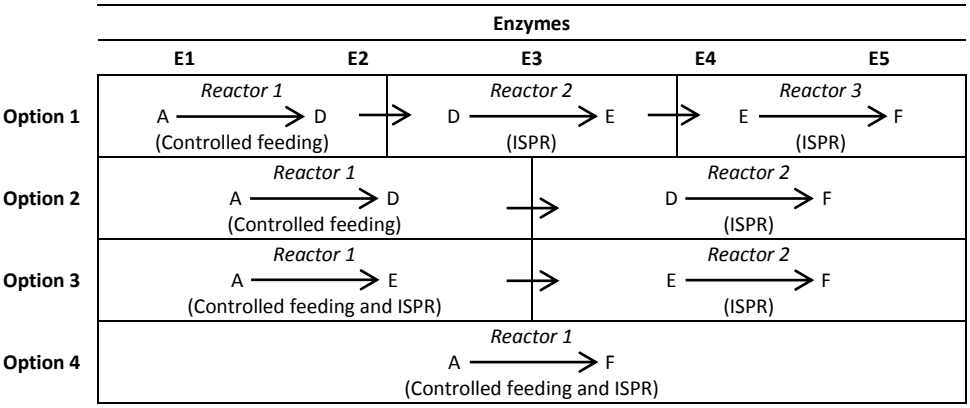


Figure 7.2. Scheme of the possible flowsheets and required process technologies to operate a 5-step cascade for production of product F (see Figure 7.1)

Multi-enzymatic processes often require combinations of enzymes from different hosts [25]. In such cases, the enzymes involved in the cascade may not share the same optimal operational conditions (pH, temperature, etc.) and the biocatalysts' reaction rate may be difficult to balance at the process conditions (substrate, product and intermediate concentrations, among others). In this case, it might be preferable to compartmentalise the different catalysts in different vessels, with a consequent increase of the capital cost (Option 1 to 3 in Figure 7.2).

When the conditions of each individual enzyme catalysed reaction are well matched, the process can be carried out by dosing the multi-enzyme system into a single reactor, in a so-called 'one-pot process' (Option 4 in Figure 7.2). In one-pot processes, the

intermediates can be consumed immediately by the subsequent enzyme, leading to low concentrations of intermediate and decreasing its inhibitory effects on the enzymes involved in the cascade. Additionally, in principle, operating in an one-pot reactor can decrease the capital costs of the process, while eliminating the separation and purification steps required to remove intermediates, leading to lower downstream processing costs [44]. Moreover, operating in a one-pot reactor allows the regulation of the catalytic activity of individual enzymes by changing the operation conditions, such as pH or temperature.

7.2.3.2 Reactor design

Great process improvement can be achieved by applying a suitable reactor design and therefore, other reactor types should be considered when proposing a new process design [218]. To date, mainly stirred tank reactors (STR, [1,219]) are used in multi-step biocatalytic reactions. However membrane reactors [215] and packed bed reactors (PBR, [220]) have also been reported. The selection of the biocatalyst can also have major implications in the reactor selection, enabling some options (e.g. packed bed reactor for immobilised enzymes), while process requirements such as oxygen supply might enable the use of other reactors, such as bubble column reactor. Process considerations for selection of the reactor design were addressed in Chapter 2 (Section 2.2.1.4).

7.3 Evaluation tools: economic and environmental assessment

Economic and environmental assessment can be used as an evaluation and decision-making tool to quantitatively estimate the expected cost structure and environmental impact of the process, respectively. Despite the uncertainty inherent to this type of evaluation at early development stage, performing such analysis can be of benefit in ranking and selecting the most promising option(s) to be further explored and to indicate the conditions for further development where research efforts should be focused. Details about the routine for simplified economic assessment of the process and environmental evaluation are given in Section 4.2.

7.4 Case study 1: Multi-enzyme system for biocatalytic production of ε-caprolactam

Polycaprolactam (6-aminohexanoic acid homopolymer) is a versatile chemical material used in several different applications (e.g. coating agents, textile fibres, engineering plastics, electronic components and food packaging) [221].

The demand and production of ε-caprolactam (the current starting material for the polymerisation to polycaprolactam) is dependent on the demand for polycaprolactam and the monomer world production is expected to be 500 000 tons/year by 2015[1] [221].

The conventional chemical synthesis by Beckmann rearrangement is a well-established industrial process for the production of ε-caprolactam worldwide [221] (Figure 7.3). However, the industrial production route results in a large quantities of (low value) ammonium sulphate [222] (4.4 kg of $(NH_4)_2SO_4$ produced per kg of ε-caprolactam [223]). Furthermore, this synthetic route takes place in very acid (pH 2 [224]) conditions and the cyclohexanone (i.e. the starting material) is toxic.

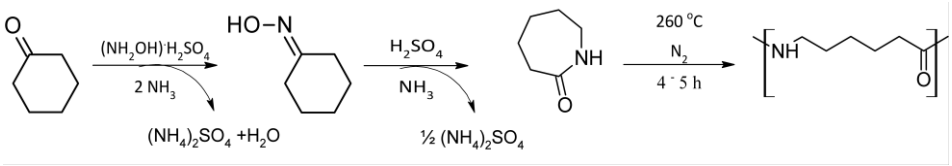


Figure 7.3. Reaction scheme for chemical synthesis of polycaprolactam

There are other alternative synthetic routes to the synthesis of ε-caprolactam using chemocatalysis [221,224,225]. However, most of these reactions have cyclohexanone as the starting material. Further, these synthetic routes still need to be improved since they produce a large amount of co-products, leading to a less effective process [221].

Since polycaprolactam is easily recyclable if a greener synthetic route could be developed, then polycaprolactam would be a potentially environmentally friendly product. Evonik Industries AG in collaboration with University of Graz has proposed a synthetic route from cyclohexanol to the polycaprolactam-monomer (6-aminohexanoic acid, 6AHA) (Jan Pfeffer, personal communication, 2012). The use of a biocatalytic route might overcome such limitations observed for the chemical synthetic routes.

This biocatalytic route provides an opportunity to improve the EHS process profile since the process runs under milder conditions when compared with the conventional chemical routes, which, ideally, will allow the process to be carried out with much reduced energy and under acid- and solvent- free conditions. Furthermore, the proposed route (Figure 7.4) starts from cyclohexanol (**1**), which can be obtained from a renewable feedstock (by pyrolysis and hydrodeoxygenation of lignin) [226] and constitutes a safer starting material than cyclohexanone. Aside from a better environmental profile, the bioprocess should also be economically competitive.

However, for the synthesis of polycaprolactam, it is necessary to convert 6AHA into ε-caprolactam. At BASF, this reaction is taking place in the liquid phase without any

[1] www.icis.com

catalyst, just containing a mixture of an organic solvent and water at temperatures of around 250°C and pressures of around 10 MPa [227,228]. Hence, when comparing the conventional chemical route and the biocatalytic route it is necessary to include the cyclisation of 6AHA into ε-caprolactam (Figure 7.4).

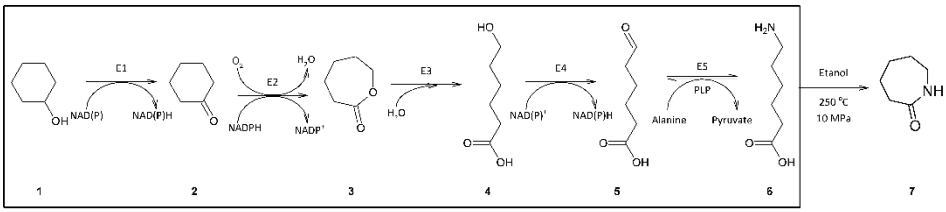


Figure 7.4. General reaction scheme for production of ε-caprolactam; Enzymes involved in the cascade: E1 alcohol dehydrogenase (ADH, EC 1.1.1.X); E2 cyclohexanone monooxygenase (CHMO, EC 1.14.13.22); E3 Lipase (EC 3.1.1.X); E4 alcohol dehydrogenase (ADH, EC 1.1.1.X); E5 ω-transaminase (TAm, EC 2.6.1.X). Compounds in the enzymatic cascade: 1) cyclohexanol 2) cyclohexanone 3) 6-hexanolactone 4) 6-hydroxyhexanoic acid 5) 6-oxohexanoic acid 6) 6AHA 7) ε-caprolactam

7.4.1 Reaction constraints

The methodology proposed in the previous section will be applied to guide process development of the biocatalytic synthesis of ε-caprolactam using the cascade proposed in Figure 7.4.

7.4.1.1 Cofactor matrix

The reaction structure is useful to fill in the cofactor matrix. Further, it is also necessary to know which type of cofactor can be accepted for each specific enzyme. Table 7.5 shows the cofactor matrix of the cascade reaction in Figure 7.4. The enzyme involved in the second catalytic step of the cascade reaction for synthesis of caprolactam is the cyclohexanone monooxygenase (CHMO). CHMO is a Baeyer-Villiger monooxygenases (BVMOs) belonging to Type I [229] requiring NADPH as source for electrons [229]. Since CHMO is strictly NADPH-dependent, the use of this enzyme requires an efficient coenzyme recycling system. This cofactor recycling can be carried out by an alcohol dehydrogenase (ADH) belonging to the group of NADP(H)-dependent ADH (EC 1.1.1.2, such as ADH from *Lactobacillus brevis*, see Chapter 9). For simplicity of the reaction design, this enzyme should catalyse an oxidation reaction within the cascade (i.e. E1 or E4 in Figure 7.4).

Table 7.5. Cofactor matrix for the enzymatic cascade for production of caprolactam; **S** substrate; **P** product; **x** no reaction

Cofactor	E1 ADH	E2 CHMO	E3 Lipase	E4 ADH	E5 Tam
ox: NAD^+	S	x	x	S	x
red: NADH	P	x	x	P	x
ox: $NADP^+$	S	P	x	S	x
red: NADPH	P	S	x	P	x

Further, on the fourth cascade step a second oxidation reaction is taking place, requiring a cofactor system for regeneration of the redox power. Since this reaction can be carried out using either NAD(H)-or NADP(H)-dependent ADH, and given that no other chemistry constraints are found, the cofactor selection must be done based on the process economics. Hence, an NAD-dependent alcohol dehydrogenase was chosen due to the cost of the nicotinamide adenine dinucleotide phosphate (NADP(H)) is about 5-fold more expensive than NAD(H) [130]). In general, NAD(H) also has an increased stability at operating conditions when compared with NADP(H) [230].

7.4.1.2 Thermodynamics of the transaminase-catalysed reaction

The second reaction consideration is related to the thermodynamic equilibrium of the ω-transaminase-catalysed reaction. ω-Transaminase is a suitable catalyst for producing chiral amines by direct asymmetric synthesis from ketones. Transaminases catalyse the transfer of an amine ($-NH_2$) group from an amine donor (e.g. alanine and propan-2-amine) to a ketone acceptor, yielding an amine and a co-product ketone (pyruvate or acetone, respectively). This reaction requires the cofactor pyridoxal phosphate (PLP) to act as a shuttle to transfer the amine group [231]. Despite the many appealing features of the ω-transaminase-catalysed reactions, thermodynamic equilibrium is a major challenge for the success of the process implementation [36].

The thermodynamic limitations encountered in transaminase-catalysed reactions can be addressed in several different ways, such as addition of excess of amine donor or application of ISPR.

Co-substrate (amine donor) excess

One of the easiest options for shifting the reaction thermodynamic equilibrium towards a high product yield is to operate with an excess of the amine donor [36]. However, this strategy is quite limited for two main reasons. First, when the production of a commodity chemical (such as ε-caprolactam) is desired, the final product concentration required is quite high (often >300 g/L). At this concentration, there will be an upper limitation of how large the excess of amine donor can be until its aqueous solubility limit is reached (for instance, aqueous solubility of alanine in 1.9 M and aqueous solubility of propan-2-amine in 16.9 M, see also Section 8.2.2.2). Secondly, the narrow margin between substrate purchasing cost and product selling

price, often observed in commodities and bulk chemical production processes (as the case with ε-caprolactam), implies a very effective amine donor recovery which can raise the overall production cost.

Additionally, for ω-transaminases the choice of the amine donor can also be discussed, since this can strongly affect the reaction equilibrium position [30]. An ideal process would use ammonium as the amine donor [232], since this is a cheap amine donor [38]. However, few reports can be found for such a reductive amination of ketones [233]. To date two main amine donors are preferred in transaminase-catalysed reactions: alanine (Ala) and propan-2-amine (IPA) [234]. The choice of the amine donor is not trivial and depends on the strategies adopted to displace the reaction equilibrium (e.g. co-product removal via conversion to nonreactive specie or recycling back to the original amine donor).

***In-situ* product or co-product removal**

A second strategy to shift the equilibrium position towards a high reaction yield is to remove the product or co-product from the medium during the reaction itself (IS(c)PR). The most suitable strategy for ISPR is dependent on the properties of the product and the other components in the reaction mixture. Regarding the product removal (IS(c)PR), this is an appealing process technology, which ideally enables the displacement of the reaction equilibrium while reducing product inhibition. However, there are some limitations for the application of IS(c)PR. A common limiting factor is related to the selectivity of the separation. Non-selective ISPR can reduce the product concentration to levels lower than its inhibitory concentration, but cannot displace the thermodynamic equilibrium. The separation selectivity becomes more challenging in multi-enzymatic systems, where the number of compounds is greater. Furthermore, the application of IS(c)PR does not solve the problem related with the regeneration (or recycling) of the amine donor and therefore it might hamper an otherwise economically feasible process if an expensive amine donor needs to be provided in (at least) stoichiometric concentrations.

Therefore, a suitable strategy is to combine the ω-transamination reaction with other enzymatic steps that convert the co-product into a nonreactive species or back to the original amine donor [36]. There is a wide range of suitable enzyme cascades that have been proven capable of converting the co-product [232,234-237]. Of particular interest is the *in-situ* recycling of the co-product back to the original amine donor. This strategy can be applied when alanine is used as amine donor, employing an amino acid dehydrogenase and ammonia [234]. Hence, when choosing this strategy, the ultimate amine donor is ammonia, which makes the process potentially more economically attractive [38]. In this system pyruvate (i.e. co-product) can then be recycled back to alanine using an alanine dehydrogenase (AlaDH, EC 1.4.1.1), which consumes ammonia and NADH [238,239]. By putting in place this strategy not only can the thermodynamic

equilibrium of the transaminase-catalysed reaction be shifted, but also the cofactor balance of the overall cascade can be closed (Figure 7.5). Table 7.6 shows the revised cofactor matrix of the cascade reaction in Figure 7.5.

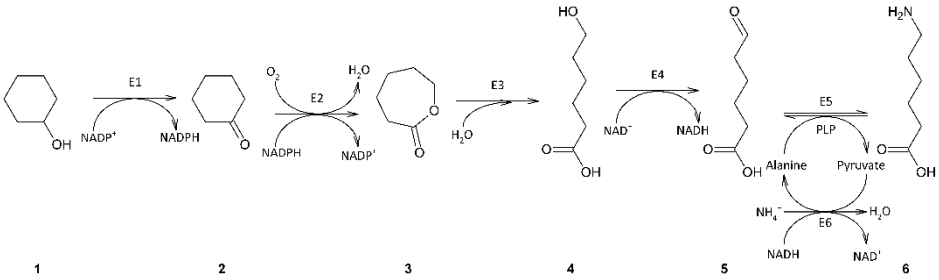


Figure 7.5. Revised enzymatic cascade for production of 6AHA; Enzymes involved in the cascade: E1 $NADP^+$-dependent alcohol dehydrogenase (ADH, EC 1.1.1.2); E2 cyclohexanone monooxygenases (CHMO, EC 1.14.13.22); E3 lipase (EC 3.1.1.X); E4 NAD^+-dependent alcohol dehydrogenase (ADH, EC 1.1.1.X); E5 transaminase (TAm, EC 2.6.1.X); E6 alanine dehydrogenase (AlaDH, EC 1.4.1.5). 1) cyclohexanol 2) cyclohexanone 3) 6-hexanolactone 4) 6-hydroxyhexanoic acid 5) 6-oxohexanoic acid 6) 6AHA

Table 7.6. Cofactor matrix for the revised enzymatic cascade for production of caprolactam; **S** substrate; **P** product; **x** no reaction

Cofactor	E1 ADH/NADP(H)	E2 CHMO	E3 Lipase	E4 ADH/NAD(H)	E5 Tam	E6 AlaDH
ox: NAD^+	x	x	x	S	x	P
red: NADH	x	x	x	P	x	S
ox: $NADP^+$	S	P	x	x	x	x
red: NADPH	P	S	x	x	x	x

From Figure 7.5 and Table 7.6, it can be concluded that, for a feasible process and independent of the catalyst formulation chosen the first two reaction steps need to be carried out together in the same vessel (one-pot) and the last three catalyst steps also need to be carried out in a one-pot reactor.

7.4.1.3 Interaction Matrix

Table 7.7 shows the interactions that exist between compounds involved in the biocatalytic synthesis of 6AHA.

Table 7.7. Interaction matrix of the enzymatic cascade for production of caprolactam; **S** substrate; **P** product; **I** inhibitor; **A** activator; **x** no reaction, no effect; [c] competing reaction (Jan Pfeffer, personal communication 2011)

Compounds	Enzymes					
	E1 ADH/NADP(H)	**E2 CHMO**	**E3 Lipase**	**E4 ADH/NAD(H)**	**E5 TAm**	**E6 AlaDH**
1) cyclohexanol	S	x	x	x	x	x
2) cyclohexanone	P	S I	x	x	x	x
3) 6-hexanolactone	x	P	S	x	x	x
4) 6-hydroxyhexanoic acid	x	x	P	S	x	x
5) 6-oxohexanoic acid	x	x	x	P, S[c]	S	x
6) 6AHA	x	x	x	x	P	x
7) ε-caprolactam	x	x	x	I	x	x
O_2	x	S	x	x, S[c]	x	x
H_2O	x	P	x	x	x	P
Alanine	x	x	x	x	S	P
Pyruvate	x	x	x	x	P	S
NH_4^+ (as NH_4Cl)	x	x	x	x	x	S
Cofactors						
NAD^+	x	x	x	S, S[c]	x	P
NADH	x	x	x	P, P[c]	x	S
$NADP^+$	S	P	x	x, S[c]	x	P
NADH	P	S	x	x, P[c]	x	S
PLP	x	x	x	x	A	x
By-products						
Adipic acid (Figure 7.6)	x	x	x	P[c] I	I	x

Table 7.7 cyclohexanone (2) has an inhibitory effect on Baeyer-Villiger monooxygenase (cyclohexanone monooxygenase, CHMO). Hence, this compound must be kept at very low concentrations. This can be achieved by balancing the relative activity of the first and the second enzyme of the multi-enzyme system (ADH and CHMO, respectively). Further, it is clear that the cyclisation of the 6AHA to ε-caprolactam cannot take place in the reaction medium, since apart from the extreme reaction conditions (250 °C which would *per se* hinder the biocatalytic synthesis) this last compound was observed to be inhibitory to the enzyme activity of the second alcohol dehydrogenase (E4). Finally, the synthesis of an undesired by-product (adipic acid) was observed (Figure 7.6). One of the possible reasons for the synthesis of adipic acid is the promiscuity of the alcohol dehydrogenase, which can transform the 6-oxohexanoic acid into adipic acid. Hence, it is necessary to search for a selective enzyme, while operating under an

inert atmosphere [240,241]. This last constraint implies that the second enzyme catalysed step, which requires oxygen, must be carried out in a separate vessel from the last three enzymatic steps. Further, in order to avoid the synthesis of adipic acid, the ADH/TAm can be adjusted in order that 6-oxohexanoic acid is promptly consumed in the transaminase-catalysed reaction.

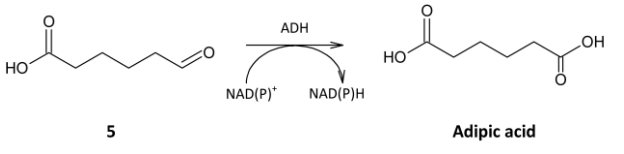


Figure 7.6. Competing side-reaction to the design ε-caprolactam, with conversion of 5) 6-oxohexanoic acid in to adipic acid [240]

7.4.2 Biocatalyst considerations

7.4.2.1 Whole-cell and isolated enzyme options

In the biocatalytic route for production of 6AHA, the choice of the biocatalyst formulation might determine in part the process design and feasibility. In general, processes running with isolated enzymes require an investment upstream of the reaction (for enzyme purification and formulation), while operating with whole-cells implies higher downstream costs [200]. Operating with whole-cell generally implies working at lower concentration and likewise side reactions may occur. Table 7.8 summarises the advantages and disadvantages of isolated enzyme or whole-cell catalyst form with respect to the limitations of 6AHA biocatalytic production.

Table 7.8. Advantages and disadvantages of whole-cell and isolated enzymes for production of 6AHA

Limitation	Enzymes affected	Whole-cell	Isolated enzyme
Enzyme activity and stability	E1 & E2 E3 E4, E5 & E6	+ Engineered cells can have improved stability at industrial conditions [200]; - Requires great effort to manipulate the expression level of each protein inside the cell.	+ Easy control of the enzyme activity; - Enzyme stability if not immobilised.
Cofactor	E1 & E2 E4, E5 & E6	+ Use of the cell-native cofactors; - Carbon source to enhance cofactor recycle.	+ Reaction control; - Requires to dose expensive unstable cofactors.
O_2 supply	E1 & E2	+ Enzymes are shielded inside the cell; - O_2 supply for reaction and maintenance [242].	+ Stoichiometric amount; - Enzyme stability due to oxidative damage of the interfacial effects [243]

When operating with whole-cells (resting cells) the host growth rate is insignificant and close to zero [140]. However, at this cell stage it is expected that there is consumption of the carbon source for cell maintenance (for ATP generation) [140], even if the

observed uptake rate is low. There are two main ATP-generating processes: respiration and fermentation [244]. Respiration is the process in which the electrons released by the electron carriers (such as NADH and NADPH) are transferred sequentially through the electron transport chain (a series of membrane-bound protein carriers) reducing a terminal electron acceptor, such as oxygen in aerobic hosts [244]. In the specific case of the biocatalytic cascade for production of 6AHA, in the cyclohexanone monooxygenase (CHMO, E2) there is a trade-off between the cell density and space-time yield due to oxygen demand, as the oxygen-transfer rate must cover both the CHMO activity and the endogenous respiration [242]. If at the operating conditions the oxygen transfer rate becomes limited, the process might be operated by applying isolated enzymes. In the absence of oxygen (such as in the case of the last three reactions of the enzymatic cascade), the host can undergo ATP-generating processes by fermentation, in which the final electron acceptor is an organic compound [244]. However, in this situation operating the process using whole-cells implies that there is synthesis of by-products, with consequent increase of the downstream process cost, which could be avoided by operating with isolated enzymes.

Perhaps the main biocatalyst challenge in multi-enzyme reactions is to regulate the activity of the enzymes involved in the cascade reaction. Operating with purified enzymes this task becomes easier to accomplish by changing the concentration of the enzymes and/or varying the reaction conditions [111]. Nevertheless, recent advances in system biology and cell engineering have led to an increased understanding of the cellular metabolic networks and cell physiology, supporting the identification of genetic targets for improved gene expression regulation, membrane stability, etc., enabling the implementation of whole-cell biocatalysts [245]. Likewise, it is important that whole-cell biocatalysts can take in substrates effectively. For many of the industrially relevant substrates there is no active transport system and diffusion across the cell membrane determines the rate of reaction [202]. Yet, permeabilisation techniques may prove useful in overcoming mass transfer limitations through the cell membrane [246].

The first step is to collect information about the enzyme activity catalysing each reaction in the cascade in order to determine the correct dosing/overexpression level of each enzyme. Ideally, the specific activities should be measured for the expected operating conditions (i.e. in the presence of the other components of the cascade reaction and according to the interaction matrix defined above). However, at an early development stage, this information might not be easily available. Furthermore, since biocatalyst specific activity is often one of the parameters subjected to development before full-scale implementation [65,247], the process engineer can use the published information in order to get a preliminary idea of the required enzyme ratio in the reactor(s). Table 7.9 compiles the published specific activities for the reactions involved in the enzymatic cascade for production of 6AHA.

Table 7.9. Specific enzyme activity for the reactions in the multi-enzyme production of 6AHA

Enzyme		Specific Activity [$mmol_{Product}/min/g_{Enzyme}$]	Reference
E1	ADH/NADP(H)	32	[248]
E2	CHMO	25	[249]
E3	Lipase	100	Assumed
E4	ADH/NAD(H)	43	[250]
E5	TAm	9.9	[196]
E6	AlaDH	50	[251]

Based on the specific activity of each individual enzyme it is necessary to determine the relative amount of each enzyme that needs to be dosed to the reactor. This issue will be raised in Section 7.5, where the different reactor configurations will be identified.

7.4.2.2 Soluble and immobilised enzyme options

The use of an immobilised catalyst in the production of 6AHA might not be a simple task, since cofactors are involved in the reaction (Section 2.2.1.2 and 7.2.2.2). Thus, the only enzymatic step that can be carried out by an immobilised catalyst is the third reaction catalysed by lipase. There are several commercial formulations of this enzyme, such as Lipozyme RM IM, Lipozyme TL IM and Novozym 435 (commercially available from Novozymes A/S). Furthermore as observed for the activity, when moving from bench-scale to an industrial scale, an increase of the biocatalyst stability is one of the parameters subject to improvement [65] by either protein engineering or immobilisation.

7.4.3 Process technology options

For the enzymatic cascade reaction for production of 6AHA, there are 16 theoretical possible flowsheets if the process is carried out in single or several reactors (see Section 7.2.3.1, 2^{5-1}=16). However, when taking into account the reaction constraints, the number of flowsheets is narrowed down to three, showing that the biocatalytic synthesis of 6-aminohexanoic needs to be carried out in a series of reactors as shown in Figure 7.7.

	Enzymes					
	E1 ADH/NADP(H)	**E2 CHMO**	**E3 Lipase**	**E4 ADH/NAD(H)**	**E5 TAm**	**E6 AlaDH**
Option 1	Reactor 1 soluble enzyme whole-cell		Reactor 2 soluble enzyme immobilised enzyme whole-cell	Reactor 3 soluble enzyme whole-cell		
Option 2	Reactor 1 soluble enzyme whole-cell			Reactor 2 soluble enzyme whole-cell		
Option 3	Reactor 1 soluble enzyme whole-cell		Reactor 2 soluble enzyme whole-cell			

Figure 7.7. Scheme of the possible flowsheets available for the biocatalytic synthesis of 6AHA

The choice of operating in batch or continuous mode in multi-step biocatalytic reactions is related to the biocatalyst formulation adopted and the required reaction yield. Operating in a continuous mode requires that the biocatalyst and cofactors can be retained inside the reactor, which might not be retained inside the reactor, which might not be economically feasible when operating with isolated free enzymes (see Section 2.2.1). Furthermore, when operating in a continuous mode a complete conversion is not achievable (unless operating in a plug-flow reactor). This last drawback of continuous operation is particularly relevant when dealing with bulk or low value fine chemicals (such as the case of ε-caprolactam), since the cost of the raw materials dominates the overall production costs, implying that high reaction yields are required for the economic viability of the process [252]. Therefore, it is assumed that the biocatalytic route for production of 6-aminhexanoic acid is carried out in a batch mode.

Regarding the reactor selection, the choice is related to the biocatalyst formulation chosen and the need to supply oxygen. Table 7.10 shows the available reactor options for the present case study.

Table 7.10. Reactor options for the biosynthesis of 6AHA

		Whole-cells	**Isolated free enzyme**	**Immobilised enzyme**
Oxygen supply required	E1 & E2	STR, BCR	STR, BCR	N/A
Oxygen supply not required	E3	STR	STR	PBR, FBR, EBR
	E4, E5 & E6	STR	STR	N/A

N/A – not applicable

It is assumed that, when operating with whole-cells and isolated enzymes, the reactor chosen is a stirred tank reactor (STR), while reactions catalysed by immobilised enzymes are carried out in a fluidised bed reactor (FBR).

Initial concentrations for all the components are reported in Section 7.6 and in Appendix 3. During the process, pH and temperature would need to be controlled and maintained.

7.5 Possible flowsheets

Selecting the most suitable process flowsheet is a central issue in process design, particularly at the initial process development stage, as the information available is often scarce. Experimental evaluation of all the different process technology options might be expensive and time-consuming due to the large number of possible combinations, resulting in an enormous number of different process designs. Reaction and biocatalyst considerations have been used to narrow down the number of alternative process flowsheets. However, apart from the arrangement of the different synthetic steps into the reactor(s) (i.e. dosing of the different biocatalysts in the reactor), there are other alternative technologies that need to be considered simultaneously (such as catalyst formulation). Hence, just by considering the different types of catalyst formulation possible in each reactor the number of flowsheets increases to 20 (Figure 7.7, Table 7.11).

The remaining challenge for the process engineer is to rank these possible alternative process technologies in terms of their economic and environmental profile. In doing so, the most environmentally friendly and economic processes at full-scale implementation would be identified. The goal of generating the flowsheets (and mass and energy balance) is not to get to a detailed design and dimensioning of the process, but instead to be able to identify the most promising solution(s) and research areas where further development is required, when limited information about the process is available. Figure 7.8 shows a possible flowsheet for biocatalytic production of 6AHA.

Based on the specific activity of each individual enzyme (Table 7.9), the relative amount of each enzyme (i.e. enzyme concentration) that needs to be dosed to the reactor can be calculated in order to adjust the overall reaction rate (Table 7.12). For this purpose, the enzyme activity of each individual enzyme is the product of enzyme concentration and its specific activity. The motivation for matching the different enzyme activities when operating with multi-enzyme systems is two-fold: to optimise the biocatalyst(s) allowable costs and; when cofactor recycling is required, to ensure that the rate of reaction is not limited by the cofactor regeneration. Table 7.12 shows the relative amount of each enzyme dosed in the biocatalytic reactors, when operating with isolated enzymes.

Table 7.11. Summary of the 20 possible flowsheets for biocatalytic production of 6AHA

Option		Reactor 1	Reactor 2	Reactor 3
Option 1	1	Isolated enzyme	Isolated enzyme	Isolated enzyme
	2	Isolated enzyme	Isolated enzyme	Whole-cell
	3	Isolated enzyme	Whole-cell	Isolated enzyme
	4	Isolated enzyme	Whole-cell	Whole-cell
	5	Isolated enzyme	Immobilised enzyme	Isolated enzyme
	6	Isolated enzyme	Immobilised enzyme	Whole-cell
	7	Whole-cell	Isolated enzyme	Isolated enzyme
	8	Whole-cell	Isolated enzyme	Whole-cell
	9	Whole-cell	Whole-cell	Isolated enzyme
	10	Whole-cell	Whole-cell	Whole-cell
	11	Whole-cell	Immobilised enzyme	Isolated enzyme
	12	Whole-cell	Immobilised enzyme	Whole-cell
Option 2	1	Isolated enzyme	Isolated enzyme	
	2	Isolated enzyme	Whole-cell	
	3	Whole-cell	Isolated enzyme	
	4	Whole-cell	Whole-cell	
Option 3	1	Isolated enzyme	Isolated enzyme	
	2	Isolated enzyme	Whole-cell	
	3	Whole-cell	Isolated enzyme	
	4	Whole-cell	Whole-cell	

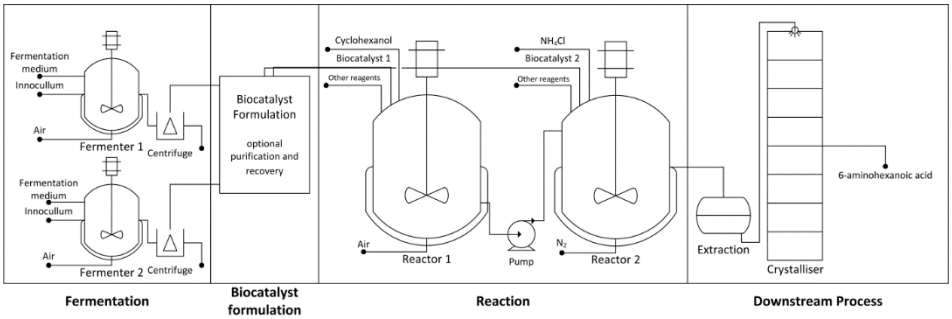


Figure 7.8. Example of a possible flowsheet for biocatalytic production of 6AHA (Option 2 or Option 3), including biocatalyst production (fermentation and biocatalyst formulation), biocatalysis (reaction) and downstream processing (recovery and purification). Note: The biocatalyst is normally produced independently from the reaction step and then stored until use [16].

The same principle can be applied to set threshold values for the overexpression level in the host cell of each individual enzyme, assuming that the relative specific activity does not change drastically. For example, the NAD(H)-dependent alcohol dehydrogenase (E4) still shows higher activity (with the same approximated activity ratio) than the transaminase (E5) inside the cell. Therefore, it is necessary to match the

overexpressed enzyme level (i.e. enzyme concentration inside the cell) assuming that 20% of the total protein content in the cell is the overexpressed enzymes [209] and the total protein content in the recombinant host is 50% of the total cell dry weight (CDW). Further, when operating with whole-cell as the biocatalyst, the overall reaction rate (and henceforth the process space-time yield) is determined by the whole-cell concentration in the reactor. Table 7.13 shows the required content of recombinant enzyme in the whole-cell (in grams of recombinant enzyme per grams of cell-dried weight) (option 2).

7.6 Process mass and energy balances

For identified process flowsheets, simulations and documented results are used to obtain the necessary process data for a process evaluation. Mass and energy balances are performed based on the candidate flowsheets generated. In the mass and energy balances, the amount of the raw materials (reagents, catalysts and reaction additives), intermediates, (co-)products and energy are calculated for each candidate flowsheet.

7.6.1 Assumptions

In order to achieve a competitive design, the process performance should (at least) match the performance of the competing technology. Therefore, a set of process performance metrics need to be defined in order to reflect the different allowable production costs (see Section 5.2.1). In this case study, the performance metrics are: reaction yield (defining the raw material allowable cost [253]); final product concentration (as an indication of the downstream process cost [254]); and space-time yield (defining labour and utilities operation costs and capital cost by the equipment occupancy [255]). Table 7.14 summarises the base case assumptions for this case study. These assumptions will define the reactor volume and reaction conditions. Aspects of current good manufacturing practice (cGMP), such as validation and qualification protocols and aseptic downstream processing have not been included in the calculations although these could be requirements at production scale. The full details of the base case are given in Appendix 3.

Table 7.12. Relative amount of enzyme dosed to each reactor for the different multi-pot flowsheets possible

	Enzyme	Specific Activity [$mmol_{Product}/min/g_{Enzyme}$]	Option 1 ($g_{Enzyme\ i}/g_{Total\ Enzyme}$)		Option 2 ($g_{Enzyme\ i}/g_{Total\ Enzyme}$)		Option 3 ($g_{Enzyme\ i}/g_{Total\ Enzyme}$)	
E1	ADH/NADP(H)	32	Reactor 1	44%	Reactor 1	38%	Reactor 1	44%
E2	CHMO	25		56%		49%		56%
E3	Lipase	100	Reactor 2	100%		12%	Reactor 2	7.0%
E4	ADH/NAD(H)	43	Reactor 3	19%	Reactor 2	19%		17%
E5	TAm	9.9		65%		65%		60%
E6	AlaDH	50		16%		16%		15%

Table 7.13. Relative amount of enzyme overexpressed in a recombinant host using whole-cell

	Enzyme	Option 1	($g_{Enzyme\ i}/g_{Total\ Protein}$)	($g_{Enzyme\ i}/g_{CDW}$)	Option 2	($g_{Enzyme\ i}/g_{Total\ Protein}$)	($g_{Enzyme\ i}/g_{CDW}$)	Option 3	($g_{Enzyme\ i}/g_{Total\ Protein}$)	($g_{Enzyme\ i}/g_{CDW}$)
E1	ADH/NADPH	Cell 1	8.8%	4.4%	Cell 1	7.6%	3.8%	Cell 1	8.8%	4.4%
E2	CHMO		11%	5.6%		10%	4.9%		11%	5.6%
E3	Lipase	Cell 2	20%	10%		2.4%	1.2%	Cell 2	1.4%	0.7%
E4	ADH/NADH	Cell 3	3.8%	1.9%	Cell 2	3.8%	1.9%		3.4%	1.7%
E5	TAm		13%	6.5%		13%	6.5%		12%	6.0%
E6	AlaDH		3.2%	1.6%		3.2%	1.6%		3.0%	1.5%

Table 7.14. Base case process assumptions for biocatalytic production of 6AHA

Annual Production (ton/year)	10000
Annual operating hours (h/year)	8000
Final product concentration (g/L)	250
Batch time (h)	8
Reaction Yield ($mol_{Product}/mol_{Starting\ material}$)	95%
Reactor Overhead (%)	20%
Glucose (% wt. of CDW)	10%
Total Turnover Number for NAD(H) ($TTN_{NAD(H)}$)	100000 [187]
$TTN_{NADP(H)}$	10000 [187]
TTN_{PLP}	90 [37]
$TTN_{Alanine}$	1

Despite the fact that these performance metrics are not often easily achieved during the early stage of process development, it is necessary to assume that these performance threshold values will be achieved when the time for implementing the process comes (i.e. before the pilot-plant tests). As the goal of the performance evaluation is to assess the potential benefits of the selected process options (put together in a flowsheet) at full-scale, these not yet achieved (but likely) assumptions will be presumed for the economic and environmental evaluation. By adopting this strategy, underdeveloped synthetic routes (such as the proposed biocatalytic route) can be compared with implemented processes (such as the synthesis of ε-caprolactam by Beckmann rearrangement). In addition, these assumptions (in line with the threshold values for the process metrics, see Section 5.2.1) define the initial operating conditions at which data and further development efforts should be focused. Hence, the true potential of further developing the proposed biocatalytic route can be assessed. However, it holds true that at the first instance with the state-of-the-art for the biocatalyst, the flowsheet chosen will not perform as defined here. The achievement of these performance metrics are thus the target for biologist, chemists and process engineers.

7.7 Process evaluation

7.7.1 Economic assessment

Economic evaluation can be used as a decision-making tool to quantitatively estimate the expected profitability of a process [16]. However, at the early development stage, this evaluation tool can select the most promising process design options, while

identifying the current process bottlenecks preventing the process commercial success. As shown in Figure 7.9 none of the evaluated processes can compete economically with the current industrial process (in which the ε-caprolactam current selling price is 2.70 €/kg).

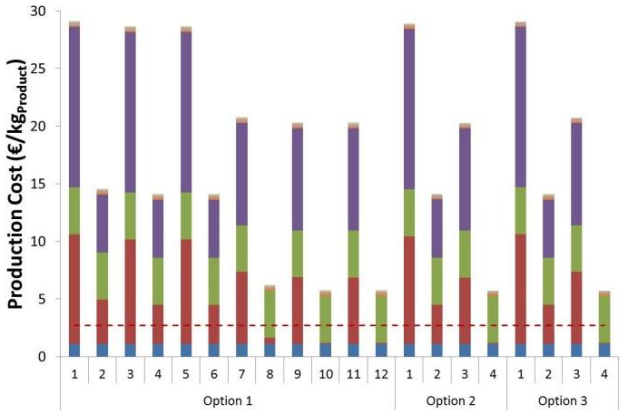


Figure 7.9. Production cost for the different process design option for production of 6AHA; Legend: cyclohexanol, biocatalyst, alanine, cofactors, glucose, NH_4Cl, utilities, labour, CAPEX and current ε-caprolactam selling price

Figure 7.9 shows clearly that there are two main process bottlenecks preventing an economically successful industrial implementation: the biocatalyst formulation (including the cofactor costs) and the efficient recycling of alanine.

Most of the reported industrial biocatalytic processes use whole-cells as catalysts [14], affecting the process economics in two ways: the possibility to use the cell's native cofactors (preventing the dosing of expensive cofactors) and the biocatalyst cost (in €/kg$_{biocatalyst}$).

From the evaluated process options, when operating with isolated enzymes (in either free or immobilised form) the cost of the biocatalyst and the cofactor can account for up to 80% of the total production costs (Figure 7.9). However, when operating with whole-cells (or partially with whole-cells) the costs are reduced by 8% (when operating partially with whole-cells, i.e. when only of the reactors is operating with whole-cells) and by 20% (when operated fully with whole-cells, i.e. when all the reactors are operating with whole-cells) when compared with the fully isolated catalyst options (options 1.1, 2.1 and 3.1). Further, it is also noticeable that the benefits of operating with whole-cells are more relevant when the last three reaction steps (E4 to E6) are performed in whole-cells, since these reactions require dosing two types of expensive cofactor (PLP and NAD(H)). The most favourable scenarios are found when both parts of the reaction that are cofactor dependent (i.e. the first two and the last three reaction steps, E1-E2 and E4-E5-E6, respectively) are performed using a whole-cell

(options 1.8, 1.10 and 1.12, option 2.4 and option 3.4). Furthermore, the assumed values for $TTN_{NAD(H)}$ and $TTN_{NADP(H)}$ are already significantly favourable for initial design stages and TTN values above the assumed are very seldom attained [187], which eliminates the option of operating with isolated enzymes.

However, for all the evaluated scenarios (including the most favourable options, i.e. options 1.8, 1.10 and 1.12, option 2.4 and option 3.4), the economic viability of the process is compromised, as the production cost is still higher than the current selling price of ε-caprolactam, since the alanine cost is the main identified bottleneck.

7.7.2 Environmental assessment

There are two sets of environmental metrics mainly used to assess the environmental profile of a production process: the green chemistry metrics and the Life Cycle Assessment (LCA) impact factors.

Green chemistry metrics can be divided into reaction-related metrics and process-related metrics. Reaction-related metrics include atom efficiency (AE) [147], reaction mass efficiency (RME) [144] and carbon efficiency (CE) [144]. These metrics are intended to quantify exclusively the greenness of the reaction chemistry and therefore are constant across the all the evaluated scenarios. Process-related metrics include water intensity (WI) [157], process mass intensity (PMI) [149] and E-factor [152]. These metrics aim at quantifying the overall process (including the reaction chemistry).

Water intensity is of special interest in biocatalysis and fermentation processes, since one of the most attractive features of bioprocesses in organic synthesis is the possibility of operating with an environmentally compatible solvent (water) [21]. However, water has become a scarce and overexploited natural resource. In addition, these processes usually lead to a large amount of wastewater that needs to be properly cleaned and this is usually very energy intensive [256]. Therefore operating with high concentrations is not only a matter of ensuring lower downstream process costs, but also an environmental issue. Despite the relevance of this green chemistry metric, it is only related to the final product concentration and consequently constant across the all the evaluated scenarios (4 $kg_{water}/kg_{product}$), due to the assumed operating conditions used to build the mass and energy balances (Table 7.14).

Process mass intensity (see Section 4.2.2.1), was also used to compare the environmental performance for the 20 different scenarios (Figure 7.10). For these flowsheets and mass balances, the reaction performance (final product concentration, space-time yield and reaction yield) are assumed constant according with Table 7.14. Hence, the variations of PMI across the evaluated scenarios (Figure 7.10) come only from the biocatalyst selected and consequently, the cofactor requirements for each

option. Since PMI is a mass-based metric (kg $_{\text{reagents and biocatalyst}}$/kg $_{\text{Product}}$), bigger mass contributors (starting material, alanine and NH_4Cl) have a bigger contribution on the PMI of the process step (reaction). Since the impact on the mass contribution of the biocatalyst is less than 2% of the total process mass (excluding water), there are no major variation in the PMI of the reaction when evaluating different catalyst options. Nevertheless, when integrating a holistic viewpoint of the overall process to the PMI calculation (i.e. including the production of the biocatalyst), the impact of the catalyst formulation has a higher impact in the PMI and leads to an increase of nearly 10% in the calculated PMI value (Figure 7.10) when compared with the calculated PMI considering only the reaction step. A further increase is expected when integrating the production of the cofactors used during the multi-enzymatic synthesis of 6AHA. However, due to the lack of inventory data regarding industrial production of cofactors, this calculation was not performed. Nevertheless, these will only accentuate the benefits of operating in a whole-cell, as shown in Figure 7.10.

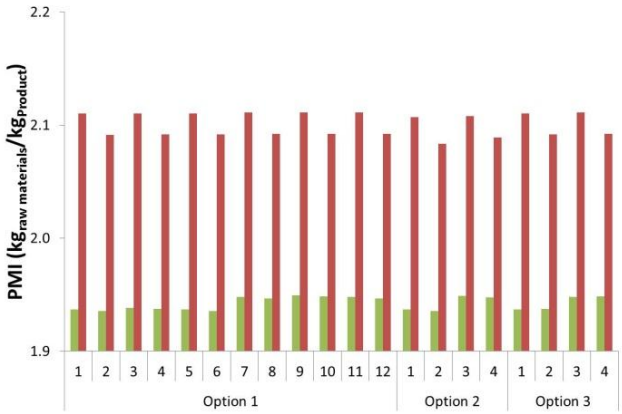


Figure 7.10. Process mass intensity (PMI, kg$_{\text{raw materials}}$/kg$_{\text{Product}}$) for production of 6AHA; Legend: ■ PMI of the reaction step; ■ PMI of the reaction step considering the fermentation and catalyst formulation for production of the catalyst

A similar trend can be observed for the E-factor. Due to the lower enzyme stability often observed when operating with isolated enzymes [16] and cost intensive enzyme-retaining techniques, biocatalyst reuse is not possible when this type of catalyst formulation is selected. Therefore, the process options that include whole-cell and/or immobilised enzymes display a more favourable E-factor. When operating with whole-cells (at least partially) resulting in a 3% decrease of the E-factor. However, as observed previously for PMI, the mass contribution of the biocatalyst is lower than the mass contribution of other waste streams containing alanine, cofactors and unreacted starting material (cyclohexanol). Pyruvate is the biggest waste contributor, since the turnover number of alanine is too low. Therefore, improving the alanine turnover

number is not only a matter of enabling an economically feasible process, but also improves its environmental profile.

The use of the aforementioned metrics is an attempt to measure the process chemistry and efficiency in a straightforward and easy to use way. Furthermore, these metrics do not require process details and are therefore attractive for initial process design decisions. However, these metrics do not distinguish between waste types and emissions [158]. Further, these metrics also do not consider the emissions, waste generated or resources used upstream or downstream of the investigated process step, nor the life cycle of the used raw materials. Life Cycle Assessment (LCA) is a more elaborate and comprehensive tool to quantify environmental effects [22], and provides detailed information about the type of emissions and the potential environmental impact over the whole life cycle of the product. Figure 7.11 displays the potential environmental impacts for the enzymatic cascade for production of 6AHA.

LCA results for the 20 evaluated process options revealed that the starting material (i.e. cyclohexanol), alanine and the catalyst are the major contributors to the process environmental impact, by contributing more than 80% of the impacts evaluated and depicted in Figure 7.11. In particular, the starting material and alanine were the biggest contributors to the environmental impacts.

Despite that the biocatalyst accounts for less than 10% of all the depicted environmental impacts, it is clear that operating with whole-cells implies savings in all the reported emissions and energy requirements. These savings are more relevant when the second half of the reaction (catalysed by E4, E5 and E6) is performed in a whole-cell by comparing the performance of option 1.2 and 1.7, option 2.2 and 2.3 or option 3.2 and 3.3, corresponding to the scenarios where the reaction catalysed by E4, E5 and E6 is conducted using a whole-cell catalyst or E1 and E2 is conducted in a whole-cell, respectively. Further, the most favourable scenarios for the environmental profile are found when both parts of the reaction that are cofactor dependent (i.e. the first two and the last three reaction steps) are performed using a whole-cell (options 1.8, 1.10 and 1.12, option 2.4 and option 3.4).

Nevertheless, more than 70% of all the reported emissions are allocated to alanine and cyclohexanol. As concluded from the process economic assessment, the low alanine turnover number leads to a high mass requirement of this amino acid, which compromises not only the economic viability, but also the environmental profile of the process. The production of cyclohexanol is currently based on the cobalt- catalysed oxidation of cyclohexane [257]. Currently this process exhibits several disadvantages: apart from numerous operational safety issues, the synthesis of cyclohexanol requires a high consumption of hydrogen for the hydrogenation step, high energy demands of the process, the oxidizing step has the disadvantage of producing multiple by-products. The aforementioned disadvantages are reflected on the cyclohexanol

environmental profile, which in turn are reflected on the studied biocatalytic process. Nevertheless, recent studies proposing new greener routes for production of cyclohexanol have been reported, opening the opportunity for improved environmental profile [257]. Further, the environmental profile of the biocatalytic process proposed can also be improved by operating at higher reaction yield, as it will be discussed later.

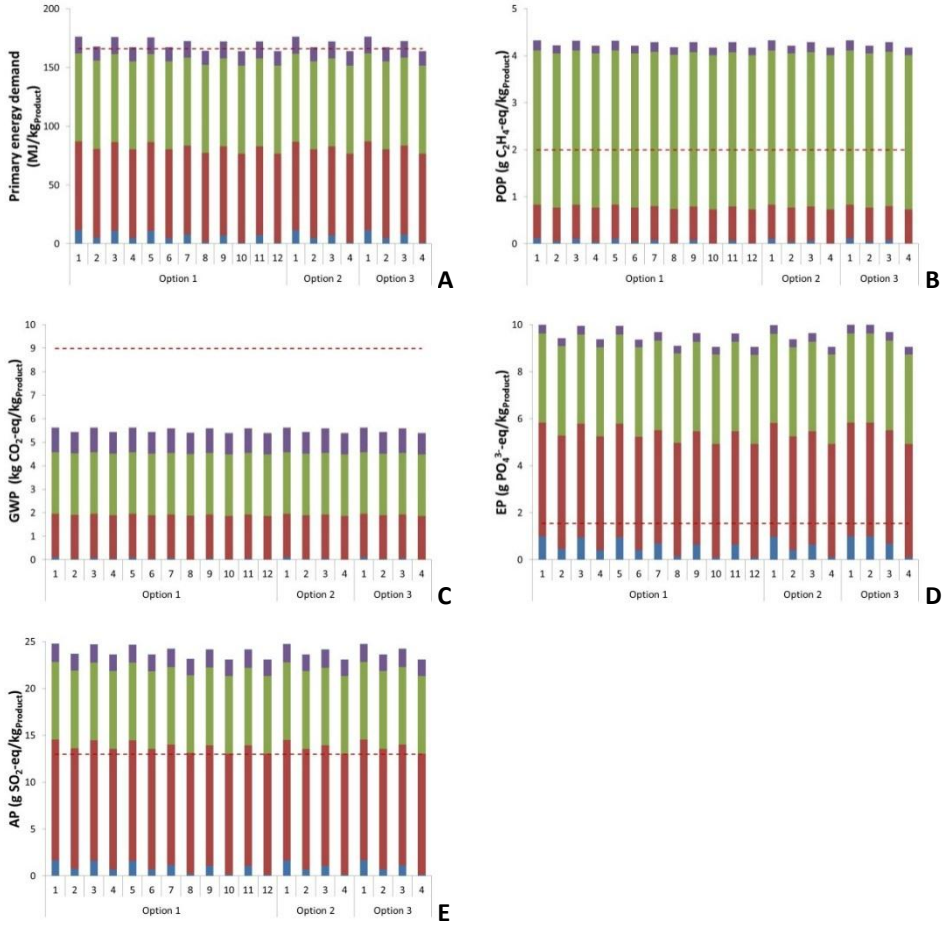


Figure 7.11. Potential environmental impacts from production of ε-caprolactam, including biocatalytic production of 6AHA and subsequent cyclisation to ε-caprolactam; **A** Primary energy demand; **B** photochemical ozone formation potential; **C** Global warming potential; **D** Eutrophication Potential; **E** Acidification potential Legend: ■ Biocatalyst (including cofactors), ■ Alanine, ■ Cyclohexanol, ■ Others and - - - ε-caprolactam production environmental impact with the Beckmann rearrangement

However, even for the identified favourable scenarios, the environmental profile for the base case is still not particularly favourable when compared to the current

chemical synthesis of ε-caprolactam. The biocatalytic route only shows savings in the primary energy demand and the greenhouse gas emissions, while for the rest of the evaluated environmental impacts (photochemical ozone creation, eutrophication and acidification potential) the performance of the biocatalytic synthetic route is worse than the environmental performance of the conventional chemical route. Contributing to the not outstanding environmental profile is the alanine and cyclohexanol production processes and the consequently the assumed values for TTN_{Ala} and reaction yield (see Table 7.14).

The above section, dedicated to process evaluation, was able to identify two main bottlenecks preventing the successful implementation of the biocatalyst process (alanine requirement and efficient conversion of the starting material). In particular, an efficient *in-situ* alanine recycle is required, in order to ensure an economically viable process. Further, the most promising solutions were identified (options 1.8, 1.10 and 1.12, option 2.4 and option 3.4) and it was shown that operating in whole-cell brings economic and environmental advantages when compared with the isolated enzyme system. From the five most promising design options, option 1.12 might result in better process design.

Option 1.12 is a three-pot process, where the catalyst used is a whole-cell for the first and the last reactor and in the second reactor, an immobilised lipase is used as the catalyst. Despite the greater capital cost of this option when compared to the scenarios operating in a two-pot process (option 2.4 and option 3.4), operating in a three reactor process can bring advantages in control as well as matching the operating conditions and overexpression level of a reduced number of enzymes might be an easier task [241]. Moreover, option 1.12 allows a greater flexibility for adapting to process modifications and/or fitting an already existing process. In addition, economic evaluation revealed that only a small percentage of the production costs arose from the process capital costs. Nevertheless, when operating with lower space-time yield (i.e. higher batch times and higher reactor occupancy and lower biocatalyst concentration) the allocated capital costs will increase and therefore operating in a battery of three reactors might not be the most economic situation. Further, when comparing scenario 1.8, 1.10 and 1.12, the economic and environmental evaluation did not identify major drawbacks to operating with an immobilised biocatalyst, as the amount of biocatalyst used is small. Hence, selecting to operate with an immobilised lipase (E3) results in higher biocatalyst stability, when compared with isolated or whole-cell (option 1.8 and 1.10, respectively), while conveying simplicity to the process.

7.8 Scenario analysis

Assuming that biocatalyst production and enzyme overexpression is not limiting the process performance, in the evaluated scenario the low efficiency of *in-situ* alanine recycling prevents the economic viability of the process.

The alanine total turnover number (TTN_{Ala}, defined as 'mole of product formed per mole of alanine') assumed is too low, leading to high alanine concentration requirements in the reactor. Previous studies using AlaDH, for alanine recycling in amination of primary alcohols [196] or asymmetric amination of ketones [258] have reported a 5-fold excess of alanine, which hinders the possibility of an economically successful scale-up. Nevertheless, the regeneration of alanine from pyruvate was reported to be a thermodynamically favourable reaction [197]. Hence, it might be possible to achieve higher TTN_{Ala} by improving the activity of alanine dehydrogenase, either by engineering its specific reaction activity via protein and/or reaction engineering (e.g. optimise pH, buffer, temperature) or by process engineering to optimise the enzyme ratio and compound concentrations in the reactor. Improving the TTN_{Ala} to 5 $mol_{product}/mol_{Ala}$ leads to 50% reduction of the production costs, enabling a profitability margin for the biocatalytic production of 6AHA, while further improvements to TTN_{Ala} to 10 $mol_{product}/mol_{Ala}$ result in 60% decrease of production costs, with a further increase of the profitability margin. Figure 7.12 displays the savings in the process production cost and in the environmental impacts of operating with higher TTN_{Ala}.

From Figure 7.12, it can be concluded that improving *in-situ* alanine recycle in order to obtain higher TTN_{Ala} than 20 $mol_{product}/mol_{Ala}$ ceases to be of any benefit to the process economics, as the production costs become dominated by cost of the starting material. Regarding the process environmental impact, there are also major benefits of operating with higher TTN_{Ala} (as shown in Figure 7.12 B), with great savings in the process energy demands and therefore, in many of energy-related environmental potentials (such as acidification potential and global warming potential).

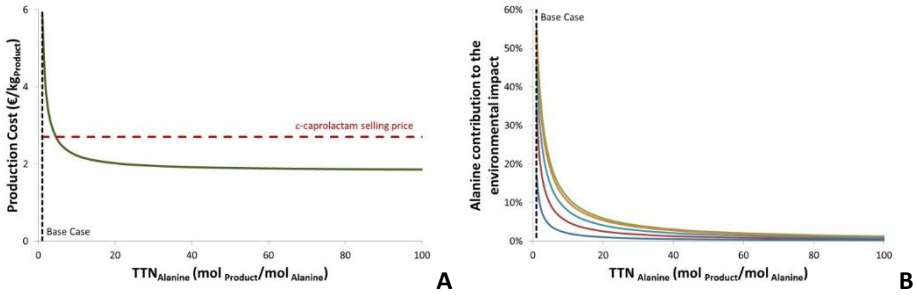


Figure 7.12. Effect of the alanine total turnover number in overall production cost (**A**) and in the process environmental impact (**B**) Legend for B: acidification potential; eutrophication potential; global warming potential; photochemical ozone creation potential; primary energy demand from renewable and non-renewable resources. Assumptions: process is performed according with option 1.12 (3-pot reactor); 95% reaction yield; 250 g/L of final product concentration; 8 hours of batch time

The second process bottleneck identified that the reaction conversion has a great impact to the process environmental impact in all of the evaluated scenarios due to the present production process of cyclohexanol, as compound is the major cost driver when using whole-cells. Therefore, it is necessary to ensure an efficient conversion of the cyclohexanol to ε-caprolactam in order to attain an economically competitive and environmentally friendly biocatalytic process. When operating with whole-cells, even at high reaction yield (99%), the cyclohexanol contributes with 50% of the total production costs (Figure 7.13 A, as commonly observed for many other bulk chemical production processes [252]). When considering the environmental impact of the process, the cyclohexanol contribution to the overall process environmental assessment is remarkable and it strongly affects the overall environmental performance (Figure 7.13 B). From the impact categories evaluated, photochemical ozone formation potential, global warming potential and primary energy demand are particularly relevant, due to the current production process of cyclohexanol, which demands intensive solvent use and large energy demands. The environmental impact for the biocatalytic production of ε-caprolactam could be greatly reduced by considering other synthetic routes for production of cyclohexanol [257].

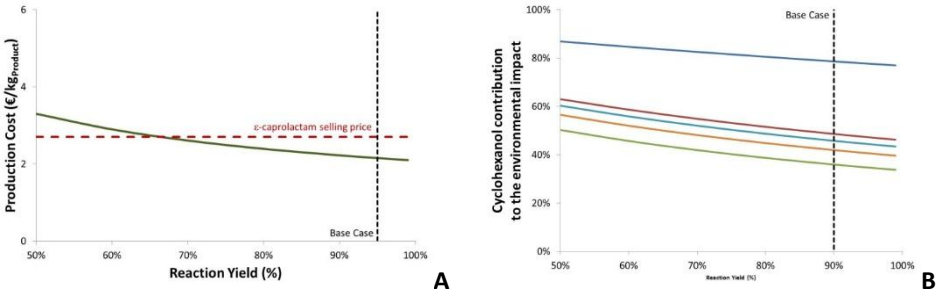


Figure 7.13. Effect of the reaction in overall production cost (**A**) and in the process environmental impact (**B**) Legend for B: acidification potential; eutrophication potential; global warming potential; photochemical ozone creation potential; primary energy demand from renewable and non-renewable resources. Assumptions: process is performed according with option 1.12 (3-pot reactor); TTN_{Ala} 10 $mol_{Product}/mol_{Ala}$; 250 g/L of final product concentration; 8 hours of batch time

In the scenario analysis, other than the identified bottleneck scenarios should be considered, in order to avoid pitfalls and sub-optimised processes. Often there is a trade-off between the amount of biocatalyst dosed and the process utilities and capital costs. For instance, operating a process for a long batch time (i.e. obtaining a small space-time yield and operating at low reaction rate) implies higher equipment occupancy and higher reactor volumes, with consequent increase of the process capital and utilities costs. However, the process reaction rates can be increased (and the batch time reduced) by dosing more biocatalyst into the reactor (i.e. increasing the biocatalyst concentration), with subsequent increase of the biocatalyst allowable cost. Hence, there is an optimal space-time yield that needs to be identified, in order to obtain the minimum production costs (Figure 7.14).

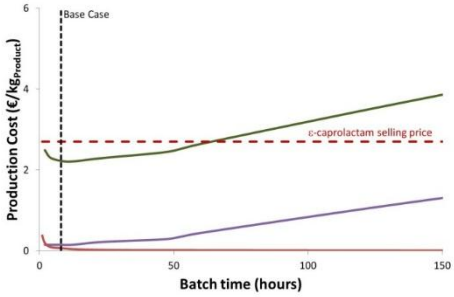


Figure 7.14. Effect of the batch time and consequent space-time yield in the reaction in overall production cost biocatalyst cost and capital costs. Assumptions: process is performed according with option 1.12 (3-pot reactor); 95% reaction yield; TTN_{Ala} 10 $mol_{Product}/mol_{Ala}$; 250 g/L of final product concentration; 8 hours of batch time

7.9 Concluding remarks

Multi-enzymatic processes can arise as a suitable and greener alternative to the conventional chemical routes. However, these processes are often very complex systems, with many interactions between enzymes, substrate(s), intermediates and product(s), affecting the catalyst(s) activities, which prevents many multi-enzymatic processes from being successfully scaled-up. Indeed, current process option selection for implementation of these processes has been performed on a case-by-case basis.

In this chapter, a rational approach for defining a development strategy for multi-enzymatic processes was presented. The proposed methodology requires a profound and structured knowledge of multi-enzyme systems, integrating chemistry, biological and process engineering. Hence, the information required (compound interaction, enzyme activity, etc.) has been listed in order to evaluate and propose a reduced number of feasible process design options, where the identified challenges are addressed. Here, this approach was used to set the mass and energy balances. This approach can also be useful in determining the biggest cost drivers and thus, proposes and guides further experimental evaluation of the process. This would lead to a reduction in expensive and time-consuming research as only the most promising need to be considered, reducing the number of experimental work.

The number of options can be further reduced, by evaluating the process economic and environmental performance. This assessment is not only able to identify the most promising process based on its economic and/or environmental performance, but it is also able to identify the current process bottlenecks and, by performing a scenario analysis, the effects of overcoming these bottlenecks can be identified.

By applying this methodology *a priori* to experimental evaluation (even at early research stage, during screening), the laboratory experts (either chemists or biologists) are able to understand the most probable operating conditions at full-scale and thus, they are able to collect information at these relevant conditions (for example, enzyme specific activity in the presence of other compounds and substrate selectivity) but also during biocatalyst development, by setting threshold values for the biocatalyst activity at relevant concentrations.

Whole-cells provide a more promising scenario for multi-enzyme systems, namely when the use of cofactors is required. Great advances have been made in understanding the complex mechanisms in protein overexpression, substrate transfer across the membrane and in knocking-out competing pathways inside the cell, in order to obtain suitable whole-cell biocatalysts for industrial processes. However, the success of whole-cell biocatalysis for multi-enzymatic processes is ultimately dependent on how enzyme activity and protein overexpression can be controlled inside the cell. Therefore, cell-free systems and artificial cells are promising alternative

to the whole-cell. However, these solutions might translate to a biocatalyst cost increase (in particular for the artificial cells).

For the specific case study presented in this chapter, some considerations were not yet taken into account that will reflect in some adjustments into the process (e.g. ISPR requirements).

For instance, the final product of the biocatalyst synthetic route (6AHA, compound **6**) has two functional groups that will probably form salts, not only with other molecules from the same compound, but also with other components present in the last reactor (alanine, pyruvate and compound **5**). This will not only affect the reaction yield, but also the downstream process. Furthermore, compound 6 is an analogue of the amino acid lysine and therefore, it may act as an enzyme inhibitor for enzymes that have this residue on their binding site. However, this effect was not observed in the interaction matrix.

Further, the values for the specific activity gathered for this example, are merely illustrative and ideally would have been collected according with the methodology at the operating conditions. However, the information used was obtained from scientific literature, where the reported enzyme-catalysed reactions were performed at different pH and temperature. Hence, when applying a second round of this methodology, the process engineer should reconsider the enzyme activities for the recombinant hosts selected and as close as possible with the defined operating conditions.

When applied to the multi-enzyme system for biocatalytic production of ε-caprolactam the proposed methodology was able:

1. To select the most favourable reactor configuration (multi-pot) in order to avoid cross interference between the different compounds in the enzymatic cascade;

2. To select the most favourable biocatalyst formulation (whole-cell) based on the economic and environmental evaluation of the process flowsheets generated;

3. To identify two main process bottlenecks that are preventing a competitive process, while indicating targets for further development:

   - Process evaluation showed that alanine has a great impact in attaining a successful process scale-up, while scenario analysis has proven the benefits of improving alanine *in-situ* regenerating system in the process viability. Therefore, it is necessary to develop process solutions to decrease the alanine requirement, increasing the TTN_{Ala} to a minimum of 10 $mol_{product}/mol_{Ala}$ by implementing an efficient regeneration system of pyruvate back to alanine.

   - Finally, the starting material (cyclohexanol) was proven to have a great impact on the environmental and economic profile of the process. Therefore,

the biocatalytic production of ε-caprolactam is stymied until greener and cheaper production routes for cyclohexanol are commercially available.

8 Windows of operation for selection of technology options

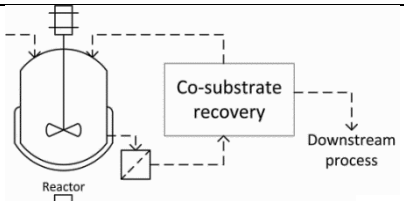
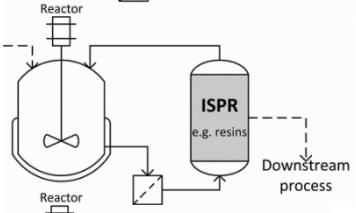
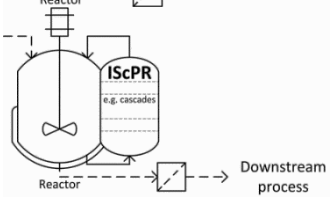
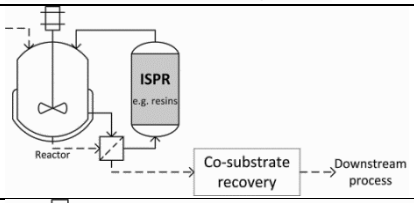
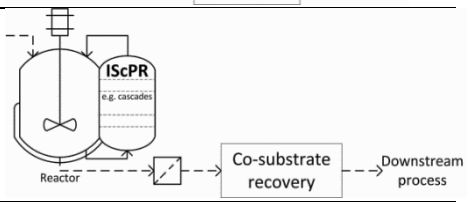
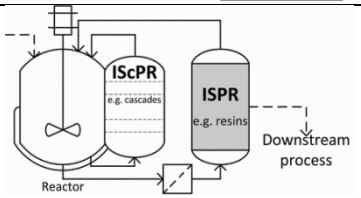
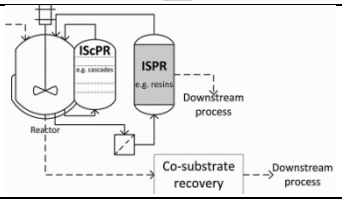
8.1 Introduction

The attractive features of biocatalytic processes (such as, exquisite selectivity, biodegradability of the biocatalyst and mild reaction conditions) have led to an increased number of processes running at a commercial scale [14,219]. Although biocatalysis has many potentially attractive features, an analysis of emerging and interesting enzyme-catalysed reactions indicates that in many cases biocatalyst yield and process intensity are frequently too low for successful industrial implementation. In fact, when a biocatalytic route is first considered, often it does not meet the required process metrics for an economically feasible scale-up (i.e. high product concentration, high enantiomeric excess, high reaction yield, high biocatalyst yield and high space-time yield) [13]. Further, for the majority of scaled biocatalytic processes, the conditions at which the enzymes are operating in the industrial reactor (e.g. substrate and product concentrations) are far different from those found in their natural environment. This incongruity can be addressed in a number of different ways (ISPR, enzyme engineering, immobilisation, etc.). A crucial step in process design is therefore to select between alternative technologies and routes to overcome this incongruity. For instance, Table 8.1 shows a list of potential process solutions that are likely to improve the process performance in biocatalytic reactions where the thermodynamic equilibrium is unfavourable.

Biocatalytic processes are still in their infancy, as many of the suitable biocatalysts are still under development and therefore, most of the reactions are neither well developed nor optimised. Furthermore, the process technologies are not yet fixed and many alternative process configurations are possible. To carry out experiments in order to evaluate all the possible set-up combinations is a costly and laborious task. Hence, it is necessary to develop a methodology to identify where the most promising process solutions lie, integrating improvements that come not only from process engineering but also from molecular biology (to improve the biocatalyst) and chemistry (to improve reaction chemistry).

The use of a conceptual process design methodology (see Chapter 3) for the development of biocatalytic processes can bring a number of advantages [69]. Nevertheless, there is still not a rational approach to option selection and development for biocatalytic processes (e.g. ISPR, IScPR, operate with co-substrate excess and catalyst formulation) where the process bottlenecks are analysed and resolved in a systematic manner. The methodology proposed in this chapter utilises

Table 8.1. Overview of potential process solutions to improve the productivity of thermodynamically challenged biocatalytic reactions

Challenge	Solution	Flowsheet
Equilibrium shifting	Co-substrate excess	
	In-situ product removal (ISPR)	
	In-situ co-product removal (IScPR)	
Equilibrium shifting Product inhibition	Co-substrate excess *In-situ* product removal	
Equilibrium shifting Co-Product inhibition	Co-substrate Excess *In-situ* co-product removal	
Equilibrium shifting Product and co--product inhibition	*In-situ* product removal *In-situ* co-product removal	
Equilibrium shifting Product and co--product inhibition	Co-substrate excess *In-situ* product removal *In-situ* co-product removal	

the principles behind windows of operation (see Section 3.2.4) as a tool to identify suitable process conditions and assess feasible process configurations. The proposed methodology aims at facilitating the development of a biocatalytic process at an early stage. The outcome of this methodology is three-fold: 1) to channel resources to collect the relevant data for process development; 2) to identify the main process technologies that will ensure a feasible process; 3) to identify further development efforts, helping to direct research and thereby, setting R&D targets that are imperative for process success.

8.2 Methodological framework

The workflow, tools and data required in the methodology for developing windows of operation are outlined in Figure 8.1. The procedure contains 7 steps and includes literature research, data collection, process modelling, data generation (through process modelling) and comparison of different scenarios, aiming to identify and set targets for improvement in the different research areas of the process design (e.g. reaction design, catalyst design, catalyst selection and ISPR, IScPR and ISSS selection).

8.2.1 Step 1. Determine reaction thermodynamic equilibrium (K_{eq})

Rationale: The reaction thermodynamic equilibrium plays a crucial role in predicting the extent of a reaction (i.e. reaction yield) and the equilibrium position for the desired process [197]. Hence, the thermodynamic equilibrium constant is a critical parameter for a rational process development strategy in reversible reactions. The thermodynamic equilibrium constant determines the substrate(s) and product(s) concentrations at equilibrium, the thermodynamic reaction yield at the equilibrium position and which process options are feasible [30]. Therefore, an early determination of the equilibrium constant (K_{eq}) is essential for a truly systematic approach for process development.

Method: Estimation tools (e.g. group contribution methods) are commonly used in the chemical process industry to predict thermodynamic properties [259]. However, to date, these have been found to be unsuitable for aqueous based biocatalytic reactions due to the poor match between the experimental and the predicted data [30]. A simple experimental methodology to accurately determine reaction thermodynamic equilibrium constant in biocatalytic reactions was proposed by Tufvesson and his co-authors [30]. Further, the Thermodynamics of Enzyme-catalysed Reactions Database (TECRDB, [176]) compiles apparent equilibrium constants and molar enthalpies of reaction on 400 different biocatalytic reactions. The output of this step is a value for the thermodynamic equilibrium constant.

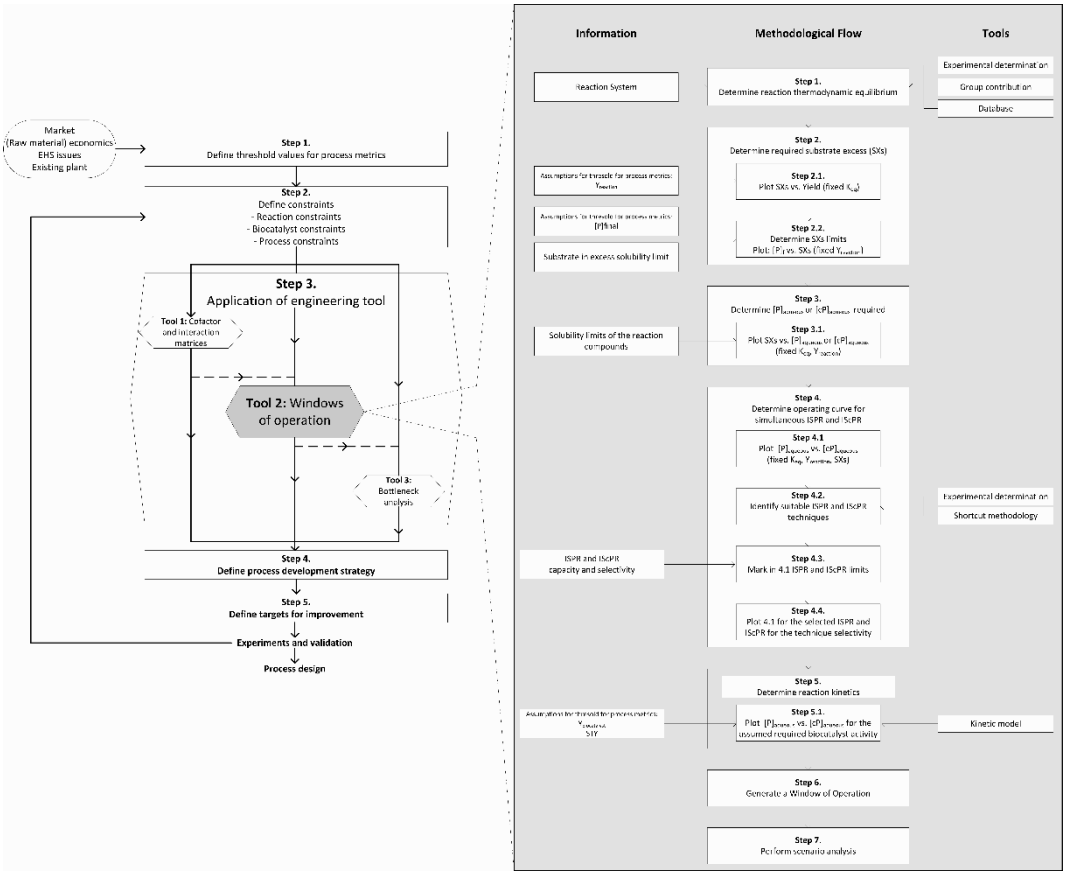


Figure 8.1. An overview of the proposed methodology to identify potential favourable scenarios

8.2.2 Step 2. Determine required co-substrate excess

Rationale: The easiest and most common strategy for shifting equilibrium is to use an excess of one of the substrates (usually the less expensive one) pulling the equilibrium towards the product side and thereby, increasing the thermodynamic reaction yield (see first entry in Table 8.1). However, operating with substrate excess increases the downstream process (DSP) costs, as the non-reacted substrate must be removed from the reactor effluent stream. Further, for a truly sustainable process, the non-reacted substrate must be recycled into the new batch, which might further increase the DSP costs.

Method: The methodology to determine the required co-substrate concentration for displacing the thermodynamic reaction equilibrium is given in Section 8.2.2.1 and 8.2.2.2.

8.2.2.1 Step 2.1. Plot required substrate excess against thermodynamic yield

For a generic reaction $A + B \rightleftarrows C + D$ given K_{eq} and initial limiting substrate concentration (A_0), the required co-substrate excess (Xs) to obtain the desired thermodynamic reaction yield ($Y_{reaction}$) by solving a system of equations (Equation 8.1).

$$\begin{cases} K_{eq} = \dfrac{C_{eq} \cdot D_{eq}}{A_{eq} \cdot B_{eq}} \\ C_{eq} = A_0 \cdot Y_{reaction} \\ D_{eq} = A_0 \cdot Y_{reaction} \\ A_{eq} = A_0 \cdot (1 - Y_{reaction}) \\ B_{eq} = A_0 \cdot (1 - Y_{reaction} + Xs) \end{cases}$$

Equation 8.1

Where A_{eq}, B_{eq}, C_{eq} and D_{eq} represent the concentration in equilibrium (reaction phase) of the reaction components and B is the co-substrate dosed in excess. Plotting the required co-substrate excess against the desired reaction yield allows a graphical visualisation of the trade-off between the desired reaction yield and the effort required to shift the equilibrium by use of co-substrate excess.

8.2.2.2 Step 2.2. Determine substrate excess limits

At industrially relevant concentrations, there is an upper constraint for the applicability of this strategy to displace the reaction equilibrium due to the water-solubility of the co-substrate dosed in excess, as well as the stability and inhibition of the biocatalyst at high co-substrate concentrations. Hence, it is necessary to determine the maximum allowable excess based on the co-substrate solubility limit and its inhibitory concentration. The physical properties of the substrates, products and reaction can be found experimentally, in the scientific literature or determined by computer aided (CAPE) software, such as ThermoData Engine software from NIST [260] and ProPred from ICAS [261].

8.2.3 Step 3. Determine product or co-product concentration required

Rationale: Many of the thermodynamically challenged reactions would require an enormous amount of substrate excess to shift the equilibrium towards the product side, which would lead to an unfeasible process. Hence, a suitable strategy is to apply

in-situ removal of product or co-product, increasing the driving force to displace the reaction equilibrium.

The potential benefits of putting in place an ISPR strategy are 4-fold: 1) it can remove the product from the reaction medium, decreasing the necessity of a substrate excess; 2) it can minimise product inhibition or stability effects on the biocatalyst [51]; 3) it can reduce product losses either by product degradation, or uncontrolled product removal from the system (e.g. by evaporation) [198] and; 4) it can decrease the DSP efforts [49].

Method: The methodology to determine the required product or co-product concentration for displacing the thermodynamic reaction equilibrium is given in Section 8.2.3.1.

8.2.3.1 Step 3.1. Plot substrate excess against (co-)product concentration in the aqueous phase

Integrating an ISPR strategy for equilibrium shift would result in a trade-off between the required (co-)product concentration in the reaction phase and the co-substrate excess. Hence, for the same generic reaction, given K_{eq}, thermodynamic reaction yield ($Y_{reaction}$) and initial limiting substrate concentration (A_0),the trade-offs where the process performance is achieved can be computed by solving a system of equations (Equation 8.2).

$$\begin{cases} K_{eq} = \dfrac{C_{eq} \cdot D_{eq}}{A_{eq} \cdot B_{eq}} \\ C_t = C_r + C_{eq} \\ C_t = A_0 \cdot Y_{reaction} \\ D_{eq} = A_0 \cdot Y_{reaction} \\ A_{eq} = A_0 \cdot (1 - Y_{reaction}) \\ B_{eq} = A_0 \cdot (1 - Y_{reaction} + Xs) \end{cases} \qquad \textbf{Equation 8.2}$$

Where C_t is the overall (co-)product production of the component C achieved for the reaction yield threshold value and C_r is the amount of (co-)product C removed from the reaction phase by ISPR (or IScPR).

The graphical representation of the required product concentration in the reaction phase and co-substrate concentration provides a preliminary indication how effective the ISPR technology put in place needs to be. However, the selection of the suitable ISPR technology will be further discussed in section 8.2.3, when *in-situ* co-product removal (IScPR) is evaluated simultaneously.

8.2.4 Step 4. Determine operating curve for simultaneous ISPR and IScPR

Rationale: For particularly thermodynamically challenged reactions, the above strategy(ies) might not yet result in a feasible process, as the concentration of the (co-)product required in the reaction phase will probably be too low to be achievable even with a very effective and selective ISPR technique. Furthermore, even at more reasonable requirements for product removal in the aqueous phase it is sensible to investigate the ease of simultaneously removing the co-product in order to alleviate the burden on one of the technologies.

Method: Hence, the trade-off between simultaneous removal of product and co-product needs to be plotted, in order to obtain a better selection of product and co-product removal technology (see Sections 8.2.4.1 - 8.2.4.3).

8.2.4.1 Step 4.1. Plot product against co-product concentration in the aqueous phase

Integrating an ISPR strategy with a simultaneous IScPR in order to shift the equilibrium would result in a trade-off between the required product and co-product concentration in the reaction phase. Therefore, for a given equilibrium constant (K_{eq}), thermodynamic reaction yield ($Y_{reaction}$), initial limiting substrate concentration (A_0) and co-substrate excess (Xs) the trade-offs where the process performance is achieved can be computed by solving a system of equations (Equation 8.3).

$$\begin{cases} K_{eq} = \dfrac{C_{eq} \cdot D_{eq}}{A_{eq} \cdot B_{eq}} \\ C_t = C_r + C_{eq} \\ D_t = D_r + D_{eq} \\ \\ C_t = A_0 \cdot Y_{reaction} \\ D_t = A_0 \cdot Y_{reaction} \\ A_{eq} = A_0 \cdot (1 - Y_{reaction}) \\ B_{eq} = A_0 \cdot (1 - Y_{reaction} + Xs) \end{cases}$$

Equation 8.3

Where D_t is the overall co-product production of the component D achieved for the reaction yield threshold value and D_r is the amount of co-product D removed from the reaction phase by IScPR.

8.2.4.2 Step 4.2. Mark in Step 4.1. the limits for ISPR and IScPR

Rationale: During the ISPR and IScPR selection, the physicochemical properties commonly investigated for separation are: volatility, water-solubility, charge, hydrophobicity and molecular size [49,50]. The sequence for selecting an appropriate separation is often determined by the separation driving force (i.e. the IS(c)PR technology chosen is that where the difference in physicochemical properties between the targeted compound to be separated and the other components in the reaction mixture is greatest) [50].

In particular, for the *in-situ* removal of the co-product, the use of enzymatic cascades to selectively remove the co-product from the reaction phase represents as a suitable solution. This IScPR technology takes advantage of the exquisite selectivity of enzymes to degrade (or recycle) the co-product. Theoretically, the co-product concentration obtained employing this process option can be as low as desired, since the rate of co-product conversion is only dependent on the amount of catalyst used for the conversion reaction(s) dosed in the reactor (due to the typically low K_m value of enzyme-catalysed reactions). However, the use of further enzymatic steps might increase the overall process cost (depending on the type of catalyst formulation adopted), as well as the complexity of the reaction system.

In most of the reported ISPR and IScPR techniques, the target molecule for selective (co-)product removal is at a higher concentration than the unreacted substrate [50]. However, these operating conditions are seldom observed in thermodynamically challenged reactions, since these processes often run under greater substrate concentration than the targeted molecule. Hence, the ISPR and IScPR techniques used in these circumstances must show high selectivity towards the product and low selectivity towards the substrates. The ideal ISPR (or IScPR) technique should show high selectivity for the target product without compromising its capacity in the presence of the other reaction compounds (reagents, co-product(s), reaction additives, etc.). However, high capacity ISPR techniques are generally associated with low selectivity towards the product [51].

Method: The adopted ISPR strategy is very much dependent on the molecules' properties and ideally should be tailor-made for the target molecule. Unless the IS(c)PR technique(s) chosen is(are) highly selective, the substrate(s) will be simultaneously removed from the reaction system, which will also affect the thermodynamic equilibrium and the reaction yield and kinetics. However, tailored-solutions are not often easy to find. To date there is no systematic approach for ISPR selection and the majority of the solutions put in place are done on a case-by-case basis.

8.2.4.3 Step 4.3. Plot product against co-product concentration in the aqueous phase for the selectivity of the selected ISPR and IScPR technique

Rationale: As mentioned earlier, one of the most important requirements for ISPR and IScPR is the selectivity of the separation technique selected (i.e. the ability to separate more effectively between the product and other compounds in the reaction phase) [50]. Operating with a non-selective IS(c)PR technology implies that other compounds in the reaction media are also removed together with the targeted molecule. Regardless the effect on the raw materials cost, applying a non-selective IS(c)PR is especially critical in thermodynamically challenged reactions as the thermodynamic equilibrium is affected.

Method: Hence, it is necessary to rectify the calculated relationship between the required product and co-product concentration in the reaction phase for the selectivity of the ISPR and IScPR methods adopted. Therefore, knowing the selectivity of the chosen ISPR and IScPR method (S_C and S_D, respectively), the equilibrium constant (K_{eq}), thermodynamic reaction yield ($Y_{reaction}$), initial limiting substrate concentration (A_0) and co-substrate excess (Xs) we can calculate the trade-off where the process performance is achieved by solving a system of equations (Equation 8.4).

Where $PC_{i,C}$ and $PC_{i,D}$ are the partition coefficient of component *i* for the ISPR and IScPR method selected, respectively ; A_t and B_t is the overall substrate and co-substrate consumed for the reaction yield threshold value; $A_{r,C}$ and $A_{r,D}$ are the amount of substrate A removed from the reaction phase when ISPR and IScPR are put in place, respectively; $B_{r,C}$ and $B_{r,D}$ are the amount of co-substrate B removed by the ISPR and IScPR, respectively; $C_{r,C}$ and $C_{r,D}$ are the amount of product C removed from the reaction phase when ISPR and IScPR are put in place, respectively; and $D_{r,C}$ and $D_{r,D}$ are the amount of substrate D removed by ISPR and IScPR, respectively. For instance, a highly selective ISPR method implies an infinite value for S_C, as the partition coefficient for the other compounds is zero.

8.2.5 Step 5. Determine reaction kinetics

Rationale: Process mathematical models provide a good process insight. Developing a kinetic model allows a rapid understanding and evaluation of the type of options required for process optimisation.

$$
\begin{cases}
K_{eq} = \dfrac{C_{eq} \cdot D_{eq}}{A_{eq} \cdot B_{eq}} \\
A_t = A_0 \cdot (1 - Y_{reaction}) \\
B_t = A_0 \cdot (1 - Y_{reaction} + Xs) \\
C_t = A_0 \cdot Y_{reaction} \\
D_t = A_0 \cdot Y_{reaction} \\
A_t = A_{r,C} + A_{r,D} + A_{eq} \\
B_t = B_{r,C} + B_{r,D} + B_{eq} \\
C_t = C_{r,C} + C_{r,D} + C_{eq} \\
D_t = D_{r,C} + D_{r,D} + D_{eq} \\
PC_{A,C} = \dfrac{A_{r,C}}{A_e} \\
PC_{A,D} = \dfrac{A_{r,D}}{A_e} \\
PC_{B,C} = \dfrac{B_{r,C}}{B_e} \\
PC_{B,D} = \dfrac{B_{r,D}}{B_e} \\
PC_{C,C} = \dfrac{C_{r,C}}{C_e} \\
PC_{C,D} = \dfrac{C_{r,D}}{C_e} \\
PC_{D,C} = \dfrac{D_{r,C}}{D_e} \\
PC_{D,D} = \dfrac{D_{r,D}}{D_e} \\
S_C = \dfrac{PC_{C,C}}{PC_{A,C} + PC_{B,C} + PC_{D,C}} \\
S_D = \dfrac{PC_{D,D}}{PC_{A,D} + PC_{B,D} + PC_{C,D}}
\end{cases}
$$

Equation 8.4

Often biocatalytic reactions are not only thermodynamically challenged but also kinetically challenged. Therefore, strategies to shift the equilibrium should be considered alongside with strategies to overcome substrate and product inhibition of the biocatalyst. A validated kinetic model is useful for predicting process performance under different operating conditions (e.g. concentration of the reaction components), in order to define an operating space, where the required biocatalyst specific activity can be achieved.

The performance of the biocatalyst can be affected mainly by stability and/or inhibition, which is ultimately reflected in the reaction rate (and biocatalyst specific activity). Moreover, the process kinetic model should ideally integrate not only the reaction kinetic model, but also dynamic models describing the mass transfer phenomena occurring in ISPR, IScPR and/or ISSS strategies adopted.

Method: The methodology is given in Section 8.2.5.1.

Step 5.1. Plot product against co-product concentration in the aqueous phase

The kinetic model can be plotted as a 3D-surface depicting the biocatalyst performance (i.e. biocatalyst specific activity) against a range of suitable operating variables (i.e. concentration of product, co-product and substrate in the aqueous phase). The biocatalyst performance metric can be determined for the assumed biocatalyst yield and process space-time yield (see Section 5.2.1). The 3D-surface is cut at the defined threshold value for the biocatalyst specific activity, creating a region where the reaction achieves the desired performance.

8.2.6 Step 6. Generate an window of operation

Rationale: All the threshold values for the process metrics and technology constraints (e.g. for ISPR and IScPR) are now plotted on common axes (operating variables, product and co-product concentration in the reaction phase) and the area where the process fulfils all the requirements (defined by the constraints and/or threshold values for process metrics) is the window of operation for the process. This methodology provides a proper visualisation: either the process is feasible (if there is a window bounded by all the aforementioned constraints and process metrics threshold values) or not (if there is no window). Moreover, by adopting this graphical methodology, the sensitivity of different metrics and constraints used as inputs to the window can be discerned.

Method: The window of operation can be obtained by superimposing the plots representing the hard constraints, i.e. the concentration of product, co-product and co-substrate to shift the equilibrium to the desired thermodynamic reaction yields,

with the limitations of the possible ISPR and IScPR techniques adopted and the kinetic plot, resulting from cutting the 3D-surface generated in Step 5. at the defined threshold performance metric for the biocatalyst specific activity).

8.2.7 Step 7. Perform scenario analysis

Rationale: In this final step, a scenario analysis (what-if analysis) is carried out. A scenario analysis is one of the most simple and straightforward forms of evaluating alternative process options not considered in the above methodological flow. This methodology allows a rapid and effective way to study the effects of potential improvements process and biocatalyst by setting threshold values for the process metrics at relevant process conditions (see Section 5.2.1).

Method: Relaxing the threshold values for the process metrics (i.e. biocatalyst yield, reaction yield, space-time yield and final product concentration) is expected to affect greatly the cost of the process. In addition, modifying these parameters will also affect the size, position and shape of the window of operation. Hence, this methodology allows a quick evaluation of the trade-offs between the production cost (in the form performance metrics) and the development cost (implicit in the size and position of the window of operation). Hence, by coupling this methodology to the development of the biocatalyst, targets for the biocatalyst development can be set, avoiding over-development, i.e. a situation where the process is no longer limited by the reaction kinetics, but instead by the thermodynamics.

Finally, the scenario analysis also provides a quick evaluation of different ISPR and IScPR technologies available at the current state-of-the-art. The methodology is able to set targets for the ISPR, IScPR and/or ISSS technique, by pinpointing the minimum required product, co-product and/or substrate concentrations in the aqueous phase. Hence, this methodology could be coupled to a rational approach for *in-situ* product removal selection.

8.3 Case study 2: Chiral amine production using ω-transaminase

8.3.1 Introduction

Biocatalytic synthesis of optically pure compounds has emerged as an attractive complement to heterogeneous and homogeneous catalysis, due to the high selectivity [38] and mild conditions, avoiding the need for protection and deprotection strategies, resulting in processes with fewer steps and potentially increasing the overall process yield whilst reducing downstream process costs [1,219,262]. Together with the

possibility to tailor the biocatalyst properties using molecular biology tools [48,65] and a potentially green profile [24], biocatalysis is a particularly attractive technology to assist in the synthesis of industrially relevant compounds, such as optically pure chiral amines.

Chiral amines are key building blocks for many pharmaceuticals (NCEs and APIs). Chiral amines can be produced both by chemical and biocatalytic synthesis [263]. In recent years, ω-transaminase-catalysed reactions have proven to be an attractive complement to the existing routes for the synthesis of optically pure chiral amines. ω-transaminases (EC 2.6.1.X, also known as aminotransferases) catalyse the transfer of an amine group from a donor molecule to a prochiral ketone (Figure 8.2) using pyridoxal-phosphate (PLP) as a cofactor [231]. Regarding the reaction step, there are two main strategies to produce chiral amines: direct asymmetric synthesis or kinetic resolution of a racemic mixture of amines. The kinetic resolution approach is a potentially industrial attractive route for the production of enantiopure chiral amines due to its favourable thermodynamic equilibrium. However, these reactions are hampered by a theoretical 50% reaction yield [36]. The alternative route, asymmetric synthesis, is the focus of this chapter. Asymmetric synthesis is generally the preferred reaction configuration due to the higher theoretical reaction yields and facilitated separation steps.

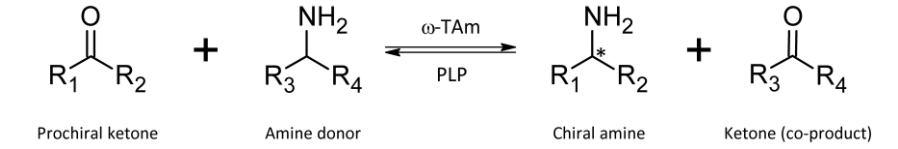


Figure 8.2. Model transaminase-catalysed reaction; * chiral centre

Nevertheless, as a relatively new technology, many of the proposed transaminase-catalysed syntheses do not yet fulfil the required economic metrics necessary for process scale-up [16]. Frequently encountered challenges for the biocatalytic synthesis of chiral amines using ω-transaminases include potentially unfavourable thermodynamic equilibrium, low biocatalyst activity and stability, as well as substrate and product inhibition (Table 8.2). To overcome these limitations there are several possible process solutions, as well as alternative solutions via biocatalyst engineering and chemistry (i.e. reaction engineering) related solutions (Table 8.2). Potential process solutions include operating at substrate excess (i.e. addition of an excess of amine donor), application of *in-situ* product removal (ISPR) and *in-situ* co-product removal (IScPR) or combination of these (Table 8.2).

Of recently published studies, only four successfully attained the process metrics suitable for a sustainable and viable process, all of them by applying the aforementioned process solutions [37,264-266] (Figure 8.3). In the studies by Savile

and co-workers [37] and Martin and co–workers [264], process feasibility was achieved by operating with an amine donor excess and using protein engineering techniques to improve biocatalyst yield and tolerance to higher concentrations of the substrate [37,264]. Interestingly, Truppo and co-workers [265] achieved process feasibility by only applying process solutions (use of enzymatic cascades and *in-situ* product removal) [265]. While in the latest successful study, Truppo and his co-workers [266] attained process feasibility following the previous work performed by Savile and co-workers [37] by applying protein engineering techniques and enzyme immobilisation while employing a greener solvent to increase substrate solubility in the reaction medium [266]. Despite the successful results, all of these studies were developed on a case-by-case basis and probably the true optimal process is yet to be designed. Ultimately, the ideal strategy would be a joint effort for process and biocatalyst improvement that should be carried out side-by-side and guided by a rational methodology in order to decrease development resources and time.

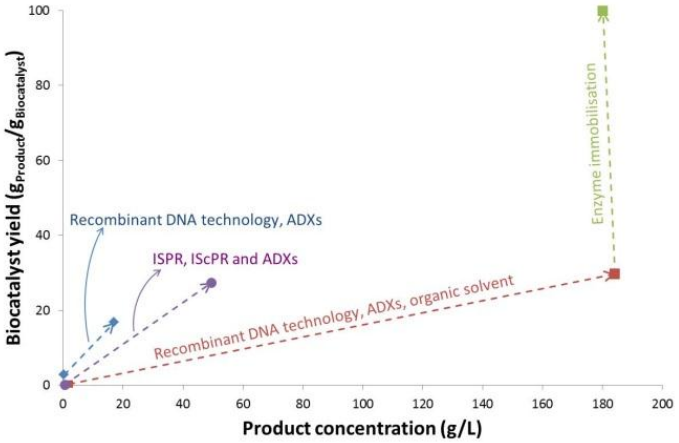


Figure 8.3. State-of-the-art of development strategies for biocatalytic processes; Legend: – – · Improvements in (*S*)-aminotetralin production by protein engineering [264] using whole-cell (biocatalyst yield in $g_{Product}/g_{CDW}$); – – · Improvements in sitagliptin production by protein engineering and 50% DMSO [37]; – – · Improvements in PEA production by ion-exchange resin for ISPR and enzymatic cascade for IScPR [265]; – – · Improvements in sitagliptin production by enzyme immobilisation and water saturated with isopropyl acetate [266] (biocatalyst yield in $g_{Product}/g_{immobilised\ enzyme}$).

Table 8.2. Bottleneck analysis. Analysis of challenges in biocatalytic transamination and implications for how suggested solutions influence which technologies that are suitable/compatible

			Challenges								
			Reaction related			Biocatalyst related				Major limitations	Implementation
			Thermodynamically challenged reaction	Low substrate solubility	Substrate/ Product liability	Inhibition	Stability	Activity	Biocatalyst separation		
Solutions	Chemistry related	Stronger amine donor	✓							Cost and availability of the donor	Selection methodology
	Process related	Excess amine donor	✓							Inhibition and stability of enzyme; Not applicable for low Keq; donor cost; DSP	Maximum defined by solubility, inhibition and stability limits (see Section 8.2.2)
		Solvent (co-solvent/2-phase)		✓						Limited enzyme stability; DSP; VOC emissions	Solvent screening
		Separation of (co-)product (ISPR and ISCPR) by a) distillation or stripping	✓							Co-distillation of water and/or other components	
		b) Extraction (solvent, membrane, resin)	✓		✓	✓				Limited selectivity between substrates and products	Screening methodology
		Controlled supply of substrate (ISSS, fed-batch)		✓	✓	✓				Limited capacity	Screening methodology
		Conversion of co-product (cascade)	✓							Compatibility; Added cost of biocatalysts; cofactors recycling	Iteration between compounds and mismatch conditions
	Biocatalyst related	Whole-cell	✓						✓	Side-reactions; separation; GMO regulations	Recombinant DNA technology (mutant screening)
		Immobilisation					✓		✓	Deactivation; development cost; higher biocatalyst cost	Screening methodology for selection of immobilization technique
		Enzyme development				✓	✓	✓		Development time and cost	Enzyme engineering (mutant screening)

8.3.2 Methodology application

In order to guide development efforts and process design at an early stage of process development a methodological approach is required. The methodology put forward in section 8.2 identifies potential windows of operation, defines guidelines for process and biocatalyst development and assists in the choice of the most suitable process option (i.e. suitable ISPR and IScPR technologies).

The proposed methodology is demonstrated for the synthesis of (*S*)-1-phenyl-ethylamine (PEA) using propan-2-amine (or isopropylamine, IPA) as the amine donor and ω-transaminase (ATA-40, c-Lecta, Leipzig, Germany) as the biocatalyst (full experimental details are given at [267]). In this system, acetophenone (APH) is the prochiral ketone (or acceptor) and acetone is the co-product (Figure 8.4).

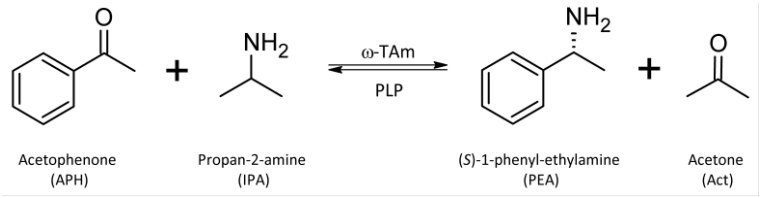


Figure 8.4. Biocatalytic production of PEA using IPA as amine donor by ω–transaminase (ATA-40)

Regardless the selection of the substrate (that is only determined by the desired product), when employing transaminases the choice of amine donor (co-substrate) is a very important issue. Different amine donors can strongly affect the thermodynamic equilibrium constant [30], the biocatalyst specific activity and may imply a different process technology(ies) and downstream operating units. Nevertheless, the use of alternative amine donors for the synthesis of PEA will not be discussed is this chapter.

8.3.2.1 Step 1. Determine reaction thermodynamic equilibrium (K_{eq})

The thermodynamic equilibrium constant for the adopted system was experimentally determined by Tufvesson and co-workers according with Equation 8.5 [30]. The thermodynamic equilibrium was found to be as 0.033 [30], i.e. strongly in favour of the reagents over the products (see also Section 8.2.1).

$$K_{eq} = \frac{[PEA]_{eq} \cdot [Ace]_{eq}}{[APH]_{eq} \cdot [IPA]_{eq}} \qquad \textbf{Equation 8.5}$$

8.3.2.2 Step 2. Determine required co-substrate excess

In order to evaluate which substrate should be dosed in excess, the process engineer must consider the cost of the substrate and the ease of recovery. In the presented case study propan-2-amine (IPA) was the substrate dosed in excess (while acetophenone (APH) is the limiting substrate), because IPA is a cheaper substrate and its recovery is potentially facilitated by applying ion-exchange chromatography, exploiting the differences of the isoelectric point of the different compounds at different pH values.

Step 2.1. Plot required substrate excess against thermodynamic yield

Based on assumed threshold values for the reaction yield (see Section 5.2.1) and in the reaction thermodynamic equilibrium (see Section 8.3.2.1), the co-substrate excess is required (Figure 8.5).

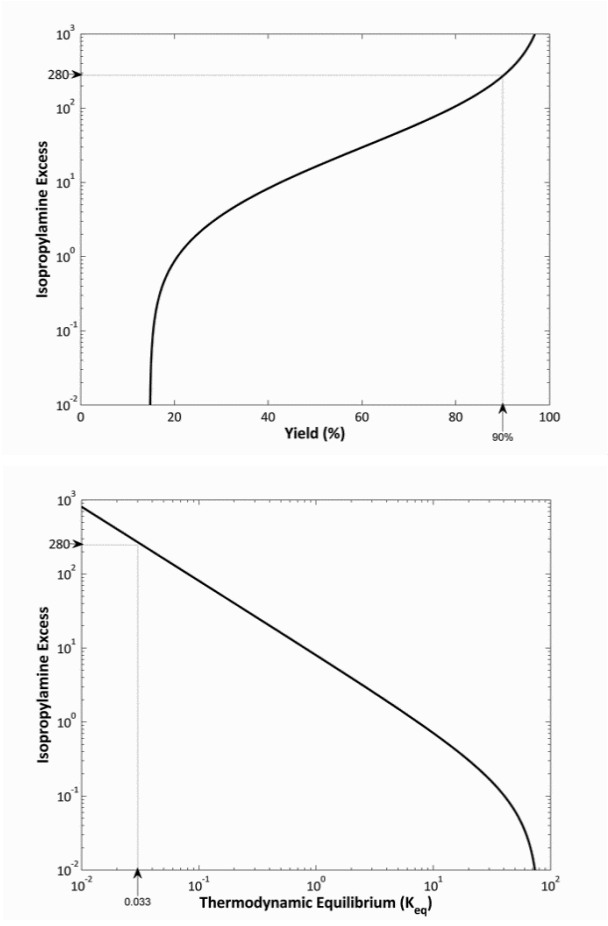


Figure 8.5. Required IPA excess to shift the thermodynamic equilibrium **A** at fixed reaction equilibrium constant aiming different reaction yield **B** at fixed reaction yield for different reaction equilibrium constants

Figure 8.5 shows that in order to obtain reaction yield of 90%, IPA should be dosed at 280-fold excess, for the determined reaction kinetic equilibrium constant. However, when relaxing the performance constraint for the reaction yield, the required IPA excess for shifting the reaction equilibrium drops according with Figure 8.5 A (see also 8.3.2.7). Furthermore, when considering other amine donors for the synthesis of 1-phenylethylamine (PEA) the reaction thermodynamic equilibrium constant differs and the co-substrate excess required will vary according with Figure 8.5 B.

Step 2.2. Determine substrate excess limits

It is necessary to evaluate to what extent co-substrate dosing is feasible, bearing in mind the co-substrate solubility limits and the effect in the reaction kinetics. The co-substrate excess is a function of the final product concentration and yield threshold values, as shown in Equation 8.6 and Equation 8.7.

$$[APH]_0 = \frac{[PEA]_f}{Yield} \qquad \textbf{Equation 8.6}$$

$$[IPA]_0 = [APH]_0 \cdot \left(1 + Xs_{IPA}\right) \qquad \textbf{Equation 8.7}$$

The initial co-substrate concentration (in the case study $[IPA]_0$) must be lower than the solubility limit (16.9 M, [36]) and it should be lower than the inhibitory concentration observed (5 M, [268]). Figure 8.6 shows the allowable co-substrate excess for the case study.

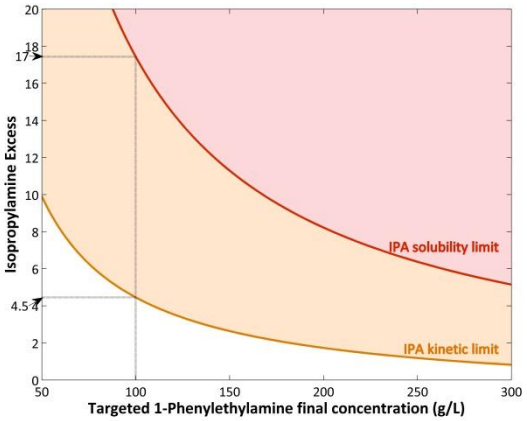


Figure 8.6. Maximum IPA excess for varying final amount of product produced per litre of reactor (assuming 90% of reaction yield) based on the solubility of the IPA (—) and inhibitory concentrations of IPA (—)

Figure 8.6 shows that the maximum co-substrate excess is a function of the performance constraint defined for the final product concentration (see also 8.3.2.7).

When a lower final product concentration is required the maximum allowable co-substrate excess increases, as shown in Figure 8.6. While at higher final product concentrations (closer to those required for bulk chemicals) the maximum allowable excess decreases and operating with very low or no co-substrate excess (i.e. at stoichiometric concentration of co-product) becomes virtually compulsory in order to avoid operating above the co-substrate solubility limit and to decrease downstream costs (particularly crucial in bulk chemicals).

Figure 8.6 shows that, for the final product concentration of 100 g/L, the maximum co-substrate excess that the reaction can be operated is 17-fold, above this value the co-substrate is not soluble and forms a second phase. Further, it is also necessary to consider the inhibitory effects of IPA concentration on the reaction kinetic performance. Hence, for a non-engineered catalyst the maximum co-substrate excess in which the reaction can be operated is 4.5-fold. However, this value can increase (up to a maximum of 17-fold, solubility limit) if the inhibition profile of the catalyst towards IPA is improved (see also 8.3.2.7).

Finally, Figure 8.6 proves that for the presented case study, the performance level defined in Step 2 (see Section 5.2.1) cannot be reached by exclusively applying co-substrate excess as a strategy for shifting the reaction equilibrium.

8.3.2.3 Step 3. Determine product or co-product concentration required

Hence, other ways to shift the reaction thermodynamic equilibrium towards the product (PEA) must be explored. Another strategy to displace the thermodynamic equilibrium towards the product side is by selectively and continuously removing the product or co-product from the reaction phase as it is formed by applying *in-situ* product removal (ISPR) or *in-situ* co-product removal (IScPR), respectively.

Step 3.1. Plot substrate excess against (co-)product concentration in the aqueous phase

The reaction equilibrium is then defined according with Equation 8.8 and Equation 8.9 for ISPR or Equation 8.10 when IScPR is applied.

$$K_{eq} = \frac{[PEA]_{aq} \cdot [Ace]_{aq}}{[APH]_{aq} \cdot [IPA]_{aq}} \qquad \textbf{Equation 8.8}$$

$$[PEA]_T = [PEA]_{aq} + [PEA]_{removed} \qquad \textbf{Equation 8.9}$$

$$[Ace]_T = [Ace]_{aq} + [Ace]_{removed} \qquad \textbf{Equation 8.10}$$

Figure 8.7 shows the trade-off between the IPA excess and the concentration of product or co-product in the aqueous (reaction) phase.

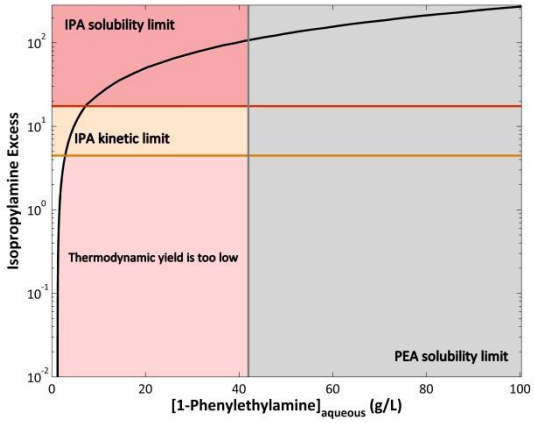


Figure 8.7. Required IPA excess at varying concentrations of **A** product and **B** co-product in the aqueous phase applying ISPR and IScPR, respectively; Assumptions: 100 g of product produced per litre of reactor; reaction yield 90%. Legend: —— IPA solubility limit; —— isopropylamine kinetic limit; —— PEA solubility limit (42 g/L, [36])

In Figure 8.7 the operating area (in white) shows the region where the performance metrics are achieved for final product concentration and reaction yield. The black curves show the operating curves of substrate excess and product or co-product removal (Figure 8.7 A or Figure 8.7 B, respectively). Below this curve, the performance metrics are not achieved (light red area). The grey area in Figure 8.7 A represents the area when the product reaches the solubility limit (42 g/L, [36]), forming a second phase in the reactor. The darker red area represents the unfeasible area for the co-substrate excess, where the co-substrate concentration is higher than its solubility limit. Finally, the orange area represents where the concentration of IPA is higher than its inhibitory limit and the reaction kinetics would be affected if operating at this co-substrate concentration. However, the feasible space for the predefined performance

metrics (Section 5.2.1) in this strategy can be enlarged if protein engineering is considered. Figure 8.7 shows the target product or co-product concentrations in the reaction phase to effectively displace the thermodynamic equilibrium and reach the threshold value for the reaction yield.

8.3.2.4 Step 4. Determine operating curve for simultaneous ISPR and IScPR

For reactions where the thermodynamic equilibrium is particularly challenged, operating with a co-substrate excess together with simultaneous removal of the product (PEA) and co-product (acetone) might constitute a suitable strategy to shift the reaction equilibrium in order to attain the defined performance metrics. The reaction equilibrium is then defined according with Equations 8.8 – 8.10.

Step 4.1. Plot product against co-product concentration in the aqueous phase

Figure 8.8 shows the trade-off between the concentration of product and co-product in the reaction phase at different IPA excess.

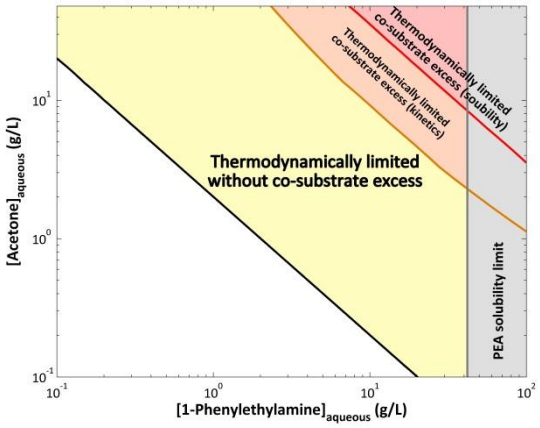


Figure 8.8. Required concentrations of product and co-product in the aqueous phase applying ISPR and IScPR, respectively; Assumptions: 100 g of product produced per litre of reactor; reaction yield 90%. Legend: —— without IPA excess —— IPA solubility limit; —— IPA kinetic limit; —— PEA solubility limit

Figure 8.8 shows how a combined strategy with simultaneous removal of product and co-product as well as an excess of amine donor can shift the equilibrium towards the product side. As expected, the requirements for ISPR and IScPR are higher when a lower excess of amine donor is used (black line, represents no IPA excess). The lines (black, orange and red) show the operating curves of PEA and acetone removal at different substrate excess (stoichiometric concentration, limit for inhibitory co-substrate excess, co-substrate excess at solubility limit, respectively). Above this curve, the performance metrics are not reached. The grey area in Figure 8.8 represents the area where the product reaches the solubility limit (42 g/L, [36]), forming a second

phase in the reactor. The red area represents the unfeasible area for the co-substrate excess, where the co-substrate concentration is higher than its solubility limit. The orange area represents the area where the concentration of IPA is higher than its inhibitory limit and the reaction kinetics would be affected if operating at this co-substrate concentration. Finally, the yellow area represents a possible operating space(s) for co-substrate excess between the stoichiometric concentration (i.e. no excess) and the excess at the kinetic limit (4.5-fold excess).

Step 4.2. Mark in Step 4.1. the limits for ISPR and IScPR

It is now necessary to define the minimum concentration that each potential process technology for ISPR or IScPR can achieve (as well as the capacity for removal). However it is very hard to generalize for a given technology because the performance (and thus, the limits) of *in-situ* (co-)product removal technology put in place is highly dependent on the physical properties of the compounds targeted for separation and the compounds present in the reaction phase.

Tufvesson and co-workers have summarised the applied ISPR strategies for the synthesis of chiral amines using transaminase [36]. Table 8.3 lists the current state-of-the-art for the process technologies available for ISSS, ISPR and IScPR for the synthesis of PEA, using IPA as amine donor.

Table 8.3. Process technologies available for the production of *(S)*-PEA by ω-transaminase using IPA as amine donor

Process technology	Motivation	Options		Disadvantages
ISSS APH feed	Avoid inhibition of the biocatalyst		Fed-batch	Limited by the concentration in the feed; Mixing
		Resins	Neutral polymeric resin	Competition with PEA
			Organic solvents	Decrease of the enzyme stability; Competition with PEA
ISPR PEA removal	Avoid inhibition of the biocatalyst; Shift the thermodynamic equilibrium	Resins	Neutral polymeric resin	Competition with APH
			Ion-exchange resin	Competition with IPA
			Organic solvents	Decrease of the enzyme stability; Competition with APH
IScPR Acetone removal	Shift the thermodynamic equilibrium	Cascade	Enzymes: ADH and GDH/FDH*	DSP; Cost of the enzymes and cofactor
			Stripping	Simultaneous removal of APH (at high [APH])

* This enzymatic cascade converts acetone into isopropyl alcohol. It requires the use of cofactor (NAD(P)H) and a final electron acceptor (glucose or formate, respectively) for cofactor regeneration; ADH – alcohol dehydrogenase; GDH – glucose dehydrogenase; FDH - formate dehydrogenase

Regarding acetone removal (IScPR), the usually applied technologies explore the difference in the vapour pressure (gas swiping or operating in vacuum), enzymatic cascade for conversion of acetone into isopropyl alcohol (Figure 8.9) and/or membrane technology. Acetone stripping or evaporation can be used to reduce acetone concentration in the reactor [269,270]. However, to achieve a low acetone concentration in the reaction phase, the amount of water that needs to be evaporated will ultimately be too high for a feasible process. Moreover, the required gas flow required for stripping will be enormous, which makes the scale-up uneconomical due to the costs of the gas compression. Other IScPR strategies such as enzymatic cascade reactions are potentially suitable when it is necessary to remove the co-product to very low concentrations [271]. For instance, ketoreductases used for co-product reduction to a secondary alcohol are active even at low co-product concentrations [237]. The relative biocatalytic activity of all the enzymes in the cascade can be adapted to the process needs by adjusting the biocatalyst amounts in the system. On the other hand, enzyme cascades will add to the process cost and need to be compatible with the process conditions for ω-transaminase reaction.

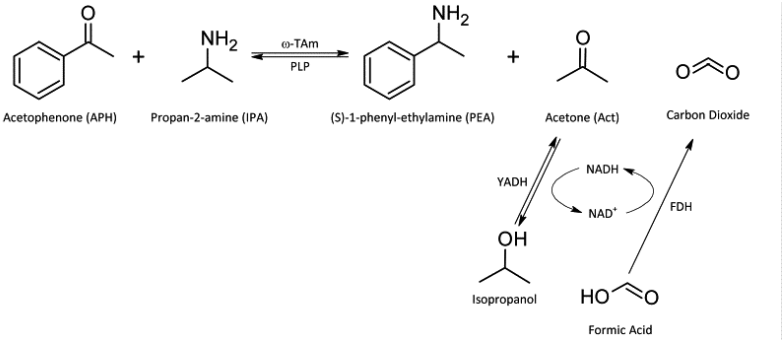


Figure 8.9. ω-Transaminase-catalysed synthesis of *(S)*-1-phenylethylamine with IPA as donor. The equilibrium displacement is attained by applying yeast alcohol dehydrogenase (YADH) and formate dehydrogenase (FDH), degrading the acetone to isopropyl alcohol and consequent cofactors regeneration

Reported ISPR and IScPR strategies and their limitations were summarised in Table 8.4. By combining the thermodynamic and biocatalyst constraints, it was possible to define the minimum requirements for ISPR and/or IScPR.

Figure 8.10 arises from marking the limits from Table 8.4 on Figure 8.8.

Table 8.4. Examples found in scientific literature for process limitations for ISPR and IScPR.

Process Limitation	Min. concentration in the reactor (g/L)	Reference
ISPR using ion-exchange resins	3.64	[265]
IScPR using acetone enzyme cascade	$1x10^{-3}$	[269]
IScPR using acetone stripping	$5.81\ x10^{-1}$	[269]

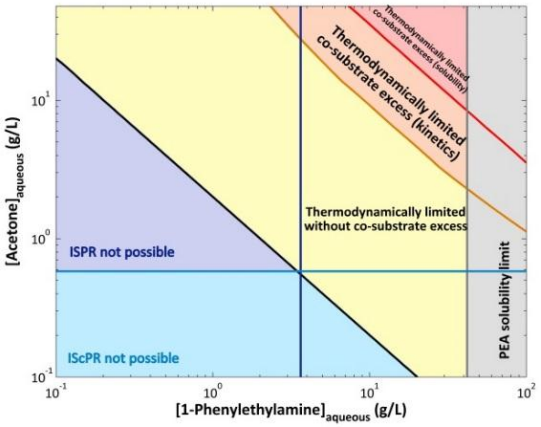


Figure 8.10. Required concentrations of product and co-product in the aqueous phase applying ISPR and IScPR, respectively; Assumptions: 100 g of product produced per litre of reactor; reaction yield 90%. —— without IPA excess; Legend: —— IPA solubility limit; —— IPA kinetic limit; —— PEA solubility limit; —— ISPR limit (ion-exchange resin); —— IScPR limit (acetone stripping)

Figure 8.10 shows that the operating region to displace the reaction thermodynamic equilibrium where the process assumptions (regarding reaction yield and concentration) are satisfied is very dependent on the amine donor excess used. Operating with stoichiometric amount of prochiral ketone and amine donor is often preferred at large-scale production (black line in Figure 8.10). However, Figure 8.10 shows that operating at this condition implies a small operating region where the assumed requirements for the performance metrics are achieved. Nevertheless, the operating region can be slightly enlarged if, instead of using acetone stripping to remove the co-product (light blue region), an enzymatic cascade is put in place. Further, operating with a value of co-substrate excess between the stoichiometric concentration and 4.5-fold excess (yellow area) can also be a suitable strategy to enlarge the feasibility region. Whereas operating at higher co-substrate excess will compromise the biocatalyst performance.

Step 4.3. Plot product against co-product concentration in the aqueous phase for the selectivity of the selected ISPR and IScPR technique

For simplicity, so far in the presented case study the ISPR and IScPR requirements plotted in Figure 8.7 and Figure 8.8 are assumed highly selective. The selectivity of the process technology adopted can highly influence its performance. However, to date not many studies have explored this drawback and there is a lack of consensus in defining the selectivity of a given ISPR or IScPR technology.

In this work, IS(c)PR selectivity towards product and co-product selectivity is defined according to Equation 8.11 and Equation 8.12, respectively.

$$S_{PEA} = \frac{PC_{PEA}}{PC_{APH} + PC_{IPA} + PC_{Ace}} \quad \textbf{Equation 8.11}$$

$$S_{Ace} = \frac{PC_{Ace}}{PC_{APH} + PC_{IPA} + PC_{PEA}} \quad \textbf{Equation 8.12}$$

Where PC_i is the partition coefficient of the compound *i* and is defined according with Equation 8.13 to Equation 8.16.

$$PC_{APH} = \frac{C_{APH_{removed}}}{C_{APH_{aq}}} \quad \textbf{Equation 8.13}$$

$$PC_{IPA} = \frac{C_{IPA_{removed}}}{C_{IPA_{aq}}} \quad \textbf{Equation 8.14}$$

$$PC_{PEA} = \frac{C_{PEA_{removed}}}{C_{PEA_{aq}}} \quad \textbf{Equation 8.15}$$

$$PC_{PEA} = \frac{C_{PEA_{removed}}}{C_{PEA_{aq}}} \quad \textbf{Equation 8.16}$$

Further, the expression for selectivity (shown in Equation 8.11) can be simplified according to the type of ISPR used. For instance, when applying neutral polymeric resins or liquid-liquid extraction to remove PEA, the ISPR technology can also remove the substrate (APH) as these ISPR technologies exploit the hydrophobicity properties of the components. Hence, a negligible amount of co-product (acetone) and co-substrate (IPA) are removed and therefore Equation 8.11 becomes:

$$S_{PEA} = \frac{PC_{PEA}}{PC_{APH}} \quad \textbf{Equation 8.17}$$

In a similar way, when applying ion-exchange resins to remove the product [265], the property explored is the isoelectric point (i.e. the protonation level) of the aminated compounds (i.e. PEA and IPA). However, this technology might imply that there is simultaneous removal of the product and the co-substrate (amine donor) and therefore Equation 8.11 becomes:

$$S_{PEA} = \frac{PC_{PEA}}{PC_{IPA}}$$ **Equation 8.18**

Figure 8.11 shows the ISPR and IScPR requirements for different ISPR selectivity (for a neutral polymeric resin). In Figure 8.11 it is assumed that the co-product removal is performed by using a highly effective enzymatic cascade for the conversion of acetone to isopropyl alcohol (S=∞).

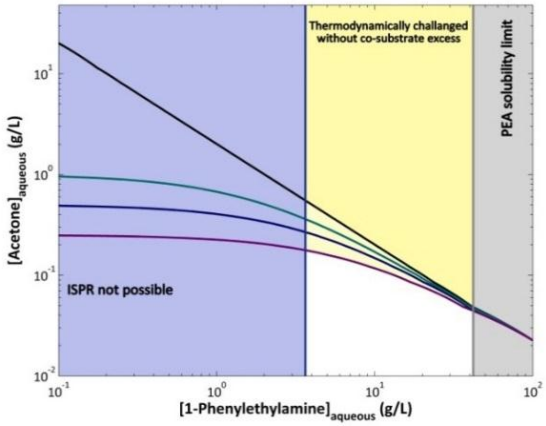


Figure 8.11. Required concentrations of product and co-product in the aqueous phase applying ISPR and IScPR, respectively with stoichiometric amounts of substrate (APH) and co-substrate (IPA); Assumptions: 100 g of product produced per litre of reactor; reaction yield 90%. Legend: highly selective ISPR S=∞; ISPR selectivity S=50; ISPR selectivity S=25; ISPR selectivity S=12.5;

As expected, the use of a less selective product removal method implies a bigger effort to displace the thermodynamic equilibrium, which is put on the co-product removal side. Hence, a lower co-product concentration in the reaction phase is required in order to achieve the performance threshold value for the reaction yield.

However, many of the studies reporting ISPR and IScPR efficiency for ω-transaminase-catalysed reactions do not give a complete characterisation of the method applied and often only capacity or concentration in the reaction phase have been reported. For simplicity in the following steps we will assume highly selective methods for PEA or acetone removal (i.e. S=∞).

8.3.2.5 Step 5. Determine reaction kinetics

The previous steps are put in place to determine suitable strategies for the displacement of the reaction equilibrium. However, it essentially considers an optimal and infinitely improved biocatalyst, as the impact of the concentration of the different reaction compounds in the biocatalyst performance is not explored. Therefore, developing a process model that can describe not only the enzyme-catalysed reaction but also the mass transfer is of great importance in enabling evaluation of the different process technologies and the catalyst formulation adopted.

Step 5.1. Plot product against co-product concentration in the aqueous phase

The model established by Al-Haque and co-workers [267] for this reaction catalysed by the ω-transaminase (ATA-40 from c-Lecta) was used to set up a window of operation. The modelled reaction system is strongly inhibited even at low substrate concentrations [267]. Therefore, a substrate feeding strategy is required to keep the substrate concentration lower than the inhibitory limits (Table 8.3). Throughout this work, the concentration of APH was kept at its solubility limit (i.e. 50 mM) by applying a substrate feeding strategy.

Furthermore, transaminase-catalysed reactions are often strongly inhibited by the product of the reaction, even at low concentrations. In order to overcome this challenge several different strategies can be applied such as using a multiphasic reaction (e.g. using a water-immiscible organic solvent or an insoluble porous resin as a second phase reservoir). Alternatively, this could also be addressed by modification to the biocatalyst itself (this option will be explored in Section 8.3.2.7). Figure 8.12 shows the kinetic profile for an ω-transaminase-catalysed reaction for the defined performance conditions.

Figure 8.12 shows the reaction specific activity for different amine donor excess at different concentrations of product and co-product in the reaction (aqueous phase). The reaction was inhibited at very low product concentrations (above 1 mM) as reflected by the kinetic parameters, namely in the Michaelis constant and product inhibition constant for PEA [267]. Therefore, a successful process requires removal of PEA, not only to shift equilibrium (as shown in the previous sections), but also to avoid product inhibition and thus achieve a higher biocatalyst yield. Further, the biocatalyst specific reaction rate was also affected by the concentration of acetone in solution and therefore IScPR should be put in place, not only to ensure a shift of equilibrium, but also to increase biocatalyst specific activity.

Figure 8.12 B and Figure 8.12 C analyse the effect of co-substrate excess on the reaction kinetics. The kinetic model used does not consider biocatalyst inhibition at high IPA concentrations (higher than 5 M, [268]). Hence, catalyst specific reaction rate

depicted in Figure 8.12 B and Figure 8.12 C was not shown to be negatively affected at high concentrations of co-substrate. This is also shown in Figure 8.13.

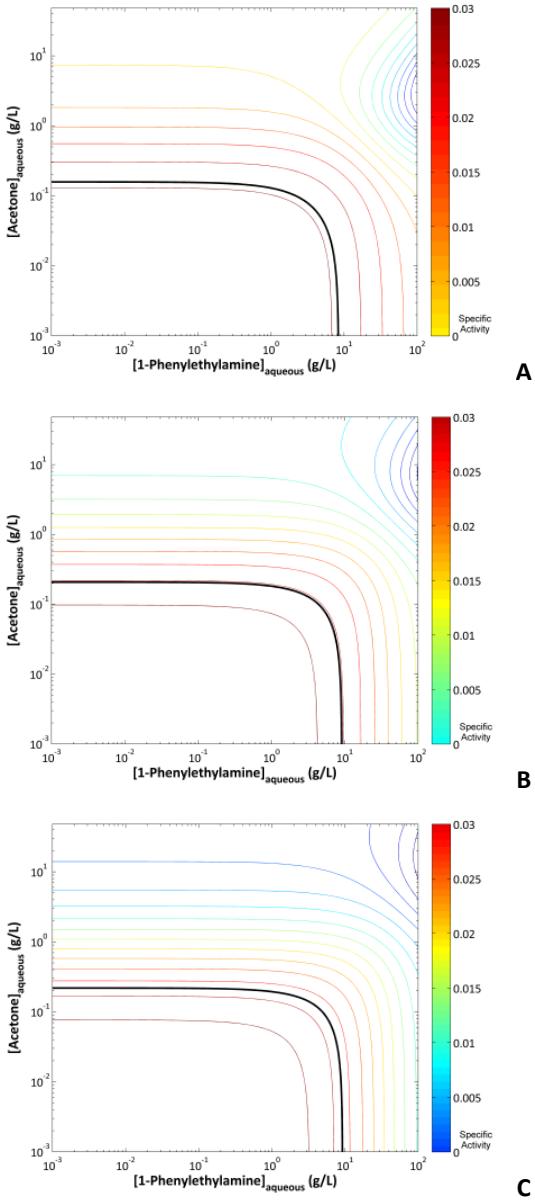


Figure 8.12. Biocatalyst specific reaction rate (expressed as g $_{product}$/(g $_{biocatalyst}$. h)) in the synthesis of PEA using IPA as amine donor **A** using stoichiometric amount of substrate and co-substrate, **B** using 4.5-fold excess of IPA (kinetic limit), **C** using 17-fold excess of amine donor (solubility limit)

The contour plots shown in Figure 8.12 can be cut at a single defined value of biocatalytic specific activity, giving a region where the threshold value for the specific activity is achieved bounded by a curve at constant specific activity (0.03 g $_{product}$/(g $_{biocatalyst}$. h), black curves in Figure 8.12). Figure 8.13 is obtained by overlapping the three black lines from Figure 8.12 A to C.

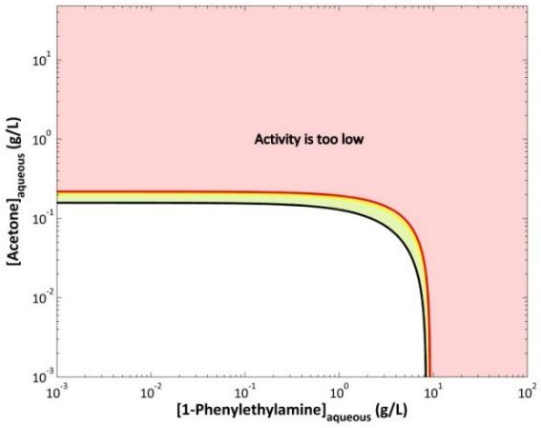


Figure 8.13. Product and co-product concentration in the reaction phase operation curve for constant biocatalyst specific reaction rate 0.03 expressed as g $_{product}$/(g $_{biocatalyst}$. h) in the synthesis of PEA using IPA as amine donor. Legend: —— without IPA excess; —— IPA concentration at solubility limit; —— IPA concentration at kinetic limit

In Figure 8.13, the red shaded area represents the operating space of different combinations of product and co-product concentrations where the biocatalyst specific activity is lower than the threshold value defined by the performance metrics (see Section 5.2.1) and thus, not a feasible region. The white area represents the range of combination of product and co-product concentration that satisfies the threshold value for the specific reaction activity (i.e. the operating area where the specific reaction activity is higher than 0.03 g $_{product}$/(g $_{biocatalyst}$. h)). The yellow area represents the enlargement of the feasible space when operating with a value of co-substrate excess between the stoichiometric concentration (no excess) and 4.5-fold excess (yellow area).

8.3.2.6 Step 6. Generate an window of operation

A window of operation can be obtained by superimposing Figure 8.10 and Figure 8.13, resulting in a window of operation (Figure 8.14) bounded by the different technology constraints and process threshold values. Windows of operation can graphically show

the operating conditions where the process can be operated to meet the defined metrics.

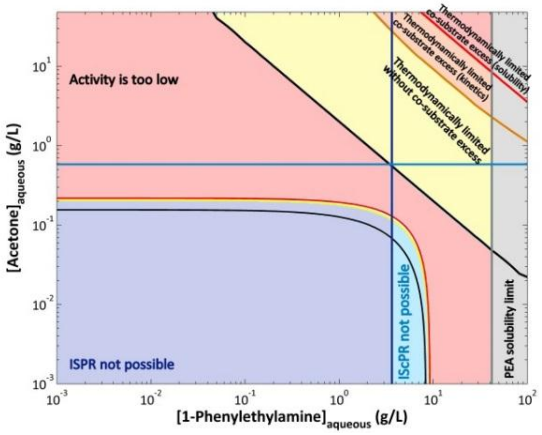


Figure 8.14. Operating space for the synthesis of PEA using soluble ω-transaminase (no window of operation can be found); Assumptions: 100 g of product produced per litre of reactor; 90% reaction yield; 0.03 g $_{product}$/(g $_{biocatalyst}$. h) of required biocatalyst activity. —— no IPA excess —— IPA solubility limit; Legend: —— IPA kinetic limit; —— PEA solubility limit; —— ISPR limit (ion-exchange resin); —— IScPR limit (acetone stripping); - - - constant specific activity at no IPA excess (0.03 g $_{product}$/(g $_{biocatalyst}$. h)) - - - constant specific activity at IPA solubility limit; - - - constant specific activity at IPA kinetic limit;

When acetone stripping is used to remove the co-product (Figure 8.14), no window of operation can be found, since the required concentration of acetone in the reaction phase is lower than the reported concentration obtained by applying this IScPR [269]. However, when enzymatic conversion of acetone is put in place (Figure 8.15), the concentration of acetone in the aqueous media can theoretically be maintained at a much lower level by adjusting the amount of the enzymes involved in the acetone-conversion cascade, matching their activity with the transaminase-catalysed reaction. Changing the IScPR technique enables a window of operation (white area in Figure 8.15) for the studied reaction.

However, the specific reaction rate considered in Figure 8.14 and Figure 8.15 does not meet the assumed requirements for an economically viable process, as the specific activity is far too low (0.03 instead of 1 g $_{product}$/(g $_{biocatalyst}$. h)). In other words, at the current biocatalyst development stage the cost contribution of the biocatalyst to the production costs is preventing an economically successful process. Hence, in order to attain an economically viable process it is necessary to improve the biocatalyst specific activity to successfully operate under more demanding operating conditions. The

benefits of altering the biocatalyst, as well as other process options, will be explored in the next section.

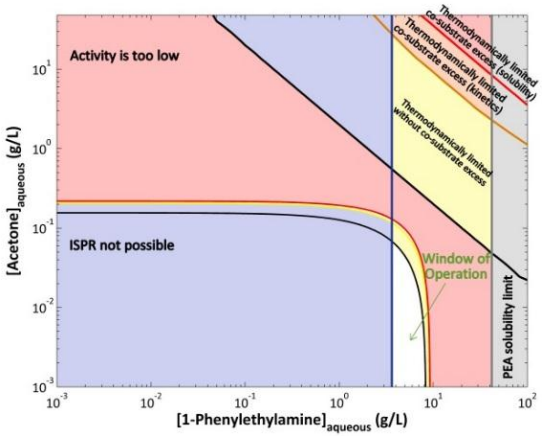


Figure 8.15. Window of operation for the synthesis of PEA using soluble ω-transaminase; Assumptions: 100 g of product produced per litre of reactor; 90% reaction yield; 0.03 g $_{product}$/(g $_{biocatalyst}$. h) of required biocatalyst activity. Legend: —— no IPA excess —— IPA solubility limit; —— IPA kinetic limit; —— PEA solubility limit; —— ISPR limit (ion-exchange resin); —— IScPR limit (enzymatic cascade); - - - constant specific activity at no IPA excess (0.03 g $_{product}$/(g $_{biocatalyst}$. h)) - - - constant specific activity at IPA solubility limit; - - - constant specific activity at IPA kinetic limit;

8.3.2.7 Step 7. Perform scenario analysis

In this step, a scenario analysis (i.e. what-if analysis) including a study of the effects of operating with a different catalyst formulation, improvement of the biocatalyst by genetic engineering, the improvement of the ISPR and IScPR technologies and modifying thresholds for performance metrics, is carried out.

Catalyst formulation

In the previous step, it is stated that the current specific activity of the catalyst is two orders of magnitude lower than desired for an economically viable process. Hence, with the present state-of-the-art it is not possible to obtain an operating space where the process is feasible and economically competitive when soluble-enzyme (ATA-40, C-Lecta, Germany) is used as the biocatalyst. One option to obtain an economically competitive process is to replace the used soluble-enzyme, by another more stable biocatalyst formulation of the same enzyme, e.g. whole-cell or immobilised enzyme.

The required catalyst specific reaction rate would then be 0.01 and 0.007 g $_{product}$/(g $_{biocatalyst}$. h), assuming a biocatalyst yield of 10 and 200 g $_{product}$/g $_{biocatalyst}$ [16], respectively and that the catalyst could be recovered and recycled 10 and 300 times, respectively (see Section 5.2.1). Mass transfer limitations, biocatalyst interface

limitation and possible adjustments of the biocatalyst kinetics have not been considered here, since the aim is to provide a quick evaluation of other process strategies.

By applying enzymatic cascades for conversion of the co-product and shifting the equilibrium, the window of operation becomes limited on the left-hand side by ISPR, on the right hand side by the product (PEA) solubility and on the top by the reaction kinetics (Figure 8.16).

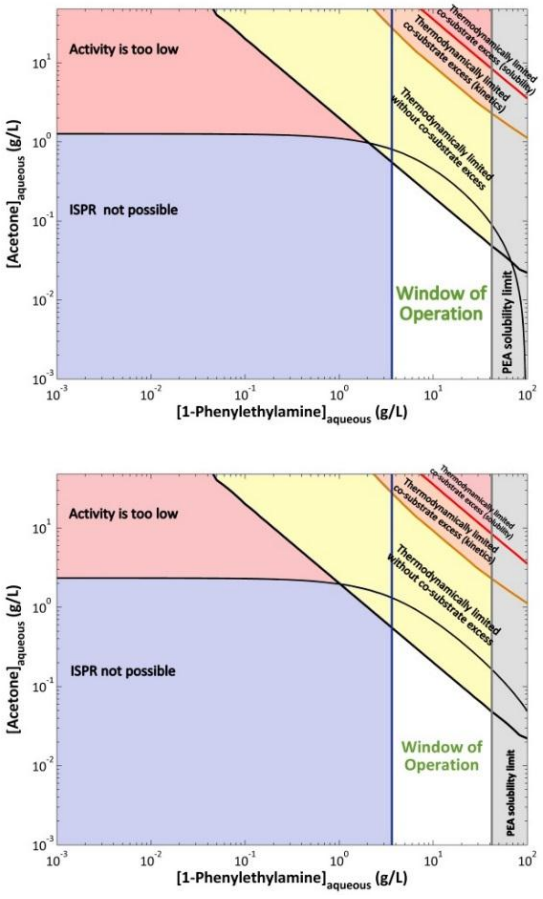


Figure 8.16. Window of operation for the synthesis of PEA using soluble ω-transaminase; Assumptions: 100 g of product produced per litre of reactor; 90% reaction yield **A** using whole-cell (biocatalyst specific activity 0.01 g $_{PEA}$/(g $_{biocatalyst}$. h)); **B** using immobilised (biocatalyst specific activity 0.007 g $_{PEA}$/(g $_{biocatalyst}$. h)) Legend: —— no IPA excess —— IPA solubility limit; —— IPA kinetic limit; —— PEA solubility limit; —— ISPR limit (ion-exchange resin);

Engineered Catalyst

Improved mutants are likely to provide a larger window of operation, allowing higher substrate and product concentrations and improving the process economy since higher biocatalyst specific activities can be attained (and consequently operating at higher space-time yield). In particular, for the base case analysed here, it could ensure the technical feasibility of the process. Figure 8.17 shows the effect of enzyme engineering on the process feasibility.

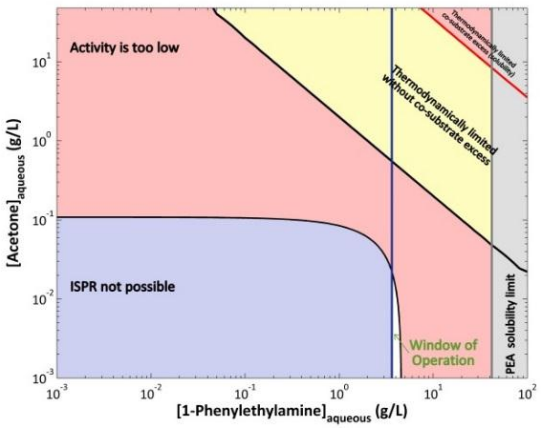


Figure 8.17. Window of operation for the synthesis of PEA using soluble improved ω-transaminase; Assumptions: 100 g of product produced per litre of reactor; 90% reaction yield; with stoichiometric amount of substrate and co-substrate; 1 g $_{PEA}$/(g $_{biocatalyst}$. h)) **A** 30-fold specific activity improvement; **B** 40-fold specific activity improvement. Legend: —— no IPA excess —— IPA solubility limit; —— PEA solubility limit; —— ISPR limit (ion-exchange resin);

When considering developing the catalyst, the goals should be as defined as possible in order to screen effectively. In the situation presented in Figure 8.17, apart from overcoming low specific activity and inhibition patterns at final product concentration, one should also consider the effect of other components in the reaction phase, such as the reaction components interfering in the acetone conversion cascade (Figure 8.9).

Further, the project developer (both process and the biocatalyst engineer) should not only consider the biocatalyst specific activity at the relevant reaction conditions (e.g. neutral pH), but also other scenarios should be evaluated. For instance, when operating the process at a higher pH, the differences in isoelectric point between the aminated compounds (i.e. co-substrate and product) are notable, enabling a more effective and selective separation of the compounds. Hence, other operating conditions not natural to the biocatalyst (but that might improve the process performance) must be considered at this stage alongside the biocatalyst development at these new operating conditions.

Hence, engineering the biocatalyst for a scalable and industrially successful process starts by defining the goal, such as increasing stability, selectivity or (often) both [65]. However, for a more cost-efficient development, the operating conditions should be defined simultaneously with the enzyme engineering goal. In other words, the goal cannot be simply "achieve 1 g $_{PEA}$/(g $_{biocatalyst}$. h))", but instead "achieve 1 g $_{PEA}$/(g $_{biocatalyst}$. h)) at 100 g/L of final product concentration, with the required concentration of substrate and co-substrate to achieve 90% reaction yield, at given pH and in the presence of acetone conversion cascade (i.e. isopropyl alcohol, formic acid and NAD(H), see Figure 8.9). In addition, trade-offs between the threshold values for the process metrics (setting the threshold value for the biocatalyst activity and the reaction conditions) and the biocatalyst engineering efforts should also be studied in order to set realistic targets for development that can be achieved in a reasonable timespan.

Modify ISPR

Operating the process with a highly effective and selective *in-situ* co-product removal technique enlarges the operating space, as shown in Figure 8.15, Figure 8.16 and Figure 8.17. The same can be applied to the ISPR technique used. For instance, the use of charged membranes constitutes a promising technology to selectively remove the chiral amine from the reaction medium, as the properties explored by this particular technology are the molecule size and the isoelectric point of the compounds present in the reaction medium [272]. Figure 8.18 shows how the operating space can be enlarged when operating with an improved ISPR (10-fold improvement) and an enzymatic cascade for acetone conversion.

The window of operation is still only limited by the biocatalyst kinetics and by the minimum concentration of product in the aqueous phase by ISPR (black and blue lines, respectively). However, there is a significant enlargement of the window, making the process operation easier to implement.

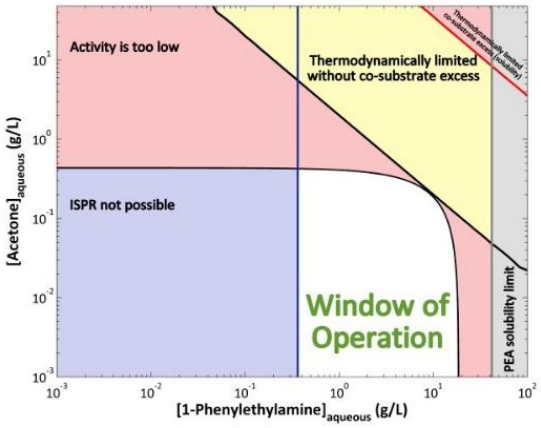


Figure 8.18. Window of operation for the synthesis of PEA using engineered ω-transaminase (40-fold improvement) with simultaneous removal of product and co-product using improved ISPR (10-fold improvement) and acetone removal for displacement of the reaction equilibrium and overcome product inhibition

Threshold for performance metrics

Naturally, the assumptions made (see Section 5.2.1) in defining the threshold for the performance metrics have a great influence on the window of operation. In this section, the effect of operating at different reaction yields, reaction times (i.e. space-time yield) and final product concentrations will be evaluated in turn. By doing so, the process engineer can assess the trade-off between operating at higher process performance and the required development.

Reaction yield

Operating at a higher reaction yield decreases the production costs associated with the raw materials and simplifies the downstream process. However, for thermodynamically challenged reactions, operating at a higher reaction yield implies a higher effort to displace the equilibrium towards the product side.

Figure 8.19 shows the substrate excess required when the reaction yield desired for the process increases from 90% to 95%.

Figure 8.19 clearly indicates that operating at a higher reaction yield is substantially more challenging, as an increase in 5% of the reaction yield implies the use of double the amount of co-substrate in excess to displace the reaction equilibrium. The same

trend is observed when other strategies for equilibrium shifting, such as selective removal of product or co-product, are considered (Figure 8.20).

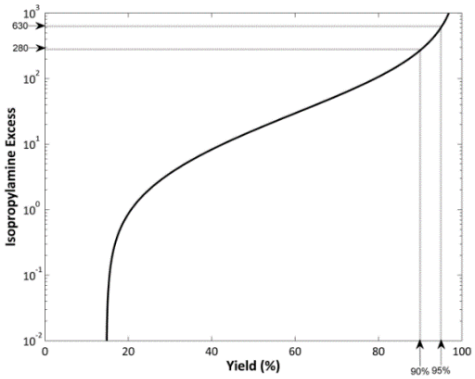
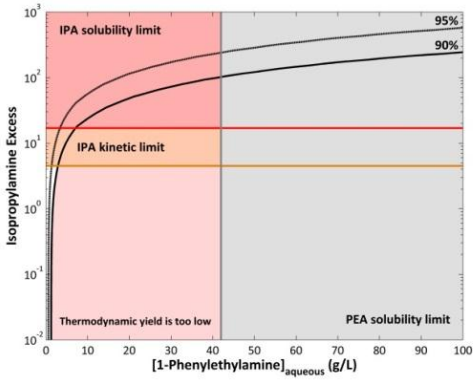
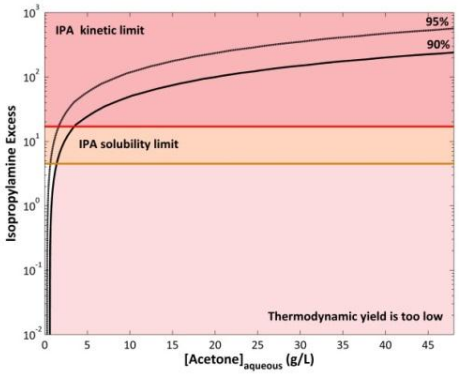


Figure 8.19. Required IPA excess to shift the thermodynamic equilibrium at fixed reaction equilibrium constant (K_{eq}=0.033) aiming different reaction yield



A



B

Figure 8.20. Required IPA excess at varying concentrations of **A** product and **B** co-product in the aqueous phase applying ISPR and IScPR, respectively; Assumptions: 100 g of product produced per litre of reactor; reaction yield 90% and 95% yield. Legend: — IPA solubility limit; — IPA kinetic limit; — PEA solubility limit (42 g/L, [36])

When simultaneous removal of product and co-product is considered as a strategy to shift the equilibrium, the choice of IScPR technique put in place and the co-substrate excess used is crucial to ensure process feasibility (Figure 8.21).

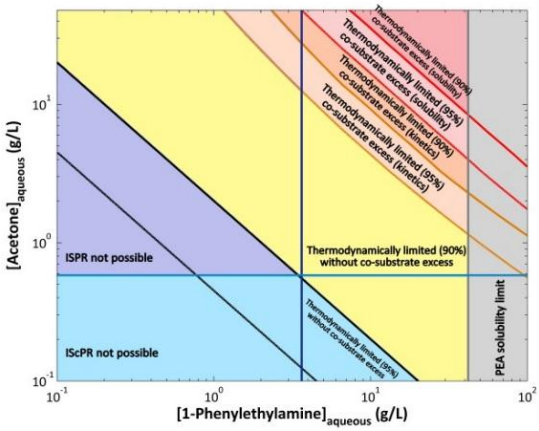


Figure 8.21. Required concentrations of product and co-product in the aqueous phase applying ISPR and IScPR, respectively; Assumptions: 100 g of product produced per litre of reactor; reaction yield 90%. Legend: —— without IPA excess —— IPA solubility limit; —— IPA kinetic limit; —— PEA solubility limit; —— ISPR limit (ion-exchange resin); —— IScPR limit (acetone stripping)

Figure 8.21 shows that operating at a stoichiometric amount of both substrates implies that more effective technologies to remove the co-product must be put in place in order to allow an operating region. Further, when the IScPR technology chosen for the removal of co-product cannot achieve the required concentration, it is necessary to operate with co-substrate excess, leading to a more challenging downstream process, and a negative impact in the overall production costs (due to an increase in cost contribution for the raw materials).

Figure 8.22 shows the operating region for the engineered catalyst (considered previously) and assumes that a low concentration of acetone can be achieved in the reaction phase, by employing the use of selective enzymatic cascades. Figure 8.22 also shows that operating at higher yield implies a size reduction of the window of operation, when compared with the situation evaluated previously in Figure 8.17. The window is limited on the right by kinetic limitations, on the left by the effectiveness of the method for the product (PEA) removal, from above by the thermodynamic equilibrium (unless the use of product excess is considered) and from beneath by the IScPR method employed and its effectiveness in removing the co-product (acetone) from the reaction phase.

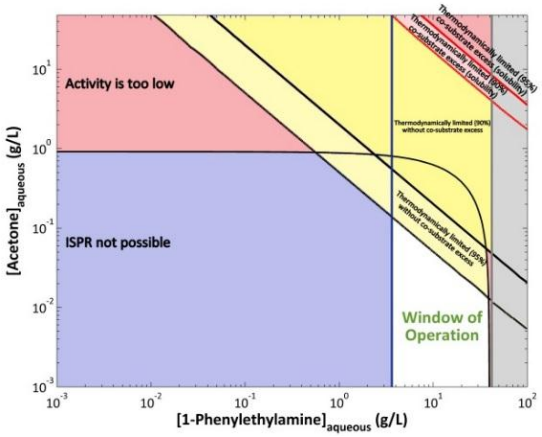


Figure 8.22. Window of operation for the synthesis of PEA using soluble improved ω-transaminase; Assumptions: 100 g of product produced per litre of reactor; 90% reaction yield; with stoichiometric amount of substrate and co-substrate; 1 g PEA/(g biocatalyst. h)) 40-fold specific activity improvement. Legend: —— no IPA excess —— IPA solubility limit; —— PEA solubility limit; —— ISPR limit (ion-exchange resin);

In the present case, further improvement of the biocatalyst specific activity can only be justified if the use of a stoichiometric concentration of the co-substrate (IPA) is considered, alongside with the use of a more effective PEA removal method.

Reaction time

The reaction time is an important constraint for an industrially viable process as it reflects directly on the capital costs and utilities costs. Regarding the operation itself, the reaction time determines how effective the catalyst needs to be, i.e. it determines the threshold value for the biocatalyst specific activity throughout the whole batch time, assuming a constant biocatalyst yield.

In this scenario, it is considered that the reaction time was reduced 4-fold when compared with the base case (24-hour batch time). This situation implies a 4-fold higher space-time yield and a specific activity of 4 g $_{PEA}$/(g $_{biocatalyst}$. h)). It is obvious that the catalyst activity needs to be developed more than 40-fold as considered in the previous scenario. Figure 8.23 shows the operating space for a 100-fold improved biocatalyst activity. Figure 8.23 shows that the operating region is mainly limited by the reaction kinetics. The expansion of the operating region is dependent on the activity improvement of the catalyst and on a later development strategy for the ISPR technique in order to reach lower concentration of product in the reaction phase.

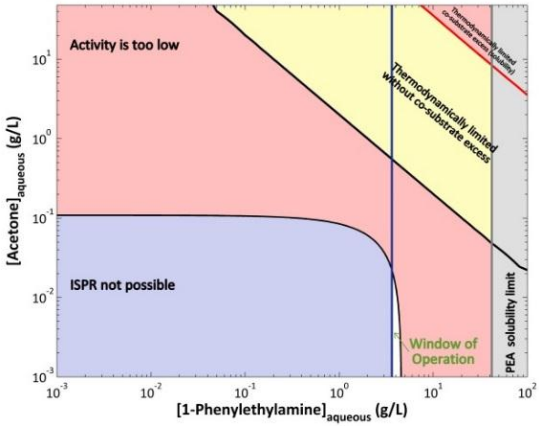


Figure 8.23. Window of operation for the synthesis of PEA using soluble improved ω-transaminase; Assumptions: 100 g of product produced per litre of reactor; 90% reaction yield; biocatalyst specific activity of 4 $g_{PEA}/(g_{biocatalyst}. h)$) for 100-fold specific activity improvement. Legend: —— no IPA excess —— IPA solubility limit; —— PEA solubility limit; —— ISPR limit (ion-exchange resin);

Final product concentration

Finally, the last process performance metric that has strong influence on the process costs is the final product concentration, i.e. the amount of product produced per litre of reactor over a batch time. The reason for defining final product concentration in such a way is, that when applying ISPR, it is desired that the final product concentration in the reaction phase is low. However, when this technique is put in place, the downstream process is often facilitated and often resulting in no overall increase of operating costs.

As mentioned earlier (see Step 2., Section 8.3.2.2), the maximum IPA excess used is a function of the final product concentration defined as performance threshold (Figure 8.6). Aiming at lower final product concentration implies that the maximum allowable co-substrate excess increases. Based on the cost of the substrate and the effort required to recycle it, the process engineer can decide to operate at a larger IPA excess, in order to decrease the effort on the ISPR and IScPR side to displace the equilibrium (Figure 8.6). Figure 8.24 shows the operating space for a 40-fold improved biocatalyst activity and applying ISPR and IScPR.

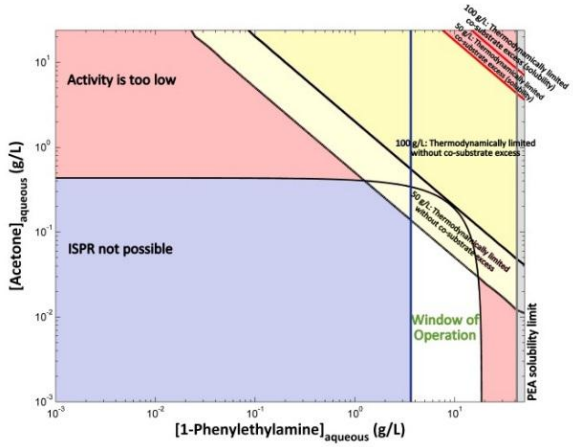


Figure 8.24. Window of operation for the synthesis of PEA using soluble improved ω-transaminase; Assumptions: 50 g of product produced per litre of reactor; 90% reaction yield; biocatalyst specific activity of 1 g $_{PEA}$/(g $_{biocatalyst}$. h)) for 40-fold specific activity improvement. Legend: —— no IPA excess 50 g/L ..... no IPA excess 100 g/L —— IPA solubility limit; —— PEA solubility limit; —— ISPR limit (ion-exchange resin);

From Figure 8.24 it can be concluded that there are no major benefits in operating at lower final product concentrations, since at the current state-of-the-art, the biocatalyst is equally inhibited at both concentrations, resulting in a similar kinetic profile. Regarding the effort for equilibrium shifting it is clear that operating with lower final product concentrations implies that more effective ISPR and IScPR methods are necessary, since a lower product and co-product concentration is required in the reaction phase. Hence, the window of operation is limited by the kinetic profile of the catalyst used (right-hand side), the effectiveness of the ISPR and IScPR technologies applied (left-hand side and beneath) and thermodynamics and the co-substrate excess used for displacing the equilibrium (above).

From the scenario analysis, it is clear that independently of the strategy chosen, for an economically viable process, the biocatalyst must be engineered, i.e. the specific activity must be enhanced at least 40-fold. Further efforts in engineering the biocatalyst, must be carefully considered alongside the improvements in process technologies used (e.g. ISPR and IScPR techniques), as these become new constraints bounding the window of operation together with the thermodynamic limitations.

Finally, a last step of this methodology should include a sensitivity and uncertainty analysis in order to identify the optimal operating space within the window, where the output variable (e.g. process performance metric for costing or environmental impact) is minimised.

8.4 Concluding remarks

The windows of operation tool presented in this chapter proved to be a valuable and convenient tool to quantify and visualise process performance and feasibility where interactions between process technologies and biocatalyst performance (or reaction) are significant. In principle, this methodology is generic. The scenario analysis in this methodology identifies the need for biocatalyst and process development (ISPR and IScPR). Furthermore, this tool can be extended to evaluate the sensitivity of each constraint in turn, pinpointing those that have the greatest impact on process feasibility as well as providing an initial estimate of the process costs.

A true integration of process modelling (i.e. kinetic modelling with dynamic models for ISSS, ISPR and IScPR) would also be beneficial for a more correct assessment of the process, since at the current stage the mass transfer phenomena were considered independently from the reaction kinetics. Furthermore, the kinetic model used in this case study only takes into account the biocatalyst specific activity, whilst stability was disregarded. Hence, it would be of great value to understand the effect of the operating conditions on the stability of the catalyst, in order to better predict the process performance.

Despite an increasing number of studies reporting improvements in the process performance by applying ISPR [68,273-278], a systematic (or short-cut methodology) to select the appropriate separation method is not yet in place. A systematic procedure that is able to select suitable separation methods confidently and rapidly would reduce the development time and help to focus on a given separation technology [49]. In addition, it will be greatly valuable that for each separation method, we could assign with a certain degree of confidence in the achievable (co-)product concentration in the reaction phase and thus, using the required (co-)product concentration as input to ISPR technology selection.

The structured approach presented in this chapter requires a profound knowledge of the process and it is based on a deep characterisation of the reaction, biocatalyst and process technologies put in place. Such characterisation requires obtaining data by reference to past information (i.e. scientific literature research) regarding suitable reaction systems (i.e. amine donors); experimental data collection to characterise the reaction (both in terms of the thermodynamic equilibrium and in the reaction kinetics); and data collection regarding the IS(c)PR technologies available. Hence, it is necessary to expand the information reported in the scientific literature and in the databases. In addition, it is essential to develop short-cut methodologies in order to select quickly between different process technologies, e.g. selection of ISPR technique based on the required product concentrations in the reaction phase.

This methodology allows the process engineer to identify the main process bottlenecks for an economically viable process implementation and provides a simple and straightforward way to analyse the outcome of the potential (but likely) improvements in the biocatalyst and process design. Furthermore, the methodological approach presented can be regarded as a feasibility analysis of different available process technologies, while assisting during data collection. Hence, the methodology constitutes a useful tool that provides interpretable results to enable rational design choices. Furthermore, this tool can be used to direct future research and assist in the evaluation of different process options on the process performance. However, integrating the cost of development of a given technology would be of great value in this tool. For example, the development of *in-situ* separation technologies does not have the same cost as developing an improved catalyst.

For innovative compounds (such as new pharmaceutical compounds) where the speed of development leads to process success, this methodology is able to detect in a straightforward and prompt manner unfeasible routes, where either the process requirements are too demanding (e.g. high demand for product removal from the aqueous phase for displacing the thermodynamic equilibrium), or the biocatalyst still requires a substantial development (low specific activity).

Moreover, the use of 'windows of operation' methodology can also assist in implementing a quality by design (QbD) in the production process as this methodology integrate the impact of raw materials and process parameters on product quality and process performance, as well as provide guidelines for a continuous monitoring of the process to assure consistent quality and a constant performance over time.

When applied to the chiral amine synthesis using ω-transaminase, two major bottlenecks were identified:

1. The biocatalyst needs to be engineered, since its specific activity needs to be improved at least 30-fold in line with the defined process metrics in order to obtain a window of operation and;
2. The feasibility of the window of operation is dependent on the minimum concentration achievable by employing suitable ISPR and IScPR technologies. Hence, highly selective ISPR and IScPR techniques must be explored (S>50, as defined in the Equations 8.11 – 8.16), as the size of the window of operation is defined by the effectiveness of ISPR and IScPR.

9 Bottleneck analysis for process optimisation

9.1 Introduction

As observed for homogeneous and heterogeneous catalysis, biocatalysts respond very sensitively to changes in reaction conditions, imposing a multivariate non-linear problem for optimising biocatalyst yield and productivity. Traditionally, in order to optimise the reaction conditions, a range of reaction conditions (e.g. pH, temperature and concentrations) and all the reaction components (i.e. substrate(s), product(s), cofactor(s), buffer(s), additive(s), solubiliser(s), etc.) must be considered. Such an approach leads to the generation of a large sampling space, which might imply that a vast number of experiments need to be carried out, often exceeding the experimental capacity (and time). In such cases, process modelling can help to reduce the experimental effort and quickly assess process alternatives or improvement strategies.

The use of process models has long been established in the chemical industry. Specifically, for biocatalytic processes, process modelling is an established engineering tool towards an effective process implementation, process control, selection and operation of the process technologies [267]. Different methodological approaches have been proposed to guide mathematical modelling for biocatalytic processes in a one-step reaction system (e.g. [267]), multi-enzyme processes (e.g. [101]) and whole-cell catalysed processes (e.g. [279]). Such models pose an excellent opportunity to assemble the available process knowledge, translated into process-relevant input (typically process variables) and output (process metrics) relationships that establish an optimal design space [100]. By using process models different scenarios can be investigated in-silico, reducing the number of time and resource consuming experiments. Hence, the use of mathematical modelling in the context of process development (and in particular for reaction engineering) can assist in the later development stages prior to pilot plant tests. Nevertheless, it is necessary to address the range of conditions (or scenarios) to which the model can be extrapolated. The extrapolation capacity of the process modelling is crucial, in order to avoid scoping for optimal scenarios where the model cannot predict the process behaviour. Therefore, at a later stage, extra experimentally collected data is required in order to validate the model-identified scenarios [100].

When applying process modelling, it is important to target on which performance or process metric the process development should be focused. For instance, the targets can be based on imposed space-time yield (STY), biocatalyst yield, reaction yield and/or product concentration, in order to improve the process economic performance (by reducing production costs), or ameliorate the process environmental profile. However, by putting the emphasis on improving one single process metric (or optimisation goal, such as $TTN_{cofactor}$) when aiming at optimising the reaction

conditions, one can fall into unidentified trade-offs leading to less economic and environmentally-friendly processes (see Table 5.3). By applying a bottleneck analysis (Figure 9.1), as a tool to evaluate the effect of modifying the reaction conditions on the performance metrics, the main process bottlenecks can be identified. In addition, suitable operating conditions can be proposed where these bottlenecks can be (partially) overcome.

9.2 Methodological framework

The proposed routine for bottleneck analysis is a structured approach to improve and develop a process based on pre-existing knowledge. Ideally, this information is gathered following the process development guidelines suggested by previously applied development tools, such as cofactor and interaction matrices (Chapter 7) and windows of operation (Chapter 8). By doing so, the process model is built closer to the expected operating conditions at full-scale and its extrapolation capacity is thus more reliable.

A methodological approach for the bottleneck analysis (Figure 9.1) proposes combining evaluation tools (economic and environmental analysis) with the predictive capabilities of process modelling by putting in place a series of 'what-if' simulations (scenario analysis) to identify optimised operating conditions. The key development in this methodology is the structured framework, rather than the tools put in place (see Section 3.2).

9.2.1 Step 1. Define flowsheet, mass and energy balances

In the first step of the bottleneck analysis, the information gathered in "Step 2. Define process constraints" of the generic methodological approach (see Figure 5.1) is put together in the form of a flowsheet (or several flowsheets). These flowsheet(s) include published scientific literature and/or the original process design. In order to compare these different process flowsheets it is necessary to calculate the corresponding mass and energy balances that characterise each operating unit of the flowsheet.

9.2.2 Step 2. Process evaluation

Tools for performance evaluation (i.e. economic and environmental assessment tools, see Section 4.2) are used in order assess process feasibility in terms of cost and environmental impact. Based on the assessment results the main cost drivers and/or contributors to the environmental profile (i.e. the process bottlenecks) are identified.

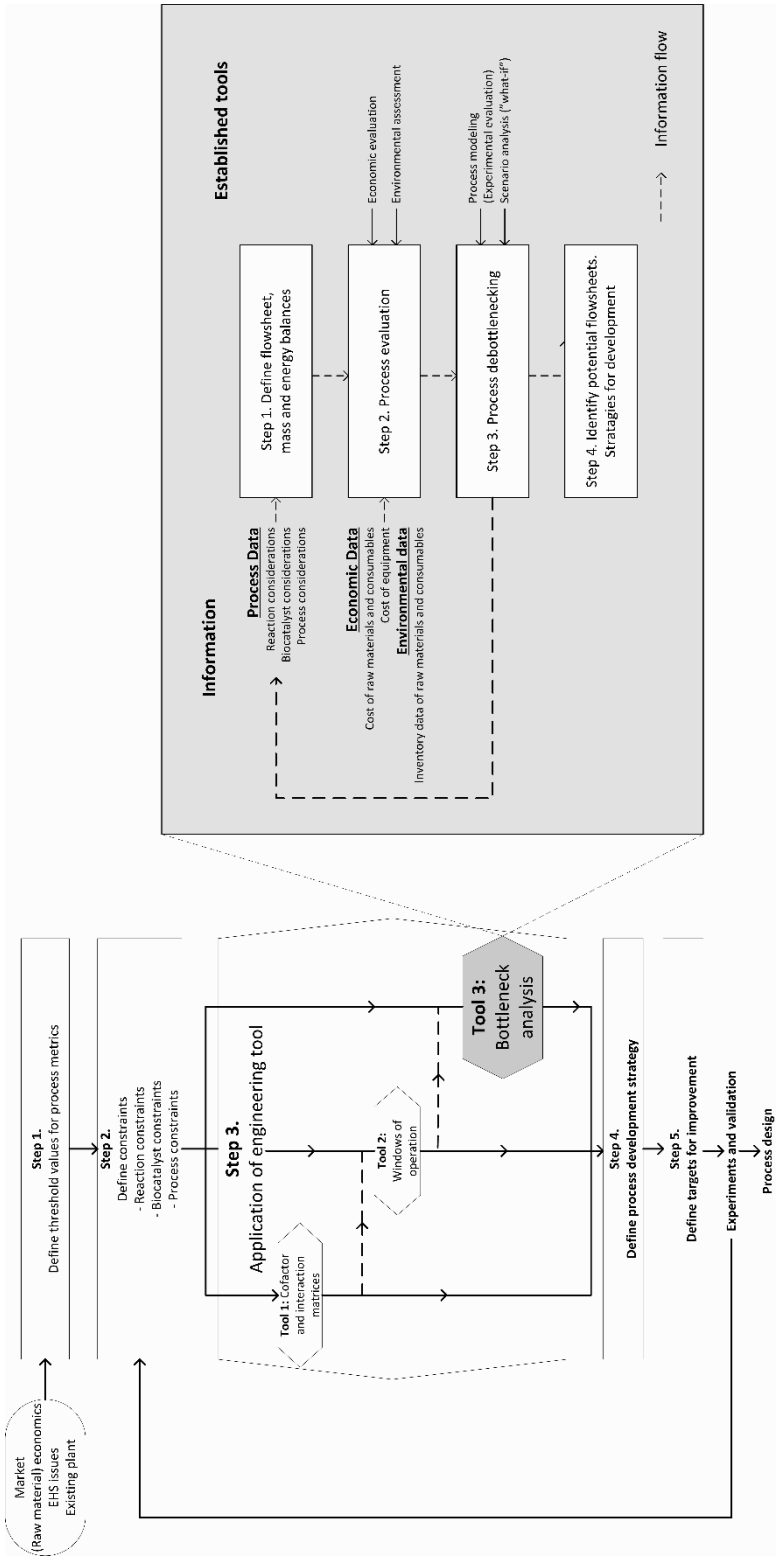


Figure 9.1. Overview of the proposed methodology for bottleneck analysis

9.2.3 Step 3. Process debottlenecking

The identified bottlenecks undergo a scenario analysis to evaluate the benefit of overcoming these. At this stage, reliable processing models can greatly assist in this task, since they allow a prompt evaluation of changes in the system (without the need for expensive and time-consuming experimental evaluation).

9.2.4 Step 4. Identify potential flowsheets and strategies for development

By using the methods described above, the potential of a process flowsheet for scale-up can be evaluated. Potential process flowsheets, improved operating conditions and designs can be identified by using the analysis in Step 3. Hence, development efforts and targets for future research are identified.

9.3 Case study 3: Chiral aliphatic alcohol production using alcohol dehydrogenase

To demonstrate the proposed methodology, the synthesis of (*R*)-2-octanol from 2-octanone using ADH as the biocatalyst was used as a case study.

The unique enantio-, regio- and stereo-selectivity of enzymes makes biocatalysis a promising technology, in particular for the fine chemical and pharmaceutical sectors. Chiral aliphatic alcohols are of special interest to these industries as they are widely applicable as building blocks for functionalised products [280]. The biocatalytic production of enantiopure alcohols displays several advantages when compared with the well-established chemical reaction routes, since it avoids the need for transition metals, high pressures and high temperatures [281]. Moreover, when compared with other chemical routes like the Corey-Bakshi-Shibata (CBS) reduction, biocatalysis can avoid the use of borane and an expensive chiral oxazaborolidine as catalyst [282]. The biocatalytic synthesis of enantiopure alcohols can be achieved either by kinetic resolution using hydrolases [14] or by direct asymmetric synthesis by employing alcohol dehydrogenases (ADH, Figure 9.2). Due to the fact that the production of chiral alcohols by kinetic resolution is hampered by the maximum yield of 50%, the asymmetric synthesis route has been regarded with great interest.

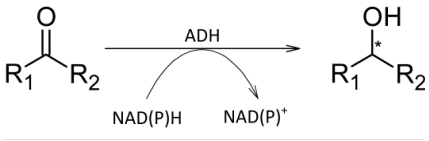


Figure 9.2. Model alcohol dehydrogenase catalysed reaction *chiral centre

Long-chain enantiopure alcohols (like (*R*)-2-octanol) are of particular interest in the fine-chemical sector due to their surfactant properties and have been used during the production of liquid crystals [280]. However, the large-scale production of chiral alcohols using ADH encounters several challenges, such as: requirement of expensive cofactor (requiring the implementation of effective cofactor recycling); industrially attractive products are often poorly water soluble ketones (leading to low productivity) and; the separation of the chiral alcohol from the reaction medium might be a laborious task.

9.3.1 Biocatalyst considerations

In recent years, the R-selective alcohol dehydrogenase from *Lactobacillus brevis* (*Lb*ADH, EC 1.1.1.2) has proven to be an attractive and outstanding biocatalyst for the enantioselective reduction of ketones, due to its wide substrate scope and high activity and stability in non-conventional reaction media (such as organic solvents, supercritical fluids and ionic liquids) [283]. *Lb*ADH requires NADP(H) as a cofactor, which is more expensive [130] and less stable [230] than NAD(H) and thus requires even more effective *in-situ* cofactor regeneration. Electrochemical methods for *in-situ* cofactor regeneration were proven to strongly affect the biocatalyst stability and reaction enantiomeric excess (*ee*) [283], and thus enzymatic cofactor regeneration can arise as suitable alternative for effective *in-situ* cofactor regeneration.

9.3.2 Reaction considerations

Enzymatic *in-situ* cofactor regeneration (in a network structure reaction) is a suitable alternative. NADP(H)-dependent glucose dehydrogenase (GDH) from *Bacillus sp.* (EC 1.1.1.47) was proven suitable for cofactor regeneration using glucose as the electron donor [284-286] (Figure 9.3). However, due to the stoichiometric formation of gluconic acid (from spontaneous hydrolysis of glucono δ-lactone, Figure 9.3), it is necessary to implement a tight pH control strategy.

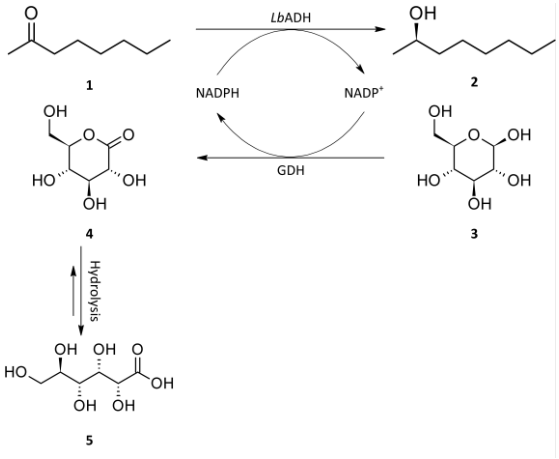


Figure 9.3. Biocatalytic production of (*R*)-2-octanol using *Lb*ADH; **1** 2-octanone, **2** (*R*)-2-octanol, **3** D-glucose, **4** Glucono δ-lactone (GDL), **5** D-gluconic acid

9.3.3 Process considerations

In order to overcome the low solubility often displayed by many of the industrially suitable ketones, the use of two-liquid-phase systems was already demonstrated [280,287]. However, products are often separated from the organic phase via distillation, implying high energy requirements [285]. Ionic liquids (ILs) have been discussed as a promising alternative to the two-phase systems, since they increase the solubility of the reaction components while improving activity, stability and selectivity of the biocatalysts [288-290]. Further, the use of a hydrophilic IL as a solubiliser was shown to increase the stability of cofactors [291].

There is a vast number of potential ILs to be used as solubilisers in biocatalytic process. However, to date, there is neither a rational approach nor enough reported data to assist the selection. However, some important considerations are solubilisation properties, enzyme activity and stability, effect on product recovery and mass transfer limitations [284]. Previous studies have reported that the use of 10% (wt.) of AMMOENG^{TM} 101 (AM-101) can increase nearly 11-fold the solubility of 2-octanone (from 7.9 mM to 94 mM [284]) with significant increase in activity and stability and lower product and substrate inhibition than observed in the buffer [284]. However, further increase in the IL concentration (to 20%) leads to a lower activity [284] and increases the solution viscosity, leading to problems in the equipment (pumps and membrane reactor) [285,286].

Due to the low solubility of long-chain chiral aliphatic alcohols, even when adopting ILs as solubiliser, it is necessary to continuously remove the product from the stream in order to increase the process productivity. The separation of long-chain chiral aliphatic

alcohols (also in IL containing solutions) can be performed using supercritical carbon dioxide ($scCO_2$). The use of $scCO_2$ in biocatalytic processes has several advantages, such as: the fact that is a non-flammable and non-toxic solvent; it is available at reasonable quantities and at a reasonable cost [292]; it can be an integration point in the plant (i.e. the CO2 produced in other points of the production site can be captured, treated and conditioned for this purpose) and; scCO2 has tuneable solvent properties, by changing the temperature and/or pressure and the reagents and products solubility can vary, facilitating the separation process [293]. However, when operating with an aqueous phase, CO2 reacts with water to form carbonic acid and thus, lowers the pH of reaction media. Further, CO2 might react with the protein amino-residues forming carbamides with consequences for the biocatalyst stability [294]. An increase in the system productivity avoiding the contact of $scCO_2$ can be attained by adsorption into a solid-phase (solid-phase extraction, SPE). Previous studies have reported that the use of polystyrene divinylbenzene copolymer-based materials can effectively remove 2-octanol and 2-octanone (product and prochiral ketone, respectively) [285,286]. Among the commercially available SPE materials, HR-P (highly porous polystyrene divinylbenzene copolymer) was identified as the best alternative [285]. However, a loss in capacity was observed when using some of the screened ILs as a reaction additive, due to its binding to the solid-phase [285]. Furthermore, the use of an IL also increases the selectivity of the SPE towards the prochiral ketone [286]. Nevertheless, the SPE material could be reused for more than 80 cycles with a product recovery higher than 65% [286] using either $scCO_2$ [285] or n-heptane/ethanol mixture [286] to elute the substrate and product from the SPE column. By adopting the aforementioned product recovery strategy, the effluent stream leaving the SPE is both product and substrate free.

Moreover, for an economically viable process, a high biocatalyst yield is required. To this end, several methods are available: immobilisation [55-57], retention by ultra- or nano-filtration membranes [295,296]. Immobilisation procedures still represent an added-cost to the process that is not always translated into more effective catalysts [217], whereas the immobilisation of the cofactor might only be cost effective if there is a significant increase of the cofactor stability [216]. Membrane technology allows a straightforward reuse of the isolated enzyme [209]. However the retention of cofactor still remains an issue. Alternatively, applying nano-filtration can partially retain the cofactors [296]. However, the use of these membranes can cause a problem if other reaction components (such as substrates, products and additives) are within the molecular weight range of the cofactors. Finally, the use of these membranes implies an increase of the utility costs (due to the high energy requirements) and the operational costs (due to the membrane fouling, reducing its lifetime). Due to reaction kinetics [285,286] the reaction was operated in a continuous mode applying two sequential enzyme membrane reactors (EMRs), allowing high reaction and biocatalyst yield.

9.3.4 Step 1. Define flowsheet, mass and energy balances

Based on the aforementioned biocatalyst, reaction and process considerations, previous studies have focused on the choice of reaction conditions, such as selection and concentration of the buffer, pH, selection and concentration of ionic liquid [285,286], leading to a very stable and promising process with more than 1000 hours of continuous operation and nearly no enzyme deactivation [286] (Figure 9.4). The conditions for the continuous synthesis of (*R*)-2-octanol taken for the base case can be found in Table 9.1.

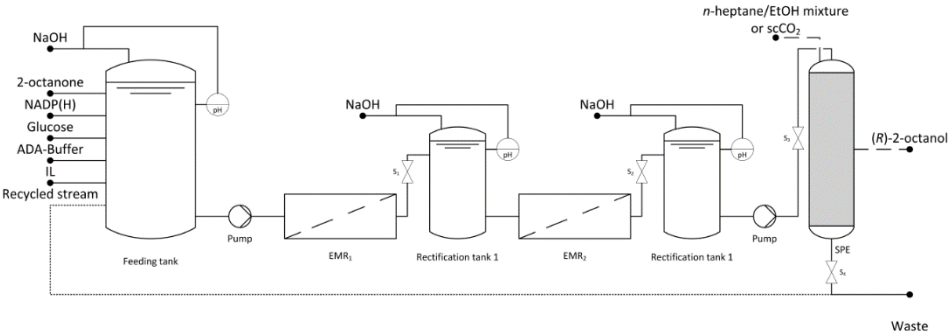


Figure 9.4. Process flowsheet for the synthesis of chiral aliphatic alcohols using *Lb*ADH; ADA- N-(2-acetamido)iminodiacetic acid; S_i- on-line sampling; EMR_i- Enzyme membrane reactor *i*; SPE- solid-phase extraction; ——Continuous — — -Batch ..... Recycled stream (Adapted from [286])

9.3.5 Step 2. Process evaluation

Evaluation tools (see Section 4.2) were used in order to identify the process bottlenecks. Since the optimisation goal in the adopted case study is to identify the reaction pitfalls and decrease the operating costs (OPEX), the economic evaluation was based on the cost of goods (i.e. substrates, cofactors, enzymes, solubiliser and buffer). Additionally, when assessing the environmental impact of the process, green chemistry metrics (GCM, Section 4.2.2.1) were preferred rather than assessing the process sustainability through LCA. Performing an LCA requires considerably more resources than calculating GCMs to collect, verify and analyse material inventory data [297]. Hence, E-factor [153,154,298] and PMI [149,150] have been chosen to evaluate the process environmental profile. Despite the disadvantages of these mass-based metrics (e.g. it does not provide a holistic viewpoint of the process and it omits specific environmental, health and safety concerns about the materials involved and the types of waste produced), these metrics are intermediate steps to calculate LCAs and footprints, without requiring a large investment of time and effort [297].

The process performance for the base case without recycling and for the case where 90% of the SPE effluent stream (2-octanone and (R)-2-octanol free) is recycled is depicted in Figure 9.5.

Table 9.1. Base case conditions for the continuous synthesis of (*R*)- 2-octanol [286]

Reaction Conditions	
Temperature (T)	25 °C
pH	7.5
Volumetric flow rate (v)	0.004 L/h
$V_{reactor}$	0.015 L
Residence time (v)	3.75 h
Enzyme loading in EMR_1 and EMR_2	5.2 mg
Maximum enzyme loading in EMR_1 and EMR_2	30 mg
E1:E2* in EMR_1 and EMR_2	19.2% (wt.)
Concentration in the inlet	
Substrate: 2-octanone	60 mM
Cofactor: NADP(H)	0.1 mM
Glucose	200 mM
ADA-buffer	150 mM
Solubiliser: AM-101	10% (wt.)
Process Metrics	
Reaction Yield	84%
STY	0.862 g/($L_{EMR1+EMR2}$.h)
$C_{(R)\text{-2-octanol, EMR2}}$	6.57 g/L (50.5 mM)
***Lb*ADH yield $_{EMR1+EMR2}$**	12900 $g_{(R)\text{-2-octanol}}/g_{LbADH}$
GDH yield $_{EMR1+EMR2}$	3080 $g_{(R)\text{-2-octanol}}/g_{GDH}$
SPE Conditions	
$V_{SPE\ column}$	13 mL
H/D $_{SPE\ column}$	30
T_{scCO2}	45 °C
P_{scCO2}	8 MPa
***n*-heptane/EtOH mixture**	50% (vv)
V_{scCO2} or $V_{n\text{-heptane/EtOH}}$	5 x $V_{SPE\ column}$

*E1:E2- Enzyme ratio $\frac{Lb\text{ADH}}{Lb\text{ADH+GDH}}$

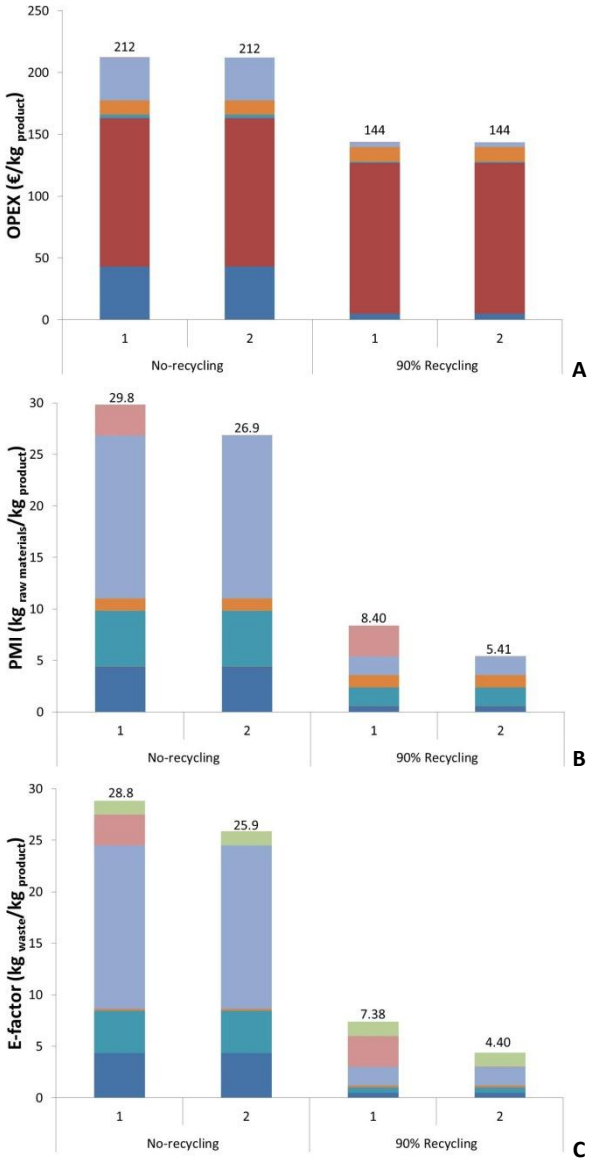


Figure 9.5. Process evaluation **A.** OPEX **B.** PMI **C**. E-factor for the base case (without recycling) and with 90% recycling of the aqueous phase when the SPE is eluted with **1**: *n*-heptane/EtOH mixture or **2**: $scCO_2$; Legend: ADA-buffer, NADP(H), *Lb*ADH, GDH, glucose, 2-octanone, AM-101, SPE elution (*n*-heptane/EtOH mixture or $scCO_2$) and gluconic acid

As expected, Figure 9.5 shows that there are major economic and environmental benefits in recycling the 2-octanone and (R)-2-octanol free stream. By partially recycling the feed-stream, the overall OPEX was reduced 33%, due to lower consumption of the IL (AM-101) and ADA-buffer. Further, when comparing the OPEX

for the different SPE elution techniques, no major differences can be seen between the use of n-heptane/EtOH mixture or $scCO_2$ to extract the product and unreacted substrate from the SPE column. However, the use of $scCO_2$ might imply an increase of the equipment cost for $scCO_2$ conditioning (i.e. heaters and compressors) and thus, when considering only the process costs, the final decision has to be taken according to the equipment available on site, the required technical efforts and the required purity of the final product. However, when focusing on the environmental evaluation, for both PMI and E-factor the use of $scCO_2$ proves more beneficial than the use of organic solvents (assuming 90% of solvent recycling [154]), for the conditions stated in Table 9.1, the *n*-heptane/EtOH mixture contributes up to 3 kg/kg $_{Product}$ for the process mass requirements.

When focusing on the OPEX (Figure 9.5 A), the cost contribution of the cofactor to the process economic performance is evident: 56% and 85%, for the base case (no recycling) and when recycling the aqueous-phase, respectively (assuming a cofactor cost of 10000 €/kg $_{NADP(H)}$ [130], see also Appendix 2). Despite the low cost contribution of both enzymes (*Lb*ADH and GDH), due to their exceptional activity and operational stability, the strictly NADP(H)-dependence of *Lb*ADH is regarded as major drawback [283]. Due to the lower costs and higher stability of NAD(H) [130,230], attempts have been made using site-directed mutagenesis to increase the NAD(H) affinity of *Lb*ADH, regrettably without any major breakthrough [299,300]. Hence, for the state-of-the-art *Lb*ADH, the optimisation goal is to improve the cofactor total turnover number ($TTN_{NADP(H)}$) by optimising the cofactor concentration and the reaction conditions, yielding higher cofactor stability in order to improve the process economic profile.

Since PMI and E-factor are process mass metrics the compounds with higher contribution to these metrics are those with a higher mass contribution in the overall process and thus, IL, ADA-buffer and glucose are the major components contributing to the process environmental profile. However, from a holistic life cycle perspective smaller mass contributors (such as cofactors) might have a higher impact in the environmental profile. Nevertheless, inventory data on these types of compounds is still scarce and difficult to model and they are often modelled using a cost allocation approach. Further, an important mass contributor in such a biocatalytic process is water. In the two scenarios depicted in Figure 9.5, the water intensity (kg $_{water}$/kg $_{product}$) was reduced 89% (from 152 kg $_{water}$/kg $_{product}$ to 17.5 kg $_{water}$/kg $_{product}$). Previous studies focused on optimising the IL [284] and buffer [286] concentrations. Hence, the optimisation goal of improving the process environmental profile must be focused on further reducing the glucose concentration, since at the base case glucose is dosed in 2.3-fold excess.

9.3.6 Step 3. Process debottlenecking

The first target of the optimisation exercise was to reduce the operating costs, by enhancing the cofactor utilisation. There are two main strategies to enhance $TTN_{NADP(H)}$: to optimise the operating conditions in order to increase the stability of the cofactors and to enhance the substrate/cofactor ratio without compromising the process performance (i.e. reaction yield).

In view of recycling, NADP(H) is known to have a limited stability in aqueous solutions [230]. In general, the cofactor stability increases when the reaction is operated at a lower temperature. The pH effect is dependent on the cofactor oxidation state: lower pH increases the stability of reduced cofactor (NADPH) and at alkaline pH oxidised cofactors ($NADP^+$) are more stable [130,216]. In particular, the reduced cofactor form (NADPH) is less stable than the oxidised one ($NADP^+$) at neutral pH [130,190,301]. Hence, in order to identify the trade-off and determine the operating pH optima, the half-life for both reduced and oxidised cofactor species were determined at the operating conditions (Figure 9.6).

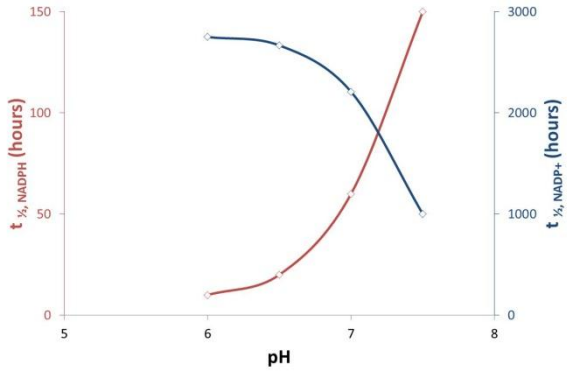


Figure 9.6. Half-life of — reduced (NADPH) and — oxidised cofactor ($NADP^+$) as a function of pH at operating conditions
(Susanne Leuchs, personal communication, 2012)

Despite the fact that the process is operated at conditions where the decay in stability of the $NADP^+$ (blue curve) is substantial, the half-life of the oxidised cofactor is longer than 1000 hours and higher than the NADPH half-life for the pH range studied and is thus not limiting. However, as previously reported [301], at neutral pH, the stability of NADPH can prevent successful cofactor recyclability. Hence, in order to improve the cofactor stability, tight pH control is required after each EMR in order to keep the reaction media at pH 7.5. Ideally, the pH control should be placed at the reactor, where conversion of glucose to gluconic acid causes a decrease in pH and thus affects the stability of the reduced cofactor. However, this would require a good

understanding of the fluid dynamics in the reactor, as well as a more vigorous agitation together with a lower concentration of base, in order to avoid enzyme denaturation when encountering an alkaline 'hotspot' in the reactor. In addition, operating with diluted acid or base for pH correction might lead to a less robust process and more difficult process control, due to great changes in the reaction volume.

Finally, varying the enzyme ratio (defined as $\frac{LbADH}{LbADH+GDH}$) in the reactors leads to an increase in the steady-state ratio of oxidised:reduced cofactor (defined as $\frac{NADP^+}{NADPH}$), increases the $NADP^+$ concentration in the reaction media, allowing higher cofactor stability throughout the process. The effects of varying the enzyme ratio on the $NADP^+$/NADPH ratio and final reaction yield (i.e. reaction yield after EMR_2) were predicted using a previously reported process model [286] (Figure 9.7, for model details see Appendix 4).

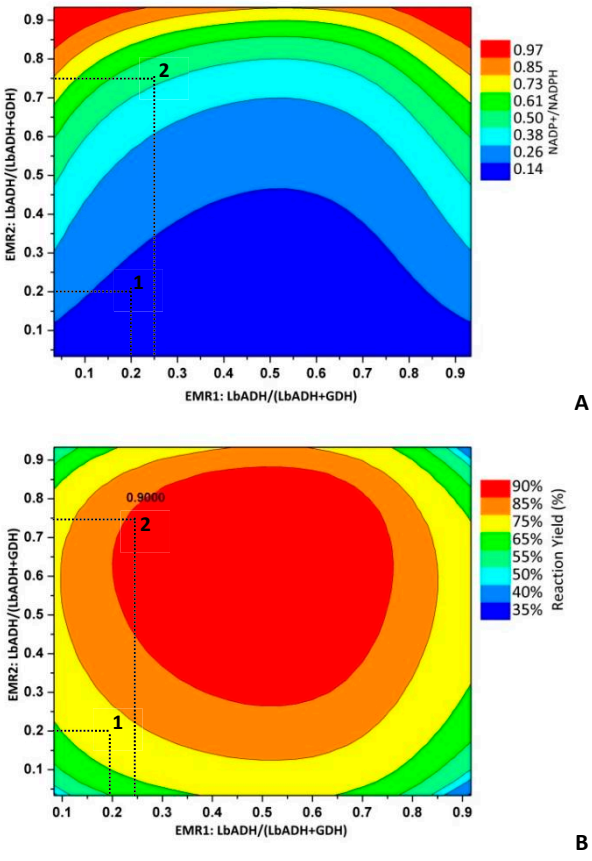


Figure 9.7. A Modelled $NADP^+$/NADPH ratio leaving EMR2 and **B** average reaction yield in EMR2 as a function of enzyme ratio in EMR_1 and EMR_2 at the operating conditions **1** base case conditions **2** improved conditions

Due to the higher cost of *Lb*ADH (in €/kg $_{catalyst}$) when compared with GDH (see Appendix 2), a low enzyme ratio in the first reactor was selected. Further, in order to increase the ratio of oxidised:reduced cofactor, without compromising the reaction yield (and consequently the SPE step), a high enzyme ratio was selected for the second reactor. Unfortunately, there is no overlapping area where the high ratio of oxidised:reduced cofactor meets high reaction yield and thus, there was a compromise between these two parameters.

In order to enhance the substrate/cofactor ratio, the cofactor concentration was optimised. Since the reaction rate depends on the concentration of the cofactor, a decrease in the concentration might lead to a lower biocatalyst specific activity (for the same operating conditions) and thus, lower reaction yield (for the same retention time). Lower reaction yields lead to a less effective process performance, due to an increase in the raw materials cost contribution and higher downstream processing costs in order to effectively separate the product and the substrate from the SPE elution stream. The process model previously developed could assist in this task (Figure 9.8). Decreasing the cofactor concentration in the feed-stream from 0.1 mM to 0.05 mM has little impact on the reaction yield in EMR_2.

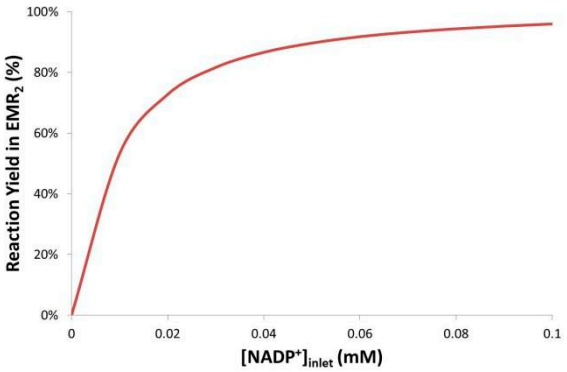


Figure 9.8. Model predictions (see Appendix 4) for the average reaction yield in EMR_2 as a function of the cofactor concentration in the feed-stream at improved conditions

The second target of the process debottlenecking is to improve the process environmental profile by optimising the concentration of glucose in the feed-stream. Previous studies have fixed the glucose concentration in the feed-stream to 200 mM [280]. Glucose was provided in excess in order to shift the equilibrium of the cofactor regenerating reaction and to ensure a stable concentration of glucose and gluconic acid in the reaction. However, this reaction is not particularly thermodynamically challenged [197] and thus the concentration of glucose can be further reduced to the stoichiometric equivalent of the product concentration, without any loss in the reaction yield (Figure 9.9).

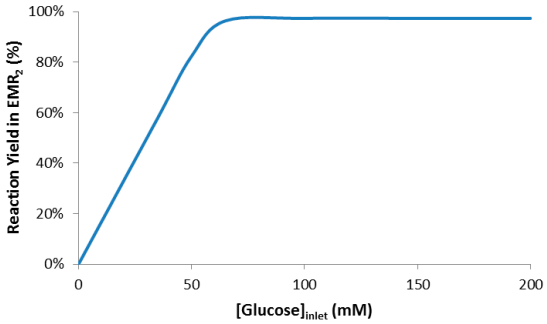


Figure 9.9. Model predictions (see Appendix 4) for the average reaction yield in EMR_2 as function of glucose in the feed-stream at improved conditions

9.3.6.1 Experimental evaluation of the improved process

Based on the identified bottlenecks and the suggested improvements for reaction optimisation (Table 9.2), an experimental evaluation of the improved process was carried out in order to validate the modelled conditions (Figure 9.10).

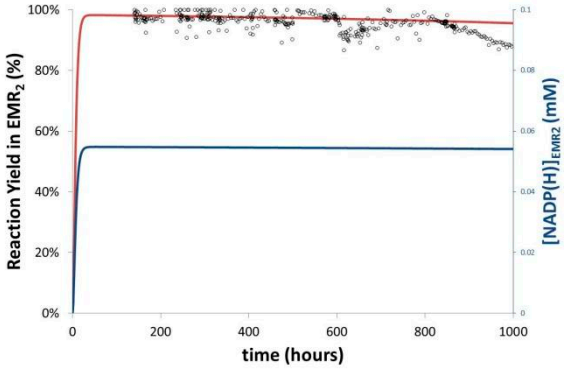


Figure 9.10. Comparison between experimental data and modelled simulated data for continuous production of (*R*)-2-octanol Legend: —— Model predictions for reaction yield and —— cofactor concentration after EMR_2 (see Appendix 4); and ○ experimental reaction yield (Susanne Leuchs, personal communication, 2012)

The use of process modelling, a well-established engineering tool, was shown to be able to predict the system behaviour at the new operating conditions for at least the first 800 hours (Figure 9.10). By reaction engineering the cofactor total turnover number ($TTN_{NADP(H)}$) was improved more than 2-fold (from 500 $mol_{(R)\text{-2-octonol}}/mol_{NADP(H)}$ in the base case to 1140 $mol_{(R)\text{-2-octonol}}/mol_{NADP(H)}$ for the improved scenario), while keeping the cofactor concentration in the system stable (Figure 9.10, blue curve).

However, for an economically feasible process, a further 10-fold increase in $TTN_{NADP(H)}$ is required [187].

Table 9.2. Suggested improved reaction conditions for the continuous synthesis of (*R*)-2-octanol

Reaction Conditions	Improved conditions
T	25 °C
pH	7.5
Volumetric flow rate (ν)	0.004 L/h
Residence time (τ)	3.75 h
Enzyme loading in EMR_1	6 mg
Enzyme loading inEMR_2	18 mg
E1:E2* in EMR_1	25% (wt.)
E1:E2* in EMR_2	75% (wt.)
Component	
Substrate: 2-octanone	60 mM
Cofactor: $NADP^+$	0.05 mM
Glucose	60 mM
ADA-buffer	150 mM
Solubiliser: AM-101	10% (wt.)
Process Metrics	
Reaction Yield	96%
STY	1.00 g/($L_{EMR1+EMR2}$.h)
$C_{(R)\text{-2-octanol, EMR2}}$	7.41 g/L (57.8 mM)
***Lb*ADH yield $_{EMR1+EMR2}$**	1980 $g_{(R)\text{-2-octanol}}/g_{LbADH}$
GDH yield $_{EMR1+EMR2}$	3300 $g_{(R)\text{-2-octanol}}$/gGDH

*E1:E2- Enzyme ratio $\frac{Lb\text{ADH}}{Lb\text{ADH+GDH}}$

Finally, the enzyme enantioselectivity was not affected at the new reaction conditions, as the measured *ee* was ≥99.5% for (*R*)-2-octanol in line with the previous findings [284-286].

9.3.6.2 Performance evaluation of the improved process

In order to identify the remaining process bottlenecks a new round of performance evaluation was carried out (Figure 9.11). In spite of the lower biocatalyst yield (Table 9.2) obtained for the improved process the cost contribution (1.3% of OPEX) of the biocatalyst is still below the recommended guidelines for fine-chemical production

[16]. The OPEX for the improved process falls within the upper limit of the market value for fine chemicals [16]. Furthermore, reaction engineering towards improved cofactor and glucose utilisation led to a significant (49%) decrease of the OPEX. In particular, the cofactor cost contribution was reduced from 83% of the total OPEX to 73%.

The observed decrease in the mass-based environmental metrics (PMI and E-factor) show the benefits of reducing the glucose concentration to the stoichiometric equivalent required to keep the cofactor regeneration system balanced. Moreover, the engineered process contributed to an improved PMI on account of the high reaction yield, leading to lower mass requirements of substrates and additives per mass unit of (*R*)-2-octanol produced. A further reduction of PMI and E-factor requires the optimisation of the ADA-buffer concentration since decreasing the IL concentration affects the substrate solubility and thus the overall reaction yield [284,285]. Another strategy to decrease PMI is to consider a different electron donor and replacing glucose (MW= 180.2 g/mol) by isopropyl alcohol (MW= 60.1 g/mol) [280]. However, this reaction system is thermodynamically challenged [302,303] and thus, an excess of isopropyl alcohol is required in order to achieve comparable reaction yields [280]. Moreover, glucose is a cheaper co-substrate than isopropyl alcohol and thus, for an economically viable process, the unreacted isopropyl alcohol must be successfully recovered and recycled back to the process.

Despite the great improvements in performance achieved with reaction engineering the process is still far from being feasible and the guidelines proposed for the process metrics (see Section 5.2.1) were not yet achieved. In particular further research efforts should be focused on improving final product concentration and space-time yield, without compromising the reaction yield and *ee*, in order to avoid jeopardising the downstream process (i.e. SPE efficiency), as with the current process highly purified product can be recovered without great effort and requiring rather simple recovery units.

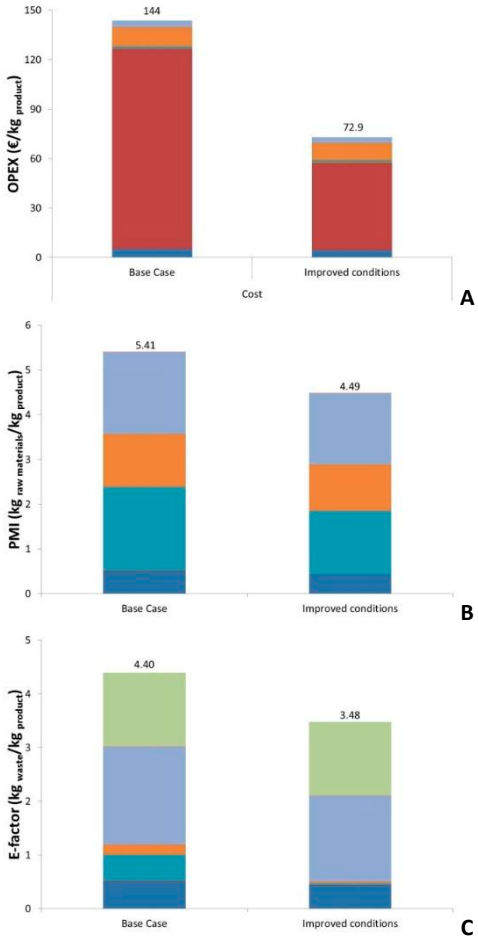


Figure 9.11. Process evaluation **A.** OPEX **B.** PMI **C**. E-factor for the base case with recycling and improved conditions with 90% recycling of the aqueous phase; Legend: ADA-buffer, NADP(H), *Lb*ADH, GDH, glucose, 2-octanone, AM-101, SPE elution (with $scCO_2$) and gluconic acid

9.3.7 Step 4. Identify potential flowsheets and strategies for development

In view of achieving the proposed guidelines for process metrics and performance increases in substrate concentration and volumetric flow rates must be addressed. Further improvements in the process would require a reconsideration of the process, by adopting a new catalyst formulation, reactor and/ or solubiliser.

Due to the restricted solubility of substrate concentration in the IL-aqueous solution, the concentration can only be increased within the substrate solubility limits. Hence, using the process model, the concentrations of 2-octanone and glucose were increased

from 60 mM to 94 mM, the maximum reported 2-octanone solubility at 10% (wt.) of AM-101. At these conditions the reaction yield was only slightly affected (94% at 94mM), but a higher final product concentration and space-time yield was obtained. The improved process metrics lead to enhanced economic and environmental performance, due to a higher utilisation of cofactor and reaction additives (IL and ADA-buffer, Figure 9.12). Hence, increasing the substrate concentration leads to a 29% decrease of the OPEX (from 72.9 €/kg$_{(R)\text{-2-octanol}}$ to 51.7 €/kg$_{(R)\text{-2-octanol}}$), mainly as a result of by a 1.5-fold increase in the $TTN_{NADP(H)}$, resulting in a lower cofactor cost contribution. While less significant to the OPEX, enhanced utilisation of IL and buffer (per product formed) in the process contributed to improved environmental performance.

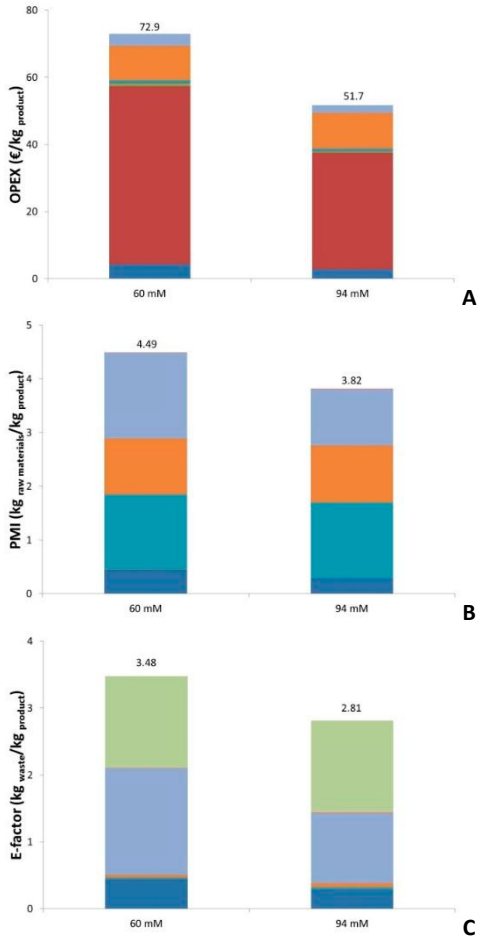


Figure 9.12. Process evaluation **A.** OPEX **B.** PMI **C**. E- factor at improved conditions ([2-octanone]$_{inlet}$=60mM) and improved concentrations ([2-octanone]$_{inlet}$=94mM); Legend: ADA-buffer, NADP(H), *Lb*ADH, GDH, glucose, 2-octanone, AM-101, SPE elution (with $scCO_2$) and gluconic acid

Increasing the IL concentration leads to an increase in the solubility of the substrate [284]. However, the viscosity of the reaction mixture increases with increasing concentrations of IL, resulting in limitations in the EMR. Ionic surfactants (such as AM-101) can interact with polyethersulfone (PES) membranes and promote its early fouling, reducing the membrane lifetime and thus increasing operating costs [284,286]. Besides, previous studies have reported a decrease in the enzyme activity and stability for concentrations of IL higher than 10% (wt.) [284]. Moreover, the efficiency of the SPE was also demonstrated to be affected by higher concentrations of IL, by affecting the partition coefficient and thus, the selectivity of the separation [286]. Hence, a further increase in the concentration requires a new round of screening for a suitable solubiliser, where the aforementioned selection criteria should be included.

Achieving higher space-time yields (STY, $g_{(R)\text{-2-octanol}}/(L_{reactors}.h)$) leads to lower capital costs (not accounted for here). STY can be increased when operating at high volumetric flow rates. However, running the process at higher flow rates implies higher biocatalyst loading in the reactor in order to avoid jeopardising the final reaction yield (Figure 9.13). Hence, there is a trade-off between the catalyst cost and the capital cost (here represented by STY), that must be achieved. Using the process model and engineering evaluation tools (economic and environmental assessment), an operating area where the process can be operated at high reaction yield and STY was found (Figure 9.13, marked with point 2).

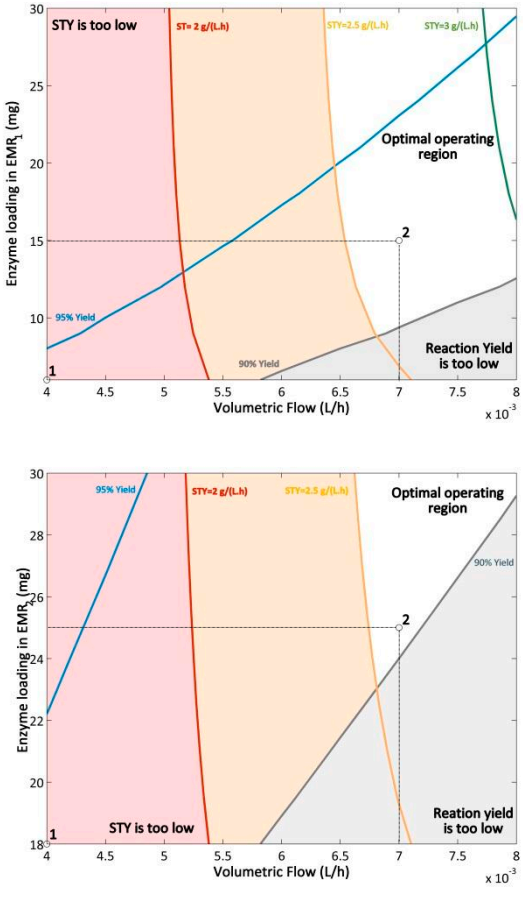


Figure 9.13. Operating space for the synthesis of (*R*)-2-octanol at **A** EMR_1 (at fixed EMR_2 enzyme loading) and **B** EMR_2 (at fixed EMR_2 enzyme loading) at different volumetric flow rate and enzyme loading; 1 conditions according with Table 9.2 and $[2\text{-octanone}]_{inlet}$=94mM and **2** optimised flow rate and enzyme loading

In order to get to the proposed optimised conditions the volumetric flow rate was fixed to a maximum of 7 mL/h since it was experimentally observed that, at a constant flow of 8 mL/h there is an increase in the reactor pressure, limiting the continuous operation of the process. A further constraint when selecting the new operating conditions was the amount of catalyst dosed to the reactor (in order to keep the catalyst cost below 5%), while keeping the enzyme ratio constant according with the conditions in Table 9.2. The newly identified operating conditions were evaluated in terms of its performance (Figure 9.14). Increasing the flow rate from 4 mL/h to 7 mL/h, together with simultaneous rise of the enzyme loading in both reactors, lead to an average reaction yield of 96% (comparable with the conditions reported in Table 9.2), while doubling the STY (2.73 $g_{(R)\text{-2-octanol}}/(L_{reactors}.h)$ at 7 mL/h, Table 9.3).

Table 9.3. Suggested improved reaction conditions for the continuous synthesis of (*R*)-2-octanol (2nd round)

Reaction Conditions	Improved conditions
T	25 °C
pH	7.5
Volumetric flow rate (ν)	0.007 L/h
Residence time (τ)	2.14 h
Enzyme loading in EMR_1	15 mg
Enzyme loading inEMR_2	25 mg
E1:E2* in EMR_1	25% (wt.)
E1:E2* in EMR_2	75% (wt.)
Component	
Substrate: 2-octanone	94 mM
Cofactor: $NADP^+$	0.05 mM
Glucose	94 mM
ADA-buffer	150 mM
Solubiliser: AM-101	10% (wt.)
Process Metrics	
Reaction Yield	96%
STY	2.73 g/($L_{EMR1+EMR2}$.h)
$C_{(R)\text{-2-octanol, EMR2}}$	11.7 g/L (89.9 mM)
***Lb*ADH yield $_{EMR1+EMR2}$**	1320 $g_{(R)\text{-2-octanol}}/g_{LbADH}$
GDH yield $_{EMR1+EMR2}$	1700 $g_{(R)\text{-2-octanol}}$/gGDH

*E1:E2- Enzyme ratio $\frac{LbADH}{LbADH+GDH}$

Despite the increase in enzyme loading (and consequently a lower biocatalyst yield, Table 9.3) this represents a minor contribution to the OPEX (Figure 9.14). Besides, when operating at higher volumetric flow rates, the amount of volume processed increased and there is even a small increment of the biocatalyst yield across the overall operating time when applying the reaction dynamic simulation. The same trend was observed for the cofactor utilisation ($TTN_{NADP(H)}$=1780 $mol_{(R)\text{-2-octonol}}/mol_{NADP(H)}$). Further, a more efficient reutilisation of process additives, as well as cofactor led to an enhanced environmental performance (Figure 9.14 B and C). Despite the small improvements in OPEX, a nearly 3-fold improvement in STY might have major implications for the capital cost, when considering a full-scale implementation.

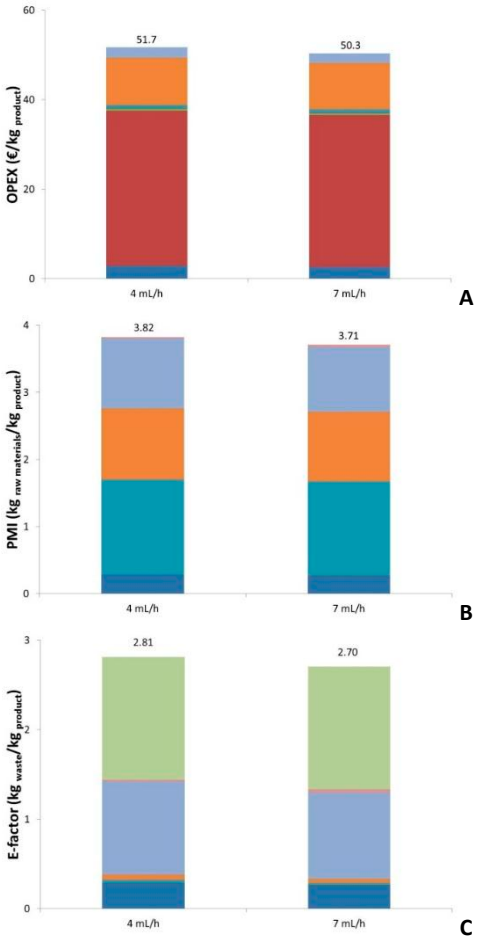


Figure 9.14. Process evaluation **A.** OPEX **B.** PMI **C**. E- factor for improved conditions and concentrations ($[2\text{-octanone}]_{inlet}$=94mM) for base case flow (4 mL/h) and 7 mL/h; Legend: ADA-buffer, NADP(H), *Lb*ADH, GDH, glucose, 2-octanone, AM-101, SPE elution (with $scCO_2$) and gluconic acid

Nevertheless, the main cost contributor remains the cofactor. One suggested strategy to lower the cofactor cost is by using NAD-kinase (EC 2.7.1.23, NADK). NADK converts NAD(H) (a cofactor 5-fold cheaper than NADP(H) [130]) into NADP(H), by catalysing the transference of a phosphate (PO_4^{3-}) from (typically) ATP to NAD^+ [304]. NADK has been used in fermentation processes to enhance the yield coefficient of the product on the substrate (Y_{sp}) [305,306]. However, in the context of a biocatalytic process using isolated enzymes [305] the introduction of a third enzyme and expensive cofactors (ATP) will certainly not bring the OPEX costs down. Hence, a suitable strategy would be to perform the enzymatic reduction of prochiral ketones using whole-cells. Apart from circumventing the use of expensive cofactors, the biocatalyst cost contribution can

also be significantly reduced [16]. Whole-cells of recombinant microorganisms overexpressing *Lb*ADH have already been reported for production of chiral alcohols [279,307-309]. However, the regeneration of the cofactor and *Lb*ADH expression were identified as limiting [279]. To avoid cofactor depletion it is necessary to co-express the NADP(H)- dependent alcohol dehydrogenase, such as formate dehydrogenase (FDH) [279] or GDH [310]. As previously mentioned (in Chapter 7), one of the remaining challenges when operating multi-enzyme systems in a whole-cell is to be able to regulate the overexpression of the two enzymes. Nonetheless, due to recent advances in metabolic engineering and increased understanding of gene expression regulation, a promising future for whole-cell biocatalytic processes is foreseen [245], enabling cost-effective processes in particular when cofactor regeneration systems are required. Moreover, the use of process additives, such as solubilisers, might present an additional challenge to the use of whole-cells, as ILs can affect the membrane integrity and thus, the biocatalyst stability [307,308].

9.4 Concluding remarks

The bottleneck analysis presented in this chapter proved to be a valuable tool for identifying the most suitable operating conditions, overcoming initial process bottlenecks and ensuring a more sustainable process. Bottleneck analysis incorporates process modelling and engineering evaluation tools (economic and environmental assessment). The combined use of process modelling and evaluation tools was able to assist in the reaction engineering, examine optimised reaction conditions leading towards a decrease in the operating costs and improved environmental performance. The real benefit of such models when integrated with evaluation tools is that they can be used to predict the process performance and identify more favourable operating conditions without requiring experimental examination and reducing the resources and time for process development.

In this chapter, bottleneck analysis has been applied to the biocatalytic synthesis of chiral alcohols. Overall, this methodology was able to indicate the operating conditions under which the OPEX is reduced 65%, from 144 €/kg$_{(R)\text{-2-octanol}}$ in the base case conditions [286] to 50.3 €/kg$_{(R)\text{-2-octanol}}$, by improving cofactor utilisation, concentration and volumetric flow rate.

At the current state-of-the-art, the cost of the cofactors can still prove a major obstacle preventing a successful large-scale implementation, as the cofactor cost contribution still represent 68% of the OPEX. Nevertheless, recent advances in recombinant DNA technology might enable the use of a whole-cell catalyst, circumventing the cofactor costs, which is often the most sustainable solution when employing biocatalysis to carry out redox reactions.

Furthermore, process development efforts envisaging scale-up should be focused on increasing the final product concentrations (at least) 10-fold by screening for a more efficient solubiliser, since with the current optimised scenario the final product concentration leaving EMR_2 is 11.7 $g_{(R)\text{-2-octanol}}$ /L. Since the recovery step (SPE) can selectively and effectively remove the product and the co-product from the EMR_2 effluent stream, it is equally important to keep a high reaction yield as this leads to a simplified downstream process. Hence, the screening criteria for a new solubiliser should include: product solubility, partition coefficient in SPE, activity and stability of the biocatalyst (isolated enzymes or whole-cell), effect of membrane fouling (if using isolated enzymes) and operating constraints (e.g. viscosity).

The structured approach for bottleneck analysis used in this case was able to:

- Optimise the cofactor utilisation, leading to savings in the operating costs.
- Reduce glucose requirements, leading to savings in the environmental profile;
- Increase the system productivity (by increasing concentration and space-time yield), leading to a more suitable process.
- Nevertheless, the final product concentration is still lower than the threshold values established in Section 5.2.1, due to the low substrate solubility and therefore it is necessary to screen for a more efficient solubiliser.

Part IV

Discussion, Conclusions and Perspectives

10 General discussion

Biocatalytic processes (as well as bioprocesses in general) have been emerging as a suitable replacement technology for conventional chemical synthesis (e.g. homogeneous and heterogeneous catalysis), driven by the need to produce chemicals from renewable raw materials, adopt greener synthetic routes, generate less toxic by-products and waste without compromising product quality. Biocatalytic processes are of particular relevance when the current process exposes considerable safety concerns (e.g. oxidation reactions in organic solvents [311]), stereo- and regio- selective synthesis is required [312], and/or there is a need to replace noble and/or transition metal catalysts (e.g. Rh, Ru, Pd and Pt), which are scarce and non-renewable resources. For some higher value market niches, biocatalytic processes provide a unique route for the synthesis of the desired product [313]. However, the most common situation is that there are other competing routes to the same product. Thus, the success of the biocatalytic process is determined by its performance when compared with the competing technologies. This thesis has suggested that the implementation of biocatalytic processes is dependent on a profound knowledge of the fundamental considerations (reaction, biocatalyst and process) that strongly influence the process viability. The application of a systematic methodology, integrating the aforementioned considerations, is of great benefit in guiding experimental work, indicating the required information for decision-making and suggesting guidelines for process metrics threshold values to be achieved with further research efforts (such as improvement in the biocatalyst activity) aiming at a full-scale implementation.

Moreover, an interesting factor for process design (although outside the scope of this thesis) is the speed of development of a process. Indeed, the process economics of a novel product may be less noteworthy than the time required to market launch or to pass Phase 1 and 2 of the clinical trials (for production of APIs). At this stage, the proposed methodology can assist in searching for the most suitable operating conditions and thus, speed up the initial development stage. As soon as the product comes off-patent or after it passes the initial phases of the clinical trial the speed of development becomes less crucial, while identifying and decreasing the production costs becomes vital for the process success. Thus, at this stage the implementation of the proposed methodology as a structured process development approach might prove beneficial in implementing a competitive process at full-scale.

10.1 Methodology

Many of the underlying constraints preventing successful process implementation can often be tackled either by improvements in the process, in the biocatalyst, or in both.

Indeed, a particular feature of biocatalytic processes is the possibility of modifying and improving the biocatalyst by advances in biochemistry, protein chemistry, molecular cloning, directed evolution, random and site-directed mutagenesis [314]. Improved enzymes may display new tolerance to reactor conditions such as temperature or pH and may also have improved selectivity or reactivity (i.e. activity) on an unnatural substrate and/or reagent. However, most of the screening efforts to tailor the catalyst properties are addressing the reaction itself by expanding the enzyme toolbox for organic synthesis. Nevertheless, future developments in this area should be addressed in the context of the industrial process in which the enzyme is applied, by trying to match high activities with the operating conditions (such as high substrate and product concentrations and presence of solvents) and providing cost-effective means for large-scale production of the biocatalyst [67].

The proposed methodology intends to understand and identify the balance between biocatalyst and process development for a competitive process at large-scale (by proposing guideline for process threshold metrics). By adopting a methodological approach during the early development stage, the type of information required for design and decision-making is identified as well as targets for further development are provided (e.g. biocatalyst screening and solvent selection). This greatly enhances the communication between those involved in process design and the chemists, protein and genetic engineers. For instance, in this thesis the lack of communication between the different disciplines is evident when the use of large amine donor excess (often more than 5-fold excess) is put in place to shift the unfavourable thermodynamic equilibrium in transaminase-catalysed reactions. This strategy, although suitable at bench-scale, has proven unfeasible at large-scale due to the donor aqueous solubility and cost.

Traditionally, developing biocatalytic processes is a time-consuming task and the holistic understanding of the process is limited. Solutions are designed for a specific reaction system and they are often non-generalizable and not able to be adapted to other similar systems. The current nature of process development in biocatalytic processes (Figure 10.1 A) is particularly disadvantageous for industries that require generic approaches and solutions that can be applied across several processes (such as the fine and pharmaceutical industries [315]). However, by applying a systematic approach to the early development stage (Figure 10.1 B) the number of experiments can be reduced, since the methodological approach is able to identify the information required for decision-making, while providing targets and guidelines for further development. This methodology is particularly relevant not only for the next generation of enzyme-catalysed reactions (see Chapter 2), catalysing particularly challenging reactions (e.g. reactions which are thermodynamically challenged or that involve hardly-water soluble substrates) where improvements in the process convey significant economic return, but also for multi-enzyme systems, due to their high complexity. Despite the time and resources consumed during *in-silico* process development and model implementation,

it is expected that the overall process development time is reduced, since these can provide a basis for experimental design, shortening the time spent in the laboratory (Figure 10.1 B). Furthermore, process modelling (including kinetic modelling) can be exploited to analyse potential scenarios at full-scale (Figure 10.1 B). In this way, the overall time and resources spent in the process development before pilot plant implementation is reduced.

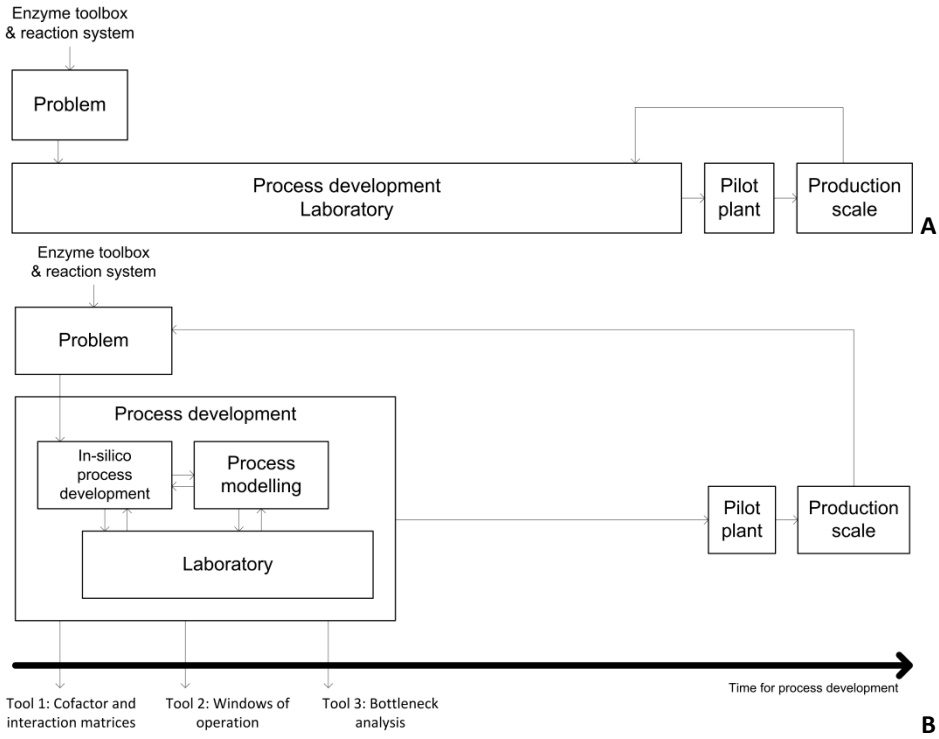


Figure 10.1. Basic steps of process development for **A** conventional process implementation and **B** the proposed process implementation

This thesis proposed three different engineering evaluation tools to be applied in different stages of the process development (cofactor and interaction matrices, windows of operation and bottleneck analysis) that were illustrated for the three different case studies (synthesis of ε-caprolactam, chiral amine and chiral aliphatic alcohols, respectively). Ultimately, a truly rational approach for process development will use all the proposed engineering tools. For instance, for the production of ε-caprolactam (see Chapter 7), the methodology was able to identify the most promising solution regarding the biocatalyst formulation. However, many questions regarding the process remained open, such as protein expression, biocatalyst activity (affecting the reaction kinetics) and mass transfer that need to be answered experimentally. This information can be fed in to a window of operation to create an operation map based upon the process technologies chosen (as shown for the biocatalytic production of chiral

amine in Chapter 8). Finally, bottleneck analysis integrating economic and environmental analysis is used to identify the most relevant parameters influencing the process viability during scale-up, where a final tuning of the process conditions can be performed (see Chapter 9). The amount of information gathered to feed each tool increases, as well as the need for reliable mathematical models describing the designed process.

Furthermore, this methodology is also able to assess the complexity of the biocatalytic process at large-scale. The process complexity is often reflected in the production costs and thus, the process development strategy is dependent on the industrial sector (bulk, fine and pharmaceutical chemicals). For bulk chemicals, the margin between raw material cost and product selling price is often quite small and therefore a competitive process implies effective conversion of the raw materials, while the allowable cost for the biocatalyst and downstream process is reduced. Therefore, for the effective production of bulk chemicals the process requires high reaction yields, high biocatalyst yields and high concentrations. Hence, the focus during process development should be on optimising the reaction conditions and the biocatalyst activity and stability (by either protein or process engineering). Due to the tight margin between the purchasing cost of the raw materials and the product selling price in bulk and commodity chemicals the implementation of process technologies (such as ISPR) is often limited for this industrial sector. At the other end of the spectrum, when developing a process for a pharmaceutical chemical, there is a bigger margin between the substrates and the product. For this industrial sector, obtaining a highly pure product is more important than obtaining a large reaction yield and thus, biocatalytic processes are especially attractive. Nevertheless, these are often challenged by either the unfavourable reaction thermodynamics, substrate(s) solubility, substrate(s) or product(s) inhibition, among others. Hence, apart from the often required biocatalyst development [47], it is also necessary to put in place multiple process technologies to overcome the challenges that are inherent to the process and reaction but are not biocatalyst-related (e.g. reaction thermodynamics, the properties of reagents and products), by putting in place, for instance, *in-situ* product and co-product removal and *in-situ* substrate supply (as shown in Chapter 8).

Finally, this methodology suggested the implementation of environmental assessment at early development stage, raising the question of comparability of the results. Despite the great and increasing concern of the chemical industry in promoting greener processes, the truth is that when comparing the environmental profile of two different synthetic routes this tool works only as a 'tiebreaker' when both processes display similar economic profiles. Nevertheless, this is not necessarily a less green management decision, as economic savings often translate into reduced emissions. Therefore, it is proposed that process evaluation at early development stage should be performed using process metrics, as they include both the economic and environmental aspects of

the process. Furthermore, the data required in the green chemistry metrics to assess the process environmental profile is based on the same original data as for process metrics.

10.2 Data collection

Step 2. of the proposed methodology (constraints definition) is considered one of the most important steps, since structured and accurate input influences the decisions in the process design and the development target setting step (Step 4.). It has also been experienced that the ranking and compiling of data about reaction, biocatalyst and process constraints is a challenging task. This thesis also proposed a structured evaluation of the constraints into hard constraints (such as reaction thermodynamic equilibrium and maximum overexpression level of the recombinant protein), soft constraints (e.g. biocatalyst activity) and an intermediate group (such as resin capacity and selectivity). Hard constraints are typically fixed boundaries of the process and thus, must be solved in the first place, as shown for the transaminase-catalysed production of chiral amine (Chapter 8), where the reaction thermodynamic equilibrium is the greatest challenge in the process. A typical soft constraint is the biocatalyst activity, as generally improved biocatalyst activity is required prior to full-scale implementation, since the conditions of the wild-type enzymes are far removed from industrially relevant conditions. In the intermediate group of constraints are those that can be partially overcome by applying a selection guide or screening. During screening trade-offs are often identified and therefore it is important to rank the scores for selection. Solvent, solubiliser or resin selection for *in-situ* product removal (ISPR) is included in this group.

The main drawback encountered in this thesis has been the low quality and quantity of suitable experimental data in the scientific literature for decision-making, such as measuring the resin selectivity and capacity at operating conditions (i.e. in the presence of other reaction compounds, Chapter 8). In the early stage of development, available databases such as the enzyme-catalysed reaction thermodynamics database (NIST Thermodynamics of Enzyme-Catalysed Reactions [176]) and enzyme activity database (BRENDA [316]) or property prediction tools might be used to overcome the lack of data. However, it is recommended that the conditions reported are as close as possible to the desired process, as much of the scientific literature is still focused on reporting new technologies at bench-scale conditions (i.e. at low concentrations) which might not always match the conditions of the designed process.

Moreover, due to the low solubility of substrates and products of relevant biocatalytic reactions (in particular for fine and pharmaceutical chemicals), many of the relevant reactions are performed in non-conventional media. Thus, solvent selection is one of the major concerns in early development [317]. To date there is a general lack of

rationale for solvent selection in biocatalytic processes. However, computer-aided property estimation (CAPE) tools for selection and design of solvents have been applied to generate a shorter list of chemicals that could be considered as potential solvents, based on environmental impact, reaction performance, separation and recovery criteria [318]. Nonetheless, when applying solvent selection rationale to the biocatalytic process framework, there is one more degree of freedom that is lost since it is necessary to ensure the solvent biocompatibility. Furthermore, the ranking of each of the factors is dependent on the stage of development of the process, as seen for the selection of the solubiliser in Chapter 9 for the production of chiral aliphatic alcohols, where the solubiliser can affect the downstream process efficiency as well as membrane fouling.

In general, there is a dearth of short-cut methods for selecting between different process technologies for biocatalytic processes. For instance, given that for a competitive process, it is necessary to implement ISPR with a given specification (i.e. required concentration in the reactive phase) the selection between membrane technology, resin or solvents can only be answered on a case-by-case approach. Experimental data collection is time-consuming and thus the development of prediction tools suitable for generating generic solutions for biocatalytic processes would be beneficial in order to narrow down the search space for experimentation. Further, computational fluid dynamics (CFD) simulations, as well as automated micro-reactor platforms offer excellent opportunities for quick data collection and to assist during formulation of process models (empirical or mechanistic). Moreover, high throughput data collection (using parallelised miniaturised systems) can be of great benefit for quick screening of a large number of different process technologies while reducing the consumable costs (e.g. only small amounts of expensive biocatalyst are consumed).

10.3 The future of chemical processes

Advances in recombinant DNA technology, combined with high throughput screening techniques, knowledge-base and statistical tools have been shown to be suitable for enzyme improvement by increasing stability at higher temperatures, in the presence of organic solvents, accepting new substrates and catalysing new non-natural reactions [65]. It is expected in the near future enzymes will be engineered at reasonable cost to fit the process specifications, making the task of the process engineer easier. Thus, it is for these engineers (process, protein and genetic engineers) that the methodological approach put together in this thesis, will be most interesting.

Further, new plant design might become a less common practice in the coming decades [319]. However, this does not necessarily mean that there is no space for novel design solutions. New synthetic processes (including biocatalytic processes) will be put in place in retrofitted plant design, either to increase capacity, improve EHS compliance, or

improve the process economics. Moreover, there is an increasing demand for modular generic solutions (building-blocks) to carry out one or more units of operation, in a standardised and well-characterised way [315] having a flexible capacity and operation, as well as being easily adaptable to new reaction chemistries. Hence, the development of systematic approaches able to assist during process design and in particular during the design of generic solutions for biocatalysis is a priority in order that these processes can gain a competitive position within the chemical industry.

This thesis has tried to cover several relevant aspects within the industrial application of biocatalysis, with application of three different case studies each representing different levels of underlying knowledge. Retrospectively, the methodological approach of (at least one of) the tools would have benefitted if the focus of the project had been on one single case study. In particular, for complex process designs (such as those proposed in Case Study 2, Chapter 8) the structured selection of the process technology(ies) for displacing the thermodynamic equilibrium was not fully achieved. However, this issue has been partly addressed in current and past PhD projects at our research group.

11 Concluding remarks and future perspectives

The main goal of this thesis was to establish a methodology to assist biocatalytic process development in its early stage. The methodology applies three different tools for different levels of knowledge that were able to assess the process feasibility at industrially relevant and competitive conditions. The difference of this methodology compared with others commonly put in place by the conventional chemical industry is that the particular features of the biocatalytic process have been introduced for each tool (e.g. catalyst formulation or catalyst improvements, etc.).

11.1 Achievements

The work done in the framework of this thesis has resulted in the following achievements:

- A general structure for a systematic **methodology for process design in biocatalysis** has been proposed. The methodology suggests guidelines for threshold process metrics (as a short-cut for integration of economic and environmental analysis), as well as the application of engineering tools for different stages of process development (Figure 11.1). The generic methodology was applied to different case studies (as sub-problem examples) bringing distinct understanding of the process and intrinsic constraints, from initial route scouting (Case Study 1, Chapter 7) to later development stages in process design (Case Study 3, Chapter 9). Although the proposed methodology is still in its infancy when compared with other PSE tools and methods, a good overview of the whole reaction system was achieved for each sub-problem, a systematic evaluation of different process options was performed and fundamental data collection for further development stages was suggested by putting in place this methodology. However, this methodology could be greatly enhanced by the implementation and integration of mechanistic models that are able to describe the mass and energy balances occurring in the reaction system.

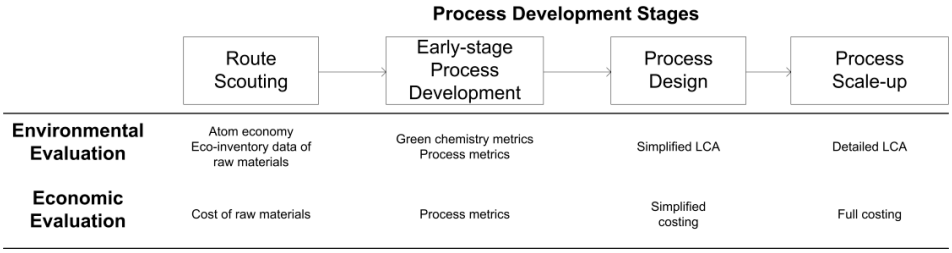


Figure 11.1. Environmental and economic evaluation tools for different development stages

The selected case studies illustrate different complexity levels and challenges and their intention is to demonstrate different tools developed to assist during process synthesis. For the case studies developed here, conclusions have been presented in the corresponding chapters. The main conclusions are:

- **Cofactor and interaction matrices** were applied to assist the selection of a suitable catalyst formulation for the multi-enzyme process yielding the synthesis of ε-caprolactam. This tool was proven to be of relevance for the understanding of the overall system, by identifying the interactions between the different reaction components and the enzymes involved in the multi-enzyme processes. Within the framework of the proposed methodology, this tool was used to: identify the requirements in cofactor regeneration; and narrow down the number of process options regarding the number of reactors required for the synthesis. The application of **evaluation tools (economic and environmental evaluation)** was able to identify the most promising catalyst formulation for each reaction (whole-cell), as well as the bottlenecks for further development (improved alanine total turnover number, TTN_{Ala}). However, rough assumptions were made in the ability of overexpression of more than one recombinant protein (enzyme) within the resting cell, as well as in the biocatalyst activity at the designed conditions. In addition, there is a trade-off not fully identified (due to the lack of data) regarding the expected lower enzyme activity (with consequent higher reactor occupancy and the possibility of side-reactions) and the use of isolated enzymes and cofactors (but with consequent increase in the purity of the product). From this case study it could also be concluded that the use of whole-cells in reactions where co-factors are required as a biocatalyst can lead to more economically competitive and greener processes, when compared with the isolated enzymes, as the cost of the co-factors often represent a big percentage of the raw materials costs. However, some disadvantages of the whole-cells were not considered (such as the transport limitations across the cell membrane).
- In the second case study, **windows of operation** were used to visualise the process performance and feasibility of the transaminase-catalysed synthesis of chiral amine. The application of this methodology requires a more in depth knowledge than required by the previous tool. This fundamental knowledge should not only cover the reaction system itself but also the process technologies put in place to attain a defined performance level. The outcome of this tool is an operating map, suggesting a combination of process technologies (i.e. different ISPR and IScPR techniques) where the process can be successfully operated. The integration of kinetic modelling in this tool enabled the identification of threshold values for biocatalyst activity improvement by protein and genetic engineering. Further, it identified a general lack of available technologies to selectively recover

the product and the co-product, essential for shifting the thermodynamic equilibrium and achieving the threshold values for reaction yield. Finally, this case study also showed that at large-scale, the success of thermodynamically challenged reactions (such as the synthesis of chiral amine) requires a combination of solutions (ISPR and IScPR), while the use of a high excess of amine donor (a more common solution in the scientific literature) might not be possible due to the amine donor solubility limit and inhibitory concentrations at relevant full-scale conditions.

- Finally, **bottleneck analysis** was applied to guide improvements in the continuous production process of chiral aliphatic alcohols in a bi-enzymatic system for cofactor regeneration (parallel reactions). The information required to apply this tool went beyond that necessary for the previously presented tools. For this case study, mass and energy balances gathered in the initial process design were compiled. The bottleneck analysis tool applies modelling, costing and environmental evaluation to identify the main process limitations. Further, application of kinetic modelling, describing the process, allows the evaluation of modifications of the process conditions and reaction optimisation *in-silico*.

11.2 Open challenges and future perspectives

The development of biocatalytic processes (as for any emerging technology) is still a challenging task that requires time to achieve a certain maturity and to become established as a competitive alternative to the current synthetic processes. It is believed that the application of a systematic approach can channel research efforts (eliminating less promising solutions). In general, it is hoped that this thesis will "catalyse" the discussion and implementation of general and systematic methodologies to improve design in biocatalytic processes. However, there are still many different fields that require further development, which can only be beneficial for future systematic frameworks for process development.

- It is necessary to develop *in-silico* predictive tools for property and thermodynamic data at operating conditions for biocatalytic processes (aqueous solutions). This can reduce the experimental work and assist in selection of process technologies (e.g. predictive tools for predict the partition coefficient and selectivity in ISPR resins and solvents).
- Most of the current mathematical models describe biocatalyst behaviour in terms of enzyme selectivity, mechanism and initial reaction rate kinetics. Further, these have generally been subject to considerable simplification and they are only able to describe the reaction kinetics at dilute conditions. There is however, a need for integration of these models with mass transfer phenomena at more

global operating conditions, e.g. ISPR mass transfer phenomena should also be integrated into the process model (Case Study 2, Chapter 8).

- Economic and environmental assessments might lead to trade-offs in the process and thus, this methodology would strongly benefit from the integration of a MINLP methods as well as an uncertainty and sensitivity analysis of the obtained results.
- For whole-cell catalysed reactions (Case Study 1, Chapter 7), the methodology would benefit from integration of models describing metabolic control in resting cells (whole-cell biocatalysts), but also the mass transfer diffusion of unnatural substrates and products across the cell membrane.
- The interest in continuous production and process intensification is increasing rapidly in the chemical industry. Hence, biocatalytic processes should also follow this trend, as it is likely that the biocatalytic step(s) would be integrated in a larger chemo-enzymatic framework for synthesis of relevant compounds.
- Many of the commonly applied PSE tools and methods for acquiring process knowledge in the conventional chemical industry (e.g. chemometrics, design of experiments, etc.) will also need to find their space within biocatalytic process design.
- Due to the huge development potential of the biocatalytic processes, target setting is essential, not only for biocatalyst development but also to the process technologies. However, these targets should not be reached at any cost. Many of the potential biocatalytic processes will simply be discontinued due to the complexity and costly research efforts required. Integrating assessment cost for development of a given technology or catalyst will be essential to support this decision.
- Finally, it would be desirable to prove the sequential application of the three developed tools and assess the development time by implementing the methodology proposed in this thesis.

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Appendices

Appendix 1: Included publications

Appendix 2: Data for economical assessment

Appendix 3: Economic and environmental assessment for biocatalytic production of ε-caprolactam

Appendix 4: MATLAB® scripts for kinetic modelling of biocatalytic production of aliphatic alcohol using alcohol dehydrogenase

Appendix 1: Included publications

Tufvesson, P., J. Lima-Ramos, M. Nordblad and J. M. Woodley (2011) Guidelines and cost analysis for catalyst production in biocatalytic processes. Org. Process Res. Dev. 15:266-274.

Tufvesson, P., J. Lima-Ramos, J. S. Jensen, N. Al-Haque, W. Neto and J. M. Woodley (2011) Process considerations for the asymmetric synthesis of chiral amine using transaminases. Biotechnol. Bioeng. 108:1479-1493.

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Guidelines and Cost Analysis for Catalyst Production in Biocatalytic Processes

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Abstract:

Biocatalysis is an emerging area of technology, and to date few reports have documented the economics of such processes. As it is a relatively new technology, many processes do not immediately fulfill the economic requirements for commercial operation. Hence, early-stage economic assessment could be a powerful tool to guide research and development activities in order to achieve commercial potential. This study discusses the cost contribution of the biocatalyst in processes that use isolated enzymes, immobilized enzymes, or whole cells to catalyze reactions leading to the production of chemicals. A methodology for rapidly estimating the production cost of the biocatalyst is presented, and examples of how the cost of the biocatalyst is affected by different parameters are given. In particular, it is seen that the fermentation yield in terms of final achievable cell concentration and expression level as well as the production scale are crucial for decreasing the total cost contribution of the biocatalyst. Moreover, it is clear that, based on initial process performance, the potential to reduce production costs by several orders of magnitude is possible. Guideline minimum productivities for a feasible process are suggested for different types of processes and products, based on typical values of biocatalyst and product costs. Such guidelines are dependent on the format of the biocatalyst (whole-cell, soluble enzyme, immobilized enzyme), as well as product market size and value. For example commodity chemicals require productivities in the range 2000−10000 kg product/kg immobilized enzyme, while pharmaceutical products only require productivities around 50−100 kg product/kg immobilized enzyme.

Introduction

Biocatalytic production holds great potential for clean and selective production processes and its application is steadily increasing in industry.[1−6] Furthermore, it is already established as a highly useful complement to conventional technologies for the production of optically pure chiral compounds in the pharmaceutical industry in particular.[1] Any new production process must pass a number of criteria to be successfully implemented. Safety, environmental, legal, economic, and throughput issues are all important aspects that need to be considered.[7] While biocatalytic processes are very competitive in terms of safety and environmental profile, one commonly discussed disadvantage is the cost of the catalyst.[8] Indeed, for lower value products, the industrial application of biocatalysis has thus far been limited, even though many potential processes have been suggested in the scientific literature.[5,9]

As has been stated elsewhere, it is frequently difficult to evaluate the cost of biocatalytic processes due to a lack of documented data on the factors contributing to the total cost.[9] Most available economic text books are focused on large-scale chemical manufacturing which makes it hard to draw parallels. Although biocatalytic processes as such can be very simple to operate, the development chain is generally more complex than for chemical processes.[2,9] It is therefore harder to estimate process cost (e.g., cost of the catalyst) and the cost of development, which in turn creates an uncertainty with respect to the risk of failure to meet the required cost of goods target. This frequently means processes may be discarded in error. For this reason there is a need for a better understanding of these costs so that the economic bottlenecks can be identified and addressed.[10]

Economic evaluation can be used as a decision-making tool to quantitatively estimate the expected profitability of a process, often alongside other criteria.[11] Cost estimates should be made throughout the early stages of a project even when comprehensive specifications (or other data) are not available.[12] However, methods for a full cost assessment are rather extensive and therefore take time to prepare. Consequently it is our contention that there is a need for methods that can simply and quickly assess not only if biocatalysis is a viable process option, but also identify the process bottlenecks. In this way guidance for research and development can be provided to give an understanding of when the process will achieve commercial success. This study presents a simplified approach for estimating the cost of different process scenarios, and ultimately the calculations can be used to evaluate process feasibility and identify bottlenecks. It should be emphasized that the results obtained should not be regarded as definitive values but as

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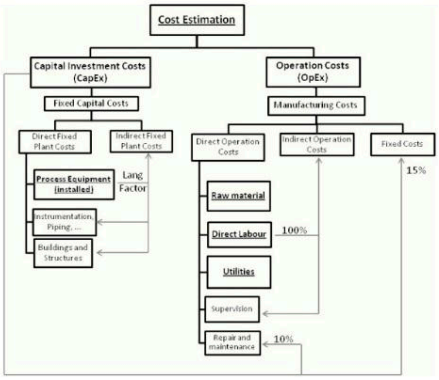

Figure 1. **Cost estimation categories and subcategories that are important for cost analysis. Underlined costs are calculated separately, while the other costs are estimated through the first ones, represented here with grey lines.**

guidelines that can serve as a starting point for other more detailed assessments.

The scope of this work is also to discuss and evaluate the cost of biocatalytic production processes with special emphasis on the cost contribution of the biocatalyst to the total production cost and the effect of scale-up, process and economic parameters. The dominating cost for different production processes and products is highly dependent upon the industry sector (i.e., pharmaceutical, fine, specialty or bulk chemical). This paper suggests minimum productivity requirements that need to be placed on the biocatalyst for processes in these different sectors (i.e., kg product/kg biocatalyst). It is well-known that process metrics such as product concentration (g/L) and space-time yield (g/L/h) are also very important for an economic evaluation but these are outside of the scope of this article.

Methodology

Cost estimation can be divided into two categories: capital investment (CapEx) and operation cost (OpEx), see Figure 1.

Capital Costs (CapEx). Fixed capital represents the capital necessary for the installed process equipment with all the accessories needed for the process start-up and operation.[11,13] In simpler approaches the calculation of CapEx is focused on the process itself, excluding site-wide auxiliaries, off-site and land-related items.[11−14] The foundation of a fixed capital estimate is equipment cost data. From this information the fixed capital investment can be calculated through the application of multipliers, such as the Lang factor.[13,15]

In order to obtain the total investment cost, the different parts of the direct CapEx (excluding equipment cost) and the indirect CapEx should be calculated separately (see Figure 1). However, in the early stages of process development the level of detail does not usually allow for an accurate and reliable calculation of these expenses. Hence, in order to obtain the total investment cost, the equipment cost is multiplied with a factor to cover the costs for all supporting equipment and services.[13] Detailed information concerning common factors used can be found in standard process design handbooks.[12,13]

The cost-capacity plot (six-tenths rule) is often applied when the effect of process scale is evaluated (see 1).

$$\text{Cost of equipment B} = \text{Cost of equipment A} \left(\frac{\text{Capacity of equipment B}}{\text{Capacity of equipment A}}\right)^{n} \quad (1)$$

where n may vary between 0.4 and 0.9, depending on the type of the equipment being costed, the operating conditions and the investigated range.[11,13]

To calculate the CapEx cost per production batch, the investment cost can be converted to an equivalent annual cost by multiplying the capital investment with an annuity factor, k (see eq 2).[11] The capital charge factor, i (or interest rate factor) is typically between 6 and 7% for the chemical industry but varies with, among other things, the risk of the project. The typical equipment economic lifetime, t, is 10 to 15 years.[13]

$$k = \frac{i}{1 - (1 + i)^{-t}} \quad (2)$$

Operating Cost (OpEx). The operating cost (OpEx) consists of direct, indirect and fixed costs. Direct operating costs includes the cost of raw materials, utilities, waste management and operating labor. Indirect and fixed operating costs can be calculated from direct labor cost and/or annual capital investment cost (see Figure 1).

The amount of raw material consumed is obtained from the process mass balances, and the cost of the most common chemicals can be obtained from the suppliers or by consulting trade journals (e.g., European Chemical News or Chemical Marketing Report).[11,13]

Utility requirements, including the cost of heating and energy for agitation, can be obtained from mass and energy balances and prices can be obtained from suppliers or purchasing agents. In fermentation processes, the dominating energy-consuming operations are often mixing and sterilization. The energy necessary for mixing can be calculated using rule-of-thumb values,[16] whereas the heat required for sterilization can be obtained using the heat capacity for water.

Although waste treatment is usually not part of the process design and cost model, waste disposal is an important process cost that should not be disregarded.[13,17] Typically wastewater treatment costs are 0.5−2 €/m^3 (depending on location), while nonhazardous solid waste disposal has a cost of around 25 €/ton.[17]

Finally, direct labor costs can be estimated from the process flowsheet based on typical labor needs for each unit operation[11] or by knowledge about labor requirements for the whole process. Labor rates can be obtained from the union contract,

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Table 1. **Summary of the considerations and source of information used in the economic model[a]**

cost	contribution to cost	consideration
CapEx	equipment cost	Matche Inc. (www.matche.com), process design software (ASPEN or SuperPro Designer)
	other capital investment costs	Lang factor:[15] 5.0 (typical for fluid processing units[18])
	annuity	From eq 2 For the base cases: $k = 0.142$, based on $i = 7\%$ and $t = 10$ years
	equipment scale-up	$n = 0.6$
OpEx	raw materials	market quotations, laboratory chemical suppliers
	utilities	0.1 €/kWh (European Energy Portal[19])
	waste handling	2 €/m[17]
	labor	30€/h (Eurostat[20])
	supervision cost and indirect opex	100% of the direct labor
	annual maintenance	10% of the annual capital investment cost
	fixed OpEx	15% of the annual capital investment cost

[a] k represents the annuity factor; i, the capital charge factor (or interest factor); t, the equipment economic lifetime.

from company labor relation supervision or from local statistical institutes (e.g., Eurostat, US Bureau of Labor Statistics).

Other operating costs can be calculated from direct labor costs or from annual capital investment. Supervision costs (direct operating costs) and indirect costs (including payroll overhead, quality control, royalties and plant overhead) normally correspond to 80 to 115% of the total direct labor costs. Annual maintenance (direct operating costs) including labor and material adds between 6 to 10% relative to the fixed capital investment.[11,13] Fixed costs are insensitive to the production scale and include depreciation, taxes, property rents, insurance, etc. corresponding to 12 to 17% the annual capital investment cost.[11,13]

Assumptions in Simplified Cost Estimation

As mentioned above, the aim of the present work is to develop a fast and accurate method for cost analysis. Since many data are not widely available, in particular when the process design is not fixed, assumptions have to be made. Table 1 summarizes the main considerations used to construct the proposed economic model.

When difficulty in obtaining raw material prices from the suppliers was experienced, the prices were estimated from laboratory chemical suppliers, by dividing the original price by 10 to 30 depending on the original package size. The uncertainty of this approach is high, but is still considered a good starting point for cost estimations. In the present case study the costs have been confirmed with industry.

The direct labor needs were determined through typical labor requirements and in discussion with industry. A value of 30€/h was assumed (Eurostat[20]) in order to calculate the cost associated with the direct labor. Labor needs are dependent on the plant scale and the degree of automation. However for processes within the same capacity range, the labor needs do not increase directly with process volume. Therefore, in this study it was assumed that labor needs did not increase with scale.

Evaluation of the costs in the preliminary design phases involves guesses and applications of rules-of thumb; therefore, the quality and accuracy of these estimations are dependent on the skill and experience of the engineer.[12] With the methodology applied in the presented study, its accuracy is considered to be on the order of ±30%. Regardless of the level of detail and complexity in an economic study and in the underlying project design, a certain degree of uncertainty will always remain.[13] This makes it is necessary to evaluate the effect of certain modifications to the original project on the total project cost.

Biocatalyst Production Costs

To determine the productivities required in a biocatalytic process to achieve a reasonable cost contribution of the biocatalyst, the manufacturing cost of the catalyst needs to be calculated. Here, these calculations have been divided into three main sections: fermentation, purification, and immobilization (see the first two sections of Figure 2). The influence of the costs on scale, accounting, and process parameters are also reported.

Fermentation. The production costs for a base case fed-batch fermentation of 10 m^3 were determined, assuming a final cell concentration of 50 g of CDW/L and 6.25 g of enzyme/L. Further, it was assumed that a single fermentation was run per week, and that the operation required a team of three full-time workers. Aspects of cGMP, such as validation and qualification protocols, and aseptic DSP processing have not been included into the calculations although these could be requirements in a final biotransformation step. The full details of the base case are given in Appendix I (Supporting Information).

Figure 3 shows the distribution of the costs and the production cost per kilogram of cells as well as per kilogram of enzyme (in the cell; nonpurified). It can be seen that in the base case the main cost drivers are equipment cost and labor costs, whereas utility costs are almost negligible. On the basis of our calculations, the production cost of one kilogram of cells is €67, corresponding to a cost per kilogram of enzyme (within the cell) of just over €500.

By analyzing the distribution of the different costs versus production volume it can be seen that the impact of the different costs varies greatly with scale. For instance at small scales (<10 m^3) the greatest cost contribution comes from labor and equipment costs, whereas at a larger scale (>50 m^3) the impact of labor is small, and the cost of the raw material becomes

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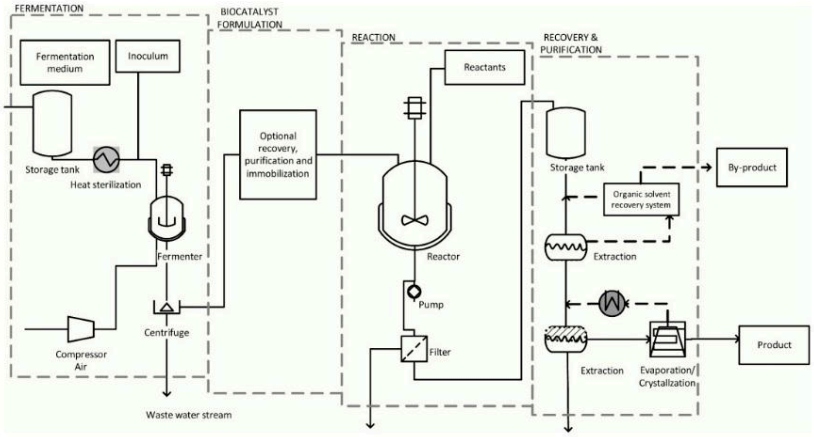

Figure 2. **Example of a theoretical biocatalytic process, including biocatalyst production (fermentation and biocatalyst formulation), biocatalysis (reaction), and downstream processing (recovery and purification). Note: The biocatalyst is normally produced independently from the reaction step and then stored until use.**

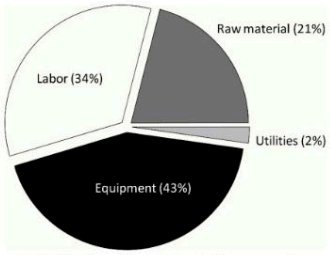

Figure 3. **Distribution of costs in the base case fermentation.**

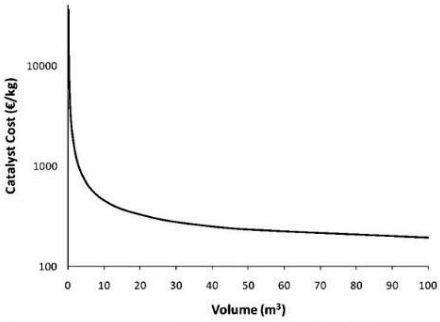

Figure 4. **Effect of scale-up in total production cost.**

dominant. The obtained trend is in accordance with other published reports.[21]

Sensitivity Analysis. Emphasizing the fact that the cost of the biocatalyst will depend on many variables, a sensitivity analysis was carried out to determine and visualize the impact of different process parameters on the biocatalyst production cost. The analysis was carried out by varying one or more input parameters in the economic model to see the effect on the costs. All figures in the sensitivity analysis section are plotted as a cost factor relative to the base case in order that these can be combined to represent a specific case.

Effect of Scale. As discussed previously, one of the most important parameters in the process is the production volume. By varying the production volume in the model, the impact on the cost per kilogram of enzyme was plotted (Figure 4). It can be seen that the production costs decrease rapidly when increasing the scale from 100 L to multiple cubic meter scale and that the cost can be more than halved when increasing the scale from 10 m^3 (the base case) to 100 m^3. However, at very high working volumes momentum, mass and gas transfer limitations are encountered in aerated fermentors. Because of this, the graph will not follow the mathematical model anymore and the points to the extreme right are speculative. On the other hand, at larger production volumes relatively lower cost of raw materials could be expected which would also reduce the total cost of the catalyst.

The general picture that cost of enzyme is dependent on scale means that the market size for a given application is of paramount importance to the selling price of the enzyme.

Effect of Equipment Cost and Utilization. As is clear from Figures 3 and 4, the equipment costs are an important contribution to the cost at practically all scales within the investigated range. A sensitivity analysis was performed directed at the assumptions controlling the equipment costs, i.e. equipment purchase cost (see eq 1), interest rate, economic lifetime of equipment (see eq 2), and equipment utilization. As can be seen from Figure 5, these assumptions also have a significant impact on the total production cost. Most notably the utilization of the equipment (i.e., the number of batches that can be run per year)

(21) Lee, S. Y. *Trends Biotechnol.* **1996**, *14*, 98.

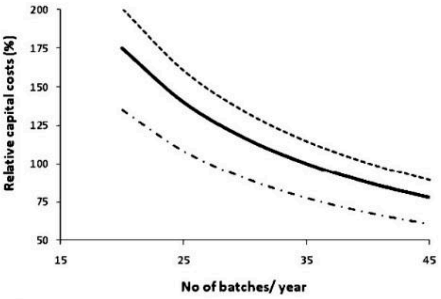

Figure 5. **Impact of equipment purchase costs, utilization, economic lifetime (depreciation), and interest rate on the cost of production in the base case $n = 10$ y, $i = 15\%$; $n = 10$ y, $i = 7\%$; and $n = 15$ y, $i = 7\%$.**

has a great impact on the cost of the enzyme, emphasizing the importance of equipment efficiency (in terms of occupancy). Hence, one can easily understand that ideally the equipment occupation time should be maximized. In our base case, calculating the full fermentation time including setup, harvesting, and cleaning is assumed to be one full working week, although the fermentation time is only 48 h. This results in a low over all productive occupancy (<30%). Indeed, for larger plant facilities the equipment can often be shared among different process lines and can therefore be used more efficiently, thereby reducing the cost of using the equipment. However, one of the main reasons for lengthy downtimes is to reduce the risk of cross contamination. This is a critical issue that needs to be properly addressed, especially in the pharma business as a consequence of GMP regulations.

It can also be seen from Figure 5 that the assumptions regarding interest rate and equipment lifetime has an effect of ~±20% on equipment costs, when varied between 7−15% interest rate and 10−15 years of plant lifetime.

Effect of Fermentation Yield. In the last part of the sensitivity analysis, the yield of enzyme in the process was varied. In an intracellular production system (e.g., *Escherichia coli*) it is possible to obtain yields up to ~15 g/L, after which the system is limited by the cell density (~100 g CDW/L) and internal protein composition (~30% of protein composition[21−23]). Higher levels also run the risk that the protein is expressed as an inclusion body. For an extracellular enzyme production system (e.g., *Pichia pastoris*) higher enzyme levels can be reached. In this study, an upper limit of 25 g/L was assumed, but even higher titers have been reported. In the base case a yield of 6.25 g enzyme/L was assumed, which would represent a somewhat optimized and reasonably successful production system.

As can be seen from Figure 6, the yield has a dramatic effect on the costs of the enzyme, particularly in combination with changes in production scale. This means that enzyme cost could easily vary between tens of thousands of euros per kilogram

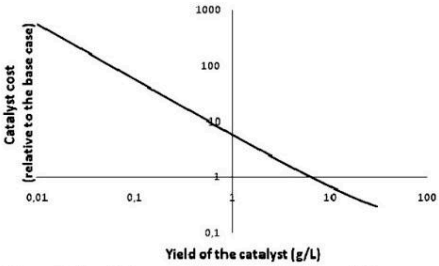

Figure 6. **Sensitivity analysis. Impact of enzyme yield on costs relative to the base case.**

down to less than 200 euros per kilogram. For instance, if the yield of enzyme is 10 mg/L instead of the base case 6.25 g/L, the cost per kilogram of enzyme is increased 500-fold. On the other hand, if the yield can be increased to 15−25 g/L the cost of the enzyme can be cut to a half or a third of base case costs.

A reasonable assumption on enzyme production cost (excluding development costs) for a developed production system on an industrial scale could therefore be between 250−1000 €/kg for an unpurified enzyme and similarly for whole-cells, 35−100 €/kg. However, some types of enzymes are more expensive to produce than others. For instance peroxidases, which require a heme group to be incorporated in the active site to be able to catalyse oxidations, are difficult to produce with high titres of active enzyme and consequently become much more expensive than the base case in this study.[24,25]

Catalyst Formulation. An important stage in the development of a biocatalytic process is to choose the form of catalyst to be used. The active enzyme can be kept inside the host cell (i.e., whole-cell catalysis), or it can be used as an isolated enzyme. If the isolated enzyme is to be used, it is also important to determine to what extent the enzyme needs to be purified, since this greatly influences the production cost.[26] The choice of catalyst form affects the process in a number of ways: the stability of the enzyme, the possibility for recycling of cofactors, selectivity, mass-transfer, etc.[8] According to an analysis performed by Straathof and co-workers,[5] about 60% of the reported industrial biocatalytic reactions use whole-cells (in either free or immobilized form) as catalysts, with the remainder using either soluble or immobilized enzymes.

As will be seen in the later sections of this article, the low allowed-cost contribution for bulk and commodity chemical production processes necessitates a high catalyst productivity, i.e. a large amount of product per kilogram of catalyst. One way of limiting the enzyme consumption would be to use very little enzyme in each reaction. However, since it is normally desirable to keep the reaction volume as low as possible, the demand for high space-time yield typically translates into a need to reuse the enzyme.[27] This means that a method for separating the enzyme from the reaction mixture is required, either by retaining the enzyme in the reactor or by separating it from the

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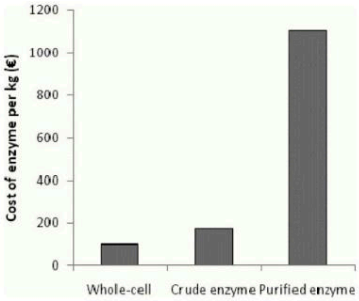

Figure 7. **Cost of purification of enzyme catalyst.**

outgoing product stream. One useful way of achieving this is by immobilizing the enzyme.[28−30] An added advantage of immobilization is that it allows enzymes to operate in systems where they are not usually soluble, such as in organic solvents.

It is important to recognize that the intrinsic enzyme properties, including activity and stability, can be quite different in an immobilized preparation compared to the soluble form.[27] Since these catalysts are heterogeneous, they will also be subject to mass transfer limitations that can reduce the overall activity and potentially selectivity of the enzyme.[31] These issues have not been considered in the present study, but are mentioned here to illustrate that modifications to a biocatalyst in order to suit a given application require additional time and cost for development and implementation.

Recovery and Purification. For whole-cell biocatalysts, application in the reactor may proceed directly (normally after centrifugation or filtration to replace the fermentation medium and/or adjust concentration). For an enzymatic catalyst (whether used in soluble or immobilized form) the costs for recovery and purification need to be estimated. In order to illustrate this, the cost of three different biocatalyst formulations was analyzed: whole-cell, crude enzyme, or purified enzyme, based on a process for manufacturing β-galactosidase in the process simulation software, SuperPro Designer.[32] The cost of the product was calculated after the different recovery and purification steps. The whole-cell biocatalyst was recovered by microfiltration; to obtain crude enzyme the cells were run through a homogenizer, centrifuged to remove cell debris, and finally submitted to ultrafiltration. Partially purified enzyme was prepared by additionally running ion-exchange and gel filtration chromatography as well as two additional ultrafiltration steps. As can be seen from Figure 7 the added cost in each step is significant. The preparation of crude enzyme from whole cells adds to the specific cost of the enzyme by a factor of almost 2. Needless to say, this value could be significantly reduced by developing an extracellular production scheme. Furthermore,

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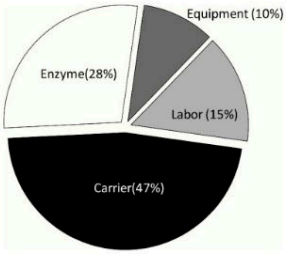

Figure 8. **Cost distribution for the base case.**

purification by chromatography adds almost an order of magnitude to the cost where the major cost contribution comes from the consumables such as the resin material. From this analysis it follows that it is very important to weigh the cost of purification against the added value of higher enzyme purity. The general rule-of-thumb is that the crudest possible form of enzyme acceptable, to maintain product quality, should be used.[10]

Immobilization of Biocatalyst. In this work immobilization by adsorption has been used as an example to illustrate the principle of costing an enzyme immobilization process, based on the parameters outlined in Supporting Information, Appendix 2. This particular immobilization procedure involves preparation of the enzyme solution and adsorption of the enzyme onto a carrier from solution, followed by filtration and drying of the preparation for storage and use.[28,31]

Figure 8 shows the distribution of costs in the base case for adsorption immobilization; e.g. raw material accounts for 75% of the costs of the catalyst. With these conditions, the immobilization increases the specific enzyme cost by a factor of 4, from 500 €/kg to 2000 €/kg, although the per kilogram cost of the catalyst is of course lower, 100 €/kg of immobilized enzyme.

Immobilization Sensitivities. The calculated 4-fold increase in enzyme cost upon immobilization for the base-case is linked to the assumptions listed in Appendix 2 (Supporting Information). Figure 9 shows the effect of variations in enzyme and material (e.g., carrier) cost, as well as labor intensity and batch size, on the final biocatalyst cost. The impact of the cost of the carrier and the labor intensity is directly proportional to the cost contribution of each in the base case. Hence, greater accuracy is required in the estimation of the material cost (a similar argument can be made for the enzyme loading on the catalyst).

The impact of production scale (batch size) is more complicated. In the base case, material costs constitute 75% of the total costs, and the final catalyst cost is thus relatively robust with respect to the production volume; reducing the batch size by 5% only increases the cost increase by 1%. Similar effects are obtained with a variation in equipment utilization (data not shown).

The relative added cost of the immobilization procedure is highly dependent on the enzyme cost, or rather the cost of the enzyme relative to the cost of added materials, labor, and equipment. For the base case, this means that the relative cost increase of the enzyme would be considerably lower for a more

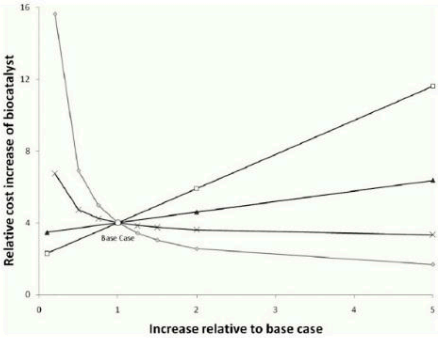

Figure 9. **Variation in relative cost increase for an immobilized enzyme with changes in cost of enzyme (◇) and other materials (□), labor intensity (▲), and batch size (×), relative to the base case.**

expensive enzyme. Although this indicates that the cost of the immobilization procedure is more critical for less expensive enzymes, the final choice of catalyst must be based on a balance between both cost and performance in the intended application.

Figure 9 shows the sensitivity of the biocatalyst cost when the key parameters are varied (cost of the free enzyme, cost of the carrier, labor intensity, and batch size). The cost variation and the slope of the variation are highly related with the cost distribution (see Figure 8), meaning that a variation in the carrier cost will strongly affect the cost of the immobilized catalyst, since it accounts for the biggest share of the total cost in this case.

By varying the different production parameters, i.e. enzyme cost, carrier cost, production scale, etc., it was found that a range of cost for the immobilized enzyme (enzyme adsorbed on resin) of 100−1000 €/kg is a reasonable assumption for further calculations.

Application of Economic Analysis

The role of economic analysis as illustrated by the above examples is three-fold. First, it can provide guidelines for targets which need to be achieved, such as the production yield (kg product/kg catalyst). Second, it can identify key bottlenecks in a process (such as the biocatalyst production or downstream process) by identifying process performance sensitivities (such as production yield, fermentation yield, and recovery yield) to operating variables and process design. Finally, the combination of these analyses leads to a strategy for process development and improvement by introducing new targets, such as increase of production yield by protein engineering,[33] improving expression system,[34] or improving product recovery steps by introducing *in situ* product removal.[35]

Guidelines for Biocatalyst Productivity Targets. As can be seen in the analysis above, biocatalysts are relatively expensive compared to other raw materials in a process in terms of cost per kilogram. However, the price of a catalyst does not mean much in itself. The important question is how much the catalyst contributes to the cost of the product compared to the added value of using biocatalysis over other production methods. The added value could be achieved through higher yield, milder reaction conditions, higher product purity, fewer reaction or purification steps, improved safety, reduced emissions to the environment, or the manufacture of a unique product.[7] In the following section the productivity requirements in terms of kilogram of product produced per kilogram of biocatalyst is calculated for different types of chemicals (bulk to pharmaceuticals) when using either whole-cell, free enzyme, or immobilized enzyme as the biocatalyst. Finally these requirements have been summarized in Table 3.

The productivity requirements (in terms of product produced per kg of biocatalyst) are related to the allowable cost contribution of the biocatalyst and the cost of the biocatalyst by the following equation:

$$\text{productivity target} = \frac{\text{biocatalyst cost}}{\text{allowable cost contribution}}$$

Different types of chemicals generally put different requirements on the allowable cost contribution of the catalyst. A high volume bulk or commodity chemical, typically priced in the range of 1 €/kg,[36] could be assumed to allow the enzyme to contribute about 5% of the selling costs, i.e. around 0.05 €/kg. For specialty or performance chemicals, such as cosmetic ingredients and food supplements, prices are somewhat higher. If a selling cost from 5 €/kg is assumed, the allowable cost of the biocatalyst could be around 0.25 €/kg.[30]

In the fine and pharmaceutical chemical segment product values are considerably higher, up to hundreds of euros per kilogram. In the framework of the present study it was assumed 15 €/kg for fine chemicals (pharmaceutical intermediates) and 100 €/kg for finished small molecules for use as pharmaceuticals. In such cases the higher-value, smaller-market, and increased process complexity would allow for a higher cost contribution of the biocatalyst. For example, if 10% is assumed, then the allowable contribution for fine chemicals is 1.5 €/kg, and for pharmaceutical intermediates, 10 €/kg.

In the case study presented here the ranges of production costs for the different forms of the biocatalyst were found to be 35−350 €/kg DCW for the whole-cell, 250−2500 €/kg for the crude isolated enzyme, and finally 100−1000 €/kg for the immobilized biocatalyst. However, the different market volumes of bulk and pharmaceutical products mean that development costs need to be shared on a widely different volume of biocatalyst and also that the production volume of the biocatalyst will be different (which also affects the biocatalyst production costs).

As can be seen from the results presented in Table 3, the required productivity targets range over several orders of magnitude, depending on the type of catalyst and product. The

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Table 3. **Required productivities for different types of processes and products, based on typical values of biocatalyst and product cost**

	typical product cost (€/kg)	allowable cost contribution of enzyme (€/kg)	biocatalyst cost	range of required productivity
pharma	>100	10	whole-cell: 100−350 €/kg DCW free enzyme: 1000−2500 €/kg enzyme immobilized enzyme: 500−1000 €/kg biocatalyst	10−35 kg product/kg dry cell weight 100−250 kg product/kg free enzyme 50−100 kg product/kg immobilized enzyme
fine chemical	>15	1.5		70−230 kg product/kg dry cell weight 670−1700 kg product/kg free enzyme 330−670 kg product/kg immobilized enzyme
specialty chemical	5	0.25	whole-cell: 35−100 €/kg DCW free enzyme: 250−1000 €/kg enzyme immobilized enzyme: 100−500 €/kg biocatalyst	140−400 kg product/kg dry cell weight 1000−4000 kg product/kg free enzyme 400−2000 kg product/kg immobilized enzyme
bulk	1	0.05		700−2000 kg product/kg dry cell weight 5000−20000 kg product/kg free enzyme 2000−10000[a] kg product/kg immobilized enzyme

[a] Productivity values similar to this have been reported in a number of well-documented commercial processes such as the production of high fructose corn syrup with glucose isomerase[37] and biocatalytic acrylamide synthesis.[38]

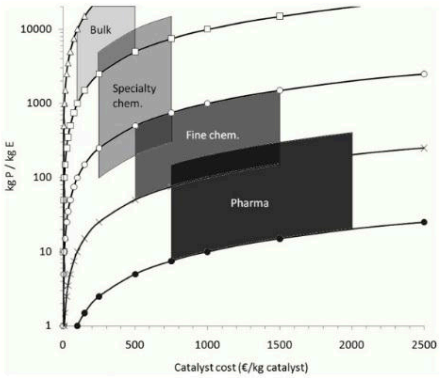

Figure 10. **Effect of biocatalyst cost and allowable cost contribution on the requirements for biocatalyst productivity in terms of kilogram of product per kilogram of biocatalyst used for production of bulk, fuel, or specialty chemicals using immobilized enzymes. Allowable cost contribution of 0.01 €/kg △, 0.1 €/kg □, 1 €/kg ○, 10 €/kg ×, 100 €/kg ●.**

large difference in productivity requirements between whole-cell and crude enzyme is striking, although this is mainly due to the difference in enzyme concentration of the two preparations. Further, as is clear from the sensitivity analysis on biocatalyst cost (discussed earlier), the cost of the biocatalyst could be orders of magnitude higher for a nonoptimized process, and in addition the added value from introducing a biocatalyst can vary very much between different processes. To illustrate this, Figure 10 shows the correlation between allowable cost contribution of the enzyme, cost of the biocatalyst, and required productivity for different types of processes, using as an example the use of an immobilized biocatalyst.

The top left box in Figure 10, represents bulk processes with an allowed cost contribution of from 1 cent per kilogram to 10 cents per kilogram and the cost of the biocatalyst in the low range (100−500 €/kg) because of the large production volumes. Slightly overlapping is the box representing specialty chemicals, which have a quite broad range of allowable cost contribution due to the many different types of chemicals in this group. The cost of the biocatalyst will probably be somewhat higher than that for bulk processes. Further down to the right are the boxes for pharmaceutical intermediates and small-molecule pharmaceutical products with allowable cost contributions of the biocatalyst much higher than that for bulk and specialty chemicals, but at the same time, higher costs for the biocatalyst. On one hand, it can be seen that for low-value bulk chemical processes (such as for biofuel) it is likely that a productivity of more than 10000 kilograms per kilogram of catalyst will be required. Even productivities that cannot realistically be achieved using biocatalysts could be required if the biocatalyst cannot be efficiently produced or if the added value to the process is very low. On the other hand, a higher-priced bulk chemical with a high margin for biocatalyst cost could allow for productivities down to about 1000 kilograms of product per kilogram of biocatalyst if a low-cost catalyst could be manufactured. For specialty chemical processes the range could be even larger, from several thousand down to less than a hundred kilograms of product per kilogram of biocatalyst. Finally, for pharmaceutical intermediates and small-molecule pharmaceutical products the required productivities are lower and lie in the range of 50−1000 for pharmaceutical intermediates and 5−100 for small-molecule pharmaceutical products.

In conclusion, more expensive products can carry a higher catalyst cost—suggesting lower productivity requirements—but these products normally have a smaller market size. Consequently, the catalyst production cost will be higher. The definitive productivity required to ensure that a process is economically viable needs to be evaluated on a case by case

basis. Nevertheless, the values suggested in Table 3 will be a useful starting point for setting development targets in different process sectors.

Towards Process Improvement. As previously mentioned, the economic analysis of a biocatalytic process is a useful tool for process improvement. Productivity targets can be set as a basis for improvement. What to improve is set by identifying the process bottlenecks (or the parts of the process preventing the process from being economic).

For example, sensitivity analysis on biocatalyst yield (see Figure 6) can be used to set the development targets for the R&D department. Subsequently, on the basis of the requirements of the specific process, a strategic decision needs to be made if the required targets can be met with reasonable development effort in terms of time and money. In the example of biocatalyst production, for a nonoptimized or wild-type expression system, quite low yields are probable; a starting point in the milligram of enzyme per liter range could be considered as reasonable. On the other hand, optimized production systems can (and must) achieve much higher yields (using genetically engineered microorganisms[21,22]). However, these high yields require highly optimized production protocols and expression systems, which normally take many weeks or months to develop.[39] This comes at a significant cost that in the end also needs to be carried by the product. However, as these costs are very difficult to estimate and the added cost per kilogram of product depends also on the sales volume, this has not been included in the current model.

Concluding Remarks

Process cost estimation is extremely useful, both in production as well as in R&D, to guide activities directed at developing, implementing, and improving processes. Much useful information can be obtained about the drivers and bottlenecks preventing the immediate implementation of an effective and economic process, even at an early stage of development (where the uncertainties are considerable). Process cost estimation can therefore be very useful as a decision-making tool.

The study we report here shows that many factors work together in determining the cost of the biocatalyst and that the range of cost is therefore rather wide (from hundreds of Euros per kilogram of enzyme up to several thousands of Euros per kilogram). In the first step of the production (fermentation), the enzyme titer is crucial; a product yield in the gram per liter range is required to avoid excessive costs. This means that almost without exception, significant effort must be put into developing the fermentation process before it is ready to be used industrially. It also means that analyzing the production cost at an early stage of process development will overestimate the cost of the mature process. Moreover, the scale of production greatly influences the production cost, especially at volumes less than ~100 kg per batch (~20 m^3). Finally, any purification steps might also increase the production costs within an order of magnitude.

As with any new technology, a cost/benefit analysis has to be performed to weigh the added cost of the biocatalyst against the value of the process improvements. This study has shown that, for low-value, large-volume products, the required biocatalyst productivity is in the range of 2000−10000 kg/kg immobilized enzyme. For higher-value products, the required productivity is, of course, lower; nevertheless, even for high-priced fine chemical compounds there are high productivity requirements, ~50−1000 kg/kg, due to the lower production volumes and thus higher cost of the biocatalyst. As proven by the number of industrially implemented biocatalytic processes, these target productivities can be reached, but low-volume specialized catalysts can only be applied to processes where they can contribute to the process via significant improvement or achievement of very high productivity. Correct assessment (as well as consistent documentation) of catalyst productivity is therefore essential to determine the viability of a biocatalytic process, and is something that should be emphasized in any study of biocatalysis.

In a biocatalytic process, directed development of the catalyst specifically for the reaction of interest is frequently required. However, some industries (such as the pharmaceutical industry) cannot afford time-consuming research on protein development, and the possibility for process development is limited. Hence, the development of industrial biocatalysis is dependent on the availability and use of already developed biocatalysts and ultimately the enlargement of technological platforms.

In many ways biocatalytic processes can still be considered a technology under development (which has not yet reached its full potential), and much work remains before platform technologies are available, allowing quick and consistent development of efficient and cost-effective biocatalytic processes. Furthermore, academia and R&D departments in industry should join efforts aimed at the development of given technological platforms embracing fermentation and biocatalyst and process development for particular reaction types. Such platforms should also be a source of information concerning the development of fermentation and catalyst production (as the pluGbug developed and commercialized by DSM), development of the catalyst (as the effort put in by Novozymes on its lipase, Novozyme 435), and process development (such as technologies for *in situ* substrate supply and product removal[3,24,35]). A range of reactions should be considered to extend the currently available technologies.

The establishment of a suitable platform might guide the development of different products and processes, leading to a common effort towards the improvement and wider application of biocatalysis in industry.

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Supporting Information Available

Appendices 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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REVIEW

BIOTECHNOLOGY AND BIOENGINEERING

Process Considerations for the Asymmetric Synthesis of Chiral Amines Using Transaminases

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ABSTRACT: Biocatalytic transamination is being established as key tool for the production of chiral amine pharmaceuticals and precursors due to its excellent enantioselectivity as well as green credentials. Recent examples demonstrate the potential for developing economically competitive processes using a combination of modern biotechnological tools for improving the biocatalyst alongside using process engineering and integrated separation techniques for improving productivities. However, many challenges remain in order for the technology to be more widely applicable, such as technologies for obtaining high yields and productivities when the equilibrium of the desired reaction is unfavorable. This review summarizes both the process challenges and the strategies used to overcome them, and endeavors to describe these and explain their applicability based on physiochemical principles. This article also points to the interaction between the solutions and the need for a process development strategy based on fundamental principles.

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KEYWORDS: biocatalysis; aminotransferase; process development; chiral amines

Introduction

Chiral amines are key building blocks for many new pharmaceuticals (NCEs and APIs). Chiral amines can be produced both by chemical and biocatalytic synthesis (Breuer et al., 2004). However, despite the great effort that has been put into developing efficient routes for chemical synthesis, this still remains a challenge (Nugent and El-Shazly, 2010). As an alternative, transaminases (EC 2.6.1.X; also known as aminotransferases) have received much attention as suitable catalysts for producing these amines either by direct asymmetric synthesis from pro-chiral ketones or by kinetic resolution of racemic amines. Transaminases catalyze the transfer of an amine ($-NH_2$) group from an amine donor, usually an amino acid or a simple amine such as 2-propyl amine, to a pro-chiral acceptor ketone, yielding a chiral amine as well as a co-product ketone or alpha-keto acid (Fig. 1). Transaminases require the cofactor pyridoxal phosphate (PLP) to act as a shuttle to transfer the amine group (Eliot and Kirsch, 2004). This cofactor is fully regenerated within the same two substrate reaction on the same enzyme, and hence does not pose the cofactor regeneration problems encountered in oxidation/reduction reactions (Hwang et al., 2005; Pannuri et al., 2003). Generally speaking, transaminases are suitable catalysts due to their high stereoselectivity, and ability to operate under environmentally mild reaction conditions. Transaminases and their function have been known for quite some time (Christen and Metzler, 1985) and the technology is already used in industry to produce selected chiral amines (Pannuri et al., 2003). Even so, in spite of the many attractive features of transaminase catalyzed reactions, there are still a number of challenges that need to be dealt with in order to make transaminase processes feasible for the production of a wider range of amines.

In the reaction step, two general strategies are used to obtain the target chiral amine; either direct asymmetric synthesis or kinetic resolution of a racemic amine. The latter alternative is the commonly used option in industry today although it is hampered by a 50% theoretical yield, unless a racemization step is included to enable a dynamic kinetic resolution (DKR). Nonetheless, using this strategy high enantiomeric excess (ee) values are easily attainable. However, in this report, the focus will be on direct asymmetric synthesis, since this is the state-of-the art of the

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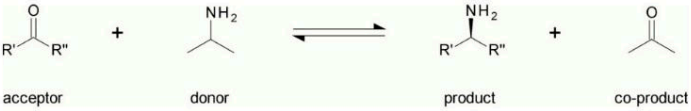

Figure 1. Overview of the biocatalytic transamination reaction.

technology and for the future the preferred reaction configuration, although more challenging than the resolution strategy.

Many of the problems encountered with transaminases are common to other biocatalytic processes and consequently many parallels can be drawn to other biocatalytic reactions for the production of chiral molecules (e.g., chiral alcohols). A number of reviews have been published describing the general features of transaminases, for instance by Taylor et al. (1998) and Stewart (2001), whereas Hwang et al. (2005) describes the different subgroups of transaminases and their substrate specificities related to the 3D structure, as well as protein engineering efforts to tailor the specificities. In a review by Koszelewski et al. (2010c) the recent developments in the field are described, with a focus on the different sources of ω-transaminases available.

The current review takes a process perspective and the focus is on the considerations for developing industrial transamination processes at large scale, summarizing the challenges and strategies to meet a number of proposed success criteria for an efficient and economic process. The article also reviews the different proposed solutions and analyzes these from a feasibility point of view supported by calculations and examples. We also suggest engineering tools to model and assess the process to move this technology towards a rational approach for developing large-scale processes.

Process Overview

The biocatalytic transaminase catalyzed production scheme consists of four major steps (Fig. 2); fermentation, biocatalyst formulation, reaction, and product recovery. Unless the biocatalyst is purchased from an external supplier (in which case the first two steps can be disregarded), the desired enzyme activity is expressed in a host microorganism to high product titer and thereafter prepared in a suitable form (biocatalyst formulation) for the reaction step (Fig. 3). To avoid unnecessary costs the biocatalyst is used in the crudest possible form; either as whole cells or cell-free extract (crude enzyme). Immobilization of the cells or enzymes can furthermore be used to facilitate recovery and improve the stability, thereby extending the use of the catalyst to multiple batches.

After the reaction is complete the biocatalyst is removed (biocatalyst separation) and the product is separated and purified. In each step of the process there are a number of factors that will determine the final economic viability of the process and the optimization of each step is essential in order to achieve a viable process.

There is little quantitative data published on the economics of biocatalytic processes and how the different performance metrics affect the cost of the total process. However, some of the metrics commonly used to benchmark the process (Bommarius et al., 2001; Straathof et al., 2002; Tufvesson et al., 2010) are summarized in Table I. In a previous article (Tufvesson et al., 2011) we have described the requirements for biocatalyst productivities for fine and pharmaceutical processes.

Many parameters work together to determine the success of an industrial biocatalytic process and clearly the limits in Table I are not absolute and should therefore be seen as general recommendations. Definitive requirements can only be determined on a case-by-case basis taking into account the added value of the process, competing technologies, and so on.

One of the most important factors is the added cost of the biocatalyst, which is why it is essential to maximize the biocatalyst productivity (g product/g biocatalyst). In addition, the product concentration is a key parameter as it determines the equipment cost and ease of downstream

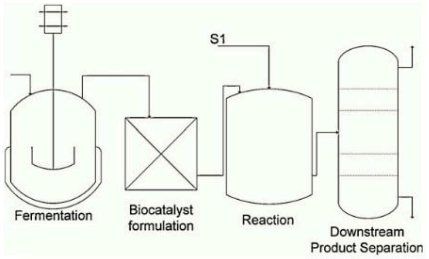

Figure 2. Overview of the biocatalytic transamination process.

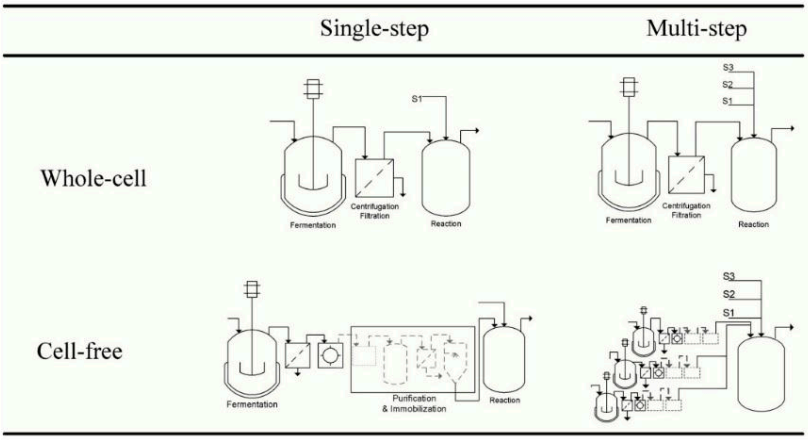

Figure 3. Detailed process design for the first three steps in the biocatalytic transamination process. S1—main feeding stream (amine donor, amine acceptor, PLP), S2—substrate for co-product removal, S3—co-factor regeneration.

separation and recovery. In order to compare how the state-of-the art transaminase technology relates to the above stated requirements, a summary of published reaction conditions and process metrics has been compiled in Tables II and III.

As can be seen from Figure 4, most studies are far from meeting the required industrial process requirements, with three notable exceptions; the work by Truppo et al. and the work by Savile et al., both reports from 2010 and the work by Martin et al. (2007). Even though none of these studies fulfill all of the guidelines in Table I, Martin et al. (2007) shows a very high catalyst productivity, especially considering that the catalyst is a whole cell. Savile et al. (2010) on the other hand demonstrate that the process can be run at substrate loadings significantly above the recommended minimum concentration. Ways for improving the reaction performance have included both biocatalyst improvements (e.g., by protein engineering or immobilization) and process improvements (e.g., by in situ product removal).

In the following sections of this article the main process challenges in biocatalytic transamination will be presented and solutions (and/or suggestions) for process improvement will be discussed.

Table I. Success factors for the economic feasibility of a biocatalytic process (Tufvesson et al., 2010).

Fermentation	
Cell titer	50–100 g CDW/L (if intra cell.)
Protein titer	1–10 g/L
Biocatalyst formulation	
Retention of activity	High
Stabilization	Improve catalyst productivity >5 times
Reaction	
Product concentration	>50 g/L
Catalyst productivity	10–35 g product/g whole cell (DCW) 100–250 g product/g free enzyme (crude) 50–100 g product/g immob. enzyme
Stereoselectivity	>98% ee
Yield	>90%

Process Challenges and Strategies

In order to meet the success criteria put forward above, a thorough knowledge of the reaction system is required relating both to the reaction thermodynamics, the physical characteristics of the reaction components and the possibilities and limitations of the given biocatalyst.

There are many challenges inherent to transaminase processes that need to be dealt with and numerous reports have been published that address one or more of these challenges. Frequently the suggested solutions, or technologies, solve more than one problem, for instance the use of an auxiliary phase may solve issues related to substrate and product inhibition as well as low water solubility, but on the other hand the solution might pose other problems such as lower biocatalyst stability. An overview of transaminase process challenges have been put together in Table IV, along with the suggested technologies or strategies used to

Table II. Summary of some the most important published transaminase reaction for the synthesis of amino acids.

Target compound	Amine donor	C amine acceptor (mM)	Ratio D/A	Temperature, pH, enzyme	Yield (%)	ee	Time (h)	Vol. prod (mM/h)	CatProd (gP/gwTAm) (gP/gCDW)	Refs.
Cell-free catalyst										
L-phosphinothricin	Glu + Asp	500	0.2 + 1.2	50, 8.0, column with immobilized phosphinothricin TAm and Glu: oxalacetate TAm	82.7	>99	24	17.2	—	Bartsch et al. (1996)
(3S)-amino-3-phenylpropionic acid	3-aminobutyrate	10	2	37, 7.5, *C. rugosa* lipase and TAm from *Mesorhizobium* sp.	20	>99	24	0.1	0.4	Kim et al. (2007b)
Ala	MBA	10	1	37, 7.0, cell extract containing ω-TA from *C. violaceum* expressed in *E. coli*	95	—	3	3.2	1.3	Kaulmann et al. (2007)
Gly		10	1		95	—	3	3.2	1.1	
L-phenylalanine	L-Asp	12.5	2	22, 7.0, free TAm and oxaloacetate decarboxylase	98.5	—	12	1.0	—	Rozzell (1985)
L-tyrosine	L-Asp	10	1	24, 7.0, TAm and oxaloacetate decarboxylase	99	—	1	9.9	0.0	
L-HPA	L-Lys	200	1.5	37, 9.0, aspartate aminotransferase (aspC from *E. coli*)	97	>99.9	24	8.1	0.9	Lo et al. (2005)
Cascades (cell-free)										
1-N-Boc-3-aminopyrrolidine	L-Ala	5	22	37, 7.0, TAm from *V. fluvialis* and PDC (Pyr decarboxylase)	80	99	1	4.0	—	Höhne et al. (2008)
2-aminobutyrate	L-Glu	50	1	37, 8.5, crude enzyme extract from *E. coli* (encoding branched-chain TAm and ornithine TAm)	91.92	—	5	9.2	7.9	Li et al. (2002)
L-*tert*-leucine	L-Glu	40	1		72.98	—	16	1.8	6.4	
Cascades (whole cell)										
Phenylalanine	Asp	100	1.5	37, 8.4, *E. coli* overexpressing Tam-AspC and phosphoenolpyr. carboxykinase PcK	93.0	—	2	46.5	3.4	Chao et al. (1999)
2-aminobutyrate	L-Asp	500	1.0	37, 7.5, *E. coli* (carrying wTAm and alsS from *B. subtilis*)	54.0	—	24	11.3	0.3	Fotheringham et al. (1999)
2-aminobutyrate	L-Asp	500	1.0	37, 7.5, *E. coli* (carrying tyrB (Tyr aminotransferase) from *E. coli* and alsS (acetolase synthase) from *B. subtilis* and ilvA (threonine deaminase) from *E. coli*)	53.7	—	24	11.2	0.3	Fotheringham (2001)
2-aminobutyrate	L-Asp	500	1.0	37, 8, *E. coli* (carrying tyrB from *E. coli*, alsS from *B. subtilis*)	58.3	—	24	12.1	0.3	
L-HPA	L-phenylalanine	40	2.5	37, 8.4, *E. coli* (AroAT from *Enterobacter* sp., carbonyl reductases, phenylpyruvate descarboxylase and GDH)	96.3	—	24	1.6	—	Hwang et al. (2009)
2-aminobutyrate	L-Asp	500	1.0	37, 7.5, *E. coli* (tyrB gene from *E. coli*, alsS gene from *B. subtilis*)	58.3	—	24	12.1	0.6	Ager et al. (2001)
2-aminobutyrate	L-Asp	500	1.0	37, 7.5, *E. coli* (ilvA gene from *E. coli*, tyrB gene from *E. coli*, alsS gene from *B. subtilis*)	53.7	—	24	11.2	0.6	
Whole cell										
beta-(2-Thienyl)-D-alanine	L-Asp	176	1.2	40, 8.0, *E. coli ATCC11303* (with tyrB), without atmospheric O2	82.0	>98	24	6.0	4.1	Meiwes et al. (1997)
L-HPA	Asp	10	1.0	37, 7.2, *E. coli* (tyrB + wtaA)	83.0	99.0	10	0.8	0.1	Cho et al. (2003)
2-aminobutyrate	Ala	10	1.0	37, 7.2, *E. coli* (avtA + wtaA)	90.0	95.0	5	1.8	0.1	
L-HPA	L-Glu	100	1.5	37, 8.5, Tyrosine-aminotransferase (glutamic-aromatic aminotransferase) from *E. coli*	95.0	>99	2	47.5	—	Chen et al. (2000)

D/A, donor to acceptor; MBA, methylbenzylamine; Ala, alanine; Asp, aspartate; Glu, glutamate; Gly, glycine; HPA, homophenylalanine IPA, 2-propyl amine; Lys, lysine; TAm, transaminase.

Table III. Summary of some the most important published transaminase reaction for the synthesis of chiral amines.

Target compound	Amine donor	C amine acceptor (mM)	Ratio D/A	Temperature, pH, enzyme	Yield (%)	ee	Time (h)	Vol. Prod (mM/h)	CatProd (gP/gwTAm) (gP/gCDW)	Refs.
Cell-free catalyst										
MBA	MPPA	5	10	30, 7, Cell-free extract from *V. fluvialis* JS17	21.4	—	24	0.04	—	Shin and Kim (1999)
MBA	3-aminoheptane	5	10		10.1	—	24	0.02	—	
MBA	*sec*-butylamine	5	10		7.7	—	24	0.02	—	
Sitagliptin	IPA	491	2	45, 7, mutant of ATA-117, 50% DMSO	92	>99	24	18.82	30.67	Savile et al. (2010)
MBA	IPA	20	50	30, 7.5, ATA-113/117	95	>99	24	0.79	0.46	Truppo et al. (2009a)
MBA	*sec*-butylamine	10	10	40, 6.0, purified TAm from *M. aurum*	19.9	>99	71	0.03	—	Takashima et al. (2004)
MBA	IPA	2	311	37, 7, *E. coli* (ω-TAm from *A. citreus*, YADH, FDH)	99.0	>99.9	24	0.07	1.35	Cassimjee et al. (2010)
Cascades (cell-free)										
MBA	L-Ala	30	10	30, 7, cell-free extract from *V. fluvialis* JS17, LDH	5.83	>99	12	0.15	—	Shin and Kim (1999)
2-butylamine	Ala	50	5	30, 7, ATA-113 + ATA-117, lactate dehydrogenase mix	99	>99	24	2.06	0.30	Koszelewski et al. (2009)
4-phenylbutylamine		50	5	30, 7, ATA-113 + ATA-117, lactate dehydrogenase mix	99	>99	24	2.06	0.62	
Mexiletine		50	5	30, 7, ATA-113 + ATA-117, lactate dehydrogenase mix	99	>99	24	2.06	0.74	
MBA	Ala	416	2	30, 7.5, ATA-113/117, GDH CDX-901, LDH-102, with ISPR (ion exchange resin)	99	>99	19	21.68	9.98	Truppo et al. (2010)
MBA	Ala	50	10	30, 7.5, ATA-103/117, LDG/GDH	96	99	10	4.80	1.16	Truppo et al. (2009a)
MBA	NH_4^+ via Ala	50	1	30, 7.5, ATA-103, LAADH-117/GDH	96	99	48	1.00	1.16	
2-aminopentane	L-Ala	76	4	30, 7, *E. coli* (BM-ω-TAm from *B. megaterium*), AADH	97.0	>99	24	3.07	0.64	Koszelewski et al. (2010b)
1-methoxy-2-propylamine		76	4		97.0	>99	24	3.07	0.66	
p-Methoxyamphetamine	L-Ala	76	4	30, 7, *E. coli* (ω-TAm from *C. violaceum*), AADH	94.0	>99	24	2.98	1.18	
2-aminopentane		76			94.0	>99	24	2.98	0.62	
1-Methoxy-2-propylamine		76			96.0	96.0	24	3.04	0.65	
2-aminopentane	L-Ala	39	4	30, 7, *E. coli* (BM-ω-TAm from *B. megaterium*), L-lactate from bovine heart	99.0	>99	24	1.61	0.67	
1-methoxy-2-propylamaine		39			94.0	>99	24	1.53	0.65	
p-Methoxyamphetamine	L-Ala	39	4	30, 7, *E. coli* (CV-ω-TAm from *C. violaceum*), L-lactate from bovine heart	94.0	>99	24	1.53	1.21	
2-aminopentane		39			99.0	>99	24	1.61	0.67	
Cascades (whole cell)										
MBA	Ala	10	10	37, 7, *E. coli* (ω-TAm from *V. fluvialis* JS17, ALS from *B. subtilis* 168)	34.0	—	20	0.17	0.21	Yun and Kim (2008)
2-amino-1,2,4-butanetriol	MBA	20	1	25, 7.0, *E. coli* (with transketolase from *E. coli* and TAm from *P. aeruginosa*)	21.0	—	62	0.07	0.85	Ingram et al. (2007)
Whole cell										
(R)-3,4-dimethoxyamphetamine	(R)-MBA	154	1	30, 8.5, *Anthrobacter* sp. KNK168	81.8	>99	40	3.15	—	Iwasaki et al. (2006)
MBA	L-Ala	30	10	30, 7, *V. fluvialis* JS17	90.2	>99	24	1.13	—	Shin and Kim (1999)
S-aminotetralin[a]	IPA	130[a]	11.5[a]	55, 7, *E. coli* containing mutant mesophilic TAm (from *A. citreus*)	88.5	>99.9	48	2.40	16.93	Martin et al. (2007)

D/A, donor to acceptor; MBA, methylbenzylamine; MPPA, 1-methyl-3-phenylpropylamine; Ala, alanine; IPA, 2-propyl amine; TAm, transaminase MPPA, 1-methyl-3-phenylpropylamine.
[a]Assumed—ketone: tetralone (MW: 147.22 g mol^{-1}) concentrations: ketone 130 mm and IPA 1,500 mm (due to lack of further information).

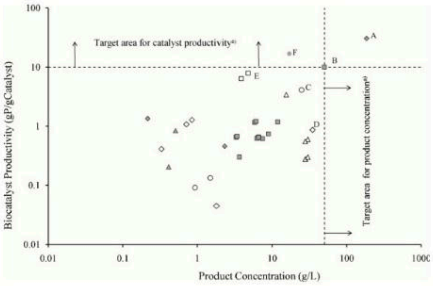

Figure 4. State of the art in transaminase reaction for chiral amines synthesis plotted AS Biocatalyst productivity versus product concentration. ◇: Cell-free catalyst; □: cascades (cell-free); Δ: cascades (whole cell); ○: whole cell; gray markers: synthesis of chiral amines; white markers: synthesis of amino acids. (A) Sitagliptin, Savile et al. (2010); (B) α-MBA, Truppo et al. (2010); (C) β-(2-thienyl)-D-alanine, Meiwes et al. (1997); (D) LHPA, Lo et al. (2005); (E) 2-aminobutyric acid, Li et al. (2002).

overcome these, as well as the further implications of using a specific technology.

Thermodynamic Limitations

A critical issue that needs to be addressed in a biocatalytic transamination reaction is the thermodynamic equilibrium of the reaction system since knowledge about the thermodynamics of the reaction will determine which process solutions are feasible on an industrial scale.

The transamination reaction is reversible and the maximum achievable conversion is thus determined by the initial concentrations and the thermodynamic equilibrium constant (K) of the reaction. K in turn is determined by the change in Gibbs free energy for the reaction, which is given by the difference in ΔG between the products and the reactants. For the amine transfer from an amino acid to an alpha keto acid to form another amino acid, the change in Gibbs free energy is small and thus the equilibrium constant is around one (Taylor et al., 1998). However, for the transfer of an amine group from an amino acid to acetophenone, a commonly investigated ketone, the equilibrium is strongly in the favor of the amino acid (amine donor). Kim and Shin

Table IV. Bottleneck analysis.

	Challenges							
	Process related			Biocatalyst related				
	Low thermodynamic equilibrium	Low substrate solubility	Substrate and product degradation	Inhibition	Stability	Activity	Separation of biocatalyst	Major limitations
Solutions								
Chemistry related								
Stronger amine donor	✓							Cost and availability of the donor
Excess amine donor	✓							Inhibition and stability of enzyme; Not applicable for low Keq; donor cost; downstream separation
Process related								
Solvent (co-solvent/2-phase)		✓						Enzyme stability; downstream separation; VOC
Separation of (co-)product by (a) distillation	✓							Co-distillation of water and/or other components
(b) Extraction (solvent, membrane, resin)	✓	✓	✓	✓				Selectivity between substrates and products
Controlled supply of substrate (fed-batch)			✓	✓				Capacity
Degradation of co-product (cascade)	✓							Compatibility; Added cost of biocatalysts; co-factor recycling
Biocatalyst related								
Whole cell	✓							Side-reactions; separation; GMO regulations
Immobilization					✓		✓	Deactivation; development cost; higher biocatalyst cost
Enzyme development					✓	✓		Development time and cost

Analysis of challenges in biocatalytic transamination and implications for how suggested solutions influence which technologies that are suitable/compatible.

(1998) report a K of about 10^{-3} (for the synthesis of α-methylbenzylamine) based on parameter estimation when performing a kinetic study. Still there are indications from other studies that it may be even more unfavorable than this, since transamination of acetophenone with 10 equivalents L-alanine was completed at 3% conversion, as opposed to the theoretical equilibrium conversion of 9% (Truppo et al., 2009a). When using 2-propyl amine instead of amino acid as the donor, the equilibrium becomes more favorable (Truppo et al., 2009a) but still the equilibrium lies strongly in favor of the reactants. Although Shin and Kim (1999) have compared the effect of different amine donors on the yield, they do not show that equilibrium has been reached. To our knowledge no other reports determining the equilibrium constants using different donors have been published. Even so, the relative amine donating potential of many compounds can be qualitatively extrapolated from literature, for instance the donor potential of α-MBA is order of magnitudes higher than for 2-propylamine, which in turn has much higher potential than alanine. It is clear that the availability of an even stronger amine donor could be very beneficial.

By knowing the reaction Gibbs free energy (ΔG), one can determine the process strategy needed to meet the requirements in terms of yield and product concentration. Different strategies inherently bring about different cost structures and therefore one can identify the reactions that are likely to be able to be scaled-up and applied in industry. Therefore knowledge of the reaction equilibrium constant and/or Gibbs free energy allows a more intelligent process design. Jankowski et al. (2008) have developed a group contribution method for estimating Gibbs free energies for biochemical reactions in aqueous solutions at pH 7 and 25°C, having a standard error of ±2 kcal/mol. This methodology was recently applied by Seo et al. (2011) in the comparison of the transamination potential of different amine donors, where 1-aminoindan was estimated to be thermodynamically favorable for the transamination of acetophenone. Considering the uncertainty in the group contribution method it was within the standard error, that an experimental yield of only 37% was obtained using four equivalents of amine donor, indicating a thermodynamically unfavorable reaction (Seo et al., 2011).

In order to overcome the thermodynamic limitations in transaminase reactions there are several solutions that have been shown to (at least) partly overcome these: addition of excess of amine donor, application of ISPR (in situ removal of product or co-product), auto-degradation of the product, use of enzymatic cascades or whole-cell catalysis.

Addition of Excess Amine Donor

The easiest option for shifting the equilibrium towards a high yield of the product would in principle be to use an excess of the amine donor. This strategy was applied by Savile et al. (2010) for the production of Sitagliptin at high substrate concentrations using approximately 10-fold excess of 2-propylamine. However, the use of this strategy is limited to those cases when the equilibrium is only slightly unfavorable. In fact, from the Savile article it can be extrapolated, that the K in this case is close to unity.

The reason for the limitation to this strategy is that if the substrate concentration is to be kept at a high level (>50 g/L), there will be an upper limitation of how large an excess of amine donor can be used, with stoichiometric equivalents in the range of 1–50 times approaching the limits of amine donor solubility. Figure 5 plots the necessary excess of amine donor required to achieve a yield of 90% at varying value of K. As can be seen from Figure 5, to achieve a yield of 90% an excess of 100-fold is required if the K value is 10^{-1}. Similarly, if K the value is 10^{-3}, an excess of 10,000-fold would be required, which for obvious reasons is unrealistic.

As a consequence of this, for transaminations where K is lower than 10^{-2} adding an excess of amine donor will not be sufficient to reach the process metrics and thus additional strategies are required.

Removal of Product or Co-Product

A second method to shift the equilibrium position in favor of the desired product is to remove the product or co-product from the media during the reaction itself, that is, in situ product removal (ISPR). Again, the equilibrium constant of the reaction determines how low a concentration of product or co-product is required to achieve the target

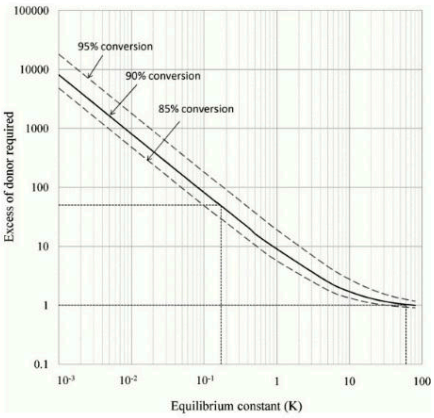

Figure 5. The equilibrium constant (K) determines the excess of amine donor required to reach a thermodynamic equilibrium of 90% (solid line). The broken lines are visual support for an excess of 10 and 50, which can be considered process boundaries.

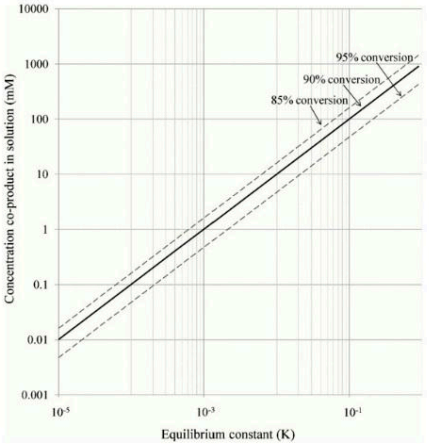

Figure 6. Concentration of co-product required to reach 90% yield when using an initial concentration of 1 mol/L ketone and a tenfold excess of amine donor for the synthesis of chiral amine.

yields. Figure 6 shows the relationship between K and co-product concentration to achieve 90% yield when using an initial concentration of 1 M ketone and a 10-fold excess of amine donor for the synthesis of a chiral amine. As can be seen in Figure 6, at K values $<10^{-3}$ the required co-product concentration will need to be <1 mM. This is important to keep in mind when considering which methods can be used to shift the equilibrium.

The best strategy for ISPR will depend on the properties of the product amine as well as the other components in the reaction mixture. In general, a strategy will be favorable when it produces a big driving force for separating the product from the other components. The physico-chemical properties that are most commonly exploited for ISPR are volatility, solubility, charge, hydrophobicity, and molecular size (Lye and Woodley, 1999).

ISPR strategies are particularly relevant when considering transamination reactions, as they enable a shift of the reaction equilibrium position as well as reducing product inhibition (as will be discussed later). There are many examples to illustrate the use of ISPR strategies in connection with transaminase catalyzed reactions. A summary of the different approaches for ISPR, including the improvement achieved and main drawbacks, can be found in Table V.

Liquid–liquid extraction is a common strategy for the downstream recovery that allows the recovery of a large range of different amines. Extraction under either acidic or basic conditions allows control of the amine product if

Table V. In situ recovery techniques applied for biocatalytic transamination.

Reaction system	Comparison of ISPR vs. without ISPR	ISPR method applied	Major drawback	Refs.
α-MBA ⟷ APH (*B. thuringiensis* JS64)	Ninefold higher reaction rate	Extraction with organic solvent	Decreased enzyme stability	Shin and Kim (1997)
α-MBA ⟷ APH (*B. thuringiensis* JS64)	99% vs. 54.7% (ee)	Membrane extraction (perstraction)	Demand for highly purified enzyme[a]	Shin et al. (2001a)
sec-But.A ⟷ 2-butanone (*E. coli* BL21)	98% vs. 32% (ee)	Evaporation of the volatile inhibitory product	Evaporation of the reaction media (e.g., water)	Yun et al. (2004)
sec-ButA ⟷ (R)*sec*-ButA (*B. megaterium* SC6394)	Enzymatic resolution of racemic mixture with 99% (ee)	Distillation of the volatile amine product	Limit number of amines can be recovered using distillation	Hanson et al. (2008)
4P2B ⟷ (R)4PB2A (ATA-117)	Achieved 92% conversion (99% ee)	Extraction with organic solvent combined pH setting[b]	Organic solvents used; potential decrease of enzyme stability	Koszelewski et al. (2008b)
APH ⟷ α-MBA (ATA-113 and ATA-117)	99% vs. 10% (*max conv.*)	Extraction with resins	Selectivity of resin	Truppo et al. (2010)

α-MBA, α-methylbenzylamine; APH, acetophenone; *sec*-But. A, *sec*-butylamine; 4P2B, 4-phenyl-2-butanone; 4PB2A, (R)-4-phenylbutan-2-amine.
[a]To reduce the residence time and consequently minimize product inhibition.
[b]Followed by evaporation under reduced pressure of the organic solvent in order to obtain the product.

protonated, and thus provides an efficient tool for separating the amine from other components in the product stream (in particular the remaining substrate ketone). For example, such an approach was applied in the post-reaction recovery of (*R*)-4-phenylbutan-2-amine by Koszelewski et al. (2008b). Integrating the extractive recovery with the reaction step would allow a shift of the equilibrium by extracting the product into the second phase, that is, in situ extraction. ISPR by employing adsorbing resins for extracting the product has also been suggested (Woodley et al., 2008). This strategy was shown by Truppo et al. (2010) to be an efficient method to also overcome product inhibition and shifting equilibrium in the production of both (*R*)- and (*S*)-methyl benzylamine. At substrate concentrations of 50 g/L (0.4 M), 200 g of ion-exchange resin was used for product adsorption, resulting in improved reaction rates and yields. This strategy further allowed the product to be easily recovered by filtration and washing of the resin. However, the added cost of using large amounts of resin needs to be considered. Multiple re-uses of the resin will be necessary for a reasonable cost contribution.

There are limitations with all separation strategies. A common limiting factor is related to the selectivity of the separation and the relative concentrations of the reaction components, including the solvent. For instance, an observed problem when using either solvent or resin extraction is that the ketones or amines have similar distribution behavior and therefore will co-extract into the solvent or resin unless another driving force is in place such as ionization. This is well illustrated in the report by Truppo et al. (2010) employing the use of resins to extract the product. The amine donor 2-propyl amine was seen to compete with the product (MBA) for binding to the resin. The similarity between the p*K*a value of the product and the amine donor (9.54 and 10.73, respectively) also excludes using ionization for separation, since at pH 7 more than 99% of both compounds are protonated. This problem was, however, alleviated in the report by changing the amine donor to alanine and implementing a cascade enzymatic system to degrade the pyruvate (as will be described below).

Evaporation of a volatile product (or co-product) may also be an option for shifting the equilibrium towards the product. This has been suggested as an option if 2-propanamine or 2-butanamine is used as the amine donor yielding acetone or butanone, respectively, as the co-substrate (Yun et al., 2004). For volatile amines, distillation could also be a possible route for product recovery in kinetic synthesis (see Table VI). For example (*R*)-*sec*-butylamine (boiling point 63°C) was recovered by distillation of the product mixture under basic conditions (Hanson et al., 2008). Also Savile et al. (2010) report a slight improvement in yield by sweeping the reactor with nitrogen gas to remove the formed acetone.

The selectivity problem is, however, also very problematic when using the evaporation strategy. Assuming ideal conditions, an estimate of the vapor composition can quickly be estimated based on Raoult's law (see Eq. 1, Table VI).

Table VI. Vapor pressure values of pure compounds at 25°C.

Compound	P_{vap} (mbar)
Acetophenone	0.53
α-methylbenzylamine	0.72
Alanine	Non volatile
Pyruvic acid	1.7
Acetone	309
2-propyl amine	773[a]
Acetaldehyde	1202
2-butyl amine	237[a]
2-butanone	121
Water	30.7

[a]At reaction conditions (pH 7) the vapor pressure of amines are negligible due to protonation of the amine.

$$x_i^{vap} = \frac{p_i^* x_i^l}{\sum p_i^* x_i^l} \quad (1)$$

x_i^{vap} molar fraction of compound i in vapor; p_i^* vapor pressure of pure compound; x_i^l molar fraction of compound i in liquid phase.

As an example, if 10 mM acetone is being removed from a water solution the relative amount of water ($C_{water} \sim 55$ M) evaporated will be over 500 times that of acetone. Hence, in a thermodynamically unfavorable system, the concentration of acetone will need to be reduced significantly beyond this point as shown previously, making the problem more difficult. Similarly, the volatility of any co-solvent and the donor amine need to be considered when using this approach.

Auto-Degradation of Co-Product

A very convenient, but not widely applicable approach is the use of a self-degrading co-product or products. Fotheringham and coworkers (Ager et al., 2001; Li et al., 2002) found that when using ornithine or lysine as amine donor, the formed amino-keto acid is cyclized spontaneously thus favoring the reaction in the direction of the amine (Ager et al., 2001; Li et al., 2002; Lo et al., 2005). Truppo et al. (2010) used a similar approach where the product cyclized, thereby shifting the equilibrium of the reaction.

Enzymatic Cascade Reactions

A much explored approach to obtain a high yield of the desired product is to couple the transamination reaction to other enzymatic steps (Fig. 3) that convert the co-product (e.g., pyruvate or acetone) into a non-reactive species or back to the original substrate. A multitude of different coupling reactions have been proposed and reported. These are summarized in Table VII and are reviewed beneath.

Table VII. Enzymatic cascades for shifting the equilibrium.

	Enzymes	Co-reactants[a]	Co-products	Refs.
Oxaloacetate degradation	PcK, PK	a-KG, ATP	Pyruvate, CO_2	Chao et al. (1999)
Pyruvate degradation	ALS	Alanine	CO_2, acetoine	Fotheringham et al. (1999)
	PDC	Alanine	CO_2, acetaldehyde	Höhne et al. (2008)
	PDC, ADH, FDH	Alanine, NADH, Formate	CO_2, ethanol	Not reported
	LDH, GDH	Alanine, Glucose, NADH	Lactic acid, gluconic acid	Shin and Kim (1999)
	LDH, FDH	Alanine, Formate, NH_4^+, NADH	Lactic acid, CO_2	Koszelewski et al. (2008b)
Co-product degradation	(Y)ADH, GDH	2-PA/BA, glucose, NADH	2-propyl/butyl alcohol, gluconic acid	Not reported
	(Y)ADH, FDH	2-PA/BA, formate, NADH	2-propyl/butyl alcohol, CO_2	Cassimjee et al. (2010)
Alanine recycling	AADH, GDH	Alanine, NH_4^+, NADPH	Pyruvate (low), H_2O	Truppo et al. (2009a,b)

ALS, acetolactate synthase; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase; FDH, formate dehydrogenase; GDH, glucose dehydrogenase; 2-PA/BA, 2-propyl amine or 2-butyl amine; PcK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase.
[a]Reactants that are required for the reaction additionally to the amine acceptor (ketone).

One early strategy, employed by Chao et al. (1999), was the combined use of phosphoenolpyruvate carboxykinase (EC 4.1.1.32; PcK) and pyruvate kinase (EC 2.7.1.40) to convert the formed oxaloacetate to pyruvate in a two-step reaction. A simpler strategy was reported by Fotheringham and co-workers (1999, 2001) in a process for making amino acids, where the transamination was coupled to acetolactate synthase (EC 2.2.1.6), which converted the formed pyruvate by-product to the non reactive acetoin. Significant yield and purity advantages over the process using the transaminase alone were reported, with an eight to tenfold increase in the ratio of product to the major impurity. Another common strategy to eliminate the pyruvate is through the addition of lactate dehydrogenase (LDH, EC 1.1.1.27), converting pyruvate to lactic acid while simultaneously oxidizing NADH to NAD^+ (Höhne et al., 2008; Hwang et al., 2009; Shin and Kim, 1999; Truppo et al., 2009a). Although the system has been shown to work effectively, the main drawback is the requirement of the co-factor NADH, which needs to be re-generated. When using cell-free transaminase, this can be achieved by adding glucose dehydrogenase (GDH, EC 1.1.99.10) or formate dehydrogenase (FDH, EC 1.2.1.2) together with glucose or formate. The same effect could also be achieved by using a whole-cell system as most organisms already have a system for pyruvate metabolism and NADH regeneration. In a report by Höhne et al. (2008) it was shown that the equilibrium can instead be shifted by the use of pyruvate decarboxylase (PDC, EC 4.1.1.1). The major argument for using this (in contrast to LDH) is that cofactor recycling is eliminated, and the reaction is practically irreversible as the products are very volatile (acetaldehyde and CO_2), and would be evaporated for the desired shift of equilibrium (Höhne et al., 2008). Truppo et al. (2009b) developed a novel system for the resolution of racemic amines using a transaminase coupled with an amino acid oxidase (AAO, EC 1.4.3.2). In contrast to previously reported approaches that use a stoichiometric amount of amine acceptor, the system described here employs a catalytic amount of amine acceptor (pyruvate) that is continuously recycled in situ by an AAO and molecular oxygen. Pyruvate can also be reconverted into L-alanine with L-alanine dehydrogenase (EC 1.4.1.5) coupled with FDH for NADH regeneration, which therefore in principle only consumes stoichiometric amounts of ammonium formate (Koszelewski et al., 2008b).

Regardless of the cascade system, the interactions and compatibility of each of the enzymes and their associated reagents need to be considered. For instance, the introduction of high concentrations of formate (for use with FDH) is likely to affect the activity and stability of the other enzymes. The interactions can be formalized in an interaction matrix table (Santacoloma et al., 2011). Characteristics related to the catalyst constrains, process modeling, and cascade or network interactions, reactor selection, monitoring and control are also described by Santacoloma et al. (2011).

Whole-Cell Biocatalysis

Despite the fact that the multi-enzyme cascade approach has the potential to be very successful (e.g., Koszelewski et al., 2008a; 2009; Truppo et al., 2010), the economical burden of using multiple enzymes is significant (Tufvesson et al., 2010). In particular the combination with the addition of co-factor (NAD(P)H) will increase the process cost, even when using low concentrations (Berenguer-Murcia and Fernandez-Lafuente, 2010). A suitable strategy to overcome this limitation is using a whole cell as the biocatalyst. Whole-cell strategies (Fig. 3) have become a very promising field especially for bioconversions which usually require a cofactor addition and/or regeneration (León et al., 1998). The wild-type microorganism containing the desired transaminase may be used, but the more common approach is to clone the desired transaminase into a host vector. For example the use of recombinant *E. coli* (Ingram et al., 2007; Koszelewski et al., 2009) or *Pichia pastoris* (Bea et al., 2010) expressing ω-transaminase, optionally following a similar approach as seen for cascades, creating so called cassettes over-expressing the production of the enzymes involved in the degradation or recycling of the co-product. Nevertheless, the number of available ω-transaminases with a known gene sequence is still rather limited (Clay et al., 2010; Koszelewski et al., 2010b).

In Tables II and III examples of the use of whole-cell systems are given. Reported yields are usually in the range of

80–99% with comparable enantioselectivity and similar reaction rates to enzyme cascades.

Several authors (Cassimjee et al., 2010; Fotheringham et al., 1999; Koszelewski et al., 2010b; Panke et al., 1999; Yun and Kim, 2008) have shown that chromosomal integration of genes under a suitable regulatory system to an *E. coli* or *P. pastoris* mutant is a very useful route for constructing a whole-cell biocatalyst that is able to synthesize chiral amines to high specific activities and that can maintain activity for extended periods under reaction conditions in the presence of an organic phase. However, the adequate expression level of each protein still remains a challenge (Kratzer et al., 2010). Other typical drawbacks found in whole-cell biocatalysis, such as uncontrolled side reaction (and consequently unwanted side products) and slower reaction rates (due to trans-membrane diffusion problems and higher metabolic burden), are also encountered in the transaminase reaction using whole cell. Consequently the lower cost of using whole cells has to be weighed against these drawbacks to find the most suitable catalyst form (Woodley, 2006).

Biocatalyst Limitations

Transaminases can be found with activity for a broad range of substrate ketones as has been recently reviewed by Kroutil et al. (Koszelewski et al., 2010c), although S-selective enzymes are more common. However, even if an enzyme with the desired specificity and selectivity can be found, the activity and stability must be high enough to allow a biocatalyst productivity that results in a feasible cost contribution from the biocatalyst (Tufvesson et al., 2010). Improvement of the biocatalyst is very often required for industrial application. For instance, poor stability of the enzyme could require it to be replenished throughout the course of reaction to maintain a sufficient rate. However, if the enzyme stability is improved to a point where it maintains a rate for a longer period of time the loading can be reduced significantly.

The cost of the biocatalyst is dependent on variables, such as expression level, efficiency of the fermentation protocol, enzyme specific activity and the form of the biocatalyst (e.g., whole cell, cell-free extract (crude enzyme), purified or immobilized enzyme). With an optimized production protocol the biocatalyst does not need to be excessively expensive, although the development of an optimized process takes time and requires many different skills (e.g., cloning, fermentation, purification/immobilization). Excluding development costs, a likely cost for an efficiently produced in house biocatalyst used for pharmaceutical production is calculated to be around 10–35 €/kg for whole cells (dry cell weight), 100–250 €/kg crude enzyme (cell-free extract) and 50–100 €/kg for an immobilized preparation (Tufvesson et al., 2011). This in turn puts requirements on the productivity of the biocatalyst in terms of product produced per amount of biocatalyst for an economical process.

A common problem is substrate and product inhibition of the enzyme. For instance, in the transamination of MBA from acetophenone, both substrates and products are known to inhibit the enzyme activity severely already at millimolar concentrations (Truppo et al., 2009a). This could be managed by multiphasic reactions, for example, using an auxiliary solvent or a resin, but it is also conceivable that this could be overcome by modifications to the enzyme itself.

Improvement of the Biocatalyst

Several recent examples illustrate very well the advances in biocatalyst improvement, such as the development of a process for the anti-diabetic drug Sitagliptin by Savile et al. (2010), and the work by Martin et al. (2007). The state-of-the-art methodology to develop the enzymes to fit process requirements is based both on random changes to the protein, combined with the addition of a selective pressure to find the improved mutants (Turner, 2009) and an understanding of the relationship between protein structure and its properties (Frushicheva et al., 2010). Approaches such as saturation mutagenesis (Reetz and Carballeira, 2007), and the use of multivariate statistical techniques, for example, ProSAR (Fox et al., 2007) has evolved into an extremely powerful tool to develop highly efficient tailor made catalyst with less effort than ever before. For instance Martin et al. (2007) managed to improve the activity of a transaminase by a factor of almost 300, while at the same time improving the stability of the enzyme toward the process conditions, yielding a much more economic process. Other examples are given in reports by Rothman et al. (2004) and Yun et al. (2005) who managed to overcome product inhibition by directed evolution, Cho et al. (2008) redesigned the substrate specificity of an ω-transaminase for the kinetic resolution of aliphatic chiral amines.

To obtain a biocatalyst with the desired properties it is important to screen under the preferred reaction process conditions. However, it is generally difficult to screen for all the desired properties simultaneously (Burton et al., 2002), why a gradual adaptation might be beneficial (Tracewell and Arnold, 2009). Also, due to the high costs associated with the techniques for biocatalyst improvement improvements in the biocatalyst should go together with process improvements.

Separation and Recycling of Biocatalyst

When the reaction is finished all detectable enzyme needs to be completely removed or eliminated to ensure product purity and also to avoid problems with emulsions being formed in the downstream processing. Fast and easy separation of the biocatalyst from the reaction medium can also be a key factor for enzymatic resolution reactions where the reaction has to be stopped at a given conversion to achieve an adequate ee of the product. In particular, when using whole cells and high concentration of organic compounds or mixing (resulting in cell lysis), the separation

can be problematic due to formation of emulsions or foaming. A simple method is to denature the enzyme to an insoluble precipitate by acidification, filtration is then sufficient to remove the majority of the enzyme precipitate (Savile et al., 2010). For high value products, discarding the enzyme after reaction can be economically feasible. However, in cases when the biocatalyst cost needs to be reduced recycling of the biocatalyst could be necessary.

Immobilization. Immobilization of enzymes can provide several advantages compared to free enzymes, including: easy recovery and reuse of enzyme, improved operational and storage stability of the enzyme, the possibility for continuous operation in packed bed reactors, and minimizing protein contamination in the product (Sheldon, 2007). Well-known problems of immobilization are loss in activity due to introduction of mass transfer limitation and by loss of active enzyme. A less discussed issue is that the required preparation step increases the cost of the enzyme. However, the cost contribution of the immobilized enzyme in the applied process has the potential to be lower than for free enzyme, since the immobilized enzyme can be reused for many reaction cycles.

Immobilization of whole cell ω-transaminase by entrapment in calcium alginate beads has been applied for the kinetic resolution of chiral amines in a packed bed reactor (Shin et al., 2001b). Entrapment of whole cells in calcium alginate beads was found to cause diffusion limitations and changes in substrate and product inhibition (Shin et al., 2001b). It was also reported that also both V_{max} and K_M changed when cells were immobilized in calcium alginate beads (Martin et al., 2007).

Immobilization of free ω-transaminases has been achieved both by covalent linkage to different solid support materials (Yi et al., 2007) and by entrapment in sol–gel matrices (Koszelewski et al., 2010a; Lee et al., 2006) with reported immobilization yields of ~20–50% protein and less than 20% activity. ω-transaminase immobilized on chitosan beads was reported to retain 77% activity after five reaction cycles, but was also susceptible to severe substrate and product inhibition (Yi et al., 2007). Immobilization of ω-transaminase in sol–gel matrices resulted in improved enzyme activity at higher pH and temperatures compared to free enzyme (Koszelewski et al., 2010a). Easy separation of product from sol–gel immobilized (*R*)-selective ω-transaminase allowed a two-step deracemization, consisting of kinetic resolution with the (*R*)-selective immobilized ω-transaminase and asymmetric synthesis with an (*S*)-selective ω-transaminase, to be carried out with a product yield of 89% (Koszelewski et al., 2010a).

When scaling up a reaction using immobilized biocatalyst the resistance of the particles to mechanical forces needs to be considered as this can limit their applicability. The use of a packed bed reactor would alleviate this problem but could be limited by the pressure drop over the bed or mass transfer (Lilly and Woodley, 1994).

Solubility Limitations and Use of Solvents

For the success of most biocatalytic routes, it is also critical to be able to supply substrates at a concentration above 50–100 g/L (Pollard and Woodley, 2007). A common characteristic inherent to aqueous biocatalytic processes is the low solubility of many substrates in water. Operating the process at too low a substrate concentration would lead to a low volumetric productivity and thereby high costs for equipment and downstream processing for product recovery. A list of some of the compounds used for transamination reactions is shown in Table VIII. From the table it is evident that for compounds such as acetophenone and homophenylalanine, a feeding strategy has to be employed to supply the substrate at a high concentration (Kim et al., 2007a). When a biocatalytic route is limited by substrate availability, whether due to low aqueous solubility, slow dissolution rate, or inhibition/toxicity, the controlled addition (feeding) of the substrate into the reaction medium is a common solution (D'Anjou and Daugulis, 2001; Doig et al., 2002; Lynch et al., 1997). This strategy can also help to minimize imine dimer formation (Savile et al., 2010, supplementary information).

Table VIII. Physical properties of different compounds at 25°C.[a]

		Aqueous solubility, S_{aq}		Critical conc. for cell, C^{aq}_{crit}	
Compound	Log *P*	g/L	mol/L	g/L	mol/L
Acetophenone	1.58	6.1	0.05	2.1	0.02
α-methylbenzylamine	1.49[b]	42	0.45	9.5	0.08
Alanine	−2.99	165	1.9	26.4	0.30
Pyruvic acid	−1.24[b]	Fully miscible	11.4	109	1.24
Acetone	−0.24	Fully miscible	17.2	100.1	1.72
2-propanamine	0.26	Fully miscible	16.9	100.5	1.70
Butanone	0.29	223	3.1	32	0.44
Butylamine	0.97	Fully miscible	1.53	105	1.44
Homophenylalanine	−1.20	5[a]	0.03	1.9	0.01
2-oxo-4-phenylbutanoic acid	0.96[b]	21.3[a]	0.12	6.1	0.03

[a]Data from EPI Suite—Estimation Software (http://www.epa.gov/oppt/exposure/pubs/episuitedl.htm).
[b]Estimated data using atom/fragment contribution method.

The substrate itself can be added beyond its solubility, thereby forming a second phase. However, this can cause toxicity and stability problems depending on the properties of the compound. The molecular toxicity, or the critical concentration (C_{crit}), is defined as the concentration at which the catalytic activity is lost (Osborne et al., 2010) or reduced by half (Vermue et al., 1993). Compounds with an aqueous solubility between 0.0003 and 1 M usually require an auxiliary phase for the purpose of in situ substrate addition (Straathof, 2003). As seen in Table VIII, this range includes for instance α-methylbenzyl amine, acetophenone, homophenylalanine and 2-oxo-4-phenylbutanoic acid which are known to have low solubility or inhibitory effects toward the catalytic activity (Kim and Shin, 1998; Lo et al., 2005).

To increase productivity, either a water miscible co-solvent (e.g., THF, iso-propanol, DMSO) that increases the solubility of the substrate in the aqueous phase can be added, or a water immiscible solvent (e.g., toluene, heptane, ethyl-acetate) can be added to act as a reservoir for the substrate. For instance Koszelewski et al. (2008b) investigated the effect of different water miscible solvents on the amination of 4-phenyl-2-butanone. It was seen that the addition of 15% DMSO gave the best activity for the enzyme. On the other hand, addition of solvents will decrease the stability of the biocatalyst and might also cause downstream problems, since the solvent needs to be separated from the product. DMSO for instance is known to be problematic to remove completely. Furthermore, water miscible solvent added up to 25% of volume generally only increases the solubility to a limited extent thereby limiting the usefulness of this approach.

An example of a 2-phase system was reported by Shin and Kim (1997), who used cyclohexanone in the resolution of MBA, which increased the reaction rate ninefold and allowed the resolution of 500 mM MBA with an ee of >95% at 51% conversion (Shin and Kim, 1997). A reported drawback was decreased stability of the enzyme due to the aqueous/organic interface. This problem was alleviated in another report by Shin and co-workers, using a reactor with the two liquid phases separated by a membrane (Shin et al., 2001a). Membrane extraction was also used in connection with a packed-bed reactor where whole cells were immobilized in calcium alginate beads (Shin et al., 2001b).

Many different solvents can be used for this purpose, although for industrial applications it is important that the solvents are generally regarded as safe (GRAS), which limits the number of available solvents. Also, the environmental impact of using solvents should be considered as solvent (volatile organic compound) emissions are one of the main contributors to the environmental impact of pharmaceutical processes (Jiménez-Gonzàlez et al., 2004). Further, the costs and efforts associated with wastewater treatment of side streams containing organic solvents are often complex and closely related with the solubility and toxicity of the solvents used (León et al., 1998).

Conclusions

Biocatalytic transamination is on the verge of taking-off as a tool for the production of chiral amines. Figure 4 points to the fact that the state-of-the art in transaminase processes has been insufficient for successful industrial application until very recently. The work by Truppo, Martin, Savile and respective co-workers could indeed indicate a breakthrough for transaminase technology. It is interesting to note that the first achieved process feasibility by reaction methods such as the use of enzymatic cascades and ISPR (Truppo et al., 2010), while the two others achieved improved process feasibility by protein engineering techniques to improve product catalyst productivity as well as tolerance to higher concentrations of the substrate (Martin et al., 2007; Savile et al., 2010). Further, the recent work on novel cascade reactions by Höhne et al. (2008) and by Koszelewski et al. (2008b) are significant contributions to the field that may in the future enable the asymmetric synthesis of products made by thermodynamically challenging reactions.

However, there is a need for the development of platform technologies to facilitate implementation and shortening of development times and uncertainties. Such technologies would include a broader availability of affordable transaminases, cascade systems or optimized whole-cell systems, preferably in an immobilized form. Also, protocols and kits for selecting the most appropriate separation procedure (e.g., resin selection for ISPR) could also simplify the development procedure. Further, the scientific community needs to be aware of the economic constraints present in industry to address the issues of biocatalyst productivity (g product/g biocatalyst), process intensity (g/L) and space time yield (g/L h). There is often a trade-off between the cost of the catalyst improvement and the benefits that can arise from such efforts. In an ideal situation, process and biocatalyst improvements should go side-by-side, in order to diminish the risk of improvement of one of the process metrics at the expense of another.

A rational process selection methodology, where the process set-up is given by the intrinsic properties of the system, for example, reaction thermodynamics, substrate solubility, enzyme kinetics (e.g., inhibition), would be desirable and would simplify and improve biocatalytic process design. However, as can be seen above, the choices made are highly interdependent and knowledge gaps still make such an approach out of reach. Even so, guidelines and rules of thumb are desirable to identify if a process is feasible allowing better choices to be made. For instance, knowledge of the thermodynamic properties of the reaction is crucial information in the early process development determining which process solutions are feasible. Still fundamental knowledge about the technologies to achieve high yields in thermodynamically unfavorable systems is lacking.

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Appendix 2: Data for economical assessment

Table A2.1. List of prices of consumables used for costing in case study 1 (Chapter 7) and case study 3 (Chapter 9)

Consumable	Cost	Reference
2-Octanone	10 €/kg	Assumed
ADA-buffer	10 €/kg	Assumed from Carl-Roth
Alanine	6 €/kg	Estimated from Sigma-Aldrich
AM-101	2.19	Our personal contact with Evonik, 2012
Carrier	50 €/kg	[16]
Cyclohexanol	1.37 €/kg	Our personal contact with Evonik, 2011
GDH	700 €/kg	Our personal contact with X-zyme, 2013
Glucose	0.5 €/kg	www.icis.com, 2010
Kanamycin	38 €/kg	Our personal contact with Evonik, 2010
*Lb*ADH	1600 €/kg	Our personal contact with X-zyme, 2012
NAD(H)	2000 €/kg	Estimated from [130]
NADP(H)	10000 €/kg	Estimated from [130]
NH_4Cl	0.38 €/kg	www.icis.com, 2011
n-heptane/EtOH mixture	0.29 €/L	www.icis.com, 2012
Peptone	3 €/kg	Our personal contact with Evonik, 2010
PLP	420 €/kg	MP Biomedicals, 2011
Salts and minerals	0.5 €/kg	Our personal contact with Evonik, 2010; www.icis.com, 2010
Utility	**Cost**	**Reference**
Electricity	0.1 €/kWh	European Energy Portal (www.energy.eu)
Process water	0.01 €/L	Our personal contact with Evonik, 2010
Wastewater handling	1 €/m^3	[141]

Appendix 3: Economic and environmental assessment for biocatalytic production of ε-caprolactam

A3.1 General Assumptions

The general assumptions for the fermentation are:

- Plant is located in Western Europe;
- The facility will be a multi-purpose plant with shared utility and services systems;
- The production of ε-caprolactam is 10 000 tons/year;
- The plant operates 8 000 hours/year
- Costs are given in Euro (€)

A3.2 Process Overview

A3.2.1 Fermentation

1. Fermenter size: A fermenter of 10m^3 with 80% working volume is used in the base case.
2. The fermenter is inoculated and run as a fed-batch fermentation for a total of 48h
3. Total process time, including preparation, fermentation, harvesting and cleaning is 3 working days.
4. Equipment is used for running 120 successful batches per year for the production of all the biocatalyst in the plant site
5. Fermentation media is prepared in a tank with a mixer and then passed through a heat exchanger for sterilization at 121°C
6. The expression of the recombinant protein(s) is done by auto-induced media
7. Aeration of 1 fermenter volume per minute is accomplished with a compressor (5kW/m^3)
8. Mixing power input is 5kW/m^3
9. Water content in the cells after centrifugation is 10% of cell dry weight
10. Fermentation metrics:
    a. Cell density: 50 g_{CDW}/L
    b. Yield coefficient of biomass on glucose (Y_{sx}) is 0.4 g $_{biomass}$/g $_{glucose}$
    c. Total Protein content in the host is 0.5 g $_{Total\ Protein}$/ g $_{biomass}$
    d. Maximum overexpressed recombinant protein(s) is 0.25 g $_{Recombinant\ Protein(s)}$/ g $_{biomass}$
    e. Yield coefficient of recombinant protein(s) on glucose (Y_{sRE}) is 0.05 g $_{Recombinant\ Protein(s)}$/g $_{glucose}$

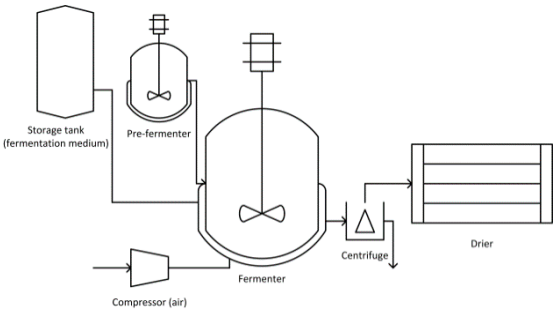


Figure A3.1. Process scheme for base case fed-batch fermentation in 10 m^3 fermenter

A3.2.1.1 Process Costs

Table A3.2. Fermentation medium (raw materials) cost

Raw materials	Weight (kg)	Price (€/kg)	Cost (€/batch)
Glucose	1250	0.50	625
Peptone	1250	3.03	3788
Kanamycin (50 mg/L)	0.5	37.80	19
Salts and minerals	200	0.49	98
Water	10000	0.01	100
Total raw material cost	**4630**		

Table A3.3. Utilities cost

Utilities		kWh	Cost (€/batch)
Sterilization	4.1813 kJ/(kg.K)	1175	118
Aeration (1 vvm)	5 kW/m^3	2400	240
Stirring (500rpm)	5 kW/m^3	2400	240
Centrifugation	2 kW/m^3	20	2
Drier	4.1813 kJ/(kg.K) 2257 kJ/kg	35	3.5
	m^3	Price (€/m^3)	Cost (€/batch)
Wastewater handling	10	1	100
Total raw material cost	**703**		

Table A3.4. Labour cost

Labour	
Labour cost	30 €/h
Labour requirements, man h/batch	Team of 2 workers for 1 week
Supervision, quality control etc.	+100%
Total labour costs	**7200 €/batch**

Table A3.5. Capital investment cost

Equipment	Size	Purchasing cost (€)	TIC [a] (€)	Annuity[b] (€/year)	Maintenance[c] (€/year)	Others[d] (€/year)	Cost (€/batch)
Storage tank	12.5 m^3	6500	32500	4627	463	694	47
Pump	5 m^3/h	7200	36000	5126	513	769	52
Heat exchanger	1 m^2	4000	20000	2848	285	427	29
Compressor	12.5 m^3/min	36000	180000	25628	2563	3844	260
Pre-Fermenter	1.25 m^3	52000	260000	37018	3702	5553	376
Fermenter	12.5 m^3	165000	825000	117461	11746	17619	1193
Centrifuge	1 m^3	12000	60000	8543	854	1281	87
Drier	10 m^2	305000	152500	21713	2171	3257	221
					Total Equipment cost (€/batch)		**2265**

[a] TIC – Total Installed cost Lang Factor = 5
[b] 7% interest rate and 10 year life-time
[c] Maintenance is 10% of TIC
[d] Other including taxes, insurance, etc. is 15% of TIC

A3.2.2 Catalyst purification

The cost of the isolated recombinant enzyme increases when compared with their whole-cell or crude extract preparation. Aiming the enzyme purification: the whole-cell was recovered by microfiltration; the crude enzyme the cells were run through a homogenizer, centrifuged to remove cell debris and finally ultrafiltration was applied. Partially purified enzyme was prepared by additionally running ion-exchange and gel-filtration chromatography as well as two additional ultrafiltration steps. The added cost in each step is significant. The preparation of crude enzyme from recombinant whole-cell adds to the specific cost of the enzyme by a factor of two. Purification by chromatography increases the cost per kg of biocatalyst 10-fold, where the major cost contribution comes from the consumables such as the resin material. [16]

A3.2.3 Immobilisation

1. Tank size: A mixing tank of 1.5 m^3 with 80% working volume is used in the base case for preparing 130 kg of biocatalyst;
2. Equipment is used for 4000h per year
3. Total process time, including preparation, immobilisation, filtering, drying and cleaning is 32 hours;
4. Immobilisation solution is prepared in a mixing tank to the concentration of 6 g/L;
5. Carrier material is added to start the immobilisation;
6. After the time required to immobilise more than 90 % of the protein, the immobilised enzyme is filtered from the immobilisation solution;
7. The catalyst is dried;
8. The equipment is emptied and cleaned;
9. The final biocatalyst has an enzyme loading of 50 $g_{enzyme}/kg_{immobilised\ biocatalyst}$.

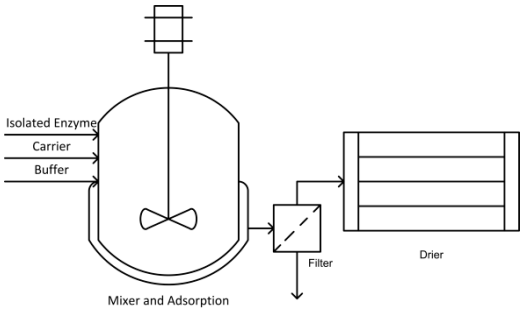


Figure A3.2. Process scheme for base case immobilised biocatalyst

A3.2.3.1 Process Costs

Table A3.6. Enzyme Immobilisation raw materials cost

Raw materials	Weight (kg)	Price (€/kg)	Cost (€/batch)
Carrier	124	50	6175
Isolated Enzyme	7.22	2368	17100
Water	1358	0.01	14
Total raw material cost	**23288**		

Table A3.7. Utilities cost

Utilities		kWh	Cost (€/batch)
Stirring (500rpm)	5 kW/m^3	170	17
Filtration	2 kW/m^3	2716	272
Drier	4.1813 kJ/(kg.K) 2257 kJ/kg	9	0.9
	m^3	Price (€/m^3)	Cost (€/batch)
Wastewater handling	1.36	1	76
Total raw material cost	**365**		

Table A3.8. Labour cost

Labour	
Labour cost	30 €/h
Labour requirements, man h/batch	1 worker for 32 hours
Supervision, quality control etc.	+100%
Total labour costs	**1920 €/batch**

Table A3.9. Capital investment cost

Equipment	Size	Purchasing cost (€)	TIC [a] (€)	Annuity[b] (€/year)	Maintenance[c] (€/year)	Others[d] (€/year)	Cost (€/batch)
Mixing tank	1.7 m^3	72000	360000	51256	5126	7688	521
Pump	5 m^3/h	7200	36000	5126	513	769	52
Filter	7 m^2	110000	550000	78308	7831	11746	795
Drier	10 m^2	34000	170000	24204	2420	3631	246
					Total Equipment cost (€/batch)		**1614**

[a] TIC – Total Installed cost Lang Factor = 5
[b] 7% interest rate and 10 year life-time
[c] Maintenance is 10% of TIC
[d] Other including taxes, insurance, etc. is 15% of TIC

A3.2.4 Reaction

1. Set of series of three or two reactors (option 1 or option 2 or 3, respectively) with 40 m3 with 80% working volume is used in the base case.
2. The reactor is dosed with the biocatalyst, substrate(s), cofactors and other process adjuvants as a batch reaction for a total of 8h
3. Total process time, including preparation, reaction and cleaning is 12h
4. Equipment is used for running 900 successful batches per year for the production of all the biocatalyst in the plant site
5. Aeration of 1 reactor volume per minute is accomplished with a compressor (5kW/m3)
6. Mixing power input is 5kW/m3
7. Cyclisation of 6AHA to ε-caprolactam reaction yield 99%
8. Biocatalytic reaction metrics:
   a. Reaction Yield: 90%
   b. For whole-cell catalyst, glucose requirement: 10% (wt.) of the CDW
   c. $TTN_{NAD(H)}$: 10^5 $mol_{Product\ formed}/mol_{NAD(H)}$
   d. $TTN_{NADP(H)}$: 10^4 $mol_{Product\ formed}/mol_{NADP(H)}$
   e. TTN_{PLP}: 90 $mol_{Product\ formed}/mol_{PLP}$
   f. TTN_{Ala}: 1 $mol_{Product\ formed}/mol_{Ala}$

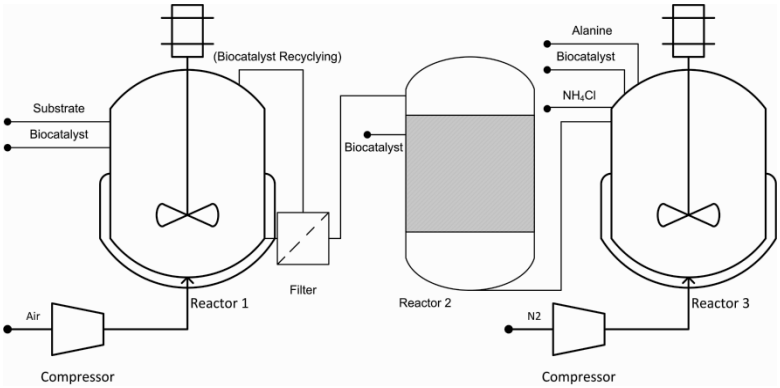


Figure A3.3. Process scheme for Option 1.12 for biocatalytic production of 6AHA

1.1.1.1 Process Costs

Table A3.10. Biocatalytic production of 6AHA raw materials cost

Raw Material	Weight (kg/batch)	Price (€/kg)	Cost (€/batch)
Whole-cell (containing ADH-NADP and CHMO)	11.41	29	332
Lipase	1.59*	4788	38
Whole-cell (containing ADH-NAD, TAm and AlaDH)	19.70	29	574
	Weight (kg/batch)	**Price (€/kg)**	**Cost (€/batch)**
Cyclohexanol	8484	1.37	11632
NH_4Cl	4531	0.38	1699
Alanine	6792	6	40752
NAD(H)	0	2000	0
NADP(H)	0	10000	0
PLP	0	420	0
Glucose	31	0.50	16
Water	40000	0.01	400
Total raw material cost			**55443**

* recycled 200 times

Table A3.11. Utilities cost

Utilities	kWh	Price (€/kWh)	Cost (€/batch)
Stirring	3200	0.10	320
Heating	698	0.10	70
Aeration for 1vvm	1274	0.10	127
N_2 Sparging	1274	0.10	127
	m^3	**Price (€/m^3)**	**Cost (€/batch)**
Wastewater handling	40	1	40
	kg	**Prices (€/kg)**	**Cost (€/batch)**
Biocatalyst waste	31	0.03	0.8
Total utilities cost			**685**

Table A3.12. Labour cost

Labour	
Labour cost	30 €/h
Labour requirements, man h/batch	3 workers for 12 hours
Supervision, quality control etc.	+100%
Total labour costs	**2160 €/batch**

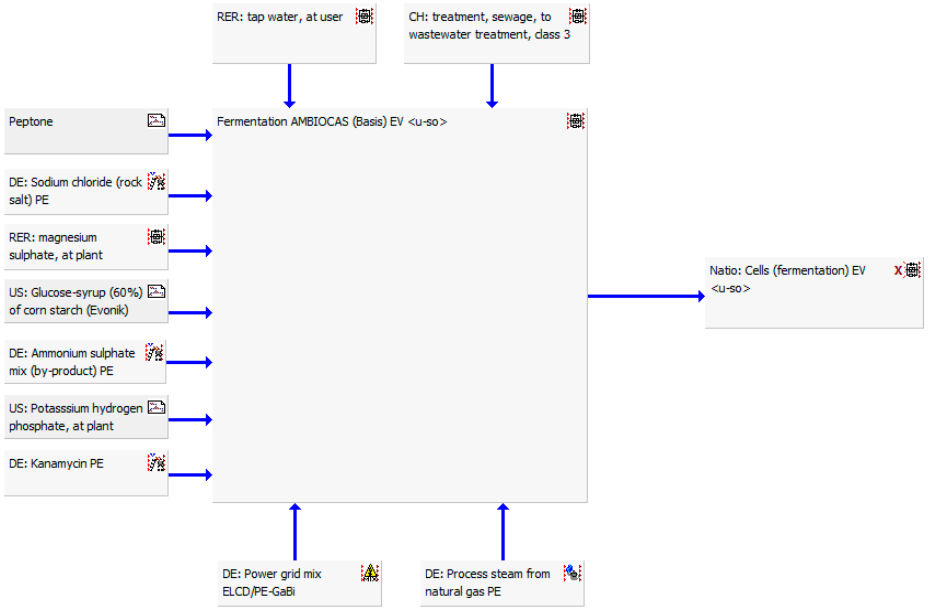
Table A3.13. Capital investment cost

Equipment	Size	**Purchasing cost (€)**	**No. units**	**TIC[a] (€)**	**Annuity[b] (€/year)**	**Maintenance[c] (€/year)**	**Others[d] (€/year)**	**Cost (€/batch)**
Storage tank	48 m^3	14000	3	210000	29899	2990	4485	47
Stirred Tank Reactor	48 m^3	430000	2	4300000	612223	61222	91834	957
Column Reactor	48 m^3	14000	1	70000	9966	997	1495	16
Compressor	40 m^3/min	150000	2	1500000	213566	21357	32035	334
Pump	10 m^3/h	11000	3	165000	23492	2349	3524	37
Filter (m2)	10 m^2	140000	2	1400000	199329	19933	29899	312
						Total Equipment cost (€/batch)		**1701**

[a] TIC – Total Installed cost Lang Factor = 5
[b] 7% interest rate and 10 year life-time
[c] Maintenance is 10% of TIC
[d] Other including taxes, insurance, etc. is 15% of TIC

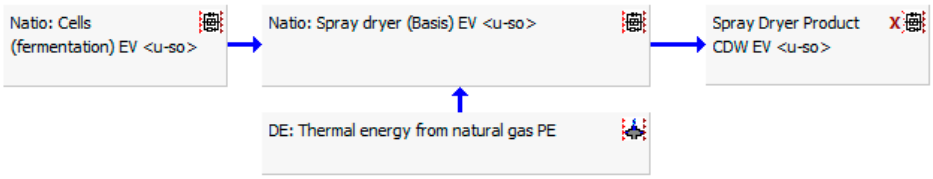
A3.3 Life cycle assessment in GaBi Product Sustainability Software (in collaboration with Evonik Industries AG)

A3.3.1 Fermentation

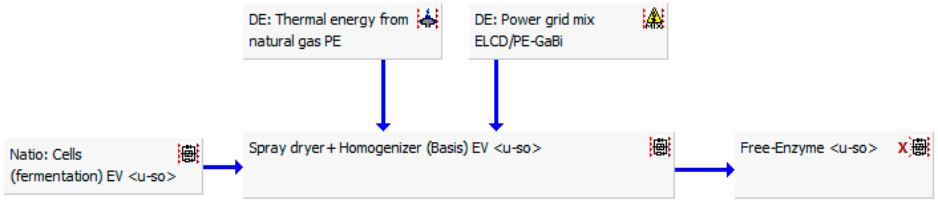


A3.3.2 Catalyst formulation

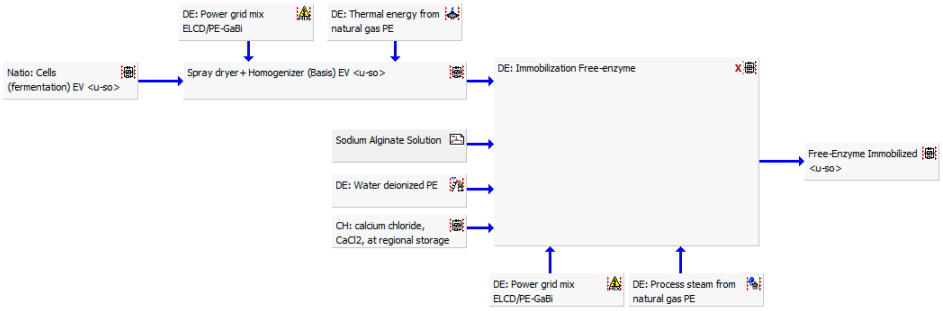
A3.3.2.1 Whole-cell



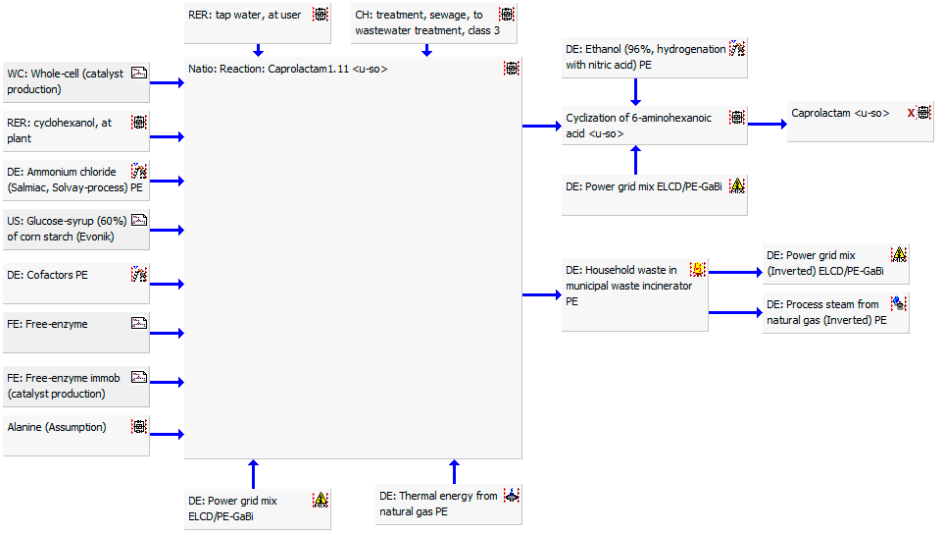
A3.3.2.2 Isolated Enzyme



A3.3.2.3 Immobilised Enzyme



A3.3.3 Reaction



Appendix 4: MATLAB® scripts for kinetic modelling of aliphatic alcohol production using alcohol dehydrogenase

```
function dy = LbADHGDH(t,y)
%  1 = NADPH
%  2 = NADP
%  3 = ON
%  4 = OL
%  5 = Gluc
%  6 = GDL
%  7 = NADPH2
%  8 = NADP2
%  9 = ON2
%  10 = OL2
%  11 = Gluc2
%  12 = GDL2
%  13 = LbADH1
%  14 = GDH1
%  15 = LbADH2
%  16 = GDH2
%  17 = Flow
%  18 = NADPinitial

dy = zeros (18,1);
```

Kinetic Parameters

```
Vmf=(17.50084735/60000)    ;%{mmol/L/s} Vmax for the forward
LbADH reaction
KMON=0.205999742           ;%{mmol/L}   KM for 2-octanone
KMNADPHON=0.037041599      ;%{mmol/L}   KM for NADPH
KP2OL=0.208178602          ;%{mmol/L}   Kp for 2-octanol
KSON=163.0470923           ;%{mmol/L}   Ks for 2-octanone
KPNADPON=0.212141656       ;%{mmol/L}   Kp for NADPH

Vb=(9.961666509/60000)     ;%{mmol/L/s} Vmax for the backward
LbADH reaction
KMOL=0.033190703           ;%{mmol/L    KM for 2-octanol
KMNADPb=0.834689069        ;%{mmol/L}   KM for NADP+
KPON=0.012225455           ;%{mmol/L}   Kp for 2-octanone
KPNADPHOL=0.358861502      ;%{mmol/L}   Kp for NADP+
KSb=6065.242184            ;%{mmol/L/s} Ks for 2-octanol

VGDH=(5.822557/60000)      ;%{mmol/L/s} Vmax for the backward GDH
reaction
KMGluc=2.729106            ;%{mmol/L}   KM for glucose
KMNADPGDH=0.025828         ;%{mmol/L}   KM for NADP+
KPNADPHGDH=0.028013        ;%{mmol/L}   Kp for NADH
KSGluc=3783.912343         ;%{mmol/L}   Ks for glucose
%Flow1=0.004/3600          ;%{L/h/s}    Flow

dy(17)=0;
dy(18)=0;
```

Initial conditions

```
NADPHinitial=0.0            ;%{mmol/L}
ONinitial=94                ;%{mmol/L}
OLinitial=0                 ;%{mmol/L}
Glucinitial=94              ;%{mmol/L}
GDLinitial=0                ;%{mmol/L}
VolReactor=0.015            ;%{L}
```

Reaction:

```
Forw1 =
Vmf.*y(13).*y(3)./(KMON.*(1+y(4)./KP2OL)+y(3).*(1+y(3)./KSON))*y(
1)./(KMNADPHON.*(1+y(2)./KPNADPON)+y(1));
Back1 =
Vb.*y(13).*y(4)./(KMOL.*(1+y(3)./KPON)+y(4).*(1+y(4)./KSb))*y(2).
/(KMNADPb.*(1+y(1)./KPNADPHOL)+y(2));
Reg1  =  VGDH.*y(14).*y(5)./(KMGluc +
y(5)*(1+y(5)./KSGluc))*y(2)./(KMNADPGDH.*(1+y(1)/KPNADPHGDH)+y(2)
);

Forw2 =
Vmf.*y(15).*y(9)./(KMON.*(1+y(10)./KP2OL)+y(9).*(1+y(9)./KSON))*y
(7)./(KMNADPHON.*(1+y(8)./KPNADPON)+y(7));
Back2 =
Vb.*y(15).*y(10)./(KMOL.*(1+y(9)./KPON)+y(10).*(1+y(10)./KSb))*y(
8)./(KMNADPb.*(1+y(7)./KPNADPHOL)+y(8));
Reg2  =  VGDH.*y(16).*y(11)./(KMGluc +
y(11)*(1+y(11)./KSGluc))*y(8)./(KMNADPGDH.*(1+y(7)/KPNADPHGDH)+y(
8));

% Cofactor stability
deacnadphr1 = y(1)*(-4.10844e-06);
deacnadphr2 = y(7)*(-4.10844e-06);
```

Mass Balance to EMR

```
%{           Input                                          Reaction
      Output              }
dy(1)=(y(17)./VolReactor.*NADPHinitial)     -Forw1 +Back1 + Reg1
- deacnadphr1 - (y(17)./VolReactor.*y(1));
dy(2)=(y(17)./VolReactor.*y(18))            +Forw1 - Back1 -Reg1
-              (y(17)./VolReactor.*y(2));
dy(3)=(y(17)./VolReactor.*ONinitial)        -Forw1 +Back1 -
(y(17)./VolReactor.*y(3));
dy(4)=(y(17)./VolReactor.*OLinitial)        +Forw1 -Back1 -
(y(17)./VolReactor.*y(4));
dy(5)=(y(17)./VolReactor.*Glucinitial)      -Reg1-
(y(17)./VolReactor.*y(5));
dy(6)=(y(17)./VolReactor.*GDLinitial)       +Reg1-
(y(17)./VolReactor.*y(6));
dy(7)=(y(17)./VolReactor.*y(1))             -Forw2 +Back2 + Reg2
- deacnadphr2-  (y(17)./VolReactor.*y(7));
dy(8)=(y(17)./VolReactor.*y(2))             +Forw2 - Back2 -Reg2
-              (y(17)./VolReactor.*y(8));
dy(9)=(y(17)./VolReactor.*y(3))             -Forw2 +Back2 -
(y(17)./VolReactor.*y(9));
dy(10)=(y(17)./VolReactor.*y(4))            +Forw2 -Back2 -
(y(17)./VolReactor.*y(10));
dy(11)=(y(17)./VolReactor.*y(5))            -Reg2-
(y(17)./VolReactor.*y(11));
dy(12)=(y(17)./VolReactor.*y(6))            +Reg2-
(y(17)./VolReactor.*y(12));
```

Deactivation LbADH (13;15) and GDH (14;16)

```
dy(13)= y(13)*(-1.925e-7);
dy(14)= y(14)*(-1.925e-7);
dy(15)= y(15)*(-1.925e-7);
dy(16)= y(16)*(-1.925e-7);

%X1=y(4)/(y(4)+y(5));
%X2=y(10)/(y(10)+y(11));
```

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```
clc
clear all
close all
A=[];
B= [];
ONinitial=94; %mM
for i=1:1
flow=[ 0.005 0.012 0.020 ];
for k=1:1; %k=1:4
%  adh=[ 50 250 500 1000];
adh=[100];
for m=1:1; %m=1:4
%  gdh=[ 50 250 500 1000];
 gdh=[300];
 for l=1:1; %l=1:4
%  adh2=[ 50 250 500 1000];
adh2=[900];
for n=1:1; %n=1:4
%  gdh2=[ 50 250 500 1000];
gdh2=[300];
 for p=6:6
 nadp=[ 0.1 0.09 0.08 0.07 0.06 0.05 0.04 0.03 0.02 0.01 ];

[t,y]=ode15s(@LbADHGDH,[0:5000:(1000*3600)],[0.0001;0.0001;0.1;0.
1;0.1;0.1;0.0001;0.0001;0.1;0.1;0.1;0.1;adh(k);gdh(m);adh2(l);gdh
2(n);flow(i)/3600;nadp(p)]);


cond=[t y];
A= mean(y);
B= [B A'] ;
R2Ol=y(:,10);
Yield=R2Ol/ONinitial;

csvwrite(['Flow_' num2str(i) num2str(k) num2str(m) num2str(l)
num2str(n) num2str(p)] ,cond)
 eval(['Flow_' num2str(i) num2str(k) num2str(m) num2str(l)
num2str(n) num2str(p) '=cond'])
 eval(['Mean_' num2str(i) num2str(k) num2str(m) num2str(l)
num2str(n) num2str(p) '=A'])
 clear ['Flow_' num2str(i) num2str(k) num2str(m) num2str(l)
num2str(n) num2str(p)]
end
end
end
end
end
end

R2Ol=y(:,10);
Yield=R2Ol/ONinitial;
```

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