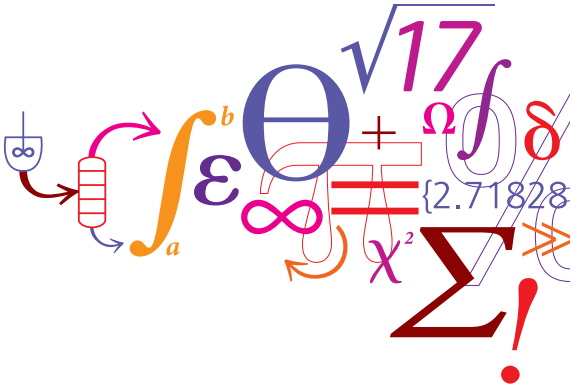
Process Considerations for the Asymmetric Synthesis of Chiral Amines using - Transaminase

Lima Afonso Neto, Watson

Publication date:
2013

Document Version
Publisher's PDF, also known as Version of record

Process Considerations for the Asymmetric Synthesis of Chiral Amines using ω-Transaminase



Watson Neto

Ph.D. Thesis

August 2013

Process Considerations for the Asymmetric Synthesis of Chiral Amines using ω-Transaminase

Ph.D. Thesis

Watson Neto

August 2013

Center for Process Engineering and Technology
Department of Chemical and Biochemical Engineering
Technical University of Denmark

Address: Center for Process Engineering and Technology (PROCESS)
Department of Chemical and Biochemical Engineering
Technical University of Denmark
Building 229
DK-2800 Kgs. Lyngby
Denmark
Phone: +45 4525 2800
Fax: +45 4593 2906
Web: www.process.kt.dtu.dk

Print: J&R Frydenberg A/S
København
December 2013

ISBN: 978-87-93054-21-9

Preface

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

The work here presented was carried out at the Center for Process Engineering and Technology (PROCESS, DTU Chemical Engineering) from June 2010 till August 2013. Professor John M. Woodley (DTU Chemical Engineering) was the principal supervisor for the project, with Dr. Pär Tufvesson (DTU Chemical Engineering) as co-supervisor.

The project was funded by Marie Curie ITN BIOTRAINS, financed by the European Union through the 7th Framework people Program (grant agreement no. 238531).

Kgs. Lyngby, 2013

Watson Neto

"*Science never solves a problem without creating ten more.*"

(George Bernard Shaw, Nobel Prize in Literature)

Acknowledgments

I would like to thank my supervisors John M. Woodley and Pär Tufvesson for the opportunity to work on this interesting project. Thank you for so many valuable, but of course never enough, input. They were very refreshing and inspiring.

Many thanks to Martin Schürmann and Lavinia Panella (DSM Innovative Synthesis, Geleen, The Netherlands) for giving me the opportunity to work at your lab and for the valuable contribution during the 4 months of my stay there. Thank you for your patience and guidance. Many thanks also to Natascha Smeets, Thomas Schmitges and Harrie Straatman who helped me finding my way in the lab.

I wish also to thank Andreas Vogel and Daniel Schwarze (c-LEcta GmbH, Leipzig, Germany) for the fruitful collaboration during these three years of project.

I would like also to acknowledge my former students Silvia Pirrung and Morten Jepsen for valuable contribution to this thesis.

These three years would not have been the same without my friends and colleagues at PROCESS extended family. I am especially grateful for the company of Aleksandar, Anna, Andrijana, Hande, Joana, Laura, Naweed and Rita. Thank you all for so many great moments and for your social and scientific inputs. *Um grande OBRIGADO* to Joana and Rita for the precious time they spent reading and correcting my thesis and for their support and company throughout these 3 years. Many thanks also to Simon for the time spent in translating the abstract to Danish and to Rob and Hema for proof reading this work.

Finalmente, um agradecimento à minha família (especialmente à minha Mãe), pelo apoio contínuo durante as minhas várias aventuras em busca de mais conhecimentos e novos horizontes. *OBRIGADO*.

Atât de mult as putea spune pentru tine. Voi spune doar că eu sunt foarte recunoscător te-am cunoscut. Mulţumesc.

Abstract

The implementation of new biocatalytic processes can be a very challenging procedure, which can require several stages of screening, characterization and evaluation prior to scale-up. Indeed, several process parameters, with different weights on the final process costs, need to be considered side-by-side. Process design and economic evaluation represent a very important part of the early process development stage. However, often the parameters set at these initial stages are based on assumptions. Therefore, a laboratory scale characterization of the biocatalyst and different process options are important in order to eliminate infeasible routes. This work illustrates the laboratory scale characterization of different process options for the asymmetric synthesis of chiral amines catalysed by ω-transaminase (ω –TAm). The studied process options include: (i) the immobilization of the biocatalyst to improve its stability and allow recycling and easy separation; (ii) the use of controlled release of substrate (fed-batch) or *in situ* substrate supply – (ISSS) to decrease substrate inhibition and deal with the substrate low solubility; and (iii) the use of *in situ* product (ISPR) and co-product removal (IScPR) to respectively alleviate product inhibition and shift the reaction equilibrium.

From an academic point of view, more important than the implementation of these technologies to a specific example, is the development of a general methodology that can be later applied in other cases. Hence, this work has also focused on development of comprehensive screening methodologies and guidelines to aid (i) the selection and characterization of suitable biocatalysts for the process; (ii) the selection and characterization of suitable carriers for immobilization of (S)- and (R)-selective ω-TAm; and (iii) the selection of suitable polymeric resins for product removal. The work has been performed in collaboration with c-LEcta GmbH (Leipzig, Germany) and DSM Innovative Synthesis (Geleen, The Netherlands) who supplied the enzymes for the case study, making possible the successful demonstration of the screening methodologies developed. Furthermore, the work addresses several practical questions regarding to the implementation of the process strategies mentioned above.

Resumé

Implementeringen af nye biokatalytiske processer kan være en meget udfordrende procedure, hvor det kan være nødvendigt med flere omgange af screening, karakterisering og evaluering før opskalering. Faktisk er det nødvendigt sideløbende at overveje adskillige proces-parametre, der har forskellig indflydelse på de endelige omkostninger. Procesdesign og økonomisk evaluering repræsenterer en meget vigtig del af de første udviklingstrin. Parametrene der er brugt på disse tidlige trin er dog ofte baseret på antagelser. Derfor er karakteriseringen af biokatalysatoren og forskellige procesløsninger på laboratorieskala vigtige for at være i stand til at eliminerer ikke levedygtige muligheder. Denne afhandling omhandler karakteriseringen af forskellige procesteknologier for den asymmetriske produktion af chirale aminer katalyseret af ω-transaminase på laboratorieskala. De undersøgte procesteknologier inkluderer: (i) Immobiliseringen af biokatalysatoren for at forbedre dens stabilitet og for at tillade genbrug og nem separation; (ii) Brugen af kontrolleret substrat frigivelse (fed-batch) eller in-situ substrat forsyning (in-situ substate supply - ISSS) for at nedsætte substrat inhiberingen og håndterer den lave opløselighed af substratet; og (iii) Bortskaffelse af produkt og co-produkt in situ (in situ product og in situ co-product removal – ISPR og IScPR) for at mindske produkt inhiberingen og forskyde reaktionsligevægten.

Fra et akademisk synspunkt er udviklingen af en generel metodik, der kan anvendes i mange tilfælde, vigtigere end implementeringen af teknologien i et specifik eksempel. Som følge af dette synspunkt har arbejdet, der danner grundlag for denne afhandling, fokuseret på udviklingen af omfattende screeningsmetodikker og retningslinjer for at hjælpe med (i) udvælgelsen og karakteriseringen af passende biokatalysator til processen; (ii) udvælgelse og karaktering af passende bærere til at immobiliserer (S) and (R) selektive ω-transaminase og (iii) udvælgelse af passende polymer resiner til produktbortskaffelse. Arbejdet har været udført i samarbejde med c-LEcta GmbH (Leipzig, Tyskland) og DSM innovative Syntesis (Geleen, Holland) som leverede enzymerne til case studiet, hvilke muliggjorte en succesfuld demonstrering af den udviklede screeningsmetodik. Derudover adresserer denne afhandling adskillelige praktiske spørgsmål angående implementeringen af ovenstående processtrategierne.

Abbreviations

Abbreviation	Description
α-KG	Alpha-Ketoglutaric acid
AAO	Amino acid oxidase
ACE	Acetone
ATP	Adenosine triphosphate
ALS	Acetolactate synthase
ButA	2-butyl amine
YADH	Yeast alcohol dehydrogenase
ADXs	Amine donor excess
ALA	Alanine
APB	3-amino-1-phenylbutane
APH	Acetophenone
BA	Benzylacetone
CAL-B	*Candida antarctica* lipase B
CFE	Cell-free extract
CO_2	Carbon dioxide
D/A	Amine donor-amine acceptor ratio
DCM	Dichloromethane
DHG	Dehydrogenase
DKR	Dynamic kinetic resolution
DMSO	Dimethyl sulfoxide
DSP	Downstream processing
E	Pyridoxal form of ω-TAm
ee	Enantiomeric excess
F	Pyridoxamine form of ω-TAm
GDH	glucose dehydrogenase
GRAS	Generally Regarded As Safe
GMOs	Genetically modified microorganism

Abbreviation	Description
HIC	Hydrophobic interaction chromatography
HPLC	High-performance liquid chromatography
Imm-CFE	Immobilized cell free extract
IPA	Isopropylamine
ISPR	*In situ* product removal
ISSS	*In situ* substrate supply
IScPR	*In situ* co-product removal
k-PBS	Potassium phosphate buffer solution
KR	Kinetic resolution
KsACE	Substrate inhibition constant for ACE
KsAPH	Substrate inhibition constant for APH
KsIPA	Substrate inhibition constant for IPA
LDH	Lactose dehydrogenase
MAN-N	Monoamine Oxidase N
MBA	Methylbenzylamine
NADH	Nicotinamide adenine dinucleotide
NLR	Non-linear regression
pKa	Acid dissociation constant
SEM	Scanning electron microscope
TCl_4	Titanium tetrachloride
THF	Tetrahydrofuran
PLP	Pyridoxal 5-phosphate
PMP	Pyridoxamine 5-phosphate
PE	Purified enzymes
PYR	Pyruvate/Pyruvic acid
(R)-	*Rectus*, Latin for right
(S)-	*Sinister*, Latin for left
STR	Stirred-tank reactor
VOC	Volatile organic compound
WC	Whole-cells
ω-TAm	ω-Transaminase

Nomenclature

Nomenclature	Description	Unit
ΔG	Variation of Gibbs free energy	$J.mol^{-1}$
A_0	enzyme's initial activity or concentration	$mmol.L^{-1}.min^{-1}$
A	enzyme's activity or concentration at time t	$mmol.L^{-1}.min^{-1}$
$[A]$	concentration of substrate A	$mmol.L^{-1}$
C_{crit}	Critical concentration for the cell	$g.L^{-1}$; $mol.L^{-1}$
$[I]$	concentration of inhibitor	$mmol.L^{-1}$
k	reaction rate constant	min^{-1}
K_A	Dissociation constant for compound A	$mmol.L^{-1}$
K_B	Dissociation constant for compound B	$mmol.L^{-1}$
Keq	Thermodynamic equilibrium constant	No units
Ki	Inhibition constant	$mmol.L^{-1}$
Km	Michaelis–Menten constant	$mmol.L^{-1}$
LogP	Partition coefficient	No units
Mw	Molecular weight	$g.mol^{-1}$
p_i^*	Vapour pressure of pure compound i	mmHG
pH	Power of hydrogen	No units
pKa	Acid dissociation constant	No units
P_{vap}	Vapour pressure	mmHG
S_{aq}	Aqueous solubility	$g.L^{-1}$; $mol.L^{-1}$
T	Temperature	˚C
t	Time	min
Tb	Boiling temperature	˚C
Tm	Melting temperature	˚C
v	reaction rate	$mmol.L^{-1}.min^{-1}$
Vmax	Maximum velocity	$mmol.L^{-1}.min^{-1}$
x_i^l	Molar fraction of compound i in liquid phase	No units
x_i^{vap}	Molar fraction of compound i in vapour phase	No units

Table of Contents

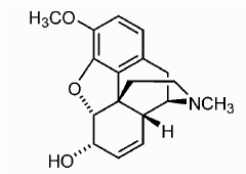
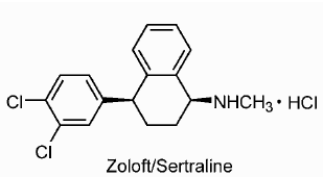
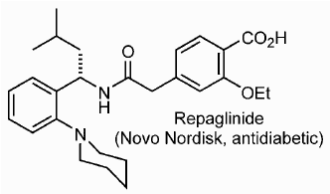
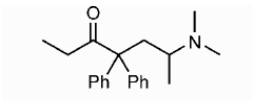
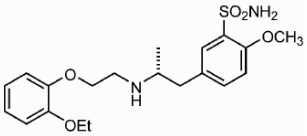
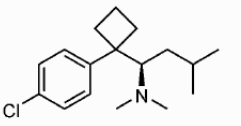
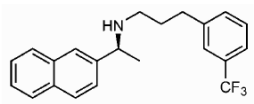
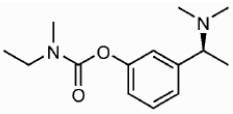
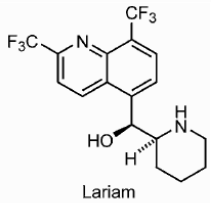
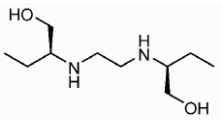
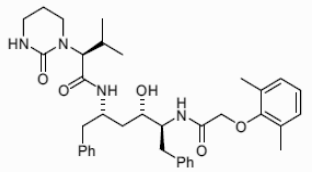
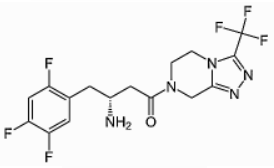
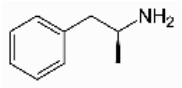
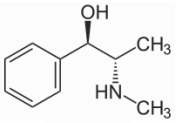
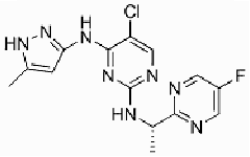
1. Thesis Introduction

Background and perspective

Optically active amines are highly valuable functionalized molecules with a wide range of applications. These include being (i) intermediates for the synthesis of pharmaceutical and agrochemical active ingredients (APIs), (ii) resolving agents for the separation of enantiomers via diastereomeric salt formation, and (iii) ligands for asymmetric synthesis using either transition metal catalysis or organocatalysis [1] .

Chiral amines integrate the backbone of several APIs used in modern medicine for treatment of a vast range of diseases and conditions [2] such as pain relievers, obesity control, treatment of cancer and diabetes as well as tuberculosis (Table 1.1). Thus, the market for these compounds is vast and their need has historically increased in the past few decades [2]. Chiral compounds have been estimated in 2000 to have a 15% fraction of the €20 billion market (total revenues from sales of pharmaceutical and agrochemical industries) [3], and it can be expected that this number has increased since then. However, despite the historical increase in demand for these compounds, their synthesis remains challenging and several laboratories around the world have ongoing research projects targeted at their efficient preparation [2-4].

Table 1.1: Examples of active pharmaceutical ingredients containing a chiral amine in their structure. Adapted from [2]. [i] these can be direcly obtained using ω-TAm.

 Codeine (Johnson Matthey, pain relief)	 Zoloft/Sertraline (Pfizer, depression)	 Repaglinide (Novo Nordisk, antidiabetic)
 Methadone - racemate (generic, narcotic analgesic)	 Flomax/Tamsulosin (Boehringer Ingelheim, prostate)	 Sibutramine (Abbott Laboratories, obesity)
 Cinacalcet (Amgen, hyperparathyroidism)	 Rivastigmine (Novartis, Alzheimer's)	 Lariam (Roche, malaria)
 Ethambutol (generic, tuberculosis)	 Lopinavir (Abbot, HIV-protease inhbitor)	 Sitagliptin/Januvia[i] (Merck, antihyperglycemic)
 Dextroamphetamine[i] (Generic, ADHD and narcolepsy)	 Ephedrine (Generic, Psychostimulant, appetite suppressant)	 AZD1480 (in clinical trial) (Astra Zeneca, leukemia treatment)

1.1. Synthesis of chiral amines

Chiral amines can be produced both by chemo- and bio-catalytic processes [2-4]. The option of preparing them using biocatalytic approaches has gained much attention from both industry and academia as a result of recent developments in biocatalyst availability, methods for improving biocatalyst stability, and the potentially high selectivity and catalytic activity that can be obtained through enzyme catalysis [1, 5]. These methods will briefly be reviewed in the following sections.

1.1.1. Chemo-catalytic processes

1.1.1.1. Crystallization with chiral carboxylic acids

One of the most conventional and widely used strategies consists of the crystallization of diastomeric salts of chiral carboxylic acids with chiral amines as depicted in Figure 1.1 [3]. This allows the isolation of (*S*) or (*R*) chiral amines as well as (*S*) or (*R*) carboxylic acids on an industrial scale [3]. For instance, (*S*)- or (*R*)-methylbenzylamine (MBA) can be obtained by crystallization either with (*S*)-malic acid or (*R*)-mandelic acid or even with (*R*, *R*)-tartaric acid. [3]. The main drawbacks are associated with the screening and/or selection of suitable resolving agents (carboxylic acid) for the amine, which can be a time consuming procedure, and the limited 50% of maximum yield that is possible to achieve.

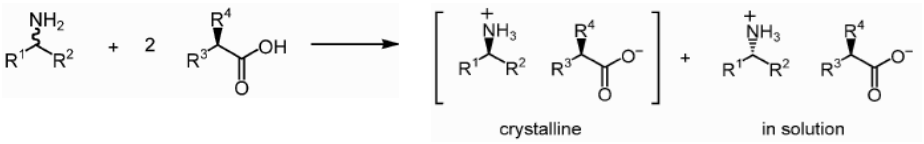


Figure 1.1: Racemate resolution through fraction crystallization of salts of racemic amines and enantiomerically pure carboxylic acids. Adapted from [3].

A prominent approach, commonly denoted as “Dutch resolution” or “The family approach” (developed by researchers at DSM and Syncom BV) [6], attempted to shorten the screening procedure for selection of a suitable resolving agent by implementing a combinatorial approach of a mixture of several optically active acids. This results in immediate precipitation of the amine in nearly pure enantiomeric form, avoiding the need for a screening for a suitable chiral carboxylic acid. This has allowed establishment of standard mixtures for resolution of amines and carboxylic acids [3].

1.1.1.2. Reduction of C=N bonds

Another option to chemically access chiral amines is through the reduction of a C=N double bond from a prochiral precursor, and the subsequent cleavage of the auxiliary group (X) to form the chiral amine (Figure 1.2). The major challenges related with this approach are the preparation of the prochiral imine precursor itself and the consecutive cleavage of the auxiliary group to give the free amine, as reviewed in several articles [2, 3, 7]. Also the screening effort for selection of a reducing agents and the use of precious metals (which increases the catalysts cost) are important disadvantages.

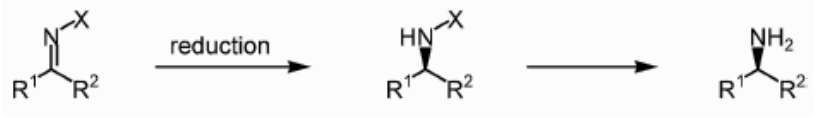


Figure 1.2: Prochiral precursor with a C=N double bond for the enantioselective synthesis of amines (X= aryl, alkyl, OR, NHR, PR_2). Adapted from [3].

1.1.2. Bio-catalytic processes

A promising and much acclaimed approach to prepare chiral amines makes use of biocatalysis to selectively yield the enantiopure amine from a wide range of precursors [1]. Biocatalytic processes have developed as an environmentally benign alternative to chemo-catalaytic methods, because the bio-catalytic process usually operates under mild conditions and avoids the need for highly flammable metal-organic reagents or heavy metal contamination [8].

Several biocatalytic routes can be employed for the synthesis of optically active amines as depicted in Figure 1.3. Some of these options employ existing and well described technologies such as the (i) kinetic resolution (KR) of racemic amines (using for instance hydrolases); (ii) dynamic kinetic resolution (DKR) of racemic mixtures using hydrolases, oxidases or transaminases; and (iii) asymmetric synthesis using transaminases [1, 4, 5]. In addition, emerging technologies such as the asymmetric reduction of imines using imine reductases or the use of decarboxylases to cleave carboxylic groups are being considered as attractive options [1]. These technologies will briefly be reviewed in the following sections.

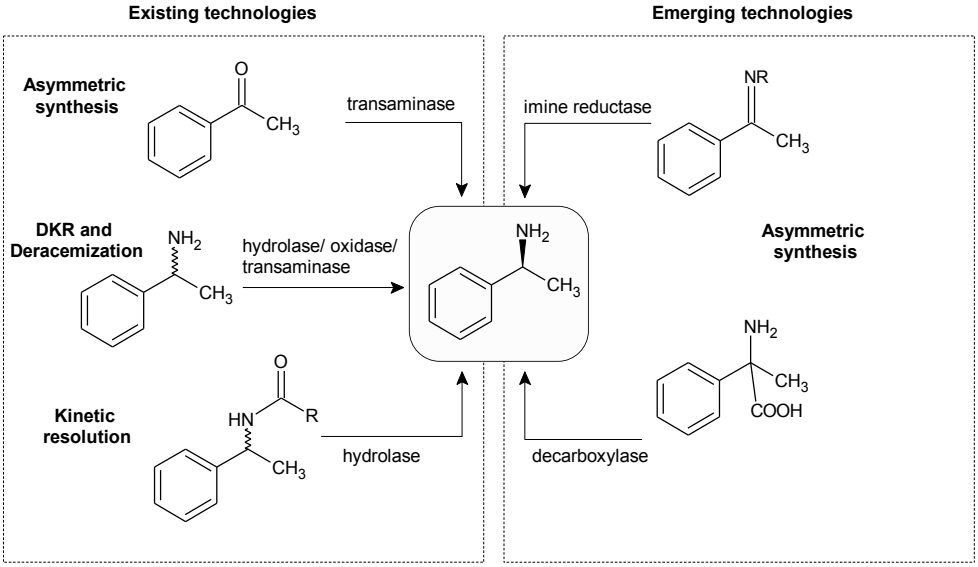


Figure 1.3: Biocatalytic approaches for the synthesis of chiral amines. Adapted from [1].

1.1.2.1. (Dynamic) kinetic resolution of racemic amines

Enzymatic kinetic resolution (KR) is the term used to refer to differentiation of two enantiomers in a racemic mixture using a biocatalyst. The two enantiomers react with different reaction rates in a chemical reaction with a chiral (bio)catalyst or reagent, resulting in an optically enriched sample of the less reactive enantiomer [9]. The classical kinetic resolution approach has a maximum theoretical yield of 50%. This is however, overcome if a racemization step is included to return the undesired product to its racemic initial form, in an approach designated as dynamic kinetic resolution (DKR). Several enzymes can be used for deracemization of racemic mixtures, as will be briefly reviewed in the following sections.

1.1.2.1.1. Hydrolytic enzymes

Hydrolytic enzymes have recently received attention for their possible application in resolution of racemic amines [10, 11]. Most of the reported examples are run under low water conditions, with *Candida antarctica* lipase B (CAL-B) (EC 3.1.1.3) as the most commonly employed hydrolase, as depicted in Figure 1.4 [1].

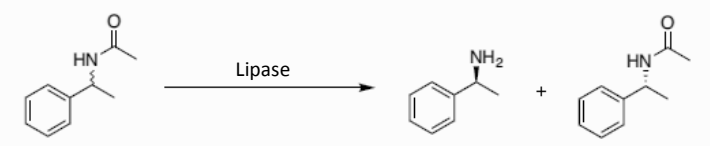


Figure 1.4: Hydrolysis of racemic N-1-phenylethylacetamide using lipase. Adapted [1]

This process is implemented at industrial scale for instance at BASF AG (Germany) for production of optically pure aliphatic amines, benzylic amines and amino alcohols [12]. Although the process is highly optimized and performed at multiton scale for a variety of amines, the yield of this kinetic resolution is limited to less than 50% [4]. As an alternative to overcome this limitation, a racemization step can be included to convert the chiral N-1-phenylethylacetamide back to its racemic form, allowing the hydrolysis to proceed. This strategy, depicted in Figure 1.5 is known as DKR. By including a racemization step, the theoretical yield could potentially reach 100%. However, it has been reported that often very harsh conditions involving metal-organic reagents are required in order to enable the racemization of the amines [13] as well as a large number of cycles, and yet it does not guarantee 100% yield.

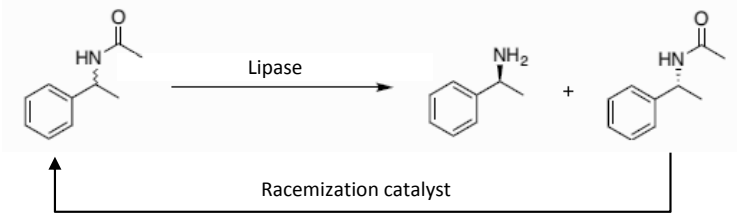


Figure 1.5: Hydrolysis of racemic N-1-phenylethylacetamide using lipase. Adapted from [1, 4]

For instance, the first DKR of amines employing CAL-B and palladium on charcoal as a racemization catalyst, was reported to yield 64% (*R*)-N-(1-phenylethyl)acetamide after eight days [14].

1.1.2.1.2. Amine oxidases

Another biocatalytic route to chiral amines employs the use of monoamine oxidase N (MAO-N) (EC 1.4.3.4). The method, as shown in Figure 1.6, was developed by Turner et al. [1, 4, 15]. In this catalytic cycle, (MAO-N) from *Aspergillus niger* catalyzes enantioselective oxidation of one enantiomer of a racemic amine to the corresponding

imine. Rather than allow the imine to simply hydrolyze to the corresponding ketone, a chemical reducing agent (e.g., ammonia borane) is added to the reaction resulting in *in situ* reduction of the imine back to the racemic amine that then undergoes another round of enantioselective oxidation to the imine. Provided that the enzyme is highly enantioselective, eventually complete deracemization of the racemate occurs yielding one enantiomer of the amine in high yield and 100% theoretical *ee* after repeated cycles [1, 4, 15].

This method allows both reactions to take place in one-pot which brings advantages in terms of reaction design. Furthermore, Turner and co-workers improved the enzyme performance by directed evolution, obtaining a MAO-N with 50-fold higher activity [16, 17]. However, the major challenges seem to be the identification or creation of an (*R*)-selective MAO to allow access to the (*S*)-enantiomer of the amine and also the limited range of substrate for (S)- and (R)- selective MAO [4].

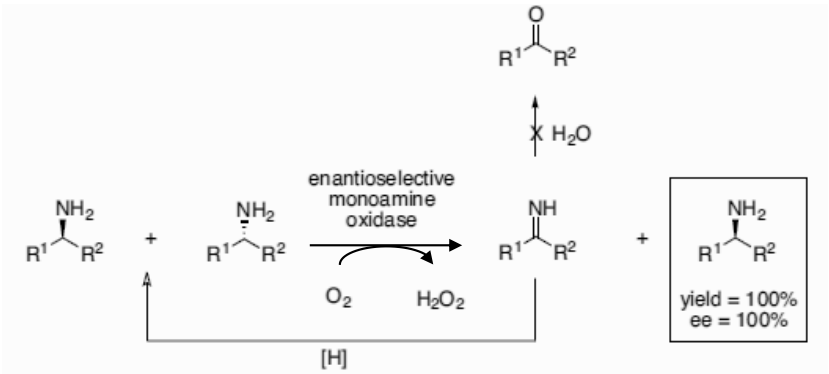


Figure 1.6: Deracemization of racemic amines by repeated cycles of enzyme-catalysed enantioselective oxidation followed by nonselective chemical reduction. Adapted from [1, 4]

1.1.2.1.3. Transaminases

The past years have been marked by an increased prevalence of biocatalytic transamination reactions for the synthesis of chiral amines from prochiral ketones, as a result of the increased availability of transaminase enzymes (also named aminotransferases, EC 2.6.1.18). ω-Transaminases (ω –TAm) can be applied to kinetic resolution and deracemisation [18] as depicted in Figure 1.7, but also in the asymmetric synthesis of chiral amines, as will be discussed later.

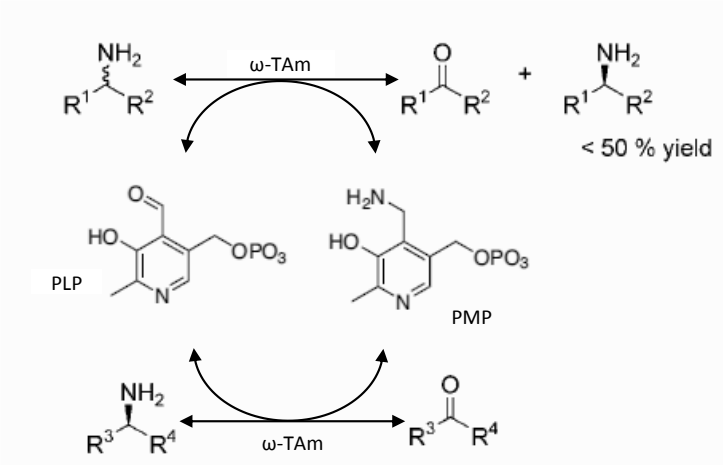


Figure 1.7: Kinetic resolution of racemic amines using transaminases (PLP= pyridoxal-5'-phosphate, PMP= Pyridoxamine 5-phosphate). Adapted from [1, 4]

ω-TAm were identified more than 50 years ago [19, 20], but only in the late 1990s they have been employed as an alternative methodology for enzymatic kinetic resolution of racemic mixtures [5]. The first significant advances for organic synthesis were achieved by Celgene Corporation (USA) [21, 22] which employed ω-TAm for the enantioselective preparation of chiral amines, via kinetic resolution of racemic amines. This process has been used on a 2.5 m^3 scale and has been demonstrated on several different aliphatic and aromatic amines. The main advantage of employing ω-TAm is that the reaction is highly enantioselective and several recombinant enzymes are readily available, most of them showing (*S*)-selectivity [5]. The lack of (*R*)-selective enzymes is however one of the major disadvantages. Furthermore, this approach is often associated with relatively low product concentrations attainable in an all-aqueous reaction system with hydrophobic compounds (since the starting pro-chiral ketone substrate often shows low solubility in water). Another disadvantage is the significant inhibition of the ω-TAm caused both by the amine products and the ketone substrates [1, 5, 22, 23]. Nevertheless, the use of ω-TAm remains a very attractive approach to access chiral amines. The main contribution for making ω-TAm so attractive for industrial application, despite these bottlenecks, is the possibility of using low-cost amine donor compounds, in conjunction with the pro-chiral ketone of interest. In particular, the broad substrate specificity [24] and the increasing availability of (*R*)-specific ω-TAm are further enabling factors.

1.1.2.2. Asymmetric synthesis

In the asymmetric synthesis, enzymes are used to yield exclusively the enantiomer of interest with a theoretical yield of 100%. Chiral amines have been reported to be obtained asymmetrically, using amine dehydrogenase (DHG) and more attractively, using ω-TAm. [1, 4]. These two technologies are relatively well known in the scientific community. Two less explored options to asymmetrically access chiral amines are the use of ketimine reductase to carry out the asymmetric reduction of prochiral imines and the use of decarboxylases to remove the carboxylic group from amino acids, yielding amines. These technologies will briefly be described in the following sections.

1.1.2.2.1. Ketimine reductase

The reduction of ketimines, as depicted in Figure 1.8, to access enantiomerically pure amines employing ketimine reductases (EC 1.5.1.25) has been rarely investigated, when compared to various chemical methods [1]. Vaijayanthi and Chadha reported that ketimines formed by the condensation of benzylamine with substituted acetophenone derivatives could selectively be reduced by applying *Candida parapsilosis* whole cells [25]. The authors reported yields of 55–80 % for the R-enantiomer, with high enantiomeric purities (95–99% *ee*). At the time of this study (2008), the enzyme was neither overexpressed nor purified and further investigations were thus required to render this approach versatile for organic synthesis. Nevertheless, the enzymatic asymmetric reduction of imines represents a potentially attractive route to chiral amines which certainly enriches the list of available technologies.

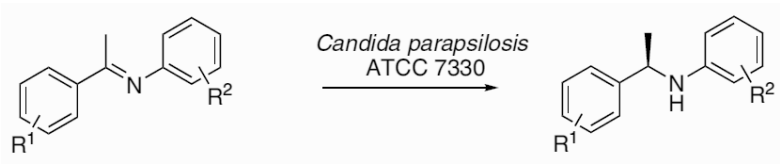


Figure 1.8 Asymmetric reduction of imines. Adapted from [1, 4]

1.1.2.2.2. Decarboxylase

Another emerging possibility makes use of decarboxylases (EC 1.4.4.X) to remove the carboxylic groups from amino-acids. This have been developed by researchers at the University of Graz, in collaboration with scientists from DSM, for the synthesis of β-amino alcohols using two different enzymes in one pot (Figure 1.9). For example, a threonine aldolase-catalysed reaction was initially used to prepare an intermediate from glycine and benzaldehyde. The intermediate was then converted to an (R)-amino alcohol in high *ee* by an irreversible decarboxylation catalysed by L-tyrosine decarboxylase [26].

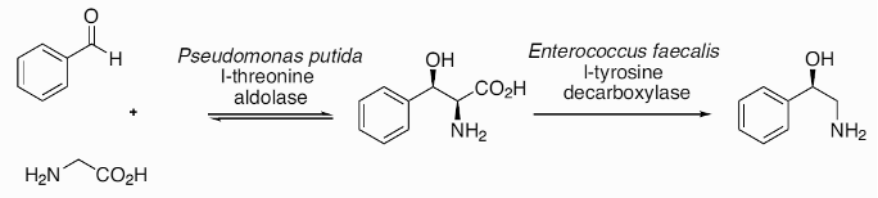


Figure 1.9: Combined use of threonine aldolase and L-tyrosine decarboxylase. Adapted from [1].

1.1.2.2.3. Amine dehydrogenase (DHG)

Similarly to MAO-N, DHG (EC 1.4.99.3) are able to oxidize amines into ketones and ammonia (Figure 1.10). This process requires the use of other redox cofactors such as copper proteins or artificial redox mediators to act as electron acceptors [27]. The main disadvantages of this process (limiting its application), is the fact that NADH-dependent enzymes cannot be widely employed. This would allow an efficient cofactor recycling by using formate and formate DGH, for example [4]. This is due to the fact that most NADH-dependent enzymes exclusively convert α-ketoacids with the exception of one amine-DHG recently isolated from *Streptomyces virginiae* which was reported to accept NADH as cofactor [28]. The authors reported that the enzyme was able to convert a wide range of substrates such as amino-alcohols, aliphatic amines, benzylic amines (although only MBA was reported) and α-amino acids. Unfortunately, the enzyme also showed very poor enantioselectivity.

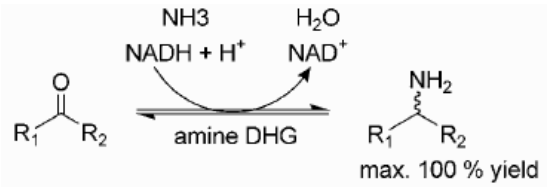


Figure 1.10: Asymmetric synthesis using amine dehydrogenase. Adapted from [4]

1.1.2.2.4. Transaminase

In turn, the use of ω-TAm eliminates the cofactor regeneration issues. The enzyme requires pyridoxal 5-phosphate (PLP) as cofactor for the transfer of the amino group. During the transamination, PLP alternates between its aldehyde form (PLP) and amino form pyridoxamine 5-phosphate (PMP) [29] as depicted in Figure 1.7 and Figure 1.11.

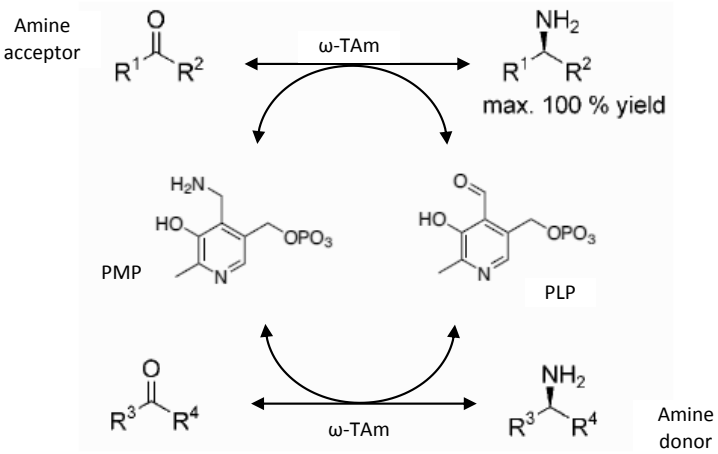


Figure 1.11: Asymmetric synthesis of chiral amines using transaminases. (PLP= pyridoxal-5'-phosphate; PMP= Pyridoxamine 5-phosphate). Adapted from [1, 4]

Briefly, the PLP dependent enzymes are reported to exist in their resting state as a Schiff base with the PLP bond with the active site lysine, forming an internal aldimine. The incoming, amine-containing substrate (the amine donor) displaces the lysine ε-amino group from the internal aldimine, and in the process the cofactor forms a new aldimine with the substrate, which is referred to as external aldimine [29], as depicted in Figure 1.12. After this step the mechanism proceeds in several different ways, depending on the enzyme employed.

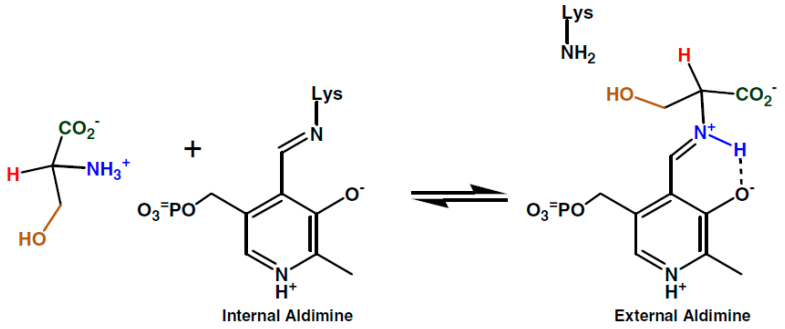


Figure 1.12: Scheme of reactions enabled by PLP. The first step of all PLP dependent reactions is the formation of an aldimine intermediate with the amine-containing substrate. Thereafter, the reactions diverge. Adapted from [29]

Although the asymmetric synthesis approach using ω-TAm theoretically allows a 100% yield of the desired optically pure product, it has been favoured less in comparison with the kinetic resolution options in past years because of the difficulties concerning reaction equilibrium and stereoselectivity and also due to intellectual property restrictions. The challenge in asymmetric syntheses that employ ω-TAm is to shift the equilibrium to the product side, especially when using an amino acid like alanine. In this case, the equilibrium is on the side of the substrates (ketone and alanine) and not on the side of the products (amine and pyruvate) [5]. Also inhibition by substrate and product, as mentioned will be discussed further in the following sections, has a strong contribution limiting its wide application at industrial scale for asymmetric synthesis today.

Table 1.2 summarizes the most important published examples of asymmetric synthesis of chiral amines using ω-TAm, where it can be seen that, with exception for cases where cascade reactions (using isolated enzymes) or large excess of amine donor (high D/A ratio) are used, the yields achieved are far from 100%. Furthermore, in most of these studies, the initial substrate concentration was beneath the solubility limit, which in turn yields low product concentrations, with a few exceptions [30, 31].

Table 1.2: Summary of some the most important published transaminase reactions for the synthesis of chiral amines. (MBA – methylbenzylamine; APB-3-amino-1-phenylbutane; ALA–Alanine; IPA–Isopropylamine; PYR–Pyruvate; ω-TAm–Transaminase. Adapted from [32].

Target compound	Amine donor (*D*)	Amine acceptor (*A*) (mM)	Ratio *D*/*A*	Temperature, pH, enzyme	Yield (%)	*ee* (%)	Time (h)	Vol. Prod (mM/h)	Refs.
MBA	APB	5	10	30, 7.0, Cell-free extract from *V. fluvialis* JS17	21.4	NR	24	0.04	[33]
MBA	3-aminoheptane	5	10		10.1	NR	24	0.02	
MBA	sec-butylamine	5	10		7.7	NR	24	0.02	
Prositagliptin	IPA	491	2	45, 7.0, mutant of ATA-117, 50% DMSO	92	>99	24	18.82	[31]
MBA	IPA	20	50	30, 7.5, ATA-113/117	95	>99	24	0.79	[34]
MBA	sec-butylamine	10	10	40, 6.0, purified ω-TA from *M. aurum*	19.9	>99	71	0.03	[35]
MBA	L-ALA	30	10	30, 7.0, Cell-free extract from *V.fluvialis* JS17, LDH	5.83	>99	12	0.15	[33]
2-butylamine	L-ALA	50	5	30, 7.0, ATA-113+ATA-117, LDH	99	>99	24	2.06	[36]
4-phenylbutylamine		50	5	30, 7.0, ATA-113+ATA-117, LDH	99	NR	24	2.06	
Mexiletine		50	5	30, 7.0, ATA-113+ATA-117, LDH	99	NR	24	2.06	
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	[30]
MBA	ALA	50	10	30, 7.5, ATA-103/117, LDG/GDH	96	99	10	4.80	[34]
MBA	PYR	50	1	30, 7.5, ATA-103, LAADH-117/GDH	96	99	48	1.00	
2-aminopentane	D-ALA	76	4	30, 7, *E.coli* (ω-TAm from *B. megaterium*), AADH	97.0	>99	24	3.07	[37]
1-methoxy-2-propylamine		76	4		97.0	>99	24	3.07	
p-Methoxyamphetamine	D-ALA	76	4	30, 7.0, *E.coli* (ω-TAm from *C. violaceum*), AADH	94.0	>99	24	2.98	
2-aminopentane		76			94.0	>99	24	2.98	
1-Methoxy-2-propylamine		76			96.0	96.0	24	3.04	
2-aminopentane	D-ALA	39	4	30, 7.0, *E. coli* (ω-TAm from *B. megaterium*), L-lactate from bovine heart	99.0	>99	24	1.61	
1-methoxy-2-propylamaine		39			94.0	>99	24	1.53	
p-Methoxyamphetamine	D-ALA	39	4	30, 7.0, *E. coli* (ω-TAm from *C. violaceum*), L-lactate from bovine heart	94.0	>99	24	1.53	
2-aminopentane		39			99.0	>99	24	1.61	
MBA	IPA	2	311	37, 7.0, *E.coli* (ω-TAm from *A.citreus*, YADH, FDH)	99.0	>99.9	24	0.07	[38]
MBA	ALA	10	10	37, 7.0, *E. coli* (ω-TAm from *V.fluvialis* JS17, ALS from *B.subtilis* 168)	34.0	NR	20	0.17	[39]
2-amino-1,2,4-butanetriol	MBA	20	1	25, 7.0, *E. coli* (with transketolase from *E. coli* and ω-TAm from *P. aeruginosa*)	21.0	NR	62	0.07	[40]
(R)-3,4-dimethoxyamphetamine	(R)-MBA	154	1	30, 8.5, *Anthrobacter sp.* KNK168	81.8	>99	40	3.15	[41]
MBA	L-ALA	30	10	30, 7.0, *V. fluvialis* JS17	90.2	>99	24	1.13	[33]

1.2. Overview and research motivation

Transaminases, in general, are highly versatile for the synthesis of optically active amines or α-amino acids [42]. While α-TAm require the presence of a carboxylic group in the α position to the keto or amine functionality, and hence only allow formation of α-amino acids, ω-TAm are much more useful as they, in principle, accept any ketone or amine [4].

The mechanism is however the same for both α- and ω-TAm. They operate by transferring an amine group from a donor substrate to an acceptor compound utilizing the cofactor pyridoxal-5'-phosphate (PLP) [32]. This can be performed either as kinetic resolution of racemic amines (as depicted in Figure 1.7) or as asymmetric synthesis starting from a prostereogenic ketone (Figure 1.11).

The enzyme is known to follow the Ping-Pong Bi Bi mechanism [43], where the first product (the keto-product, P) is released before the second substrate (the prochiral ketone/amine acceptor, B) binds to the enzyme, as depicted in Figure 1.13.

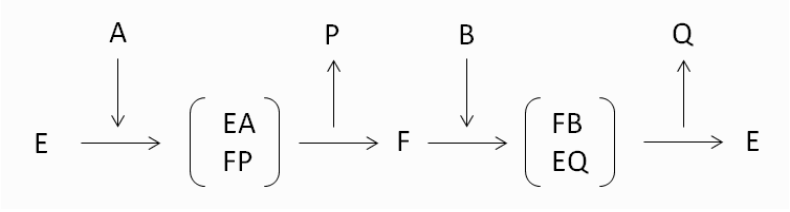


Figure 1.13 – Ping pong Bi Bi mechanism. Adapted from [43, 44]

This is common for all transaminases and for both kinetic resolution or asymmetric synthesis [43]. Interestingly, by employing the same ω-TAm in a kinetic resolution or in an asymmetric synthesis, amines with opposite absolute configurations are accessible; for example, if the ω-TAm leads to the (S)-enantiomer product during asymmetric synthesis, the (R)-enantiomer can be obtained in the kinetic resolution [5].

While the use of ω-TAm to access chiral amines is very attractive to industry, the limitations inherent with the whole process development, makes it very challenging. These include strong inhibition caused by substrates and products, potential

unfavourable thermodynamic equilibrium in the direction of interest and low water solubility of the ketone substrates [32]. Hence, several factors need to be considered and several strategies need to be applied in order to obtain high product concentrations ($g_{Product}/L$).

Protein engineering tools such as directed evolution and/or rational design, have the potential to enable wild-type enzymes to be used outside their natural environment (where the substrate concentrations and reaction rates are low), as demonstrated by several authors [31, 45-47]. On the other hand, process engineering tools can help to overcome some of the limitations that protein engineering cannot, e.g. the unfavourable thermodynamic equilibrium, as mentioned before. It is thus evident that the two technologies can be used in a smart manner, allowing high productivity to be achieved by balancing the contribution of each. Table 1.3 summarizes the different methodologies that can be used to improve biocatalytic process. These include process, reactor, reaction and biocatalyst engineering. For simplicity, the term "process engineering" is used throughout this thesis to refer to any manipulation in the process which does not cause alteration to the biocatalyst, hence, this includes for instance: reaction and reactor engineering strategies. These will be further developed in Chapter 5.

Driven by the attractiveness of accessing chiral amines using ω-TAm, several research works (as shown in Table 1.2) have been carried out aiming at understanding and overcoming the various bottlenecks limiting the industrial implementation of ω-TAm catalysed reactions. While some of these works have focused on the enzyme discovery and/or development (biocatalyst engineering), others have focused on process engineering strategies to overcome the above mentioned disadvantages [1, 32]. The latter, is of particular interest since it has the potential to overcome several limitations without altering the enzyme structure, hence avoiding the need to change multiple amino acids in the protein and screening among various mutants for the different features which can be a very labour (and cost) intensive task [48, 49]. Furthermore, limitations such as unfavourable equilibrium or low solubility of the substrates cannot be directly solved by protein engineering strategies, hence the solution, at least to some extent, relies on process engineering strategies. It is however important, to mention that protein engineering can be used to indirectly solve the equilibrium related issues.

For instance, the enzyme can be engineered to operate at a higher temperature which would influence the thermodynamic equilibrium.

Table 1.3 Description of potential methodologies, tools and the respective technologies for use in biocatalysis

Eng. Methodologies	Options (tools)	Example of Technologies
Process Engineering	*In situ* product removal (ISPR)	- Resins - Solvents - Membrane - Distillation
	In situ substrate supply (ISSS)	- Resins - Solvents
	In situ co-product removal (IScPR)	- Resins - Solvents - Membrane - Distillation/evaporation - Enzymatic cascade
	Immobilization	- Binding to carrier - Crosslinking - Encapsulation
	Biocatalyst formulation	- Whole-cell (WC) - Cell-free extract (CFE) - Purified enzymes (PE) - Immobilized biocatalyst
Reactor Engineering	Selection of operating mode	- Batch - Fed-batch - Continuous
	Hydrodynamic	- STR - Plug-flow
	Optimization of stirring speed	- Optim. of stirring speed
Reaction Engineering	Optimization reaction condition	- Optim. pH, T and P - Optim. of [Biocatalyst] - Stoichiometry/Molar ratio - Substrate selection - Co-solvent
Biocatalyst Engineering	Protein improvement	- Rational design - Directed evolution
	Optimization of expression	- Enhance expression level - Co-expression of enzymes - Host selection

This strategy has been explored and demonstrated, for instance, by Shin and Kim who developed various methods aimed at increasing the product concentrations of ω-TAm catalysed amine resolutions, through the continuous removal of product ketone from the reaction using an aqueous/organic two-phase system. This was applied to the resolution of racemic MBA and was found to yield superior product concentration than those obtained in an single aqueous-phase system [50, 51]. However, a drawback of the biphasic system was found to be the increased enzyme deactivation rate compared to the aqueous-only system due to the aqueous/organic interface, which is expected if the enzyme is not developed to operate at such conditions. Another reported disadvantage was the significant inhibition of the ω-TAm by the amine acceptor substrate (pyruvate) used [18], suggesting the need for further strategies to fully enable the reaction potential. These bottlenecks, as mentioned before, are well known to affect this enzyme when used in both kinetic resolution and asymmetric synthesis at process condition [32]. This suggests the need for further work in the field to help understanding the potential of the different methodology in the process development for ω-TAm catalysed reactions.

This thesis was motivated by the lack of a work where these different methodologies were considered in parallel. Focusing mainly on Process and Protein engineering methodologies and the integration of the two, using ω-TAm as case study, this work aims at bringing up the discussion on the role of process and protein engineering in the biocatalysis. In 2011, we published a review summarizing the state of the art of ω-TAm catalysed reactions where this discussion was initiated [32]. This thesis aims at becoming an experimental extension of that work, focusing on the early process development stages where various screenings and characterizations at lab scale are required in order to both gather information regarding the reaction parameters (e.g. biocatalyst data, reaction conditions and separation options) and to eliminate infeasible process options.

In summary, it is part of the objective of this thesis to support the process development for ω-TAm catalysed as well as other similar biocatalytic processes which can also benefit from this work.

1.3. Model reaction systems

In this thesis, 4 main reaction systems are used to characterize the process at the different stages. These include two amine donors (Isopropylamine - IPA and Alanine -ALA) and two prochiral ketones/amine acceptors (Acetophenone-APH and Benzylacetone-BA), yielding Methylbenzylamine (MBA) and 3-amino-1-phenyl butane (APB), as depicted in Figure 1.14. Throughout the experimental work in this thesis at least one of these reactions systems will be used. These systems were selected because they deal with non-toxic and inexpensive compounds and these compounds' properties fairly represent the main challenges faced in the asymmetric synthesis of chiral amines catalysed by ω-TAm (low solubility, potentially unfavourable thermodynamic equilibrium and enzyme inhibition) [32]. Furthermore, the similarity in properties between the substrates and products (which is very common in transamination) brings several challenges to downstream processing (which is also represented by the choice of these reaction systems). Two different amine donors were selected to demonstrate their importance to process. The choice of amine donor affects not only the thermodynamic equilibrium but also enables different product removal and DSP options, since different co-products are formed depending on the donor.

For instance, the use of IPA as amine donor is often reported, mostly due to its low cost [32]. In this case acetone (ACE) is formed as the co-product, which can potentially be evaporated by stripping using an inert gas such as nitrogen [52] allowing, in principle at least, an equilibrium shift as will be discussed later.

On the other hand, the use of ALA as the amine donor opens the possibility of carrying out cascade reactions to remove the formed pyruvate co-product [30], which is a great advantage since the thermodynamic equilibrium for reactions featuring ALA as amine donor are very low in comparison to those featuring IPA (for example: Keq for synthesis of MBA is $4.03E^{-05}$ and $3.33E^{-02}$ for ALA and IPA as amine donors, respectively [53]).

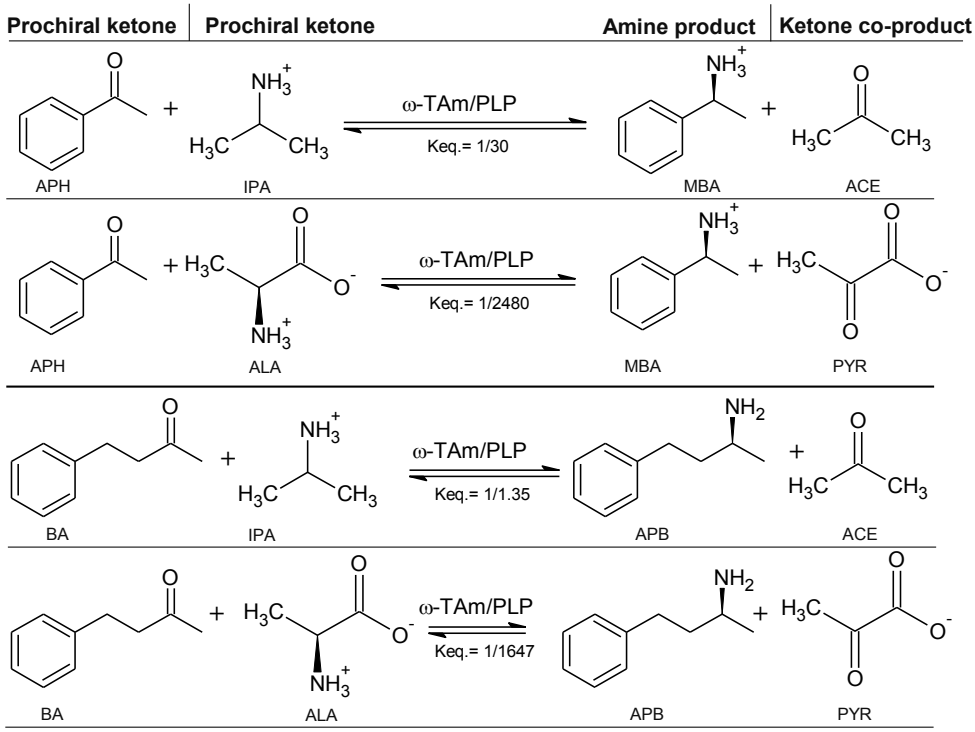


Figure 1.14 Overview of the model reactions catalysed by ω-TAm. APH – Acetophenone, BA – Benzylacetone, IPA – Isopropylamine, ALA – Alanine, MBA – Methylbenzylamine, APB – 3-amino-1-phenyl butane. Keq. obtained from [53]

1.4. Specific research goals

This thesis aims at developing strategies to improve the production process of chiral amines through the asymmetric synthesis using ω-TAm. The thesis identifies the main challenges inherent with different stages of the process development and gives a theoretical as well as experimental overview of the possible solutions for the different challenges as well as their limitations. Process engineering strategies such as *in situ* product and co-product removal (ISPR and IScPR) were used to overcome the product inhibition and unfavourable thermodynamic equilibrium limitations.

Several screening methodologies were developed and implemented with the objective of aiding the selection of: i) a suitable biocatalyst for the process; ii) a suitable carrier for enzyme immobilization; and iii) a suitable support for product removal. The work follows a comprehensive approach throughout these screening methodologies, defining several criteria that can potentially be used at the different stages.

In summary, the goals established for this thesis were:

- to overview the integration of process engineering strategies in biocatalysis;
- to integrate these with protein engineering and identify the role of each of these two technologies as well as their limitations;
- to establish guidelines and develop screening methodologies to aid the selection of a suitable biocatalyst to be used in process;
- to overview the different biocatalyst formulations that can be used in biocatalysis and study their influence in the process;
- to overview the main bottleneck affecting the development of biocatalytic processes;
- study the influence of these on the process (e.g. the influence of (i) amine donor, (ii) catalyst formulation on the inhibition);
- to review the downstream processing challenges and the different options, and;
- to overview and implement strategies to improve yield of the asymmetric production of chiral amines.
- to summarize in one work the all the important considerations for the entire biocatalytic process development for the asymmetric synthesis of chiral amines.

1.5. Structure of the PhD thesis

This thesis is divided in seven main chapters, four of them being theoretical and serving as introduction and conclusion for the experimental chapters. In that regard, the chapter's title and content are as follows:

Chapter 1: Thesis introduction

- Introduction to chiral amines and ω-transaminase;
- Definition of model reactions systems used in the thesis;
- Identification of the challenges and research motivation;
- Identification of the thesis goals.

Chapter 2: Biocatalyst considerations

- Overview of the challenges inherent with biocatalysis in general and ω-TAm catalysed reactions in particular;
- Overview of the potential of protein and process engineering tools and the integration of both;
- Overview of the different biocatalyst formulations.

Chapter 3: Screening and characterization of biocatalyst

- Development of screening methodology for selection and characterization of ω-TAm enzymes based on the inhibition and stability profile of the enzymes;
- Study of the influence of the catalyst formulation on inhibition;
- Study of the influence of the amine donor choice on inhibition;

Chapter 4: Immobilization of ω-TAm

- Development of screening methodology for immobilization carriers;
- Characterization of free and immobilized ω-Tam;
- Economical evaluation of immobilization.

Chapter 5: Process considerations

- Overview of the main challenges affecting the ω-TAm catalysed reactions: unfavourable thermodynamic equilibrium, substrate and product inhibition;
- Overview of the process engineering tools used to overcome these limitations;
- Overview of the challenges inherent with downstream processing.

Chapter 6: Implementation of process engineering strategies

- Overview of the different technologies used as auxiliary phase (polymeric resins and organic solvents);
- Screening of auxiliary phase (polymeric resins) for *in situ* product removal;
- Development of rapid screening mythology for process strategies at small scale;
- Implementation of process engineering tools to attain high product concentration.

Chapter 7: Conclusions and future perspectives

- Concluding remarks and future perspective.

1.6. Contributions

This project was greatly enriched by feedback obtained at different conferences and seminars attended and also the collaborations established. All these events resulted in important contributions for the conclusion of this work. The list of the most relevant contributions follows, divided in journals papers, oral presentations and poster presentations.

1.6.1. Journal papers

The following submitted publications have resulted from work presented in this thesis. Parts or these have been reproduced in Chapters 2 and 5. Copy of these publications are presented in the Appendixes 1A, B and C. References to more recent studies were included when suitable.

Tufvesson, P., Lima-Ramos J., Jensen J. S., Al-Haque N., Neto, W., Woodley, M. J. (2011). Process considerations for the asymmetric synthesis of chiral amines using transaminases. *Biotechnology and Bioengineering.* 108 (7), 1479-1493.

Cárdenas-Fernández, M., Neto, W., López, C., Álvaro, G., Tufvesson, P., Woodley, J. M. (2012) Immobilization of *Escherichia coli* containing ω-transaminase activity in LentiKats®; *Biotechnology Progress.* 28 (3), 693-698.

Al-Haque, N., Santacoloma, P. A., Neto, W., Tufvesson, P., Gani, R., Woodley, J. M. (2012) A Robust Methodology for Kinetic Model Parameter Estimation for Biocatalytic Reactions. *Biotechnology Progress*. 28(5), 1186-1196

1.6.2. Conference oral presentations

Neto, W., Al-Haque, N., Tufvesson, P. and Woodley, J. M., *"Integrated downstream processing for biocatalytic reactions."* Presented at BIOTRAINS biannual scientific meeting, December 2010. Basel, Switzerland.

Neto, W., Tufvesson, P. and Woodley, J. M., *"Strategies for integrated product removal applied to production of chiral amines."* Presented at Frontiers in White Biotechnology, June 2011. Delft, The Netherlands

Neto, W., Al-Haque, N., Tufvesson, P. and Woodley, J. M., *"Process considerations when implementing two-liquid phase biocatalytic reactions."* Presented at BIOTRAINS biannual scientific meeting. February 2012, York, United Kingdom

Neto, W., Anderssen, M., Schwarze, D., Tufvesson, P., Vogel, A. and Woodley, J. M. *"Process considerations for ω-transaminase: Towards integrating process and protein engineering."* Presented at ANQUE ICCE 2012. June 2012, Seville, Spain

Neto, W., Tufvesson, P. and Woodley, J. M. "*Process considerations for ω-transaminase*" Presented at BIOTRAINS biannual scientific meeting. September 2012, Copenhagen, Denmark

1.6.3. Conference poster presentations

Neto, W., Al-Haque, N., Tufvesson, P., Woodley, J. M. *"Study of the inhibitory effect present in the ω-Transaminase catalysed processes."* Presented at EFB BEC 2010, September 2010, Brac, Croatia.

Neto, W., Al-Haque, N., Tufvesson, P., Woodley, J. M. *"Evaluation of the inhibitory effect present in the ω-Transaminase catalysed processes – The importance of in situ product removal."* Presented at ESOF 2010, July 2010. Torino, Italy.

Neto, W., Al-Haque, N., Tufvesson, P., Woodley, J. M. *"Evaluation of the inhibitory effect present in the Transaminase catalysed processes – The importance of in situ product removal."* Presented at biannual scientific meeting, December 2010. Basel, Switzerland.

Neto, W., Tufvesson, P., Woodley, J. M. *"Strategies for integrated product removal applied to production of chiral amines."* Presented at CAPEC-PROCESS Annual Meeting, June 2011, Borupgaard, Denmark.

Neto, W., Tufvesson, P., Woodley, J. M. *"Strategies for integrated product removal applied to production of chiral amines"*. Frontiers in White Biotechnology. June 2011, Delft, The Netherlands

Neto, W., Tufvesson, P., Woodley, J. M. *"Polymeric resins as strategy for Removal of Chiral amines, Produced using omega-Transaminase."* Presented at BIOTRANS 2011, October 2011. Sicily, Italy.

Neto, W., Pirrung, S., Tufvesson, P., Woodley, J. M. *"Process strategies for implementing ω-transaminase catalysed reactions."*; Presented at 1st International Symposium on Transaminase Biocatalysis. Feb 2013, Stockholm, Sweden.

Neto, W, Schwarze, D., Vogel, A., Panella, L., Schürmann, M., Tufvesson, P. Woodley, J. M. *"Process strategies for implementing ω-transaminase catalysed reactions."* Presented at BIOTRANS 2013, July, Manchester, United Kingdom.

2. Biocatalyst Considerations

Summary

In this chapter, a brief overview of the most important considerations regarding the biocatalyst are discussed. The chapter serves as the introduction to Chapter 3, where the screening and characterization of a suitable biocatalyst will be carried out following a methodology developed for that purpose. Hence, this chapter overviews the main challenges associated with biocatalysis in general and ω-TAm catalysed reactions in particular. These challenges include for instance the stability issues faced by many enzymes and the inhibition caused by substrates and/or product, which as mentioned before, greatly affects the ω-TAm catalysed reactions. In addition, the role of both protein and process engineering to address these issues is discussed.

The chapter gives an overview of the most important considerations regarding to the biocatalyst, from the estimation of the kinetic parameters to the choice of biocatalyst formulation as well as the influence of each of these on the process design.

2.1. Introduction

Over the past decades, the use of biocatalysis to produce fine chemicals and pharmaceutical intermediates has increased significantly. It is believed that several hundred biocatalytic processes operate at an industrial scale, which is testament to the interest (both economic and environmental) for the implementation of such processes [54, 55]. A major contribution to this trend has been the continuous discovery and isolation of new enzymes from a variety of different biological sources. This has broadened the scope of biocatalysis which nowadays allows the regio- and enantio-selective synthesis of many compounds, through processes with potentially green credentials [56-59]. The technology therefore has many potential advantages over classical chemical synthesis to prepare fine chemical and pharmaceutical intermediates. The list of advantages includes, for instance, lower energy demand since biocatalytic processes often run at mild temperatures and pressures, reduced number of side products and increased stereo-selectivity, among others [60].

2.1.1. Challenges in biocatalytic processes

Despite these advantages, there are several challenges associated with this technology. First it is necessary to find a suitable enzyme or group of enzymes that are able to catalyse the synthesis of the desired compound. Secondly, often the naturally occurring enzyme does not meet the requirements of the process conditions, where high substrate and product concentrations as well as high stereoselectivity and biocatalytic yield ($g_{product}/g_{biocatalyst}$), are key to economic feasibility at an industrial/commercial level [32]. Table 2.1 summarizes the most common advantages and disadvantages associated with the use of enzyme and cells as biocatalysts.

ω-TAm are well known to be affected by several of these issues, as introduced in Chapter 1. Inhibition caused both by substrates and products, unfavourable equilibrium and low stability under process conditions are the most commonly reported in the scientific literature [47, 61, 62].

In a recent work, we have reviewed all the bottlenecks affecting the process development of ω-TAm catalysed reactions and suggested solutions for these different limitations [32].

These were divided into those which are related to the biocatalyst itself and those related to the process, as illustrated in Table 2.2.

This chapter and chapter 3 focus on the biocatalyst related challenges (with the exception of enzyme immobilization/separation which will be addressed in Chapter 4) while the process related challenges will be addressed in Chapters 5 and 6.

Table 2.1- Advantages and disadvantages of cell and enzymes in biocatalysis, Adapted from [60].

Advantages	▪ Stereo and regio-selective ▪ Ambient temperatures required ▪ Low energy consumption ▪ Fewer side products ▪ Can be potentially re-used ▪ Can be degraded biologically [i] ▪ Nontoxic (when correctly used) [i]
Disadvantages	▪ Wild type enzymes are often [ii]: - unstable at high temperature [iii] - unstable at extreme pH values [iii] - unstable to aggressive solvents [iii] - hydrolysed by proteases - low activity in non-natural environment - unstable in non-natural environment - exhibit poor *ee* in process conditions ▪ Some enzymes [ii]: - are very expensive - require expensive cofactors - are inhibited by substrates and/or products

[i] in comparison with organo-metals used as catalyst in classical chemical approach. Although GMOs also require special handling.
[ii] often the wild type enzyme needs to be engineered to overcome these limitations
[iii] these are only disadvantages in the rare cases where these conditions are required in the process

Table 2.2 - Analysis of challenges in biocatalytic transamination and implications to the process for the different suggested solutions. Adapted from [32]

			Challenges							
			Process related			Biocatalyst related				
			Low thermodynamic equilibrium	Low substrate solubility	Substrate and product degradation	Inhibition	Low Stability	Low Activity	Separation of biocatalyst	**Major limitations**
Solutions	Process or reaction related	Stronger amine donor	✓							Cost and availability of the donor
		Excess amine donor	✓							Inhibition and stability of enzyme; donor cost; down-stream processing
		Solvent (co-solvent/ 2-phase system)		✓						Enzyme stability; down-stream separation; VOC
		Separation of (co-) product by distillation, extraction (solvent, membrane, resin)	✓	✓	✓	✓				Co-distillation of water and/or other components , selectivity between substrates and products
		Controlled supply of substrate or fed-batch			✓	✓				Reservoir capacity,
		Degradation of co-product (cascade)	✓							Compatibility; Added cost of biocatalysts; co-factor recycling
		Immobilization					✓		✓	Deactivation; higher biocatalyst cost
	Biocatalyst related	Whole-cell catalysis	✓							Side-reactions; separation; GMO regulations
		Enzyme development				✓	✓	✓		Time and cost

VOC: Volatile organic compounds; GMO: Genetically modified organism

2.1.1.1. Stability related issues

When enzymes are used outside of their natural environment (where the conditions are mild and operation is at low substrate concentrations and for a short period of time) their activity is often compromised by the process or media conditions. This is due to a loss of their native quaternary stable structures (denaturation) which are sensitive to conditions which are not similar to the ones in their biological environment [60]. Table 2.3 summarizes the most common causes of denaturation of enzymes in biocatalysis as well the most common strategies used to minimize these.

Table 2.3: Most common enzyme denaturation factors and solutions often implemented (Adapted from [60])

Factor / (Objective)	Cause of denaturation	Alleviated by
Temperature increase / (increase rates an yields)	Unfolding or chemical modification	Use enzyme's optimum temperature or use improved enzyme
Shear stress caused by agitation / (increase mass transfer)	Unfolding	Immobilize the enzyme
pH increase or decrease / (Increase rates and yields)	Unfolding when pH>>or<<pI	Immobilized enzyme or use improved enzyme
O_2 / (increase rates with oxidases)	Oxidations of –SH or methionine	Use improved enzymes
Higher substrate concentration/ (increase yields, reduce DSP costs)	Chemical modification	Use improved enzyme
Organic solvents / (increase substrate/product solubility)	Unfolding	Immobilize enzyme

The deactivation/denaturation by temperature and pH of several enzymes has been reported to follow a first-order process [63, 64] as described by Equation 2.1:

$$\frac{A}{A_0} = exp^{-kt} \tag{2.1}$$

where, A and A_0 are the enzyme's activity or concentrations at time t and initial activity or concentrations, respectively, A/A_0 represents residual enzymatic activity at time t (min), and k (min^{-1}) is the reaction rate constant at a given temperature, pH or other condition. By estimating k one can in theory create models to estimate the deactivation under different conditions.

2.1.1.2. Inhibition by substrates and products

Inhibition is an issue in several enzyme catalysed reactions and as mentioned before, this is one of the most discussed issues inherent to transaminase catalysed reactions.

Enzyme inhibition occurs when a compound, the inhibitor, reversibly binds to the enzyme slowing down the reaction rate. The higher the concentration of the inhibitor the slower the rate becomes [65]. There are three types of reversible enzyme inhibition defined in scientific literature [66, 67]:

i. Competitive: where the inhibiting molecule is competing with the substrate to bind the enzyme. It occupies the active site and forms a complex with the enzyme, preventing the substrate from binding to the enzyme itself. This results in an apparent increase in the enzyme–substrate dissociation constant (K_m) (i.e., an apparent decrease in the affinity of the enzyme for the substrate) without affecting the enzyme's maximum velocity (Vmax);
ii. Uncompetitive: the inhibitor binds to the enzyme on a site distinct from the site which binds the substrate. This, results in an apparent decrease in both Vmax and K_m. The apparent increase in affinity of the enzyme for the substrate (i.e. a decrease in K_m) is due to unproductive substrate binding, resulting in a decrease in free enzyme concentration. Half-maximum velocity, or half-maximum saturation, will therefore be attained at a relatively lower substrate concentration;
iii. Non-competitive (or linear mixed): the inhibitor binds to the enzyme, either to the free enzyme or to a complex, on a site distinct from the active site. Substrate can still bind; however, the enzyme is inactivated. This results in an apparent decrease in Vmax and an apparent increase in K_m.

These three types of inhibition can be graphically distinguished using the linear form of Michaelis–Menten expression (Equation 2.2), commonly designated as Lineweaver–Burk equation (Equation 2.3), where v represents reaction rate, K_m Michaelis–Menten constant, $[A]$ is the concentration of substrate and Vmax the maximum velocity.

$$v = \frac{Vmax \times [A]}{K_m + [A]} \tag{2.2}$$

$$\frac{1}{v} = \frac{K_m}{Vmax}\left(\frac{1}{[A]}\right) + \frac{1}{Vmax} \tag{2.3}$$

Adding the factor $\left(1 + \frac{[I]}{Ki}\right)$ to the slope, or to the intercept or to both terms in Equation 2.3, equations can be obtained describing the competitive, the uncompetitive and non-competitive inhibitions respectively, as depicted in equations 2.4, 2.5 and 2.6.

$$\frac{1}{v} = \frac{K_m}{Vmax}\left(1 + \frac{[I]}{Ki}\right)\left(\frac{1}{[A]}\right) + \frac{1}{Vmax} \tag{2.4}$$

$$\frac{1}{v} = \frac{K_m}{Vmax}\left(\frac{1}{[A]}\right) + \frac{1}{Vmax}\left(1 + \frac{[I]}{Ki}\right) \tag{2.5}$$

$$\frac{1}{v} = \frac{K_m}{Vmax}\left(1 + \frac{I}{Ki}\right)\left(\frac{1}{[A]}\right) + \frac{1}{Vmax}\left(1 + \frac{[I]}{Ki}\right) \tag{2.6}$$

Where [*I*] represents the inhibitor concentration, which can be a molecule which is not part of the reaction or one of the reaction substrates or products and *Ki* represents its dissociation constant [65, 67].

2.1.1.2.1. Substrate inhibition

Substrate inhibition is observed when the initial rates do not increase asymptotically with higher substrate concentrations to a specific maximum value but instead decrease after reaching a certain substrate concentration.

There are several possible causes for this [44]:

i) the substrate can combine as a dead-end inhibitor with an enzyme form with which it is not supposed to react;
ii) high levels of substrate can cause an altered order of addition of reactants, or in any other way generate an altered reaction pathway;
iii) the substrate may combine at an allosteric site and cause either total or partial substrate inhibition;
iv) higher levels of substrate may cause nonspecific inhibition as a result of increased ionic strength to a toxic level;

More specifically, in the case of ω-TAm which follows a Ping-Pong Bi Bi mechanism [43] as represented in Figure 2.1. During the reaction, the enzyme oscillates between two main forms represented by *E* and *F* (where E represents the pyridoxal form and *F*, the pyridoxamine form of the enzyme) as also described in Chapter 1.

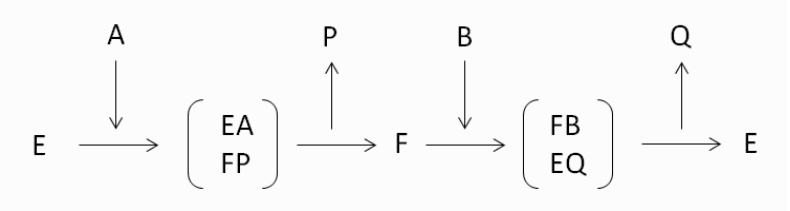


Figure 2.1 – Ping pong Bi Bi mechanism. Adapted from [43, 44]

The substrate *A* is meant to react with the *E* form of the enzyme and *B* with the *F* form. However, considering the similarity between the two forms, it is reasonable to expect that *B* has also some affinity for the form *E* and likewise *A* for *F* (if the active site is able to accommodate *A*). These unconventional combinations will result in lower reaction rates at a certain substrate level caused by competitive substrate inhibition (where *B* competes with *A* for the active site of the *F* form and less commonly, *A* competing with *B* for the active site of *E*). Other type of substrate inhibition are unlikely to be expected for transaminase [44]. This can be kinetically represented by multiplying Michaelis–Menten constant for substrate *A* and *B* (K_A and K_B) terms in the denominator of the rate equation by $\left(1+\frac{[A]}{KiA}\right)$ and $\left(1+\frac{[B]}{KiB}\right)$ respectively [43, 65, 68], as previously explained for Equation 2.3.

2.1.1.2.2. Product inhibition

For similar reasons, product inhibition occurs as the result of the interaction of substrate in the reverse reaction with the wrong enzyme form (i.e. *Q* interacting with *F* instead of *E*). This leads to formation of a complex that cannot further react [65, 69].

2.1.1.3. Kinetics of ω-transaminase (parameter estimations)

Knowing the kinetic parameters of an enzyme allows accurate quantification of the biocatalyst effectiveness and provides guidance for biocatalyst improvement [70]. The kinetic parameters can also be used in a process model to describe the dynamic behaviour

of the reaction and in this way be used to evaluate opportunities for process integration (e.g. *in situ* product removal) [71], process control and operational optimization [72] and derive the reaction equilibrium [32].

Several methods have been developed that allow estimation of dissociation and inhibition constants. Graphical methods using reciprocal plots have been used for several decades now and several books and publications have detailed the stepwise procedure allowing its implementation, as well as details of the experiments (initial rate measurements) needed [43, 44, 65-67, 69]. Although relatively easy to implement, this method requires a large amount of experimental data. Furthermore, this method is known to be inaccurate as small experimental errors will drastically affect the estimated parameters [73, 74]. Furthermore, where there is significant inhibition of the substrates, the plots are no longer linear and therefore assumptions of linear regions are not valid [67]. Another often used methodology is non-linear regression (NLR). It relies on minimizing the margin of error between the model outputs (or model predictions) and the corresponding experimentally measured values. Often, this procedure is carried out using an optimization routine such as the least squares method. This is clearly an improvement on the graphical method since no model linearization is required, although usually mathematical software with curve fitting or an optimization toolbox is needed as well as expertise on using these. The major advantage of the NLR method is that it can be applied for both initial rate data (as the graphical method) and a set of reaction progress curves. However, a difficulty in using the NLR method is the necessity of good initial guesses for the kinetic parameters [75]. In a recent publication [76], these issues have been minimized by combining both methodologies, *i.e*. using the graphical method to obtain a good initial guess of the parameters and using non-linear regression to fit the reaction rate equations to the experimental data (both initial rates measurements at different substrates and product concentrations and multiple reaction progress curves). Regardless of the method used to estimate the parameters, it is important to know the kinetic mechanism followed by the enzyme and to derive the reaction rate expression. In the cited work the kinetic model was derived based on the King–Altman method [43, 66] using the reaction system featuring IPA as amine donor and APH as the amine acceptor (Figure 2.2). The mechanism includes the formation of four non-productive complexes E-

PLP-APH, E-PMP-IPA, E-PMP-MBA, and E-PLP-ACE, which are characterized by a substrate inhibition constant KsAPH and KsIPA in the forward direction and KsMBA and KsACE in the reverse direction.

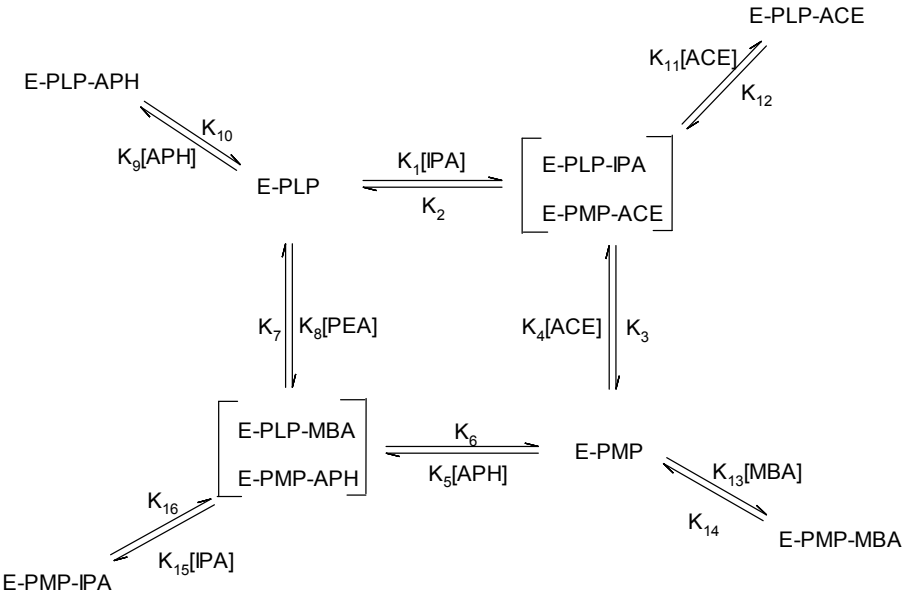


Figure 2.2 King-Altman representation of Ping Pong Bi Bi mechanism for transamination of isopropylamine (IPA) and acetophenone (APH) to form methylbenzylamine (MBA) and acetone (ACE). Adapted from [43].

Estimating the full list of kinetic parameters involves a large set of experiments. In the above work, approximately 60 experiments were performed, while for graphical methods and non-linear regression, an average of 130 experiments are required [76]. In order to estimate the kinetic parameters for different enzyme mutants or for different reaction system, these numbers of experiments are multiplied by the number of variables (mutants or reactions systems). For this reason this will not be pursued in this thesis. Here, the inhibition profile for different mutants and different reaction system will be compared based on primary Michaelis-Menten plots and its calculated constants (for substrate inhibition) and the direct result (reaction rate) of increased initial product concentration in the system (for product inhibition), rather than using the full list of parameters. Regardless of the inaccuracy that might be associated with this methodology, it considerably reduces the number of experiments required while giving a fair overview of the differences between the compared variables, as will be demonstrated and discussed in Chapter 3.

2.1.2. Protein engineering

As discussed in the previous section (see Table 2.2), several of the biocatalyst limitations (i.e. inhibition, low stability, low activity and poor enantioselectivity) can be alleviated or improved by engineering the biocatalyst and this is often (or even nearly always) necessary in order to meet process requirements[49]. Throughout the history of protein engineering, three main approaches have been pursued to obtain improved biocatalysts [55]. Early work was focused on rational design and thereby restricted to enzymes where there was a considerable knowledge of structure. In the following generation, the emphasis was on the development of diversity via random mutagenesis to create large libraries. Whilst in the last decade this type of library has developed such that a greater diversity is used as the starting point, also using natural diversity [77, 78]. The libraries are screened and, via repeated cycles, evolution is directed at the required properties [49, 79-82]. This has allowed several improved catalysts to be obtained showing remarkable new features, such as higher stability, higher activity and even the inversion of the enantioselectivity has been demonstrated [83-85]. A well-known example in the context of ω-TAm, has been achieved by Merck and Co (Rahway, NJ, USA) together with Codexis, Inc. (CA, USA) where the combination of substrate walking, modelling and mutation was used to obtain marginal activity toward the ketone required in a transamination for the synthesis of the pharmaceutical Sitagliptin. This formed the basis for further development of the enzyme via directed evolution to enable operation at adequate concentration (200 g/L), ee (99.95%) and yield (92%), representing a major achievement [86]. However, this technology also has its limitations. Firstly the cost, the time and the human resources that are required in order to change numerous amino acids in a protein and/or screen among thousands of generated mutants for different enhancements is a major drawback, especially if more than one trait is sought, thus requiring various cycles of mutations [48]. Furthermore, the substrate scope that the enzyme can operate with and the process flexibility might be compromised by developing the enzyme in favour of one substrate as in the example carried out by Merck. And finally, some of the limitations inherent with some biocatalytic processes cannot be solved by improving the enzyme. This is the case with unfavourable equilibrium, which is known to affect the ω-TAm in the synthetic direction of interest [55].

2.1.3. Process engineering

In such cases, one solution is to make use of process engineering tools to complement protein engineering and help to overcome this limitation (see Table 2.2). These tools include, for instance, the use of a feeding strategy such as *in situ* substrate supply (ISSS) or fed-batch and *in situ* product removal (ISPR) to respectively overcome substrate and product inhibition [30, 87-89] and the use of substrate excess and *in situ* co-product removal to shift the equilibrium in favour of product formation [30, 38]. The use of these technologies in biocatalysis to address unfavourable thermodynamic equilibrium as well as product and substrate inhibition is very well described in scientific literature [90, 91]. They can be divided into two groups: (i) those that both alleviate the product inhibition and shift the equilibrium, such as using an auxiliary phase (solid, liquid or gas) to remove the inhibitory product and (ii) those that only shift the equilibrium such as the removal of co-product or its conversion through enzymatic cascades into a less toxic compound, and/or the use of one substrate in excess. These will further described in Chapter 5.

2.1.4. Methodologies to implement biocatalytic processes

Until this point, there were two main routes for designing biocatalytic processes and dealing with wild type enzyme limitations: (i) design the process around the limitations of the enzyme (sacrificing process yields and productivity), and (ii) to engineer the enzyme to fit the process specifications (sacrificing the enzyme scope and process flexibility) as depicted in Figure 2.3 A and B. This has been reviewed and properly discussed by Burton and co-workers [49] and more recently by Bornscheuer and co-workers [48] and by Woodley [55].

In the present work, a third option is introduced for designing biocatalytic processes, an option where protein and process engineering are considered in parallel during process development (Figure 2.3 C). This option capitalizes upon the protein engineering tools which can be used to broaden the operating window, e.g. decrease substrate and/or product inhibition, while process engineering tools can be used to enhance stability (through immobilization), shift equilibrium (through substrate excess and/or product

and co-product removal) and further alleviate inhibition (controlled release of substrate and *in situ* product removal).

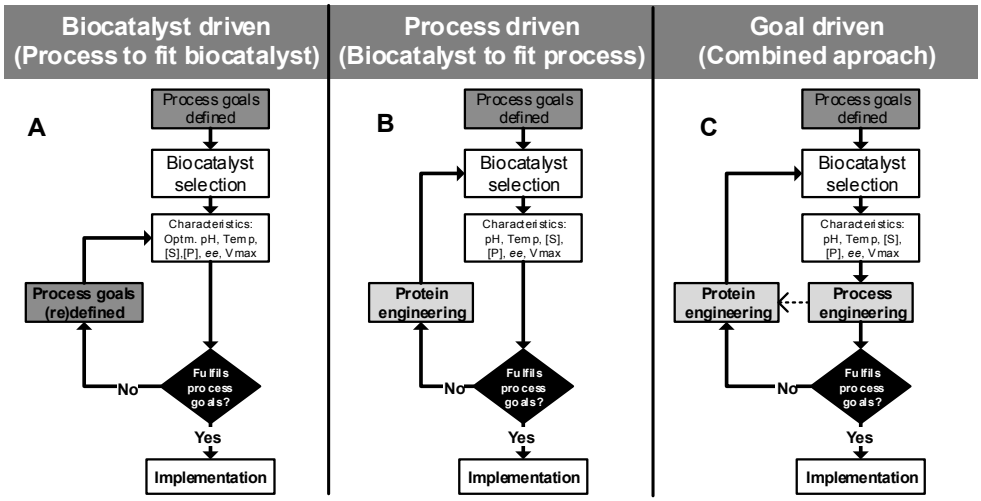


Figure 2.3: Routes for implementation of a biocatalytic process. (Filled arrows) process flow; (dotted arrow) information flow. **A) Process to fit biocatalyst:** the process goals are defined in the beginning, followed by a search for the most suitable biocatalyst (based on the process goals). After the characterization of the selected biocatalyst, the process goals are re-defined again in order to fit the enzyme optimum conditions. As a result, the process goals might be decreased, sacrificing the yields. **B) Biocatalyst to fit process:** Process goals are defined followed by a search for the most suitable biocatalyst. If the biocatalyst does not allow the fulfilling of the process objectives, it goes into a loop of improvements (through protein engineering technology) and screening until a catalyst with the desired property (ies) is found. This might take several cycles, since often various new properties are needed to achieve the process goals. Furthermore it might result in a very process-specific biocatalyst. **C) Combined approach:** process goals are defined and the most suitable biocatalyst is selected. Based on the biocatalyst characteristics, process engineering tools are used to improve process. If the process objectives are not achieved, the biocatalyst is marginally improved so that in combination with process engineering tools the process goals can be achieved. This is expected to reduce the number of rounds needed for the enzyme development and to eventually reduce the costs as well as allowing obtaining a more flexible biocatalyst.

In the context of ω-TAm, there are some successful examples in which engineered enzymes have showed improved performance when combined with process engineering tools (Figure 2.4). In summary, the process can be improved vertically (towards higher biocatalytic yield – $g_{product}/g_{biocatalyst}$) through improved expression, enzyme purification and through immobilization and re-use, and horizontally (towards higher yield and product concentration) by alleviating the inhibition and increasing the stability which can be initially achieved with protein engineering but should be further enhanced using process engineering tools.

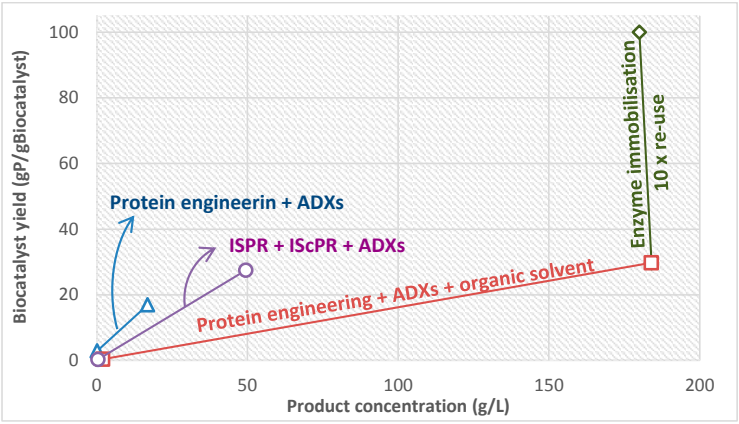


Figure 2.4 – Examples of combined approach (protein and process engineering) applied to ω-transaminase catalysed reactions (△ Martin et al. 2007 [45]; ○ Truppo et al. 2010 [30]; □ Savile et al. 2010 [31]; ◇ Truppo et al. 2012 [92]). ADXs = Amine donor excess; ISPR = *In situ* product removal and IScPR = *In situ* co-product removal.

2.2. Biocatalyst formulations

A biocatalyst can be used in several different formulations. Choosing the right one is an important part of the development of a biocatalytic processes [93]. To some extent this is determined by the type of industry in which the biocatalyst will be used (whether it is for pharmaceutical, fine chemicals or bulk chemical production). For example, if cascade reactions and/or co-factor regeneration are required, it would justify the use of whole-cells (WC) or cell-free extract (CFE). On the other hand, if the formation of side products and mass transfer are issues, it would justify the use of purified enzymes (PE) [94]. Ultimately, any of these formulations can be presented as a liquid solution, lyophilized powder or immobilized preparation. In Chapter 4 the use of immobilized enzyme preparations will be discussed, while the Chapter 3 will focus on the use of WC and CFE.

The formulation and the level of purity required for the enzyme greatly influences the production cost [95]. According to an analysis performed by Straathof and co-workers in 2002, it was suggested that about 60% of the reported industrial biocatalytic reactions use WC (in either free or immobilized form) as catalysts, with the remaining 40 % using either soluble or immobilized enzymes [96]. This difference can be related to the cost associated to prepare enzymes as depicted in Figure 2.5. The more units of operations that are necessary to produce and formulate the biocatalyst the higher the production cost becomes. This accounts for example for the cost associated with labour, chemical, carriers and energy.

Often the biocatalyst production cost (excluding development costs) for a developed production system on an industrial scale is reported to vary from 35-100 €/kg for WC and 250-1000 €/kg for PE [97]. Using WC translates into a cheaper solution, as it eliminates the need to disrupt the cells, separate the debris and concentrate the enzyme solution. However it has increased mass transfer limitation and higher chances of side products formation. Indeed this assumes that is not exported outside the cell. For enzymes exported outside the cell, the whole scenario changes and the costs are reduced since the need for cell disruption and debris separation is eliminated and less purification steps are required since most of the undesired enzymes will be retained inside the cells.

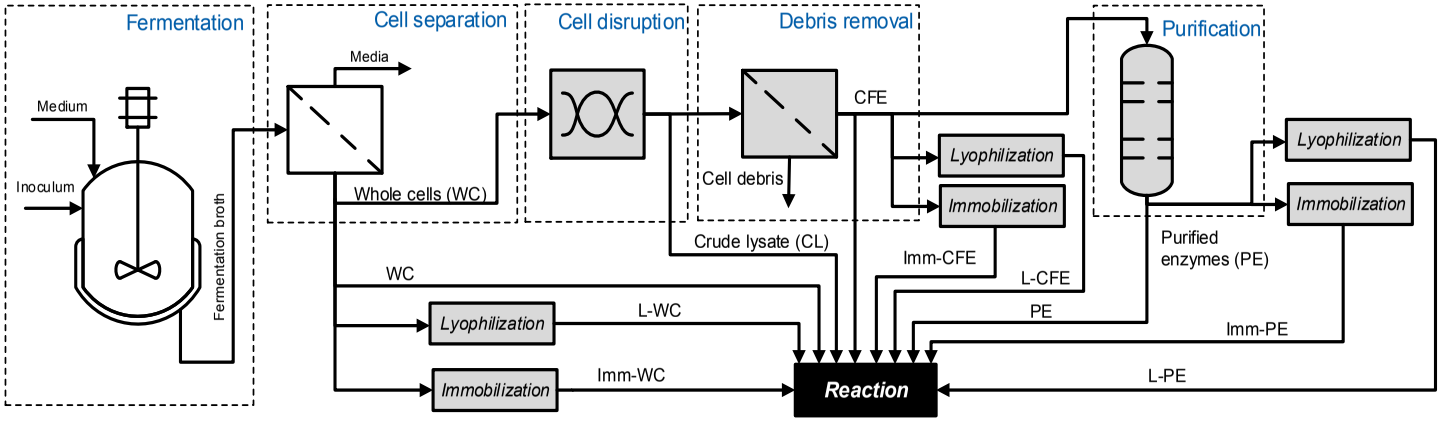


Figure 2.5: Common biocatalyst formulations used in bulk, fine chemicals or pharmaceutical industries for intracellular enzymes. WC= whole-cells; Imm-WC=Immobilized whole-cells; L-WC= Lyophilized whole-cells; CL=Cell lyase; CFE=Cell-free extract; Imm-CFE= Immobilized cell-free extract; L-CFE= Lyophilized cell-free extract; PE=Purified enzymes; Imm PE=Immobilized purified enzymes; L-PE=Lyophilized purified enzymes. Formation of inclusion bodies was not considered for the simplicity of this scheme.

3. Screening and Characterization of Biocatalyst

Summary

The implementation of new biocatalytic processes can be a challenging procedure which can require several stages of characterization and evaluation prior to scale up. One of the main challenges is to find a suitable enzyme to perform a specific reaction step. When this is done, the selected enzymes often demonstrate poor performance under process conditions, where high substrate and product concentrations and aggressive media composition contrasts with the enzyme's natural environment. Protein engineering tools (e.g. directed evolution) are able to tackle these limitations and develop enzymes with improved activity, stability, and enantioselectivity under process conditions. Often, a screening procedure needs to take place in order to select the most capable mutants among a library. Ideally this procedure should be as fast and robust as possible.

This chapter focuses on the step of process development which includes the selection and characterization of a suitable biocatalyst. This was done by identifying the main issues affecting ω-transaminase (e.g. inhibition, stability and activity under process conditions) and characterizing a mutant library according to these issues. The library included initially 5 ω-transaminase enzymes developed by c-LEcta GmbH (Leipzig, Germany) which were compared with respect to inhibition caused by substrates and products, pH and temperature stability and with respect to their performance at high substrate concentration. Furthermore, aspects such as the biocatalyst formulation and choice of amine donor were investigated with respect to their effect on inhibition. This was done by employing in total 4 reaction systems featuring two amine donors (isopropylamine - IPA and alanine - ALA) and two amine acceptors (acetophenone – APH and benzylacetone - BA) and two biocatalyst formulations (lyophilized whole cells and cell-free extract).

3.1. Rapid enzyme screening

The screening of a large library of enzymes can be extremely time consuming if a logical and simplified screening methodology is not followed. Biocatalysts can be compared in regard to several criteria (Box 3.1). An efficient way (i.e. fast with reliable output) is to select a small number of properties that correspond to the process conditions sought and use them as screening criteria. For example, if the process is desired to run at pH 8 (because the product is unstable at acidic pH, for example) then it would make sense to screen the biocatalyst that has good activity at pH 8.

The screening criteria can be applied in several steps or combined in a few steps. They can also be applied with increasing complexity. For example, a large window (e.g. activity at a very low substrate concentration) can be defined as the first criterion in order to reduce the library size and then a second criterion can be applied. The same parameter can be used but with a narrower window to further tune the library (e.g. by increasing the substrate concentration desired). When the library is reduced to a small number of biocatalysts then the characterization procedure can be started. In this step all the required information for the process should be collected. This can include kinetics parameters, performance in special conditions or any other criteria which were not used in the screening phase. This is represented in Figure 3.1 where the methodological approach used for mutant screening is depicted.

- ☐ **Activity**
- ☐ **Stability**
  - to pH
  - to temperature
  - in organic solvents
  - buffers strength
- ☐ **Inhibition**
  - by substrates
  - by products
  - by other reaction component
- ☐ **Selectivity (*ee*)**
- ☐ **Half life time**
- ☐ **Substrate range**

Box 3.1 Example of criteria for enzyme screening

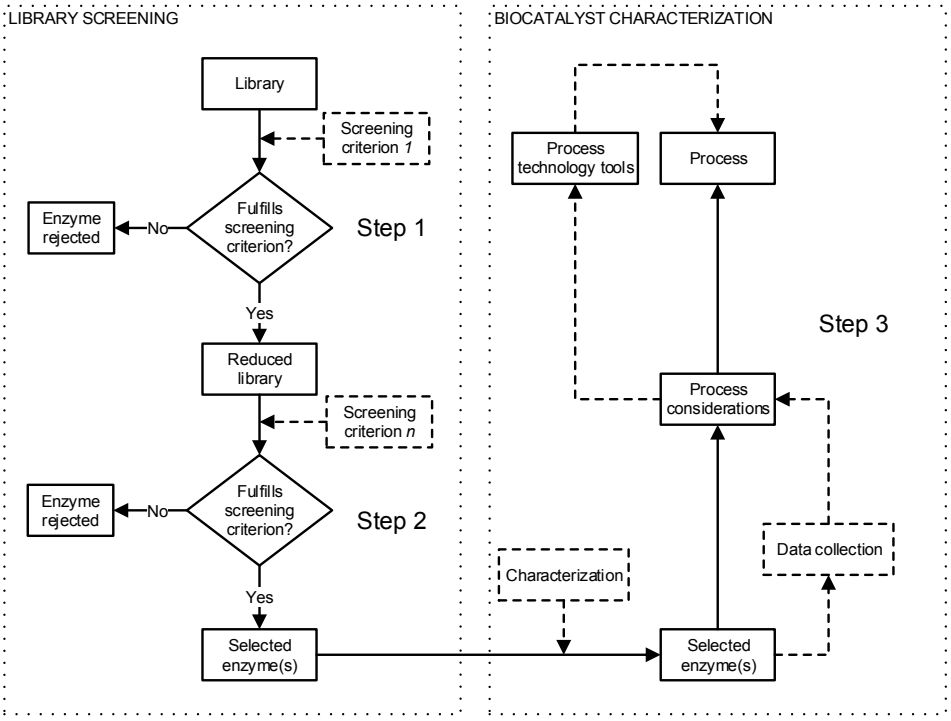


Figure 3.1 Diagram for a rapid mutant screening and characterization. (Filled arrows – screening flow; dotted arrows – information flow)

In this chapter, activity (at high substrate concentration), inhibition by products and substrates and stability to pH and high temperatures were used as screening criteria. The screening was divided in two parts: in the first part (which is not shown in this thesis), a library of 5 ω-TAm mutants was screened with regard to substrate inhibition/activity at 30 mM of substrates (APH). This concentration was chosen for being close to the water solubility limit for this substrate (this corresponds to Step 1 in the screening diagram depicted in Figure 3.1). From this step, three most capable enzymes (showing higher activity) were selected for the second part of the screening (Step 2 in Figure 3.1). Finally one enzyme was selected and further characterized (Step 3).

3.2. Materials and methods

3.2.1. General

E. coli BL21 cells over expressing (ATA 40, ATA 44 and ATA 47) and corresponding semi-purified CFE preparations were obtained from c-LEcta GmbH, Leipzig, Germany. The cells were grown and lyophilized in house. All the chemicals were obtained from Sigma-Aldrich (Buchs, Switzerland).

3.2.2. Production of ω-TA (ATA 40, ATA 44 and ATA 47)

The culture medium (*ZYM505*) was composed by *ZY* with 1x *M*, 1x *505* and 2 mM MgSO4, where *ZY* was composed by 5 g/L of Yeast extract and 10 g/L of Tryptone; *M* was composed by 0.5 M of Na_2HPO_4, 0.5 M KH_2PO_4, 1 M NH_4Cl and 0.1 M Na_2SO_4; and *505* was composed by 250 g/L of Glycerol and 25 g/L of Glucose.

Pre-cultures: One colony from LB-agar plates was cultivated in complex media ZYM505 supplemented with 50 µg/ml kanamycin and incubated overnight at 37 °C (150 rpm). The pre-cultures were used to inoculate fermentations with an initial OD_{600} of 0.1.

Fermentations: Fermentation was performed at 30°C, pH 7 in fermenters with 1L working volume with complex media supplemented with 50 µg/ml kanamycin. The cultures were induced with 1mM IPTG at early exponential phase and harvested by centrifugation, 4000 rpm 20min, 20h after inoculation. The cell pellet was washed with 50 mM potassium phosphate buffer (k-PBS) pH 7.4 and stored at -80°C and freeze-dried.

3.2.3. Freeze drying

The frozen cells (at -80 ˚C) were lyophilised at -54 ˚C for 6 hours under vacuum (10^{-2} mBar) using a Heto LyoLab 3000 from Thermo Scientific (Massachusetts, USA). The resulting lyophilized powder was stored at -5 ˚C before use.

3.2.4. Protein expression level

Cell samples were sonicated for 10 min at an amplitude of 50 % and a cycle of 0.5. Afterwards, the extraction mixture was cooled in an ice bath. The cell debris was removed by centrifugation at 14000 rpm for 5 min. 5 µL of loading buffer (4x) and 2 µL of 500 mM DTT were added to 13 µL of supernatant. After mixing, the sample was incubated on a thermoblock at 95°C for 5 min. A 10x dilution was prepared.

The samples and 5 µL of Precision Plus Protein™ Standard were loaded onto a polyacrylamide gel. A SDS-PAGE running buffer 1x was used. A constant voltage was applied for 1 hour.

The gel was stained with coomassie blue. The gel was analysed using the software GelAnalyzer 2010. The molecular weight of TAm was determined using a log linear calibration curve to be 48 kDa, which is in accordance with previously published reports [98, 99] (Appendix 3A).

3.2.5. Activity assay

Activity assays were performed in a thermoshaker (HLC Biotech Model 11, Pforzheim - Germany) at 30 ˚C with orbital shaking of approximately 400 rpm. Samples of 100 µL were taken at minute 1, 5 and 10 and added to 400 µL of 1 N HCl to stop the reaction. The samples were then centrifuged for 5 minutes at 14100 rpm (MiniSpin plus, Eppendorf AG, Hamburg, Germany) and analysed by HPLC.

3.2.6. Analysis:

All samples were analysed using HPLC by measuring the concentration of MBA and/or APB using an Ultimate 3000 HPLC (Dionex, Sunnyvale, CA, USA). The compounds were separated on a Luna 3 µm C18(2) 100 Å (50 x 4.6 mm) column (Phenomenex, Torrance, CA, USA) at a flow rate of 2 mL/min using a multi-step gradient flow of aqueous 0.1% v/v trifluoroacetic acid and acetonitrile, with the following percentage of acetonitrile: 0 min (0%), 1 min (10%), 2.5 min (10%), 5.9 min (60%), 6 min (0%), 7 min (0%). Compounds were detected at 210 nm (3.9 min for MBA and 5.9 for APB). The quantitative analysis was performed from peak areas by external standards (Appendix 3B).

3.2.7. Step I – Initial screening of library of 5 mutants

In the first step of the screening (following the diagram in Figure 3.1), 5 ω-TAm mutants (all produced by c-LEcta GmbH, Leipzig, Germany) were characterized for their activity at 30 mM APH (and 1 M IPA as mine donor). From this step 3 mutants were selected for further characterization (Steps II and III of the screening diagram Figure 3.1).

3.2.8. Step II – Further screening of library of 3 mutants

3.2.8.1. Substrate and product Inhibition

Five reaction mixtures containing increasing concentrations of APH of 0-30 mM (for substrate inhibition experiments) and five with increasing concentrations of (*S*)-MBA of 0-10 mM (for product inhibition experiments) were prepared with 2 mM PLP, 1 M IPA and 100 mM k-PBS pH 7. Prior to its addition, IPA was neutralized using 6 M HCl. The vials containing 2.5 mL reaction mixture were tightly closed and pre-incubated for 5 minutes at 30 ˚C with continuous agitation of approximately 500 rpm (HLC Biotech, Model 11, Pforzheim, Germany) prior to addition of lyophilized CFE (2.1 g/L of ATA 40, 1.18 g/L of ATA 44 and 0.85 g/L of ATA 47, dried weight) which were diluted in 0.5 mL 100 mM k-PBS (pH 7) was added and the activity assay initialized.

3.2.8.2. pH stability

2.94 g/L of whole cells (ATA 40, ATA 44 and ATA 47) were incubated at pH 6-10 (using a wide range buffer prepared from boric acid, citric acid and trisodium phosphate – Appendix 3C) and samples were taken at times 0, 0.5, 5 and 24 hours to run the activity assay. Prior to addition of the biocatalyst, the reaction mixture was pre incubated at assay conditions for 5 minutes.

3.2.8.3. Temperature stability

2.94 g/L of WC (ATA 40, ATA 44 and ATA 47) were incubated at 30-60 ˚C in a buffer solution (0.1 M k-PBS, pH 7) and samples were taken at times 0, 0.5, 5 and 24 hours to run the activity assay. Prior to addition of the biocatalyst, the reaction mixture was pre-incubated at assay conditions for 5 minutes.

3.2.8.4. Effect of DMSO on inhibition

For the experiments carried out with DMSO, the same procedure as described in the previous section was followed with an additional amount of DMSO being added to the reaction mixtures (25 v/v% in total)

3.2.8.5. Performance at high substrate concentration:

Lyophilized WC (4.06, 3.44 and 4.42 g/L for ATA40, ATA44 and ATA47 respectively – dry weight) were run in reaction for 30 h. The reaction mixture consisted of 1 M of IPA, 300 mM APH, 2 mM PLP, 100 mM k-PBS pH 7. The temperature was fixed at 30 ˚C and agitation was 400 rpm. Samples were centrifuged for 5 minutes at 14100 rpm (MiniSpin plus, Hamburg, Eppendorf AG, Germany) and analysed by HPLC.

3.2.8.6. Enantiomeric excess determinations

The enantiomeric excess was analysed using a Chiralpak IB (Daicel Group, Cedex, France) column and a mobile phase of 98% n-hexane, 2% isopropanol and 0.1% ethylenediamine with a flow rate of 1 ml/min and oven temperature of 35°C. Compounds were detected at 254 nm. The samples analysed resulted from a 30 hours reaction of 1 M IPA and 300 mM. Samples had also 2 mM PLP and 100 mM k-PBS pH 7.2.

3.2.9. Step III - Effect of amine donor and catalyst formulation on inhibition

3.2.9.1. Substrate inhibition

Using fixed amounts of either 1 M of IPA or 1 M of ALA, the concentration of amine acceptor was varied from 2 mM to 30 or 60 mM for APH while for BA it was varied from 2 mM to 10 mM or 30 mM and the activity assay proceeded as described for Step I. This was done using both WC (5 g/L) and CFE (0.85 g/L)

3.2.9.2. Product inhibition

30 and 10 mM of APH and BA respectively (approximately corresponding to their solubility limit) was used for reaction featuring IPA (1 M) and 10 and 5 mM of APH and BA respectively were used for reactions featuring ALA (1 M). The product (MBA and APB) concentrations were varied from 0 to 10 mM and the activity assay proceeded as described for Part I using both whole cells (5 g/L) and cell-free extract (3 g/L).

3.3. Results and discussion

The results are divided in the 3 Steps (as depicted by the screening diagram in Figure 3.1). In Step I, a library of 5 ω-TAm mutants (c-LEcta GmbH, Leipzig, Germany) was screened based on the enzymes' activity at 30 mM (data not shown). From this step, 3 ω-TAm mutants were selected for further screening (Step II). In Step II, the mutants were compared with respect to degree of inhibition caused both by substrate and products, to their performance at high pH and temperatures values, their performance in a water miscible organic solvent (DMSO) and finally to their performance at high substrate concentrations (300 mM APH).

In Step III, the best mutant was selected for further characterization where the influence of biocatalyst formulation and the amine donor on substrate and product inhibition were investigated.

3.3.1. Step I – Initial screening of library of 5 enzymes

From the first screening step, three mutants (ATA 40, ATA 44 and ATA 47) were selected and carried to Step II. These mutants showed relatively better performance in the presence of 30 mM of substrate (APH).

3.3.2. Step II – Further screening of library of 3 enzymes

3.3.2.1. Substrate inhibition

Figure 3.2 shows results for substrate inhibition for all the three enzymes (ATA 40, ATA 44 and ATA 47). It was observed that the inhibition caused by APH was significant for ATA 40 and ATA 44, with the latter having its activity decreased by more than 50% at the substrate concentration of 30 mM, in comparison with the 25% loss observed for ATA 40 (Figure 3.2). However, despite this significant loss in relative activity (%), in terms of specific activity (μmol $_{MBA}$.min^{-1}.g$_{CFE}^{-1}$), ATA 44 showed at 30 mM of substrate about 12 μmol $_{MBA}$.min^{-1}.g$_{CFE}^{-1}$, compared to 8 μmol $_{MBA}$.min^{-1}.g$_{CFE}^{-1}$ observed for ATA 40. This difference could be related to a better protein expression for ATA 44. In order to confirm this the quantification of protein content between the mutants could have been carried out, however this was not pursued.

On the other hand, no substrate inhibition was observed for ATA 47 at concentrations of APH up to the solubility limit (approximately 30 mM at 25 ◦C). The reaction rate increased with the increase of substrate concentration. Also the specific activity was found to be more than 10 fold higher than ATA 40 and approximately 5 fold higher than ATA 44.

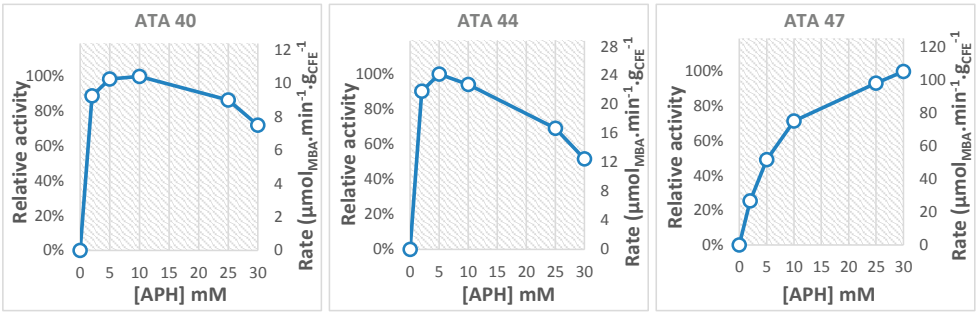


Figure 3.2: Substrate inhibition for ATA 40, ATA 44 and ATA 47. Reactions were ran with concentrations of APH ranging from 0 to 30 mM, and with 1 M of IPA, 2 mM of PLP, 0.1 M of k-PBS pH 7 and CFE concentrations (dry weight) were 2.1 g/L ATA 40, 1.18 g/L ATA 44 and 0.85 g/L ATA 47.

The improvements in substrate inhibition could be related to improvements in the catalytic centre that may have reduced the affinity towards the substrate. This could be observed by the dissociation constant (K_m) which was calculated for the 3 enzymes using the graphical method (details in Appendix 3D). The K_m was found to be 0.34, 0.39 and 7.99 mM for ATA 40, ATA 44 and ATA 47 respectively. These were calculated using the data point for the non-inhibitory regions only, hence being subject to some errors [67] but allowing a qualitative comparison. With this, the conclusion can be drawn that ATA 40 and ATA 44 have higher affinity to the substrate than ATA 47, which could be related to an increased pocket size.

3.3.2.2. Product inhibition

With respect to product inhibition, it was observed that the relative activity loss when operating with initial product concentration of 10 mM is roughly similar for the three enzymes (more than 80%). However, ATA 47 being a more active mutant, presented higher activity at a given product concentration than the other two mutants (approximately 10 fold higher), Figure 3.3. Similar to the results for substrate inhibition, these experiments were also made using initial rate measurements in order to avoid the effect of equilibrium on these results. However, the increasing concentration of product

added to the reaction, affected the equilibrium position which became more unfavourable with the addition of more product. This had a strong influence on this results.

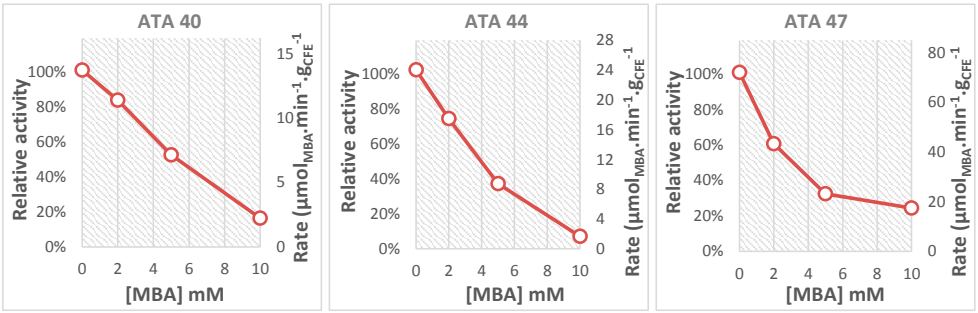


Figure 3.3: Product inhibition for ATA 40, ATA 44 and ATA 47. Reactions were run with APH fixed at 10 mM and with initial concentrations of MBA ranging from 0 to 10 mM. IPA was fixed at 1 M, PLP at 2 mM and pH at 7 using 0.1 M k-PBS. The CFE concentration was (dry weight) 2.1 g/L ATA 40, 1.18 g/L ATA 44 and 0.85 g/L ATA 47.

3.3.2.3. pH stability

The three enzymes were compared in terms of pH stability. A slightly better stability could be observed for ATA 44 which at pH 9 and 10 showed superior performance than ATA 40 and ATA 47 (Figure 3.4). In summary, the pH stability of ATA 44>ATA 47>ATA 40.

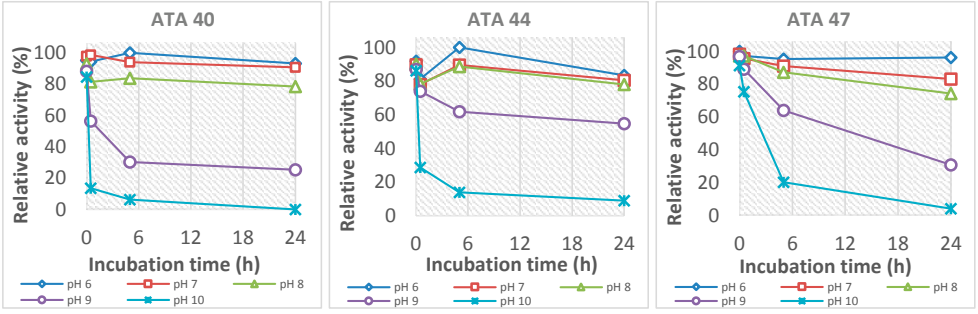


Figure 3.4: pH stability: 2.94 g/L of whole cells (ATA 40, ATA 44 and ATA 47) were incubated at pH 6, 7, 8, 9 and 10 (0.05 M wide range buffer) and samples were taken at times 0, 0.5, 5 and 24 hours to run the activity test at 30 ˚C (1 M of IPA, 5 mM APH, 2 mM of PLP, 0.1 M of k-PBS pH 7).

3.3.2.4. Temperature stability

Similarly to what was observed for pH stability, also in terms of temperatures stability ATA 44 showed a slightly superior performance, while ATA 40 and ATA 47 were strongly affected by temperatures superior than 30 °C, especially for incubation periods longer than 6 hours (Figure 3.5). In summary the temperature stability of ATA 44>ATA 47>ATA 40.

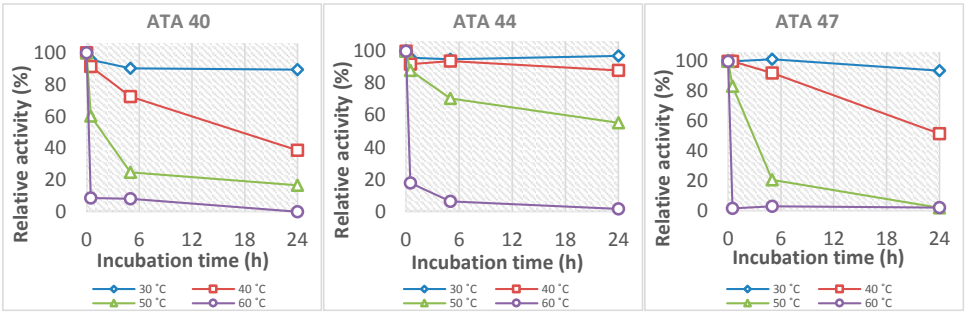


Figure 3.5: Temperature stability: 2.94 g/L of whole cells (ATA 40, ATA 44 and ATA 47) were incubated at 30, 40, 50 and 60 ˚C in a buffer solution (0.1 M potassium phosphate buffer pH 7) and samples were taken at times 0, 0.5, 5 and 24 hours to run the activity test at 30 ˚C (1 M of IPA, 5 mM APH, 2 mM of PLP, 0.1 M of k-PBS pH 7)

3.3.2.5. Effect of Solvent on inhibition

Supported by previous results where K_m was observed to be superior for the mutant affected the least by substrate inhibition (ATA 47 – section 3.3.2.1), it was hypothesised that having a reaction media more favourable to the substrate would allow decreasing the concentration of substrate at the enzyme's catalytic centre, hence decreasing the substrate inhibition. This effect can be simulated by adding a water miscible solvent such as Dimethyl sulfoxide (DMSO) to the media, making it more favourable for the hydrophobic substrate.

In order to test this theory, the three mutants were characterized for substrate inhibition in the presence of 25% DMSO and the results were compared to those obtained in the absence of DMSO (section 3.3.2.1) and are shown in Figure 3.6. For both ATA 40 and ATA 44, the presence of DMSO had a strong negative effect on the activity. The rates lowered when DMSO was added. Between these two enzymes, ATA 44 seemed to have better tolerance for DMSO since the difference between the reaction rate in the organic solvent and in the buffer were lower than those obtained for ATA 40. The ATA 47 on the other hand showed only a minor decrease in activity in the presence of DMSO suggesting higher tolerance. In respect to the effect of DMSO on the substrate inhibition, the results obtained for ATA 40 and ATA 44 suggested that inhibition by the substrate APH was decreased when DMSO was present in the media. In both cases the reaction's rate increased asymptotically with the increase in the substrate concentration until Vmax was achieved and from that point it remained constant, compared to the results obtained in the buffer media, where the reaction rate decreased after Vmax was achieved.

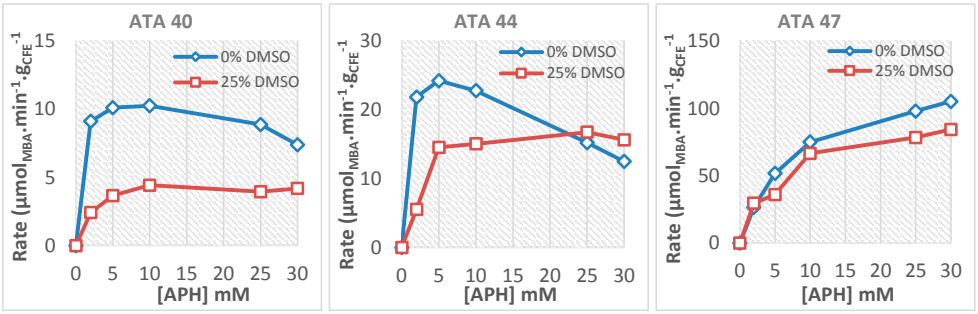


Figure 3.6: Effect of DMSO on substrate inhibition for ATA 40, ATA 44 and ATA 47. Reactions were ran with concentrations of acetophenone ranging from 0 to 30 mM, and with 1 M of isopropylamine, 2 mM of PLP, 0.1 M of potassium phosphate buffer pH 7 and crude cell extract (2.1 g/L ATA 40, 1.18 g/L ATA 44 and 0.85 g/L ATA 47; c-LEcta GmbH).

This was not observed for ATA 47 as this enzyme was not affected by substrate inhibition. In order to fully study this on ATA 47 it would have been necessary to use substrate concentration above the solubility limit in the presence of DMSO. However this experiment was not performed.

Similarly to what was performed in section 3.3.2.1, the K_m and Vmax were calculated for the 3 enzymes also in the presence of 25% DMSO and the results are compared in Table 3.1. The apparent decrease in Vmax observed for all three mutants confirms the negative effect of DMSO on the enzyme activity while the increase of K_m for all the enzymes (with the exception of ATA 47) confirms the decrease of substrate affinity, hence decrease in inhibition. The parameters calculated for ATA 47 are to be assumed underestimated since the maximum rate obtained is not a result of Vmax being achieved but due to the solubility limit. Substrate concentrations above this limit did not further increase the reaction rate, as will be shown further.

Table 3.1 – Km and Vmax in presence of 0% and 25% of DMSO for ATA 40, ATA 44 and ATA 47. The arrows reflects the comparison between the reaction in the presence of 0% and 25% of DMSO

	0% DMSO		25 % DMSO	
Enzyme	**Km (mM)**	**Vmax (µmol$_{MBA}$.min^{-1}g$_{CFE}^{-1}$)**	**Km (mM)**	**Vmax (µmol$_{MBA}$.min^{-1}g$_{CFE}^{-1}$)**
ATA 40	0.34	10.70	2.58 ↑	5.58 ↓
ATA 44	0.39	26.11	5.46 ↑	20.75 ↓
ATA 47	7.99	133.34	4.43 ↓	95.24 ↓

3.3.2.6. Performance at high substrate concentration

The three enzymes were used in a 30 hour reaction with 300 mM of APH and 1 M of amine donor IPA. They were compared with respect to the final product concentration as well as the biocatalytic yield and the result is presented in Figure 3.7, where it can be seen that ATA 47 showed approximately 24-fold higher product concentration (4.7 g MBA/L) in comparison with the ATA 40 and about 4 fold higher than ATA 44. The *ee* was found to be 99.9% for all the three enzymes.

With these results, ATA 47 was selected for further characterization. Although this enzyme showed superior performance, it should be noted that the final product concentration obtained is still below the equilibrium yields of 9.5 g MBA/ L. This suggests that product inhibition is a major issue, as shown in section 3.3.2.2.

In Chapter 5, the way in which process engineering tools could play an important role in solving this, will be discussed.

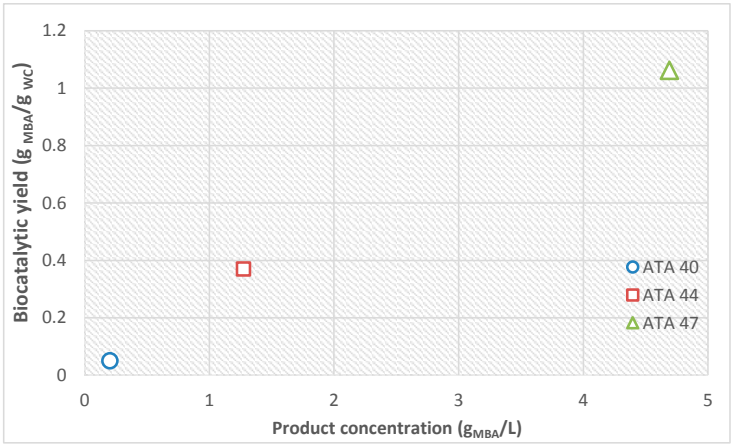


Figure 3.7: Performance at higher substrate concentration. Lyophilized WC (2.94 g/L ATA 40, 3,44 g/L ATA 44 and 4.42 g/L ATA 47) were run for 30 hours with 1 M of IPA, 300 mM APH, 2 mM of PLP, 0.1 M of k-PBS pH 7. The temperature was fixed at 30 ˚C and agitation was approximately 500 rpm.

3.3.3. Step III – Biocatalyst characterization

3.3.3.1. Influence of catalyst formulation on inhibition

In this section, whole cells (WC) and cell-free extract (CFE) formulations of the enzyme selected from the previous step (ATA 47), were compared in respect to substrate and product inhibition in order to understand whether the presence of the cellular membrane (in a WC) would have any effect on inhibition since the enzymes are not directly exposed to the media in this type of formulation. These were made using two prochiral ketones/ amine acceptor (APH and BA) and IPA as amine donor.

3.3.3.1.1. Substrate inhibition

The inhibition profile for WC and CFE presented in Figure 3.8 show very similar trends for the two formulations, suggesting no major differences in substrate inhibition. The rates initially increase with increasing substrate concentrations until the solubility limit is reached. At higher concentrations, the enzyme experiences only the concentration of substrate in the aqueous phase and therefore the rate does not increase further. This is observed for both substrates.

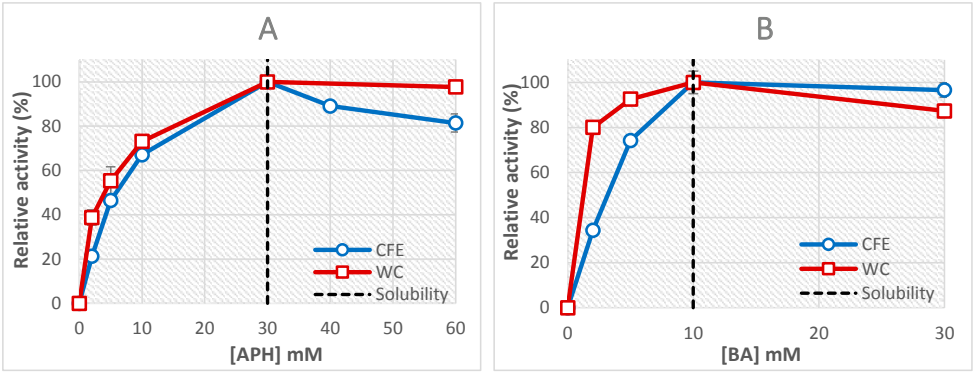


Figure 3.8: Substrate inhibition profile as function of biocatalyst formulation; A) varying concentration of APH (2-60 mM) and B) varying concentration of BA (2-30 mM) run with 1 M of IPA and 2 mM of PLP at 30 ˚C and pH 7. (Squares): WC 5 g.L^{-1} (dry weight) and (circles): CFE 0.85 g L^{-1} (dry weight); (dashed line): experimental solubility limit at room temperature.

3.3.3.1.2. Product inhibition

Similar to what was observed for substrate inhibition, also inhibition by product seems to equally affect both catalyst formulations in both reaction systems. These results, together with those obtained for substrate inhibition (section 3.3.2.1) suggest that inhibition affects equally both biocatalyst formulations, and that the presence of cellular membrane (in WC) does not help in alleviating the inhibition caused by substrate and product. This could be related to the fact that both formulations were used as lyophilized powder. It is reasonable to assume that the cellular membrane (or at least the proteins responsible for transportation across the membrane) are destroyed during the lyophilisation process, allowing free circulation of compounds in and outside the cell.

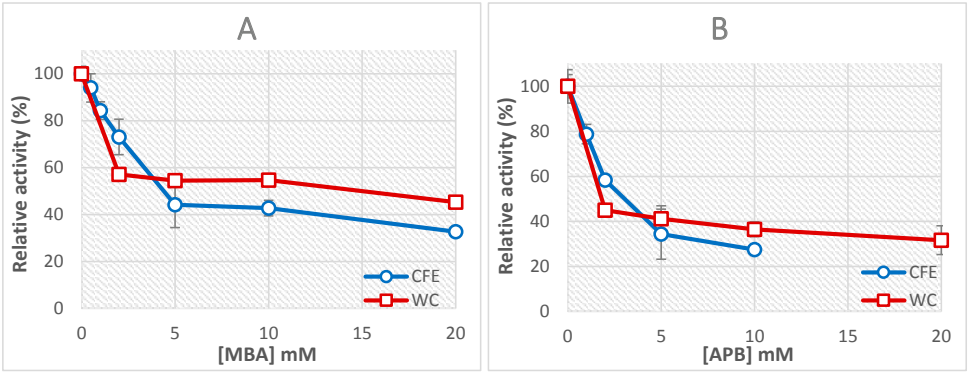


Figure 3.9: Product inhibition profile as function of biocatalyst formulation; A) varying concentration of MBA and B) varying concentration of APB (0 – 10 mM for both) were run with 1 M IPA, 2 mM PLP and 30 mM APH (for A) or 10 mM BA (for B). Reaction were run at 30 ˚C and pH 7; (squares): WC 5 g.L^{-1} (dry weight) and (circles): CFE 3 g L^{-1} (dry weight); (dashed line): experimental solubility limit at room temperature.

3.3.3.2. Influence of amine donor on inhibition

In this section, the effect of amine donor on the inhibition was investigated. This was done by comparing substrate and product inhibition profiles using 1 M of ALA and 1 M of IPA.

3.3.3.2.1. Substrate inhibition

The results depicted in Figure 3.10 suggest that substrate inhibition is not influenced by the use of ALA or IPA as the amine donor, at least in the evaluated concentration range of substrates. The main difference observed was regarding the solubility limits of APH and BA which seemed to be lowered when ALA was used as the amine donor. The solubility of both APH and BA decreased from 30 and 10 mM to approximately 20 and 6 mM, respectively (visually observed – data not shown).

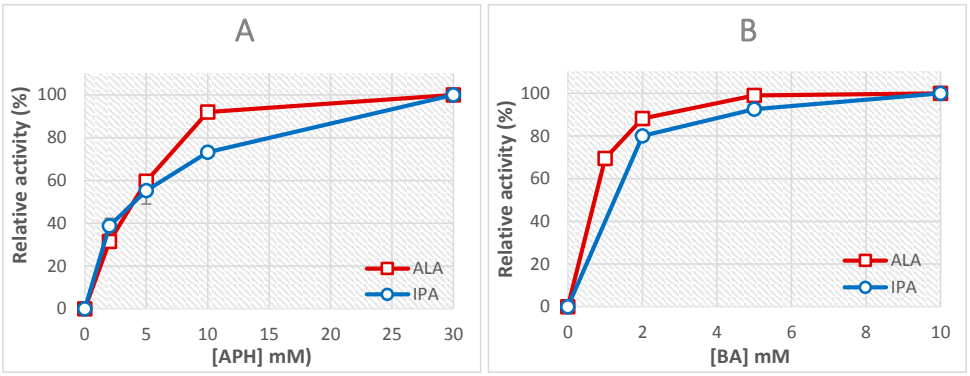


Figure 3.10: Substrate inhibition profile as function of amine donor; A) varying concentration of APH (0-30 mM) and B) varying concentration of BA (0-10 mM) were run with 1 M of ALA as amine donor (squares) or 1 M of IPA as amine donor (circles) and 2 mM of PLP. Reactions were at 30 ˚C and pH 7 using 5 g L^{-1} WC (dry weight) as catalyst.

3.3.3.2.2. Product inhibition

On the other hand, substantial differences in the degree of product inhibition were observed between the two amine donors. Product inhibition was more prominent when ALA was used as amine donor with the reaction rate dropping to zero already at 3 mM of MBA and 10 mM of APB, as can be observed in Figure 3.11.

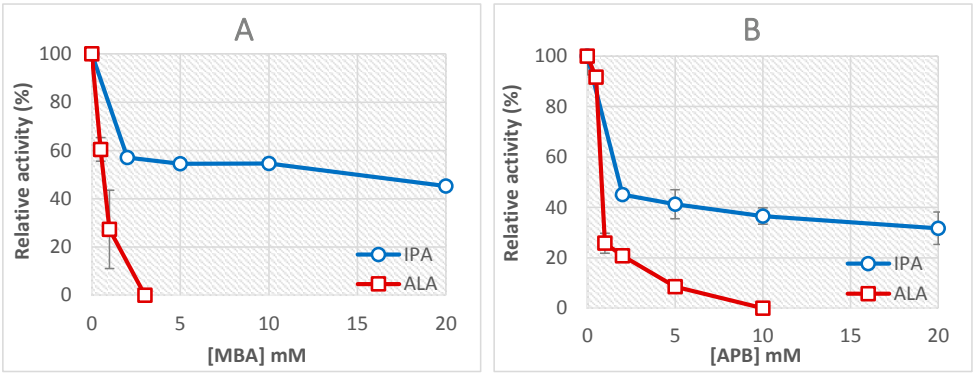


Figure 3.11 Product inhibition profile as function of amine donor; A) varying concentration of MBA (0-20 mM) and B) varying concentration of APB (0-20 mM) were run with 1 M of ALA as amine donor (squares) or 1 M of IPA as amine donor (circles). Reactions were at 30 ˚C and pH 7 using 2 mM of PLP and 5 g L^{-1} WC (dry weight) as catalyst.

A possible explanation for this could rely on the interaction of the amine donor with the enzyme. As described before (section 2.1.1.2), product inhibition can be caused by the interaction of the product with the wrong form of the enzyme in the reverse reaction (i.e. *Q* interacting with *F* in Figure 2.1) which would cause the formation of an abortive complex stopping the reaction. However, it is also reasonable to assume that the products (MBA and APB) have higher affinity (lower K_m) to the enzyme's catalytic centre than then amine donors (ALA and IPA). This would make it difficult for the amine donors to bind to the enzyme afterwards if the product is still occupying the catalytic centre (since both the amine donor and the product bind to the same form of the enzyme, *E*). So it can be hypothesised that IPA is able to cause detachment of the products from the catalytic centre and bind to the enzyme better than ALA does. This could have been confirm by measuring the K_m for both the amine donors (IPA and ALA) and the products (MBA and APB). However this was not investigated.

In addition, as mentioned before, this experiment is also influenced by the equilibrium position. Increasing concentration of the product will inevitably change the equilibrium position, and therefore the reaction rate decreases. This is more expressive for reactions where ALA is used as amine donor which are more affected by the equilibrium. This could have been avoided if a constant substrate/product rate were maintained at the different initial product concentration. Nevertheless, these findings emphasize the need for process engineering tools to deal with the decreased activity in the presence of product.

3.4. General discussion:

The screening of biocatalysts can be simplified if a methodology is followed. This process needs to be efficient, fast and reproducible. Here, a methodological approach has been suggested consisting of the following steps: i) create a list of screening constraints or criteria that represent challenges to the process to be developed; ii) use one or a few of these criteria to reduce the library, which can be done in more than one step; and iii) after reducing the library use the remaining constraints to further characterize the remaining biocatalysts.

In the present study, enzyme activity at 30 mM was used as first criterion to evaluate the initial library consisting of 5 enzymes and this allowed the reduction of the library to 3 enzymes. Inhibition by substrates and products was used as a second criterion, which allowed selection of one suitable biocatalyst, the ATA 47. This enzyme showed an improved performance at high substrate concentrations without being inhibited at substrate concentrations up to the solubility limit. It also showed a slightly improved stability at high pH, and improved enantioselectivity. However, product inhibition continues to be a major issue. This will be dealt with in Chapter 5 by employing the ISPR technology. Nevertheless, the enzyme was selected for further characterization.

Considering that ATA 47 had an increased stability in presence of high substrate concentrations it was expected that it also had a slightly improved stability in presence of organic solvents. This was investigated and confirmed by adding DMSO (25%) to the reaction mixture. ATA 47 showed similar performance in the presence of DMSO as in the presence of buffer. This opened the possibility of using water miscible solvents to increase substrate and products solubility. However, in order to obtain high concentrations of product (e.g. 30-50 g/L), higher concentrations of substrate are required in the aqueous phase and consequently much more DMSO would be needed in order to solubilize it. It was measured that circa 50% DMSO would be needed to solubilize about 200 mM (~25 g/L) of APH (Appendix 3F) and this would require an even more stable enzyme. Nevertheless, the use of DMSO was confirmed to decrease substrate inhibition to a certain degree as was demonstrated here for ATA 40 and ATA 44.

Selection of the right biocatalyst formulation is crucial, as it has a strong impact on the process cost, as discussed earlier in Chapter 2. The more the number of steps or unit operations required to produce the catalyst, the higher the cost. Indeed these steps bring benefits to the process, such as more selective catalysts and less side products (as in using purified enzyme), or increased storage stability and easier handling (lyophilized biocatalyst), easy separation and ability to re-use the biocatalyst (in case of immobilization of biocatalyst) or *in situ* co-factor regeneration (WC). Besides the obvious advantages of the different formulations, very little is known regarding the performance of the different formulations with respect to inhibition for example. Often, questions arise on whether the presence of a cell wall would have a beneficial effect on the inhibition due to selective mass transport across the membrane. This question was addressed in this chapter by comparing the substrate and product inhibition profiles when using both WC and CFE (both lyophilized) and no major difference was observed between the two (section 3.3.3.1). It would have been interesting to include non-lyophilised WC in this study in order to fully study the role of the cellular membrane on inhibition. This was however not performed.

Another important question often raised in ω-TAm catalysed reactions lies on the choice of amine donor. This has great influence not only on the DSP but also on the process options that can be used to alleviate limitations such as the thermodynamic equilibrium. IPA is often used since it is a relatively inexpensive substrate [32]. When used, it forms the co-product acetone, which can theoretically be removed by vacuum or nitrogen sweeping [52] in order to shift the equilibrium. These strategies are not selective though and could potentially cause also the removal of the the ketone substrate, as will be discussed in Chapter 5. Alternatively cascade reactions can be used to selectively remove acetone [32]. ALA is another commonly used amine donor [30]. The major advantage of using it relies on the fact that it forms pyruvate as co-product, which can be easily removed by the lactate dehydrogenase (LDH)/glucose dehydrogenase (GDH) cascade system [30]. These two amine donors were tested for their effect on substrate and product inhibition. While no major difference in substrate inhibition was found, the opposite was observed regarding product inhibition. The use of ALA worsened the inhibitory effect of both products to a critical point, with reactions rates dropping to zero at very small product concentration (3.3.3.2). However, has discussed earlier, it is possible that the equilibrium position is negatively influencing these results.

3.5. Conclusion

Inhibition is heavily present in the transaminase catalysed reactions. The chiral product competes with the ketone substrate (amine acceptor) for the catalytic centre, and the opposite happens in the reverse reaction. This, alongside with the unfavourable thermodynamic equilibrium, represents the major drawbacks to these reactions as it was here demonstrated. Protein engineering has the potential to alleviate the inhibition by substrates and/or products and broaden the operating space. With continuous improvements and with a rational screening methodology, improved mutants can be identified. The use of a screening methodology can help simplifying this process while allowing an efficient stepwise screening. In this study, a methodology was development and successfully demonstrated.

Despite the improvements that can be achieved by engineering the biocatalyst, this will never affect directly the thermodynamic equilibrium since it is a function of the substrates only. In such case, process engineering tools such as ISPR can help overcoming this, as it will be further discussed in Chapter 5.

The choice of the amine donor is of extreme importance, as it was role demonstrated. It affects not only the thermodynamic equilibrium, but also the inhibition profile. This was very clear with especially emphasis for the inhibition caused by the products (MBA and APB). When ALA was used as amine donor, the product inhibition was found to be more prominent, with the reaction rates reaching zero at very low product concentrations. This difference in product inhibition profile as function of the choice of amine donor has never been observed or discussed before in scientific literature.

The thermo stability of the selected mutant was however found to be slightly inferior in comparison to other mutants present in the initial library. Immobilisation of the biocatalyst can help to increase the stability but also allow the re-use of the biocatalyst as well as facilitate the DSP. This will be pursued in Chapter 4.

4. Immobilization of ω-Transaminase

Summary

In this chapter, the results regarding the experimental work carried out to screen and select suitable supports to immobilize ω-TAm enzymes are presented and discussed. A screening methodology was developed and applied to 20 synthetic macroporous carriers, representing 3 types of interactions with the enzymes: i) hydrophobic interactions, using octadecyl functional groups; ii) covalent bonding, using epoxy functional groups and iii) ionic interaction, using amine functional groups present on the surface of the resin. The influence of the following parameters on the immobilization efficiency were studied: particle and pore size, length of functional group and the immobilization mechanism.

Two types of ω-TAm enzymes were immobilized: a (*S*)-selective, developed and supplied by c-LEcta GmbH, Leipzig , Germany (hereafter designated as "S-TAm") and a (*R*)-selective, developed and supplied by DSM Innovative Synthesis BV, Geleen, Netherlands (hereafter designated as "R-TAm"). The immobilized biocatalysts were tested for their activity throughout many cycles of 24 hours under process conditions, as well as for their activity in non-conventional conditions such as: i) high temperature, which could potentially allow increasing the reaction rate and also the evaporation of volatile co-products such as acetone (to shift equilibrium); ii) reaction in organic solvents, which could allow reactions in pure solvents to increase the substrate and product solubility or to carry out 2 liquid-liquid phase systems as an ISPR/ISSS strategy. Suitable carriers were found following the screening methodology developed here. The selected carrier allowed retention of the enzyme activity over a long period of operation under process conditions, it allowed re-use of the enzyme for several cycles, and it increased the biocatalyst storage period at ambient temperature. The outcome of these results will assist during the process considerations chapter (Chapter 5).

4.1. Introduction

Documented attempts to immobilize enzymes date back as far as 1916, when Nelson and Griffin reported that an invertase immobilized on charcoal was still active after the immobilization process [100]. Since then, several immobilization techniques have been described in the scientific literature for different enzymes. However, only a few of these immobilized preparations have made their way towards industrial implementation [101]. Most of the immobilized enzymes used at industrial scale are glucose isomerase (immobilized on inorganic carriers) for production of fructose corn syrup, penicillin acylase (covalently attached to polyacrylate carriers) for production of semi-synthetic penicillins, lactase (immobilized on an ion exchange resin) for production of low-lactose milk, lipase from *Thermomyces lanuginosus* (immobilized on silica) for fat modification and lipase B from *Candida Antarctica* (immobilized on polyacrylate) for use in resolutions in the manufacture of pharmaceutical intermediates [23].

Main advantages of immobilization

- easy recovery and reuse
- improved activity
- improved operational stability
- improved storage stability
- possibility for continuous operation in packed bed reactors,
- minimization of protein contamination in the product

Box 4.1: Example of advantages offered by immobilization.

Considering the long list of advantages that the use of an immobilized biocatalyst offer compared to its free formulation (see Box 4.1), it would be expected that a large number of processes would be running using immobilized biocatalysts as well as a large number of commercially available immobilized enzymes. However, this is not the reality. Lipase is probably the most successful case of a commercially available immobilized enzyme which is supplied by several companies such as Novozymes A/S, Denmark (Novozym®435 and Lipozyme® TL IM), Roche Molecular Biochemicals, Germany (Chirazyme L-2), as well as similar preparations from SPRIN Technologies S.p.A (Italy), c-LEcta GmbH (Germany) and CLEA Technologies B.V (The Netherlands) [102].

A possible reason for this trend can be the lack of a general, and simple to use, method for immobilization that can be applied to any enzyme. Hence, a trial and error approach often is required whenever one wishes to utilize immobilized enzymes.

Furthermore, the loss in activity due to introduction of mass transfer limitation and loss of active enzymes by leaching or irreversible denaturation, which are commonly observed in immobilization of enzymes, can be a limiting factor. Another disadvantage which could limit the use of immobilized enzymes is the increase of the biocatalyst cost contribution in the production cost. However, the cost contribution of the immobilized enzyme for an implemented full scale process can potentially be lower than for a free enzyme, since the immobilized enzyme can be reused for many reaction cycles [32].

4.1.1. Immobilization of ω-TAm

For challenging processes, such as the synthesis of chiral amines using ω-TAm, where several process strategies need to be applied in order to achieve higher biocatalytic yield ($g_{product}/g_{biocatalyst}$), higher product concentration ($g_{product}/L$) and reaction yield (%), immobilizing the catalyst can be decisive for the process feasibility. As will be discussed in Chapter 5, some of the strategies necessary to shift equilibrium and overcome product inhibition makes use of external agents such as organic solvents, or polymeric resins which, in theory, can decrease the biocatalyst stability and activity or interfere with its availability in the reactor (by binding onto column resin for example). In both cases, immobilizing the biocatalyst can be advantageous, especially when operating with cell-free extract (CFE) or purified enzymes (PE) which are more exposed to media condition than when formulated as whole cells (WC). Moreover, the use of a immobilized biocatalyst can drastically reduce the costs associated with the biocatalyst and downstream processing, as immobilization would ease the separation of the biocatalyst from the products, and would also allow its re utilization for several cycles, increasing the biocatalytic yield (as discussed in Chapter 2).

There is a limited amount of scientific literature reporting the use of immobilized ω-TAm with focus on both CFE and WC. For instance, immobilized WC of ω-TAm by entrapment in calcium alginate beads has been applied by Shin and co-workers in the kinetic

resolution of chiral amines in a packed bed reactor [103]. The authors reported diffusional limitations and changes in substrate and product inhibition. In another study it was also reported that both Vmax and K_m changed when WC were immobilized in calcium alginate beads, indicating diffusional limitations [104]. This has been overcome in another unrelated work where WC and permeabilized cells have been immobilized by entrapment in PVA-gel (Lentikats®) with no diffusional limitations and with an immobilization efficiency of 100% (observed activity/activity immobilized) [105]. Attempts to immobilize *E. coli* WC by crosslinking with glutaraldehyde, or by entrapment with carrageenan and polyacrylamide were reported unsuccessful, with more than 50% of activity being lost in the case of crosslinking and entrapment with polyacrylamide, while the entrapment using carrageenan resulted in a mechanically unstable preparation [106]. In the same work, the authors reported immobilization of WC using hydrous titanium oxide (surface adsorption), calcium alginate (entrapment) and chitosan (by cell flocculation). The former was reported to show very poor loading capacity (less than 0.1 $g_{WC}/g_{carrier}$, dry weight) and consequently decreased immobilization yield, while the preparation made with calcium alginate had decreased activity due to mass transfer limitations already at small loading as 0.2 $g_{WC}/g_{carrier}$. This contrasted with the preparation made using chitosan which allowed loadings up to 3.2 g WC/g carrier (dry weight) and more than 60% residual activity.

Immobilization of CFE of ω-TAm has been achieved both by covalent linkage to different solid support materials and by entrapment in sol–gel matrices [107, 108] with reported immobilization yields of 20–50% protein (immobilized protein/total protein) and less than 20% of remaining activity [109]. Others works have reported low immobilization yields as well as poor residual activity (<50%) after immobilization by covalent attachment to carriers [110-112]. On the other hand, the same studies reported increased storage stability, often explained by the immobilization of the native proteases from the CFE which then become unable to degrade the ω-TAm.

Enzyme recycle studies have also been reported with immobilized transaminase enzymes. Preparation of ω-TAm immobilized on chitosan beads was reported to retain 77% activity after five reaction cycles of 8 hours each under the process conditions (25 mM MBA, 25 mM PYR, 0.1 M k-PBS and 1 mM PLP) however it was also susceptible to

severe substrate and product inhibition [109]. Cárdenas-Fernández and co-workers also reported 5 times re-use (in cycles of 3 hours) of ω-TAm WC with retention of 80% of initial activity [105]. Another study reports an improvement in the operational stability of the enzyme as result of the immobilization [108]. The enzyme was immobilized in sol–gel matrices and was reported to have a slightly improved activity at higher temperatures compared to free enzyme, although the activity at higher pH (9-11) was significantly inferior. Moreover, it was possible to re-use the preparation in 8 cycles of 24 hours each with a decrease of about 20% in the maximum conversion achievable.

In a recent work, Truppo and co-workers reported the development of an immobilized ω-TAm capable of operating in organic solvents (namely isopropyl acetate, isopropanol and toluene) [113]. However, it is not clear how much this is a result of the immobilization itself or a result of the prior improvements to the enzyme achieved through protein engineering techniques [31], as previously mentioned in Chapter 2. Nevertheless, the immobilized preparation allowed 10 times recycling in optimized reaction conditions, corresponding to ca. 200 hours of operation. While the stability can be associated with the protein engineering, the ability to filter, recover and re-use the biocatalyst is a feature that only immobilization makes possible.

4.1.2. Motivation for this work:

In some of the mentioned work, immobilization techniques using supports such as calcium alginate, hydrogels, hydrous titanium oxide and others, which require prior preparation are reported. Moreover, some of these compounds, or the preparation steps they are involved in, require special handling for being toxic or exothermic. This is the case of preparation of hydrous titanium oxide for instance, which is carried out by employing the exothermic reaction between hydrochloric acid (HCl) and titanium tetrachloride (TCl_4) [114]. This can be a disadvantage for large scale production of immobilized enzymes where the procedures should be quick, robust, scalable and reproducible, while preserving the enzyme activity [115]. Moreover, working environment issues such as the handling of chemicals and dust-producing materials should be considered at such scale [115]. In addition, the mechanical stability of the immobilized preparation needs to be considered when scaling up a reaction using

immobilized biocatalyst. The carrier should be resistant to mechanical forces, as this can limit their applicability [116]. These concerns suggest the need for a different immobilization solution which is easier to use while allowing reproducibility, retention of the enzyme activity and ultimately providing mechanical rigidity to the enzyme. Furthermore, a structured screening approach procedure for immobilization carriers featuring ω-TAm is lacking. This work addresses these issues and gives an economic evaluation of the immobilization using the selected carriers.

4.1.3. Overview of immobilization methods

Over the last century, several methods have been developed to immobilize both free enzymes and whole cells. Immobilization of enzymes (CFE) will be the focus of this study. The immobilization methods for enzymes can be basically organized in three main groups (as depicted in Figure 4.1): i) immobilization by binding to a solid support (carrier); ii) immobilization by cross-linking and; iii) immobilization by entrapment (encapsulation) [117].

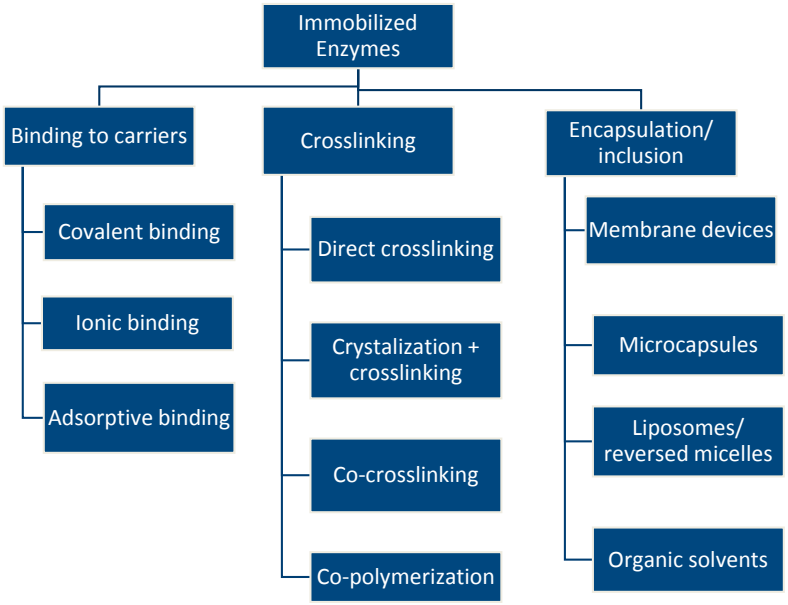


Figure 4.1 Classification of immobilization methods. Adapted from [117].

4.1.3.1. Binding to carriers:

Enzymes can be physically attached to supports through hydrophobic or van der Waals interactions, or chemically attached through ionic or covalent interactions. Physical interactions are however, generally too weak to keep the enzyme fixed to the carrier under industrial conditions (vigorous mixing, high reactant and product concentrations and high ionic strength), leading to enzyme leaching and consequently to a decrease in the activity. On the other hand, chemical interactions are stronger, being covalent interactions stronger than the ionic interactions, and thus significantly reduce or eliminate the risk of enzyme leaching from the support. One of the main drawbacks often associated with this type of immobilization is the high cost for the carriers, especially the ones of synthetic origin [118]. Nevertheless, the immobilization of enzymes using porous support such as polymeric resins, are free of intensive labour for carrier preparations, as these carriers are commercially available and ready to use. They are very versatile as it is possible to have different functional groups which can interact with enzymes in different manners as well as different particle and pore sizes which provide a wide range of possible immobilization mechanism for different enzymes at different operating conditions. Furthermore resins are mechanically stable supports, which allow the immobilized enzyme to be loaded in packed bed columns, for example [60, 117-119].

4.1.3.2. Cross-linking

When cross-linked, the enzymes are covalently bond using a di-functional agent such as glutaraldehyde. In this method the enzyme acts as its own carrier, without requiring a support, which can bring several economic benefits. The immobilization is achieved through generation of enzyme aggregates or crystals of enzymes add mix those with precipitants such as acetone, ammonium sulphate, ethanol or 1,2-dimethoxyethane, followed by addition of a crosslinker, commonly glutaraldehyde [60, 117-119]. This type of immobilization brings some disadvantages such as the time consuming and labour intensive procedure to achieve the immobilization, the often reported loss of activity due to chemical changes caused to the enzymes when crosslinking them, the impossibility of loading the biocatalyst in columns and finally the often reported activity loss upon recycling [120].

4.1.3.3. Entrapment (Encapsulation)

Enzymes (and whole cells) can also be immobilized by entrapment using a polymer network such as an organic polymer, a silica sol-gel, or a membrane device such as a hollow fibre or a microcapsule. Encapsulation is one of the best means of avoiding any negative influence on the enzyme structure. However, mass transfer limitations for the substrates diffusion are often observed in this type of immobilization [60, 117-119].

4.1.4. Selection of suitable supports for immobilization

Considering the multiple options for enzyme immobilization, as depicted in Figure 4.1, it can be difficult to select the most suitable method for an enzyme of interest. Trial and error approaches can be very labour intensive and time consuming, hence, guidelines and constraints are required to simplify the carrier selection procedure.

As discussed earlier, at full scale immobilization procedures should be quick and robust. It is also crucial to avoid the handling of dangerous chemicals and dust-producing materials which are often used in immobilization by entrapment and crosslinking. Finally, the immobilization is desired to provide an increase of mechanical stability to the catalyst. For this reason this work focuses on the immobilization of enzymes through binding to solid carriers.

Some guidelines for selection of solid carriers can be found in scientific literature focusing on the ideal carrier properties which appear to be suitable to most of the enzymes [60, 117]. These include the following list of guidelines:

- the driving force for enzyme-carrier binding interaction should be as mild as possible. A harsh condition (e.g: extreme pH or temperature) can lead to loss of activity due to change in the enzyme conformation or complete denaturation;
- the support should be highly porous so the enzymes and substrates can easily diffuse. Pore sizes of 10-100 nm appear to make the internal surface accessible for immobilization of most enzymes;
- the surface area should be large (>100 m^2g^{-1}) as it increases the number of functional groups per unit of space and possibly allowing higher enzyme loading;

- the carrier itself should be mechanically stable and insoluble under the reaction conditions in order to prevent both enzyme loss or/and contamination of product, especially if stirred tank reactors are used as the shear forces can destroy the carrier. Carriers stable in the temperatures ranges of 10-60 ˚C and pH of 5-10 should be suitable for most biocatalytic processes;
- the shape of the support is preferred to be spherical particles with very low swelling properties;
- the particle size should not be too small. This influences the type of filtration sieves required for biocatalyst separation when working on repeated batch mode in stirred tank reactors or using column reactors in batch and continuous modes, since small particles can result in high back pressures. Particles with diameter between 0.1 to 0.8 mm appear to be suitable for most enzymes;

These guidelines give already an excellent overview on the desired carrier properties which satisfy most of the enzymes. There are several commercially available supports which successfully fulfil these characteristics. They can be divided into those of inorganic and organic origin. Examples of inorganic origin supports are: Silica gel (commercially available under the names Spherosil®, Pall, USA or Aerosil®, Evonik Industries AG, Germany) and Organopolysiloxanes (commercially available as Deloxan®, Evonik Industries AG, Germany). Organic supports can be of natural occurrence, such as chitosan and dextran, or more mechanically stable and more commonly used, of synthetic origin such as polymeric resins. These are mainly made of polystyrene, polyacrylate, polyvinyls, polyamide and polypropylene. They are commercialized as purely adsorptive supports, as ion exchangers or with epoxy functional groups (allowing covalent bonding). These supports are commercialized by several companies, under different trade names such as Amberlite, Duolite and Dowex (Dow Chemicals. USA), Lewatit (Lanxess, Germany), Diaion, Sepabeads and Relyzmes (Resindion, Italy), Purolite ECR® (Purolite, USA), among others [60].

The work by Truppo and co-workers [92], featured ω-TAm immobilized on a macroporous hydrophobic support, Sepabeads EXE 120 (Mitsubishi, Japan). As mentioned before the preparation showed remarkable stability in organic solvent and good residual activity after 10 batch-cycles. This work opened a new door regarding the

immobilization of ω-TAm on rigid synthetic polymers. However, the carrier used is not commercially available and is protected by a patent application [121]. Therefore, a step-wise characterization of various commercially available polymeric supports (hereafter simply referred to as "resins") is required to screen and select a suitable carrier that can potentially be applied to ω-TAm.

4.2. Resins library

The resin library was built based on the guidelines discussed in section 4.1.3. The library comprised 20 commercially available macroporous polymeric resins composed by a rigid polymethacrylate polymer matrix (Figure 4.3). The library contained resins establishing in total 3 different types of interaction with the enzyme (Figure 4.2):

i. hydrophobic interactions using otcadecyl functional groups present on the surface of the resin;
ii. covalent bonding using epoxy functional groups present on the surface of the resin, and;
iii. ionic interaction using the amine functional groups present on the surface of the resin.

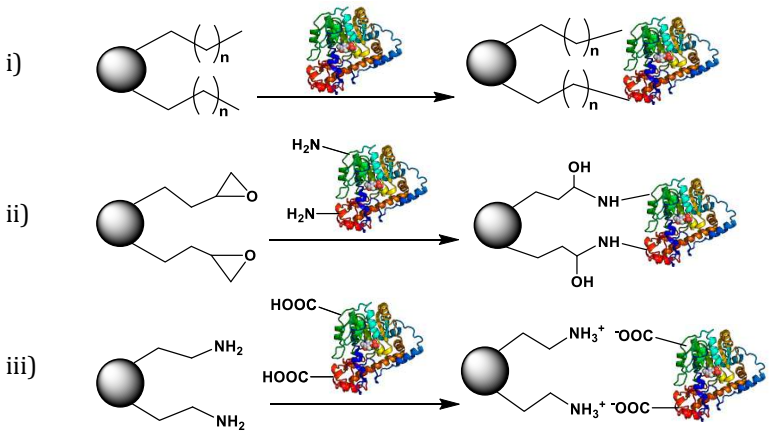


Figure 4.2: Immobilization mechanism. i) hydrophobic interactions using octadecyl functional groups; ii) covalent bonding using epoxy functional groups; iii) ionic interaction using the amine functional groups;

Besides the multiple options for immobilization mechanisms, the library was also comprised of resins with different functional group lengths as well as different bead and pore size grades in order to understand the effect of these properties on the immobilization of transaminase (Table 4.1).

The library was characterized with regard to loading capacity, activity retention, enzyme leaching, re-usability, solvent, temperature stability and storage stability. Based on these, a screening methodology was developed which is discussed in the following section.

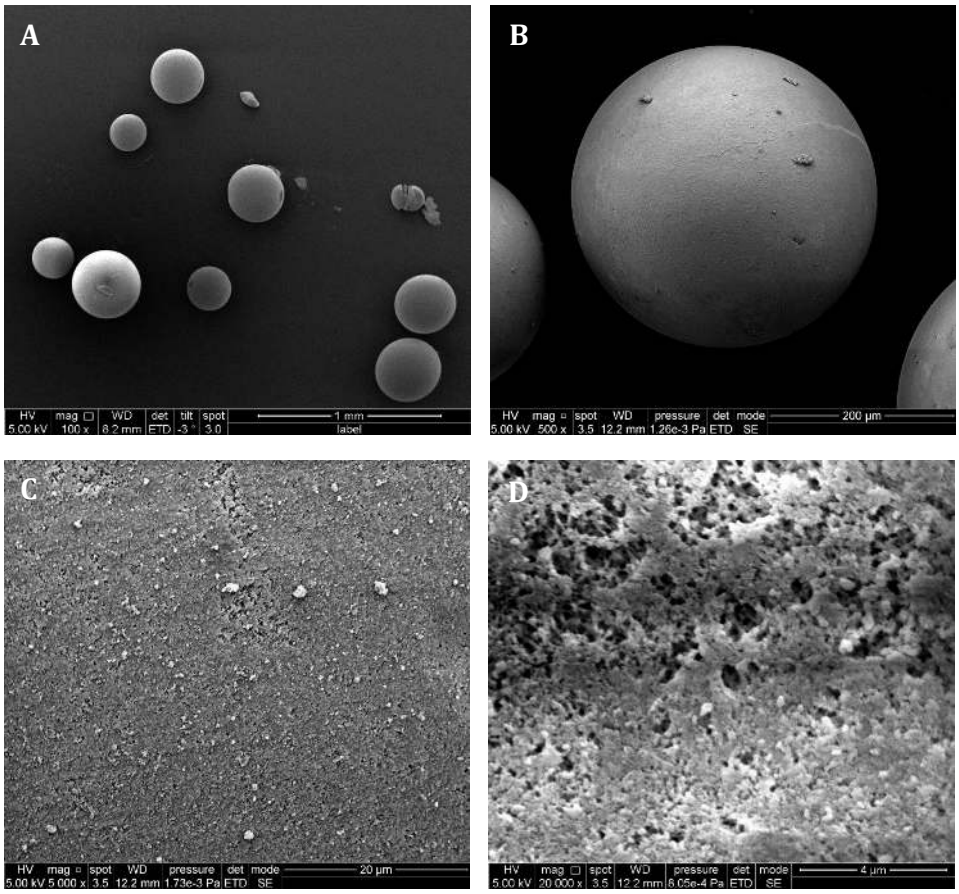
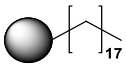
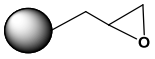
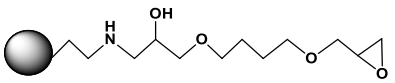
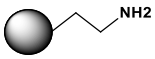
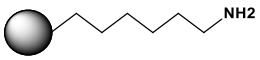


Figure 4.3 SEM pictures of macroporous polymeric resin (Relizyme OD403/m). (A = 100x magnification, B = 500x, C = 5000x and D = 20.000x)

Table 4.1: List of resins for enzyme immobilization and their properties. "*s*" grades correspond to bead sizes in the range of 0.1-0.3 mm and "*m*" grade to bead size in the range of 0.2-0.5. All the Relizymes have pore diameters between 40-60 nm and Sepabeads 10-20 nm. Carriers B and C are resins establishing hydrophobic interaction with the enzyme; D to G are resins establishing covalent bonding with the enzymes and H to K establish ionic interaction. Specfications according to the manufacturer (Resindion Srl, Italy)

	Structure and functional group	Resin ID	Resin name	Pore ⊘ (nm)	Beads ⊘ (mm)
Hydrophobic	 Octadecyl	B-s	Relizyme OD403/s	40-60	0.1-0.3
		B-m	Relizyme OD403/m		0.2-0.5
		C-s	Sepabeads EC-OD/s	10-20	0.1-0.3
		C-m	Sepabeads EC-OD/m		0.2-0.5
Covalent interaction	 Epoxy	D-s	Relizyme EP403/s	40-60	0.1-0.3
		D-m	Relizyme EP403 /m		0.2-0.5
		E-s	Sepabeads EC-EP /s	10-20	0.1-0.3
		E-m	Sepabeads EC-EP/m		0.2-0.5
	 Amino-Epoxy	F-s	Relizyme HFA403 /s	40-60	0.1-0.3
		F-m	Relizyme HFA403/m		0.2-0.5
		G-s	Sepabeads EC-HFA/s	10-20	0.1-0.3
		G-m	Sepabeads EC-HFA/m		0.2-0.5
Ionic interaction	 Ethylamino	H-s	Relizyme EA403/s	40-60	0.1-0.3
		H-m	Relizyme EA403/m		0.2-0.5
		I-s	Sepabeads EC-EA/s	10-20	0.1-0.3
		I-m	Sepabeads EC-EA/m		0.2-0.5
	 Hexaethylamino	J-s	Relizyme HA403/s	40-60	0.1-0.3
		J-m	Relizyme HA403/m		0.2-0.5
		K-s	Sepabeads EC-HA/s	10-20	0.1-0.3
		K-m	Sepabeads EC-HA/s		0.2-0.5

4.3. Screening methodology

Suitable supports for immobilization can be selected based on different factors, depending on the features desired. Often, the immobilization of enzymes is carried out to increase the enzyme stability, especially in non-conventional media (e.g. organic solvent, high pH or temperature), while in other cases the possibility of re-using the biocatalyst is more emphasized.

In a challenging process such as the synthesis of chiral amines using ω-TAm, where the biocatalyst contribution cost is presumably high, the main advantage that immobilization can bring is the possibility to re-use the biocatalyst [32]. Furthermore, in order to implement the process engineering strategies mentioned in Chapter 2, it would be an advantage if immobilization could also bring operational stability. With this in mind, a screening methodology was elaborated in order to select suitable supports to immobilize both (*S*)- and (*R*)-selective ω-TAm. The methodology is divided in 4 steps of characterization as described below and illustrated in Figure 4.4:

- Step I: Prior to any experimental work, the library should be screened according to the carrier stability (pH and temperature). These properties (carrier operational pH and temperature) should match the reaction conditions. Resins showing these properties lower than the reaction conditions are discarded;
- Step II: The resins are tested for: a) capacity to immobilize the enzyme, this can be evaluated through the loading capacity ($g_{Imm\text{-}CFE}/g_{resin}$) and/or immobilization yield ($g_{Imm\text{-}CFE}/g_{CFE}$ x 100 or $activity_{Imm\text{-}CFE}/activity_{CFE}$); and b) capacity to retain enzyme's activity ($activity_{Imm\text{-}CFE}/activity_{CFE}$). A threshold of 0.9 ($activity_{Imm\text{-}CFE}/activity_{CFE}$) was defined in this study in order to select only the most suitable resins. Preparations showing residual activity or immobilization yields <0.9 were discarded. The pre-selected resins are expected to have properties favourable to the enzyme, thus, other type (e.g. *S* or R-ω-TAm), or ω-TAm from other microorganism sources (with similar Mw) can be introduced in the screening at this stage;

- Step III: The pre-selected preparations are tested for their activity throughout several cycles in reaction conditions. In the current study, a total of 8 cycles were made and preparations showing residual activities ($activity_{Imm\text{-}CFE}/activity_{CFE}$) lower than 0.5 were discarded.
- Step IV: The suitable selected resins are tested for activity at high temperatures, activity in organic solvent and activity after a long storage period at room temperature.

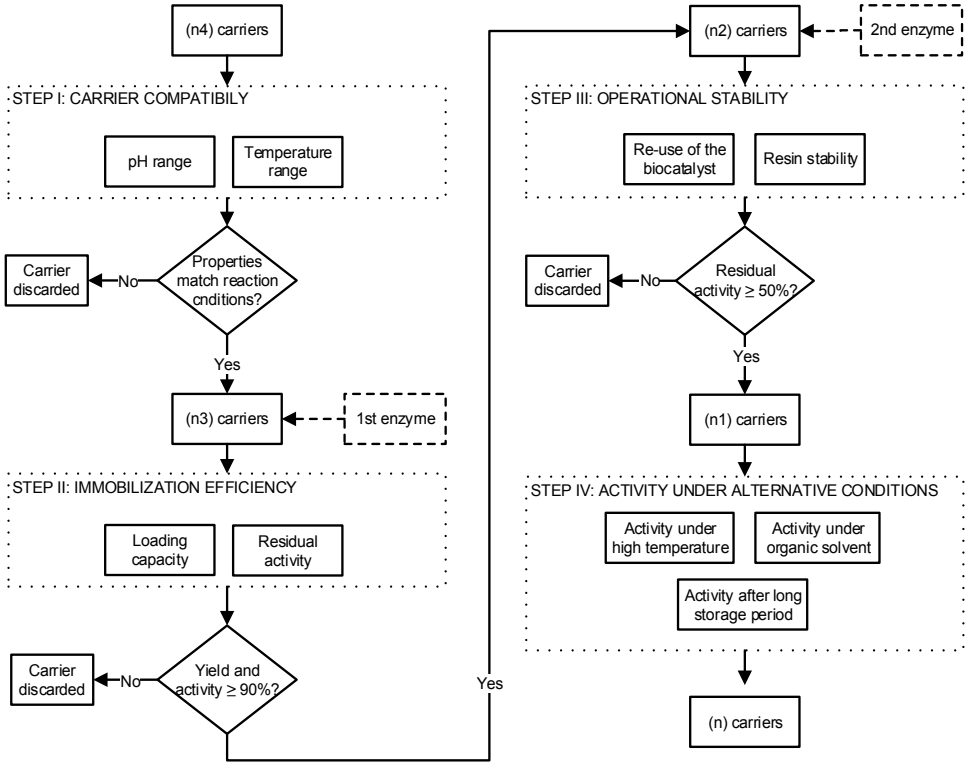


Figure 4.4: Screening diagram ($n_4>n_3>n_2>n_1$)

4.4. Materials and methods

All the resins were purchased from Resindion S.R.L (Milan, Italy). The chemicals were all obtained from Sigma-Aldrich (Buchs, Switzerland). The S-TAm, formulated as lyophilized cell-free extract, was purchased from c-LEcta GmbH (Leipzig, Germany) and the R-TAm (WC) was kindly supplied by DSM Innovative Synthesis (Geleen, The Netherlands). With exception for assays regarding temperature stability, all the experiments were run in duplicate.

4.4.1. Pre-treatment of S-TAm enzymes:

The enzymes were obtained already formulated as a lyophilized CFE powder. An enzyme solution of 5 gCFE/L (dry weight) was diluted in 100 mM k-PBS pH 7 containing 8 mM of PLP and 1 mL of this solution was added to the resins.

4.4.2. Pre-treatment of R-TAm enzymes:

The R-TAm was obtained as frozen centrifuged fermentation broth. The cells were re-suspended in 100 mM k-PBS pH 7 containing 8 mM of PLP solution (with a buffer/WC cell mass ratio of 2:1). The OD at 620 nm was measured to be approximately 0.3 (after 30 times dilution). The suspension was sonicated using a Sonics vibra-cell TM, sonicator Model"CV.18 9836A (USA), set for 10 minutes with 75% of amplitude and a pulse of 10. The OD was once again measured to be approximately 0.03 (after 30 times dilution). The sonicated was then centrifuged (Eppendorf Model 5415 R, Hamburg - Germany) for 10 minutes at 1300 rpm and the pellet was discarded. The supernatant was diluted with 100 mM k-PBS pH 7 containing 8 mM of PLP with a ratio 1:9. The final solution corresponded to approximately 7.9 gCFE/L (dry weight) when lyophilized.

Lyophilisation: The sample were frozen -80 ˚C overnight and lyophilised at -54 ˚C for 6 hours under vacuum ($10E^{-2}$ mBar) using a Heto LyoLab 3000 from Thermo Scientific (Massachusetts, USA).

4.4.3. Immobilization procedure:

The resins (50 mg for S-TAm and 100 mg for R-TAm) were washed with 1 mL 100 mM k-PBS pH 7 at room temperature. After 1 minute the washing buffer was discarded by means of a syringe with needle. Afterwards, 1 mL of the enzyme solution was added to the resins. The preparations were placed on an orbital shaking incubator (IKA ® KS, Model 130 Basic - Germany) at room temperature (25-27 ˚C) for 48 h at 400 rpm.

4.4.4. Activity assay:

The activity assay was carried out in 4 mL vials at 30 ˚C at approximately 400 rpm (IKA ® KS, Model 130 Basic - Germany). The reaction mixture (1 mL final volume) for S-TA was composed of 1 M IPA, 30 mM APH, 2 mM PLP and 100 mM k-PBS pH 7. For R-TA, the reaction mixture (2 mL) was composed of 50 mM DL-(-)-MBA, 10 mM BA, 2 mM PLP and 100 mM k-PBS pH 7. Samples (100 µL) were taken at hours 1.5, 3, 6 and 24, diluted into 400 µL of 1 M HCl and centrifuged for 10 minutes (Eppendorf Model 5415 R - Germany) at 1300 rpm prior to analysis.

4.4.5. Analytical:

All the samples were analysed by measuring the concentration of (S)-MBA (for reactions catalysed by S-TAm) and (R)-APB (for reactions catalysed by R-TAm) using HPLC (Agilent 1100 Series). The compounds were separated on a Prevail C18 250x4.6 mm, 5 µm column (Alltech Associates Inc., Illinois, USA) at a flow rate of 2 mL/min using a multi-step gradient flow of aqueous 100 mM Perchloric acid and pure acetonitrile, with the following percentage of acetonitrile: 0 min (0%), 1 min (10%), 2.5 min (10%), 5.9 min (60%), 6 min (0%), 7 min (0%). Compounds were detected at 210 nm. The quantitative analysis was performed based on peak areas from external standards.

4.4.6. Step I of screening – Carrier compatibility

The MSDS (material safety data sheet) obtained from the manufacturer was used to find the carrier proprieties. For all the resins establishing a covalent interactions with the enzymes (resins D, E, F and G from Table 4.1), the temperature and pH operational stability was found to be in the range of 0-10 ˚C and 5-8, respectively. For all the other carriers, the operational stability reported for temperature and pH is 2-60 ˚C and 1-14, respectively. Considering that most of ω-TAm catalysed reactions reported are run in temperatures between 25 ˚C and 60 ˚C and pH from 6 to 8, the covalent resins were considered not compatible with the process. Therefore they were discarded.

4.4.7. Step II of screening: Immobilization efficiency

After the immobilization, samples were taken from the supernatant and assayed for residual activity in order to estimate the amount of enzymes that did not bind to the carrier. The remaining supernatant was discarded and the resins were washed twice (for 2 minutes and 1h30 respectively) with 100 mM k-PBS pH 7.0 under mild agitation at room temperature (25-27 ˚C). Samples were taken from the supernatant after the second washing and analysed for activity in order to quantify the amount of enzyme that might have leaked out of the carrier (leakage quantification). The remaining washing buffer was discarded and reaction mixture was added to the resin and activity assay was carried out to estimate the residual activity in the immobilized. Free enzymes treated in the same conditions were used as reference.

4.4.8. Step III of screening: Operational stability

Both (S)- and (R)-selective ω-TAm immobilized, as previously described, were re-used for 8 cycles of 24 hours each (with exception for cycle number 5 which lasted 72 hours). Samples were taken at hours 1.5, 3, 6, and 24 or 72. After each cycle, the remaining reaction mixture was discarded and the resins were washed once with 1 mL of 100 mM k-PBS pH 7. The washing buffer was removed (by means of a syringe with a needle) and fresh reaction mixture was added to start the new cycle.

4.4.9. Step IV of screening: Activity under alternative conditions

4.4.9.1. Activity under high temperature

Free enzymes were incubated for 12 hours at 50 °C in 100 mM k-PBS pH7 solution containing 2 mM PLP. After the incubation, reaction mixture was added to the enzymes and the activity assay was initiated at 50 °C. The immobilized enzymes were incubated in the reaction mix used for the activity assay (as described in 4.4.4). After the incubation, the liquid was discarded and the immobilized enzymes were rinsed with 100 mM k-PBS pH7 solution before fresh reaction mixture was added to start the activity assay.

4.4.9.2. Activity under organic solvents

Lyophilized enzymes (5 mg for S-TAm and 7.9 mg fo R-TAm - dry weight) and immobilized enzymes (prepared as previously described and followed by a drying step with Nitrogen sweeping) were tested for activity in water saturated toluene, isopropyl acetate, cyclohexane and 50 % isopropanol. The solvents were saturated with equal volume of aqueous solution (100 mM k-PBS pH 7, containing 2 mM of PLP) for 72 hours under vigorous mixing to ensure proper saturation of both phases. The aqueous phase was discarded and IPA and APH (final concentrations of 1 M and 30 mM, respectively) were added to the saturated organic phase for the reaction using S-TAm, while DL-MBA and BA (final concentration of 50 mM and 10 mM respectively) were added to reactions using R-TAm. The activity assay was carried out in 4 mL vials at 30 ˚C at approximately 400 rpm (IKA ® KS, Model 130 Basic - Germany). Samples of 100 µL were taken at 1.5, 3, 6 and 24 hours, diluted in 400 µL acetonitrile, centrifuged and analysed by HPLC.

4.4.9.3. Activity after storage:

Free and immobilized enzymes (prepared as described in 4.4.1) were left in the fume hood for 20 days at room temperature (which oscillate between 25-27 °C). The free enzymes were prepared in 100 mM k-PBS pH 7.0, 8 mM PLP solution, while the immobilized enzymes were left in a semi wet state (the excess of immobilization buffer was removed by means of a syringe).

In addition, the immobilized enzymes previously used in Step III of characterization (section 4.4.8) were washed with 100 mM k-PBS pH 7.0, 8 mM PLP solution after the 8th cycle, the excess washing buffer was then removed by means of a syringe and the immobilized preparations were left in the fume hood (25-27 °C) for 60 days. After the resting period, the biocatalysts were added to the reaction mixtures and the activity assay was initiated.

4.5. Results and Discussion

4.5.1. Step I of screening – Carrier stability

Based on the MSDS files obtained from the manufacturer, it was observed that for all the resins establishing covalent interaction with enzyme (resins D, E, F and G), the temperature and pH operational stability was in the range of 0-10 ˚C and 5-8, respectively. For all the other carriers, the operational stability was in the range of 2-60 ˚C and 1-14, for temperature and pH respectively. Considering that most of ω-TAm catalysed reactions reported are run in temperatures between 25 and 60 ˚C and pH from 6 to 8, it was concluded that these resins were not compatible with the process conditions. This was experimentally confirmed in an assay where these resins dissolved in the reaction solution after 24 h of operation (data not shown). Resins B, C, H, I, J and K were selected for the next step of the screening.

4.5.2. Step II of screening – Immobilization efficiency

The immobilization yield ($activity_{Imm\text{-}CFE}/activity_{CFE}$), together with the loading capacity ($g_{Imm\text{-}CFE}/g_{Resin}$) of the carrier is of high importance, since low immobilization yield and low loading capacity is often translated into an economically infeasible immobilization process. Often, a reference loading capacity of 0.1 $g_{Imm\text{-}CFE}/g_{Resin}$ is used in scientific literature as ideal [60, 102].

In this step, these parameters were used to evaluate the performance of the different preparations and the results are summarized in Figure 4.5, and are discussed in the following sections.

4.5.2.1. Leakage quantification

For all the resins the results suggested no significant enzyme leakage after 1h30min washing with 100 mM k-PBS pH 7, since no activity was detected in the washing buffer (data not shown).

4.5.2.2. Diffusional limitations

No mass transfer limitations or diffusional effects were observed. For all the resins, the activity missing in the carrier (characterized by the low activity in the immobilized) was traced back in the supernatant (corresponded activity in the aqueous phase) as it can be observed in Figure 4.5. For some of the preparations, the biocatalytic activity appeared to be enhanced as result of the immobilization. This was observed, for instance, with carriers H, I, J and K where the sum of the activity in the supernatant and the activity in the immobilized exceed 100%.

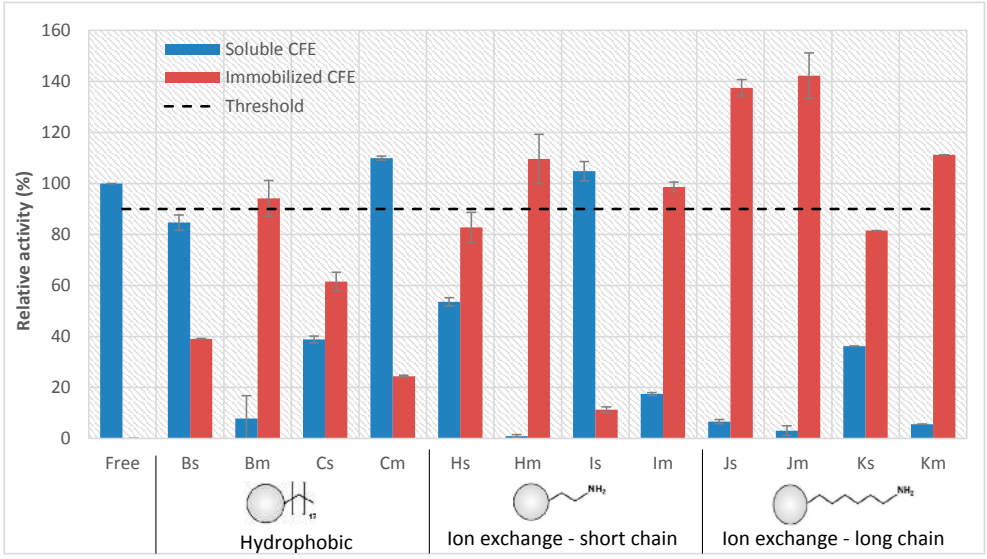


Figure 4.5 – Relative activity in the immobilized and in the supernatant. Blue bars - relative activity in the supernatant (not immobilized enzyme); Red bars - relative activity in the immobilized, dashed line – threshold for screening. Results based on initial rate measurements for supernatants and immobilized, in comparison with initial rate measurements for free enzyme.

4.5.2.3. Effect of functional group and its length on the immobilization yield:

The results obtained suggested better loading capacity for resins establishing ionic interactions with the enzyme in comparison with those establishing hydrophobic interactions. Within this group, the resins having long length functional groups appeared to have better loading capacity for the enzyme. This can be observed for the resins Jm, Js, Ks and Km which showed the highest activity in the immobilized (and also the least activity in the supernatant), while the resins showing short length functional groups

showed a slightly inferior loading activity in the immobilized and in average more activity in the supernatant (resins Hs, Hm, Is and Im). In theory this could be related to the obstruction of the enzyme's catalytic centre upon immobilization in case of short length functional group due to proximity of the enzyme to the carrier surface. This is often overcome by the use of spacers [60, 117, 120]. However, if this would have been the case, it would have resulted in low activity both in the supernatant and in the immobilized, since enzymes would be still loaded onto the carrier but not active due to a inaccessible catalytic centre or wrong conformation, and this was not observed. Hence, it can be hypothesized that there is an easier interaction of long length functional groups of the resin with groups on the enzyme as observed for the carriers J and K in comparison with H and I. Fs, Jm, Js, Fm and Gs (Figure 4.5). This was also observed for the covalent resins (data not shown), where the resins F and G (long chain functional group) showed better residual activity and loading capacity in comparison with resins D and E (short chain function group).

4.5.2.4. Effect of particle size on the immobilization yield:

The particle size also appear to have an effect on the immobilization yield as well, with the "m" grade carriers (0.2-0.5 nm mean particle size) showing in general higher residual activity hence loading capacity than the "s" grade (0.1-0.3 nm). This can be observed for all the resins (Figure 4.5). This is potentially due to the higher surface area, which also corresponds to higher functional group density.

4.5.2.5. Effect of pore size on the immobilization yield:

Finally, the results also indicate an effect of pore size on the immobilization yield. Resins showing larger pore size (40-60 nm) showed in general better immobilization yield than those having a smaller pore size (10-20 nm). For instance, immobilizations with carrier B (hydrophobic resins) showed higher immobilization yield than immobilization with C. The same is observed for H and I, and finally for J and K, respectively (Figure 4.5)

4.5.2.6. Summary of the results obtained in Step II

Considering these results, the influence of the different properties on the immobilization of ω-TAm can be organized as follow (based on immobilization yield):

- ***length of functional group:*** long > short;
- ***immobilization mechanism:*** covalent[1] > ionic > hydrophobic;
- ***particle size:*** 0.2-0.5 mm > 0.1-0.3 mm;
- ***pore diameter***: 40-60 nm > 10-20 nm.

This suggests the ideal carrier for immobilization of ω-TAm should be particles with mean diameter in the range of 0.2-0.5 mm, with long functional group chain and a pore diameter in the range of 40-60 nm. The larger particle size is convenient since it will influence the type of filters required to separate the biocatalyst in the downstream processing phase, as previously discussed.

Together with these criteria, a threshold of 90% for residual activity and immobilization yield was set and all the preparations showing lower residual activity were discarded. In summary, the following resins were selected for Step III: Bm, Hm, Im, Jm and Km. All these resins had a loading capacity comparable to the reference of 0.1 $g_{Imm\text{-}CFE}/g_{Resin}$ used in literature [60, 102], as can be observed in Table 4.2.

Table 4.2 Summary of residual activity ($activity_{Imm\text{-}CFE}/activity_{CFE}.100$) and loading capacity ($g_{Imm\text{-}CFE}/g_{Resin}$) for the pre-selected resins. The "$activity_{Imm\text{-}CFE}$" was obtained from the difference between the activity of free CFE and the activity in the supernatant (after immobilization).

Resin #	Residual activity (%)	Loading capacity ($g_{Imm\text{-}CFE}/g_{Resin}$)
Bm	92.2	0.09
Hm	99.0	0.10
Im	82.5	0.08
Jm	96.9	0.10
Km	94.4	0.09

[1] Although not shown here, immobilization was also carried out using covalent resins. Excellent immobilization yields were observed. However, after 24 hours under the reaction condition the preparation dissolved in the reaction mixture.

4.5.3. Step III of screening – Operational stability

Equally important as the loading capacity, the ability to re-use the biocatalyst is in most cases the key advantage pursued by industry with regard to immobilization. The five pre-selected resins from Step II were re-used for 8 cycles of 24 hours each under the reaction condition (with exception for cycle number 5 which lasted 72 hours instead of 24). In total the five resins were subjected to approximately 250 hours of operation (pH 7, and temperature of 30 ˚C).

A second ω-TAm (R-TAm, from DSM) was introduced at this stage of the screening. Although this enzyme was from a different organism, it was assumed that the five selected resins would have similar affinity to this new enzyme. Both initial rate and production over time were used to evaluate the biocatalysts performance. The results are presented in Figure 4.6 and Figure 4.7, for production over time and initial rate measurements experiments, respectively.

4.5.3.1. Immobilization yields:

The immobilization yield for S-TAm was similar or superior to the ones obtained in Step II (approximately 100% for all the selected 5 resins) while for R-TAm the immobilization yield were around 50% (Appendix 4B), despite the fact double amount of resins was used. On the other hand, the amount of R-TAm (CFE) use was superior to the one used for S-TAm (7.9 g/L in comparison with 5 g/L - dry weight), hence the protein content in the R-TAm solution immobilized can be expected to be superior. It is also possible that the expression level and the purification level in both cases are different since both enzymes were prepared by different laboratories, being the S-TAm supplied in a semi-purified formulation, which could explain the difference in immobilization yield.

4.5.3.2. Operational stability (biocatalyst re-cycle)

The results obtained for biocatalyst re-use for both S-TAm and R-TAm were evaluated by following the conversion over time for both enzymes throughout 8 cycles (Figure 4.6 A and Figure 4.6 B) and also by following the initial rate measurements for each cycle (Figure 4.7 A and Figure 4.7 B for S-TAm and R-TAm, respectively).

Based on the maximum possible conversions achieved at the end of each cycle, the preparations using the resins Bm, Jm and Km showed better performance among the 5 for S-TAm. For these 3 preparations, the maximum possible conversions (comparing to the free enzymes) were achieved at the end of all the cycles (Figure 4.6 A). For R-TAm, all the 5 preparations showed maximum possible conversion after 24 hours in all the cycles (Figure 4.6 B), suggesting an excellent activity retention despite the lower immobilization yield.

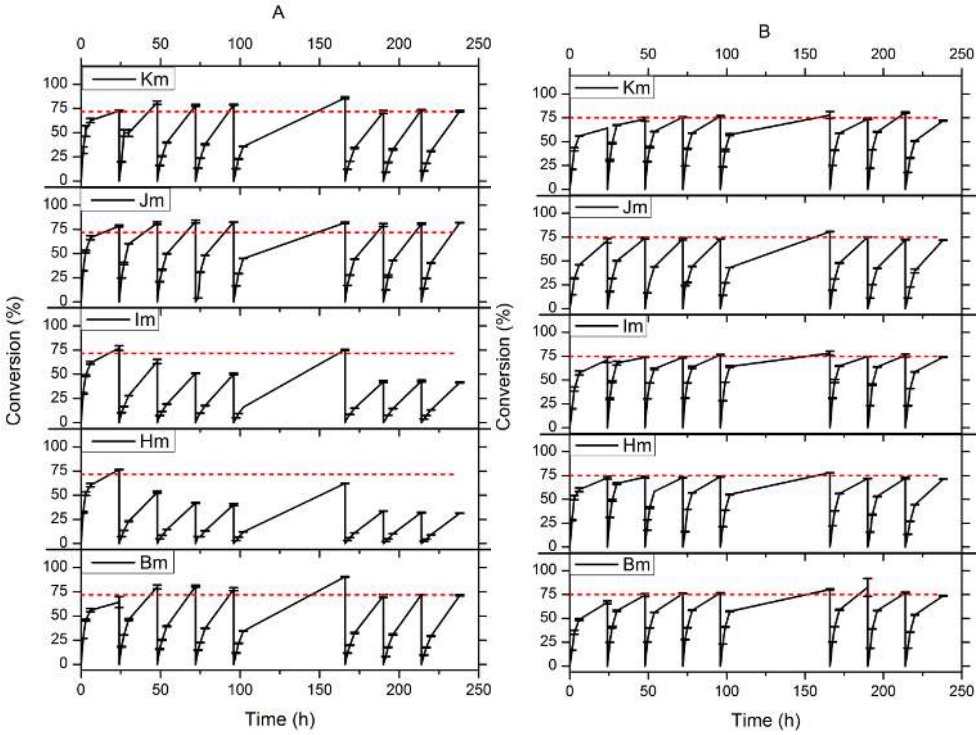


Figure 4.6: Conversion over time for the different cycles (A: using immobilized S-TAm; B: using immobilized R-TAm). Dashed line marks the maximum conversion after 24 hours achieved for free enzyme (used as reference) under similar conditions.

However, these results do not say much about the enzyme activity at each cycle and how much of the activity is lost. A different picture would be obtained if higher substrates concentration were used and if thermodynamic equilibrium and product inhibition were not limiting these reactions. In order to better evaluate the performance throughout the different cycles, initial rate measurements were plotted for both enzymes and are presented in Figure 4.7. As expected, due to the high immobilization yield achieved, all the preparations for S-TAm presented in the first cycle an activity which is comparable

to the free enzyme activity (illustrated by the dashed line in Figure 4.7 A). However, in the second cycle the activity decreases almost in 50% for all of the preparations and from this point the loss of activity for the following 7 cycles is not so accentuated, especially for the preparations using Bm, Jm and Km which kept 50-60% of the initial activity after the 8 cycles (about 250 hours of operation).

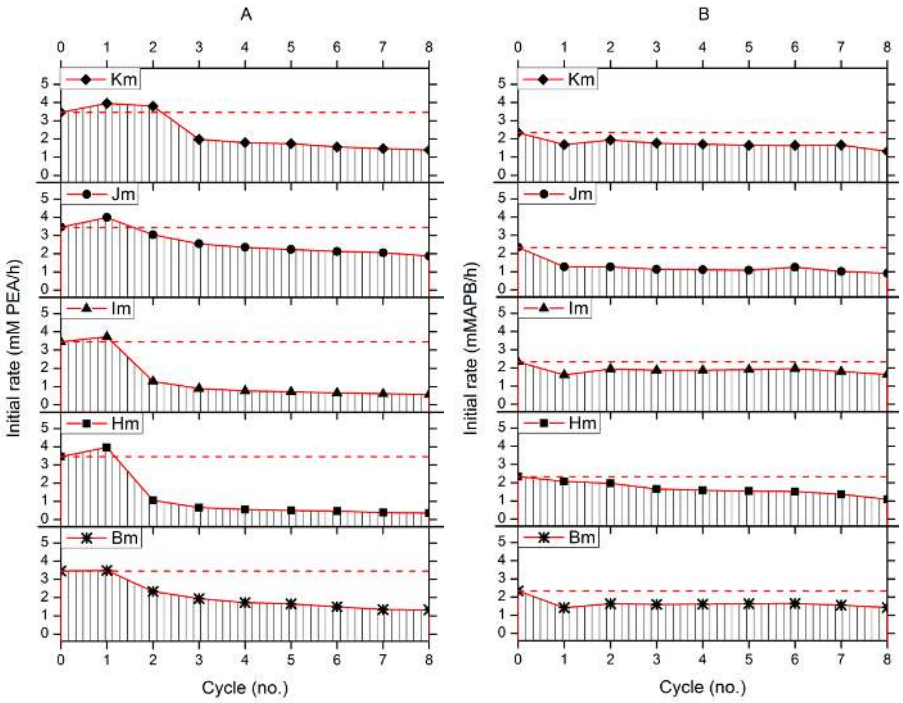


Figure 4.7: Initial rate measurements for different cycles. A: using immobilized S-TAm; B: using immobilized R-TAm). Dashed line marks the maximum conversion after 24 hours achieved for free enzyme (used as reference) under similar conditions.

Since protein quantification studies were not performed between the cycles, it is not clear whether the accentuated loss of activity from the first to the second cycle is due to enzyme leaching or deactivation. Enzyme leaching would not be expected to occur with carriers establishing ionic interactions, as previously discussed.

The results for initial rate measurements for R-TAm showed a better conservation of activity throughout the 8 cycles for all the preparations, with special emphasis to preparations featuring the resins Bm and Im which retained more than 90% of the activity (Figure 4.7 B).

Based on these results, the resin library was reduced to 3 carriers: Bm, which showed good activity retention for both enzymes; Jm and Im, which showed good performance for S-TAm and R-TAm, respectively.

4.5.4. Step IV of screening –Activity under alternative conditions

Stability is one of the improvements often used to justify the need for immobilization. Many authors reported enhancement of the biocatalyst stability as result of immobilization using many different enzymes reported [122, 123].

Temperature stability is one of the improvements which can be achieved through immobilizing the enzyme. A more thermo stable biocatalyst may open the possibility of carrying out reactions at higher temperatures, which can be translated into higher reaction rates and the possibility of new process options such as the evaporation of volatile co-products such as acetone in order to shift reaction equilibrium [32].

Furthermore, the activity in non-conventional conditions (or alternative conditions), such as in organic solvents, is often discussed and reported in several scientific literature [92, 122, 124]. As previously discussed, having biocatalyst which can operate in organic solvents can make possible the use of ISPR using liquid-liquid separation or reaction in pure organic phase which could bring advantages such as an increased substrate and product solubility [32].

Finally, the possibility to keep the immobilized preparation for long period of time can also be advantageous and it is equally widely discussed and pursued [117, 119, 122, 125, 126]. It can save time and allow immobilization of large amount of enzyme in advance for future utilization as well as it opening the possibility for commercialization of the immobilized enzyme.

In order to fully explore the improvements in the biocatalyst resulting from the immobilization, temperature, solvent and storage stability experiments were carried out to the preparations of S-TAm and R-TAm immobilized in the 3 pre-selected resins. The results are presented and discussed in the following sections.

4.5.4.1. Activity under high temperature

The immobilized enzymes were incubated for 24 hours at 50 °C and compared to free enzymes incubated at the same temperature but for 12 hours. Both were finally compared to the free enzymes incubated at 30 °C for 24 hours. The results illustrated in Figure 4.8 A and Figure 4.8 B for S-TAm and R-TAm respectively, show that free enzymes incubated at 50 °C lose about 80% and 100% of its activity (respectively). None of the immobilized preparations of S-TAm showed substantial improvements under the assay conditions (50 °C), when compared with the free enzyme treated under the same conditions. The best result for the S-TAm was obtained with the preparations of the resin Jm (long chain ionic exchange resin) which showed slightly higher residual activity in comparison to the free enzymes (Figure 4.8 A).

On the other hand, the results were more prominent for the R-TAm, which was found to retain 80% of its activity in the immobilization preparation of resin Bm (hydrophobic) and about 30% with the Im (short chain ionic exchange), in comparison with the 0% of residual activity measured in free enzymes (Figure 4.8 B). These encouraging results illustrate an enhanced stability at 50 °C, achieved as result of immobilization.

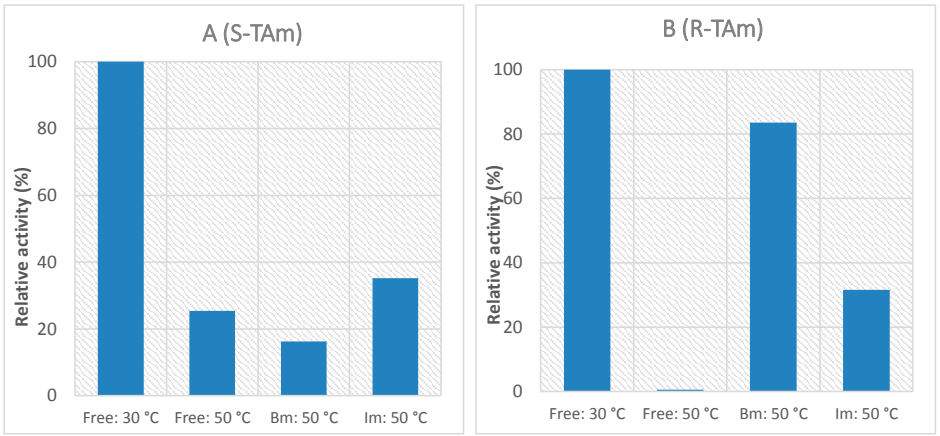


Figure 4.8: Temperature stability for S-TAm (A) and R-TAm preparations (B).

4.5.4.2. Activity under organic solvent

Free and immobilized enzymes were used to run reactions in water saturated organic solvents, both water-miscible and water-immiscible. Similar experiment have been previously reported with excellent results [92]. However, the results obtained here suggest that the biocatalyst activity in organic phase was not enhanced as result of immobilization. None of the immobilized preparations performed better than free enzymes in any of the solvents with exception for the assay performed under 50% isopropanol, where almost 5% of residual activity was found in the preparation of S-TAm immobilized with resin Jm (Figure 4.9). As previously mentioned, a good performance in organic solvent is related to the activity of the enzyme itself under the organic solvent. On the other hand, these results could also be related to a poor mass transfer of the substrate from the organic phase to the biocatalyst itself, or the lack of the cofactor (PLP) in the reaction mixture, since PLP is not soluble in organic phase. However, this possibility was not further experimentally explored.

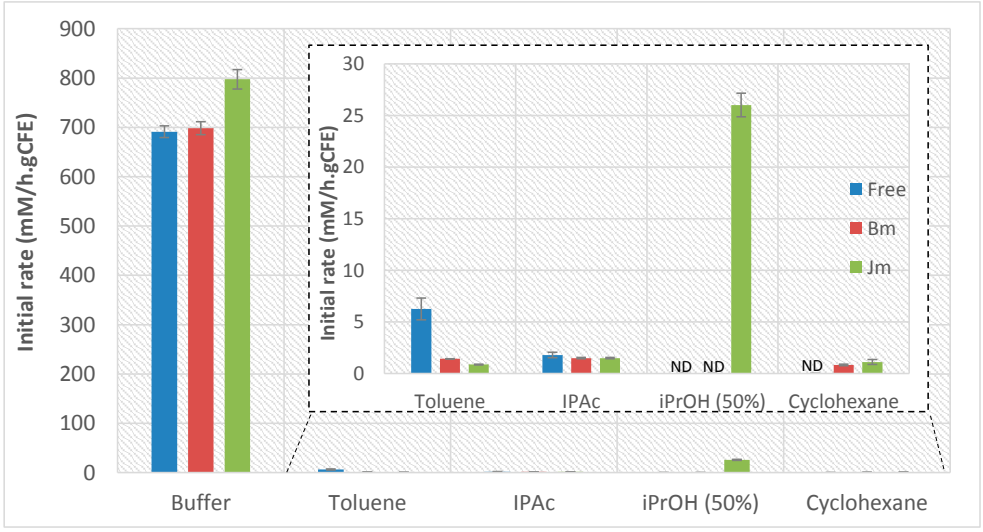


Figure 4.9: Activity of S-TAm under organic solvent. IPAc (isopropylacetate), iPrOH (isopropyl alcohol). ND – No activity detected.

4.5.4.3. Activity after storage period

The results for the storage stability presented in Figure 4.10 show an improved stability at room temperature for all the immobilized preparations, since these retained more activity than the free enzyme formulations of both S-TAm and R-TAm.

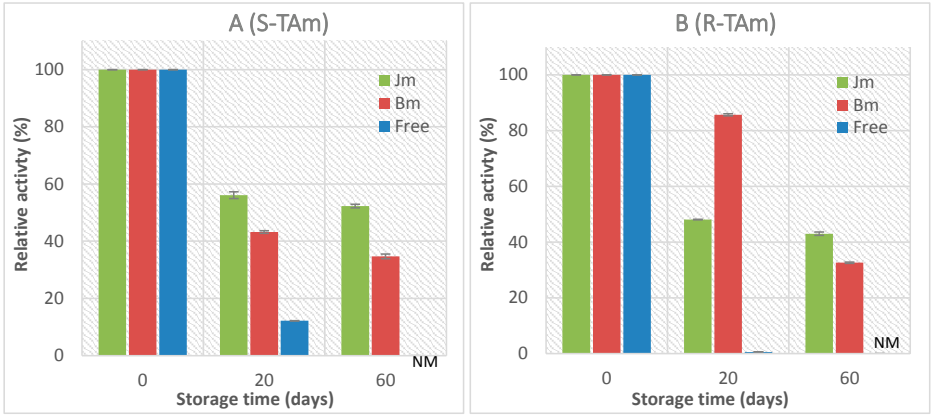


Figure 4.10: Relative activity after storage at room temperature for S-TAm (A) and R-TAm (B). NM - Not measured.

Similarly to what was observed for the operational stability experiment (section 4.5.3), the storage stability of the immobilized preparation also appears to slightly lose activity in the first cycles/days, stabilising from that point. Both immobilized enzymes (using both hydrophobic and ionic exchange resins) retained more activity after 20 and 60 days in comparison with their free formulations. In general, the ion exchange resins (Im and Jm) allow more activity retention than the hydrophobic resins (Bm) after 60 days. More than 50 % and 40% of residual activity was observed for S-TAm and R-TAm immobilized on the ionic exchange support, after 60 days. These results are more encouraging than those previously reported where losses of 30 % and 92% of activity of ω-TAm immobilized on chitosan and Eupergit® C, respectively, after 3.5 weeks (c.a. 24 days) under 4 °C were obtained [109]. The same study reports a possible benefit effect of the co-factor PLP on the enzyme stability over time. This was not investigated in this thesis.

The outcome of this experiment allows a better understanding of the period the immobilized preparations can be stored. A better activity retention is expected if the preparations were stored at 4-5 °C.

4.5.5. Immobilization costs:

The cost associated with the introduction of the immobilization step in the catalyst formulation (Figure 4.11) can be decisive for the success of the whole immobilization process, especially when using a synthetic carrier, where the costs are often reported as the highest for an immobilization carriers [20]. Factors such as the immobilization yield, the loading capacity of the carrier and the number of times that it is possible to re-use the preparation have a significant impact in the biocatalyst cost contribution to the process. In some biocatalytic processes the cost associated with the biocatalyst (in house production and formulation) can be about 35%, as described by Tuvfesson and co-workers [97] for lipase catalysed reactions. It can be expected that for ω-TAm catalysed reactions, this fraction could be even higher if one considers that the biocatalyst production costs for ω-TAm are expectedly higher than for lipase-catalysed reactions (since processes featuring ω-TAm are relatively new).

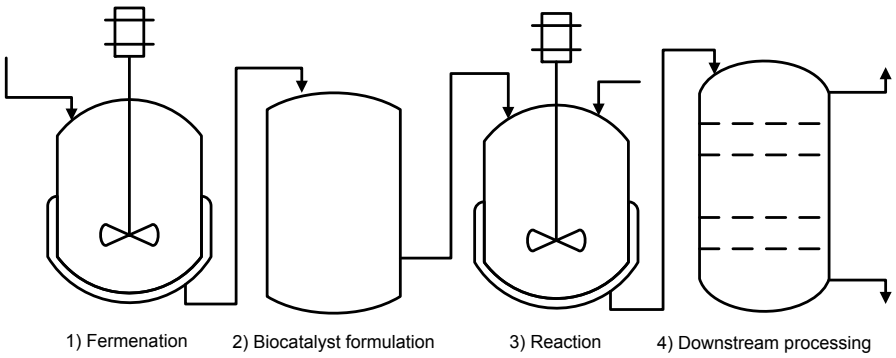


Figure 4.11: Overall Biocatalysis process scheme

In the next sections, the immobilization costs associated with the use of resins to immobilize ω-TAm will be discussed. The calculations were made with exclusive focus on the cost associated with the enzyme and carrier. Costs associated with capital expenses (e.g. equipment) and utilities (e.g. electricity), other raw materials or labour required to implement the process depicted in Figure 4.12 have not been considered.

Unless stated otherwise, all the calculations were based on the following process characteristics:

- Number of cycles/batches= 10
- Reaction volume: 10 L (pilot scale)
- Enzyme concentration in the process: 10 g_{CFE}/L (dry weight)
- Resin loading capacity = 0.1 g_{CFE}/g_{Resin} (assumed 100% immobilization yield)
- Enzyme cost: 100-1000 €/Kg

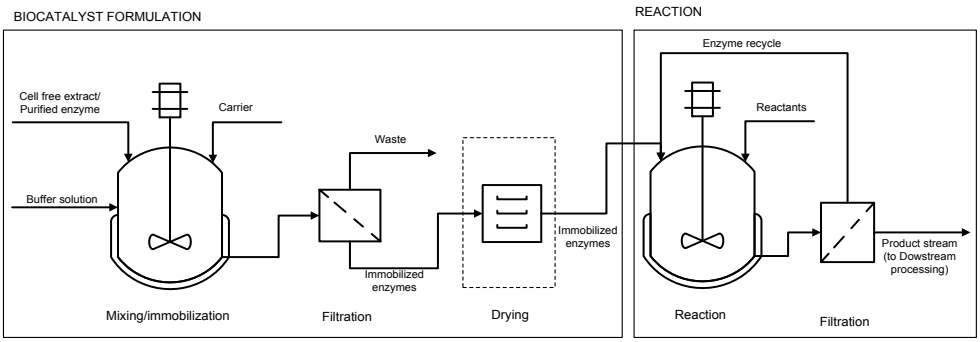


Figure 4.12: Overall immobilization process scheme.

4.5.5.1. Carrier cost:

The cost associated with the porous synthetic polymers to immobilize enzymes is reported to vary from 20 to 200 €/Kg [23]. As mentioned before, several manufacturers supply suitable similar carriers as the ones used here, and the prices vary from manufacturer to manufacturer. Since a rigorous investigation was not performed regarding to the price difference between them, it is difficult to fairly discuss about which supplier has the best offer. For this work, all the carriers were obtained from Resindion Slr. (Milan, Italy). The price of resins used in this work vary from 180 to approximately 720 €/Kg (full price presented in Appendix 4C). These prices were however calculated based on the manufacturer's price for 5 and 10 Kg packages, which, in theory, is enough to immobilize up to 1 Kg of enzyme (assuming a loading of 0.1 g_{CFE}/g_{Resin} considered in this study). It is reasonable to assume that purchase of larger amounts would significantly reduce the price per kilogram.

The type of immobilization mechanism established by the carrier has a significant impact on the price. According to the data collected (Appendix 4C), the covalent resins seem to be the most expensive, followed by the hydrophobic ones. Or another way of looking at this is to look at the length of the functional group: carriers with longer functional group chains are in general more costly than carriers having shorter ones. This can be observed among the covalent resins, for instance where F and G (long length functional groups) are in average 3 times more expensive than the resins D and E (short length functional groups). Also the particle size and pore size seems to have an influence on the price. The smaller the particles and/or pore size, the more expensive the resin becomes. Nevertheless, the resin costs can be disregarded if a high loading capacity or various number of cycles are possible, as will be discussed in the following sections. The 3 selected carriers (Bm, Im and Jm) will be used to perform the economic evaluation. The purchasing cost for these resins can be found in Table 4.3

Table 4.3: Resins prices. Calculated from 5 and 10 Kg packages obtained from the supplier homepage (http://www.resindion.com)

Resin	Resin name	Cost (€/kg)
B-m	Relizyme OD403/m	576
I-m	Sepabeads EC-EA/m	183
J-m	Relizyme HA403/m	221

4.5.5.2. Influence of enzyme production or purchase cost:

As mentioned in previous sections, immobilization can be a great advantage especially in processes where the cost of the biocatalyst represents a large part of the operating costs. This is illustrated in Figure 4.13 where the cost associated with the biocatalyst production/purchase required to run a 10 cycles in a 10 L scale was compared between free and immobilized enzymes. The real cost associated with production or purchase of large amount of biocatalyst is not clear as there is no consensus in the scientific literature. Different authors suggest different price ranges, nevertheless this often goes from 100€/kg up to 1000 €/Kg for CFE [32, 97, 102]. For this reason, this range has been chosen in this work to illustrate the effect of enzyme cost on the process operating costs.

The biocatalyst production or purchase costs is known to be scale depended (i.e. the larger is the production scale of the biocatalyst, the less is the production/purchase cost)

From the Figure 4.13, it clear that for a biocatalyst where production or purchase cost is about 100 €/Kg or less, the costs associated with the immobilization (using any of the three carriers) surpass the cost of using free enzymes (600 € and 200 € for preparations using the hydrophobic and ionic resins, respectively, in comparison with 100 € for the use of free enzymes). On the other hand, as the cost associated with the production or purchase of biocatalyst increases the more advantageous it becomes to immobilize it.

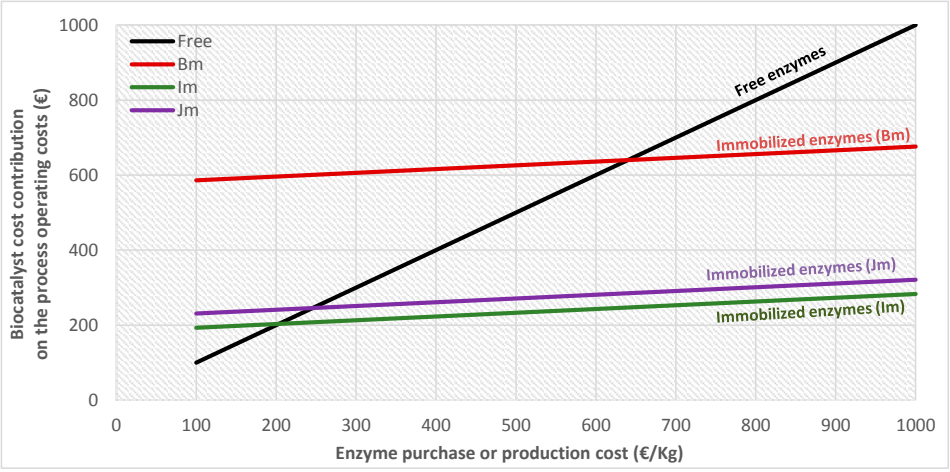


Figure 4.13 Biocatalyst cost contribution to the process operating costs (€).Calculations based on a 10 L scale reactor, an enzyme concentration of 10 g_{CFE}/L, carrier loading of 0.1 $g_{Imm\text{-}CFE}/g_{Resin}$ (assumed 100% immobilization yield) and 10 recycles.

This is mainly due to the high cost associated with the carrier which is more significant for the hydrophobic carrier (Bm). In cases where the enzyme production or purchase costs are low, the carrier has the greater cost contribution to the cost associated with preparing the immobilized biocatalyst. However, when the enzyme cost increases, it becomes more advantageous to immobilize the enzyme, since this will allow using the same preparation for 10 cycles and avoid using large amount of enzyme (which would multiply the costs by a factor of 10). This reinforces the advantage of using immobilized biocatalyst when operating with potentially expensive biocatalyst.

4.5.5.3. Influence of number or cycles achievable:

On the other hand, another way to justify the use of immobilized enzymes is to increase the number of cycles achieved with its immobilized formulation. This is demonstrated in Figure 4.14 where the enzyme purchase or production cost was varied from 100 to 1000 €/kg (x-axis) and the number of cycles it was possible to achieve was varied from 10 to 50. For a scenario where the enzyme cost is 100€/kg (the lowest here considered), at least 20 cycles are required in order to make immobilization economically attractive when using the ionic exchange resins (Im and Jm), and more than 50 recycles when considering the use of hydrophobic carrier (Bm)

As the enzyme cost increases, fewer number of cycles are required to justify the immobilization, as discusses in previous section.

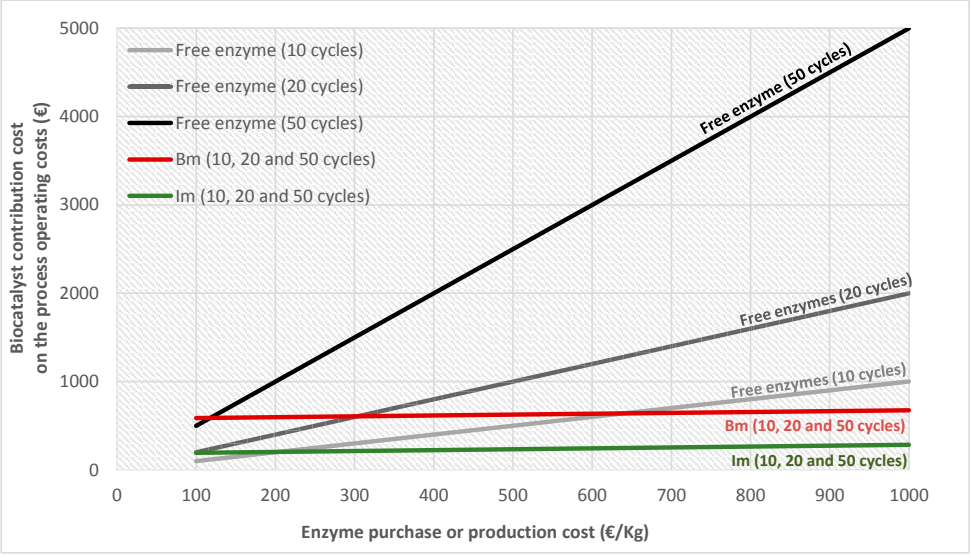


Figure 4.14: Cost associated with the biocatalyst in the process (y-axis) as function of the production or purchase cost of biocatalyst (x-axis) and the number of cycles. Calculations based on a 10 L scale reactor, an enzyme concentration of 10 g (DW)/L, carrier loading of 10 g resin/ g protein (assumed 100% immobilization yield) and 10 to 50 recycles.

4.5.6. Volume occupied by the preparation in the reactor:

Another important parameter that needs to be kept in mind when considering the use of immobilized biocatalysts, is the volume the immobilized preparation occupies in the reactor. An overloaded reactor can limit the stirring and consequently the mass transfer.

In order to illustrate the impact of the resin loading capacity on the reactor volume, a calculation was made and presented in the Figure 4.15, considering a 10 L reactor with an CFE concentration ranged from 10 to 60 g/L and a resin loading capacity of 0.1 $g_{Imm\text{-}CFE}/g_{Resin}$ and 0.2 $g_{Imm\text{-}CFE}/g_{Resin}$ (with 100% of theoretical immobilization yield) and a resin density of 1.1 g/mL (data obtained from the manufacturer MSDS files).

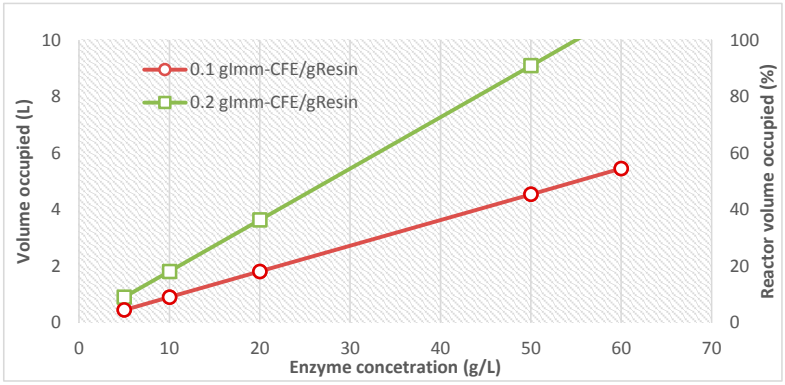


Figure 4.15: Influence of enzyme concentration and loading capacity of the resin in the volume occupied by the preparation in the reactor.

From Figure 4.15 it can be observed that higher enzyme concentrations in the reactor will require a higher amount of carrier, which will consequently occupy more space in the reactor. Higher enzyme concentrations can be an option for accelerating slow reaction rates. Furthermore, the need for a second or third enzyme such as in the case of enzymatic cascades reactions, used for instance to shift equilibrium [30, 32], can increase the amount of enzymes required in the vessel. In such case it would be desirable to use a carrier with a high loading capacity, which can potentially allow co-immobilization of enzymes. Another strategy that can help decrease the amount of carrier required is to immobilize purified enzymes instead of CFE. In addition, packed bed reactors (PBS) can be employed to solve the issues related with the volume occupied by the biocatalyst in the stirred tank reactor (STR).

4.6. General discussion

The immobilization of ω-TAm on macroporous polymeric resins was achieved with excellent activity retention (Figure 4.5). Physical properties of the carries were found to have a strong influence on the immobilization yield with carriers having longer functional groups showing better affinity to the enzyme than the ones having shorter functional groups. This is often related to obstruction of the catalytic centre of the enzyme when immobilization occurs too close to the resin surface or related to potential utilization of critical residues of amino acids in the enzyme (necessary for the catalytic activity). Often, spacers are introduced in order to increase the space between the enzyme and the carrier surface [60, 117, 120]. Regarding the particle size, it was observed that larger particle size resulted in higher immobilization yields.. Larger pore size (40-60 nm rather then 10-20 nm) seems to be preferred by the transaminase which is in the range of what has been established as the common pore size for most of enzymes (30-60 nm) [60, 117]. This gives a clear indication of properties required to immobilize ω-TAm from other sources with a similar molecular weight.

The screening methodology developed allowed selection of 3 suitable resins for immobilization of both (R)- and (S)-selective ω-TAm and enhancing their performance. The methodology is composed by 4 steps. In Step I, the carrier properties are evaluated and compared with the reaction conditions. Carriers showing operational stability lower than those required by the process (i.e. lower pH and temperature stability) were discarded. In this step, all the covalent carriers were rejected due to very poor mechanical and operational stability. These carriers were stable only at temperatures below 10 °C and dissolved in the reaction after 24 hours under process conditions (data not shown). As an alternative, carriers from another supplier showing better operational stability, such as the Purolite® ECR series (Purolite, UK), which are reportedly stable under stronger shear forces and able to sustain pH values up to 9 and temperatures up to 50 °C (according to the MSDS data file supplied by the manufacturer) could have been considered. Another possibility, would be to pre-activate the ionic exchange resins using glutaraldehyde, turning them into a covalent carrier, as demonstrated in several studies [120, 122, 126-128]. However, due to time restrictions neither approaches were tried.

Based on the constraints set for carrier selection from Step II to Step III (>90% immobilization yield), 5 resins were selected: Bm, Jm, Im Hm and Km. The resins were successfully re-used for 8 cycles of 24 hours in Step III. The results suggested that more cycles could have been achieved if the experiment had been allowed to proceed further (Figure 4.6 and Figure 4.7). This was more evident for the preparation of R-TAm which, despite the lower immobilization yield obtained, retained more than 90% of the initial activity after 250 hours in continuous operation. It is possible that these results could have been improved if a longer immobilization time would have been considered. Higher operational stability as result of a longer immobilization time has previously been reported [117]. This was however not tried in this work. From this Step, 3 resins were selected considering that they had more than 50% of residual activity after 8 cycles in operation (Bm, Im, and Jm).

In Step IV, the activity under alternative conditions and storage stability of both enzymes immobilized on the three pre-selected carriers were tested. Preparations were evaluated for activity after exposure to high temperature (50 ˚C), to organic solvents and to long resting periods at ambient temperature. The results for temperature stability revealed an excellent performance for preparations of R-TAm immobilized on the hydrophobic carrier (Bm), which had more than 80% of residual activity after 24 hours incubated at 50 ◦C, while no activity was found for free enzyme incubated for 12 at the same temperature. However, the same carrier did not show similar enhancement for S-TAm which retained only approximately 16% of its initial activity. It should be noted that this experiment was not a conventional temperature stability experiment, where the biocatalyst is incubated at the desired temperature and the activity assay is run at the optimum temperature. In this case both the incubation and the activity assay were performed at 50 °C. This has over challenged the biocatalysts and the carrier. Nevertheless, the results obtained suggested an improvement in stability achieved through immobilization. This trend was not observed for solvent stability experiments, where the results suggest a rather poor performance with all the preparations showing lower than 10% of residual activity, contrasting with previous work [92]. This could be explained by the fact that the enzymes used were not developed to operate in organic solvents, while in the cited work the biocatalyst used is known to be engineered to

operate under high substrate concentrations and organic solvents [31]. Furthermore, the mass transfer of the substrates from the organic phase to the biocatalyst could be reduced as the presence of water is crucial. Some authors have achieved good results with ω-TAm (of another origin) by adjusting the water activity in the reactor to 0.6 [129]. This guaranteed that only the minimum amount of water required was present in the reactor.

Another possible reason for the low performance in the solvent phase could be related to the lack of the co-factor PLP in the reaction mixture. Although the solvents were saturated in a buffer solution containing PLP, one should expect that a very small amount of it was present in the organic phase, since the water content in this phase was also very small, as shown in Appendix 4A, and considering that PLP is not soluble in the organic phase. This issue has not been discussed in any of the published works featuring reaction in organic solvents.

The results obtained for the activity after storage at room temperature show that the immobilized enzymes were able to be stored at room temperature (25-27 °C) for period of 20 days with 40-90% of its initial activity retained and 30-50% after 60 days (Figure 4.10). These results are among the highest reported for immobilized transaminase [103, 106, 111]. Results obtained with another enzyme (penicillin G amidase) showed an enhancement in the storage stability when enzymes were allowed to interact with the carrier for longer period of time[130]. This could be an option to improve the storage stability for ω-TAm. Furthermore, the cofactor seems to have a great influence on the enzyme stability [109]. An optimization of the co-factor concentration could have possibly improved these results.

It is important to consider the costs associated with the immobilization as this will be the decisive factor for the immobilization. The cost contribution from the immobilization is related to the number of cycles that is possible to achieve with the preparation (the more cycles achieved the less the impact of the carrier cost on the immobilization), as well as the cost associated with the production or purchase of the enzyme.

4.7. Conclusions

Immobilization of ω-TAm by adsorption onto rigid supports was achieved in this chapter. It was demonstrated that immobilization granted an improved performance of both S-TAm and R-TAm. The immobilized preparation showed great performance at high temperature, as well as an increased storage stability and good retention of activity after 8 cycles of 24 hours in reaction conditions, corresponding to about 250 hours of operation.

A screening methodology was developed and successfully demonstrated for the screening and selection of suitable carriers to immobilize ω-TAm. With this methodology three carries were successfully selected and used in reaction conditions. This work demonstrates the first documented step-wise screening of commercially available enzyme carriers for ω-TAm following a screening methodology, and the first documented attempt to immobilize (*R*)-selective ω-TAm.

In summary, with respect to the immobilization of ω-TAm CFE using macroporous polymeric resins, it can be concluded that:

- the ideal resin for immobilization of ω-TAm should be particles with diameter in the range of 0.2-06 mm or more, with a pore diameter in the range of 40-60 nm and preferably with long chain functional groups;
- the selected resins allowed a loading capacity of approximately 0.1 g_{CFE}/g_{Resin};
- the selected resins allowed the re-use of the enzyme for 8 cycles of 24 hours each, corresponding to c.a. 250 hours of continuous operation, with more than 90% of the initial activity being retained at the end (for the best case, R-TAm);
- the immobilization of enzymes using the selected resins allowed the retention of c.a. 90% of the initial activity after a 24 hours incubation at 50 °C (R-TAm immobilized on hydrophobic carrier), while the free formulation treated under similar conditions showed no activity;
- the immobilization did not enhance the performance of the enzymes in organic solvents;
- the immobilization improved the storage stability of the biocatalyst.

5. Process Considerations

Summary

ω-Transaminases are strongly affected by product inhibition and unfavourable thermodynamic equilibrium, as demonstrated in Chapter 3. This, combined with the low water solubility of the substrate often reported, makes the process development for this enzyme a real challenge. As discussed in Chapter 2 and 3, protein engineering has the potential to solve some of these issues, however, in some cases process engineering tools also need to be implemented. Many strategies to overcome these issues can be found in the scientific literature. In this chapter these strategies are reviewed. Their potentials and limitations are discussed. These tools include, for instance, the use of *in situ* substrate supply (ISSS) and product removal (ISPR) to respectively control substrate and product toxicity and the use of substrate excess and *in situ* co-product removal (IScPR) to shift the equilibrium in favour of product formation and also biocatalyst immobilization to improve stability.

5.1. Introduction

Over the last few decades, several strategies have been suggested and implemented in biocatalysis with the objective of increasing the stability, activity and productivity. Contrasting with the enzyme development strategies (such as recombinant DNA technology, directed evolution or random mutagenesis, which were discussed in Chapter 2), process engineering tools do not seek alter the structure of the biocatalyst. Instead, these strategies manipulate the media conditions or reactor configuration, for example, in order to attain higher process or biocatalytic performance. As discussed in Chapter 1, these strategies are divided as follows:

i. **reaction engineering strategies:** which, in the context of ω-TAm, it would consider the change of amine donors or the use of amine donor excess to make the process more thermodynamically favoured;
ii. **process engineering strategies:** which would include strategies such the use of an auxiliary phase (solid, liquid or gas) to deal with equilibrium issues and/or product inhibition), and also immobilization of the biocatalyst to improve stability and allow its re-use, and;
iii. **reactor engineering strategies:** which considers, for instance, the use of fed-batch reactors to alleviate substrate inhibition or low-water solubility issues, the use of membrane reactors to decrease product contamination, or new reactor designs to accommodate process engineering strategies.

Biocatalyst stability:
- Immobilization
- Reaction design

Product inhibition:
- *In situ* product removal

Substrate inhibition
- Fed-batch
- *In situ* substrate supply

Unfavourable equilibrium
- Excess of one substrate
- *In situ* product removal
- *In situ* co-product removal

Low substrate solubility
- Water miscible solvents
- *In situ* substrate supply
- Fed-batch

Enzyme separation/re-use
- Membrane reactor
- Immobilization

Box 5.1 Process engineering strategies used in biocatalysis

For simplicity, these three strategies will be treated as one in this thesis and designated as "process engineering" strategies. Some of these strategies have already been introduced in the previous chapters. These include the immobilization of the biocatalyst to improve stability and allow re-use of catalyst, the use of *in situ* substrate supply (ISSS) and product removal (ISPR) strategies to respectively control substrate and product toxicity and the use of substrate excess and *in situ* co-product removal (IScPR) to shift the equilibrium in favour of products formation. ISSS strategies have the advantage of both alleviating substrate inhibition as well as solving the problem of low substrate solubility, as will be discussed in the coming sections, as well as ISPR which besides alleviating product inhibition also allows shifting the equilibrium. A list including the most common strategies used for different limitations can be found in Box 5.1.

In this chapter, these strategies will be tested and discussed with the main focus on product inhibition and equilibrium shifting strategies. These can be divided into two groups: (i) those that both alleviate the product inhibition and shift the equilibrium, such as using water-immiscible organic solvents or polymeric resins to remove the inhibitory product as soon as it is formed (ISPR) and (ii) those that only shift the equilibrium such as the evaporation of co-product or its selective conversion through enzymatic cascades to a non-inhibitory compound (IScPR) (Table 5.1).

Table 5.1: List of common methods for *in situ* product and co-product removal in transamination.

	Product removal (adsorption onto polymeric resins)	**Product removal (extraction with organic solvent)**	**Co-product removal (evaporation)**	**Co-product removal (enzymatic cascades reactions)**
Alleviate product inhibition	✓	✓	×	×
Shift equilibrium	✓	✓	✓	✓
Property used	Ionic or hydrophobic interactions	Hydrophobicity	Volatility, vapour pressure	Functional groups
Methods	Adsorption onto a water-insoluble polymeric carrier	Extraction with water-immiscible solvent	Distillation, vacuum, gas stripping, or evaporation	Selective chemical or biocatalytic reactions
Advantages/ Limitations	Low selectivity, (hydrophobic resins), may require immobilized biocatalyst	Low selectivity, hard to find suitable non-toxic solvent, may require immobilized biocatalyst	Not selective, applicable to limited number of compounds, can harm biocatalyst	Very selective, Applicable to limited number of compounds
Improvements/ Reference	Conv. improved Truppo et al. (2010)[30]	Reaction rate improved Shin and Kim (1997)[50]	Yield and *e.e* improved Yun et al. (2004)[131]	Yield improved Shin and Kim (1999)[33]

5.1.1. Thermodynamic equilibrium

The transamination reaction is reversible and the maximum achievable conversion is thus determined by the initial concentrations and the thermodynamic equilibrium constant (Keq) of the reaction. Keq 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 [132]. However, for the transfer of an amine group from an amino acid to acetophenone (APH) for instance, the equilibrium is strongly in the favour of the amine donor. For instance, a Keq of about 10^{-3} has been reported for the synthesis of methylbenzylamine (MBA) using IPA as the amine donor, based on experimental determination and parameter estimation from kinetics results [53, 76]. This becomes even more critical when ALA, for example, is used as the amine donor (as discussed in Chapter 2). Truppo and co-workers have reported that the transamination of APH with 10 equivalents of L-ALA was completed at 3% conversion, as opposed to the theoretical equilibrium conversion of 9% [34]. In a recent publication, experimental values of Keq for several ω-TAm catalysed reactions have been reported [53]. 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 (Gibbs free energy) allows a more intelligent process design.

5.1.1.1. Determination of equilibrium constant of reaction (Keq)

There are several methods to estimate or experimentally calculate the equilibrium constants in a reaction. A small overview is given in the following sections.

5.1.1.1.1. Kinetic parameters estimation:

The equilibrium constant can be calculated through estimation of the kinetic parameters of a reaction (Chapter 2). By knowing the various reaction parameters (Km, Vmax and

Ki), one can calculate the equilibrium constant using the Haldane relationship (equation 4.1) [43, 76]:

$$K_{EQ} = \left(\frac{K_{cat}^{f}}{K_{cat}^{r}}\right)^{2} \cdot \frac{K_M^P K_M^Q}{K_M^A K_M^B} = \left(\frac{K_{cat}^{f}}{K_{cat}^{r}}\right) \cdot \frac{K_M^Q K_i^P}{K_M^B K_i^A} = \left(\frac{K_{cat}^{f}}{K_{cat}^{r}}\right) \cdot \frac{K_M^P K_i^Q}{K_M^A K_i^B} = \frac{K_i^P K_i^Q}{K_i^A K_i^B} \quad (4.1)$$

where K_{cat}^{f} and K_{cat}^{r} are catalytic turnover of the reaction; K_M^A, K_M^B, K_M^Q and K_M^P are the Michaelis parameters for amine donor, amine acceptor, amine product and the ketone co-product, respectively; and K_i^A and K_i^Q are the inhibition parameters for the amine donor and amine product respectively. The determination of Keq constant is however affected by errors in the parameter estimation. If the parameters are miscalculated, the accumulated error will eventually be represented in the estimated equilibrium constant.

5.1.1.1.2. Group contribution:

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 (c.a. 8.37 kj/mol) [133]. The method consist of estimating the $\Delta_r G$ (Gibbs energy of reaction) based on the molecular structures of the compounds involved in the reaction. Hence, the molecular structure of a single compound is decomposed into a set of smaller molecular substructures based on the hypothesis that $\Delta_r G$ and $\Delta_f G$ (Gibbs energy of formation) can be estimated using a linear model where each model parameter is associated with one of the constituent molecular substructures (or groups) that combine to form the compound. 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 favourable for the transamination of APH [134].

5.1.1.1.3. Experimental determination:

A more conventional method for the estimation of equilibrium constants is to allow reactants to reach equilibrium from both directions of the reaction [135, 136]. However this approach was argued by Tufvesson et al (2012) to be difficult to reproduce due to slow reaction rates and the occurrence of phenomena, such as degradation or evaporation of the reactants [53].

The authors suggest an alternative approach which is a modification of the former, consisting of observing the reaction rate using varying compositions of the reactants and products to find the point where the forward and reverse reactions converge to give a zero net reaction. This was done by calculating the reaction quotient Q (Equation 4.2) over a time period long enough to allow the reaction to occur (normally between 1 and 4 h), for mixtures of reactants and products at different concentrations.

$$Q = \frac{[Q]\times[P]}{[A]\times[B]} \tag{4.2}$$

where [A], [B], [Q], and [P] correspond to the concentration of the reactants. Then, Q_t/Q_0 is plotted against Q_0 and the equilibrium constant is obtained from a power curve fitting by calculating the value of Q_0 corresponding to $Q_t/Q_0 = 1$ [53].

5.1.2. Strategies to shift the thermodynamic equilibrium

In order to overcome the thermodynamic limitations in transaminase catalysed reactions there are several solutions that have been shown to (at least) partly overcome these: i) addition of excess of amine donor, ii) application of ISPR using an auxiliary phase such as organic solvents or polymeric resins, ii) auto-degradation of the product and use of enzymatic cascades or whole-cell catalysis. These will be further explained in the coming sections.

5.1.2.1. Addition of excess of 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 IPA [31]. However, the use of this strategy is limited to those cases when the equilibrium is only slightly unfavourable. In fact, from the Savile article it can be extrapolated that the Keq 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 $g_{product}$/L), there will be an upper limitation to 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. In Figure 5.1 the necessary excess of amine donor required to achieve a yield of 90% at varying Keq values is plotted. As can be seen, to achieve a yield of 90% an excess of 100-fold is required if the Keq value is 10^{-1}. Similarly, if the Keq value is 10^{-3} (which is the case of production of MBA using IPA as amine donor), an excess of 10,000-fold would be required, which for obvious reasons is unrealistic. As a consequence, for transaminations where Keq 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.

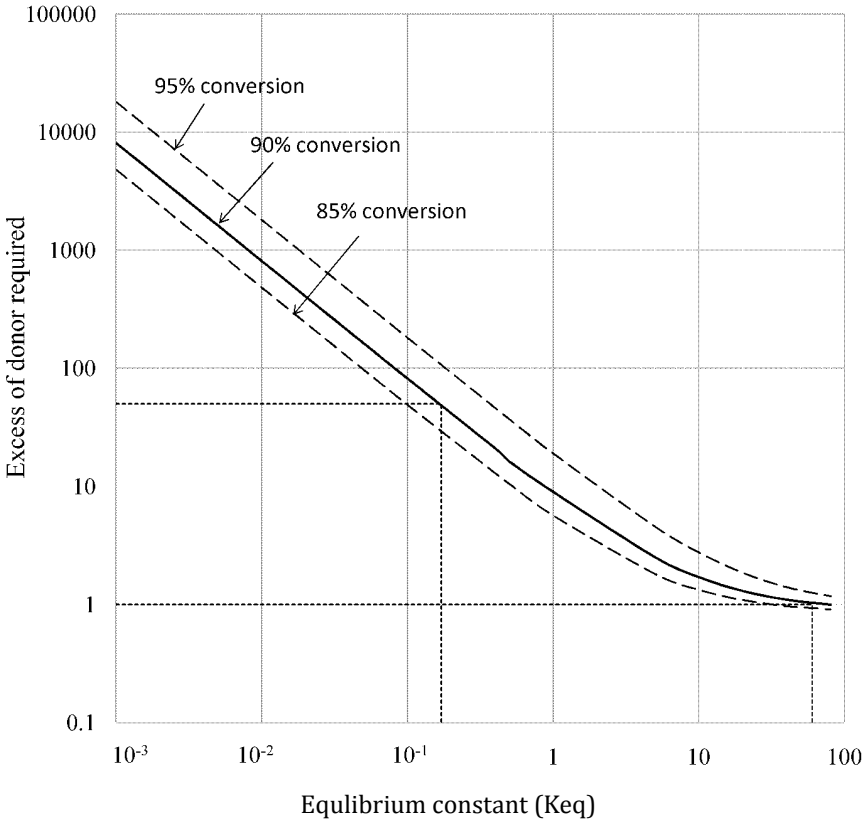


Figure 5.1 The equilibrium constant (Keq) determines the excess of amine donor required to reach a thermodynamic equilibrium of 90% (solid line). The broken lines are visual support for an excess of 1 and 50, which can be considered process boundaries. Adapted from [32]

5.1.2.2. Removal of (co-) product (ISPR and IScPR) to an auxiliary phase

A second method to shift the equilibrium position in favour 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) and co-product removal (IScPR). Again, the equilibrium constant of the reaction determines how low a concentration of product or co-product is required to achieve the target yield. Figure 5.2 shows the relationship between Keq 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 it can be seen, at Keq values $<10^{-3}$ the required co-product concentration will need to be <1 mM.

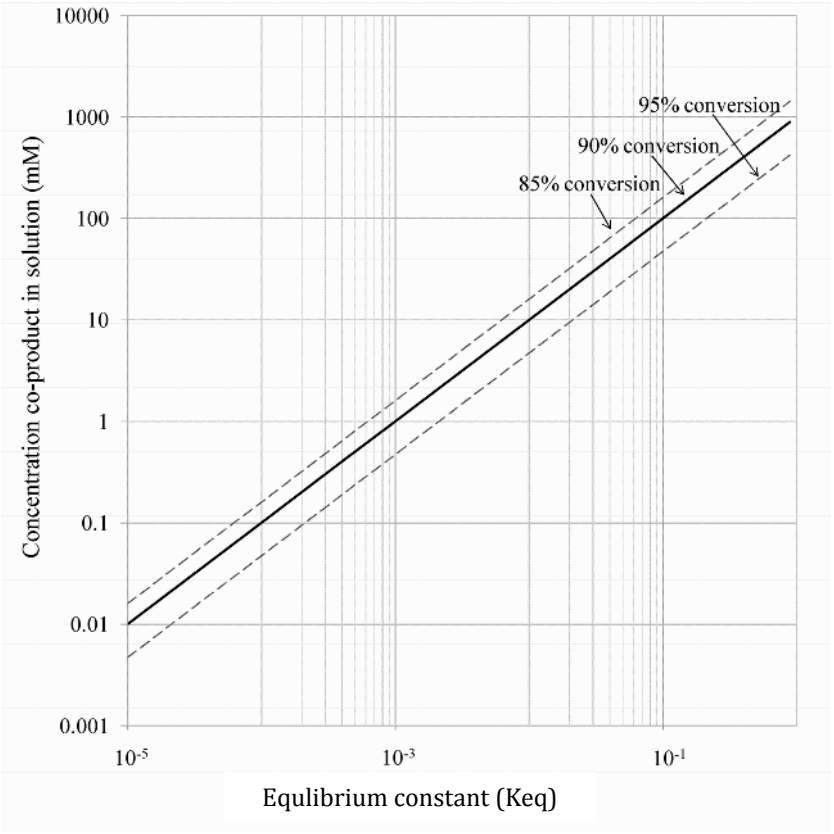


Figure 5.2: Concentration of co-product required to reach 90% yield when using an initial concentration of 1 mol/L ketone and a tenfold excess of amine donor for the synthesis of chiral amine.

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 favourable 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 [137]. 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 mentioned before. There are many examples to illustrate the use of ISPR strategies in connection with transaminase catalysed reactions. A summary of the different approaches for ISPR, including the improvements achieved and main drawbacks, can be found in Table 5.2.

Table 5.2: *In situ* recovery techniques applied for biocatalytic transamination.

Reaction system	Comparison ISPR vs. without ISPR	ISPR method applied	Major drawback	References
α-MBA ⇌ APH (*B. thuringiensis* JS64)	nine fold higher reaction rate	Extraction with organic solvent	Decreased enzyme stability	Shin and Kim, 1997 [50]
α-MBA ⇌ APH (*B. thuringiensis* JS64)	99% *vs.* 54,7% (*ee*)	Membrane extraction (perstraction)	Demand for highly purified enzyme [a]	Shin *et al*, 2001 [103]
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 [131]
sec-ButA ⇌ (R)sec-ButA (*B. megaterium* SC6394)	Enzymatic resolution with 99% (*ee*)	Distillation of the volatile amine product	Limit number of amines can be recovered using distillation.	Hanson *et al.* 2008 [138]
BA ⇌ (R)-APB (ATA-117)	92% conversion obtained (99% *ee*).	Extraction with organic solvent combined pH setting [b]	Organic solvents used: potential decrease of enzyme stability	Koszelewski *et al.* 2008 [139]
APH ⇌ α-MBA (ATA-113 and ATA-117)	99% *vs.* 10% (*max conv.*)	Extraction with ion exchange resins	Dimension and cost of resins (fine particles)	Truppo *et al*, 2010 [30]

α-MBA: α-methylbenzylamine; APH: acetophenone; sec-But.A: sec-butylamine; BA: Benzylacetone; APB: 3-amino-1-phenylbutane. 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.

For instance, integration of extractive recovery with the reaction step allows the shifting of the equilibrium by extracting the product into the second phase. The second phase can be a liquid (liquid-liquid extraction) as employed by Shin and Kim 1997 and Koszelewski et al. 2008 [50, 139], or a solid resin (solid-liquid extraction) as suggested by Woodley and co-workers 2008 [91], and employed by Truppo et al. (2010) [30]. The latter was shown to be an efficient method to also overcome product inhibition and shifting

equilibrium in the production of both (R)- and (S)-MBA. A substrate concentration of 50 g/L (0.4 M) and about 200 g of ion-exchange for product adsorption resulted in an improved reaction rate and 100% theoretical yield. 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, especially considering that the resin cost varies as function of several parameters such as particle size, functional groups and pore size. Multiple re-uses of the resin will be necessary for a reasonable cost contribution. Another common limiting factor regarding this technology, is related to the selectivity of the separation and the relative concentration of the reaction components, including the solvent. For instance, an observed problem when using either solvent or resin extraction is that the ketones and the amines have similar distribution behaviour and therefore will co-extract into the solvent or resin unless another driving force is put in place. This is well illustrated in the report by Truppo et al. (2010) employing the use of resins to extract the product. The amine donor IPA was seen to compete with the product (MBA) for binding to the resin. The similarity between the pKa 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 later).

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 IPA or 2-butanamine are used as the amine donor yielding ACE or butanone, respectively, as co-substrates [131]. For volatile amines, distillation could also be a possible route for product recovery in transamination. For example (R)-sec-butylamine (boiling point of 63°C) was recovered by distillation of the product mixture under basic conditions [138]. Also Savile et al. 2010 reported a slight improvement in yield by sweeping the reactor with nitrogen gas to remove the formed ACE [31]. The selectivity is, however, also very problematic when using the evaporation strategy. Assuming ideal conditions, an estimate of the vapour composition can quickly be estimated based on Raoult's law (Eq. 4.3),

$$x_i^{vap} = \frac{p_i^* \times x_i^l}{\sum p_i^* \times x_i^l} \quad (4.3)$$

where, x_i^{vap} is molar fraction of compound *i* in vapour phase; p_i^* is the vapour pressure of pure compound (Table 5.3) and x_i^l is the molar fraction of compound *i* in liquid phase. As an example, if 10 mM of ACE is being removed from a water solution the relative amount of water (C_{water}=55 M) evaporated will be over 500 times that of acetone. Hence, in a thermodynamically unfavourable 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.

Table 5.3: Vapour pressure values of pure compounds at 25 °C. Adapted from [32].

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 vapour pressure of amines are negligible due to protonation of the amine.

5.1.2.3. Auto degradation of Co-Product

A very convenient, but not widely applicable approach is the use of a self-degrading co-product or products. It was demonstrated that when using ornithine or lysine as amine donor, the formed amino-keto acid is cyclized spontaneously, thus favouring the reaction in the direction of the amine [140-142]. Also Truppo et al. (2010) used a similar approach where the product cyclized, thereby shifting the equilibrium of the reaction [30]. This strategy is of course limited to use of these compounds as amine donor.

5.1.2.4. Enzymatic Cascades Reactions

A much explored approach to obtain a high yield of the desired product is to couple the transamination reaction to other enzymatic steps that convert the co-product (e.g. pyruvate or acetone) into a non-reactive species or back to the original substrate (Figure 5.3). One early strategy, employed by Chao et al. (1999), was the combined use of phosphoenolpyruvate carboxykinase (PcK, EC 4.1.1.32) and pyruvate kinase (EC 2.7.1.40) to convert the formed oxaloacetate to pyruvate in a two-step reaction [143]. A simpler strategy was reported by Fotheringham and co-workers (1999) 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 co-product to the non-reactive acetoin [144]. Significant yield and purity advantages over the process using the transaminase alone were reported, with an eight to ten fold 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^+ [33, 34, 145, 146]. 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 (Figure 5.3). 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 Hohne 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 [145].

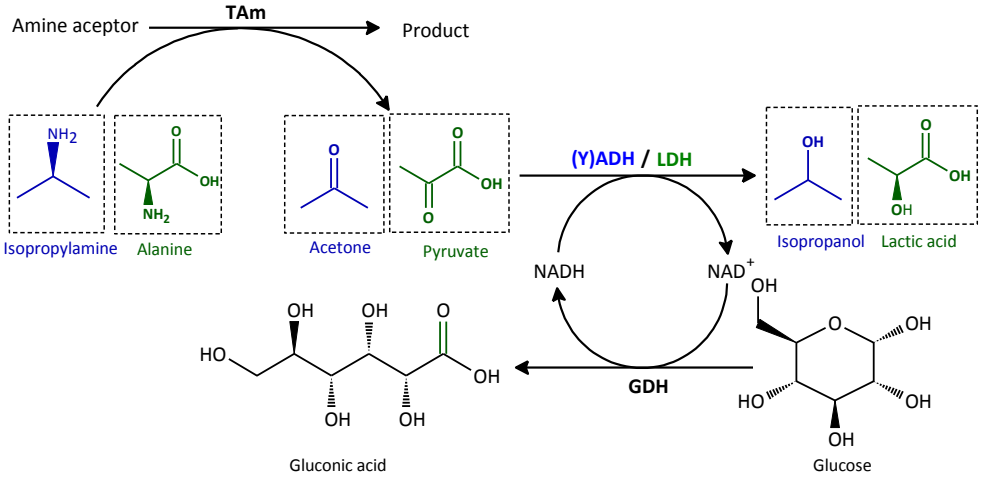


Figure 5.3: Example of enzymatic cascade reactions used in transamination (Tam: Transaminase, (Y)ADH: Yeast alcohol dehydrogenase; LDH: Lactose dehydrogenase; GDH: Glucose dehydrogenase.

Truppo et al. (2009) 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 [147].

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 [139].

These strategies are summarized in Table 5.4. Regardless of the cascade system employed, 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 as well as the formation of high concentrations of isopropanol (formed when YADH is used to convert acetone) [38]. So it is crucial to weigh the pros and cons of introducing a cascade strategy. Also the costs associated with introduction of more enzymes and cofactors and the respective downstream processing costs need to be considered.

Table 5.4 Enzymatic cascades for shifting the equilibrium

	Enzymes	Co-reactants [a]	Co-products	Refs.
Oxaloacetate degradation	PcK, PK	α-KG, ATP	Pyruvate, CO_2	[143]
Pyruvate degradation	ALS	Alanine	CO_2, acetoin	[144]
	PDC	Alanine	CO_2, acetaldehyde	[145]
	PDC, ADH, FDH	Alanine, NADH, Formate	CO_2, ethanol	N/R
	LDH, GDH	Alanine, Glucose, NADH	Lactic acid, gluconic acid	[33]
	LDH, FDH	Alanine, Formate, NH_4^+, NADH	Lactic acid, CO_2	[139]
Co-product degradation	(Y)ADH, GDH	IPA/ButA, glucose, NADH	2-propyl/butyl alcohol, gluconic acid	N/R
	(Y)ADH, FDH	IPA/ButA, formate, NADH	2-propyl/butyl alcohol, CO_2	[38]
Alanine recycling	AADH, GDH	Alanine, NH_4^+, NADPH	Pyruvate (low), H_2O	[147]

[a] Reactants that are required for the reaction additionally to the amine acceptor (ketone) ALS - Acetolactate synthase; PDC-Pyruvate decarboxylase; ADH-Alcohol dehydrogenase; FDH-Formate dehydrogenase; GDH-Glucose dehydrogenase; IPA/ButA: isopropylamine or 2-butyl amine; PcK- Phosphophenol pyruvate carboxykinase; PK-pyruvate kinase. α-KG - Alpha-Ketoglutaric acid; ATP - Adenosine triphosphate

5.1.2.5. Whole Cell catalysis

Despite the fact that the multi-enzyme cascade approach has the potential to be very successful, the economic burden of using multiple enzymes is significant [23]. In particular the combination with the addition of co-factor (NAD(P)H) will increase the process cost, even when using low concentrations [148]. Co-immobilizing the enzymes and/or the co-factors [149] could help to lower the costs associated with the biocatalysts, as previously discussed in Chapter 4.

Also, using a WC as the biocatalyst could be a suitable strategy to overcome the limitations associated with co-factor recycling and multiple enzyme usage. Whole cell strategies have become a very promising field especially for biocatalytic reactions which usually require co-factor addition and/or regeneration [150]. The wild-type microorganism containing the desired ω-TA may be used, but the more common approach is to clone the desired ω-TAm into a host vector. For example the use of recombinant *E. coli* [36, 40] or *Pichia pastoris* [151] expressing ω-TAm, 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-TAm with a known gene sequence is still rather limited [37, 152].

Several authors 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 [37-39, 144, 153]. However, the adequate expression level of each protein still remains a challenge [154]. Other typical drawbacks found in whole-cell biocatalysis, such as uncontrolled side reactions (and consequently unwanted side products) and slower reaction rates (due to trans-membrane diffusion problems and higher metabolic burden), are also encountered in the ω-TAm 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 [93].

5.1.3. Substrate inhibition and low water solubility

For the success of most biocatalytic routes, it is also critical to be able to supply substrates at concentration above 50–100 g/L [155]. A common characteristic inherent to aqueous biocatalytic processes is the low solubility of many substrates in water. Operating the process at too low substrate concentration would lead to a low volumetric productivity and thereby high costs for equipment and downstream processing for product recovery. A list of solubility of some of the compounds used for transamination reactions is shown in Table 5.5 from where it is evident that for compounds such as APH and BA, which are used as case studies in this thesis, a feeding strategy has to be employed to supply the substrate at a high concentration [89], if the biocatalyst is not developed to tolerate high concentrations of these compounds. 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 [156-158]. This strategy can also help to minimize imine dimer formation [31] and increase the enantiomer excess obtained [159].

Table 5.5 Data for solubility of some compounds used in transamination.

		Aqueous solubility[a] (S_{aq})		Critical conc. for cell[b] (C_{crit})	
Compound	**Log P**	**(g/L)**	**(mol/L)**	**(g/L)**	**(mol/L)**
Acetophenone	1.58	4.48	0.04	1.63	0.01
Benzylacetone	1.96	1.63	0.01	0.76	0.005
α-methylbenzylamine	1.49	42	0.35	9.55	0.08
Alanine	-2.96	165	1.85	26.38	0.30
Pyruvic acid	-1.24	1000	11.36	109.25	1.24
Acetone	-0.24	1000	17.22	100.10	1.72
Isopropylamine	0.26	1000	16.92	100.47	1.70
Butanone	0.29	223	3.09	32.01	0.44
sec-Butylamine	0.74	112	1.53	18.64	0.25

a: estimated based on Log P using EPI Suite (http://www.epa.gov/oppt/exposure/pubs/episuitedl.htm)
b: estimated based on aqueous solubility using the correlation: $\mathbf{log}(C_{\mathbf{crit},}) = \mathbf{0.79} \times \mathbf{log}(S_{aq.}) - \mathbf{0.74}$. [88]

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 [160] or reduced by half [161]. Compounds with an aqueous solubility between 0.0003 and 1 M usually require an auxiliary phase for the purpose of *in situ* substrate supply [88]. As seen in Table 5.5, this range includes for instance APH and BA which are known to have low water solubility and have an inhibitory effect on the catalytic activity [51, 142] as was shown in Chapter 2.

To increase productivity, despite the use of an improved biocatalyst able to tolerate higher substrate concentrations (as discussed and demonstrated in Chapter 2 and 3), three other main strategies can be applied, as illustrated in Figure 5.4:

i. the substrate can be fed at an optimized rate to the media using a precision pump (Fed-batch mode);
ii. a water miscible co-solvent (e.g., THF, iso-propanol, DMSO) that increases the solubility of the substrate in the aqueous phase or aqueous media saturated with a water immiscible solvent (e.g., toluene, heptane, ethylacetate) can be used;
iii. an auxiliary phase saturated with the substrate can be used to act as a reservoir for the substrate. The auxiliary phase can be a liquid (water immiscible solvent

such as toluene, heptane or ethylacetate) or a solid (such as polymeric resins). If the auxiliary phase has equal affinity to the product, a substrate feeding-product removing strategy using the same support can be employed, as described by Hilker and co-workers 2004 [162].

The effect of different water miscible solvents on the amination of BA was investigated by Koszelewski et al. 2008 [139]. It was seen that the addition of 15% DMSO resulted in a better enzymatic activity. This result, together with the ones obtained in Chapter 3, highlight the potential of this strategy. However, as also demonstrated, there are issues related to the amount of the solvent that are required to solubilize the desired concentration of substrate and also concerns related with the biocatalyst stability that need to be considered. The addition of solvents can decrease the stability of the biocatalyst and might also cause downstream processing problems, since the solvent needs to be separated from the product and preferably recycled.

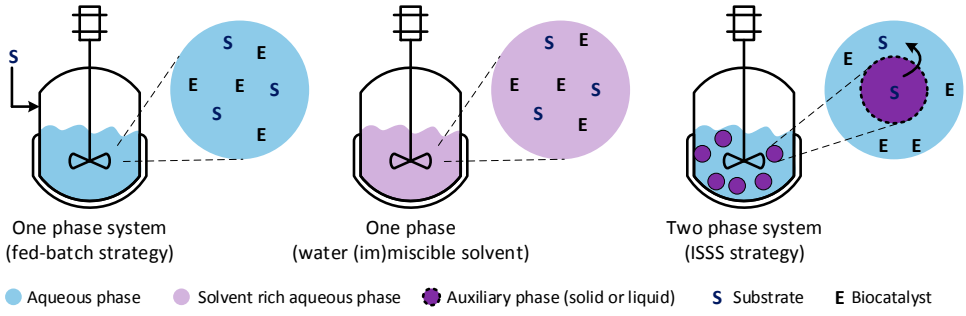


Figure 5.4: Representation of substrate supply strategies.

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 nine fold and allowed the resolution of 500 mM MBA with an *ee* of >95% and 51% conversion [50]. 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 [62]. Membrane extraction was also used in connection with a packed-bed reactor where whole cells were immobilized in calcium alginate beads [103].

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 VOC (volatile organic compound) emissions are one of the main contributors to the environmental impact of pharmaceutical processes [163]. 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 [150].

Another alternative would be use polymeric resins as reservoir for substrates. This would minimize the concern and the costs associated with downstream processing and waste water treatment as resins are insoluble and therefore easily separated from the media by means of a filter. A major limitation for this strategy is the limited capacity of the resins towards the substrate and the enormous space they may occupy in the reactor. This can be however solved by using an external column packed with the resins.

5.1.4. Product Inhibition and *in situ* product removal

As mentioned in the previous section, strategies to alleviate product inhibition also have the potential to shift the equilibrium in favour of products. The advantages introduced by including an *in situ* product removal step go beyond the decrease in product inhibition and equilibrium shift. The whole downstream process is facilitated with ISPR. If the right configuration is used, the product-biocatalyst separation is immediately guaranteed, reducing the total number of steps required. Furthermore, also the reactor volume required can be reduced since larger amounts of product can be obtained and stored in the ISPR support. This decreases the reaction time (equilibrium

Benefit	Impact
Increased product concentration (g_P/L)	Reduced reactor volume, easier DSP
Increased biocatalytic yield (g_P/g_B)	Reduced catalyst costs
Increased yield on substrate (g_P/g_S)	Reduced substrate costs
Increased volumetric productivity (g_P/l.h)	Reduced reactor volume and/or processing time, easier DSP

Abbreviations: DSP, downstream processing; g_B, grams biocatalyst; g_P, grams product; g_S, grams substrate.

Box 5.2: The potential benefits of *in situ* product removal adapted from [15]

shift), allows higher product concentration to be obtained with less biocatalyst and improves the conversion (Box 5.2).

ISPR strategies can be applied in several configurations. They have been reviewed by Woodley et. al 2008 and divided in a simplified fashion in:

i. those where the removal support (liquid or solid) is in direct contact with the biocatalyst, and
ii. those where the support does not contact directly with the biocatalyst (indirect contact), often achieved by immobilizing the biocatalyst or physically separating them using a membrane reactor or similar.

Inside each of these configurations, the removal support can be located inside the reactor or externally, the latter requiring the use of an external column or second tank and solid removal supports [91]. The process itself can be carried out in batch mode, semi-batch or even in continuous mode with substrate being continuously added and the product (plus ISPR support) being continuously removed and fresh supports being added (Figure 5.5).

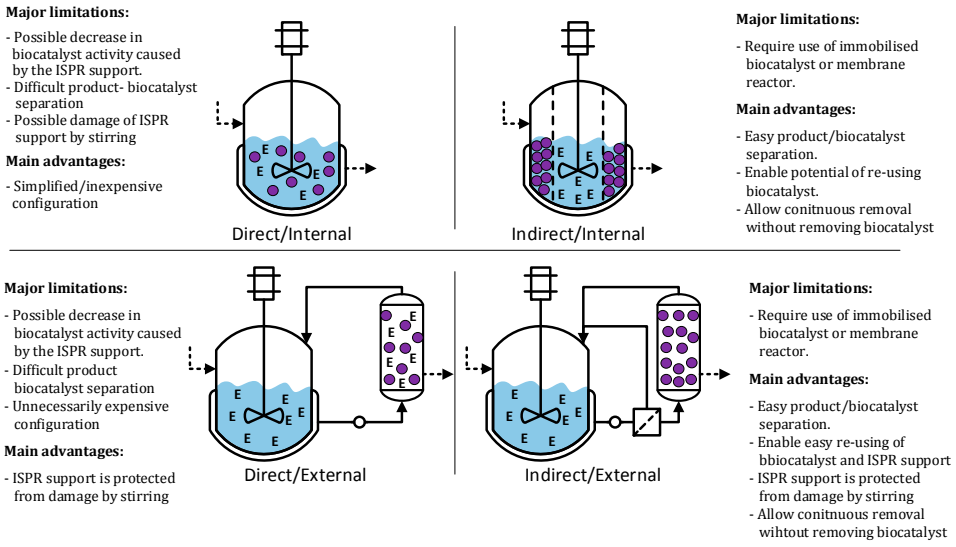


Figure 5.5: Schematic flow-sheet showing internal and external modes of ISPR operation with direct and indirect cell contact. Adapted from [91].

Independently of the configuration chosen, the main challenge associated with ISPR is the selection of a separation method. This is chosen based on key properties (either physical or chemical) that the compound to be separated needs to have distinct from any other compound involved in the reaction in order to allow implementation of ISPR. Some examples of ISPR applied to transamination making use of hydrophobicity (extraction with solvent) and charge difference (extraction using ion exchange resins) have already been mentioned. Evaporation also has been reported, although this is not applicable to the product since it would be lost (Table 5.2). Alternatively, distillation could be used in theory, however this hasn't been reported in the context of ω-TAm catalysed reactions. Methods for ISPR should be easy to implement and reproducible with great product recovery capacity. Selecting a method for ISPR follows the same principle as selecting the method for downstream processing with the difference that the unit operation for separation is integrated in the reaction step, so the presence of the biocatalysts should be considered as it is desired that they remain active during the ISPR process. This concern as regards biocatalyst viability is what makes ISPR more challenging than the downstream processing (DSP) itself, but both are intrinsically related.

5.2. Downstream Processing

In a reaction such as the transamination, where products and substrates are so similar in properties, DSP can be a great challenge. Substrates and products are similar in their hydrophobicity (ketone substrate and amine product), net charge (amine donor and amine product) and volatilities (amine donor – at pi and ketone co-product). The molecular weights between the species are also very similar since only an amine group is transferred from one compound to another. This creates a major challenge to purify the product, hence even more of a challenge to integrate this into the reactor (for means of ISPR). The first step is to separate the biocatalyst from the media, or to first disrupt the cells before separation, if whole cells are used and the product is intracellular. In case of ω-TAm catalysed reactions employing the use of *E. coli* WC or CFE, the products are extracellular, avoiding the need for cell disruption, and the media can be easily separated from the biocatalyst by means of centrifugation or filtration which, in case of immobilized biocatalyst, should be sufficient to remove most of the proteins. In case of an ISPR

strategy being implemented, the biocatalyst is in theory retained in the reactor, facilitating the separation process as discussed before. After this, the real challenge begins: the separation of products from the substrates and from the rest of media components. In order to do this, it is important to study the chemical and physical properties of all the compounds involved in the reaction, since different properties will allow different types of separation strategy (Table 5.6).

Table 5.6 . Classification of key substrate and product separation technique. Adapted from [137]

Driving force	Example techniques
Physical properties	
Volatility	Distillation, Gas stripping
Molecular weight or size	Membranes (MF, UF etc.), Centrifugation, Size exclusion
Solubility	Pervaporation or Perstraction Extraction (solvents, SCCO2) Precipitation,Crystallization
Chemical properties	
Charge	Ion-exchange, Electrodialysis
Hydrophobicity	HIC, Adsorption

Table 5.7 summarizes the chemical and physical properties of the compounds involved in the four model reactions systems used in this thesis (Chapter 1). It can be observed that the substrates and products present several similar properties, as already mentioned: the amine donors (ALA and IPA) and the ketone co-product (PYR and ACE), respectively, share similarities in molecular weights (Mw), boiling points (although the amine donors charge can be manipulated by pH adjustments), similarities in the hydrophobicity (logP) and solubility in water (S^{aq}). In the same way, the ketone substrates (BA and APH) and the amine products (APB and MBA), respectively, present similar molecular weight, boiling points, solubility and hydrophobicity. This suggest that more than one strategy is required to fully distinguish products from substrates.

The choice of amine donor has a very important weight on this. As mentioned before, the choice of IPA brings the advantage of making possible the use of an excess of a relatively inexpensive substrate to help shift equilibrium, despite the discussed limitations. However, it also increases the difficulty of the product separation.

Table 5.7: Chemical and physical properties for compounds separation

	Mw (g/mol)	Tb (˚C)	Tm (˚C)	pKa	LogP	P ^{vap} (mmHG)	S ^{aq} (g/L)
Alanine	89.09	380.28	292.0	2.33, 9.71	-2.96	2.68E-8	165
Isopropylamine	59.11	31.7	-95.1	10.73	0.26	5.80E+2	1000
Benzylacetone	148.21	233.5	-13.0	N/A	1.96	6.51E-2	3.26
Acetophenone	120.15	202.0	-9.86	N/A	1.58	3.97E-1	6.13
α-Methylbenzylamine	121.18	185	-65.0	9.75	1.49	5.00E-1	43.0
3-amino-1-phenyl butane	149.24	223	22.46	10.63	2.12	6.72E-3	12.0
Acetone	58.08	55.5	-98.3	N/A	-0.24	2.32E+2	1000
Pyruvic acid	88.06	186.79	13.8	2.30	-1.24	1.29E+0	1000

This is illustrated in Figure 5.6 where two properties (hydrophobicity and molecular charge) are used to evaluate the separation of the components involved in two reactions systems: (i) ALA and BA reacting to form PYR and the APB, and; (ii) IPA and BA reacting to form ACE and APB. This considers separation *in situ*, where pH values cannot be manipulated to extreme values in order to preserve the biocatalyst activity. From the figure, it becomes obvious that the product (APB) can easily be separated from all the other components using hydrophobicity in both reaction systems, except for the ketone substrate (BA) which is as hydrophobic as the product (APB) (Figure 5.6 A and B). This suggests that: i) for ISPR using hydrophobicity, the separation might not be efficient and also result in the removal of the ketone substrate; and ii) if hydrophobicity is used as a first step of the DSP, another technique (e.g. separation by charge) needs to be applied afterwards to distinguish between substrate and product.

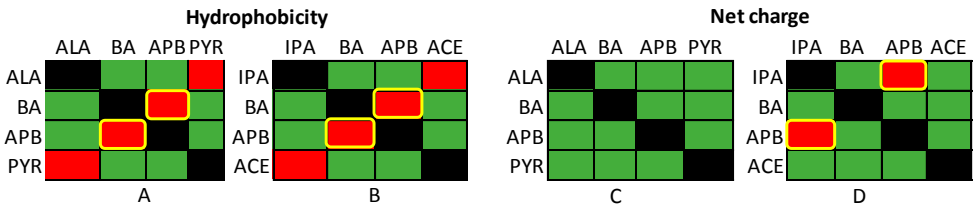


Figure 5.6: Interaction matrix for species separation under reaction conditions (pH ~7). Red – impossible to separate; green – separation is possible; black – not applicable. ALA=Alanine, IPA= Isopropylamine, BA=Benzylacetone, APB= 3-amino-1-phenylbutane; PYR = Pyruvic acid, ACE= Acetone. Based on logP and pka values.

On the other hand, if the separation by charge is considered in first place, the product (APB) can be easily separated from all the other components (with no exception) in cases when ALA is used as the amine donor (Figure 5.6 C). This contrasts with the scenario in which IPA is used as the amine donor, where the separation between the amine product (APB) and the amine donor (IPA) is compromised (Figure 5.6 D). This difference lies in the amphoteric properties of ALA (and all amino acids in general). ALA has 2 levels of protonation associated with pKa values of 2.33 (for protonation of the carboxylic group) and 9.71 (for protonation of amino group). This means that ALA has an isoelectric point (Pi) of c.a. 6.02 and at pH values around the Pi, the net charge of the compound is zero, contrasting with IPA which has a pKa = Pi of 10.63 and is fully protonated at reactions pH (c.a. 7), as depicted in Figure 5.7.

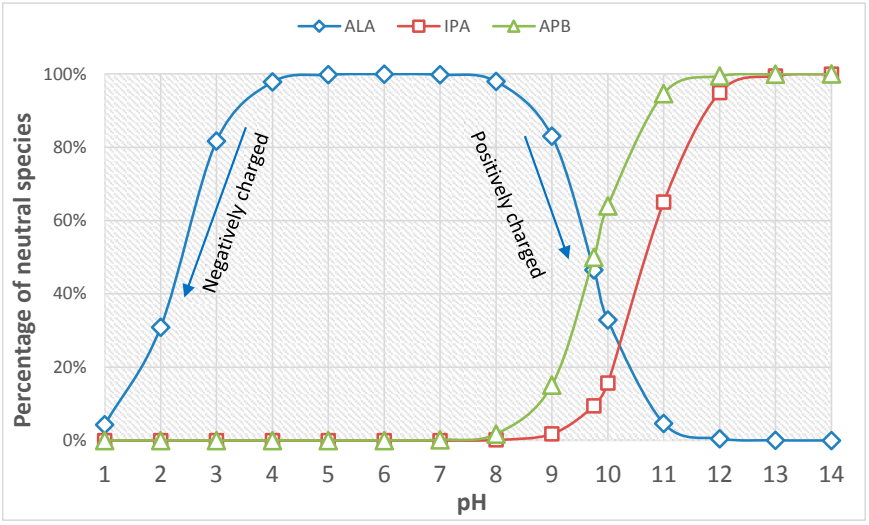


Figure 5.7 Percentage of species with neutral net charge. Created based on pKa values.

Liquid–liquid extraction is a common strategy for the downstream recovery in transamination that allows recovery of a large range of different amines. Extraction under either acidic or basic conditions allows control of the amine product if 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)-APB by Koszelewski and co-workers [37, 139]. The reaction involved the use of ALA as the amine donor and BA as

substrate. After the reaction the authors implemented a pH adjustment by adding 5 M of HCL, which lowered the pH to 1, causing the protonation of the amine donor and the product (becoming positively charged). Afterwards dichloromethane (DCM) was added to remove the ketone substrate (by means of hydrophobicity) while both the amine donor and the product remained in aqueous phase due to their charged properties. Afterwards a new pH adjustment was applied to the remaining aqueous phase, increasing the pH to 12 (10 M NaOH), which caused the deprotonation of the amine product (net charge become null) while the remaining amine donor became negatively charged due to deprotonation of the carboxylic group. This allowed exclusive removal of the amine product to the organic phase (DCM) which is later separated from the product by means of distillation (Figure 5.8). Such an approach would not be successful if IPA was used as the amine donor since this would not be completely removed in step 2 due to its low hydrophobicity. Only in the last step (distillation) it would have been completely separated from the amine product (due to its low boiling point) as depicted in Figure 5.9.

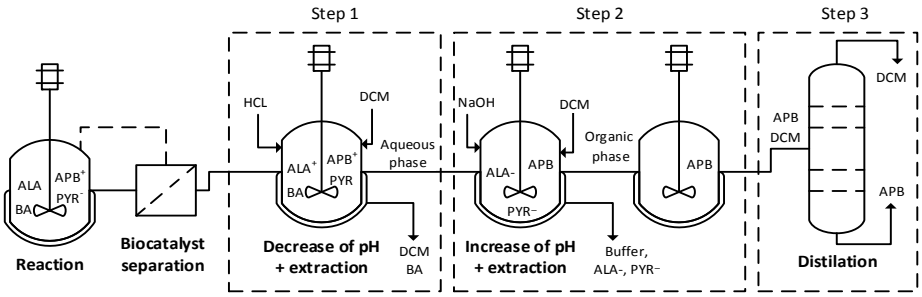


Figure 5.8: Schematic overview for DSP featuring Alanine as amine donor (ALA= Alanine, BA= Benzylacetone, APB= 3-amino-1-phenylbutane, PYR= Pyruvic acid, HCl = Hydrochloric acid, DCM= Dichloromethane. The charge of different species at different stages is represented by the presence of (+) for positively charged, (-) for negatively charged and no sign for neutral net charged species.

In both cases, the first step could be replaced by an adsorptive process (by means of hydrophobic polymeric resins) in order to avoid the use of large amounts of solvents as discussed earlier. This step could also be integrated in the reaction (ISPR) as it will be demonstrated later. In the second step, on the other hand, the solvent cannot be replaced by a solid support, since it is important to have the product in organic phase for the distillation.

Also ion exchange resins could be used to separate the charged product from the remaining reaction components in the first step, in the case where ALA is used as amine donor. The 2 last steps of DSP would remain unchanged.

In conclusion, different amine donors require different DSP options. The costs and time can be reduced by evaluating the impact of different amine donors upfront, designing the reaction based on the DSP options in what could be called reverse process design.

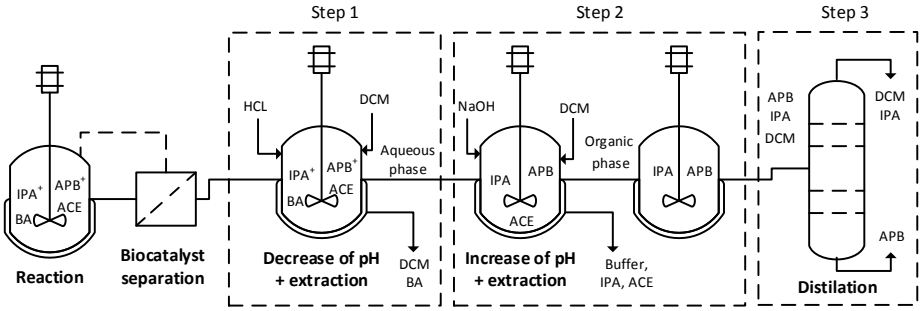


Figure 5.9 Schematic overview for DSP featuring Isopropylamine as amine donor (IPA= Isopropylamine, BA= Benzylacetone, APB= 3-amino-1-phenylbutane, ACE= Acetone, HCl = Hydrochloric acid, DCM= Dichloromethane. The charge of different species at different stages is represented by the presence of (+) for positively charged, (-) for negatively charged and no sign for neutral net charged species.

6. Implementation of Process Eng. Strategies

Summary

In this chapter, the strategies overviewed in Chapter 5 are implemented with the objective of increasing the productivity in the selected model reactions. This chapter focuses on the description and screening of suitable auxiliary phase (resins) for implementation of ISPR/ISSS strategies, as well as the laboratory scale demonstration of the potential and limitations of the different strategies.

The following strategies were considered in this chapter:

i) use of amine donor excess (ADXs) and co-product removal (IScPR) to help displacing the thermodynamic equilibrium.
ii) the selection of amine donor (IPA *vs.* ALA) to enable the removal of ACE by nitrogen sweeping (in case when IPA is used) or the enzymatic removal of PYR through enzymatic cascade reaction (in case when ALA is used);
iii) the removal of product (ISPR) using hydrophobic and ion exchange polymeric resins to aid in displacing equilibrium as well as alleviating product inhibition;
iv) The use of fed-batch or *in situ* substrate supply (ISSS) to overcome the ketone substrate solubility issues.

Excellent results were obtained by combining the following strategies: 1) Fed-batch + ISPR using hydrophobic resins + ACE removal using nitrogen sweeping, when IPA was used as the amine donor, and 2) ISPR using ion exchange resins + enzymatic removal of pyruvate, when ALA was used as the amine donor.

6.1. Description of auxiliary phases:

The auxiliary phase used for ISPR, ISSS or IScPR can be a liquid (such as organic solvent), a solid (such polymeric resins) or a gas (such as nitrogen used to evaporate volatile compounds), as described in Chapter 5. While the use of gas auxiliary phases has only been demonstrated to evaporate the co-product and not to recover the product itself, the use of both liquid and solid phases can easily be applied to remove the product of interest and/or act as reservoir for the substrate, as also discussed and demonstrated in Chapter 5. The following sections give an overview of these two possible technologies for ISPR/ISSS.

6.1.1. Extraction using organic solvents

The use of organic solvents in chemical and pharmaceutical industries is well established. Organic solvents play a very important role in these industries. As time went by, and due to pressure and enormous effort from governments and environmental/health institutions, some of the most toxic and dangerous solvents have been eliminated, replaced by those regarded as safer or greener and also recycled to minimize the amount of solvents used, hence reducing their impact on health and environment [164].

There are several published guidelines for solvent selection. Most of them focus on the health risks, environmental impact and operational safety and these criteria are used to classify the different solvents [165]. The most famous and widely used is the GlaxoSmithKline's (GSK) solvent selection guide which gives an overview about the safety issues for a range of solvents. However, to be used in ISPR, the solvent also needs to present other key features besides the operational safety, which can be controlled by using solvents with high flash points and low vapour pressures.

In addition, the solvent needs to be insoluble in water and have good partition towards the product, which is often characterized by logP values higher than 1. At the same time the solvent needs to be biocompatible (low toxicity towards the biocatalyst). Solvents

with logP > 4 are often considered to be biocompatible as will be demonstrated below. Lastly, it should be possible to separate the product from the solvent itself. This can be easily achieved through distillation, however it is important that the solvent has a different boiling point than the product and that both do not form and azeotropic mixture (Box 6.1).

Guidelines for solvent selection:

Product extraction:
- Insoluble in water (LogP>1),

Operational safety:
- High flash point;
- Low P^{vap}
- inert (non-reactive)

Biocompatibility:
- Low toxicity to biocatalyst (LogP >4)

Separation (Distillation):
- Tb solvent ≠ Tb product
- Do not form azeotrope with the product

Box 6.1: Criteria for solvent selection for ISPR

One can easily elaborate a list of solvents that fulfils these criteria, however, it is important to note that these all have to be met at the same time, and often the list of solvents available is drastically reduced due to incompatibility between the criteria. The most obvious example is the incompatibility between good partition and biocompatibility. Often the solvents that show high capacity towards the products are the one that are more aggressive towards the biocatalyst. This can be in theory overcome by immobilization, as it was demonstrated by Truppo and co-workers, 2012 [92]. However, as concluded in Chapter 4, the immobilization not always enhance the stability in presence of solvents. This is mainly due to poor solvent stability of the biocatalyst itself which is severely affected, especially by solvents with LogP values lower than 4. This can be observed Appendix 6A, which is the result of study where toxicity of different solvents towards the biocatalyst was tested in respected to product and substrate partition. For this reason, the use of organic solvents for ISSS and ISPR were not considered in this thesis.

A possible solution to overcome this biocompatibility *vs.* partition issue would be to design a reactor that guarantees a good mixture between the aqueous and organic phase (ensuring good mass transfer) while preventing the biocatalyst directly contacting with the solvent or the interface between the two phases. Inspired by the technology available for continuous liquid-liquid extraction (Appendix 6B-1, 2), a design for a reactor system integrated with a liquid- liquid extraction apparatus was proposed (Appendix 6B-3), but never tested.

6.1.2. Adsorption onto a solid support – Polymeric resins

The use of *sorbents* date back several centuries, although the term itself was only introduced in 1909 by J. W. McBrain [166]. By definition, the sorbed solute (which can be a gas or a liquid) is denominated as *sorbate* and the sorbing agent (liquid or solid) is referred to as *sorbent.* This designation applies to Liquid-solid, liquid-liquid, gas-liquid or gas-solid interactions.

6.1.2.1. Hydrophobic polymeric resins

On the other hand, adsorption processes are designated as *sorption* operations in which components of a fluid phase (solutes) are selectively transferred to insoluble, rigid particles suspended in a vessel or packed in a column. Thus, adsorption is a designation which applies only to liquid-solid or gas-solid interactions [166]. More precisely, in the adsorptive process molecules, atoms or ions, in a gas or liquid phase, diffuse to the surface of a solid, where they bond with the solid surface or are held by weak intermolecular forces (physical interaction). The solid support (the *adsorbent*) is normally a small-diameter particle composed by interconnected pores where the solute (or the *adsorbate*) is adsorbed. The presence of pores combined with the small diameter of the particles provide a large surface area for adsorption per unit of volume. The solute can be externally adsorbed on the particle surface (1), internally adsorbed on the adsorbent (2), or in the inter pore space (3). The pores diameter can also be used as a size exclusion factor which can prevent bulky molecules (e.g. proteins) of being adsorbed (4), as depicted in Figure 6.1.

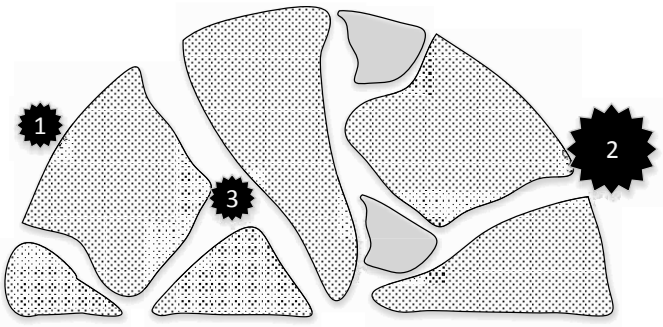


Figure 6.1 Schematic representation of the adsorption processes. Shaded surface – Adsorbent; (1) solute adsorbed on the surface of the particle, (2) bulky solute retained on the surface of the particle (3) solute retained in the inner pore space,.

Adsorption has been used for centuries to improve the taste of water by using charred wood, or for the decolorization of liquids by adsorption with bone char and other materials. Adsorption (of gas) was first described in 1773, but it was only in 1960s that its industrial utilization escalated with the invention of molecular sieves zeolites [167]. The most common solid adsorbents are: activated alumina, silica gel, activated carbon, molecular sieves and polymeric resins.

Polymeric resins are typically spherical particles produced by polymerization of styrene and divinylbenzene (Figure 6.2), or by polymerization of acrylic esters for adsorbing polar solutes. Nowadays, these particles have several applications such as, immobilization of enzymes as shown in Chapter 4; gas purification (e.g. removal of organics from vent streams) or liquid purification (e.g. removal of organics from water, vice-versa). In this chapter they will be used for ISPR and ISSS.

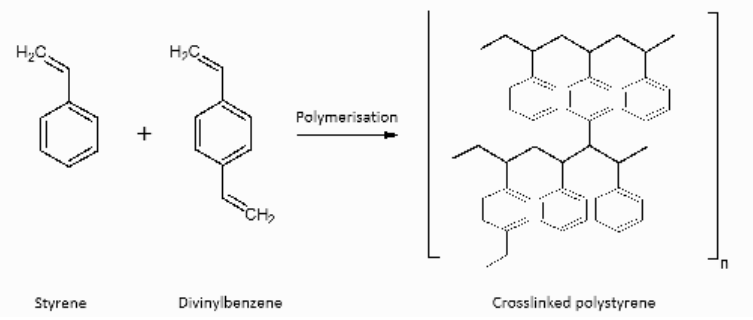


Figure 6.2: Polymerisation of cross linked polystyrene out of styrene and divinylbenzene

6.1.2.2. Ion exchange polymeric resins

In an ion-exchange process, ions of positive charge (cations) or negative charge (anions) in a liquid solution, replace dissimilar and replaceable ions, called *counterions,* of the same charge present. These counterions are coupled to an immobile, insoluble and permanently bound co-ion of the opposite charge [166], as illustrated in Figure 6.3. Ion exchangers can be defined as insoluble materials that carry exchangeable ions, either cations or anions. By treatment of an ion exchanger with a solution containing ions of the same charge, the ions in solution can replace the ones bound to the resin for a stoichiometrically equivalent amount. In most cases, this process is reversible and the ion exchanger can be regenerated. Ion exchange is a diffusion process and therefore it does

not follow usual chemical reaction kinetics. Ion exchangers can be separated into cationic, anionic or amphoteric ion exchangers that are capable of exchanging cations as well as anions. Additionally, there are several types of materials that can be used as an ion exchanger including minerals, coals, resins and synthetic inorganic ion exchangers [166].

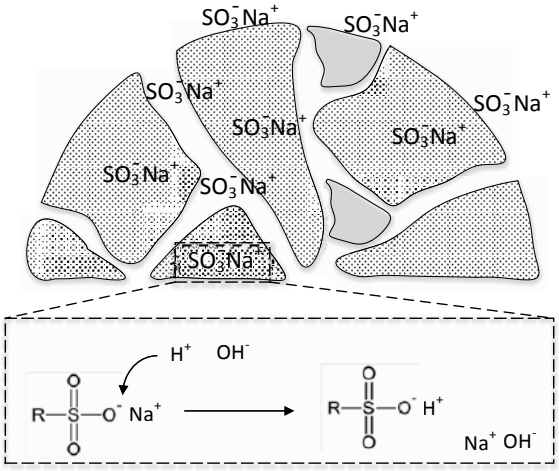


Figure 6.3 Schematic representation of the ion exchange process. Shaded surface correspond to the surface of the particle covered by the co-ion or ion exchange (SO_3^-).

In this work, polymeric ion exchange resins were compared with hydrophobic resins with respect to their capacity to remove the amine products. Ion exchange resins can be obtained by the same process as described for hydrophobic ones, by the polymerization of styrene and divinylbenzene. The type of functional group present on the resin determines which type of ion exchange it belongs to. They can be i) weak-base anionic exchange resins, ii) strong-base anionic exchange resins, iii)weak-acid cationic exchange resins and iv) strong-acid cationic exchange resins [166]. Due to the strong acidity of the functional group, strong-acid cationic exchange resins can operate over a wide pH range. The most common functional group in this type of ion exchangers is sulfonic acid (Figure 6.3). Strong-acid cationic exchange resins are used commercially in many areas, including etherification (mainly for the production of methyl *tert*-butyl ether, MTBE), dehydration, alkylation and condensation reactions, as well as for water softening and demineralization [166, 168]. They are fairly selective, ranging somewhere between electrolytes and highly selective enzymes. Regeneration of the resin is possible by reintroduction of the functional groups or by treatment with acids or solvents [166, 169].

6.2. Screening of suitable resins

The selection of suitable polymeric resins to be used for ISPR or at DSP follows similar principles, with the exception that ISPR requires the resin to be biocompatible, similar to what was discussed for organic solvents in the previous section. Furthermore, some of the criteria used for the screening of immobilization carriers (Chapter 4) also applies in this case.

The biocompatibility, or non-interaction of the resin with the biocatalyst can achieved by either using resins with small pore (preventing the biocatalyst from being adsorbed), or by physically separating the resin from the biocatalyst by means of external column, membrane or immobilization of the biocatalyst. Other important criteria are presented in Box 6.2.

Guidelines for sorbent selection:

The ideal sorbent should have:

- **High selectivity:** to enable an efficient separation;
- **High capacity:** to minimize the amount of sorbent needed;
- **Favourable kinetic:** for a rapid sorption;
- **Chemical and thermal stability**
- **Extremely low solubility in the media;**
- **Mechanical stability:** to prevent crushing or and erosion;
- **Capability to be regenerated**

Box 6.2: Criteria for resin selection. Adapted from [55].

In this work, the resins selected had very similar properties. They were similar in the matrix composition, particle size and average pore diameter. This allows the conclusion that the cost per Kg does not vary much inside the selected library. For this reason, and for a matter of simplicity, the resins were screened based on their capacity to adsorb the different compounds in the reaction. This was done by exposing isolated compounds under vigorous mixing with the resins and measuring afterwards the amount of compound adsorbed. While this allows a rapid selection of the resin with the highest capacity towards the products, it does not give a precise quantification of the real selectivity of the resins towards the different compounds in the reaction, nor information regarding the mass transfer kinetics. However, it gives a fair overview of the resins' affinity towards the different compounds. This will be further extended in the results section.

6.2.1. Resins library

Two ion exchange resins were used for screening (Table 6.1). Both resins were strong acid cationic exchangers, stable at temperatures range from -20 to 125 °C and pH of 0 to 14 (according to the MSDS date obtained from the manufacturer). Both resins are widely used for demineralization of water for production of industrial steam (according to the manufacturer: LANXESS AG, Germany) and none have been previously used for ISPR in ω-TAm catalysed reactions. In the work by Truppo *et al*, implementing ISPR using an ion exchange resin [30], the reported resin (Amberlite XAD1180N) was found to have hydrophobic properties rather than the reported ionic exchange properties. Therefore, a comparison between the selected resins and the one used in the above cited work was not possible. This resin was however included in the library of hydrophobic resins (Table 6.2).

Table 6.1: Library of cation exchange resins. SDB: Styrene-Divinyl Benzene

Resin	Ionic form	Functional group	Matrix	Avg. pore d. (nm)	Surface area [m^2/g]	Avg. particle d. (mm)
LEWATIT MonoPlus SP 112	Na^+	SO_3^-	SDB	-	-	0.65
LEWATIT K 2629	H^+	SO_3^-	SDB	33	40	0.5

The library of neutral, hydrophobic resins were composed of 9 resins, from 3 different manufactures (LANXESS – Lewatit; DOW chemicals – Amberchrom, Amberlite and Dowex; Mitsubishi Chemical – Diaion and Sepabeads) (Table 6.2). Industrial applications for these resins varies from enzyme immobilization, polishing of water streams, adsorption of traces of organic compounds. Similarly to the ion exchange, these ones have also not previously been used for ISPR in ω-TAm catalysed reactions.

Table 6.2 Library of neutral hydrophobic resins. SDB: Styrene-Divinyl Benzene

Resin	Matrix composition	Avg. pore d. (nm)	Min. surface area [m^2/g]	Avg. particle d. (mm)
Amberchrom CG300	SDB	300	700	~ 0.12
Dowex Optipore L-493	SDB	46	1100	0.42 - 0.85
LEWATIT AF 5	Carbon	8	1500	0.4 - 0.8
LEWATIT VP OC 1600	methacrylate	130	150	0.32 - 0.45
Amberlite XAD1180N	SDB	300	600	0.35 - 0.60
LEWATIT VP OC 1064 MD PH	SDB	50	800	0.44 - 0.54
Diaion HP-20SS	SDB	260	500	~ 0.5
Amberlite XAD7HP	Acrylic ester	90	450	0.56 - 0.71
Sepabeads SP850	SDB	38	1000	~ 0.5

6.3. Rapid characterization of process strategies

The selected resins were tested in various process options with the objective of studying their effect on the productivity.

A set up at 2 mL scale was developed to simultaneously test the different options featuring hydrophobic resins (Figure 6.4): (A) control reaction (reaction without ISPR/ISSS/IScPR); (B) reaction with ISPR only; (C) reaction with ISPR combined with ISSS; D) reaction with ISPR combined with Fed-batch; (E) reaction with ISPR combined with Fed-batch and acetone removal (IScPR); and (F) reaction with ISPR combined with Fed-batch and acetone removal (IScPR) using immobilized enzymes and external column to accommodate the ISPR resins. These were tested using the (S)-selective ω-TAm (c-LEcta GmbH, Leipzig, Germany) selected from Chapter 3 and the model reaction system featured IPA as the amine donor and BA or APH as substrate.

The ion exchange resins were tested at 10 mL scale. The model reaction used featured ALA as the amine donor and BA as ketone substrate. The reaction was catalysed by a (R)-selective ω-TAm (DSM Innovative Synthesis, Geleen, The Netherlands). Three scenarios were compared: (G) control reaction (without ISPR/ISSS/IScPR); (H) reaction with IScPR only (PYR removal through cascade reactions using LDH/GDH system); and (I) reaction with ISPR (ion exchange resin) and IScPR (PYR removal through cascade reactions using LDH/GDH system). The scenario H was run using isolated enzymes and whole cells for comparison.

These nine experiments allowed a good understanding of the effect of the different process strategies on the productivity and on maximum product concentration achievable.

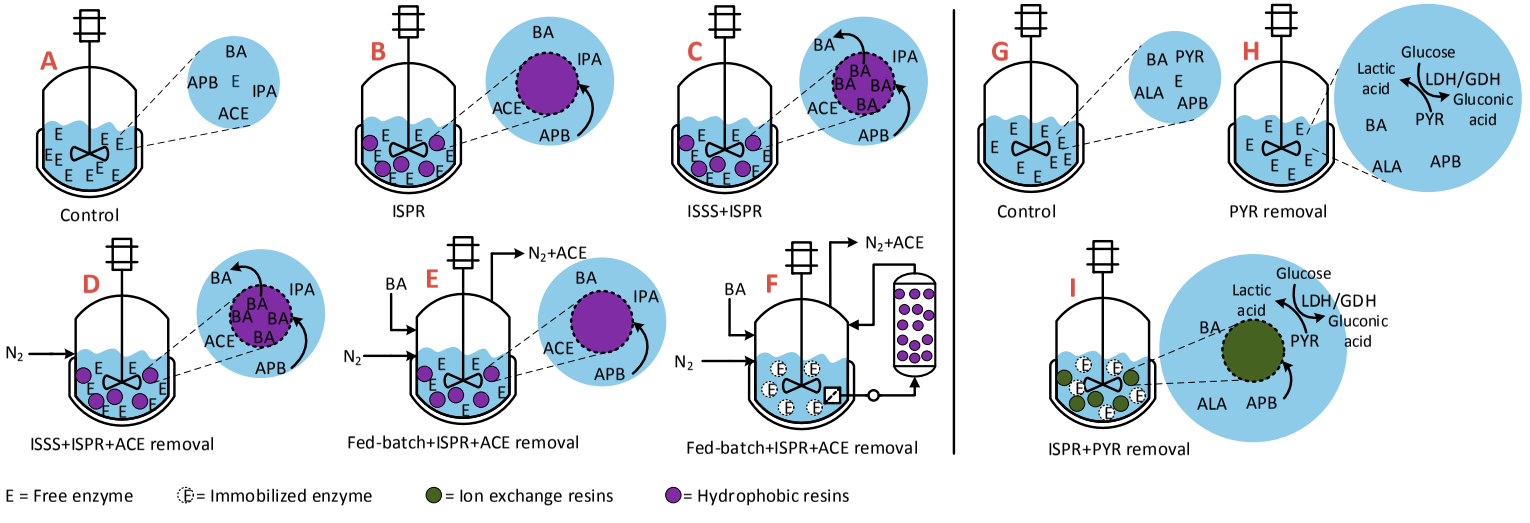


Figure 6.4 Process strategies used to characterize different solutions to increase yield and product concentration.

6.4. Materials and methods

Commercial-grade reagents and solvents were purchased from Sigma-Aldrich (Buchs, Switzerland) and used without further purification. The enzyme ω-TAm (ATA 47) which came as lyophilized CFE powder was supplied by c-LEcta GmbH (Leipzig, Germany). The WC of *E. coli* over expressing ω-TAm (ATA 47) were grown in-house as described in Chapter 2. In all experiments the biocatalyst amount refers to grams of lyophilized CFE or WC powder. Resins were kindly provided by Sigma Aldrich (Buchs, Switzerland) and LANXESS AG (Germany).

HPLC

Samples were measured using an Ultimate 3000 HPLC (Dionex. Sunnyvale, CA, USA). The compounds were separated on a Luna 3 μm C18(2) 100 Å (50 x 4.6 mm) column (Phenomenex, Torrance, CA, USA) at a flow rate of 2 mL/min using a multi-step gradient flow of aqueous 0.1% v/v trifluoroacetic acid and acetonitrile, with the following percentage of acetonitrile: 0 min (0%), 1 min (10%), 2.5 min (10%), 5.9 min (60%), 6 min (0%), 7 min (0%). Compounds were detected at 210 nm (3.9 min for MBA, 5.9 for APB, 7.3 for APH and 7.9 for BA). The quantitative analysis was performed from peak areas by external standards

6.4.1. Screening of polymeric resins for ISPR/ISSS

The screening of hydrophobic and ion exchange resins was done by quantifying their loading capacity towards the products (MBA and APB) and the substrates (APH and BA).

6.4.1.1. Screening of hydrophobic resins

Approximately 50 mg of each of the nine hydrophobic polymeric resins: Lewatit AF 5, Lewatit VP OC 1064 MD PH, Lewatit VP OC 1600 (LANXESS AG, Leverkusen, Germany), Amberchrom CG300, Amberlite XAD1180N, Amberlite XAD7HP, Dowex Optipore L-493 (Dow Chemical, Midland, MI, USA) and Diaion HP-20SS and Sepabeads SP850 (Mitsubishi Chemical, Tokyo, Japan) were initially washed with 100 mM k-PBS (pH 7) and kept in solution for 30 min at room temperature and with soft agitation (~400 rpm - HLC Biotech, Model 11, Pforzheim, Germany) to allow swelling of the resins. After that the buffer

solution was removed and to each of the resins was added approximately (in separated vessels): 0.3 M MBA, 0.05 M APH, 0.04 M of APB, 0.01 M of BA, 2.1 M ACE and 2 M IPA, all prepared in 100 mM k-PBS (pH 7) to a volume of 2 mL. The solutions were mixed for 24 hours, and after that 500 μL samples from the aqueous phase were taken and analysed. The amount adsorbed onto the solid phase was calculated by difference from the starting concentrations. The concentration of IPA was measured ex-situ on an UV 1800 Spectrophotometer (Shimadzu, Kyoto, Japan) quantified at wavelength of 590 nm with fixed temperature of 30 °C using protocols developed by Rahman and co-workers [170]. The concentration of ACE was measured ex-situ on an UV 1800 Spectrophotometer (Shimadzu, Kyoto, Japan) quantified at wavelength of 280 nm with fixed temperature of 30 °C using an internal standard. The concentrations of BA, APH, MBA and APB were measured by HPLC as described above.

6.4.1.2. Optimization of hydrophobic resin loading

In order to understand how the loading of the selected resin (Lewatit AF 5) affected the adsorption of products and substrates, approximately 0.4 M of BA, APH, MBA and APB (prepared in 100 mM k-PBS pH 7 to a volume of 2 mL) were incubated with 100 g/L and 150 g/L of resin for 24 hours at approximately 400 rpm and 30 °C (HLC Biotech, Model 11, Germany). Samples (500 μL) were taken from the aqueous phase, diluted in acetonitrile and analysed by HPLC.

6.4.1.3. Screening of ion exchange resins

Approximately 50 mg of each of the two ion exchange resins (Lewatit MonoPlus SP 112 and Lewatit K 2629) were initially rinsed once with 1M HCl and afterwards with 100 mM with k-PBS (pH 7) in order to remove the HCl and ensure neutral pH for 30 min at room temperature and with mild agitation (~400 rpm - HLC Biotech, Model 11, Pforzheim, Germany). After that the buffer solution was removed and to each of the resins was added approximately (in separated vessels) 0.3 M MBA, 0.05 M APH, 0.05 M of APB, 0.014 M of BA, all prepared in 100 mM k-PBS (pH 7) to a volume of 2 mL. The solutions were mixed for 24 hours, and after that 500 μL sample from the aqueous phase were taken and analysed by HPLC.

6.4.1.4. Optimization of ion exchange resins resin

Increasing loadings (50-800 g/L) of the selected resin (Lewatit MonoPlus SP 112) were initially washed with 1 M HCl and afterwards with 100 mM with k-PBS (pH 7) in order to remove the HCl and ensure neutral pH. Afterwards, approximately 0.35 M of BA and 0.24 M of APB were prepared separately to a volume of 2 mL in 100 mM k-PBS pH 7, was added to each of the vials containing different resin loadings. The solution were mixed for 24 hours at 400 rpm and 30 °C (HLC Biotech, Model 11, Pforzheim, Germany). After that, samples of 500 µL were taken from the aqueous phase and analysed by HPLC.

6.4.2. Rapid characterization of process strategies

6.4.2.1. Process strategies featuring hydrophobic resins and ACE removal

In all the experiments the resins were washed with 100 mM k-PBS for 1 hour to allow swelling prior usage. The excess of liquid was removed using syringes.

For experiments where nitrogen was used to evaporate the ACE, the reactor was pre-installed with an open output where the gas could freely flow out the vessel to an exhaust installed above the setup.

The biocatalyst used in this section was a S-TAm (ATA 47 from c-LEcta GmbH, Germany) formulated as CFE or WC lyophilized powders.

6.4.2.1.1. Acetone removal assay

The effect of nitrogen sweeping to remove the ACE was tested in the production of MBA. The nitrogen was supplied at a flow rate of c.a. 1.7×10^{-6} $m^3.s^{-1}$ and saturated in two consecutive vessels containing water vigorously mixed prior to its addition to the reactor (Appendix 6C). The reaction mixture was composed by 1 M IPA, 0.5 M APH, 2 mM PLP, and 0.1 M k-PBS (pH 7) and 27.4 g/L WC was used. The temperature at the saturation vessels and reactor were kept at 30 ˚C. Reaction were ran at 2 mL scale with agitation kept at 400 rpm (HLC Biotech, Model 11, Pforzheim, Germany). Samples were taken at 2, 4, 7 and 24 hours and analysed by HPLC.

6.4.2.1.2. Process characterization assay

A setup was prepared to test the 6 process options simultaneously (Appendix 6C). For all the reactions the pH was kept at 7 using 100 mM k-PBS (pH 7), the temperature at 30 ˚C and agitation at 400 rpm (HLC Biotech, Model 11, Germany). The co-factor (PLP) concentration was 2 mM. In the reactions A-E 5 g/L of CFE was used. For reaction F 5 g/L of CFE were immobilized using 50 mg of Relizyme HA403/M (with approximately 100% of immobilization yield).

The reactions conditions for each of the scenario (A-F) are summarized in Table 6.3, where the ISPR resins refers to Lewatit AF5 (LANXESS, Germany) and the feed in scenarios E and F was guaranteed using a syringe pump (Harvard Apparatus, Model 11 Plus, Holliston, MA, USA) filled with pure BA (c.a. 6..66 M) (Appendix 6C).

Table 6.3: Conditions for the different process options. i: BA was fed for 24.5 h, but reaction proceeded for 48 h in total; ii: BA was fed for 48 h, but reaction proceeded for 65 h in total, the recirculation through the column was made at a flow of 1.5 ml/min.

	Process strategies					
	A	**B**	**C**	**D**	**E**	**F**
[BA] M	0.4	0.4	0.4	0.4	0.42	0.22
[IPA] M	1	1	1	1	1	1
Volume (mL)	2	2	2	2	2	4
ISPR Resin (g/L)	0	100	100	100	250	250
Reaction time (h)	48	48	48	48	48[i]	65[ii]
Feed (µmol BA/h)	-	-	-	-	3.68E-02	1.93E-02

In the end of the reaction, samples were taken from the aqueous phase, diluted and analysed by HPLC. The resins were recovered by removing the aqueous phase using a syringe. 4 mL of acetonitrile was added to each of the vials containing resin and mixed vigorously for 2 hours. Afterwards samples were taken from the liquid phase and once again analysed by HPLC. The liquid was again removed using a syringe and fresh acetonitrile was added and the elution process was repeated two more times.

6.4.2.2. Process strategies featuring ion exchange resins and PYR removal

In all the experiments, the resins were pre-washed with 1 mM HCl for 5 minutes and the liquid was removed afterwards. In order to re-set pH to 7, the resins were washed several times using 100 mM k-PBS, pH 7. The excess of liquid was removed afterwards using a syringe.

6.4.2.2.1. Whole-cell catalysis

The WC experiments were run using 1 M ALA and 30 mM APH as substrates in 2 mL scale. The PLP concentration was 2 mM in all the cases. The glucose concentration in the three scenarios was: 0 mM, 31.5 mM and 100 mM. The WC concentration was 10 g/L. Samples were taken over a period of 24 hours and analysed by HPLC.

The PYR concentration was measured by HPLC using a Rezex ROA-Organic Acid H+ (300x7.8 mm) column (Phenomenex, Torrance, CA, USA) at a flow rate of 1 mL min^{-1} using an isocratic gradient flow of 0,025 M sulphuric acid.

For the ISPR reaction involving WC, 400 g/L of resin was added to reaction mixture. 100 In the end of reaction 100 µL sample was taken and diluted in 400 µL 1 M HCl. The remaining supernatant was discarded. The resin was washed multiple times with 2 mL 1 M HCl.

6.4.2.2.2. Process characterization assay

The experiments G and H were carried out by Harrie Straatman at DSM Innovative Synthesis, Geleen, The Netherlands. The data and the following protocol was kindly supplied as result of the collaboration established with the company for means comparison. For all the reactions the temperature was kept at 30 ˚C and the pH at 7.5 by automatically controlling the supply of 1 M NaOH (pH-stat Tititrino plus, 877, Metrohm, Switzerland). The reaction volume was 10 mL and the CFE of (R)-ω-TAm (DSM) concentration was 47.4 g/L for reactions G and H and 40.02 g/L for I (immobilized on 9.1 g of Sepabeads EC-EA/M with an equivalent immobilization yield of 84.4%). The PLP concentration was 1 mM.

The overall reaction conditions for each of the experiments (G-I) are summarized in Table 6.4, where the ISPR resins refers to Lewatit MonoPlus SP 112 (LANXESS, Germany). The LDH was obtained from Codexis (California, USA) and GDH was obtained from DSM Innovative Synthesis (Geleen, The Netherlands). Homogenous samples (of 100 uL to 500 uL) were taken during the reaction (at 0, 2, 4 and 6 hours), diluted with acetonitrile (in 10 to 25 mL volumetric flasks) and analysed by HPLC. In the end of the reaction (24h) the pH of the reaction mixture was increased to 11 by adding 1 M NaOH in order to release the product from the resins and afterwards the entire content of the reactor was diluted in acetonitrile (500 mL) and vigorously mixed for 2 hours. Samples were taken from the resulting liquid phase and analysed by HPLC.

Table 6.5 Conditions for the different process options using enzymatic cascades and ion exchange resins.

	Process strategies		
	G	**H**	**I**
[BA] M	0.10	0.18	0.2
[ALA] M	0.19	0.36	0.4
Volume (mL)	10	10	10
LDH/GDH (g/L)	-/-	5/5	1/1
NAD (g/L)	-/-	5/5	1/1
CFE (g/L)	47.4	47.4	40.02
ISPR Resin (g/L)	0	100	100
Reaction time (h)	24	24	24

6.5. Results and discussion

The results are presented divided in two parts. In the first part it is presented the results for the screening of suitable polymeric resins for implementing ISPR and ISSS (section 6.5.1), and in the second the suitable resins were used to implemented the different process strategies (section 6.5.2).

6.5.1. Screening of polymeric resins for ISPR/ISSS

6.5.1.1. Screening of hydrophobic resins

The nine resins composing the hydrophobic resins library (Table 6.2) were screened for their capacity to adsorb the substrates and products involved in the two model reactions system in study (production of MBA and APB using IPA as the amine donor).

After 24 hours mixing the compounds in the presence of the different resins, it was expected that mass transfer equilibrium was achieved. The concentration of compounds in the aqueous phase was used to estimate the amount adsorbed onto the resins. The results are shown in Figure 6.5 A (for the amine products) and B (for the ketone substrates).

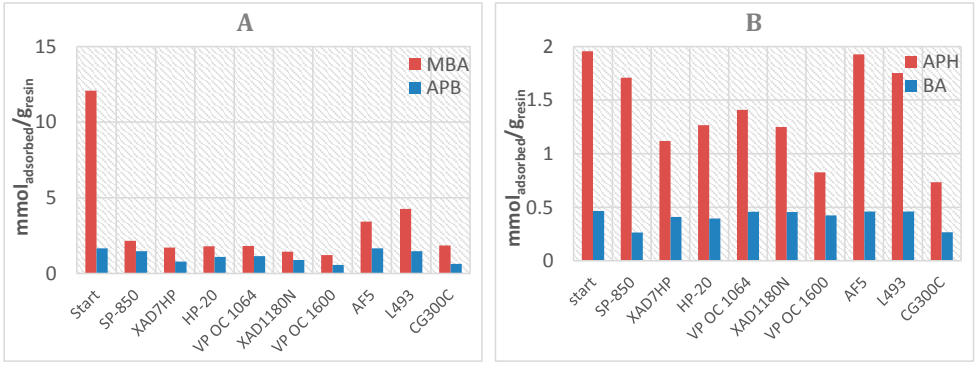


Figure 6.5: Screening of hydrophobic resins based on their adsorption capacity towards MBA and APB (A) and APH and BA (B). Resin = 25 g/L; MBA = 0.3 M; APB = 0.04 M; APH = 0.05 M and BA = 0.01 M.

Two resins (Lewatit AF5 and Dowex Optipore L493) were observed to have a superior capacity to adsorb both the amine products and the substrates better than all the other

resins. This is likely to be related to the superior surface area of these two resins in comparison to the others (Table 6.2).

To select between these two resins, the amine donor (IPA) and the co-substrate (ACE) were evaluated for their affinity towards each of the resins. Ideally, only a small concentration of these compounds would be expected to have affinity towards the resins since they are fully miscible in aqueous phase with LogP values lower than 1 (Table 5.7). However, it was observed that both resins adsorbed some of these compounds (Figure 6.6). This is in part due to the high concentrations these compounds that was used in this assay (2 M for both compounds). Nevertheless, a difference in affinity was observed. The resin Lewatit AF5 showed slightly a lower adsorptive capacity towards both IPA and ACE.

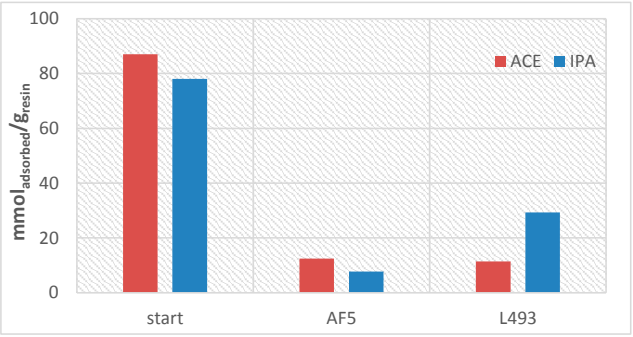


Figure 6.6: Adsorption of IPA and ACE on the resins AF5 and L493.
Resin = 25 g/L; IPA = ~2 M and ACE = ~2.1 M

Considering these results, the resin Lewatit AF5 (Figure 6.7) was selected from the library and used in the next set of experiments where the different process strategies featuring this resin is tested.

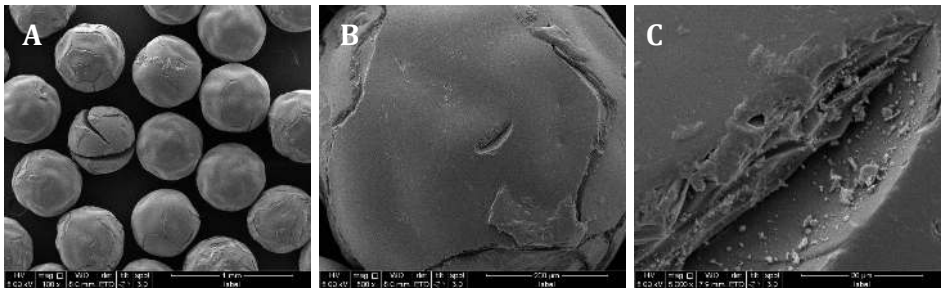


Figure 6.7: SEM pictures of Lewatit AF5 (A = 100 x magnification, B = 500x and C = 5000 x)

6.5.1.2. Optimization of hydrophobic resin loading

As demonstrate in Chapter 3, small concentrations of products caused severe inhibition to the enzyme. For this reason, in order to be effective, the ISPR strategy needs ensure low product concentrations in the media. As the results in the previous sections demonstrates, the hydrophobic resins also have great affinity towards the ketone substrates considering their hydrophobic characteristics (Table 5.7). This suggest a potential substrate-product separation issue, as discussed in Chapter 5. This can potentially make difficult to guarantee an efficient and complete removal of the product without compromising the availability of substrates.

To better illustrate this, two resin loadings (100 g/L and 150 g/L) were used in the presence of approximately 0.4 M of each of the ketone substrates and amine products, and the results are shown in Figure 6.8.

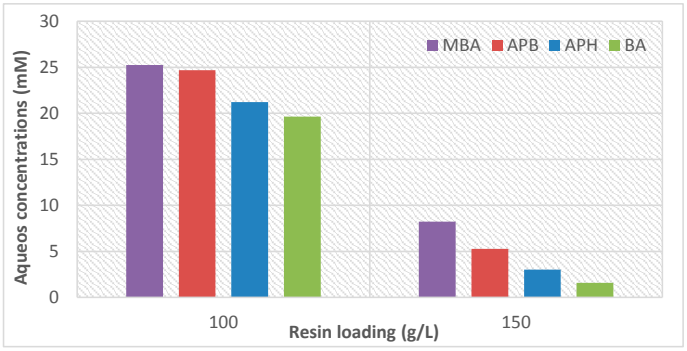


Figure 6.8 Adsorption of approximately 0.4 M of APB, MBA, APH and BA in function of resin (Lewatit AF5) loading after 24 hours equilibration.

As it can be observed, with a resin loading of 100 g/L, the aqueous concentration of the inhibitory products are approximately 25 mM for both APB and MBA. Considering the results obtained in Chapter 3, it can extrapolated that at this product concentration the inhibition will affect 100% of the enzymes (lowering the reaction rate to zero). On the other hand, at this resin loading the substrates (APH and BA) concentration in the aqueous phase is ideal to guarantee maximum reaction rate. With a resin loading of 150 g/L, the products concentration reduce to approximately 5 mM and 8 mM for APB and MBA respectively. However, also the substrates concentrations in aqueous phase decrease to concentrations that would limit the reaction rate.

This leads to a bottleneck where a compromise between the resin loading and the desired product concentration in the aqueous phase is difficult to achieve. A possible solution could be to use the highest resin loading, since it is important to keep the product concentration in aqueous phase low to prevent the inhibition issues, and supply the substrate either in a fed-batch mode or as a combined ISSS/ISPR strategy. These were tested and the results will be shown and discussed further in this chapter.

6.5.1.3. Screening of ion exchange resins

The two ion exchange resins were screened based on their adsorptive capacity for the ketone substrate (APH) and the corresponding amine product (MBA). Both resins were similar in properties (matrix and particle size), thus it was expected that both would have similar adsorptive capacity towards these compounds. This is illustrated in Figure 6.9, where this can be observed.

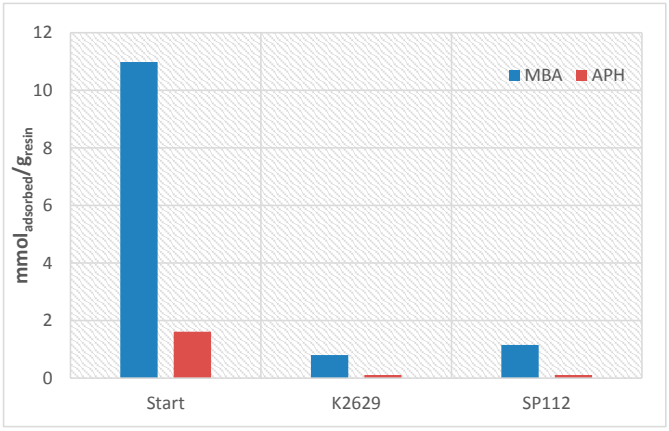


Figure 6.9: Adsorption of approximately 0.3 M of MBA and 0.04 M of APH onto the 25 g/L of resins.

A major difference between this result and the one obtained for the hydrophobic resins, is that the ion exchange resins show lower adsorptive capacity. This is due to the lower surface area of these resins in comparison with the hydrophobic ones (c.a. 40 m^2/g for Lewatit Mono Plus SP 112 in comparison with 1500 m^2/g for Lewatit AF5) although their particle sizes are very similar.

Between the two ion exchange resins, the Lewatit Mono Plus SP 112 (Figure 6.10) was selected for further experiments, since it showed slightly higher adsorptive capacity and it has a slightly larger particle size which can be an advantage for separation.

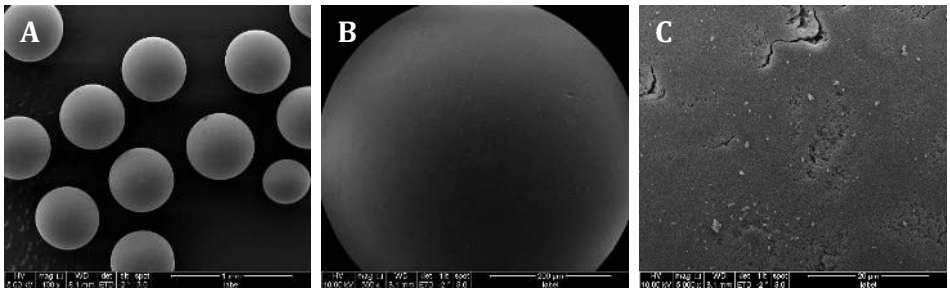


Figure 6.10 SEM pictures of Lewatit Mono Plus SP112 (A = 100 x magnification, B = 500x and C = 5000 x)

6.5.1.4. Optimization of ion exchange resin loading

In order to understand the resin loading required to adsorb the products, a fixed concentration of products APB (c.a. 0.25 M) and MBA (c.a. 0.35 M) were mixed with increasing resins loading. The results are summarized in Figure 6.11 where it can be observed that a loading of about 800 g_{Resin}/L is requited to lower the product concentration to a value below 10 mM. Considering the space this resin loading might occupy in the reactor, a possible strategy would be use an external vessel for the resins (e.g. packed bed column or second tank) where the resins would be retained.

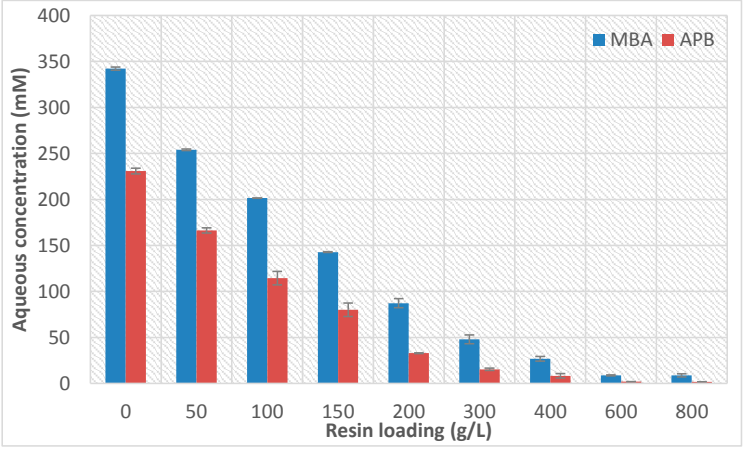


Figure 6.11 Adsorption of c.a. 0.35 M of MBA and 0.25 M APB for increasing resin loading (Lewatit Mono Plus SP 112).

6.5.2. Characterization of process strategies

In this section, it is presented the results regarding the different process strategies used to improve the productivity.

Subsection 6.5.2.1 summarizes the results regarding ISPR, ISSS, IScPR and fed-batch strategies applied to the production of MBA using IPA as the amine donor. ISPR using the hydrophobic resins was implemented to control the product inhibition and ACE removal by means of nitrogen sweeping was applied in order to remove the formed ACE and help displacing the equilibrium. To avoid excessive evaporation of substrate (APH) which occurred when nitrogen sweeping was used, the feeding strategies ISSS and fed-batch were tested.

In subsection 6.5.2.2, the results for ISPR using ion exchange resins are presented. The assay were carried out at 10 mL scale for the reaction system producing APB with ALA as amine donor. This was coupled with IScPR through cascade reactions using the LDH/GDH system. In addition, results regarding whole cell catalysis as a possible alternative for isolated enzymes are shown. This was done using the production of MBA and ALA as the amine donor.

6.5.2.1. Process strategies featuring hydrophobic resins and ACE removal

6.5.2.1.1. Acetone removal assay

As discussed in Chapter 5, the use of an inert gas such as nitrogen to remove the volatile co-substrate (ACE) is not selective resulting in the evaporation of the ketone substrate as well. This is illustrated in Figure 6.12 where it can be seen that the reaction equipped with nitrogen sweeping strategy had most of the substrate (APH) evaporated in the first 6 hours of the reaction. After 24 hours, only a residual concentration of the substrate was left in the reactor. Considering that the substrate disappearance did not correspond to product formation, it can be assumed that the loss occurred as result of evaporation. This clearly demonstrate the non-selective characteristic of this strategy and it seems to be even more critical when insoluble concentrations of the substrate are used.

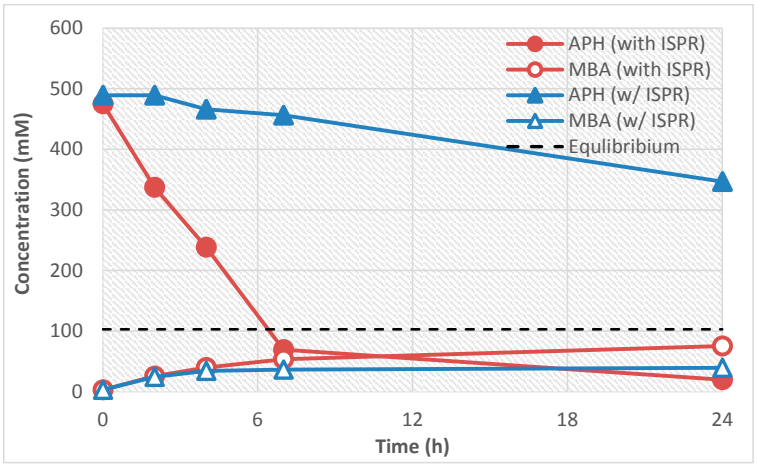


Figure 6.12: Production of MBA over time for ATA 47 with ACE removal (circles) and without ACE removal (triangles). Whole cells (27.4 g/L) were added to 1 M IPA, 0.5 M APH, 2 mM PLP, and 0.1 M potassium phosphate buffer pH 7. The temperature was fixed at 30 ˚C. (dashed line) equilibrium conversion.

In another work where water soluble concentration of APH (c.a. 30 mM) were used instead, it was observed that the degree of substrate lost was inferior (data not shown). This suggest that using a feeding strategy such as ISSS or fed-batch would help overcoming this issue and minimize substrate evaporation.

6.5.2.1.2. Process characterization using hydrophobic resins

The results for the rapid characterization of process strategies featuring hydrophobic resins are presented in Figure 6.13, where it can be seen that the control reaction (A) stopped at approximately 5 g/L of product concentration. Considering that this value is far from the theoretical equilibrium concentrations (c.a. 40 g/L), and based on results observed in Chapter 3, this low performance can be directly associated to inhibition caused by the product. The strategies B to D were carried out using 100 g/L of resins loading. However, this did not result in major improvements in final product concentration obtained. This is most likely due to the resin selectivity issues, as previously discussed. This was observed in strategy B (ISPR only) where large amount of substrate was removed from the aqueous phase upon addition of resin. After 48 hours of reaction, very small concentration of substrate was found left in the aqueous phase while a large amount was recovered from solid phase (data not shown).

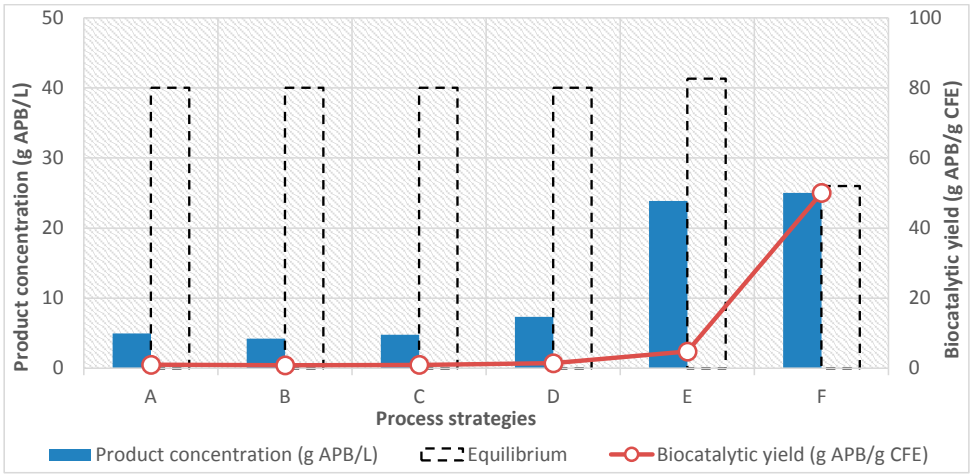


Figure 6.13 Biocatalytic yield and product concentration achieved with different process strategies. (A) reaction without ISPR/ISSS/IScPR (control), (B) ISPR only, (C) ISPR combined with ISSS, (D) ISPR combined with Fed-batch, (E) ISPR combined with Fed-batch and IScPR (acetone removal), and (F) ISPR combined with Fed-batch and IScPR using immobilized enzymes . Approximately 5 g/L (s)-ω-TA CFE (ATA 47, c-LEcta, Germany), 1 M IPA and 0.4 APH, 150 g/L resins (B-D) and 250 g/L (E-F) (Lewatit AF 5).

Loading the substrate onto the resin in advance and implementing the ISSS/ISPR combined strategy (C) helped in controlling the concentration of substrate in the aqueous phase, however, again due to the large amount of product that also accumulated in the aqueous phase, the conversion did not improve further.

In strategy D, nitrogen sweeping was added to the ISSS/ISPR strategy to remove the formed acetone and therefore help shifting the equilibrium position. A slight improvement was observed in comparison to options without nitrogen sweeping (A, B and C). However, the results obtained were still far from the equilibrium yields. Product inhibition and substrate evaporation prevented this strategy to yield better results.

In strategies E and F, the substrate feeding was done using a syringe pump (fed-bath mode instead of ISSS). This allowed a better control of the substrate supply rate. When combined with acetone sweeping using nitrogen, it resulted in better performance. In both cases the product concentration obtained was 5 fold higher than the control (A). However, in case of E, the theoretical equilibrium was still not achieved. This could to be related to the feeding rate of BA which seemed to have been too fast for the reaction rate, resulting in the adsorption of the substrate to the resin. In strategy F, the feeding rate was decreased and immobilized enzymes were used in order to make possible the use of

external column to where the reaction media was re-circulated. This resulted in a better performance with the reaction reaching equilibrium yields, but not continuing further, which suggest inefficiency of the co-product (ACE) removal strategy.

6.5.2.2. Process strategies featuring ion exchange resins and PYR removal

Ion exchange resins combined with cascade reactions were used in the reaction system featuring ALA as amine donor. As previously discussed, reactions featuring ALA as amine donor have a very low thermodynamic equilibrium in comparison with those featuring IPA. On the other hand, the formed PYR can be selectively and completely removed using cascade reactions as introduced in section 5.1.2.4. This have been investigated using whole cells (WC) catalysis and isolated enzymes and the results are presented in the following sections.

6.5.2.2.1. Whole-cell catalysis

In order to investigate whether the use of WC allow removal of pyruvate, and how the initial concentration of glucose (Glu) influences the reaction efficiency, three assays were prepared: i) reaction without initial Glu, ii) with 31.5 mM and ii) with 100 mM. The results are shown in Figure 6.14.

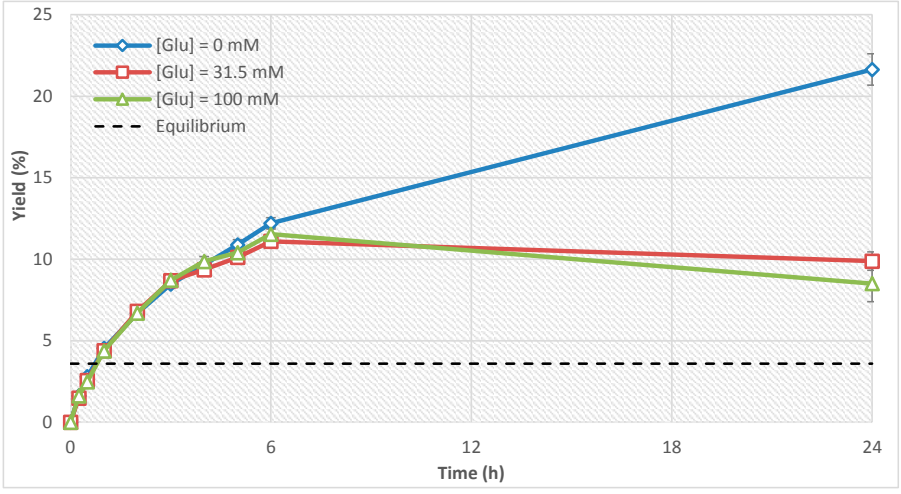


Figure 6.14 Yield of (S)-MBA over time for different initial concentrations of Glucose (0, 31.5 and 100 mM). 1 M ALA and 30 mM APH as substrates; 10 g/L WC.

For all the three scenarios, the reaction proceeded beyond the equilibrium threshold, which suggest a functional mechanism for PYR removal. However, the results suggest that the reaction achieve higher yields when no Glu (0 mM) was added, contrasting with reactions where 31.5 and 100 mM of Glu were used. In order to better understand the reason behind this, the concentration of PYR was followed during the three reactions and the results presented are presented in Figure 6.15 where it can be seen that when the Glu was not added, the PYR was continuously removed throughout the 24 hours of reaction, contrasting with reactions where 31.5 and 100 mM of Glu was added causing PYR production after 6 hours of reaction.

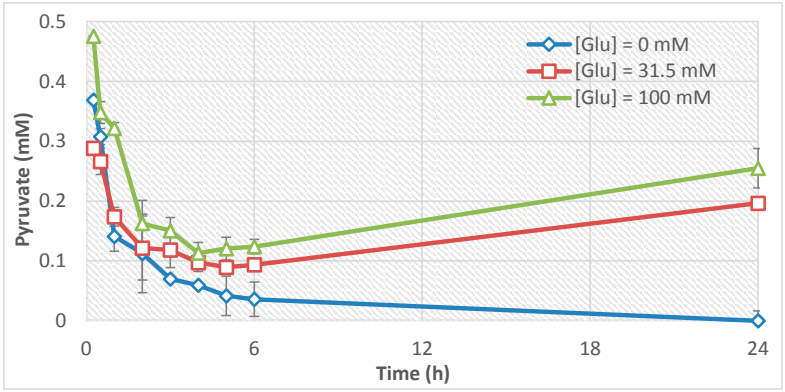


Figure 6.15: Pyruvate concentration over time for reactions using 10 g/L WC, 1 M ALA, 30 mM APH, and Glu concentrations of 0, 31.5 and 100 mM.

Other studies showed before that the metabolism of living cells (*Vibrio fluvialis*) were able to remove pyruvate internally [33]. In this case, lyophilized resting *E. coli* cells were used and still these results suggest that they are metabolically active and therefore, able to remove the co-product PYR internally. The use of resting cells bring the advantage of avoiding biomass formation and potentially increase of product yields on carbon and energy sources [171].However, the results shown in Figure 6.15 also suggest that this cells were also able to undergo through other(s) pathways in the presence of Glu, causing formation of PYR, which affected the final yields as was observed in Figure 6.14.

The cell growth was not measured as this was not expected considering that lyophilized cells were used in this study and the reaction media was not favourable for cell growth.

Lyophilized cells are thought to not be active due to the harsh process they are subjected to during the drying process [172]. If bacterial growth is occurring, it can be also possible that the amine group that is supplied by the amine donor is being used for formation of other metabolites instead. The product can also be degraded by the cells to form other intermediates. Cell growth of supposedly resting cells was also observed in other studies when glucose was added to the reaction medium [173].

An option to ensure that the desired cascade reaction system is predominately working in the cell, is to overexpress all of the enzymes involved in the cascade (LDH and GDH) alongside with ω-TAm. This approach was pursued by several groups [38, 39]. This can also be achieved by using isolated enzymes as it will be demonstrated in the following section.

6.5.2.2.2. Process characterization using ion exchange resins

In contrast with the reactions featuring IPA which are primarily affected by inhibition since the equilibrium is slightly more favourable, in the reactions where ALA is used as the amine donor, the equilibrium becomes the main issue. Without a strategy to displace it, the reaction will not even proceed to the point where inhibition becomes an issue. This can be observed in options G in Figure 6.16 where reaction without any process strategy was carried out.

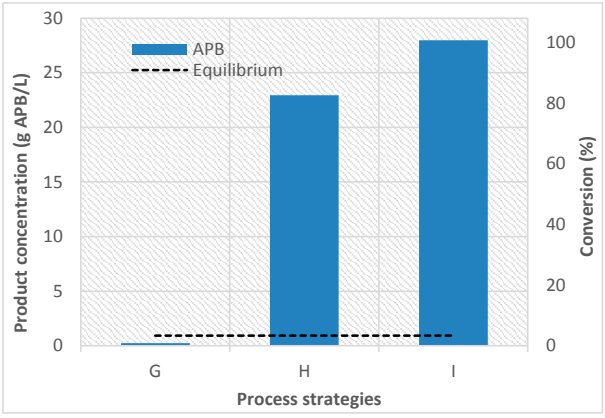


Figure 6.16: Process strategies using (R)-ω-TAm (DSM), LDH/GDH cascade and ion exchange resins. Reactions were run for 6 hours using ~ 0.2 M BA (10 mL scale). G – control reaction; H – reaction with IScPR (LDH/GDH cascade); I – reaction with IScPR (LDH/GDH cascade) and ISPR (ion exchange resins)

On the other hand, when cascade reactions were used (H), higher product concentrations were obtained. However, the reaction did not proceed to completion, due to product inhibition. This was overcome when the ion exchange resins were introduced in the reaction, allowing the reaction to proceed to 100% yield in very short period of time (approximately 4 hours) (I). The production overtime for this assay can be seen in Appendix 6D.

In addition, WC combined with resins were investigated. This was done using ALA as amine donor and APH as amine acceptor. The results are summarized in Figure 6.17 where it can be observed that upon addition of 400 g/L of resins, the final yield is decreased in comparison to the reaction without resin. This result is unexpected since the addition of resin to the isolated enzymes assay showed excellent results. A possible explanation for this could be related to the shear forces caused by the resins to the cells, this could have damaged the cells and the relevant mechanism for PYR transportation and reaction, or other unknown experimental error that may have harmed the cells.

The PYR concentration was measured and found to be 2.90± 0.38 mM, which is very similar to the final product concentration of 3.89 mM. This confirms that the mechanism to remove PYR was not functional, opposite to what was previously observed in absence of resins. The use of a more moderate stirring speed or an external column filled with the resins could be an option to overcome this issue. However, this was not investigated.

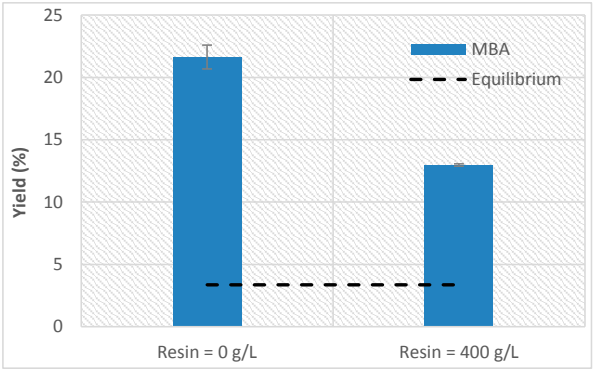


Figure 6.17: Yield of MBA after 24 hours reaction using 1M ALA, 30 mM APH and 10 g/L L-WC

6.6. General discussion

Process engineering strategies have a realistic potential to overcome many of the issues inherent to transaminase catalysed reactions. In this chapter these strategies were successfully demonstrated using polymeric resins to overcome inhibition and nitrogen sweeping or cascade reactions combine with amine donor excess to displace equilibrium. It was made clear that the selectivity of the technology is a very important parameter as it will dictate the efficiency of the implemented strategy. In order to guarantee the use of the right strategy, it is important to implement a smart but also rapid screening process.

In this work, the polymeric resins were screened using mainly their loading capacity towards the compounds. This approach disregards the selectivity of these resins and their adsorptive/adsorptive kinetics, but on the other hand guarantees a very fast screening among a library of resins with similar properties.

In the case of hydrophobic resins, the screening process is challenging since these resins has good affinity for both the ketone substrate and the amine product. This issue is also present in cases when organic solvents are used for ISSS and/or ISPR, although this also has the additional challenge of toxicity for the biocatalyst.

The ion exchange resins combined with the use of ALA s the amine donor, guaranteed a better selectivity towards the products. The main disadvantage of using ALA is the unfavourable equilibrium which becomes critical. However, as it was here demonstrated, using cascade reactions to remove the co-product PYR through the use of LDH/GDH cascade system can solve this issue and make the reaction go even faster than some other reaction system with better thermodynamic equilibrium.

From this work it also became clear how the choice of amine donor influences the process and the strategies needed to solve the several bottleneck inhered with transamination. For instance, when IPA is used as the amine donor, the equilibrium constant, which is still unfavourable towards the products, is however higher by ratio of 1.000-10.000 than the equilibrium constant when ALA is used as donor. This difference causes a tremendous change in the driving bottleneck for the process. When ALA is used, the main driving

bottleneck is the equilibrium which prevents the reaction to even yield products concentration that can inhibit the reaction. On the other hand, when IPA is used as amine donor, the equilibrium ceases to be the main bottleneck giving place to the inhibition instead, which in turn forces the reaction to stop long before equilibrium yields are achieved.

Nevertheless, in this work it has been demonstrated that ISPR and IScPR can play an important role. The use of excess of amine donor was used in every experiments here reported, however it is the removal of the products and co-products that have the major impact.

Figure 6.18 A (reaction using IPA as amine donor and hydrophobic resins) and B (reaction using ALA as amine donor and ion exchange resins) summarizes the major enhancements obtained with the different strategies. The combination of product and co-product removal allow obtaining higher product concentration, while the use of immobilized biocatalyst allow obtaining higher biocatalytic yield (considering the possible re-utilization of the biocatalyst).

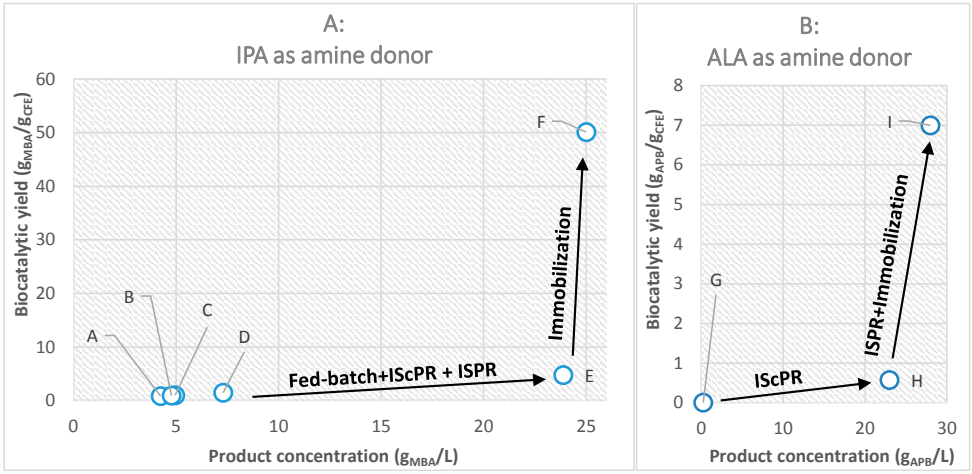


Figure 6.18: Summary of results obtained using the different strategies. (A) Control reaction (without ISPR/ISSS/IScPR); (B) ISPR only; (C) ISPR combined with ISSS; (D) ISSS/ISPR combined IScPR (acetone removal using nitrogen sweeping); (E) ISPR combined with Fed-batch and IScPR (acetone removal using nitrogen sweeping); and (F) ISPR combined with Fed-batch and IScPR (acetone removal using nitrogen sweeping) using immobilized enzymes. (G) Control reaction (no IScPR/ISPR); (H) reaction with IScPR (LDH/GDH cascade); (I) reaction with IScPR (LDH/GDH cascade) and ISPR (ion exchange resins).

6.7. Conclusion

This work allows the conclusion that ISPR and IScPR tools are able to effectively improve productivity in transaminase catalysed reactions. The main challenge associated with these technologies is the need for screening and selection of a suitable auxiliary phase, which can be solid, liquid or gas. Two main criteria can be considered to be important in the screening process: the toxicity of the support towards the biocatalyst and the selectivity towards the product in detriment of the substrates.

Ion exchange resins were found to be more suitable for ISPR in transaminase catalysed reactions when ALA was used as the amine donor, in comparison with the hydrophobic resins (used when IPA was employed as the amine donor). The former is more selective and allowed selective removal of the protonated product. Similarly, the use of cascades reactions to remove the co-product PYR when ALA is used as amine donor was shown to be more selective and efficient than using nitrogen to remove the ACE when IPA was used as amine donor.

The process engineering strategies here discussed are able to overcome the challenges associated with transamination catalysed by ω-TAm. The feeding of the substrate, product and co-product removal allow achieving higher product concentration (g/L) while the use of immobilized biocatalyst allowed obtaining higher biocatalytic yield ($g_{product}/g_{biocatalyst}$).

7 Conclusions and future perspectives

Biocatalysis offers a wide range of possibilities to access many interesting compounds with key function in the modern days. The technology has great potential as well as many open challenges that need to be addressed so that full advantage of its benefit can be seen.

This thesis focused on the interesting case study of ω-transaminase catalysed reactions which is one of the most acclaimed processes in the past decade to asymmetrically access chiral amines. The enzyme allow access to enantiomerically active amines which are the important building blocks of many pharmaceutical intermediates. Throughout this thesis, an overview of the main challenges associated with the process development for this enzyme was made. Among these, product inhibition and the unfavourable thermodynamic equilibrium are to be highlighted. Each of these limitations prevent the maximum theoretical yield to be achieved and the combination of both directly translate in the process infeasibility.

It was demonstrated in this thesis that these two limitations affected the enzyme at different degree, depending on the amine donor chosen for the process. For instance:

- **when isopropylamine was used as the amine donor** the inhibition seemed to be the major issue, preventing the reaction to reach the theoretical equilibrium yields;
- on the other hand, **when alanine was used as the amine donor**, the thermodynamic equilibrium constants drastically lowers (1000-10000 fold compared to when isopropylamine is used). Thus the reaction stop before product inhibition becomes an issue.

A key finding in this thesis, was the difference in the inhibition level depending on the choice of the amine donor. Reactions featuring alanine was shown to be more severely affected by inhibition than those featuring isopropylamine. The reaction rate rapidly dropped to zero with small product concentrations. However, these results also suggest the need for further research on his matter as the equilibrium might as well be the responsible for this behaviour.

Interestingly, the choice of amine donor also influences the type of technology that can be used to overcome these limitations. The use of isopropylamine prevents the use of the *in situ* product removal strategy using ion exchange resins, due to similar charge properties between the amine donor and the amine product. As alternative hydrophobicity can to be used to remove the product. However, also this option revealed to be inefficient due to selectivity issues between the ketone substrate and the chiral amine, since both are similar in terms of hydrophobicity. This issue can potentially be solved by employing a controlled feeding strategy, and synchronize this with the enzyme reaction rate, in order to ensure that the supplied substrate is converted into product before being adsorbed onto the resin. In order to this, it is important know the kinetic of the adsorption beforehand. This possibility is an open challenge that is part of future work.

Another possibility, also demonstrated in this thesis, is to use alanine as the amine donor. Being alanine an amino-acid, it has two protonation levels which provide an isoelectric point of c.a. 6. This can be used to neutrally charge the compound, allowing the use of ion exchange resins for ISPR. This was demonstrated here with excellent results, although an optimization of the enzyme loading is required in order to increase the biocatalytic yield ($g_{product}/g_{biocatalyst}$) which was slightly lower than those obtained in the experiments featuring hydrophobic resins.

With the results here obtained it can be concluded that alanine is a better amine donor than isopropylamine as it allows the use of cascade reactions using LDH/GDH system to selectively remove the co-substrate pyruvate (IScPR) and also allow using ion exchange resins to selective remove the formed chiral amine product (ISPR).

It was demonstrated that the key factor for an efficient implementation of the product and co-product removal strategies is the selectivity of the technology used. This was evident specially when using acetone removal strategy (to shift equilibrium when isopropylamine was used as the amine donor). Although a substantial improvement in the yield and final product concentration was observed (for the combination of fed-batch/ISPR/acetone removal), the process resulted in major loss of the ketone substrate by evaporation. On the other hand, the use of enzymatic cascade reactions represent a more efficient strategy. The major limitation of this technology can be the selection of suitable enzymes to remove the co-product and the need for co-factor regeneration, which often requires the use of extra enzymes, increasing the process costs. Therefore, it is important to balance the benefits with the costs.

An options to overcome this issue, can be to immobilize the enzymes in the same support. This allow the possibility of re-using the biocatalysts for many cycles, as was demonstrated, which would help reducing the costs. A still open challenge in this topic relies on the co-immobilization of many enzymes in the same support. This can be crucial in order reduce the space the immobilized biocatalysts occupy in the rector. Co-immobilization of the co-factors is also a possibility that can be explored to reduce the costs.

In this thesis, ω-TAm enzymes have been immobilized using commercially available polymeric resins. It was possible to re-use the immobilized enzyme over 8 times which corresponds to more. 250 hours of operation in reaction condition with more than half of its initial activity left. More importantly, the outcome of this work opened the possibility to a more efficient process and allow a comprehensive step-wised carrier selection for immobilization of ω-TAm. The screening methodology used can be implemented for other enzymes and type of carriers. The properties identified in this work are believe to be equally suitable to other forms of this enzyme, from other microorganism. In summary, **particles with diameter in the range of 0.2-06 mm or higher, with a pore diameter in the range of 40-60 nm and preferably with long chain functional groups** should be suitable for other ω-TAm of similar molecular weight.

7.1. Future works

Several open challenges and potential future works have become apparent during the writing of this thesis. These have not been pursued due to time limitation.

- Characterization of the biocatalyst: During the comparison of the three ω-TAm mutants, the reaction rates were found to be very different for ATA 40, ATA 44 and ATA 47. While the latter was demonstrated to have better tolerance towards substrate and products, it would have been useful to measure the protein content for each of these enzymes in order to understand if the difference in activity was anyhow related to a better expression of ω-TAm in ATA 47;
- The same could have been done during the immobilization process, between the cycles. A protein quantification in the aqueous phase would have allowed understanding if the activity loss throughout the cycles were related with enzyme leaching or simply enzyme deactivation.
- Also in the immobilization work, it would have been interesting to include covalent resins from other manufacturer in the study for better quality of this work. Another alternative that was not explored is the derivatization of the ionic exchange resins using glutaraldehyde. This would have allow covalent immobilization of the enzymes.
- The co-immobilization of cascade enzymes (LDH and GDH) as well as the co-factors was attempted, but never completed, remaining as an open challenge.
- The screening methodology used to select resins for ISPR and ISSS, allowed a very quick selection a suitable resin. However, the addition of a step where further characterization of the selected resin were performed would have allowed a better understanding of the potential of this technology. For instance, isotherms could have been used to understand the adsorption/desorption kinetics. This would have been of great help during the process characterization step, where the lack of this information possibly resulted in the major adsorption of the substrates onto the hydrophobic resin.

- For reactions where fed-batch were approach were used, it is believed that further optimization of the substrate supply rate (matching this with the reaction rate for instance) would have resulted in a better performance of the system.
- The experiments involving nitrogen sweeping could have been further optimized if the gas flow rate for instance have been investigated in order to find the most suitable for the scale used.
- The results obtained using the ion exchange and the hydrophobic resin cannot be compared, considering that different biocatalysts and different reaction systems were used in each. The work successfully demonstrates the potential of each of these technologies and could have improved if all these technologies were implemented using both enzymes and both reaction systems.
- The importance of selecting a suitable amine donor was made clear in this work. The results here obtained suggests the need for further investigation of the effect of each amine donor on inhibition.
- Economic evaluation and scale up are very important part of the early stage process development. However, it was not possible to pursue any of these. The quality of this work would have been enhanced if the influence of each process strategy (tested in Chapter 6) on the economics would have been investigated.
- Finally, the scale up of the most successful strategies (Fed-batch/ISPR/acetone removal for reactions using IPA and ISPR/cascade reactions for reaction using ALA) would have been useful to validate these strategies and further optimize them.

References

[1] N.J. Turner, M.D. Truppo, Biocatalytic Routes to Nonracemic Chiral Amines, *Chiral Amine Synthesis*, 2010, 431-459.

[2] T.C. Nugent, M. El-Shazly, Chiral Amine Synthesis – Recent Developments and Trends for Enamide Reduction, Reductive Amination, and Imine Reduction, *Advanced Synthesis & Catalysis* 352 (2010) 753-819.

[3] M. Breuer, K. Ditrich, T. Habicher, B. Hauer, M. Keßeler, R. Stürmer, T. Zelinski, Industrial Methods for the Production of Optically Active Intermediates, *Angewandte Chemie International Edition* 43 (2004) 788-824.

[4] M. Hoehn, U.T. Bornscheuer, Biocatalytic Routes to Optically Active Amines, CHEMCATCHEM 1 (2009) 42-51.

[5] D. Koszelewski, K. Tauber, K. Faber, W. Kroutil, ω-Transaminases for the synthesis of non-racemic α-chiral primary amines, *Trends in Biotechnology* 28 (2010) 324-332.

[6] T. Vries, H. Wynberg, E. van Echten, J. Koek, W. ten Hoeve, R.M. Kellogg, Q.B. Broxterman, A. Minnaard, B. Kaptein, S. van der Sluis, L. Hulshof, J. Kooistra, The Family Approach to the Resolution of Racemates, *Angewandte Chemie International Edition* 37 (1998) 2349-2354.

[7] A. Johansson, Methods for the asymmetric preparation of amines, *Contemporary Organic Synthesis* 2 (1995) 393-407.

[8] K. Faber, W. Kroutil, New enzymes for biotransformations, *Current Opinion in Chemical Biology* 9 (2005) 181-187.

[9] H.B. Kagan, J.C. Fiaud, Kinetic Resolution, *Topics in Stereochemistry*, 1988, 249-330.

[10] F. van Rantwijk, R.A. Sheldon, Enantioselective acylation of chiral amines catalysed by serine hydrolases, *Tetrahedron* 60 (2004) 501-519.

[11] E. García-Urdiales, V. Gotor, I. Alfonso, Enantioselective enzymatic desymmetrizations in organic synthesis, *Chemical Reviews* 105 (2005) 313-354.

[12] F. Balkenhohl, K. Ditrich, B. Hauer, W. Ladner, Optically active amines via lipase-catalyzed methoxyacetylation, *Journal Fur Praktische Chemie-Chemiker-Zeitung* 339 (1997) 381-384.

[13] Y. Ahn, S.-B. Ko, M.-J. Kim, J. Park, Racemization catalysts for the dynamic kinetic resolution of alcohols and amines, Coordination *Chemistry Reviews* 252 (2008) 647-658.

[14] M.T. Reetz, K. Schimossek, Lipase-catalyzed dynamic kinetic resolution of chiral amines: Use of palladium as the racemization catalyst, *CHIMIA* 50 (1996) 668-669.

[15] N. Turner, I. Fotheringham, R. Speight, Novel Biocatalyst Technology for the Preparation of Chiral Amines, *ChemInform* 36 (2005).

[16] R. Carr, M. Alexeeva, A. Enright, T.S.C. Eve, M.J. Dawson, N.J. Turner, Directed Evolution of an Amine Oxidase Possessing both Broad Substrate Specificity and High Enantioselectivity, *Angewandte Chemie -International Edition* - 42 (2003) 4807-4810.

[17] R. Carr, M. Alexeeva, M.J. Dawson, V. Gotor-Fernández, C.E. Humphrey, N.J. Turner, Directed Evolution of an Amine Oxidase for the Preparative Deracemisation of Cyclic Secondary Amines, *ChemBioChem* 6 (2005) 637-639.

[18] C.M. Clouthier, J.N. Pelletier, Expanding the organic toolbox: a guide to integrating biocatalysis in synthesis, *Chemical Society Reviews* 41 (2012) 1585-1605.

[19] F.F. Noe, W.J. Nickerson, Metabolism Of 2-Pyrrolidone And Gamma-Aminobutyric Acid by *Pseudomonas-Aeruginosa*, *Journal of Bacteriology* 75 (1958) 674-681.

[20] K.H. Kim, Purification And Properties Of A Diamine Alpha-Ketoglutarate Transaminase from *Escherichia Coli*, *The Journal of biological chemistry*. 239 (1964) 783-786.

[21] D. Stirling, A. Zeitlin, G. Matcham, Enantiomeric enrichment and stereoselective synthesis of chiral amines, in: EPO (Ed.), Celgene, 1990.

[22] G.W. Matcham, A.R.S. Bowen, Biocatalysis for chiral intermediates: Meeting commercial and technical challenges, *Chimica Oggi-Chemistry Today* 14 (1996) 20-24.

[23] P. Tufvesson, W. Fu, J.S. Jensen, J.M. Woodley, Process considerations for the scale-up and implementation of biocatalysis, *Food and Bioproducts Processing* 88 (2010) 3-11.

[24] J. Ward, R. Wohlgemuth, High-Yield Biocatalytic Amination Reactions in Organic Synthesis, *Current Organic Chemistry* 14 (2010) 1914-1927.

[25] T. Vaijayanthi, A. Chadha, Asymmetric reduction of aryl imines using Candida parapsilosis ATCC 7330, 19 (2008) 93-96.

[26] J. Steinreiber, M. Schürmann, M. Wolberg, F. van Assema, C. Reisinger, K. Fesko, D. Mink, H. Griengl, Overcoming Thermodynamic and Kinetic Limitations of Aldolase-Catalyzed Reactions by Applying Multienzymatic Dynamic Kinetic Asymmetric Transformations, *Angewandte Chemie International Edition* 46 (2007) 1624-1626.

[27] M. Iwaki, T. Yagi, K. Horiike, Y. Saeki, T. Ushijima, M. Nozaki, Crystallization and properties of aromatic amine dehydrogenase from Pseudomonas sp, 220 (1983) 253-262.

[28] N. Itoh, C. Yachi, T. Kudome, Determining a novel NAD+-dependent amine dehydrogenase with a broad substrate range from Streptomyces virginiae IFO 12827: purification and characterization, 10 (2000) 281-290.

[29] M.D. Toney, Reaction specificity in pyridoxal phosphate enzymes, *Archives of Biochemistry and Biophysics* 433 (2005) 279-287.

[30] M.D. Truppo, J.D. Rozzell, N.J. Turner, Efficient Production of Enantiomerically Pure Chiral Amines at Concentrations of 50 g/L Using Transaminases, *Organic Process Research & Development* 14 (2010).

[31] C.K. Savile, J.M. Janey, E.C. Mundorff, J.C. Moore, S. Tam, W.R. Jarvis, J.C. Colbeck, A. Krebber, F.J. Fleitz, J. Brands, P.N. Devine, G.W. Huisman, G.J. Hughes, Biocatalytic Asymmetric Synthesis of Chiral Amines from Ketones Applied to Sitagliptin Manufacture, *Science* 329 (2010).

[32] P. Tufvesson, J. Lima-Ramos, J.S. Jensen, N. Al-Haque, W. Neto, J.M. Woodley, Process considerations for the asymmetric synthesis of chiral amines using transaminases, *Biotechnology and Bioengineering* 108 (2011) 1479-1493.

[33] J.S. Shin, B.G. Kim, Asymmetric synthesis of chiral amines with omega-transaminase, *Biotechnology and Bioengineering* 65 (1999).

[34] M.D. Truppo, J.D. Rozzell, J.C. Moore, N.J. Turner, Rapid screening and scale-up of transaminase catalysed reactions, *Organic & Biomolecular Chemistry* 7 (2009) 395-398.

[35] S. Mitsuda, Y. Takashima, M. Wieser, Protein capable of catalyzing transamination stereoselectively, gene encoding said protein and use thereof, *Sumitomo Chemical Company, Limited,* 2002.

[36] D. Koszelewski, D. Clay, D. Rozzell, W. Kroutil, Deracemisation of alpha-Chiral Primary Amines by a One-Pot, Two-Step Cascade Reaction Catalysed by omega-Transaminases, *European Journal of Organic Chemistry* (2009) 2289-2292.

[37] D. Koszelewski, M. Goritzer, D. Clay, B. Seisser, W. Kroutil, Synthesis of Optically Active Amines Employing Recombinant omega-Transaminases in E. coli Cells, *Chemcatchem* 2 (2010) 73-77.

[38] K.E. Cassimjee, C. Branneby, V. Abedi, A. Wells, P. Berglund, Transaminations with isopropyl amine: equilibrium displacement with yeast alcohol dehydrogenase coupled to in situ cofactor regeneration, *Chemical Communications* 46 (2010) 5569-5571.

[39] H. Yun, B.G. Kim, Asymmetric Synthesis of (S)-alpha-Methylbenzylamine by Recombinant Escherichia coli Co-Expressing Omega-Transaminase and Acetolactate Synthase, *Bioscience Biotechnology and Biochemistry* 72 (2008) 3030-3033.

[40] C.U. Ingram, M. Bommer, M.E.B. Smith, P.A. Dalby, J.M. Ward, H.C. Hailes, G.J. Lye, One-pot synthesis of amino-alcohols using a de-novo transketolase and beta-alanine: Pyruvate transaminase pathway in Escherichia coli, *Biotechnology and Bioengineering* 96 (2007) 559-569.

[41] A. Iwasaki, Y. Yamada, N. Kizaki, Y. Ikenaka, J. Hasegawa, Microbial synthesis of chiral amines by (R)-specific transamination with Arthrobacter sp. KNK168, *Applied Microbiology and Biotechnology* 69 (2006) 499-505.

[42] J.D. Stewart, Dehydrogenases and transaminases in asymmetric synthesis, 5 (2001) 120-129.

[43] I.H. Segel, Enzyme Kinetics, Wiley, New York, 1993.

[44] W.W. Cleland, Substrate inhibition, Methods in enzymology. 63 (1979) 500-513.

[45] A.R. Martin, R. DiSanto, I. Plotnikov, S. Kamat, D. Shonnard, S. Pannuri, Improved activity and thermostability of (S)-aminotransferase by error-prone polymerase chain reaction for the production of a chiral amine, Biochemical Engineering Journal 37 (2007) 246-255.

[46] S.C. Rothman, M. Voorhies, J.F. Kirsch, Directed evolution relieves product inhibition and confers in vivo function to a rationally designed tyrosine aminotransferase, *Protein Science* 13 (2004) 763-772.

[47] H. Yun, B.Y. Hwang, J.H. Lee, B.G. Kim, Use of enrichment culture for directed evolution of the Vibrio fluvialis JS17 omega-transaminase, which is resistant to product inhibition by aliphatic ketones, *Applied and Environmental Microbiology* 71 (2005) 4220-4224.

[48] U.T. Bornscheuer, G.W. Huisman, R.J. Kazlauskas, S. Lutz, J.C. Moore, K. Robins, Engineering the third wave of biocatalysis, *Nature* 485 (2012) 185-194.

[49] S.G. Burton, D.A. Cowan, J.M. Woodley, The search for the ideal biocatalyst, *Nature Biotechnology* 20 (2002).

[50] J.S. Shin, B.G. Kim, Kinetic resolution of alpha-methylbenzylamine with omega-transaminase screened from soil microorganisms: Application of a biphasic system to overcame product inhibition, *Biotechnology and Bioengineering* 55 (1997).

[51] J.-S. Shin, B.-G. Kim, Kinetic modeling of ω-transamination for enzymatic kinetic resolution of α-methylbenzylamine, *Biotechnology and Bioengineering* 60 (1998) 534-540.

[52] M. Zanfir, X. Sun, A. Gavriilidis, Microstructured Mesh Contactor for Asymmetric Transfer Hydrogenation with Simultaneous Stripping: Modeling and Experiments, *Industrial & Engineering Chemistry Research* 47 (2008) 8995-9005.

[53] P. Tufvesson, J.S. Jensen, W. Kroutil, J.M. Woodley, Experimental determination of thermodynamic equilibrium in biocatalytic transamination, *Biotechnology and bioengineering* 109 (2012) 2159-2162.

[54] J.M. Woodley, Advances in enzyme technology--UK contributions, *Advances in biochemical engineering/biotechnology* 70 (2000) 93-108.

[55] J.M. Woodley, Protein engineering of enzymes for process applications, *Current Opinion in Chemical Biology* 17 (2013) 310-316.

[56] A. Schmid, F. Hollmann, J.B. Park, B. Buhler, The use of enzymes in the chemical industry in Europe, *Current Opinion in Biotechnology* 13 (2002) 359-366.

[57] P.L. Rogers, Y.J. Jeon, C.J. Svenson, Application of biotechnology to industrial sustainability, *Process Safety and Environmental Protection* 83 (2005) 499-503.

[58] J.P. Rasor, E. Voss, Enzyme-catalyzed processes in pharmaceutical industry, *Applied Catalysis a-General* 221 (2001) 145-158.

[59] J.M. Woodley, New opportunities for biocatalysis: making pharmaceutical processes greener, *Trends in Biotechnology* 26 (2008).

[60] K. Buchholz, V. Kasche, U.T. Bornscheuer, Biocatalysts and enzyme technology, *Wiley-VCH Verlag, Weinheim*, 2005.

[61] H. Yun, B.K. Cho, B.G. Kim, Kinetic resolution of (R,S)-sec-butylamine using omega-transaminase from Vibrio fluvialis JS17 under reduced pressure, *Biotechnology and Bioengineering* 87 (2012) 772-778.

[62] J.S. Shin, B.G. Kim, A. Liese, C. Wandrey, Kinetic resolution of chiral amines with omega-transaminase using an enzyme-membrane reactor, *Biotechnology and Bioengineering* 73 (2001) 179-187.

[63] A. Chitnis, A. Sadana, Ph-dependent enzyme deactivation models, *Biotechnology and Bioengineering* 34 (1989) 804-818.

[64] V. Sant'Anna, A.P.F. Corrêa, D.J. Daroit, A. Brandelli, Kinetic modeling of thermal inactivation of the Bacillus sp. protease P7, *Bioprocess and Biosystems Engineering* 36 (2013) 993-998.

[65] W.W. Cleland, The kinetics of enzyme-catalyzed reactions with two or more substrates or products. II. Inhibition: nomenclature and theory, *Biochimica et biophysica acta*. 67 (1963) 173-187.

[66] M.M. Vrvic, Comprehensive enzyme kinetics by V. Leskovac, *Journal- Serbian Chemical Society* 68 (2003) 1011-1012.

[67] M. van Boekel, Enzyme Kinetics: a Modern Approach. AG Marangoni. John Wiley & Sons, Ltd, Chichester, 2002. 248 pp ISBN 0-471-15985-9, *Journal of the Science of Food and Agriculture* 85 (2005).

[68] V. Leskovac, Comprehensive enzyme kinetics electronic resource, *Kluwer Academic/Plenum Pub*, New York, 2003.

[69] F.B. Rudolph, L.P. Daniel, [16] Product inhibition and abortive complex formation, Enzyme Kinetics and Mechanism Part A Initial Rate and Inhibitor Methods, *Academic Press*, 1979, pp. 411-436.

[70] R.J. Fox, M.D. Clay, Catalytic effectiveness, a measure of enzyme proficiency for industrial applications, *Trends in Biotechnology* 27 (2009) 137-140.

[71] G. Sin, J.M. Woodley, K.V. Gernaey, Application of modeling and simulation tools for the evaluation of biocatalytic processes: A future perspective, *Biotechnology Progress* 25 (2009) 1529-1538.

[72] Z. Findrik, Đ. Vasic'-Rački, S. Lütz, T. Daußmann, C. Wandrey, Kinetic Modeling of Acetophenone Reduction Catalyzed by Alcohol Dehydrogenase from Thermoanaerobacter sp, *Biotechnology Letters* 27 (2005) 1087-1095.

[73] J. Nielsen, J. Villadsen, E. Keshavarz-Moore, Bioreaction Engineering Principles, *Trends in Biotechnology* 13 (1995).

[74] J. Peter, J.E. Prenosil, J.R. Bourne, Comparison of various methods to determine kinetic constants for β-galactosidase in soluble and immobilised states, *Journal of Chemical Technology and Biotechnology* 31 (1981) 705-709.

[75] M. Zavrel, K. Kochanowski, A.C. Spiess, Comparison of different approaches and computer programs for progress curve analysis of enzyme kinetics, *Engineering in Life Sciences* 10 (2010) 191-200.

[76] N. Al-Haque, P.A. Santacoloma, W. Neto, P. Tufvesson, R. Gani, J.M. Woodley, A robust methodology for kinetic model parameter estimation for biocatalytic reactions, *Biotechnology Progress* 28 (2012) 1186-1196.

[77] P. Lorenz, K. Liebeton, F. Niehaus, J.r. Eck, Screening for novel enzymes for biocatalytic processes: accessing the metagenome as a resource of novel functional sequence space, *Current Opinion in Biotechnology* 13 (2002) 572-577.

[78] N.J. Turner, Directed evolution of enzymes for applied biocatalysis, Trends in Biotechnology 21 (2003) 474-478.

[79] M. Adamczak, S.H. Krishna, Strategies for improving enzymes for efficient biocatalysis, *Food Technology and Biotechnology* 42 (2004) 251-264.

[80] D.N. Bolon, C.A. Voigt, S.L. Mayo, De novo design of biocatalysts, *Current Opinion in Chemical Biology* 6 (2002) 125-129.

[81] U.T. Bornscheuer, M. Pohl, Improved biocatalysts by directed evolution and rational protein design, *Current Opinion in Chemical Biology* 5 (2001) 137-143.

[82] S.B. Rubin-Pitel, H.M. Zhao, Recent advances in biocatalysis by directed enzyme evolution, *Combinatorial Chemistry & High Throughput Screening* 9 (2006) 247-257.

[83] P.N. Bryan, Protein engineering of subtilisin, *Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology* 1543 (2000) 203-222.

[84] O. May, P.T. Nguyen, F.H. Arnold, Inverting enantioselectivity by directed evolution of hydantoinase for improved production of L-methionine, *Nature Biotechnology* 18 (2000) 317-320.

[85] K. Liebeton, A. Zonta, K. Schimossek, M. Nardini, D. Lang, B.W. Dijkstra, M.T. Reetz, K.E. Jaeger, Directed evolution of an enantioselective lipase, *Chemistry & Biology* 7 (2000) 709-718.

[86] C.K. Savile, J.M. Janey, E.C. Mundorff, J.C. Moore, S. Tam, W.R. Jarvis, J.C. Colbeck, A. Krebber, F.J. Fleitz, J. Brands, P.N. Devine, G.W. Huisman, G.J. Hughes, Biocatalytic Asymmetric Synthesis of Chiral Amines from Ketones Applied to Sitagliptin Manufacture, *Science* 329 (2010) 305-309.

[87] K. Schmoelzer, K. Maedje, B. Nidetzky, R. Kratzer, Bioprocess design guided by in situ substrate supply and product removal: Process intensification for synthesis of (S)-1-(2-chlorophenyl)ethanol, *Bioresource Technology* 108 (2012) 216-223.

[88] A.J.J. Straathof, Auxiliary phase guidelines for microbial biotransformations of toxic substrate into toxic product, *Biotechnology Progress* 19 (2003).

[89] P.-Y. Kim, D.J. Pollard, J.M. Woodley, Substrate supply for effective biocatalysis, *Biotechnology Progress* 23 (2007).

[90] A. Freeman, J.M. Woodley, M.D. Lilly, In-Situ Product Removal As A Tool For Bioprocessing, *Bio-Technology* 11 (1993).

[91] J.M. Woodley, M. Bisschops, A.J.J. Straathof, M. Ottens, Future directions for in-situ product removal (ISPR), *Journal of Chemical Technology and Biotechnology* 83 (2008).

[92] M.D. Truppo, H. Strotman, G. Hughes, Development of an Immobilized Transaminase Capable of Operating in Organic Solvent, *Chemcatchem* 4 (2012) 1071-1074.

[93] J.M. Woodley, Microbial biocatalytic processes and their development, in: A.I. Laskin, S. Sariaslani, G.M. Gadd (Eds.), *Advances in Applied Microbiology*, Vol 60, 2006,. 1-15.

[94] J.D. Rozzell, Commercial scale biocatalysis: myths and realities, 7 (1999) 2253-2261.

[95] J.-P. Lange, Lignocellulose conversion: an introduction to chemistry, process and economics, *Biofuels, Bioproducts and Biorefining* 1 (2007) 39-48.

[96] A.J.J. Straathof, S. Panke, A. Schmid, The production of fine chemicals by biotransformations, 13 (2002) 548-556.

[97] P. Tufvesson, J. Lima-Ramos, M. Nordblad, J.M. Woodley, Guidelines and cost analysis for catalyst production in biocatalytic processes, *Organic Process Research and Development* 15 (2011) 266-274.

[98] H. Hayashi, H. Wada, T. Yoshimura, N. Esaki, K. Soda, Recent Topics in Pyridoxal 5'-Phosphate Enzyme Studies, *Annual Review of Biochemistry* 59 (1990) 87-110.

[99] S. Schätzle, M. Höhne, E. Redestad, K. Robins, U.T. Bornscheuer, Rapid and Sensitive Kinetic Assay for Characterization of ω-Transaminases, *Analytical Chemistry* 81 (2009) 8244-8248.

[100] J.M. Nelson, E.G. Griffin, Adsorption of Invertase, *J. Am. Chem. Soc.* 38 (1916) 1109 – 1115.

[101] R. DiCosimo, J. McAuliffe, A.J. Poulose, G. Bohlmann, Industrial use of immobilized enzymes, *Chemical Society Reviews* (2013).

[102] P. Tufvesson, U. Törnvall, J. Carvalho, A.J. Karlsson, R. Hatti-Kaul, Towards a cost-effective immobilized lipase for the synthesis of specialty chemicals, 68 (2011) 200-205.

[103] J.S. Shin, B.G. Kim, D.H. Shin, Kinetic resolution of chiral amines using packed-bed reactor, *Enzyme and Microbial Technology* 29 (2001) 232-239.

[104] A.R. Martin, D. Shonnard, S. Pannuri, S. Kamat, Characterization of free and immobilized (S)-aminotransferase for acetophenone production, *Applied Microbiology and Biotechnology* 76 (2007) 843-851.

[105] M. Cardenas-Fernandez, W. Neto, C. Lopez, G. Alvaro, P. Tufvesson, J.M. Woodley, Immobilization of Escherichia coli containing omega-transaminase activity in LentiKats (R), *Biotechnology Progress* 28 (2012) 693-698.

[106] G. Rehn, C. Grey, C. Branneby, L. Lindberg, P. Adlercreutz, Activity and stability of different immobilized preparations of recombinant E. coli cells containing ω-transaminase, 47 (2012) 1129-1134.

[107] C.. Lee, S.-S. Yi, J. Kim, Y.-S. Lee, B.-G. Kim, Improved immobilized enzyme systems using spherical micro silica sol-gel enzyme beads, *Biotechnology and Bioprocess Engineering* 11 (2006) 277-281.

[108] D. Koszelewski, N. Muller, J.H. Schrittwieser, K. Faber, W. Kroutil, Immobilization of omega-transaminases by encapsulation in a sol-gel/celite matrix, *Journal of Molecular Catalysis B-Enzymatic* 63 (2010) 39-44.

[109] Y. Song-Se, L. Chang-won, K. Juhan, K. Dohyun, K. Byung-Gee, L. Yoon-Sik, Covalent immobilization of ω-transaminase from Vibrio fluvialis JS17 on chitosan beads, *Proc Biochem* 42 (2007) 895-898.

[110] J. David Rozzell, M. Klaus, Immobilized aminotransferases for amino acid production, Immobilized Enzymes and Cells, Part C, *Academic Press*, 1987, 479-497.

[111] A. Schulz, P. Taggeselle, D. Tripier, K. Bartsch, Stereospecific production of the herbicide phosphinothricin (glufosinate) by transamination: isolation and characterization of a phosphinothricin-specific transaminase from Escherichia coli, *Applied and Environmental Microbiology* 56 (1990) 1-6.

[112] K. Bartsch, R. Schneider, A. Schulz, Stereospecific production of the herbicide phosphinothricin (Glufosinate): Purification of aspartate transaminase from Bacillus stearothermophilus, cloning of the corresponding gene, aspC, and application in a coupled transaminase process, *Applied and Environmental Microbiology* 62 (1996) 3794-3799.

[113] M.D. Truppo, H. Strotman, G. Hughes, Development of an Immobilized Transaminase Capable of Operating in Organic Solvent, *ChemCatChem* (2012) 1-4.

[114] J.F. Kennedy, J.M.S. Cabral, Use of titanium species for the immobilization of cells, *Transition Metal Chemistry* 15 (1990) 197-207.

[115] O. Kirk, M.W. Christensen, Lipases from Candida antarctica: Unique Biocatalysts from a Unique Origin, *Organic Process Research & Development* 6 (2002) 446-451.

[116] M.D. Lilly, J.M. Woodley, A structured approach to design and operation of biotransformation processes, *Journal of Industrial Microbiology* 17 (1996) 24-29.

[117] W. Tischer, F. Wedekind, Immobilized enzymes: Methods and applications, Biocatalysis - from Discovery to Application 200 (1999) 95-126.

[118] R.A. Sheldon, Enzyme Immobilization: The Quest for Optimum Performance, *Advanced Synthesis & Catalysis* 349 (2007) 1289-1307.

[119] J.E. Přenosil, Ö.M. Kut, I.J. Dunn, E. Heinzle, Immobilized Biocatalysts, Ullmann's *Encyclopedia of Industrial Chemistry*, 2000.

[120] U. Hanefeld, L. Gardossi, E. Magner, Understanding enzyme immobilisation, *Chemical Society Reviews* 38 (2009) 453-468.

[121] M. D. Truppo, J. M. Janey, G. Hughes, Immobilized transaminases and process for making and using immobilized transaminase, 2012.

[122] P.V. Iyer, L. Ananthanarayan, Enzyme stability and stabilization - Aqueous and non-aqueous environment, *Process Biochemistry* 43 (2008).

[123] T. Gitlesen, M. Bauer, P. Adlercreutz, Adsorption of lipase on polypropylene powder, Biochimica Et Biophysica Acta-Lipids and Lipid Metabolism 1345 (1997) 188-196.

[124] L. Betancor, H.R. Luckarift, Bioinspired enzyme encapsulation for biocatalysis, *Trends in biotechnology* 26 (2008) 566-572.

[125] P. Monsan, D. Combes, Enzyme Stabilization by Immobilization, *Methods in Enzymology* 137 (1988) 584-598.

[126] H. Bayraktar, M. Serilmez, T. Karkaş, E.B. Çelem, S. Önal, Immobilization and stabilization of α-galactosidase on Sepabeads EC-EA and EC-HA, 49 (2011) 855-860.

[127] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Improvement of enzyme activity, stability and selectivity via immobilization techniques, *Enzyme and Microbial Technology* 40 (2007) 1451-1463.

[128] N. Prlainović, Z. Knežević-Jugović, D. Mijin, D. Bezbradica, Immobilization of lipase from Candida rugosa on Sepabeads®: the effect of lipase oxidation by periodates, *Bioprocess and Biosystems Engineering* 34 (2011) 803-810.

[129] F.G. Mutti, W. Kroutil, Asymmetric Bio-amination of Ketones in Organic Solvents, Advanced Synthesis & Catalysis 354 (2012) 3409-3413.

[130] W. Tischer, F. Wedekind, Immobilized Enzymes: Methods and Applications, *Topics In Current Chemistry* 200 (1999) 95-126.

[131] H. Yun, B.-K. Cho, B.-G. Kim, Kinetic resolution of (R,S)-sec-butylamine using omega-transaminase from Vibrio fluvialis JS17 under reduced pressure, *Biotechnology and Bioengineering* 87 (2004) 772-778.

[132] P.P. Taylor, D.P. Pantaleone, R.F. Senkpeil, I.G. Fotheringham, Novel biosynthetic approaches to the production of unnatural amino acids using transaminases, *Trends in Biotechnology* 16 (1998) 412-418.

[133] M.D. Jankowski, C.S. Henry, L.J. Broadbelt, V. Hatzimanikatis, Group contribution method for thermodynamic analysis of complex metabolic networks, *Biophysical Journal* 95 (2008) 1487-1499.

[134] J.-H. Seo, D. Kyung, K. Joo, J. Lee, B.-G. Kim, Necessary and sufficient conditions for the asymmetric synthesis of chiral amines using ω-aminotransferases, *Biotechnology and Bioengineering* 108 (2011) 253-263.

[135] Y.B. Tewari, R.N. Goldberg, J.D. Rozzell, Thermodynamics of reactions catalysed by branched-chain-amino-acid transaminase, *Journal of Chemical Thermodynamics* 32 (2000) 1381-1398.

[136] R.N. Goldberg, Y.B. Tewari, T.N. Bhat, Thermodynamics of enzyme-catalyzed reactions: Part 7 - 2007 update, *Journal of Physical and Chemical Reference Data* 36 (2007) 1347-1397.

[137] G.J. Lye, J.M. Woodley, Application of in situ product-removal techniques to biocatalytic processes, *Trends in biotechnology* 17 (1999) 395-402.

[138] R.L. Hanson, B.L. Davis, Y. Chen, S.L. Goldberg, W.L. Parker, T.P. Tully, M.A. Montana, R.N. Patel, Preparation of (R)-Amines from Racemic Amines with an (S)-Amine Transaminase from Bacillus megaterium, *Advanced Synthesis & Catalysis* 350 (2008) 1367-1375.

[139] D. Koszelewski, I. Lavandera, D. Clay, D. Rozzell, W. Kroutil, Asymmetric Synthesis of Optically Pure Pharmacologically Relevant Amines Employing ω-Transaminases, *Advanced Synthesis & Catalysis* 350 (2008) 2761-2766.

[140] D.J. Ager, T. Li, D.P. Pantaleone, R.F. Senkpeil, P.P. Taylor, I.G. Fotheringham, Novel biosynthetic routes to non-proteinogenic amino acids as chiral pharmaceutical intermediates, *Journal of Molecular Catalysis B-Enzymatic* 11 (2001) 199-205.

[141] T. Li, A.B. Kootstra, I.G. Fotheringham, Nonproteinogenic α-Amino Acid Preparation Using Equilibrium Shifted Transamination, *Organic Process Research & Development* 6 (2002) 533-538.

[142] H.H. Lo, S.K. Hsu, W.D. Lin, N.L. Chan, W.H. Hsu, Asymmetrical synthesis of L-homophenylalanine using engineered Escherichia coli aspartate aminotransferase, *Biotechnology Progress* 21 (2005) 411-415.

[143] Y.-P. Chao, Z.J. Lai, P. Chen, J.-T. Chern, Enhanced Conversion Rate of l-Phenylalanine by Coupling Reactions of Aminotransferases and Phosphoenolpyruvate Carboxykinase in Escherichiacoli K-12, *Biotechnology Progress* 15 (1999) 453-458.

[144] I.G. Fotheringham, N. Grinter, D.P. Pantaleone, R.F. Senkpeil, P.P. Taylor, Engineering of a novel biochemical pathway for the biosynthesis of L-2-aminobutyric acid in Escherichia coli K12, *Bioorganic & Medicinal Chemistry* 7 (1999) 2209-2213.

[145] M. Hohne, S. Kuhl, R. Karen, U.T. Bornscheuer, Efficient asymmetric synthesis of chiral Amines by combining transaminase and pyruvate decarboxylase, *Chembiochem* 9 (2008) 363-365.

[146] J.Y. Hwang, J. Park, J.H. Seo, M. Cha, B.K. Cho, J. Kim, B.G. Kim, Simultaneous Synthesis of 2-Phenylethanol and L-Homophenylalanine Using Aromatic Transaminase With Yeast Ehrlich Pathway, *Biotechnology and Bioengineering* 102 (2009) 1323-1329.

[147] M.D. Truppo, N.J. Turner, J.D. Rozzell, Efficient kinetic resolution of racemic amines using a transaminase in combination with an amino acid oxidase, *Chemical Communications* (2009) 2127-2129.

[148] A. Berenguer-Murcia, R. Fernandez-Lafuente, New Trends in the Recycling of NAD(P)H for the Design of Sustainable Asymmetric Reductions Catalyzed by Dehydrogenases, *Current Organic Chemistry* 14 (2010) 1000-1021.

[149] M.Q. Zheng, S.P. Zhang, G.H. Ma, P. Wang, Effect of molecular mobility on coupled enzymatic reactions involving cofactor regeneration using nanoparticle-attached enzymes, *Journal of Biotechnology* 154 (2011) 274-280.

[150] R. Leon, P. Fernandes, H.M. Pinheiro, J.M.S. Cabral, Whole-cell biocatalysis in organic media, *Enzyme and Microbial Technology* 23 (1998) 483-500.

[151] H.S. Bea, Y.M. Seo, M.H. Cha, B.G. Kim, H. Yun, Kinetic Resolution of alpha-methylbenzylamine by Recombinant Pichia pastoris Expressing omega-transaminase, *Biotechnology and Bioprocess Engineering* 15 (2010) 429-434.

[152] D. Clay, D. Koszelewski, B. Grischek, J. Gross, I. Lavandera, W. Kroutil, Testing of microorganisms for omega-transaminase activity, *Tetrahedron-Asymmetry* 21 (2010) 2005-2009.

[153] S. Panke, V. de Lorenzo, A. Kaiser, B. Witholt, M.G. Wubbolts, Engineering of a stable whole-cell biocatalyst capable of (S)-styrene oxide formation for continuous two-liquid-phase applications, *Applied and Environmental Microbiology* 65 (1999) 5619-5623.

[154] R. Kratzer, M. Pukl, S. Egger, B. Nidetzky, Whole-cell bioreduction of aromatic alpha-keto esters using Candida tenuis xylose reductase and Candida boidinii formate dehydrogenase co-expressed in Escherichia coli, *Microbial Cell Factories* 7 (2008) 12.

[155] D.J. Pollard, J.M. Woodley, Biocatalysis for pharmaceutical intermediates: the future is now, *Trends in Biotechnology* 25 (2007) 66-73.

[156] M.C. D'Anjou, A.J. Daugulis, A rational approach to improving productivity in recombinant Pichia pastoris fermentation, *Biotechnology and Bioengineering* 72 (2001) 1-11.

[157] S.D. Doig, P.J. Avenell, P.A. Bird, P. Gallati, K.S. Lander, G.J. Lye, R. Wohlgemuth, J.M. Woodley, Reactor Operation and Scale-Up of Whole Cell Baeyer-Villiger Catalyzed Lactone Synthesis, *Biotechnology Progress* 18 (2002) 1039-1046.

[158] R.M. Lynch, J.M. Woodley, M.D. Lilly, Process design for the oxidation of fluorobenzene to fluorocatechol by Pseudomonas putida, 58 (1997) 167-175.

[159] P. Darrigo, G.P. Fantoni, S. Servi, A. Strini, The effect of absorbing resins on substrate concentration and enantiomeric excess in yeast reduction, *Tetrahedron-Asymmetry* 8 (1997) 2375-2379.

[160] S.J. Osborne, J. Leaver, M.K. Turner, P. Dunnill, Correlation of biocatalytic activity in an organic-aqueous two-liquid phase system with solvent concentration in the cell membrane, *Enzyme and Microbial Technology* 12 (1990) 281-291.

[161] M. Vermue, J. Sikkema, A. Verheul, R. Bakker, J. Tramper, TOXICITY OF homologous series of organic-solvents for the gram-positive bacteria arthrobacter and nocardia sp and the gram-negative bacteria acinetobacter and pseudomonas sp, *Biotechnology and Bioengineering* 42 (1993) 747-758.

[162] I. Hilker, V. Alphand, R. Wohlgemuth, R. Furstoss, Microbial transformations, 56. Preparative scale asymmetric Baeyer-Villiger oxidation using a highly productive "two-in-One" resin-based in situ SFPR concept, *Advanced Synthesis & Catalysis* 346 (2004) 203-214.

[163] C. Jimenez-Gonzalez, A.D. Curzons, D.J.C. Constable, V.L. Cunningham, Cradle-to-gate life cycle inventory and assessment of pharmaceutical compounds, *International Journal of Life Cycle Assessment* 9 (2004) 114-121.

[164] A.D. Curzons, D.C. Constable, V.L. Cunningham, Solvent selection guide: a guide to the integration of environmental, health and safety criteria into the selection of solvents, *Clean Products and Processes* 1 (1999) 82-90.

[165] C. Jiménez-González, A.D. Curzons, D.J.C. Constable, V.L. Cunningham, Expanding GSK's Solvent Selection Guide—application of life cycle assessment to enhance solvent selections, *Clean Technologies and Environmental Policy* 7 (2004) 42-50.

[166] E.J. Henley, J.D. Seader, D.K. Roper, Separation process principles, Wiley, Hoboken, N.J., 2011.

[167] R.M. Milton, Drying Cracked Gas, in: U.C. Corporation (Ed.), United States of America, 1964.

[168] M.A. Harmer, Q. Sun, Solid acid catalysis using ion-exchange resins, Applied *Catalysis a-General* 221 (2001) 45-62.

[169] A. Chakrabarti, M.M. Sharma, Cationic ion-exchange resins as catalyst, *Reactive Polymers* 20 (1993) 1-45.

[170] N. Rahman, M. Kashif, Application of ninhydrin to spectrophotometric determination of famotidine in drug formulations, *Il Farmaco* 58 (2003) 1045-1050.

[171] M.K. Julsing, D. Kuhn, A. Schmid, B. Bühler, Resting cells of recombinant E. coli show high epoxidation yields on energy source and high sensitivity to product inhibition, *Biotechnology and Bioengineering* 109 (2012) 1109-1119.

[172] R.S. Pembrey, K.C. Marshall, R.P. Schneider, Cell surface analysis techniques: What do cell preparation protocols do to cell surface properties?, *Applied and Environmental Microbiology* 65 (1999) 2877-2894.

[173] Z. Jinhua, S. Junling, L. Yanlin, Bioconversion of resveratrol using resting cells of non-genetically modified Alternaria sp, *Biotechnology and Applied Biochemistry* 60 (2013) 236-243.

Appendix

Appendix 1A – Publication 1

REVIEW

BIOTECHNOLOGY and BIOENGINEERING

Process Considerations for the Asymmetric Synthesis of Chiral Amines Using Transaminases

Pär Tufvesson, Joana Lima-Ramos, Jacob S. Jensen, Naweed Al-Haque, Watson Neto, John M. Woodley

Department of Chemical and Biochemical Engineering, Technical University of Denmark, Søltofts Plads, DK-2800 Lyngby, Denmark; telephone: 45-4525-2926; fax: 45-4593-2906; e-mail: pt@kt.dtu.dk

Received 11 February 2011; revision received 25 March 2011; accepted 28 March 2011
Published online 31 March 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.23154

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.

Biotechnol. Bioeng. 2011;108: 1479–1493.

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 prochiral 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

Correspondence to: P. Tufvesson
Contract grant sponsor: European 6th Framework Programme
Contract grant number: EIB.08.016
Contract grant sponsor: European Union 7th Framework Programme
Contract grant number: 245144
Contract grant sponsor: 7th Framework People Programme
Contract grant number: 238531

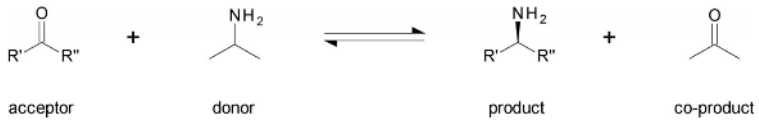


Figure 1. Overview of the biocatalytic transamination reaction.

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

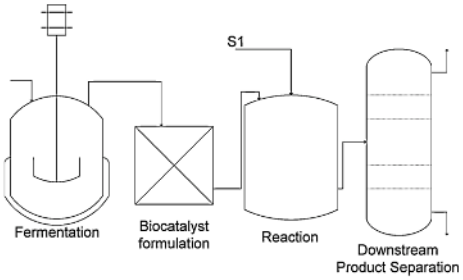


Figure 2. Overview of the biocatalytic transamination process.

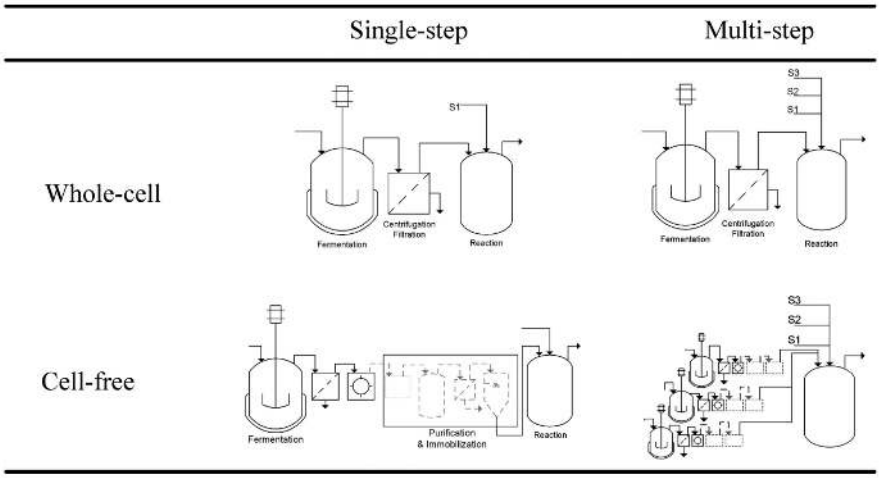


Figure 3. Detailed process design for the first three steps in the biocatalytic transamination process. S1—main feeding stream (amine donor, amine acceptor, PLP), S2—substrate for co-product removal, S3—co-factor regeneration.

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

Biotechnology and Bioengineering, Vol. 108, No. 7, July 2011

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	30, 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. violaceum* lipase and TAm from *Mesorhizobium* sp.	20	>99	24	0.1	0.4	Kim et al. (2007a)
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	21, 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)	89	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		72.92	—	16	1.8	0.4	
Cascades (whole cell)										
Phenylalanine	Asp	100	1.5	37, 8.4, *E. coli* overexpressing TAm-AspC and phosphoenolpyr. carboxylinase 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	0.8	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	0.8	Fotheringham (2001)
2-aminobutyrate	L-Asp	500	1.0	37, 8, *E. coli* (carrying tyrB from *E. coli*, alsS from *B. subtilis*)	58.8	—	24	12.1	0.8	
L-HPA	L-phenylalanine	10	2.5	37, 8.1, *E. coli* (AroAT from *Enterobacter* sp., carbonyl reductases, phenylpyruvate decarboxylase and GDH)	96.8	—	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)-L-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)	88.0	99.0	10	0.8	0.1	Cho et al. (2006)
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*	97.0	>99	2	17.3	—	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).

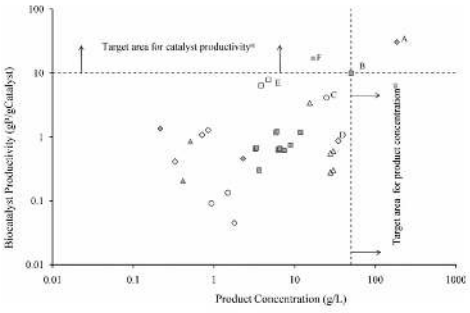


Figure 4. State of the art in transaminase reaction for chiral amines synthesis plotted AS Biocatalys. productivity versus product concentration. ◇: Cell-free catalyst; ▽: cascades (cell-free); Δ: cascades (whole cell); ○: whole cell; gray markers: synthesis of chiral amines; white markers: synthesis of amino acids. (A) Sitagliptin; Savile et al. (2010); (B) α-MBA, Truppo et al. (2010); (C) 3-(2-thienyl)-L-alanine, Meilves et al. (1997); (D) L-HPA, 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

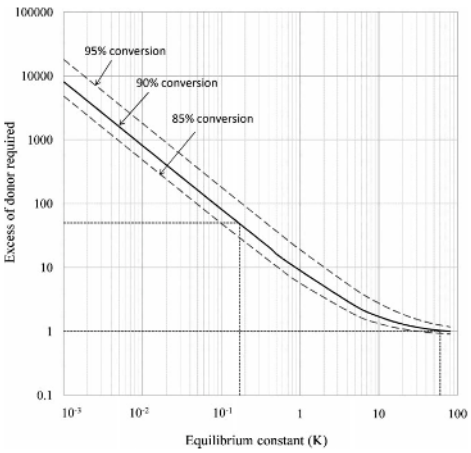


Figure 5. The equilibrium constant (K) determines the excess of amine donor required to reach a thermodynamic equilibrium of 90% (solid line). The broken lines are visual support for an excess of 10 and 50, which can be considered process boundaries.

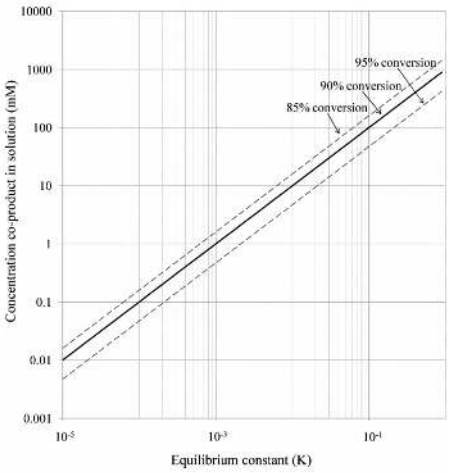


Figure 6. Concentration of co-product requires to reach 90% yield when using an initial concentration of 1 mol/L ketone and a ten-fold excess of amine donor for the synthesis of chiral amine.

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* IS64)	Ninefold higher reaction rate	Extraction with organic solvent	Decreased enzyme stability	Shin and Kim (1997)
α-MBA ⟷ APH (*B. thuringiensis* IS64)	99% vs. 54.7% (oc)	Membrane extraction (pertraction)	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. 50% (mass 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 pKa 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	$P_{A,p}$ (mbar)
Acetophenone	0.53
α-methyl benzylamine	0.72
Alanine	Non volatile
Pyruvic acid	1.7
Acetone	309
2-propyl amine	775[a]
Acetaldehyde	1202
2-butyl amine	287[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	α-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, NAD(H)	Lactic acid, gluconic acid	Shin and Kim (1999)
	LDH, FDH	Alanine, formate, NH_4^+, NAD(H)	Lactic acid, CO_2	Koszelewski et al. (2008a);
Co-product degradation	(S)ADH, GDH	2-PA/BA, glucose, NAD(H)	2-propyl/butyl alcohol, gluconic acid	Not reported
	(S)ADH, FDH	2-PA/BA, formate, NAD(H)	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 diminated 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]

Compound	Log *P*	Aqueous solubility, S_{aq} g/L	Aqueous solubility, S_{aq} mol/L	Critical conc. for cell, $C^{aq}_{i,cr}$ g/L	Critical conc. for cell, $C^{aq}_{i,cr}$ mol/L
Acetophenone	1.58	6.1	0.05	2.1	0.02
α-methylbenzylamine	1.19[b]	12	0.15	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]	21.5[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-González 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.

PT wishes to acknowledge the financial support from the ERA-IB project "Eng Biocat" (Registration Number: ERB.08.01.01). PT, ISI and NA-II acknowledge the project AMBIOCAS financed through the

European Union 7th Framework Programme (grant agreement no.: 245144). JL-R and WN acknowledge support from BIOTRAINS Marie Curie ITN, financed by the European Union through the 7th Framework people Programme (grant agreement no.: 238531).

References

Ager DJ, Li T, Pantaleone DP, Senkpeil RF, Taylor PP, Fotheringham IG. 2001. Novel biosynthetic routes to non-proteinogenic amino acids as chiral pharmaceutical intermediates. J Mol Catal B Enzym 11:199–205.

Bartsch K, Schneider R, Schulz A. 1996. Stereospecific production of the herbicide phosphinothricin (Glufosinate): Purification of aspartate transaminase from *Bacillus stearothermophilus*, cloning of the corresponding gene, aspC, and application in a coupled transaminase process. Appl Environ Microbiol 62:3794–3799.

Bea HS, Seo YM, Cha MH, Kim BG, Yun H. 2010. Kinetic resolution of alpha-methylbenzylamine by recombinant *Vibrio fluvialis* expressing omega-transaminase. Biotechnol Bioprocess Eng 15:429–434.

Berenguer-Murcia A, Fernandez-Lafuente R. 2010. New trends in the recycling of NAD(P)H for the design of sustainable asymmetric reductions catalyzed by dehydrogenases. Curr Org Chem 14:1000–1021.

Bommarius AS, Schwarm M, Drauz K. 2001. Comparison of different chemoenzymatic process routes to enantiomerically pure amino acids. Chimia 55:50–59.

Breuer M, Ditrich K, Habicher T, Hauer B, Kesseler M, Stürmer R, Zelinsky T. 2004. Industrial methods for the production of optically active intermediates. Angew Chem Int Ed 43:788–824.

Burton SG, Cowan DA, Woodley JM. 2002. The search for the ideal biocatalyst. Nat Biotechnol 20:37–45.

Cassimjee KE, Branneby C, Abedi V, Wells A, Berglund P. 2010. Transaminations with isopropylamine: Equilibrium displacement with yeast alcohol dehydrogenase coupled to in situ cofactor regeneration. Chem Commun 46:5569–5571.

Chao YP, Lai XJ, Chen P, Chern JT. 1999. Enhanced conversion rate of L-phenylalanine by coupling reactions of aminotransferases and phosphoenolpyruvate carboxykinase in *Escherichia coli* K-12. Biotechnol Prog 15:453–458.

Chen ST, Tseng M-J, Suokhteo B. 2000. Facile synthesis of L-homophenylalanine by equilibrium shift enzymatic reaction using engineered tyrosine aminotransferase. US Patent no: 6146839B.

Cho BK, Cho HJ, Park SH, Yun H, Kim BG. 2003. Simultaneous synthesis of enantiomerically pure (S)-amino acids and (R)-amines using coupled transaminase reactions. Biotechnol Bioeng 81:783–789.

Cho B-K, Park H-S, Seo J-H, Kim J, Kang T-J, Lee B-S, Kim B-G. 2008. Redesigning the substrate specificity of ω-aminotransferase for the kinetic resolution of aliphatic chiral amines. Biotechnol Bioeng 99:275–284.

Christen P, Metzler DE, editors. 1985. Transaminases. Hoboken, NJ: Wiley.

Clay D, Koszelewski D, Grischek B, Gross J, Lavandera I, Kroutil W. 2010. Testing of microorganisms for ω-transaminase activity. Tetrahedron-Asymm 21:2005–2009.

D'Anjou MC, Daugulis AJ. 2001. A rationale approach to improving productivity in recombinant *Pichia pastoris* fermentation. Biotechnol Bioeng 72:1–11.

Doig SD, Avenell PJ, Bird PA, Gallati P, Lander KS, Lye GJ, Woltdgemuth R, Woodley JM. 2002. Reactor operation and scale-up of whole-cell Baeyer-Villiger catalysed lactone synthesis. Biotechnol Prog 18:1069–1046.

Eliot AC, Kirsch JF. 2004. Pyridoxal phosphate enzymes: Mechanistic, structural, and evolutionary considerations. Annu Rev Biochem 73:383.

Fotheringham IG. 2001. Transaminase biotransformation process. US Patent no: 6197558B1.

Fotheringham IG, Grinter N, Pantaleone DP, Senkpeil RF, Taylor PP. 1999. Engineering of a novel biochemical pathway for the biosynthesis of L-2-amino butyric acid in *Escherichia coli* K12. Bioorgan Med Chem 7:2209–2213.

Fox RJ, Davis SC, Mundorff EC, Newman LM, Gavrilovic V, Ma SK, Chung LM, Ching C, Tam S, Muley S, Grate J, Gruber J, Whitman JC, Sheldon RA, Huisman GW. 2007. Improving catalytic function by ProSAR-driven enzyme evolution. Nat Biotechnol 25:338–344.

Frushicheva MP, Cao J, Chu ZT, Warshel A. 2010. Exploring challenges in rational enzyme design by simulating the catalysis in artificial kemp eliminase. Proc Natl Acad Sci 107:16869–16874.

Hanson RL, Davis BL, Chen Y, Goldberg SL, Parker WL, Tully TP, Montana MA, Patel RN. 2008. Preparation of (R)-amines from racemic amines with an (S)-amine transaminase from *Bacillus megaterium*. Adv Synth Catal 350:1367–1375.

Höhne M, Kühl S, Robins K, Bornscheuer UT. 2008. Efficient asymmetric synthesis of chiral amines by combining transaminase and pyruvate decarboxylase. ChemBioChem 9:363–365.

Hwang BY, Cho BK, Yun H, Koteshwar K, Kim BG. 2005. Revisit of aminotransferase in the genomic era and its application to biocatalysis. J Mol Catal B Enzym 37:47–55.

Hwang JY, Park J, Seo JH, Cha M, Cho BK, Kim J, Kim BG. 2009. Simultaneous synthesis of 2-phenylethanol and L-homophenylalanine using aromatic transaminase with yeast Ehrlich pathway. Biotechnol Bioeng 102:1323–1328.

Ingram CU, Bommer M, Smith MEB, Dalby PA, Ward JM, Hailes HC, Lye GJ. 2007. One-pot synthesis of amino-alcohols using a de-novo transketolase and beta-alanine: Pyruvate transaminase pathway in *Escherichia coli*. Biotechnol Bioeng 96:559–569.

Iwasaki A, Yamada Y, Kizaki N, Ikenaka Y, Hasegawa J. 2006. Microbial synthesis of chiral amines by (R)-specific transamination with *Arthrobacter* sp. KNK168. Appl Microbiol Biotechnol 69:499–505.

Jankowski MD, Henry CS, Broadbelt LJ, Hatzimanikatis V. 2008. Group contribution method for thermodynamic analysis of complex metabolic networks. Biophys J 95:1487–1499.

Jiménez-González C, Curzons AD, Constable DJC, Cunningham VL. 2004. Cradle-to-gate life cycle inventory and assessment of pharmaceutical compounds. Int J Life Cycle Assess 9(2):114–121.

Kaulmann U, Smithies K, Smith MEB, Hailes HC, Ward JM. 2007. Substrate spectrum of ω-transaminase from *Chromobacterium violaceum* DSM30191 and its potential for biocatalysis. Enzyme Microb Tech 41:628–637

Kim J-S, Shin B-G. 1998. Kinetic modeling of ω-transamination for enzymatic kinetic resolution of α-methylbenzylamine. Biotechnol Bioeng 60:534–540.

Kim P-Y, Pollard DJ, Woodley JM. 2007a. Substrate supply for effective biocatalysis. Biotechnol Prog 23:71–82.

Kim J, Kyung D, Yun H, Cho BK, Seo JH, Cha M, Kim BG. 2007b. Cloning and Characterization of a Novel β-Transaminase from *Mesorhizobium* sp. Strain LUK: A new biocatalyst for the synthesis of enantiomerically pure β-amino acids. Appl Environ Microbiol 73:772–1782.

Koszelewski D, Lavandera I, Clay D, Guebitz GM, Rozzell D, Kroutil W. 2008a. Formal asymmetric biocatalytic reductive amination. Angew Chem Int Ed 120:9477–9480.

Koszelewski D, Lavandera I, Clay D, Rozzell D, Kroutil W. 2008b. Asymmetric synthesis of optically pure pharmacologically relevant amines employing omega-transaminases. Adv Synth Catal 350:2761–2766.

Koszelewski D, Clay D, Rozzell D, Kroutil W. 2009. Deracemisation of alpha-chiral primary amines by a one-pot, two-step cascade reaction catalysed by omega-transaminases. Eur J Org Chem 14:2289–2292.

Koszelewski D, Müller N, Schrittwieser JH, Faber K, Kroutil W. 2010a. Immobilization of ω-transaminases by encapsulation in a sol–gel/celite matrix. J Mol Catal B-Enz 63:39–44.

Koszelewski D, Goritzer M, Clay D, Seisser B, Kroutil W. 2010b. Synthesis of optically active amines employing recombinant ω-transaminases in *E. coli* cells. ChemCatChem 2:73–77.

Koszelewski D, Tauber K, Faber K, Kroutil W. 2010c. ω-Transaminases for the synthesis of non-racemic alpha-chiral primary amines. Trends Biotechnol 28:324–332.

Kratzer R, Pohl M, Egger S, Nidetzky B. 2010. Whole-cell bioreduction of aromatic alpha-keto esters using *Candida tenuis* xylose reductase and *Candida boidinii* formate dehydrogenase co-expressed in *Escherichia coli*. Microbial Cell Fact 7: 37.

Lee CW, Yi SS, Kim J, Lee YS, Kim BG. 2006. Improved immobilized enzyme systems using spherical micro silica sol-gel enzyme beads. Biotechnol Bioprocess Eng 11:277–281.

León R, Fernandes P, Pinheiro HM, Cabral JMS. 1998. Whole-cell biocatalysis in organic media. Enzyme Microb Technol 23:483–500.

Li T, Kootstra AB, Fotheringham IG. 2002. Nonproteinogenic alpha-amino acid preparation using equilibrium shifted transamination. Org Process Res Dev 6:533–538.

Lilly MD, Woodley JM. 1994. A structured approach to design and operation of biotransformation processes. J Ind Microbiol Biotechnol 17:24–29.

Lo HH, Hsu SK, Lin WD, Chan NL, Hsu WH. 2003. Asymmetrical synthesis of l-homophenylalanine using engineered *Escherichia coli* aspartate aminotransferase. Biotechnol Prog 21:411–415.

Lye GJ, Woodley JM. 1999. Application of *in-situ* product removal techniques to biocatalytic processes. Trends Biotechnol 17:395–402.

Lynch RM, Woodley JM, Lilly MD. 1997. Process design for the oxidation of fluorobenzene to fluorocatechol by *Pseudomonas putida*. J Biotechnol 58:167–175.

Martin A, DiSanto R, Plotnikov I, Kamat S, Shonnard D, Pannuri S. 2007. Improved activity and thermostability of (S)-aminotransferase by error-prone polymerase chain reaction for the production of a chiral amine. Biochem Eng J 37:246–255.

Meilwes J, Schudok M, Kretzschmar G. 1997. Asymmetric synthesis of 2-thienylalanines. Tetrahedron-Asymm 8:527–536.

Nugent TC, El-Shazly M. 2010. Chiral amine synthesis—Recent developments and trends for enamide reduction, reductive amination, and imine reduction. Adv Synth Catal 352:753–819.

Osborne SJ, Leaver J, Turner MK, Dunnill P. 2010. Correlation of biocatalytic activity in an organic-aqueous two-liquid phase system with solvent concentration in the cell membrane. Enzyme Microb Tech 12:281–287.

Panke S, Lorenzo V, Kaiser A, Witholt B, Wubbolts MG. 1999. Engineering of a stable whole-cell biocatalyst capable of (S)-styrene oxide formation for continuous two-liquid-phase applications. Appl Environ Microbiol 65:2637–5643.

Pannuri S, DiSanto R, Kamat S. 2003. Biocatalysis. Kirk-Othmer Encyclopedia of Chemical Technology. Hoboken, NJ: Wiley.

Pollard DJ, Woodley JM. 2007. Biocatalysis for pharmaceutical intermediates: The future is now. Trends Biotechnol 25:66–73.

Reetz MT, Carballeira JD. 2007. Iterative saturation mutagenesis (ISM) for rapid directed evolution of functional enzymes. Nat Protoc 2:891–903.

Rothman SC, Voorhies M, Kirsch JF. 2004. Directed evolution relieves product inhibition and confers in vivo function to a rationally designed tyrosine aminotransferase. Protein Sci 13:763–772.

Rozzell D. 1988. Production of l-amino acids by transamination. US Patent no: 4518692.

Santacoloma PA, Sin G, Gernaey KV, Woodley JM. 2011. Multienzyme-catalyzed processes: Next-generation biocatalysis. Org Process Res Dev 15:203–212.

Savile CK, Janey JM, Mundorff EC, Moore JM, Tam S, Jarvis WR, Colbeck JC, Krebber A, Fleitz FJ, Brands J, Devine PN, Huisman GW, Hughes GJ. 2010. Biocatalytic Asymmetric synthesis of chiral amines from ketones applied to Sitagliptin manufacture. Science 329:305–309.

Seo JH, Kyung D, Joo K, Lee J, Kim BG. 2011. Necessary and sufficient conditions for the asymmetric synthesis of chiral amines using ω-transaminases. Biotechnol Bioeng 108:253–263.

Sheldon RA. 2007. Enzyme immobilization: The quest for optimum performance. Adv Synth Catal 349:1289–1307.

Shin JS, Kim BG. 1997. Kinetic resolution of alpha-methylbenzylamine with omega-transaminase screened from soil microorganisms: Application of a biphasic system to overcome product inhibition. Biotechnol Bioeng 55:348–358.

Shin JS, Kim BG. 1999. Asymmetric synthesis of chiral amines with omega-transaminase. Biotechnol Bioeng 65:206–211.

Shin JS, Kim BG, Shin DH. 2001a. Kinetic resolution of chiral amines using packed-bed reactor. Enzyme Microb Tech 29:232–239.

Shin JS, Kim BG, Liese A, Wandrey C. 2001b. Kinetic resolution of chiral amines with omega-transaminase using an enzyme-membrane reactor. Biotechnol Bioeng 73:179–187.

Stewart JD. 2001. Dehydrogenases and transaminases in asymmetric synthesis. Curr Opin Chem Biol 5:120–129.

Straathof AJJ. 2003. Auxiliary phase guidelines for microbial biotransformations of toxic substrate into toxic product. Biotechnol Prog 19:755–762.

Straathof AJJ, Panke S, Schmid A. 2002. The production of fine chemicals by biotransformations. Curr Opin Biotechnol 13:548–556.

Takashima Y, Mitsuda S, Wieser M. 2004. Protein capable of catalyzing transamination stereoselectively; gene encoding said protein and use thereof. US Patent no: 6727083 B2.

Taylor PP, Pantaleone DP, Senkpeil RF, Fotheringham IG. 1998. Novel biosynthetic approaches to the production of unnatural amino acids using transaminases. Trends Biotechnol 16:412–418.

Tracewell CA, Arnold FH. 2009. Directed enzyme evolution: Climbing fitness peaks one amino acid at a time. Curr Opin Chem Biol 13:3–9.

Truppo MD, Rozzell JD, Moore JC, Turner NJ. 2009a. Rapid screening and scale-up of transaminase catalysed reactions. Org Biomol Chem 7:395–398.

Truppo MD, Turner NJ, Rozzell JD. 2009b. Efficient kinetic resolution of racemic amines using a transaminase in combination with an amino acid oxidase. Chem Commun 2127–2129.

Truppo MD, Rozzell JD, Turner NJ. 2010. Efficient production of enantiomerically pure chiral amines at concentrations of 50 g/l. using transaminases. Org Process Res Dev 14:234–237.

Tufvesson P, Fu W, Jensen JS, Woodley JM. 2010. Process considerations for the scale-up and implementation of biocatalysis. Food Bioprod Process 88:3–11.

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

Turner NJ. 2009. Directed evolution drives the next generation of biocatalysts. Nat Chem Biol 5:567–573.

Vermue M, Sikkema J, Verheul A, Bakker R, Tramper J. 1993. Toxicity of homologous series of organic solvents for the gram positive bacteria *Arthrobacter* and *Nocardia* sp. and the gram negative bacteria. Biotech Bioeng 42:716–778.

Woodley JM. 2008. Microbial biocatalytic processes and their development. Adv Appl Microb 64:1–15.

Woodley JM, Bisschops M, Straathof AJJ, Ottens M. 2008. Future directions for *in-situ* product removal (ISPR). J Chem Technol Biotechnol 83:121–123.

Yi SS, Lee CW, Kim J, Kyung D, Kim BG, Lee YS. 2007. Covalent immobilization of omega-transaminase from *Vibrio fluvialis* JS17 in chitosan beads. Process Biochem 42:895–898.

Yun H, Kim BG. 2008. Asymmetric synthesis of (S)-alpha-methylbenzylamine by recombinant *Escherichia coli* co-expressing omega-transaminase and acetolactate synthase. Biosci Biotech Biochem 72:3080–3083.

Yun H, Cho BK, Kim BG. 2004. Kinetic resolution of (R,S)-sec-butylamine using omega-transaminase from *Vibrio fluvialis* JS17 under reduced pressure. Biotechnol Bioeng 87:772–778.

Yun H, Hwang BY, Lee JH, Kim BG. 2005. Use of enrichment culture for directed evolution of the *Vibrio fluvialis* JS17 ω-transaminase, which is resistant to product inhibition by aliphatic ketones. Appl Environ Microbiol 71:4220–1221.

Appendix 1B - Publication 2

Immobilization of *Escherichia Coli* Containing ω-Transaminase Activity in LentiKats®

Max Cárdenas-Fernández
Dept. of Chemical Engineering, Applied Biocatalysis Unit associated to IQAC (UAB-CSIC), School of Engineering, Universitat Autònoma de Barcelona, 08193-Bellaterra (Cerdanyola del Vallès), Catalunya, Spain

Watson Neto
Dept. of Chemical and Biochemical Engineering, Center for Process Engineering and Technology, Technical University of Denmark, DK-2800 Lyngby, Denmark

Carmen López and Gregorio Álvaro
Dept. of Chemical Engineering, Applied Biocatalysis Unit associated to IQAC (UAB-CSIC), School of Engineering, Universitat Autònoma de Barcelona, 08193-Bellaterra (Cerdanyola del Vallès), Catalunya, Spain

Pär Tufvesson and John M. Woodley
Dept. of Chemical and Biochemical Engineering, Center for Process Engineering and Technology, Technical University of Denmark, DK-2800 Lyngby, Denmark

DOI 10.1002/btpr.1538
Published online May 21, 2012 in Wiley Online Library (wileyonlinelibrary.com).

Whole Escherichia coli cells overexpressing ω-transaminase (ω-TA) and immobilized cells entrapped in LentiKats® were used as biocatalysts in the asymmetric synthesis of the aromatic chiral amines 1-phenylethylamine (PEA) and 3-amino-1-phenylbutane (APB). Whole cells were permeabilized with different concentrations of cetrimonium bromide (CTAB) and ethanol; the best results were obtained with CTAB 0.1% which resulted in an increase in reaction rate by 40% compared to the whole cells. The synthesis of PEA was carried out using isopropyl amine (IPA) and L-alanine (Ala) as amino donors. Using whole cell biocatalysis, the reaction with IPA was one order of magnitude faster than with Ala. No reaction was detected when permeabilized E. coli cells containing ω-TA were employed using Ala as the amino donor. Additionally, the synthesis of APB from 4-phenyl-2-butanone and IPA was studied. Whole and permeabilized cells containing ω-TA and their immobilized LentiKats® counterparts showed similar initial reactions rates and yields in the reaction systems, indicating 100% of immobilization efficiency (observed activity/activity immobilized) and absence of diffusional limitations (due to the immobilization). Immobilization of whole and permeabilized cells containing ω-TA in LentiKats® allowed improved stability as the biocatalyst was shown to be efficiently reused for five reaction cycles, retaining around 80% of original activity. © 2012 American Institute of Chemical Engineers *Biotechnol. Prog.*, 28: 693–698, 2012
Keywords: whole cell biocatalysis, ω-transaminase, asymmetric synthesis, chiral amines, cell permeabilization, immobilization by entrapment, LentiKats®

Introduction

The production of chiral amines is of great interest in synthetic chemistry as they find wide application as chiral auxiliaries and intermediates for a wide variety of pharmaceutical and agrochemical targets. In fact, chiral amine synthesis has been identified by the pharmaceutical industry as one of the top research priorities for synthetic chemistry.[1] Chiral amines can be produced either by chemical or biocatalytic means, although the implementation of effective methods remains a challenge.[2] Biocatalytic methods to produce optically pure chiral amines include kinetic resolution of racemic amines using transaminases or hydrolases.[3]

ω-Transaminases (ω-TAs) are pyridoxal-5′-phosphate (PLP)-dependent enzymes that catalyze the transfer of an amine group (at the non-α-position) from an amino-donor, usually an arylamine or amino acid, to a prochiral acceptor ketone, yielding a second chiral amine and a ketone by-product.[4] The use of ω-TAs has gained attention because of its potential for the resolution of racemic amines as well as in the asymmetric synthesis of optically pure amines.[5–7] Today, asymmetric synthesis is a necessity for economic reasons. Nevertheless, despite many advantages from an industrial viewpoint, such as the high enantioselectivity and broad substrate range of ω-TAs, the unfavorable equilibrium and substrate or product inhibition problems are frequently major

Correspondence concerning this article should be addressed to Pär Tufvesson at pt@kt.dtu.dk.

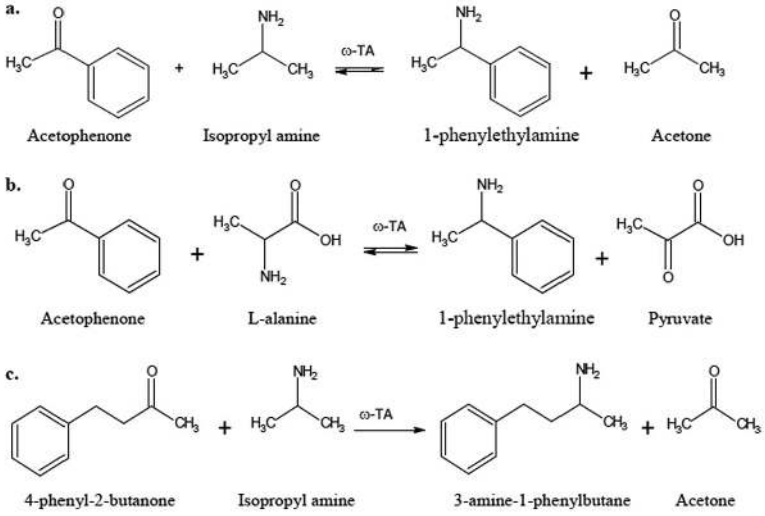


Figure 1. Reactions for the synthesis of aromatic chiral amines 1-phenylethylamine (PEA) and 3-amino-1-phenylbutane (APB) catalyzed by *E. coli* cells containing ω-transaminase activity (ω-TA).

drawbacks.[8] However, these constraints can be overcome by removing the product or by-product during the reaction (either in situ or by coupled enzymatic cascade reactions).[5,9] A further challenge is low catalyst productivity (gram product per gram biocatalyst), which leads to high process costs.[10] A recent analysis of published reports on transaminase processes shows that low catalyst productivity remains a serious limitation to the technology.[9]

Because of the lower work-up costs using whole cells as a biocatalyst, compared to isolated enzymes, it is frequently advantageous from an economic perspective. Further advantages of using cells in bioprocesses are regeneration of cofactors (e.g., NADH), using cell metabolic systems to carry out complex enzymatic reactions or removing by-products, etc.[11] However, undesirable reactions and formation of by-products could take place, which can be overcome by using a proper strain with a highly expressed enzymatic activity (recombinant cell) to increase the reaction rates of interest and decrease side reactions.[9] Often whole-cell biocatalysts are strongly affected by the inner and outer cell membranes that act as a permeable barrier against the diffusion of substrates and products, and as a consequence the reactions rates can be drastically reduced, especially for non-natural substrates. Potentially, this can be overcome by permeabilizing the cells with organic solvents (e.g., ethanol) or surfactants [e.g., cetrimonium bromide (CTAB)] that could facilitate the diffusion of substrates and products through the cell membranes.[12]

Immobilization is a very useful technique for reducing the cost contribution from the biocatalyst. It has the potential to make the biocatalysts more stable and facilitate ease of repeated use. There are many methods for immobilizing cells and immobilization by entrapment is one of the most commonly used methods; κ-carrageenan, alginate, and chitosan are the most popular matrices when immobilizing cells.[11,12] LentiKats® is a relatively new commercial matrix which is based on the controlled drying of the nontoxic and inert polyvinyl alcohol hydrogel and subsequent chemical stabilization, leading to lens-shaped (oblate spheroidal) particles.[14] Because immobilization in LentiKats® is carried out under mild conditions, it allows the preservation of the cells. Furthermore, due to the geometry of the particles, the negative effects of diffusional limitation may be minimized in contrast to spherical Ca-alginate beads.[15,16] Microorganisms such a bacteria and yeast have been successfully immobilized in LentiKats® and due excellent mechanical properties have been used in different bioreactor configurations.[15-18]

The aim of the work reported here is to immobilize recombinant *E. coli* cells containing overexpressed ω-TA activity by entrapment in LentiKats® and to study the effects of permeabilization on free cells and their immobilized counterparts. To test the cells they have been applied in the synthesis of the aromatic amines 3-amino-1-phenylbutane (APB), a building-block for the synthesis of the antihypertensive dilevalol, and 1-phenylethylamine (α-methylbenzylamine, PEA) (Figure 1). Finally, the reusability of free and immobilized cells, permeabilized and nonpermeabilized, has been evaluated.

Materials and Methods

Materials

All chemicals were of analytical grade and were purchased from Sigma-Aldrich (St Louis, MO). LentiKats® gel and LentiKats-printer® were obtained by GeniaLab (Braunschweig, Germany). The recombinant enzyme ω-TA denoted TA-D44 (ω-TA) was cloned in *E. coli* BL21(D3) and supplied by c-LEcta (Leipzig, Germany) and grown at Department of Chemical Engineering at Autonomous of Barcelona University (Spain).

Analytical

PEA and APB concentrations were analyzed on an Ultimate 3000 HPLC (Dionex Sunnyvale, CA). The compounds were separated on a Luna 3 μm C18(2) 100 Å (50 × 4.6 mm) column (Phenomenex, Torrance, CA) at a flow rate of 2 mL/min using a multistep gradient flow of aqueous 0.1% (v/v) trifluoroacetic acid and acetonitrile, with the following percentage of acetonitrile: 0 min (0%), 1 min (10%), 2.5 min (10%), 5.9 min (60%), 6 min (0%), and 7 min (0%). PEA and APB were detected at 210 nm after 2.1 min and 254 nm after 4.1 min, respectively.

Expression of ω-transaminase

Recombinant cells containing ω-TA were grown on a defined mineral medium[19] containing 0.05 g/L kanamycin at pH 7 and 37°C. Expression of ω-TA was induced by adding 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) in the middle of the logarithmic phase of growth (after 6.5 h cultivation and on OD_{600} of 10.2) and maintained at 30°C for 17.5 h. Cultivation was carried out in a 5-L bioreactor (Biostat® B; Sartorius, Germany), the pO_2 value was maintained at 30% of air saturation at 1 vvm via adjustment of the agitation rate between 200 and 900 rpm. pH was controlled by adding 30% NaOH or 2 M H_2SO_4, as required. The cells were harvested by centrifugation (3600g, at 4°C for 15 min) and washed twice with 0.1 M potassium phosphate buffer pH 7.

Permeabilization treatment of cells containing ω-TA

A cell suspension (0.1 g/mL) was treated with 4 mL of CTAB (dissolved in 50 mM phosphate buffer pH 8) to give a final concentration of 0.1, 0.2, and 0.4% (w/v), or with 4 mL of ethanol to give a final concentration of 20, 40, and 60% (v/v). The mixture was gently shaken (200 rpm) at 25°C for 20 min. The cells were centrifuged at 7200g at 4°C for 10 min, and then washed with 0.1 M potassium phosphate buffer pH 7.

Immobilization of whole and permeabilized cells containing ω-TA by entrapment in LentiKats®

The immobilization of wet cells containing ω-TA by entrapment in LentiKats® was carried out according to the GeniaLab protocol (*LentiKats® tips and tricks*, Braunschweig, Germany) as follows; LentiKats® gel was heated at 95°C and then cooled to 35°C. Subsequently, 2 mL of whole cells (0.8 g/mL) or permeabilized cells (0.3 g/mL) were mixed with 8 mL of LentiKats® liquid, before formation of the lens-shaped particles using the GeniaLab LentiKats-printer®. Drops (3–4 mm in diameter) were kept in Petri dishes and maintained at room temperature until about 70% of the water had been evaporated. Finally, the stabilizing solution was added and kept at room temperature for 2 h. After this time, the solution was filtered, and the immobilized cells were washed five times with 0.1 M potassium phosphate buffer pH 7, before collection and storage at 4°C. Immobilization efficiency was calculated as the quota between the activity at the end of the immobilization process and the offered initial activity.

Transaminase-catalyzed synthesis of 1-phenylethylamine and 3-amino-1-phenylbutane

Synthesis was carried out with whole cells and permeabilized cells and their respective immobilized counterparts in LentiKats® (0.02 g of wet cells per mL of reaction). PEA was synthesized by means of two reaction systems using 6 mM acetophenone and either 1 M isopropyl amine (IPA) or 0.1 M L-alanine as amino donors. APB was synthesized using 6 mM 4-phenyl-2-butanone (PB) and 1 M of IPA as the amino donor. All reactions were carried out in 0.1 M potassium phosphate buffer pH 7 containing 2 mM PLP, at 500 rpm in orbital agitation and 30°C in a thermoshaker from HLC (Bovenden, Germany) and 2 mL of final volume reaction. The initial rates, increase of the molar concentration of PEA or APB per unit of time, and reaction yields were calculated for each reaction. One unit of activity was defined as the amount of cells that produces 1 μmol of amino product (PEA or APB) per minute at 6 mM of ketone substrate and 1 M of IPA or 0.1 M Ala.

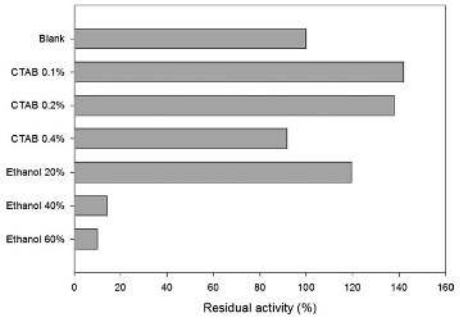


Figure 2. Permeabilization of cells ω-TA with different concentrations of surfactant cetrimonium bromide (CTAB; phosphate buffer 50 mM pH 8), and solvent ethanol.

All the cases, 0.1 g wet cells ω-TA were mixed within 4 mL of permeabilizing agent, and kept at 25°C for 20 min.

Reusability studies

Whole and permeabilized cells (0.05 U per mL of reaction) and their immobilized counterparts (0.03 U per mL of reaction) were reused in five reaction cycles for the synthesis of APB using the above reaction conditions. At the end of each batch, whole and permeabilized cells were recovered by centrifugation at 7200g at 4°C for 10 min and washed with 0.1 M potassium phosphate buffer pH 7. Immobilized derivatives were filtered and washed with the same buffer. The initial rates and reaction yield after 3 h were determined in each batch reaction.

Results and Discussion

To increase the transaminase activity of cells containing ω-TA, the effect of different cell membrane permeabilization agents such as ethanol (20, 40, and 60%) and CTAB (0.1, 0.2, and 0.4%) was evaluated. Figure 2 shows that low concentrations of CTAB have a positive effect on cell permeabilization, increasing the observed reaction rate by around 40%. Although the observed reaction rate was increased by 20% when 20% ethanol was added, as expected higher concentrations of the solvent seem to affect the activity negatively. Thus, further experiments on cell permeabilization

Table 1. Immobilization of Whole and Permeabilized Recombinant *E. coli* Cells Containing ω-Transaminase Activity (ω-TA) by Entrapment in LentiKats®

	Cells ω-TA	
	Whole	Permeabilized
Initial activity (U/gcell)*	1.69	2.16
g of cells/g of LentiKats®	0.17	0.06
Offered activity (U/g LentiKats®)	0.28	0.13
Obtained activity (U/g LentiKats®)	0.31	0.13
Process time (h)	3	3

*The initial activity considering the reaction with acetophenone and isopropyl amine, for both whole and permeabilized cells ω-TA.

Table 2. Synthesis of 1-Phenylethylamine (PEA) and 3-Amino-1-phenylbutane (APB) Catalyzed by Free-Whole and Permeabilized Cells

	Free cells ω-TA			
	Whole		Permeabilized	
Substrates	r_0 (U/gcells)	Yield* (%)	r_0 (U/gcells)	Yield* (%)
APH+IPA	1.69	58.2	2.16	61.3
APH+ALA	0.13	31.3	N.R	N.R
PB+IPA	2.88	91.6	3.49	91.9

Reactions were carried out with cells containing ω-TA (0.02 g of cells per mL of reaction) in buffer potassium phosphate 0.1 M pH 7 containing 6 mM ketone substrate (APH or PB), 1 M of IPA or 0.1 M ALA and 2 mM pyridoxal-5′-phosphate.

APH: acetophenone; IPA: isopropyl amine; ALA: D-alanine; PB: 4-phenyl-2-butanone; r_0: initial rate; N.R: no reaction detected.

*Reaction yield after 24 h reaction.

were performed with 0.1% CTAB in 50 mM buffer phosphate pH 8, at 25°C for 20 min.

Immobilized LentiKats® derivatives of both whole cells and permeabilized cells were prepared (Table 1) and used in the asymmetric synthesis of aromatic optically pure chiral amines and compared with freely suspended whole and permeabilized cells. In the first part of the work, the production of PEA was used as a model reaction (Figures 1a,b), using low concentrations (6 mM) of acetophenone (APH). High concentration of the two amino donors 1 M IPA and 0.1 M L-alanine were used to displace the equilibrium in the synthetic direction.

Previous studies have shown that transamination of acetophenone to PEA is thermodynamically unfavorable and is constrained by strong inhibition by products and by the ketone substrate even at very low concentrations.[8,20] Thermodynamic constraints can be overcome by removing the by-product in situ or by means of a coupled reaction to remove the product. Acetone and pyruvate are the two by-products formed by deamination of IPA and Ala, respectively. Acetone can in principle be evaporated, although it presents a great challenge to achieve the low concentrations required for high yields.[9] Using a cascade enzymatic system to degrade the formed pyruvate therefore appear like a promising alternative.[5,21-23] However, the principal drawback is the regeneration of the cofactor NADH which leads to increased process costs. Using a whole cell biocatalysis can therefore be an alternative solution for elimination of the by-product pyruvate by using the existing cell metabolism since pyruvate is an intermediate metabolite of many enzymatic cellular pathways. However, in the present study, when comparing the initial rates for whole cell biocatalysis (Table 2), the reaction rate with IPA was seen to be a magnitude faster than with Ala. The reaction yields after 24 h reaction when using IPA as donor were also higher, 58.2% as compared to 31.3% when using Ala (Figure 3). The lower yield cannot be explained only by the lower initial reaction rate. In a previous work by Shin and Kim,[6] similar reaction yields were obtained by using Ala as the amino donor, although this was increased to 90% by increasing the cell concentration in the reaction medium. In the same study, it was also demonstrated that whole cell biocatalysis improves the reaction yield considerably compared with the cell-free extract using Ala as donor. The results can be explained by slow uptake or metabolism of Ala by other cellular metabolic pathways decreasing its availability for ω-TA, or accumulation of pyruvate that is not metabolized as expected, which would lead the equilibrium to be favored for the ketone/alanine side rather than amine/pyruvate.[24] This would in turn imply that in the case of using Ala as donor, the reaction bottleneck would not be the activity of the transaminase, although to validate this is beyond the scope of the current study. Furthermore, it was seen that the conversion of acetophenone was significantly higher than the yield of PEA (conversion 55% as compared to 31% yield) when using Ala as amine donor, whereas when IPA was used as donor this effect was less marked but still significant (ca. 71% conversion as compared to 58% yield). For the conversion of PB to APB, the yield matched the conversion, which could be explained by the faster reaction rate, or alternatively that PB is less prone to degradation or side reactions.

Comparing the reaction rate and yield measured for permeabilized cells (Table 2) shows, as expected, that the reaction rate with acetophenone and IPA was higher than for the nonpermeabilized whole-cells. The reaction yield was similar to nontreated cells. However, no reaction occurred when Ala was used as the amino donor. Yun and Kim who obtained low reaction yield when using similar concentration of substrates explained this phenomenon by poor secretion of the

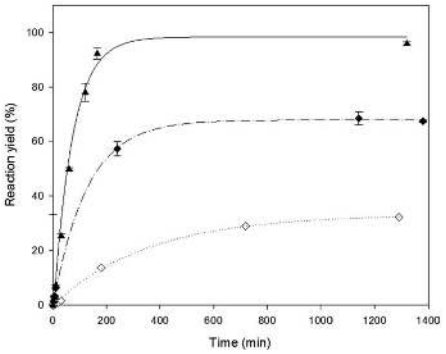


Figure 3. Conversion courses of synthesis of aromatic chiral amines: (◆) Reaction a; 6 mM acetophenone and 1 M isopropyl amine; (◇) Reaction b; 6 mM acetophenone and 0.1 M L-alanine; (▲) Reaction c; 6 mM 4-phenyl-2-butanone and 1 M isopropyl amine.

The reactions were carried out with cells ω-TA (0.02 g of wet cells per mL of reaction) in buffer potassium phosphate 0.1 M pH 7 containing 2 mM pyridoxal-5′-phosphate at 500 rpm orbital agitation and 30°C. Error bars represent standard error of two measurements.

Table 3. Synthesis of 1-Phenylethylamine (PEA) and 3-Amino-1-phenylbutane (APB) Catalyzed by Whole and Permeabilized Cells Immobilized in LentiKats®

	Immobilized Cells ω-TA in LentiKats®			
	Whole		Permeabilized	
Substrates	r_0 (U/gcells)	Yield* (%)	r_0 (U/gcells)	Yield* (%)
APH+IPA	1.68	68.8	2.11	68.4
PB+IPA	2.61	96.7	3.21	99.6

Reactions were carried out with a proper weight of LentiKats® (0.02 g of cells ω-TA per mL of reaction medium) in buffer potassium phosphate 0.1 M pH 7 containing 6 mM ketone substrate (APH or PB), 1 M of IPA and 2 mM pyridoxal-5'-phosphate.

APH: acetophenone; IPA: isopropyl amine; PB: 4-phenyl-2-butanone; r_0: initial rate.

* Reaction yield after 24 h reaction.

product PEA.[23] However, this is not a likely explanation in the present study as this problem was not observed when the reaction with IPA was carried out. Another possibility could be that the permeabilization disrupts the uptake of Ala into the cells. However, neither of these explanations was further investigated.

In the second part of the study, amination of PB, to synthesize APB, was studied using IPA as an amino donor (Figure 1c). PB has been described to have higher amino acceptor reactivity, a more favorable equilibrium and a weaker inhibitory effect than acetophenone.[6] The results show an initial rate 1.7-fold higher than that with acetophenone and with a very high reaction yield, over 90%, after 3 h of reaction when using whole and permeabilized cells (Table 2).

Whole and permeabilized cells were then immobilized by entrapment in LentiKats®. Initial reaction rates and yields were similar to free cells when the three reactions were performed (Table 3), although slightly lower for the reaction with APH and Ala (data not shown; this reaction was discarded from further studies due to the low yield). Hence the immobilization efficiency was 100% which indicates an absence of diffusional limitations in accordance with the previous studies when immobilizing microorganisms in LentiKats®.[15,17,25] This is an improvement on previously reported methods for immobilizing cells with ω-TA activity. In a report by Martín et al., who immobilized *E. coli* spray-dried cells with ω-TA activity in a calcium alginate support, a significant increase of the K_M (fivefold) pointing to diffusional limitations was described.[26] Furthermore, immobilization of the purified ω-TA from *Vibrio fluvialis* JS17 in supports such as chitosan, Eupergit® C[27] and micro silica sol-gel[28] have been reported with low immobilization efficiency.

Because of fast reaction rate and high reaction yield (Figure 3), the synthesis of APB using whole and permeabilized cells immobilized in LentiKats® was selected to examine the repeated use of the biocatalysts. The different preparations were used in consecutive reaction batches, and the immobilization efficiency was studied and compared to that of the free cells. As can be seen in Figure 4, free-permeabilized cells lost around 65% of activity already after the first batch, while whole cells lost 61% in five batches. The loss of activity might be due to recovery losses (centrifugation and washing), and cell-lysis during the reaction. This effect is likely to be more severe for permeabilized cells because of the weakness of the cell membranes that make it more susceptible to lysis. Immobilized whole and permeabilized cells lost

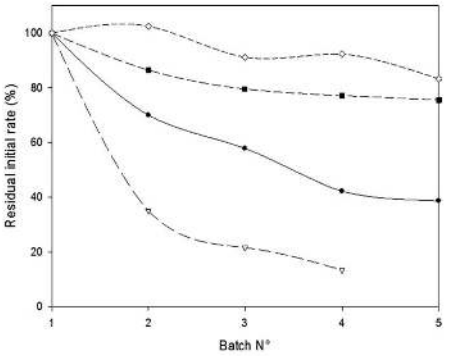


Figure 4. Recycling of free cells ω-TA and LentiKats® immobilized derivatives in the synthesis of 3-amino-1-phenylbutane (APB).

(◇) Permeabilized cells immobilized in LentiKats®; (■) whole cells immobilized in LentiKats®; (●) free-whole cells; (▽) free-permeabilized cells. Initial activities for immobilized derivatives (0.03 U per mL of reaction) and free-cells (0.05 U per mL of reaction).

only 24 and 17% of activity, respectively, after five batches. Although immobilization could improve the stability of the cellular structure, cell-lysis, or leakage of the cells out the support cannot be discarded, which could explain the small activity losses.

Conclusions

Recombinant *E. coli* cells overexpressing ω-TA (cells-ωTA) were found to be an excellent biocatalyst for the synthesis of the aromatic chiral amine APB with high reaction yield (>90%). Permeabilization with the surfactant CTAB 0.1% was the best option to increase the observed reaction rate. IPA was found to be an effective amino donor when cells containing ω-TA were used as the biocatalyst. To the best of our knowledge, this is the first time that the immobilization by entrapment in LentiKats® of cells with ω-TA activity has been reported. Immobilization of cells containing ω-TA in LentiKats® was found to be a fast, easy and suitable method with absence of diffusional limitations and no loss of activity, thereby showing a considerable improvement on previously reported studies. Further, immobilization in LentiKats® allowed significantly increasing the stability of whole and permeabilized cells under reaction conditions. Whole and permeabilized cells containing ωTA immobilized in LentiKats® could be used for five reaction cycles synthesis of APB with only a minor loss in activity indicating that the procedure could be effective for improving the catalyst productivity and thereby the process economy for transaminase processes. Further studies on prolonged process stability and process scale-up will be the next steps to validate the improvements in process cost structure.

Acknowledgments

This work was supported by the ERA-IB project EngBiocat (Spanish MICINN EUI2008-03615 and Danish Registration Number EIB.08.016) and by DURSI 2009SGR281 Generalitat

de Catalunya. Max Cárdenas-Fernández thanks the financial support of a predoctoral grant from AECID (Spanish MAEC).

Literature Cited

1. Constable D, Dunn P, Hayler J, Humphrey G, Leazer J, Linderman R, Lorenz K, Manley J, Pearlman B, Wells A, Zaks A, Zhang T. Key green chemistry research areas—a perspective from pharmaceutical manufacturers. *Green Chem.* 2007;9:411–420.
2. Nugent TC, El-Shazly M. Chiral amine synthesis—recent developments and trends for enamide reduction, reductive amination, and imine reduction. *Adv Synth Catal.* 2010;352:753–819.
3. Panke S, Held M, Wubbolts M. Trends and innovations in industrial biocatalysis for the production of fine chemicals. *Curr Opin Biotechnol.* 2004;15:272–279.
4. Shin JS, Kim BG. Exploring the active site of amine:pyruvate aminotransferase on the basis of the substrate structure-reactivity relationship: how the enzyme controls substrate specificity and stereoselectivity. *J Org Chem.* 2002;67:2848–2853.
5. Koszelewski D, Tauber K, Faber K, Kroutil W. ω-Transaminases for the synthesis of non-racemic α-chiral primary amines. *Trends Biotechnol.* 2010;28:324–332.
6. Shin JS, Kim BG. Asymmetric synthesis of chiral amines with ω-transaminase. *Biotechnol Bioeng.* 1999;65:206–211.
7. Shin JS, Kim BG, Shin DH. Kinetic resolution of chiral amines using packed-bed reactor. *Enzyme Microb Technol.* 2001;29:232–239.
8. Shin JS, Kim BG. Comparison of the ω-transaminase from different microorganisms and application to production of chiral amines. *Biosci Biotechnol Biochem.* 2001;65:1782–1788.
9. Tufvesson P, Lima-Ramos J, Jensen JS, Al-Haque N, Neto W, Woodley JM. Process considerations for the asymmetric synthesis of chiral amines using transaminase. *Biotechnol Bioeng.* 2011;108:1479–1493.
10. Tufvesson P, Lima-Ramos J, Nordblad M, Woodley JM. Guidelines and cost analysis for catalyst production in biocatalytic processes. *Org Process Res Dev.* 2011;15:266–274.
11. Karel SF, Libicki SB, Robertson CR. Immobilization of whole cells: the engineering principle. *Chem Eng Sci.* 1985;40:1321–1354.
12. Chen RR. Permeability issues in whole-cell bioprocesses and cellular membrane engineering. *Appl Microbiol Biotechnol.* 2007;74:730–738.
13. Dervakos GA, Webb C. Viable cell immobilization. *Biotechnol Adv.* 1991;9:559–612.
14. Fernandes P, Marques MPC, Carvalho F, Cabral JMS. A simple method for biocatalyst immobilization using PVA-based hydrogel particles. *J Chem Technol Biotechnol.* 2009;84:561–564.
15. Durieux A, Nicolay X, Simon JP. Continuous malolactic fermentation by *Oenococcus Oeni* entrapped in LentiKats®. *Biotechnol Lett.* 2000;22:1679–1684.
16. Parascandola P, Branduardi P, Alteriis E. PVA-gel (Lentikats®) as an effective matrix for yeast strain immobilization aimed at heterologous protein production. *Enzyme Microb Technol.* 2006;38:184–189.
17. Rebros M, Rosenberg M, Stloukal L, Kristofıkova L. High efficiency ethanol fermentation by entrapment of *Zymomonas mobilis* into LentiKats. *Lett Appl Microbiol.* 2005;41:412–416.
18. Tippkötter N, Roikaew W, Ulber R, Hoffmann R, Denzler HJ, Buchholz H. *Paracoccus denitrificans* for the effluent recycling during continuous denitrification of liquid food. *Biotechnol Prog.* 2010;26:756–762.
19. Ruiz J, Pinsach J, Álvaro G, González G, de Mas C, Resina D, López-Santín J. Alternative production process strategies in *E. coli* improving protein quality and downstream yields. *Process Biochem.* 2009;44:1039–1045.
20. Shin JS, Yun H, Jang JW, Park I, Kim BG. Purification, characterization, and molecular cloning of a novel amine:pyruvate transaminase from *Vibrio fluvialis* JS17. *Appl Microbiol Biotechnol.* 2003;61:463–471.
21. Cassimjee KJ, Branneby C, Abedi V, Wellsd A, Berglund P. Transaminations with isopropyl amine: equilibrium displacement with yeast alcohol dehydrogenase coupled to in situ cofactor regeneration. *Chem Commun.* 2010;46:5569–5571.
22. Hohne M, Kuhl S, Robins K, Bornscheuer UT. Efficient asymmetric synthesis of chiral amines by combining transaminase and pyruvate decarboxylase. *Chem Bio Chem.* 2008;9:363–365.
23. Yun H, Kim BG. Asymmetric synthesis of (*S*)-α-methylbenzylamine by recombinant *Escherichia coli* co-expressing omega-transaminase and acetolactate synthase. *Biosci Biotechnol Biochem.* 2008;72:3030–3033.
24. Koszelewski D, Gçritzer M, Clay D, Seisser B, Kroutil W. Synthesis of optically active amines employing recombinant ω-Transaminases in *E. coli* cells. *Chem Cat Chem.* 2010;2:73–77.
25. Rosenberg M, Rebros M, Kristofıkova L, Malatova K. High temperature lactic acid production by *Bacillus coagulans* immobilized in LentiKats®. *Biotechnol Lett.* 2005;27:1943–1947.
26. Martin AR, Shonnard D, Pannuri S, Kamat S. Characterization of free and immobilized (*S*)-aminotransferase for acetophenone production. *Appl Microbiol Biotechnol.* 2007;76:843–851.
27. Yi SS, Lee CW, Kim J, Kyung D, Kim BG, Lee YS. Covalent immobilization of ω-transaminase from *Vibrio fluvialis* JS17 on chitosan beads. *Process Biochem.* 2007;42:895–898.
28. Lee CW, Yi SS, Kim J, Lee YS, Kim BG. Improved immobilized enzyme systems using spherical sol-gel enzyme beads. *Biotechnol Bioprocess Eng.* 2006;11:277–281.

Manuscript received Dec. 16, 2011, and revision received Feb. 26, 2012.

Appendix 1C - Publication 3

A Robust Methodology for Kinetic Model Parameter Estimation for Biocatalytic Reactions

Naweed Al-Haque, Paloma A. Santacoloma, Watson Neto, Pär Tufvesson, Rafiqul Gani and John M. Woodley

Dept. of Chemical and Biochemical Engineering, Technical University of Denmark, Lyngby DK-2800, Denmark

DOI 10.1002/btpr.1588
Published online July 25, 2012 in Wiley Online Library (wileyonlinelibrary.com).

Effective estimation of parameters in biocatalytic reaction kinetic expressions are very important when building process models to enable evaluation of process technology options and alternative biocatalysts. The kinetic models used to describe enzyme-catalyzed reactions generally include several parameters, which are strongly correlated with each other. State-of-the-art methodologies such as nonlinear regression (using progress curves) or graphical analysis (using initial rate data, for example, the Lineweaver-Burke plot, Hanes plot or Dixon plot) often incorporate errors in the estimates and rarely lead to globally optimized parameter values. In this article, a robust methodology to estimate parameters for biocatalytic reaction kinetic expressions is proposed. The methodology determines the parameters in a systematic manner by exploiting the best features of several of the current approaches. The parameter estimation problem is decomposed into five hierarchical steps, where the solution of each of the steps becomes the input for the subsequent step to achieve the final model with the corresponding regressed parameters. The model is further used for validating its performance and determining the correlation of the parameters. The final model with the fitted parameters is able to describe both initial rate and dynamic experiments. Application of the methodology is illustrated with a case study using the ω-transaminase catalyzed synthesis of 1-phenylethylamine from acetophenone and 2-propylamine. © 2012 American Institute of Chemical Engineers *Biotechnol. Prog.*, 28: 1186–1196, 2012
Keywords: biocatalysis, parameter estimation, kinetic modeling, omega-transaminases

Introduction

Biocatalysis is continuing to attract a growing interest in the chemical and pharmaceutical industries and is now becoming a key component in the toolbox of industrial process chemists.[1] Often the green credentials and effective utilization of the raw materials are cited as the most important positive features of biocatalysis. However, in order for the process to be economically feasible (as well as green), high catalyst productivity (mass of product per mass of catalyst) is required.[2] To achieve this, as for any chemical process, detailed knowledge of the reaction kinetics is essential for effective implementation and operation of the process technology. Furthermore, the kinetic parameters provide an insight into the biocatalytic reaction mechanisms (such as inhibition).[3] Often, the ratio of $E_0 k^+_{cat}/K^A_M$ is used to describe the biocatalyst performance. However, for more complex biocatalytic reactions it is important to include also the inhibition parameters of the substrate(s) and product(s) to accurately quantify the catalyst effectiveness and to provide guidance for biocatalyst improvement.[3] The kinetic parameters can also be used in a process model to describe the dynamic behavior of the reaction and in this way be used to evaluate opportunities for process integration (e.g., in situ product removal),[6] process control and operational optimization.[7] Furthermore, the reaction equilibrium can be derived from the kinetic parameters, which is essential in selecting suitable process operating strategies.[8]

Biocatalytic reactions are frequently considered to obey mixed order (between 0 and 1) kinetics similar to the Langmuir-Hinshelwood model denoted as the Michaelis and Menten kinetics.[9] The principles of the Michaelis and Menten kinetics have been further expanded to describe multisubstrate reactions with complex reaction behavior. For example, a generic equilibrium controlled bisubstrate reaction can be formulated as Eq. 1:

$$A + B \leftrightarrow P + Q \qquad (1)$$

The kinetic model (full) for Eq. 1 which follows a ping pong bi-bi mechanism in which substrate (A) is bound first, while the coproduct (P) is released before the second substrate (B)

Correspondence concerning this article should be addressed to J. M. Woodley at: jmw@kt.dtu.dk.

and the final product (Q) leaves the enzyme last,[10] can be formulated as Eq. 2[11]:

$$r_Q = -r_A = \frac{E_0 K^f_{cat} K^r_{cat} \left(\gamma_A [B] - \lambda \frac{[P][Q]}{K_{EQ}}\right)\left(1 - \frac{[I]}{K^I_{Si}}\right)}{K^r_{cat} K^B_M \gamma [A] \quad K^f_{cat} K^A_M \gamma [B] \left(1 + \frac{K^f_{cat} K^Q_M}{K_{EQ}} \lambda [P] \quad \frac{K^f_{cat} K^P_M}{K_{EQ}} \lambda [Q]\right)}$$

$$- K^r_{cat} \gamma A [B] - \frac{K^I_{cat} K^Q_M}{K^A_I K_{EQ}} \gamma \lambda A [P] + \frac{K^I_{cat}}{K_{EQ}} \lambda P [Q]$$

$$\frac{K^r_{cat} K^A_M}{K^Q_I} \gamma \lambda B [Q] \qquad (2)$$

The dynamic equation consists of nine parameters including terms such as the catalytic turnover of the reaction (K^r_{cat},K^f_{cat}), Michaelis parameters (K^A_M, K^B_M, K^P_M, K^Q_M), inhibition parameters (K^A_I,K^Q_I) that are derived from the core mechanism, uncompetitive substrate inhibition parameter (K^I_{Si}) due to formation of nonproductive complexes and the binary reaction direction indicator (λ and γ) thus forming a complex model.

The chemical equilibrium can be represented with a constitutive equation using the Haldane relationship. The relationship is shown in Eq. 3:

$$K_{EQ} = \left(\frac{K^f_{cat}}{K^r_{cat}}\right)^2 \cdot \frac{K^P_M K^Q_M}{K^A_M K^B_M} - \left(\frac{K^I_{cat}}{K^r_{cat}}\right) \cdot \frac{K^Q_M K^P_I}{K^B_M K^A_I} - \left(\frac{K^f_{cat}}{K^I_{cat}}\right) \cdot \frac{K^P_M K^Q_I}{K^A_M K^B_I} - \frac{K^P_I K^Q_I}{K^A_I K^B_I} \qquad (3)$$

Although enzymes have evolved to operate very effectively at low concentrations of substrates in a natural environment, for industrial applications, high concentrations of substrates are required to ensure that capital and subsequent downstream processing costs are manageable. For this reason, the effects of higher concentrations of substrate (and product) on the enzyme kinetics are critically important. In fact, under industrial conditions it is normally the case that the enzyme is inhibited by the substrate and the product or even other components present in the reaction medium. These excess inhibition effects are introduced into the kinetic expression through an inhibition relationship $\left(1 + \frac{[I]}{K_I}\right)$ factored into Eq. 2 either in the velocity term or the Michaelis constant term or both, depending upon whether the reaction exhibits uncompetitive, competitive or noncompetitive inhibition, respectively.[12] For example, a reaction exhibiting uncompetitive inhibition with compound I, would result in the inhibition relationship factored into the numerator as shown in Eq. 2. When attempting to estimate the parameters in Eq. 2, it is often recognized that the parameters may be strongly correlated, thereby compromising the uniqueness of the parameter values.[13] This means that the physical meaning of the parameters is lost and that many different sets of parameter values can fulfil the requirements of the given equations. For instance, different initial guesses for the parameters will result in completely different values of the parameters. There is, therefore, a need to address the problem of determining the kinetic parameters for biocatalytic reactions in a systematic and efficient manner.

To reduce the number of parameters to be estimated simultaneously, it is therefore suggested to decompose the full model to the initial rate model by designing experiments where samples are taken at the initial period of the experiment. During this initial time, it can be assumed that the influence of product and reversibility of the reaction is negligible. Therefore, the terms related to product accumulation and the equilibrium relationships can be omitted. For equilibrium controlled reactions, in the absence of products, Eq. 2 can be decomposed into Eqs. 4a and 4b by considering $\gamma = 1$ and $\lambda = 0$ to get equation for $-r_A$ and considering $\lambda = 0$ and $\gamma = 1$ to get equation for r_Q:

Forward direction (Eq. 4a):

$$r_Q = -r_A = \frac{E_0 K^r_{cat} [A][B]}{K^A_M [B] - K^B_M [A] - [A][B]} \left(1 + \frac{[I]}{K^I_{Si}}\right) \qquad (4a)$$

Reverse direction (Eq. 4b):

$$r_A = -r_Q = \frac{E_0 K^r_{cat} [P][Q]}{K^P_M Q \quad K^Q_M P] \quad P][Q} \left(1 - \frac{[I]}{K^I_{Si}}\right) \qquad (4b)$$

where Eq. 4a represents the consumption of substrate A for the forward direction, while Eq. 4b represents the consumption of substrate Q when running the reaction in the reverse direction. The most commonly used methodologies to estimate the parameters for enzymatic reactions are the graphical plotting (GP) method and nonlinear regression (NLR) method. The former requires a linearization of the kinetic expression at different initial rate conditions to obtain linear plots from which the kinetic parameters can be determined. Some of the commonly used plots are the Lineweaver-Burke, Hanes and Dixon plots.[14] Although simple to use, these methods introduce inaccuracy in the parameter estimation as the errors increase significantly at low concentrations of the substrate.[15,16] Furthermore, where there is significant inhibition of the substrates, the plots are no longer linear and therefore assumptions of linear regions are not valid.

The NLR method, on the other hand, relies on minimizing the margin of error between the model outputs or model predictions and the corresponding experimentally measured values. Often, this procedure is carried out using an optimization routine such as the least squares method. This is clearly an improvement on the GP method since no model linearization is required, although usually mathematical software with curve fitting or an optimization toolbox is needed. The major advantage of the NLR method is that it can be applied for both initial rate data (as the GP method) and a set of reaction progress curves. However, a difficulty in using the NLR method is the necessity of good initial guesses for the kinetic parameters. A local minimum of the objective function is frequently found if the initial guesses are poor and the kinetic model is complex.[17]

An alternative approach developed by Chen et al.[18] proposed a methodology, which simplifies the kinetic expression into regions of negligible and non-negligible inhibition. However, the assumption of including a region of negligible inhibition is rather subjective, leading to an inherent weakness in this approach. For reaction systems with severe inhibition, using data from the low concentration range (where inhibition is assumed to be negligible) is likely to result in erroneous estimations and may result in overestimation of the Michaelis parameters. Furthermore, the method was validated for an irreversible biocatalytic reaction with the assumption that the coproduct was completely removed in situ. On the other hand, many industrially relevant

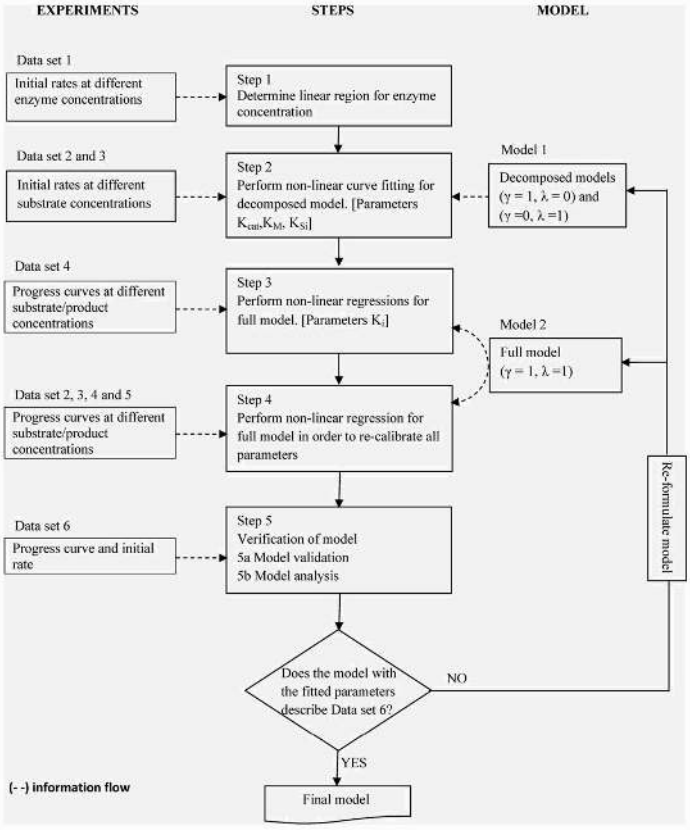


Figure 1. Proposed methodology for kinetic parameter estimation.

biocatalytic reactions (e.g., reactions catalyzed by transaminases, transketolases, and transaldolases) are equilibrium controlled reactions involving two substrates being converted into two corresponding products for which the optimization of the parameters have to be treated differently.

In this article, a systematic methodology to estimate the kinetic parameters of enzymatic systems which exhibit substrate/product inhibition is presented. The methodology incorporates the advantages of each of the different approaches, thus enabling estimation of the parameters with the highest reliability. Likewise, the methodology avoids the assumptions made by Chen et al.[18] to construct a negligible inhibition region. An experimental framework is presented to provide guidelines for the type of experiments required to acquire the kinetic parameters according to the model selected. The outcome of the new approach will provide a platform for good practice for estimating kinetic parameters of biocatalytic reactions. In this article, the methodology is exemplified stepwise using the ω-transaminase catalysed reaction between acetophenone and 2-propylamine for the asymmetric synthesis of (*S*)-1-phenylethylamine as a demonstration.

Proposed Methodology for Kinetic Parameter Estimation

To deal with the problems discussed above, a robust systematic methodology for kinetic parameter estimation has been developed. This is built upon the previously documented methodology of Chen et al.[18] The parameter estimation problem is decomposed into five hierarchical steps where the solution of each of the steps becomes the input for the subsequent step. The available data is broken down into six subsets to match the different steps of the estimation. Figure 1 illustrates the proposed methodology.

Description of the methodology

In step 1, data set 1 contains the results of experiments where the initial rate of reaction is measured as a function of enzyme concentration. Often, the rate of reaction increases linearly with enzyme concentration until a saturation limit is reached where mass-transfer limitations begin to influence the overall reaction rate. Subsequent addition of enzyme will

increase the overall rate of reaction in a nonlinear manner. The objective is therefore to determine this linear region to guide the experiments such that all subsequent experiments for parameter estimation must be carried out with enzyme concentrations within the enzyme linear region as the nonlinear effect of the enzyme concentration is not considered in this study.

In step 2 the kinetic model (full model) (Eq. 2) is decomposed into two initial rate models for the forward and reverse reactions (Eqs. 4a and 4b) to represent the initial period of the experiment. In this way, the numbers of parameters to be estimated simultaneously are reduced. Data collected from initial rate experiments (data sets 2 and 3) at different substrate concentrations in both directions are used to regress the parameters (K_{cat}^{f}, K_{cat}^{r}, K_M^A, K_M^B, K_M^P, K_M^Q, K_{Si}^{f}) in the decomposed models (Eqs. 4a and 4b). The objective function (Eqs. 5a and 5b) for both forward and reverse reactions which is calculated as the sum of the squares of the deviations between model prediction and experimental result (least squares) is minimized for both the forward and reverse reaction subject to that parameter values are greater than 0 ($\theta > 0$, where θ represents a vector of parameters).

$$J_1(\theta) = 1/2\sum_{j=1}^{N_r}\left(r_{A,exp,j} - r_{A,j}(\theta)\right)^2 \tag{5a}$$

$$J_2(\theta) = 1/2\sum_{j=1}^{N_r}\left(r_{Q,exp,j} - r_{Q,j}(\theta)\right)^2 \tag{5b}$$

A common challenge in kinetic parameter estimation for biocatalytic reactions is the high degree of correlation between parameters, which often results in a local minimum in the objective function. Therefore, it is required to solve the optimization problem for different initial parameter values to increase the probability of finding a global minimum in the objective function. The optimization is solved within a given range to obtain the local minimum for each evaluated set. The final estimated parameters correspond to the minimal of the local optimization. These parameters can be considered to the global optimal solution.

In step 3 the additional core inhibition parameters (K_i^A and K_i^Q) from the full kinetic model (Eq. 2) are regressed using data set 4 (dynamic experiments where the product accumulation and substrate depletion is followed over time) keeping all the other parameters fixed at the values obtained in step 2. Similar to step 2, the optimization problem is solved several times with different initial values. Equation 2 represents the reaction rate which is used to formulate the mass balances for each of the reactants in the process. Consequently, the process model is expressed as a set of ordinary differential equations (ODEs), which must be solved to obtain the prediction for the substrate and product concentrations as a function of time. The expressions for product formation and substrate consumption have the same form only differing in a plus sign for product formation and minus sign for substrate consumption. The objective function (Eq. 6) is formulated by the least squares method as the sum of squared error between the model prediction and experimental data of the different compound concentrations $y_{i,}$. Furthermore, the optimization is subjected to the constraint that parameter values are greater than 0 ($\theta > 0$. θ now represents the additional parameters).

$$J(\theta) = 1/2\sum_{j=1}^{N}\left([y_i]_{j,exp} - [y_i]_{j,model}(\theta)\right)^2 \tag{6}$$

where, n represents the number of different compounds present in the reaction

In step 4 the parameters that have been estimated in steps 2 and 3 are used as initial estimates and using the full data set (sets 2, 3, 4, and 5), they are regressed to obtain the final model (Eq. 2). The final model with the regressed parameters is able to describe the initial rate data sets as well as the progress curve data sets. The knowledge of the equilibrium constant if available can be included into the full model to reduce the search space for the estimation. The value of the equilibrium constant (K_{EQ}) may be obtained experimentally[19,20] or by using group contribution methods.[21] However, this data is generally not available for enzymes exhibiting slow reaction rates because performing equilibrium experiments would require a lot of enzymes which is relatively expensive. K_{EQ} can then be calculated using the estimated parameters by following Eq. 3. Two parameters (K_i^B and K_i^P) which are not included in the full model (Eq. 2) are calculated using the relationship shown in Eq. 3, the estimated parameters and the value of the K_{EQ}.

Once the final model with the corresponding parameters is obtained, it is verified with new data and analyzed before making it ready for different applications. In step 5a, verification of performance is made through data set 6 (progress curves and initial rate data with different initial conditions). In step 5b, the objective is to understand the correlation between parameters, thereby identifying the relationship between the parameters. A linear approximation of the covariance matrix of parameter estimators, COV(θ), was used to estimate the correlation matrix. The covariance matrix of the estimated parameters was formulated in Eq. 7[22]:

$$\mathrm{Cov}(\theta) = \frac{\min J(\theta)}{N-P}\left[\left(\frac{d\,y}{d\theta}\right)^T \frac{1}{\sigma^2}\left(\frac{d\,y}{d\theta}\right)\right]^{-1} \tag{7}$$

where min $J(\theta)$ corresponds to the minimum value obtained from the objective function (Eq. 6), N is the number of data points and P is the number of estimated parameters, and $\left(\frac{dy}{d\theta}\right)$ corresponds to the Jacobian matrix which is also the local sensitivity of model variable y to parameters θ.

The importance of the covariance matrix is to calculate both the confidence intervals of the parameters and the correlation matrix of the estimated values.

The confidence interval of parameters is determined with a confidence level of (1 α) corresponding to the 95th percentile of the t-distribution value calculated at the $\alpha/2$ percentile with $N - p$ degrees of freedom, as described in Eq. 8.

$$\theta_{1-\alpha} = \theta \perp \sqrt{\mathrm{diag}(\mathrm{Cov}(\theta))} \cdot t\left(N-p,\frac{\alpha}{2}\right) \tag{8}$$

In Eq. 8, the diag(Cov(θ)) takes into account only the diagonal values of the covariance matrix of parameters (see Eq. 7) for this calculation.

The linear relationship between the parameters is analyzed with the correlation matrix COR(θ_i, θ_j) as shown in Eq. 9, thus[22]:

$$\mathrm{Cor}(\theta_i, \theta_j) = \frac{\mathrm{Cov}(\theta_i, \theta_j)}{\sqrt{\mathrm{Cov}(\theta_i, \theta_i)\mathrm{Cov}(\theta_j, \theta_j)}} \quad (9)$$

Here, correlation coefficients are calculated: a negative coefficient between two parameters indicates that as one parameter value increases, the other decreases; while a positive coefficient indicates that both parameter values increase or decrease simultaneously. A value of zero denotes a lack of correlation. Even though it is preferable that correlation between parameters does not exist, this is usually not the case since the parameters in multisubstrate enzymatic reactions are generally correlated with each other. At the end of step 5, the model is ready for use in different applications.

Type of experiments

The experiments required to collect the six data sets can be summarized in Table 1 as follows:

Table 1. Collected Data Sets for the Proposed Methodology

Variables	Initial Rate* Enzyme concentration	Initial Rate† Substrate concentration	Progress Curve‡ Substrate or product concentration over time
Data set 1	✓		
Data sets 2 and 3		✓	
Data sets 4 and 5			✓
Data set 6§		✓	✓

* Initial rate of reaction is measured for varying enzyme concentration at a fixed substrate concentration. † Initial rate of reaction is measured for varying substrate concentration for forward and reverse direction at a fixed enzyme concentration. ‡ Concentration of substrates and products are measured as a function of time (until it approaches equilibrium) at a fixed enzyme concentration. § Independent data set which is not used for parameter estimation.

Case Study: Kinetic Modeling of ω-Transamination for the Synthesis of 1-phenylethylamine

To illustrate the application of the systematic methodology for kinetic model parameter estimation, the synthesis of 1-phenylethylamine (PEA, also know as α-methylbenzene amine) and coproduct acetone (ACE) from acetophenone (APH) and 2-propylamine (IPA) in the presence of the ω-transaminase (EC 2.6.1.X) (TAm, ATA - 040) has been studied. In the synthesis of optically pure chiral amines using TAm, the reaction is catalyzed by the transfer of an amine ($-NH_2$) group from an amine donor, to a pro-chiral acceptor ketone, yielding a chiral amine as well as a coproduct ketone[11] (see Figure 2). The enzyme requires pyridoxal phosphate (PLP) as a cofactor to act as a shuttle to transfer the amine moiety between the molecules.[24]

Experimental section

Reagents and Enzyme. Commercial-grade reagents and solvents were purchased from Sigma-Aldrich (Buchs, Switzerland) and used without further purification. The unpurified enzyme ω-transaminase (ATA-040) which came as lyophilized powder was kindly supplied by c-LEcta Gmbh (Leipzig, Germany). In all experiments enzyme amount refer to gram of lyophilized powder.

Type of Experiments. Unless otherwise mentioned, all reactions were carried out in 4 mL vials with an operating volume of 3 mL. The experiments were performed in a thermoshaker (Model 11, HLC Biotech, Bovenden, Germany) at 30°C with orbital agitation of ~400 rpm. The pH was maintained at 7 by addition of 100 mM of potassium phosphate buffer (pH 7). The concentration of cofactor PLP in the vial was 2 mM. Samples of 200 μL were taken at the specified time intervals and added to a centrifuge tube containing 800 μL of 1 N HCL to stop the reaction. The samples were centrifuged for 5 mins at 14,100 rpm (MiniSpin plus, Eppendorf AG, Germany) and analyzed by HPLC with a final dilution of 50-fold. The experiments required to collect the six data sets are summarized in Table 2 as follows:

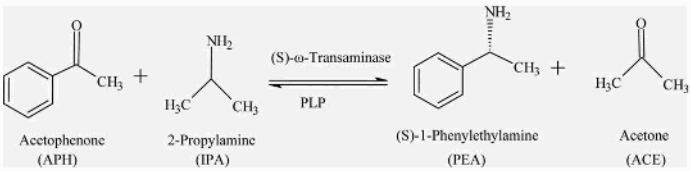


Figure 2. Transamination catalysed by ω-transaminase illustrating the synthesis of 1-phenylethylamine (PEA) and co-product acetone (ACE) from the substrates acetophenone (APH) and 2-propylamine (IPA) in the presence of the enzyme TAm.

Table 2. Data Collection for the Application of the Proposed Methodology

Data Set	Variable: Enzyme (g/L)	IPA (mM)	APH (mM)	MBA (mM)	ACE (mM)	Sampling Time (min)
1	0–10	1,000	5	–	–	1, 3, 5
2	1.8	100, 300, 500	1–10	–	–	1, 3, 5
3	1.8	–	–	1–30	50, 100, 500	1, 3, 5
4	1.8	1,000	1.8	0.8	–	Intervals of 30 min until equilibrium
5	1.8	1,000	1.7	0.5	–	Intervals of 30 min until equilibrium
6a	3.6	–	–	5	1,000	Intervals of 30 min until equilibrium
6b	1.8	–	–	1–30	1,000	1, 3, 5
Parity plot	1.8	1,000	2	–	–	Intervals of 30 min until equilibrium

g represents the grams of lyophilized powder of the enzyme

Analytical. Samples were measured *ex situ* on an Ultimate 3000 HPLC (Dionex, Sunnyvale, CA), equipped with a UV detector and a photodiode array detector. The compounds were separated on a Luna 3 μm C18(2) 100 Å (50 × 4.6 mm) column (Phenomenex, Torrance, CA) at a flowrate of 2 mL/min using a multistep gradient flow of aqueous 0.1% v/v trifluoroacetic acid and acetonitrile, with the following percentage of acetonitrile: 0 min (0%), 1 min (10%), 2.5 min (10%), 5.9 min (60%), 6 min (0%), 7 min (0%). The compounds were quantified at the following wavelengths: acetophenone (280 nm) and 1-phenylethylamine (210 nm), with retention times of 5 and 2.1 mins, respectively.

Reaction kinetic modeling

The ω-transaminase-catalyzed reaction is known to follow the so-called "ping-pong bi-bi" mechanism.[25,26] The reaction is heavily influenced by (competitive) inhibition of the substrate acetophenone (APH) and product 1-phenylethylamine (PEA), as well as having unfavorable reaction equilibrium. Consequently, there is a strong motivation to go forward with the formulation and analysis of a kinetic model to predict reaction behavior under different process conditions.

The kinetic model to describe the reaction mechanism is derived based on the King–Altman method[11,27,28] as shown in Figure 3. Alternatively, kinetic models from the scientific literature if available can also be used.

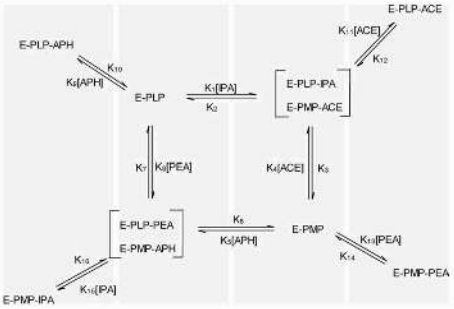


Figure 3. King-Altman representation of the ω-transaminase reaction mechanism.

As can be seen in Figure 3, the mechanism includes the formation of four nonproductive complexes E-PLP-APH, E-PMP-IPA, E-PMP-PEA, and E-PLP-ACE, which are characterized by a substrate inhibition constant K_{Si}^{APH} and K_{Si}^{IPA} in the forward direction and K_{Si}^{PEA} and K_{Si}^{ACE} in the reverse direction. The derived rate equation is as follows (Eq. 10)[11] ($\lambda = \gamma = 1$):

$$r_{[PEA]} = -r_{APH} = \frac{[E_0]K_{cat}^f K_{cat}^r\left(\lambda[IPA][APH] - \gamma\frac{[ACE][PEA]}{K_{EQ}}\right)}{K_{cat}^r K_M^{APH}\lambda[IPA]\left(1+\gamma\frac{[PEA]}{K_{Si}^{PEA}}+\frac{[IPA]}{K_{Si}^{IPA}}\right)+K_{cat}^r K_M^{IPA}[APH]\lambda\left(1+\frac{[APH]}{K_{Si}^{APH}}+\gamma\frac{[ACE]}{K_{Si}^{ACE}}\right)} + K_{cat}^f\frac{K_M^{PEA}[ACE]}{K_{EQ}}\gamma\left(1+\frac{[APH]}{K_{Si}^{APH}}+\lambda\frac{[ACE]}{K_{Si}^{ACE}}\right)+K_{cat}^r\frac{K_M^{ACE}[PEA]}{K_{EQ}}\gamma\left(1+\frac{[PEA]}{K_{Si}^{PEA}}+\lambda\frac{[IPA]}{K_{Si}^{IPA}}\right) + K_{cat}^r\lambda[IPA][APH]-K_{cat}^f\frac{K_M^{PEA}\lambda\gamma[IPA][ACE]}{K_{EQ}K_i^{IPA}}+K_{cat}^f\gamma\frac{[ACE][PEA]}{K_{EQ}}+K_{cat}^r\frac{K_M^{IPA}\lambda\gamma[APH][PEA]}{K_i^{PEA}} \quad (10)$$

The constitutive equation of the chemical equilibrium is formulated using the Haldane relationship in Eq. 11:

$$K_{EQ} = \left(\frac{K_{cat}^f}{K_{cat}^r}\right)\frac{K_M^{PEA}K_M^{ACE}}{K_M^{APH}K_M^{IPA}} = \left(\frac{K_{cat}^f}{K_{cat}^r}\right)\frac{K_M^{ACE}K_i^{PEA}}{K_M^{IPA}K_i^{APH}} = \left(\frac{K_{cat}^f}{K_{cat}^r}\right)\frac{K_M^{PEA}K_i^{ACE}}{K_M^{APH}K_i^{IPA}} = \frac{K_i^{PEA}K_i^{ACE}}{K_i^{APH}K_i^{IPA}} \quad (11)$$

Equation 10 is further decomposed into Eqs. 12a and 12b by considering the initial period of the experiment. In this case study, the time interval for initial rate was considered to be the first 5 mins of reaction time. During this time period, it was assumed that the concentration of product was extremely small and thus the terms related to product and equilibrium in Eq. 7 could be neglected. In this way, the rate equation in the absence of products for both forward, $-r_{APH}$, ($\gamma = 1$, $\lambda = 0$) and reverse direction, $-r_{PEA}$, ($\gamma = 0$, $\lambda = 1$) was decomposed into Eqs. 12a and 12b.

Forward direction (Eq. 12a):

$$r_{[PEA]} = -r_{[APH]} = \frac{[E_0]K_{cat}^f([APH][IPA])}{K_M^{APH}[IPA]\left(1+\frac{[IPA]}{K_{Si}^{IPA}}\right)+K_{IPA}[APH]\left(1+\frac{[APH]}{K_{Si}^{APH}}\right)+[IPA][APH]} \quad (12a)$$

Reverse direction (Eq. 12b):

$$r_{[APH]} = -r_{[PEA]} = \frac{[E_0]K_{cat}^r([PEA][ACE])}{K_M^{PEA}[ACE]\left(1+\frac{[ACE]}{K_{Si}^{ACE}}\right)+K_M^{ACE}[PEA]\left(1+\frac{[PEA]}{K_{Si}^{PEA}}\right)+[PEA][ACE]} \quad (12b)$$

Unlike the methodology of Chen et al.,[18] the simplified model used here takes into account substrate inhibition for all components involved in the reaction removing the risk of assuming a negligible inhibition region.

Application of the proposed methodology

Step 1: Determine Linear Region for Increasing Enzyme Concentration. Plotting the initial rate of reaction vs. the concentration of the enzyme from data set 1 (see Figure 4), shows that the rate of reaction loses its linearity after an enzyme concentration of around 5 g/L. Further addition of the enzyme increases the rate of reaction but not in a linear manner with respect to enzyme concentration and therefore also the model predictions were limited to rates of reactions carried out with enzyme concentration of up to 5 g/L.

Step 2: Perform Nonlinear Curve Fitting for Decomposed Model. The kinetic model (Eq. 10) was decomposed into two initial rate models as shown in Eqs. 12a and 12b. The objective function was formulated as shown in Eqs. 5a and 5b. The objective function was evaluated for different initial parameter values within the range of 0–1,000. The new estimated parameters are selected as established in the methodology and listed in Table 3 along with the 95% confidence interval (CI) calculated for each of the estimated parameters. The optimization was solved in Matlab® (The Mathworks, Natick, MA) using the built-in least square function with a tolerance of 1.0E-06. Figure 5 displays the performance of the model predictions with the estimated parameters compared to the experimental data (data sets 2 and 3). From Table 3 it could be seen that the parameters, K_{Si}^{IPA} and K_{Si}^{ACE}, which lies in the denominator of the fraction in Eq. 7, were extremely large compared to the operating concentration of the reactants. The significance of these terms could thus be considered negligible and were omitted from the kinetic model (Eq. 10). It can be noted that a very good fit of the experimental data has been achieved.

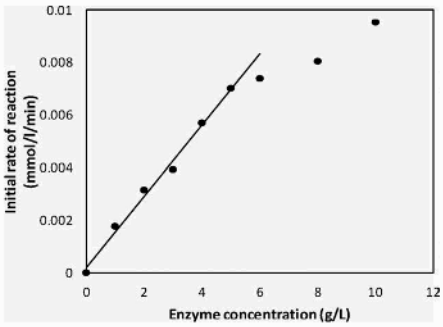


Figure 4. Enzyme linear region.

Reaction condition: 1000 mM IPA, 5 mM APH, 2 mM PLP, 100 mM phosphate buffer.

Table 3. Estimated Parameter Values from Step 2 Using the Proposed Approach

Parameters	Estimated Values	95% CI
Rate constants (min^{-1})		
K_{cat}^{f}	0.0025	±7E-5
K_{cat}^{r}	0.023	±4E-4
Michaelis constants (mM)		
K_{M}^{APH}	1.50	±0.10
K_{M}^{IPA}	89.77	±10.93
K_{M}^{ACE}	240.62	±9.41
K_{M}^{PLA}	2.41	±0.14
Substrate inhibition constants (mM)		
K_{Si}^{APH}	1.23	±0.16
K_{Si}^{PLA}	6.01	±0.21
K_{Si}^{IPA}	7.2E4	±7E5
K_{Si}^{ACE}	1.1E4	±1E4

Step 3: Perform Nonlinear Regression for Full Model. The previously determined parameters (K_{cat}^{f}, K_{cat}^{r}, K_{M}^{APH}, K_{M}^{IPA}, K_{M}^{PLA}, K_{M}^{ACE}, K_{Si}^{APH}, K_{Si}^{PLA}) were fixed and the additional parameters (K_{i}^{IPA} and K_{i}^{PLA}) were regressed with data set 4 using the full kinetic model (Eq. 10). The objective function was formulated as shown in Eq. 6. The full dynamic process model represented by the set of ODEs was solved in Matlab® (The Mathworks, Natick, MA) using the ODE45 solver which was based on the Runge-Kutta algorithm for solving ODEs. Additionally, integration accuracy was set to 1.0E-06 to maintain accuracy in the application of the solver.

Table 4 gives the parameter values obtained by minimization of Eq. 6. Using initial rate experiments is not advised in this step as it does not include the effect of the reaction equilibrium and may compromise the value of core inhibition parameters. In a report by Shin and Kim,[29] initial rate data was used to estimate the kinetic parameters for ω-transaminase (from the source *Bacillus thuringiensis* JS64) catalyzed resolution of 1-phenylethylamine. However, when the estimated parameters were analyzed, it was evident that the predictions were erroneous. This could be confirmed from the significantly deviated predictions of the equilibrium constant when compared to literature.[19]

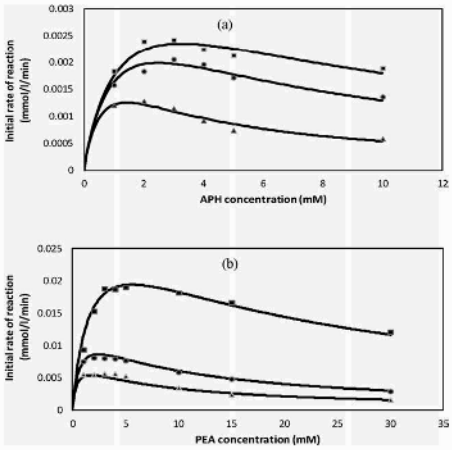


Figure 5. Experimental data (symbol) and model prediction (solid line) using estimated parameters from step 2.

Reaction conditions: (a) Forward direction: E_0 is 1.8 g/L, concentration of IPA is fixed at 500 mM [ ], 300 mM ○ and 100 mM △, 2 mM PLP, 100 mM phosphate buffer. (b) Reverse direction: E_0 is 1.8 g/L, concentration of ACE is fixed at 500 mM [ ], 100 mM ○ and 50 mM △, 2 mM PLP, 100 mM phosphate buffer.

Step 4: Perform Nonlinear Regression for Full Model for Recalibrating all Parameters. The final model and fitted parameters were obtained by regressing all the parameters from steps 2 and 3 using data sets 2, 3, 4, and 5. In this case study, the knowledge of the equilibrium constant was acquired from literature.[19] The remaining parameters (K_i^{APH} and K_i^{ACE}) which were not included in the model (Eq. 10) were then additionally calculated using Eq. 11.

The final estimates of the parameters are listed in Table 4 and Figure 6 illustrates the model predictions using the estimated parameters. From Figure 6 it can be seen that the model predictions fitted very well the experimental data for the progress curve and the initial rate data sets. From Table 4, it can be seen that the parameter value K_{cat}^r which is the catalytic turnover of the reaction in the forward direction is much smaller than the parameter value K_{cat}^s which is the catalytic turnover of the reaction in the reverse direction. This was not surprising as this parameter describes the rate of catalyst turnover and from experimental observations the reverse reaction is more favourable which is reflected by the difference in the K_{cat}^r and K_{cat}^s values. This is also consistent with a study reported by Shin and Kim.[29] The Michaelis constant of the cosubstrates (K_M^{IPA} and K_M^{ACE}) is higher than the corresponding reactants, which reflects the need to add an excess of the cosubstrate in the reaction media to drive the reaction forward, which is consistent with other studies reported on transaminases.[30] Further it can be seen from the substrate inhibition constant of APH, K_{Si}^{APH} and PEA, K_{Si}^{PEA} that inhibition contributes significantly to the reduction of the rate of reaction which implies the need for a feeding strategy to alleviate the substrate inhibition problem. The core inhibition constant for IPA and ACE (K_i^{IPA} and K_i^{ACE}) are extremely high which was expected as they did not pose any inhibitory affect towards the rate of reaction. However the value of the core inhibition constant of PEA, K_i^{PEA} was significantly low which also is consistent with studies reported by other researchers. This confirms, what is experimentally observed, that the rate of reaction is significantly reduced with the build up of the product PEA when running the reaction in the forward direction.[29,31] The relatively low value of the core inhibition constant indicates the necessity of an effective in situ product removal technique to run the reaction to achieve high levels of conversion.

Table 4. Estimated Parameters Values from Steps 3 and 4 Using the Proposed Approach

	Estimated Values	95% CI	Estimated Values	95% CI
Parameters	Step 3		Step 4	
Rate constants (min^{-1})				
K_{cat}^r	0.0025	±7E-5	0.0078	±7E-5
K_{cat}^s	0.023	±4E-4	0.013	±0.007
Michaelis constants (mM)				
K_M^{APH}	1.50	±0.10	1.85	±1.78
K_M^{IPA}	89.77	±10.93	101.28	±38.23
K_M^{ACE}	240.62	±9.41	148.99	±2.91
K_M^{PEA}	2.41	±0.11	0.12	±0.01
Substrate inhibition constants (mM)				
K_{Si}^{APH}	1.23	±0.16	1.15	±3E-4
K_{Si}^{PEA}	6.01	±0.21	10.38	±3E-4
Core inhibition constants (mM)				
K_i^{APH}	–	–	0.09	–
K_i^{IPA}	4,281	±0.03	4,281	±0.63
K_i^{ACE}	1.55	±0.01	0.11	±0.01
K_i^{PEA}	–	–	1E5	–
Equilibrium constant				
K_{eq}	–		0.033	

Table 4 shows the comparison of model predictions and experimental data for a different set of reaction conditions. As can be seen, the agreement between the simulated data (solid line) and experimental data (symbols) is very good.

Step 5a: Model Validation. To further confirm the validity of the methodology, the estimated parameters used in the model were checked against new data (data set 6) of progress curve and initial rate experiments, carried out using different initial conditions as shown in Figure 7.

Step 5b: Model Analysis. In this study, the standard deviation of measurements σ was assumed to be 5% of the average measured concentration during the batch. The standard deviation of measurements was assumed to be identical at each time instant. The correlation matrix for the case described here is shown in Figure 8. As expected (for these type of systems), parameters are significantly correlated. It can be especially seen in the parameters K_M^{IPA} and K_N^{APH} displayed strong correlation of around 0.99, which is reflecting the high confidence interval of the estimated parameters. The true kinetic parameter values are strongly influenced by the initial values used in the parameter estimation step. That is,

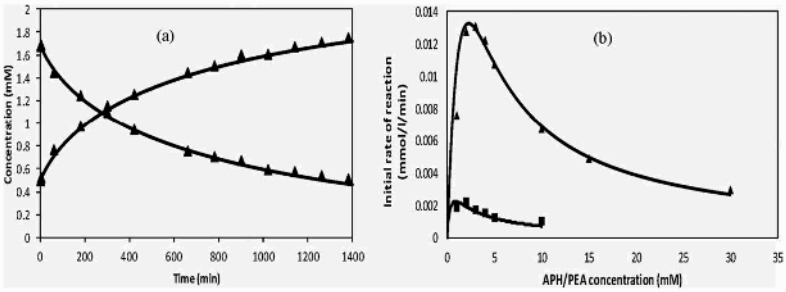


Figure 6. Model predictions using the estimated parameters of the model (solid line) and experimental data (symbols).

Reaction conditions: (a) $E_0 = 1.8$ g/L, $C_{IPA} = 1000$ mM, $C_{APH} = 1.70$ mM, $C_{PEA} = 0.50$ mM, 2 mM PLP, 100 mM phosphate buffer (b) $E_0 = 1.8$ g/L, C_{IPA} is fixed at 500 mM □ and C_{APH} is varied from 1 to 10 mM, 2 mM PLP and buffer 100 mM. C_{ACE} is fixed at 100 mM (triangle) and C_{PEA} is varied from 1 to 30 mM, 2 mM PLP, and buffer 100 mM.

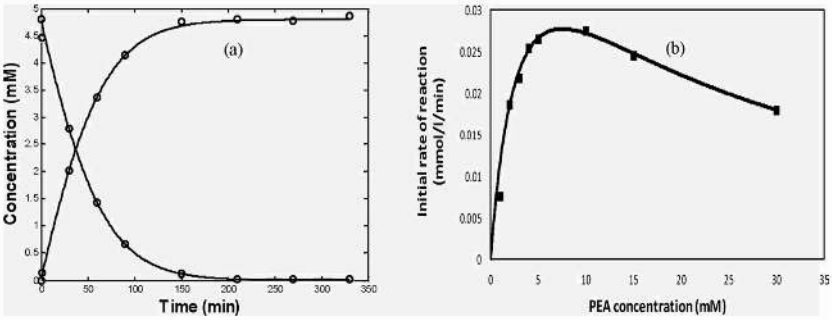


Figure 7. Model predictions using the estimated parameters of the model (solid line) and experimental data (symbols).

Reaction conditions: (a) $E_0 = 3.6$ g/L, $C_{APH} = 0$ mM, $C_{PEA} = 5$ mM, $C_{ACE} = 1000$ mM, 2 mM PLP, 100 mM phosphate buffer. (b) $E_0 = 1.8$ g/L, concentration of ACE is fixed at 1000 mM, 2 mM PLP, 100 mM phosphate buffer.

	K_{si}^{r}	K_M^{IPA}	K_M^{APH}	K_{Si}^{APH}	K_{si}^{r}	K_M^{PEA}	K_M^{ACE}	K_{Si}^{PEA}	K_i^{IPA}	K_i^{PEA}
K_{si}^{r}	1									
K_M^{IPA}	-0.67	1								
K_M^{APH}	0.26	-0.59	1							
K_{Si}^{APH}	0.02	0.34	-0.02	1						
K_{si}^{r}	-0.52	0.35	-0.42	0.09	1					
K_M^{PEA}	-0.27	0.39	0.03	0.23	0.18	1				
K_M^{ACE}	-0.47	0.84	-0.85	0.41	0.41	0.08	1			
K_i^{IPA}	-0.19	0.26	-0.12	-0.44	0.01	0.01	0.01	1		
K_i^{PEA}	-0.65	0.99	-0.59	0.34	0.34	0.39	0.84	0.26	1	
K_{PEA}	-0.29	0.62	-0.99	0.05	0.43	0.01	0.87	0.12	0.62	1

Figure 8. Correlation matrix of the estimated parameters.

Negative coefficient between two parameters indicates that as one parameter value increases, the other decreases. Positive coefficient indicates that both parameter values increase and decrease simultaneously. Zero denotes a lack of correlation.

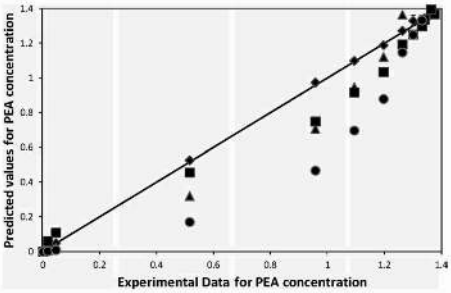


Figure 9. Parity plot of different estimation methodologies.

Proposed methodology (diamond), graphical approach (triangle), NLR (square) and Chen et al. (circle). Reaction conditions: $E_0 = 1.8$ g/L, $C_{IPA} = 1000$ mM, $C_{APH} = 2$ mM, 2 mM PLP, 100 mM phosphate buffer.

the reason why the proposed methodology reduces the possibility of accumulating errors in the parameter estimation and at the same time provides accurate estimates.

Discussion

The methodology presented in this article builds on previously established methods, to provide reliable estimation of kinetic model parameters. In the current approach, model decomposition along with NLR of both initial rate data and progress curves has been incorporated in the methodology. Although, NLR is a quicker method which utilizes all the information from the experiments, it rarely results in a global optimum because of the existence of the strong correlation between the parameters which results in erroneous predictions. Thus a step-wise estimation is used in the current approach, which assists in increasing the probability of finding the global optimum. The Chen methodology uses the advantages of NLR, as can be seen in Figure 9, however, the early assumption of a negligible inhibition region results in the Michaelis and rate parameters to be overestimated because of the use of data points at a very low concentration to estimate K_{ii}^{r} and K_{si}^{r}. The methodology developed for the approach reported in this article builds on the NLR method as well as the Chen et al. method with a more systematic and robust approach. The final model with the fitted parameters is able to describe both type of experiments (initial rate and dynamic experiments). The methodology is approached in a systematic and step-wise manner such that the parameters are reconciled at every step to minimize the estimation errors. Figure 9 illustrates the robustness of this technique. The model predictions of the concentration of PEA with the different methodologies are illustrated with a new set of experiments.

In Figure 9, a parity plot of the different methodology is shown. The figure clearly illustrates the reliability of the current approach where the model predictions fall on the experimental data line. It can be seen that while using the other methodologies, the predictions deviate over time as opposed to the current approach which displays a better fit to describe the experiment. In the Chen methodology, the predictions fit quite well in the initial time period, but with increasing time, the reversibility of the reaction combined with the substrate and product inhibition contribute to deviations from the prediction.

Apart from the robustness of the technique, it is valuable to examine the number of experiments required. Table 5

Table 5. Different Methodologies to Estimate Kinetic Parameters for Equilibrium Reactions

Type of experiment	Methodology	Number of experiments	Limitations
Initial rates	Graphical plotting	High*	Applicable only for reactions that follows simple Michaelis-Menten kinetics. High estimation errors
	Nonlinear regression	High†	Low accuracy when parameters are correlated
Progress curves	Nonlinear regression	Low‡	Reliability is dependent on the need for good initial guess of parameters due presence of local minima
Initial rates and progress curves	Chen and coworkers	Intermediate§	Unreliable for equilibrium reactions including regions of negligible inhibitory region
	Proposed approach	Intermediate¶	A robust method though it is dependent on the knowledge of the equilibrium constant which is used for final parameter calibration

* Gyamerah and Willems used 130 experiments to determine the parameters for a transketolase reaction.[33] † Shin and Kim used 125 experiments to determine the parameters for an ω-transaminase reaction.[23] ‡ The minimum number of data points corresponds to the number of parameters. § Chen et al. used 18 experiments to determine the parameters for a transketolase reaction.[18] ¶ 32 experiments were used to determine the parameters for the ω-transaminase reaction.

tabulates the type of experiment, number of experiments and limitations of the studied methodologies.

A global sensitivity analysis using the Morris method[22] was performed on the estimated parameters involved in the reaction model shown in Eq. 10. The Morris method estimates the effect of the model parameters by calculating for each parameter the elementary effect of varying the other parameter. This analysis is not part of the methodology developed here, but, it was performed to gain further understanding of the significance of the parameters in the model output. The algorithm for performing the analysis was implemented in Matlab® (The Mathworks, Natick, MA). From the Morris screening, it was identified that most of the parameters contributed significantly in the model. This phenomenon was expected, since it could be seen from the correlation matrix (Figure 8), that some of the parameters were strongly correlated with each other. However, three parameters K_I^{Cal}, K_M^{APR}, and K_M^{PYR} did not significantly influence the model output. Therefore, with this knowledge, if the model was to be recalibrated with a new set of experimental data, these three parameters can be fixed and only the remaining seven need be re-estimated. In this way, the parameter space can be reduced.

The sensitivity analysis also forms the basis for the type of experiments that need to be made. Specifically in this TAm case study, it was found that the progress curve provided more information (dynamic behavior). Initial rate experiments are laborious and in addition provide less information (1 data point per experiment). More information can be gathered if the intended initial rate experiments were continued over a longer time interval (until steady state). The sensitivity analysis can provide information about which time intervals it would be desirable to take measurements and further the initial conditions (reactant concentrations) required for the experiments. In this case study, if the model were to be recalibrated, further experiments (progress curves) could be conducted with varying concentrations of the acetophenone and fixed concentrations of 2-propylamine without the need for adding 1-phenylethylamine to the initial reaction mixture.

Conclusion

A method to estimate the kinetic parameters of (ping-pong bi-bi mechanism) biocatalytic reactions suffering from substrate and product inhibition has been developed. The methodology is exemplified with the case study of an ω-transaminase-catalyzed reaction which displays severe substrate and product inhibition. The methodology takes into account the advantages of previously developed methodologies and aims to avoid assumptions which lead to erroneous estimates. The methodology decomposes the kinetic model into initial rate models to reduce the number of parameters involved. The optimization is solved for different initial values of the catalytic turnover, Michaelis parameters and substrate inhibition parameters to increase the probability that the final regressed parameters correspond to global optimal solution. The parameters are then fixed and the remaining core inhibition parameters are determined by NLR using progress curves on the full model. Finally, all the parameters are reconciled (re-estimated) using all the data sets (initial rate and progress curve) to obtain the final kinetic model with the corresponding fitted parameters. The final model with the regressed parameters is able to describe initial rate and progress curve data sets. The methodology is highly reliable which is further validated by comparing predicted values of product concentration with different sets of experimental results. In the proposed approach the number of experiments required is considerably less than the linear plotting method and more robust and reliable.

We believe that the proposed method will significantly simplify and improve the estimation of kinetic parameters for challenging reactions such as ω-transaminases, which can be used in reaction models to evaluate the implications of improved enzymes and processes.

Acknowledgments

The authors N.A.H. and P.A.S. kindly acknowledges Technical University of Denmark. N.A.H. and P.T. acknowledges project AMBIOCAS financed through the European Union Seventh Framework Programme (Grant Agreement no. 245144) for the financial support. W.N. acknowledges support from BIOTRAINS Marie Curie ITN, financed by the European Union through the 7th Framework people Programme (Grant agreement no.: 238531). P.T. wishes to acknowledge the financial support from the ERA-IB project "Eng Biocat" (Registration Number: EIB.08.016).

Notation

[A] — concentration of substrate A, mM
[B] — concentration of substrate B, mM
[P] — concentration of product P, mM
[Q] — concentration of product Q, mM
$[E_0]$ — concentration of enzyme, g/L
[I] — concentration of inhibitory compound, mM

K_{cat} — rate of catalyst turnover, 1/min
K_M — Michaelis constants, mM
K_i — core inhibition constants, mM
K_S — substrate inhibition constants, mM
K_{EQ} — chemical equilibrium constant
γ, λ — reaction direction indicator
ν — model variable
σ — standard deviation
α — significance level
COV — covariance
θ — parameter
J — Jacobian matrix
N — number of data points
p — number of parameters to be estimated
TAm — ω-transaminase
PLP — pyridoxal 5′ phosphate
[APH] — concentration of acetophenone, mM
[IPA] — concentration of 2-propylamine, mM
[PEA] — concentration of 1-phenylethylamine, mM
[ACE] — concentration of acetone, mM

Superscripts

f — forward
r — reverse
A — compound A
B — compound B
P — compound P
Q — compound Q
I — inhibitory compound
APH — compound acetophenone
IPA — compound 2-propylamine
PEA — compound 1-phenylethylamine
ACE — compound acetone

Subscripts

n — number of compounds in reaction
i, j — index

Literature Cited

1. Woodley JM. New opportunities for biocatalysis: making pharmaceutical processes greener. *Trends Biotechnol.* 2008;26:321–327.
2. Tufvesson P, Lima Ramos J, Nordblad M, Woodley JM. Guidelines and cost analysis for catalyst production in biocatalytic processes. *Org Process Res Dev.* 2011;15:266–274.
3. Straathof AJJ, Rakels JLL, Heijnen JJ. Kinetics of the enzymatic resolution of racemic compounds in bi bi reaction. *Biocatal Biotrans.* 1992;7:13–27.
4. Koshland DE. The application and usefulness of the ratio kcat/Km. *Biorg Chem.* 2002;30:211–213.
5. Fox RJ, Michael DC. Catalytic effectiveness, a measure of enzyme proficiency for industrial applications. *Trends Biotechnol.* 2009;27:137–140.
6. Sin G, Woodley JM, Gernaey KV. Application of modelling and simulation tools for the evaluation of biocatalytic processes: a future perspective. *Biotechnol Prog.* 2009;25:1529–1538.
7. Findrik Z, Vasic-Racki D, Lutz S, Daussmann T, Wandrey C. Kinetic modelling of acetophenone reduction catalyzed by alcohol dehydrogenase from *Thermoanaerobacter* sp. *Biotechnol Lett.* 2005;27:1087–1095.
8. Tufvesson P, Lima-Ramos J, Jensen JS, Al-Haque N, Neto W, Woodley JM. Process considerations for the asymmetric synthesis of chiral amines using transaminases. *Biotech Bioeng.* 2011;108:1479–1493.
9. Michaelis L, Menten ML. Kinetik der Invertinwirkung. *Biochem Z.* 1913;49:333–369.
10. Walsh C. *Enzymatic Reaction Mechanisms*. New York: W.H. Freeman; 1998.
11. Leskovac V. *Comprehensive Enzyme Kinetics*. New York: Plenum; 2003.
12. Cornish Bowden A. *Fundamental of Enzyme Kinetics*, 3rd ed. London: Portland Press; 2004.
13. Santacoloma PA, Sin G, Gernaey KV, Woodley JM. Sensitivity analysis of a kinetic model describing the bi enzymatic synthesis of lactobionic acid. In: *Proceedings of the 20th European Symposium on Computer Aided Process Engineering, Ischia, Italy*; 2010:1491–1496.
14. Ranaldi F, Vanni P, Giachetti. What the student must know to determinate enzyme kinetic parameters. *Biochem Educ.* 1999;27:87–91.
15. Nielsen J, Villadsen J, Liden G. *Bioreaction Engineering Principles*. New York: Plenum; 2003.
16. Peter J, Prenosil JE, Bourne JR. Comparison of various methods to determine kinetic constants for β galactosidase in soluble and immobilised states. *J Chem Tech Biotechnol.* 1981;31:705–709.
17. Zavrel M, Kochanowski K, Spiess AC. Comparison of different approaches and computer programs for progress curve analysis of enzyme kinetics. *Eng Life Sci.* 2010;10:191–200.
18. Chen BH, Hibbert BG, Dalby PA, Woodley JM. A new approach to bioconversion reaction kinetic parameter identification. *AIChe J.* 2008;54:2155–2163.
19. Tufvesson P, Jensen JS, Kroutil W, Woodley JM. Experimental determination of thermodynamic equilibrium in biocatalytic transamination. *Biotech Bioeng.* Vol. 109, No. 8, August, 2012.
20. Tewari YB, Goldberg RN. Thermodynamics of reactions catalysed by branched chain amino acid transaminase. *J Chem Thermodyn.* 2000;32:1381–1398.
21. Jankowski MD, Henry CS, Broadbelt LJ, Hatzimanikatis V. Group contribution method for thermodynamic analysis of complex metabolic networks. *Biophys J.* 2008;95:1487–1499.
22. M. Omlin, P. Reichert. A comparison of techniques for the estimation of model prediction uncertainty. *Ecol Model.* 1999;115:45–48.
23. Sin G, Gernaey KV, Lantz AE. Good modeling practice for PAT applications: propagation of input uncertainty and sensitivity analysis. *Biotech Bioeng.* 2009;103:1018–1035.
24. Sin G, Olsman P, Petersen N, Lantz AE, Gernaey KV. Matrix notation for efficient development of first-principles models within PAT applications: integrated modelling of antibiotic production with *Streptomyces coelicolor*. *Biotech Bioeng.* 2008;101:153–171.
25. Hwang BY, Cho BK, Yun H, Koteshwar K, Kim BG. Revisit of aminotransferase in the genomic era and its application to biocatalysis. *J Mol Catal B: Enzym.* 2005;37:47–55.
26. Segel LH. *Enzyme Kinetics*, 1st ed. New York: Wiley; 1975.
27. Hayashi H, Mizuguchi H, Miyahara I, Islam MM, Ikushiro H, Nakajima Y, Hirotsu K, Kagamiyama H. Strain and catalysis in aspartate aminotransferase. *Biochim Biophys Acta.* 2003;1647:103–109.
28. Segel IH. *Enzyme Kinetics. Behaviour and Analysis of Rapid Equilibrium and Steady State Enzyme Systems*. New Jersey: Wiley; 1993.
29. Laidler KJ, Bunting PS. *The Chemical Kinetics of Enzyme Action*. 2nd ed. Oxford: Clarendon; 1973.
30. Shin J S, Kim B G. Kinetic modeling of ω transamination for enzymatic kinetic resolution of α methylbenzylamine. *Biotech Bioeng.* 1998;60:534–540.
31. Kuramitsu S, Hiromi K, Hayashi H, Morino Y, Kagamiyama H. Pre-steady-state kinetics of *Escherichia coli* aspartate aminotransferase catalyzed reactions and thermodynamic aspects of its substrate specificity. *Biochemistry.* 1990;29:5469–5476.
32. Truppo MD, Rozzell JD, Turner NJ. Efficient production of enantiomerically pure chiral amines at concentrations of 50 g/L using transaminases. *Org Process Res Dev.* 2010;14:234–237.
33. Gyamerah M, Willetts AJ. Kinetics of overexpressed transketolase from *Escherichia coli* JM 107/pQR 700. *Enzyme Microbial Technol.* 1997;20:127–134.
34. Morris MD. Factorial sampling plans for preliminary computational experiments. *Technometrics.* 1991;33:161–174.

Manuscript received Mar. 14, 2012, and revision received May 31, 2012.

Appendix 3A - SDS-PAGE Analysis

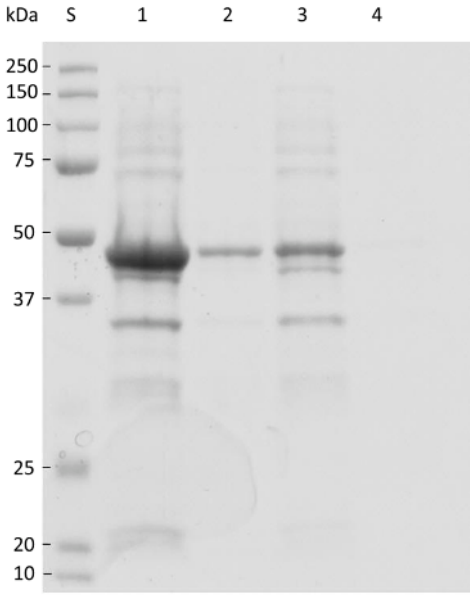
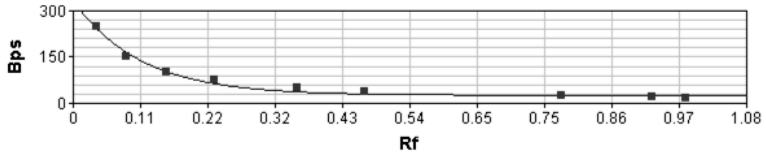


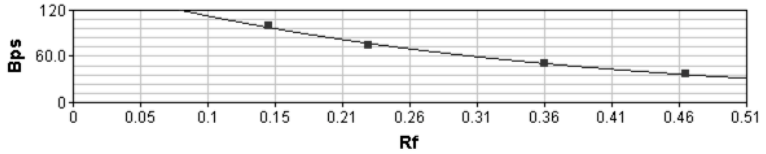
Figure 3A1: SDS-PAGE; S: Standard; 1: Total Protein; 2: Total Protein 10x Dilution; 3: Supernatant; 4: Supernatant 10x Dilution



$y = 305.62976 * e^{(-9.255051 * x)} + 26.370014$

$R^2 = 0.983$

Figure 3A2: MW Calibration Curve; Rf: Relative Mobility



$\ln(y) = -3.108823 * x + 5.0432696$

$R^2 = 0.995$

Figure 3A3: MW Calibration Curve in the desired range; Rf: Relative Mobility

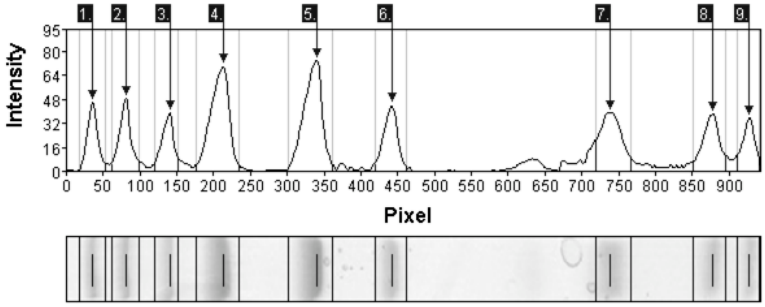


Figure 3A4: Gel analysis of lane 1

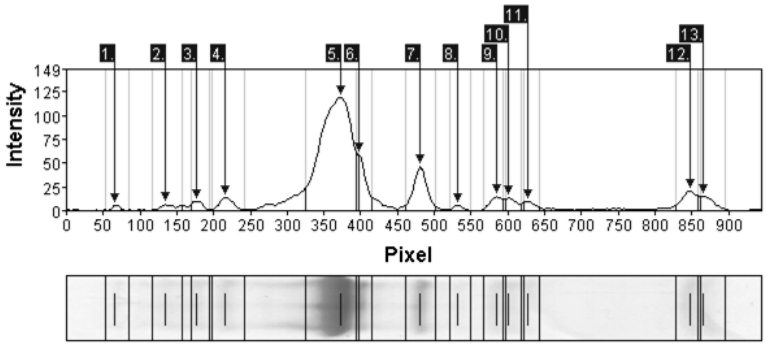


Figure 3A5: Gel analysis of lane 2

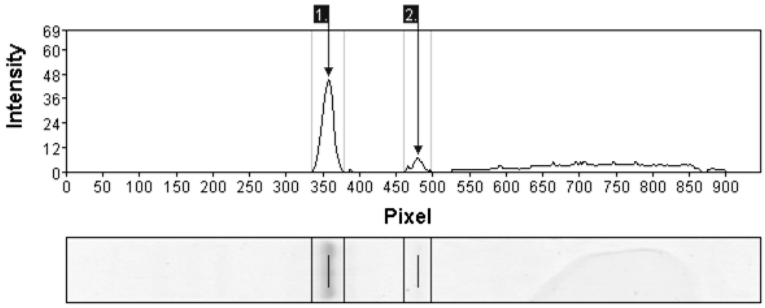


Figure 3A6: Gel analysis of lane 3

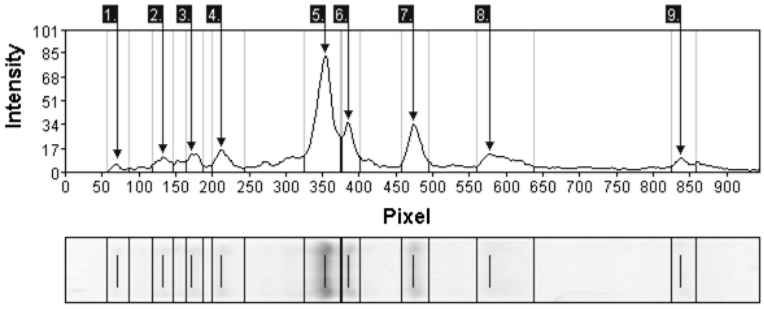


Figure 3A7: Gel analysis of lane 4

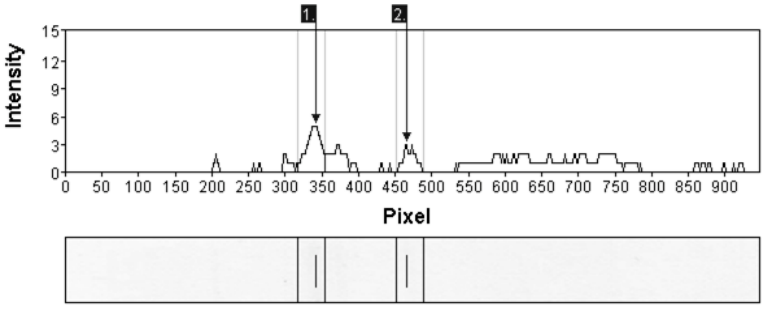


Figure 3A8: Gel analysis of lane 5

Table 3A1: Peak area analysis; MW: calibrated over whole range; MW cal: calibrated in desired range

Lane	Peak	Rf	Raw Volume	MW	MW cal
1.	1.	0.038	757	250	138
1.	2.	0.085	887	150	119
1.	3.	0.149	724	100	**100**
1.	4.	0.225	2060	75	**75**
1.	5.	0.359	2155	50	**50**
1.	6.	0.466	985	37	**37**
1.	7.	0.78	1331	25	14
1.	8.	0.926	993	20	9
1.	9.	0.979	695	15	7
2.	1.	0.071	76	185	124
2.	2.	0.143	172	108	99
2.	3.	0.189	163	80	86
2.	4.	0.23	309	63	76
2.	5.	0.395	5830	34	45
2.	6.	0.422	701	33	42
2.	7.	0.509	975	29	32
2.	8.	0.563	88	28	27
2.	9.	0.619	271	27	23
2.	10.	0.636	228	27	21
2.	11.	0.664	170	27	20
2.	12.	0.896	464	26	10
2.	13.	0.916	326	26	9
3.	1.	0.378	826	36	48
3.	2.	0.505	114	29	32
4.	1.	0.076	102	180	122
4.	2.	0.142	236	109	100
4.	3.	0.184	243	82	87
4.	4.	0.226	386	65	77
4.	5.	0.376	2205	36	48
4.	6.	0.408	613	33	44
4.	7.	0.502	743	29	33
4.	8.	0.612	695	27	23
4.	9.	0.886	240	26	10
5.	1.	0.362	118	37	50
5.	2.	0.493	54	30	33

Appendix 3B – Chromatogram

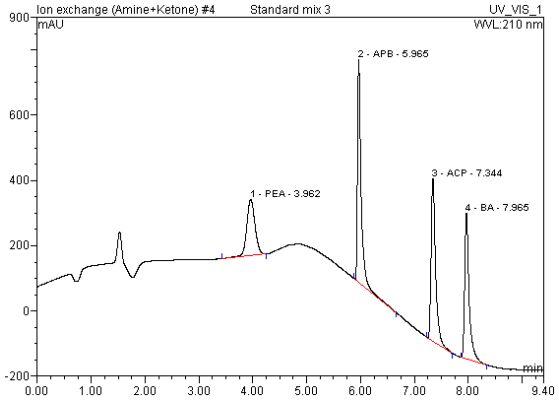


Figure 3B1: Spectrum obtained by using a standard solution containing MBA, APB, APH and BA

Appendix 3C - Wide range buffer

Different pH values were obtained by mixing the following mass of Boric acid (Mw: 61.83 $g.mol^{-1}$), Citric acid.H_2O (Mw: 210.14 $g.mol^{-1}$) and $Na_3PO_4 \cdot 12H_2O$ (Mw: 380.13 $g.mol^{-1}$) in water to a total volume of 50 mL:

Table 3C1: Buffer composition

	Volume of buffer	50 mL; 1M	
pH	$m_{Boric\ Acid}$ [g]	$m_{Citric\ Acid}$ [g]	m_{Na3PO4} [g]
2	2.4481	2.0801	0.1930
3	2.3453	1.9927	0.9831
4	2.2159	1.8828	1.9776
5	2.0661	1.7555	3.1283
6	1.9353	1.6443	4.1341
7	1.7564	1.4924	5.5084
8	1.6047	1.3635	6.6741
9	1.4057	1.1944	8.2038
10	1.1882	1.0096	9.8753
11	1.0228	0.8690	11.1467
12	0.4661	0.3961	15.4244

Appendix 3D - Estimation of Km and Vmax

1/vo was plotted against 1/[S] according to the Lineweaver–Burk equation:

$$\frac{1}{v} = \frac{Km}{Vmax}\left(\frac{1}{[A]}\right) + \frac{1}{Vmax}$$

Where the intercept with x-axis y(0) equals to $-\frac{1}{Km}$ and the intercept with y-axis is equal to $\frac{1}{Vmax}$ (using non-inhibitory concentrations – linear zone only)

ATA 40:

Table 3D1: Data for ATA 40

APH (mM)	1/APH	µmol/min.g	1/vo
2	0.5	9.112254443	0.109742
5	0.2	10.11412535	0.098872
10	0.1	10.25724977	0.097492
25	0.04	8.873713751	0.112692
30	0.033333	7.394761459	0.135231

Km = 0.34 mM, Vmax = 10.7 µmol/min.g

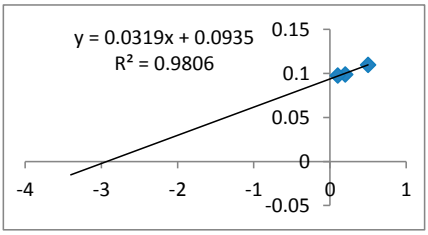


Figure 3D1: Lineweaver–Burk for ATA 40

ATA 44

Table 3D2: Data for ATA 44

APH (mM)	1/APH	µmol/min.g	1/vo
0	1E+20	0	1E+20
2	0.5	21.845	0.045777
5	0.2	24.225	0.04128
10	0.1	22.78	0.043898
25	0.04	15.215	0.065725
30	0.033333	12.495	0.080032

Km = 0.39 mM, Vmax = 26.11 µmol/min.g

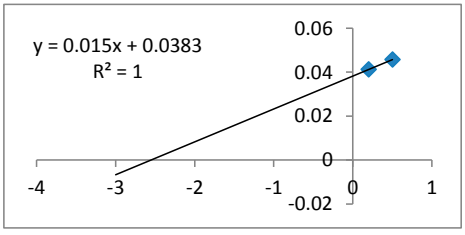


Figure 3D2: Lineweaver–Burk for ATA 44

ATA 47

Table 3D3: Data for ATA 47

APH (mM)	1/APH	µmol/min.g	1/vo
0	1E+20	0	1E+20
2	0.5	26.62412993	0.03756
5	0.2	51.7099768	0.019339
10	0.1	75.02088167	0.01333
25	0.04	97.97679814	0.010206
30	0.033333	105.0765661	0.009517

Km = 7.99 mM, Vmax = 133.3 µmol/min.g

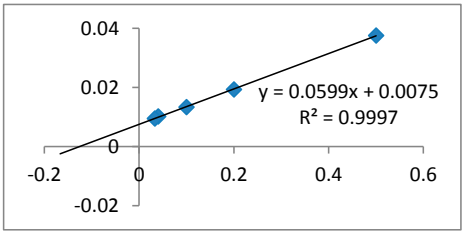


Figure 3D3: Lineweaver–Burk for ATA 47

Appendix 3E – Enantiomeric excess

Table 3E1 – Enantiomeric excess for ATA 40, 44 and 47.

	% ee MBA	% ee APB	[PEA] (g/L)	Yield (%)
ATA040	99.9	88.4	0.20	0.65
ATA044	99.9	63.5	1.37	4.0
ATA047	99.9	99.9	4.70	18

Appendix 3F - Solubility of APH in presence of DMSO

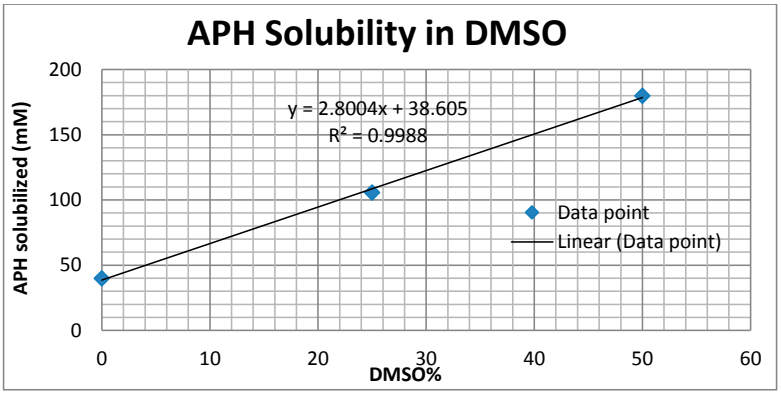


Figure 3F1 – Solubility of APH in DMSO. Increasing concentration of APH was mixed with 25 and 50% DMSO until formation of two a second was observed.

Appendix 4A - Water content in pure and water saturated organic solvents

Table 4A1 – water content in pure and saturated organic solvents

	% water Pure	% water in saturated
Toluene	0.0094	0.1602
IPAc	0.0929	1.6869
Cyclohexane	0.000	0.0137

Appendix 4B - Immobilization yield for Step II of screening

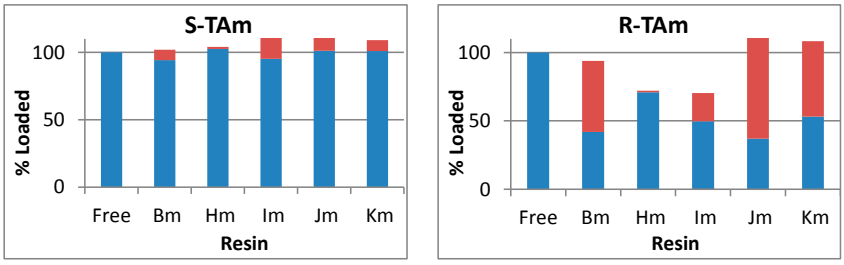
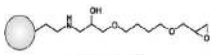
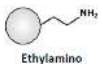
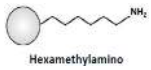


Figure 4A1 and 4A2 – Immobilization yield for S-TAm and R-TAm, respectively.

Appendix 4C – Resins costs

Table 4C-1-: Resins prices. Calculated from 5 and 10 Kg packages obtained from the supplier homepage (http://www.resindion.com)

Structure and functional group	Resin ID	Resin name	Cost (€/kg
 Octadecyl	B-s	Relizyme OD403/s	668
	B-m	Relizyme OD403/m	576
	C-s	Sepabeads EC-OD/s	549
	C-m	Sepabeads EC-OD/m	474
 Epoxy	D-s	Relizyme EP403/s	232
	D-m	Relizyme EP403 /m	199
	E-s	Sepabeads EC-EP /s	194
	E-m	Sepabeads EC-EP/m	167
 Amino-Epoxy	F-s	Relizyme HFA403 /s	722
	F-m	Relizyme HFA403/m	625
	G-s	Sepabeads EC-HFA/s	603
	G-m	Sepabeads EC-HFA/m	522
 Ethylamino	H-s	Relizyme EA403/s	258
	H-m	Relizyme EA403/m	221
	I-s	Sepabeads EC-EA/s	215
	I-m	Sepabeads EC-EA/m	183
 Hexamethylamino	J-s	Relizyme HA403/s	258
	J-m	Relizyme HA403/m	221
	K-s	Sepabeads EC-HA/s	215
	K-m	Sepabeads EC-HA/s	183

Appendix 6A – Solvent toxicity *vs.* partition of products and substrates

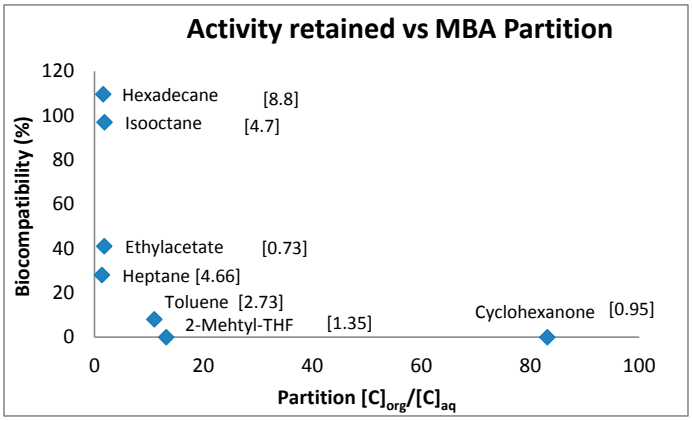


Figure 6A1: Biocompatibility plotted against partition of MBA. The numbers between brackets are LogP values.

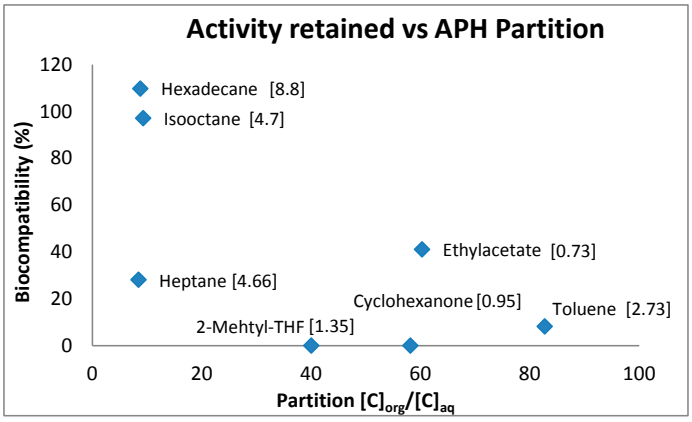


Figure 6A2: Biocompatibility plotted against partition of APH. The numbers between brackets are LogP values.

Biocompatibility was measured by preparing about 40 mg WC (ATA 44, C-LEcta GmbH, Germany) in 750 µL of water and vigorously mix this with equal volume of different solvents for 1 hours and 30 °C. Afterwards cells were separated from solvent by mean of centrifugation (20 min at 10.000 rpm) at room temperature and the pellet were

resuspended in 500 µL buffer (pH 7) and tested for activity (1 M IPA, 5 mM APH, 2 mM PLP and 100 mM k-PBS pH 7). Results are based in initial rate measurements.

The partition was measured by vigorously mixing 1 mL of 150 mM of MBA or APH prepared in 100 mM k-PBS pH 7 with equal volume of different solvent for 20 hours at 30 °C. Samples were taken from the water phase and analysed by HPLC.

Appendix 6B – Design for Integrated Liquid-Liquid extraction

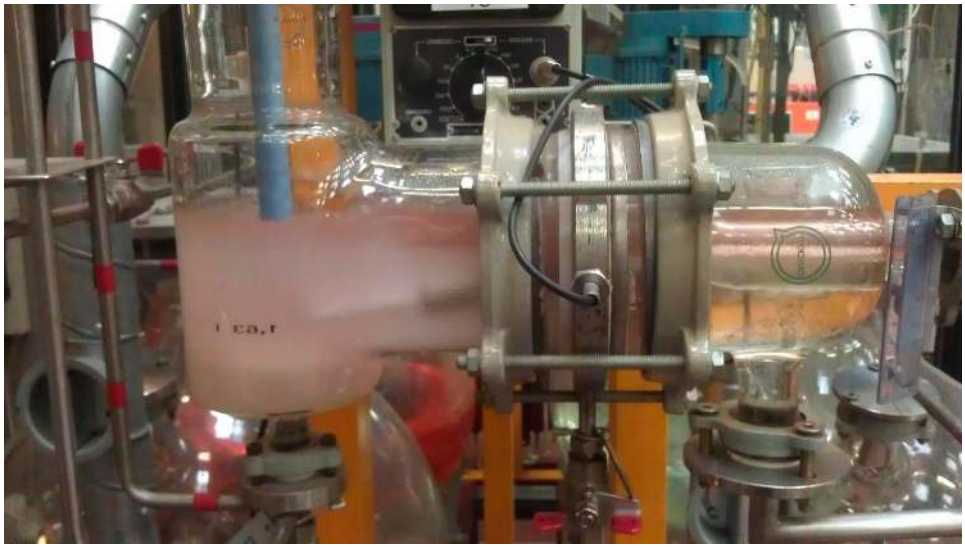


Figure 5B1: Still of a continuous liquid-liquid separator.

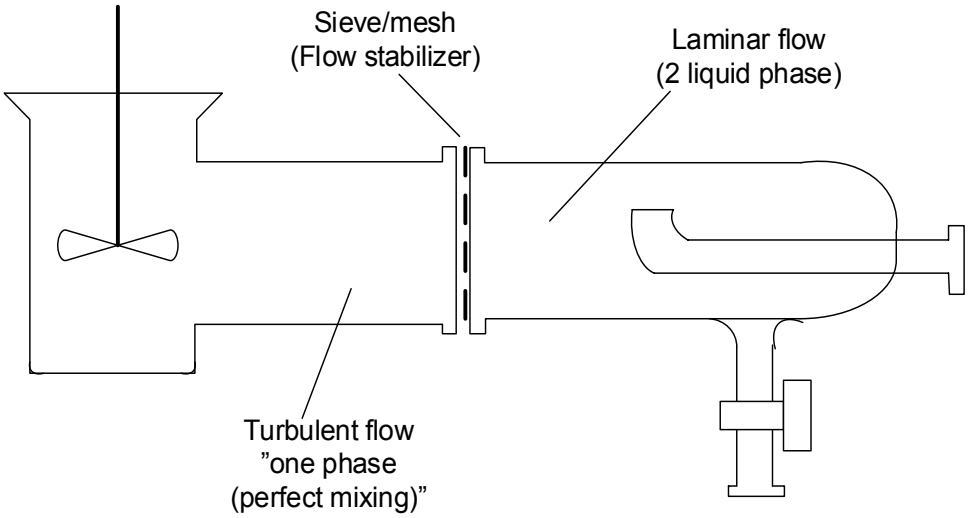


Figure 6B2: Continuous liquid-liquid separator.

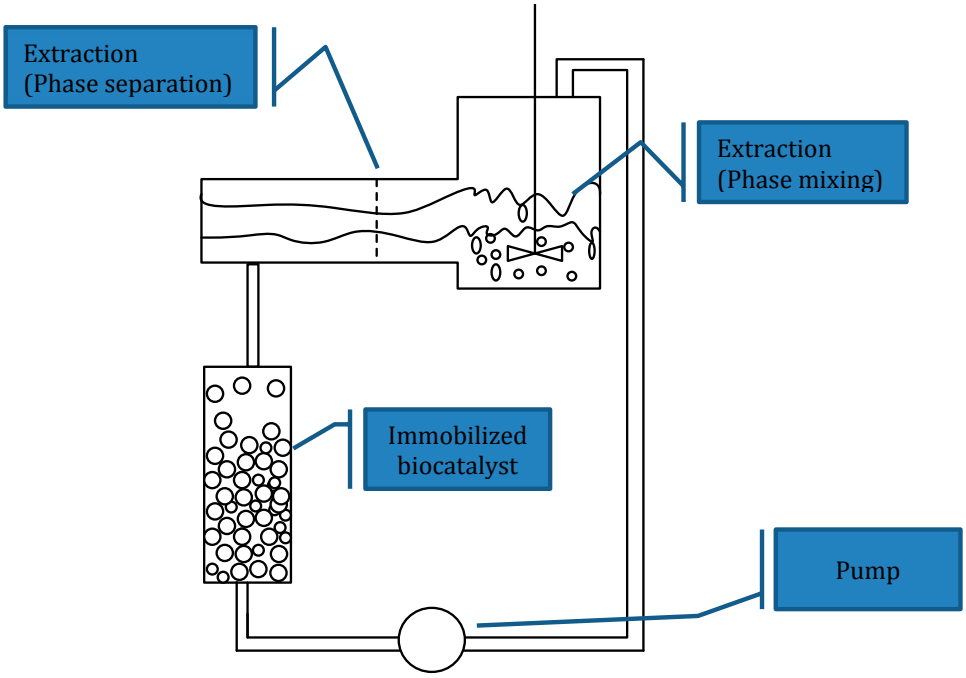


Figure 6B2: Schematic overview of a potential liquid-liquid ISPR/ISSS.

Appendix 6C – Set up for ISPR/ISSS/IScPR characterization

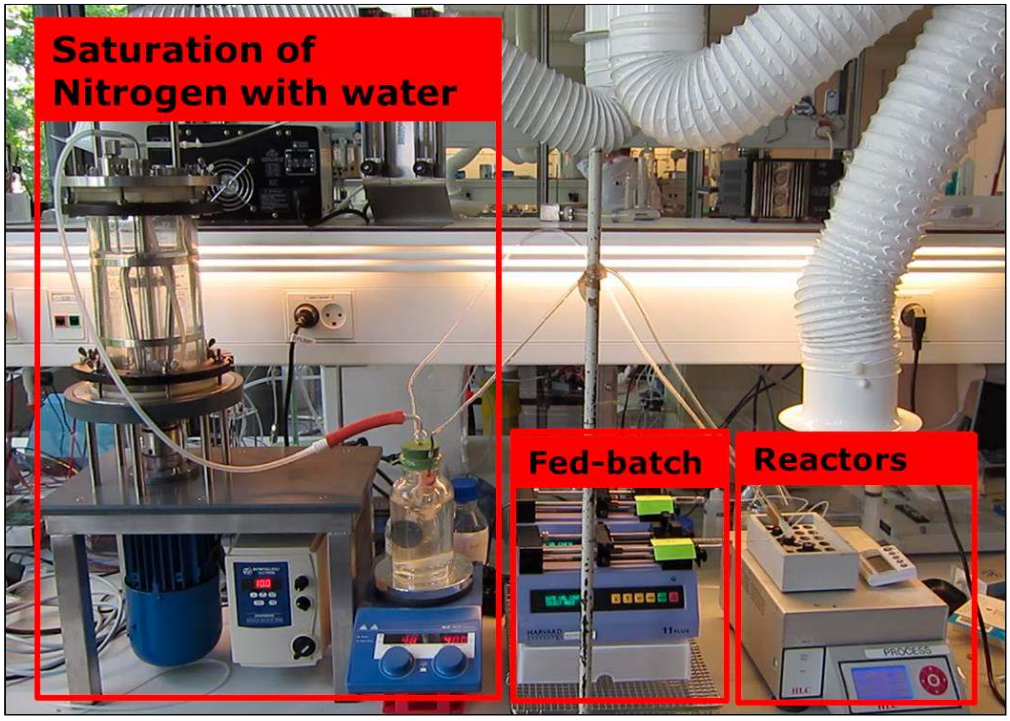


Figure 6C1: Setup for process characterization featuring ACE removal and hydrophobic resins.

Appendix 6D – Results for experiments featuring ALA

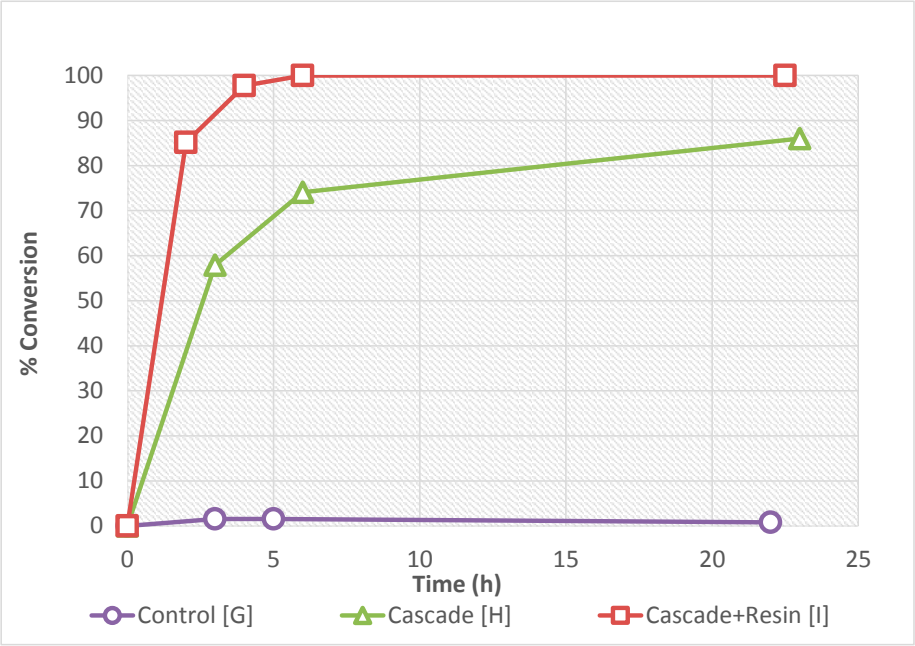


Figure 6D1: Reaction over time for scenario without cascade and ISPR (G), with cascade but without ISPR (H) and with cascade and ISPR (I).

Center for Process Engineering and Technology (PROCESS)
Department of Chemical and Biochemical Engineering
Technical University of Denmark
Søltofts Plads, Building 229
DK-2800 Kgs. Lyngby
Denmark

Phone: +45 4525 2800
Fax: +45 4525 2906
Web: www.process.kt.dtu.dk

ISBN : 978-87-93054-21-9