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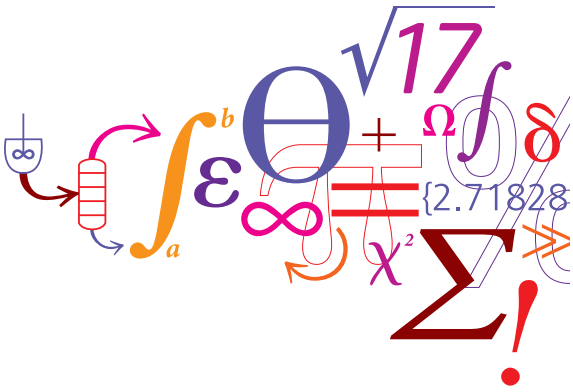

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Ph.D. Thesis
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INTEGRATING POROUS RESINS IN ENZYMATIC PROCESSES

PhD thesis

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Preface

This thesis is written in partial fulfilment of requirements for the degree of Doctor of Philosophy (PhD) at the Technical University of Denmark (DTU). The work has been carried out in the Center for Process Engineering and Technology (PROCESS) at the Department of Chemical and Biochemical Engineering from November 15 2009 to 31 December 2012 under the supervision of Professor John M. Woodley, Professor Rafiqul Gani (CAPEC) and Dr. Pär Tufvesson. The work has been funded by the Technical University of Denmark (KT and CAPEC) and the project AMBIOCAS (EU seventh framework programme – grant nr. 245144).

Acknowledgement

The conclusion of my PhD thesis marks the beginning of a new era in my life. I can confidently say the PhD was an exciting challenge and helped to develop myself as a researcher and as a person. A number of people have contributed to this for which I am ever grateful. A special thanks to my supervisor Professor John Woodley for giving me the opportunity to work under his supervision. He has been a never ending inspiration, a friend and an excellent support. I could not ask for anyone better. I thank my co-supervisor Dr. Pär Tufvesson for being there to answer all my silly questions, motivate me and discuss my problems. I would further like to thank Professor Rafiqul Gani for all the fruitful discussions and critical reviews.

I would like to thank my mother, me doing a PhD has been a dream that we both shared and I am extremely glad that I could keep up to her expectations. Her unconditional love and belief, kept me going during the tough times.

My wife, Agnieszka, has been a constant support throuout my Ph.D. Her love, laughter and constant encouragement made this roller coaster ride easier. I would not have been able to complete my Ph.D without her next to me. Thank you for being a part of my life.

My sincere thanks go to my other family members, my brothers and sister-in-laws, and especially Joy bhaiya for his constant support and advice. He has always been there for me through all stages of my life. Thank you for everything.

I would like to express my gratitude to Bapi for all the love he gave me. I would further like to acknowledge the support from my Polish family, thank you for making me feel at home.

I would like to acknowledge my father, though he passed away when I was young, but I believe he is out there smiling down at me.

During my stay in PROCESS, I had the privilege of sharing an office with two fantastic ladies, Joana and Paloma. Thanks for making this short period a lot of fun. I will miss the good times! We have developed a friendship which I am sure will stay even after DTU. I had the opportunity to meet some more wonderful people at PROCESS, whom I consider my friends and excellent researchers. Thanks for all the great discussions and the fun social gatherings.

Summary

Increasing pressure mandated by different government policies, for developing sustainable chemical processes for the synthesis of optically pure compounds, has resulted in increased considerations of biocatalysis as a viable option by many industries. Biocatalysis, with its exquisite selective properties and potential 'green' attributes, presents it as a sustainable alternative. Today, the role of biocatalysis is most evident in the pharmaceutical industry and is currently extending towards fine and bulk chemical production as well. The use of hydrolytic enzymes (lipases) is well established in several chemical industries, though certain challenges persist in other types of enzymes (transferases and ketoreductases), thus limiting their implementation in industry. Inhibition by substrate and product as well as low aqueous solubility of substrates has constrained the full potential of these enzymes to be harnessed.

To overcome these challenges, different process strategies are required to obtain high yields. A number of different challenges and proposed solutions are discussed in chapter one of this thesis and have also been published as a review. In recent years, integrating porous resins as an auxiliary phase in enzymatic processes, to non-selectively bind the substrate and product as a means to alleviate substrate and product inhibition, has gained considerable recognition. The resins act as a reservoir for the inhibitory substrate and a sink for the inhibitory product and simultaneously attain the required high substrate loading to make the process economically feasible. In this way the potential benefit of the enzyme can be exploited.

Porous resins as opposed to other auxiliary phases, for example organic solvents, are non-bioavailable, biocompatible and offer simpler operational handling (no foaming and emulsification). This strategy has been applied effectively to single substrate – single product systems (oxidation,

microbial degradation and hydrolysis). However, this concept has not been extended to other industrially relevant reactions which are two substrate – two product systems.

In this thesis, a methodological framework has been successfully developed to aid in implementing the strategy of integrating porous resins for multi-component systems. In this manner, a generic platform has been established for biocatalytic reactions that require the integration of this strategy. The framework identifies the key information about the reaction and the process using a step-wise protocol with the required tools. It includes the use of kinetic modelling in characterizing the reaction kinetics, a heuristic approach for screening resins and a model based approach for evaluating the process. Greater knowledge about the enzymatic processes with integrated porous resins can therefore be gained and thus the efficiency of process development with respect to time and resources required (reduced number of experiments) could be increased.

Estimating kinetic model parameters for enzymatic reactions is quite complex and frequently leads to identifiability issues. In order to understand the different techniques to estimate the parameters, a number of concepts are discussed in chapter four of this thesis. This knowledge has contributed to the development of a robust methodology for the estimation of kinetic model parameters for biocatalytic reactions, which has also been published in a peer reviewed journal.

Screening resins for moderately hydrophobic multi-component systems is challenging. Often it is found that the capacity of the resin is inversely related with product selectivity. Therefore a trade-off has to be made between these parameters which can be crucial from an economic point of view. A low resin capacity points towards the need for higher resin loading, which in turn determines the equilibrium concentration of the substrate in the reactor and the type of reactor that can be used (stirred tank reactor or packed bed reactor). Similarly low product selectivity would result in higher product concentration in the reactor and thus not aid in alleviating inhibition. Further considerations

of resin stability and cost also have to be taken into account in the screening procedure. The screening therefore becomes a multi-objective task that has to be solved simultaneously. Such an approach has been applied in the method formulated in this framework.

Process modelling is a very effective tool in evaluating a process. Critical information about the process can be gained by means of simulations, which can further be re-used to tune the reaction or process conditions to harness the full potential of the enzyme. State-of-the-art mathematical techniques for model quality evaluation, such as uncertainty and sensitivity analysis, have been included in this analysis in order to identify the key model parameters for better understanding of the process.

Three case studies were used to illustrate the applicability of the methodology to fulfil different objective requirements. The case studies were selected for not only being industrially relevant but as well as having certain limitations which contributed in developing the tools and strategies to overcome them. The asymmetric synthesis of 1-phenylethylamine using ω-transaminse, the asymmetric synthesis of 1-methyl-3-phenylpropylamine using ω-transaminse and enantioselective synthesis of 2-octanol using alcohol dehydrogenase were selected.

Dansk resumé

Et stigende pres fra offentlig politik med hensyn til udviklingen af bæredygtige kemiske processer til syntese af optisk rene forbindelser, har resulteret i øgede overvejelser om biokatalyse som en farbar vej af mange brancher. Biokatalyse, med sin udsøgte selektive egenskaber og potentielle grønne attributter, præsenterer sig som et bæredygtigt alternativ. Biokatalyse er i dag mest tydelig i den farmaceutiske industri og strækker sig i øjeblikket mod fine og bulk kemikalie produktion. Brugen af hydrolytiske enzymer (lipaser) er veletableret i flere kemiske industrier, der er dog stadig visse udfordringer med andre typer af enzymer (transferaser og ketoreduktaser), hvilket begrænser deres gennemførelse i industrien. Inhibering af substrat og produkt samt lav vandopløselighed af substrater har begrænset realisering af disse enzymers fulde potentiale.

For at overvinde disse udfordringer, kræves der forskellige proces strategier for at opnå høje udbytter. En række forskellige udfordringer og forslag til løsninger er diskuteret i kapitel ét af denne afhandling og er også blevet offentliggjort som et review. I de seneste år er det blevet populært at integrere porøse resiner som en ekstra fase i enzymatiske processer. Resinerne vil uden specifik selektivitet binde substrat og produkt og virke som et middel til håndtering substrat og produkt inhibering, dette har opnået betydelig anerkendelse. Resinerne virker som et reservoir for det hæmmende substrat og en vask for det hæmmende produkt. Her kan der også opnås den nødvendige høje koncentration af substrat i resinet som gør processen økonomisk mulig. Potentialet som enzymet har kan på denne måde blive udnyttet.

De porøse resiner er i modsætning til andre hjælpe faser, for eksempel organiske opløsningsmidler, ikke-biotilgængelige, biokompatible og kan tilbyde enklere operationel håndtering (ingen skumdannelse og emulgering). Denne strategi er blevet anvendt effektivt til enkelt substrat- enkelt

produkt-systemer (oxidation, nedbrydning og hydrolyse). Imidlertid har dette koncept ikke blevet udvidet til at omfatte andre industrielt relevante reaktioner, som er to substrat- to produkt-systemer.

I denne afhandling, er der udviklet en metodologisk skelet til at hjælpe med at gennemføre strategien til integration af porøse resiner i multi-substrat og multi-produkt-systemer. På denne måde, er der blevet fastlagt en generisk platform for biokatalytiske reaktioner, som kræver integration af denne strategi. Platformen identificerer de vigtigste oplysninger om reaktionen og processen ved hjælp af en trinvis protokol med de nødvendige værktøjer. Den omfatter anvendelse af kinetiske modeller til at karakterisere de reaktionskinetiske forhold, en heuristisk metode til screening resiner og en modelbaseret fremgangsmåde til vurdering af processen. Større viden om de enzymatiske processer med integrerede porøse resiner kan derfor også opnås, dermed bliver effektiviteten af procesudviklingen med hensyn til tid og ressourcer øget.

Estimering kinetiske modelparametre for enzymatiske reaktioner er temmelig kompleks og fører ofte til et spørgsmål om identificerbarhed. For at forstå de forskellige teknikker til at estimere de parametre, findes der er en række begreber, disse diskuteres i kapitel fire af denne afhandling. Denne viden har bidraget til udviklingen af en robust metode til beregning af kinetiske modelparametre for biokatalytiske reaktioner, dette er også blevet offentliggjort i et peer-reviewed tidsskrift.

Screening af resiner til moderat hydrofobe flerkomponentsystemer er udfordrende. Ofte er det sådan at kapaciteten af resinet er omvendt proportional med produktselektivitet. Der skal derfor foretages et trade-off mellem disse parametre, og dette kan være afgørende ud fra et økonomisk synspunkt. En lav resin kapacitet peger mod behovet for højere resin kapacitet, som igen bestemmer ligevægtskoncentrationen af substratet i reaktoren og den type reaktor, der kan anvendes (Stirred Tank Reaktor eller Packed Bed Reaktor). Tilsvarende lav produkt selektivitet ville resultere i højere

produktkoncentration i reaktoren og er derfor således ingen hjælp til at lindre inhiberingen. Yderlige betragtninger af resiners stabilitet og omkostninger skal også tages i betragtning i screeningsproceduren. Screeningen bliver derfor en multi-objektiv opgave, der skal løses samtidig. En sådan strategi er blevet anvendt i den metode, som er formuleret i dette skelet.

Procesmodellering er et meget effektivt værktøj til at vurdere en proces. Kritisk information om processen kan opnås ved hjælp af simulationer, som yderligere kan genbruges, til at tune reaktionsbetingelserne eller procesbetingelserne til at opnå det fulde enzym potentiale. State-of-the-art matematiske teknikker til model kvalitetsevaluering, såsom usikkerheds-og følsomhedsanalyse, er blevet inkluderet i denne analyse med henblik på at identificere de vigtigste modelparametre til en bedre forståelse af processen.

Tre casestudier blev brugt til at illustrere anvendeligheden af metodens evne at opfylde forskellige objektive krav. Casestudierne blev ikke kun udvalgt for at være industrielt relevante, men også for at have visse begrænsninger, dette bidrog til udviklingen af de værktøjer og strategier som er behøvet for at overvinde dem. Asymmetrisk syntese af 1-phenylethylamin med ω-transaminse, asymmetrisk syntese af 1-methyl-3-phenylpropylamin hjælp ω-transaminse og enantioselektiv syntese af 2-octanol med alkohol-dehydrogenase blev valgt.

Abbreviation

[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_{Si}	Substrate inhibition constants (mM)
K_{EQ}	Chemical equilibrium constant
γ, λ	Reaction direction indicator
y	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	Compound acetophenone

IPA	Compound 2-propylamine
PEA	compound 1-phenylethylamine
ACE	compound acetone
PB	compound 4-phenyl-2-butanone
MPP	compound 1-methyl-3-phenylpropylamine
NADP	nicotinamide adenine dinucleotide phosphate
$NADP^+$ - NADPH	Nicotine amide
LbADH	alcohol dehydrogenase from *Lactobacillus brevis*
GDH	glucose dehydrogenase from *Bacillus spec*
ON	Compound 2-octanone
OL	Compound 2-octanol
Glu	Compound Glucose
Gdl	Compound Gluconic acid-δ-lactone
ADA	N-(2-acetamido)iminodiacetic acid
[APH]	Concentration of acetophenone (mM)
[IPA]	Concentration of 2-propylamine (mM)
[PEA]	Concentration of 1-phenylethylamine (mM)
[ACE]	Concentration of acetone (mM)
[ON]	Concentration of 2-octanone (mM)
[OL]	Concentration of 2-octanol (mM)
[Glu]	Concentration of glucose (mM)
[Gdl]	Concentration of gluconolactone (mM)
ISSS	*in-situ* substrate supply
ISPR	*in-situ* product removal
Aq	aqueous phase
Sol	resin phase

Eq	Equilibrium
γ, λ	reaction direction indicator
SS	substrate supply
PR	product removal
Q	substrate flux (mmol/min)
V	volume of reactor (L)
R	rate of reaction (mM/min)
Mass	mass of resin (g)
q*	reactant adsorbed per gram of resin (mmol/g)
C	concentration (mmol/L)
T	time (mins)
kLA	mass transfer coefficient (m/min)
A	surface area (m^2)
Qmax	maximum adsorption capacity (mmol/g)
P	Langmuir constant (mmol/L)
N	unit less
V	velocity of reaction (U/mg)
K_M	Michaelis constant (mM)
K_P	core inhibition constant (mM)
K_i	substrate surplus inhibition constant (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
ON	Compound 2-octanone
OL	Compound 2-octanol
Glu	Compound glucose
Gdl	Compound gluconolactone

Subscripts

N	number of compounds in reaction
i, j	Index

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Chapter 1

Introduction

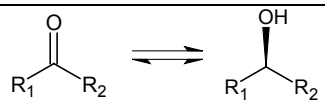
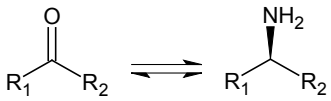
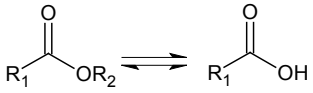
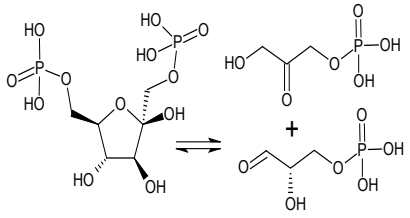
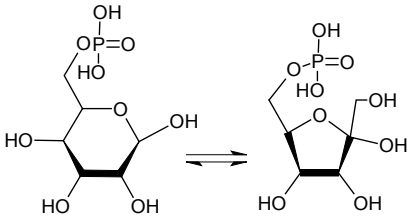
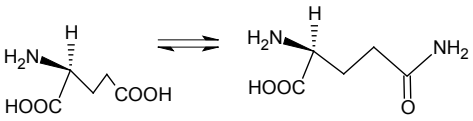
1.1 Biocatalysis

In recent years there is a great societal expectation that chemists and chemical engineers should produce greener and more sustainable chemical processes (Dunn, 2012). Part of this awareness comes from increasing pressure from different governmental policies (U.S., E.U. and Japan) to synthesize optically pure compounds along with reduced waste. (Muñoz Solano et al., 2012). Biocatalysis, with its exquisite properties stereo- and regio- selectivity (Turner and Carr, 2007) and potential green (mild operating conditions and reactions performed in water) attributes (Jegannathan and Nielsen, 2013; Tao and Xu, 2009; Wu and Tao, 2012), presents it as a sustainable alternative (Schmid et al., 2001; Schoemaker et al., 2003). The role of biocatalysis is most evident in the pharmaceutical industry (Pollard and Woodley, 2007) and its repertoire currently extending towards the industrial synthesis of fine chemicals, agrochemical intermediates, food ingredients and bulk chemicals (Busacca et al., 2011; Clouthier and Pelletier, 2012; Jackson, 2011; Li et al., 2010; Nestl et al., 2011; Nielsen et al., 2008; Patel, 2011). Some examples of industrial biocatalysis include hydroxylation of steroids (Znidarsic-Plazl and Plazl, 2010), penicillin G acylase to make semisynthetic penicillin (Elander, 2003), lipase-catalysed resolution of chiral precursors for synthesis of diltiazem (a blood pressure drug) (Matsumae et al., 1993), hydroxynitrile-lyase-catalysed synthesis of intermediates for herbicides (Griengl et al., 2000), carbonyl-reductase-

catalysed synthesis of enantiopure alcohols for cholesterol-lowering statin drugs (Liljeblad et al., 2009), lipase catalysed synthesis of wax esters such as myristyl myristate or cetyl ricinoleate for cosmetics (Hills, 2003). Nature has provided several classes of enzymes which can perform complex chemistry in a single step (Wohlgemuth, 2010). In this context the protection and de-protection in chemical synthesis can be avoided resulting frequently in a single step reaction for the generation of new chiral centres via asymmetric reactions, such as reduction with dehydrogenases, to resolve an existing chiral center, such as kinetic resolution with a lipase, or, C-C bond formation by lyases (Woodley, 2008a). From a process perspective this means that biocatalysis has the potential in creating new green chemical synthetic processes.

The different types of enzymes found in nature have been categorized into six groups based on the type of chemistry that can be performed (Webb, 1992) as shown in Table 1.1.

Table 1.1 Different categories of enzymes (modified from Faber, 2011)

Category	**Enzyme examples**	**Example**
Oxidoreductase	Alcohol dehydrogenase (EC 1.1.1.1)	 Ketone reduction
Transferase	ω-transaminase (EC 2.6.1.18)	 Transfer of amine groups
Hydrolases	Lipase (EC 3.1.1.3)	 Hydrolysis
Lyase	Aldolase (EC 4.1.2.13)	 Cleaves carbon-carbon bond
Isomerases	Phosphoglucose isomerase (EC 5.3.1.9)	 Structural rearrangement of isomers
Ligases	L-glutamine synthetase (EC 6.3.1.2)	 Forms carbon-nitrogen bond

1.2 Challenges in biocatalytic reactions

The opportunity of incorporating a biocatalytic step in chemical synthesis has been exploited by several chemical industries (Wohlgemuth, 2007). Companies such as BASF, DSM and Lonza have established large scale (> 1000 tons) production facilities which include a biocatalytic step in the process (Ghisalba et al., 2010). An effective integration of a biocatalytic step in chemical synthesis is indeed paramount because for many complex syntheses only a few steps will be biocatalytic while the majority will be chemical (Hailes et al., 2007). It is realized from the companies' product portfolios and filed patents that the industries use mainly hydrolytic enzymes (lipases) in their processes. These enzymes are extremely robust (high stability and tolerance to substrates and products), and moreover not constrained by equilibrium limitations. On the other hand, certain challenges persist in other types of enzymes (transferases and ketoreductases), thus limiting their implementation in industry. Some of the major challenges associated with these enzymes are listed in Table 1.2.

Table 1.2 Challenges for biocatalytic reactions

Limitations	**Possible solutions**	**Reference**
Substrate is inhibitory, toxic or insoluble	Use of an auxiliary phases or fed batch reactor	(Hilker et al., 2004)
Product is inhibitory or toxic	Use of *in-situ* product removal	(Woodley et al., 2008b)
Equilibrium controlled	Use of *in-situ* product removal or excess of substrate	(Tufvesson et al., 2012)

From Table 1.2, it can be seen that a number of challenges are posed towards these enzymatic reactions for which possible solutions are suggested. The scope of the list has been limited to reaction considerations. Process factors, specifically liquid hetrogeneity due to poor mixing, oxygen

transfer rate and pH control should be considered during scaling up of a bioprocess (Marques et al., 2010b; Takors, 2012; Tufvesson et al., 2010). Similarly, further enzyme related limitations such as poor enzyme stability and low activity (Ma et al., 2010; Pavlova et al., 2009; Sheldon, 2007) have to be taken into account. In the following subsections the challenges encountered in biocatalytic reactions will be discussed in more details.

1.2.1 Substrate concentration

In many cases, the appropriate substrate feeding technology is dependent on the type of biocatalyst, either in the form of isolated enzyme, immobilized enzyme or whole cell. The different biocatalysts face different challenges for which tailored solutions have to be provided. For instance, isolated enzyme benefits the reaction with an easy control of kinetics (Woodley, 2006). However, the enzyme is often denatured in the presence of high concentration of non-natural substrate or organic solvents (Kohlmann et al., 2011). The stability of the biocatalyst and tolerance to high substrate concentration can be enhanced by means of immobilization, such as adsorption on a porous support (Kourkoutas et al., 2004), entrapment inside a polymer (Cardenas-Fernandez et al., 2012) or by crosslinking the enzyme (Sheldon, 2011). The stability in many cases is greatly enhanced but it can lead to mass transfer limitations, if the reaction is fast. Whole cells are the most economical choice with respect to the absolute cost of the catalyst (Tufvesson et al., 2011) but could be challenged with increased mass transfer limitations as well as undesired side reactions. Some of the major challenges which are characteristic of biocatalytic reactions with regards to substrate concentration are highlighted in Table 1.3.

Table 1.3 Challenges associated with biocatalytic reactions with regard to substrate concentration

Challenges	Problem	Reference
Low enantiomeric excess	High substrate concentration could lead to low substrate selectivity	(D'Arrigo et al., 2010; Houng and Liau, 2003)
Substrate inhibition/toxicity	The rate of reaction is compromised at high concentrations of the substrate	(Al-Haque et al., 2012; Rojanarata et al., 2004)
Equilibrium limitations	Adding excess of substrate in not sufficient for K_{EQ} lower than 0.5	(Tufvesson et al., 2011)
Downstream processing	Product intensity is low because of poor aqueous solubility	(Baldwin and Woodley, 2006)
Biocatalyst stability	Enzymes are not evolved for high substrate concentrations	(Schmoelzer et al., 2012)

1.2.2 Product concentration

For a biocatalytic process to be industrially feasible, high product concentration has to be achieved. For instance in the pharmaceutical industry a minimum of 50 – 100 g/L of product concentration is required for it to be considered for scaling-up (Pollard and Woodley, 2007), while for fine and bulk chemicals the minimum requirements is many fold higher (Panke et al., 2004; Straathof et al., 2002). Such high concentrations are required to minimise downstream processing cost (Luetz et al., 2008; Ruinatscha et al., 2006; Straathof et al., 2002; Woodley, 2008b). However, operating a reaction at such concentration, in many cases may display inhibitory effects (reversible) or could be even toxic (irreversible) (Lye and Woodley, 1999). Similarly, effective product recovery can be limited due to low product intensity leading to the requirement of multiple steps for product recovery. Furthermore, in some cases product degradation can be a limitation for which additional measures have to be taken to remove the product simultaneously as the reaction proceeds (Woodley et al., 2008a). Some of the major challenges which are characteristic of biocatalytic reactions with regards to product concentration are highlighted in Table 1.4.

Table 1.4 Challenges associated with biocatalytic reactions in regards to product concentration

Challenges	Problem	Reference
Product inhibition/toxicity	The rate of reaction is compromised at high concentrations of the product	(Al-Haque et al., 2012; Rojanarata et al., 2004)
Product degradation	High product concentration is limited by unstable product	(Hofstetter et al., 2004)
Product separation	Multi-step separation required if product concentration is low	(Bruggink et al., 2003)

1.2.3 Equilibrium limitations

No reaction is truly irreversible. Reactions called irreversible occur very far from the equilibrium or the reaction is "pulled" (for example carbon dioxide cleaved off from a substance and then leaving the reaction system) so that only reaction in one direction can be observed. Typically in biocatalytic processes, as mentioned earlier, hydrolytic enzymes have been studied the most (Straathof et al., 2002). The concentration of water is 55 M (1000 g/L) providing the necessary push to make the reaction irreversible. However for other industrially important enzyme types such as transferases and lyases, water is not a substrate. Therefore the maximal conversion is bound by the equilibrium constant, K_{EQ}. This parameter is independent of the biocatalyst and therefore protein engineering techniques cannot overcome this hurdle. Process strategic solutions are required to drive the reaction to product synthesis (Tufvesson et al., 2011). Recent publication illustrated methods to quantify the equilibrium constant value experimentally (Tufvesson et al., 2012), using group contribution method (Jankowski et al., 2008; Rother et al., 2010) and by calculating it from the kinetic model parameters using the Haldane relationship (Al-Haque et al., 2012). It is a powerful parameter which can indicate the required effectiveness of the different process strategies to displace the equilibrium towards the synthetic direction. Table 1.5 lists the reaction equilibrium constant value for different substrate combinations. From Table 1.5 it can be seen that the K_{EQ} value

can range from very small to larger than unity, providing indications that some process strategies need to be applied to attain yields not limited by equilibrium.

Table 1.5 Reaction equilibrium value for substrates of different enzyme classes in the synthetic direction

Enzyme	Substrate 1	Substrate 2	K_{EQ}*	Reference
alcohol dehydrogenase (EC 1.1.1.2)	1-Phenyl-1-pentanone	2-propanol	0.48	(Tewari et al., 2006)
ω-transaminase (EC 2.6.1.X)	Cetophenone	2-propylamine	0.03	(Tufvesson et al., 2012)
triacylglycerol lipase (EC 3.1.1.3)	Butyl decanoate	H_2O	0.71	(Tewari and Bunk, 2001)
carnitine dehydratase (EC 4.2.1.89)	L-carnitine	N/A	1.5	(Jung et al., 1989)
xylose isomerase (EC 5.3.1.5)	D-Glucose	N/A	1.21	(Converti and Borghi, 1997)
isoleucine-tRNA ligase (EC 6.1.1.5)	L-isoleucine	ATP + $tRNA^{Ile}$	0.61	(Airas, 2006)

*value is dependent on the reaction condition. N/A stands for not applicable

1.3 Proposed strategies to overcome biocatalytic reaction bottlenecks

1.3.1 Substrate feeding

The substrate feeding strategy for biocatalytic reactions depend on the natural, pre-existing phase of the substrate (Kim et al., 2007). Different methods, such as fed-batch or adding an auxiliary phase, can be applied for controlled substrate supply if the substrate has low aqueous solubility or is toxic/inhibitory at high concentration towards the biocatalyst. The various substrate delivery techniques are shown in Figure 1.1. Different configurations for substrate supply have its

limitations, as listed in Table 1.6. Careful consideration should be taken to select the substrate supply technique.

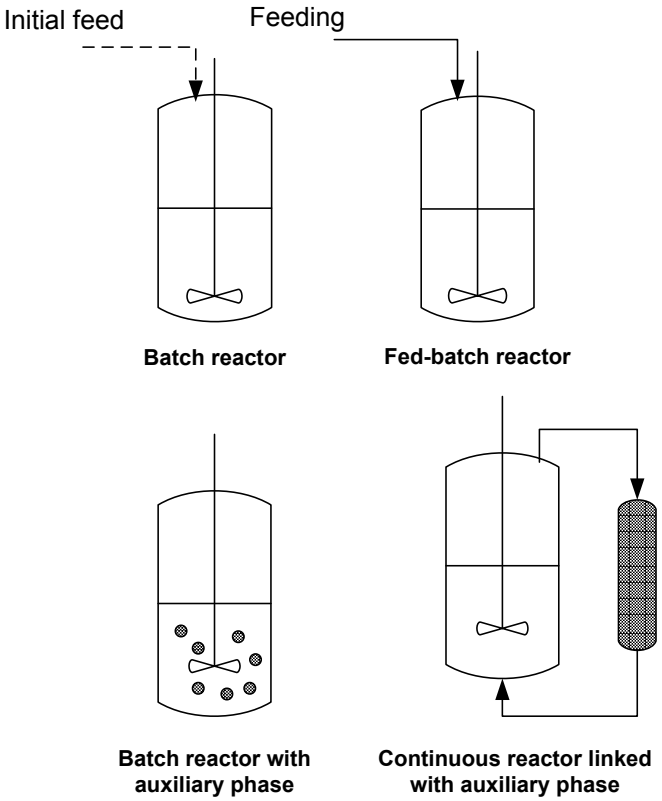


Figure 1.1 Substrate feeding techniques (shaded area indicates substrate loaded auxiliary phase)

In a batch reactor, all the substrate is fed at the start. If the substrate is liquid with high aqueous solubility and negligible inhibition at required substrate concentration then a batch mode would be feasible. However, if the substrate poses inhibitory effects or has low aqueous solubility then it should be considered to feed the substrate in a fed-batch mode (Amanullah et al., 2002; Doig et al., 2002). The complexity arises when the substrate is solid. Dissolution of solid substrates can often limit the reaction (Wick et al., 2002) and therefore it would be required to dissolve the substrate in a water-miscible co-solvent if a two phase reaction is not desired.

The use of an auxiliary phase to supply substrate is an alternative option for substrate feeding as a means of controlling the concentrations around a biocatalyst (Dafoe and Daugulis, 2011; Straathof, 2003). The auxiliary phase can be solid (porous resin), liquid (organic solvent and ionic liquid) or gas (Daugulis and Janikowski, 2002; Ferloni et al., 2004; Lilly, 1982; Roberts and Lye, 2002; Weuster-Botz, 2007) for which special considerations have to be taken into account. Further details about auxiliary phase will be explained in the following chapter.

Table 1.6 Substrate delivery configurations

Techniques	**Methodology**	**Limitations**	**References**
Batch	All substrates are fed at start-up	Not possible to supply substrate above critical concentration	(Walsgrove et al., 2002)
Fed-Batch	Substrate/co-substrate is fed over a time interval	Solubility of substrate in feed sets limit of aqueous concentration in reactor	(Alphand et al., 2003)
Organic Solvent	Substrate is fed via diffusion	Organic solvent may be toxic towards biocatalyst and also increases complexity of product recovery	(Truppo et al., 2012)
Ionic liquid	Substrate is fed via diffusion	Ionic liquid has to be recycled more than 200 times to be considered economic	(de los Rios et al., 2012)
Resin	Substrate is fed by desorption and diffusion	Vigorous stirring may damage the resins	(Held et al., 2000)

1.3.2 In-situ product removal (ISPR)

An innovative technique to overcome product inhibition is to remove the product *in-situ* during reaction (Freeman et al., 1993; Stark and von Stockar, 2003; Woodley et al., 2008a). In this way the biocatalyst experiences low product concentration and simultaneously leads to higher product

yields, because in-situ product removal is applied to minimize product inhibition or degradation, or reaction reversibility (Woodley et al., 2008b). The product removal can be carried out internally or externally as shown in Figure 1.2. In Figure 1.2 the ISPR is shown as a batch process, however, it is possible to continuously extract product out of the removal system eg. pervaporation.

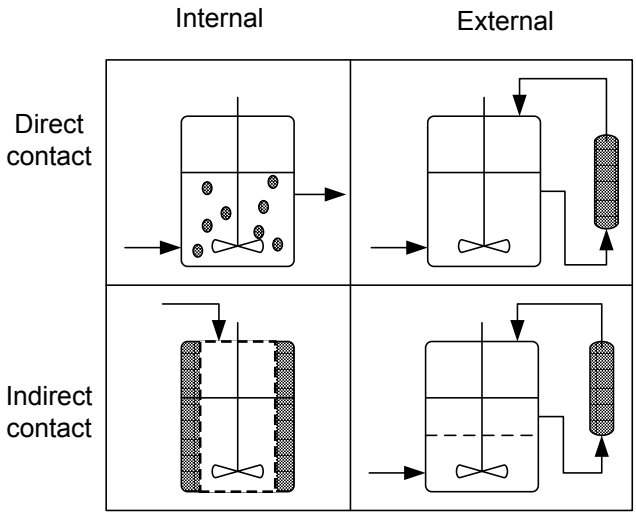


Figure 1.2 Schematic flow-sheet showing internal and external modes of ISPR operation (shaded area indicates the ISPR)

Implementation of an ISPR technique is of key importance for many reaction systems. Many examples can be seen where the indroctuion of an ISPR technique has increased product yields by many folds (Bechtold and Panke, 2009; Heerema et al., 2011; Suwannakarn et al., 2009; Vilt and Ho, 2011). However, what is more important is the need to understand how to select between them confidently and rapidly.

Lutze et al., 2012 presented a methodology for the selection of processes based on integrating unit operations (reaction – separation) (Lutze et al., 2012). Alternatively, the selection can be done based

on the physical and chemical properties of the different components (Jaksland et al., 1995; Lye and Woodley, 1999). The different techniques applicable to biocatalysis are listed in Table 1.7.

Table 1.7 *In-situ* product removal techniques applicable for biocatalysis

Separation basis	Technique	Important properties	References
Volatility	Reactive distillation Evaporation Pervaporation	Boiling point	(Etschmann et al., 2005; Tang et al., 2005; Yun et al., 2004)
Solubility	Crystallization Precipitation Extraction Stripping Absorbtion	Solubility point	(Buque-Taboada et al., 2006)
		Partition coefficient	(Khan and Daugulis, 2011a; Lye, 1997; Yun and Kim, 2008)
Molecular weight	Filtration Membrane	Size, molecular weight	(Heerema et al., 2011)
Charge/hydrophobicity	Ion exchange Electrodialysis Adsorption	pKa, LogP	(Dafoe and Daugulis, 2011)
Chemistry	Cascade reaction	Gibbs energy of formation	(Karmali and Coelho, 2011; Li et al., 2012)
Other	Hydrolysis, cyclisation, complexation		(Liting et al., 2009; Truppo et al., 2010)

1.3.3 Protein engineering

Protein engineering can be used to develop enzymes by either modifying the protein structure of the enzyme. The development of improved enzymes requires advanced knowledge in protein engineering (including directed evolution) (Kazlauskas and Bornscheuer, 2009; Turner, 2009), gene synthesis, sequence analysis, bioinformatics tools (Rothlisberger et al., 2008) and computer modelling (Bornscheuer et al., 2012). The consequence of this is that new and useful libraries of

enzymes can be 'designed' which can perform efficient catalysis in conditions that nature did not intend it to do. The biocatalyst can be engineered to fit the process conditions (Figure 1.3).

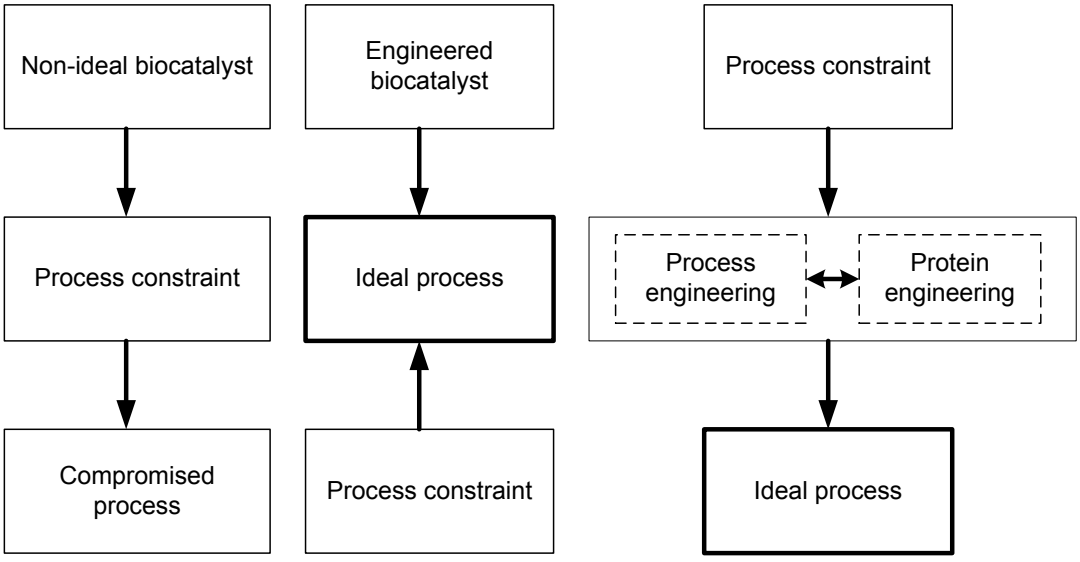


Figure 1.3 The search for the ideal process

The implications are that the biocatalyst can be designed to withstand high concentrations of substrate and product and even to catalyse reactions under elevated temperature and non-conventional media. Some examples can be seen in the synthesis of Januvia sitagliptin, a drug to treat type 2 diabetes, using (R)-transaminase. In that case, the enzyme was improved by directed evolution with initial activity 40,000 folds higher than the wild type and moreover was tolerant to around 100 g/L of substrate, temperature of around 60 ^{o}C and 20% (V/V) co-solvent DMSO (Savile et al., 2010). A further example can be seen in the synthesis of (R)-4-cyano-3-hydroxybutyric acid, precursor for the cholesterol lowering drug Lipitor, using nitrilase afforded eanantioselectivity of only 88%. Through gene site saturation mutagenesis, the enantiomeric excess could be improved to 98.5% at 3 M substrate loading and a volumetric productivity of 619 g/L-day (DeSantis et al.,

2003). However, there are also limitations for engineering enzymes and the protocol for finding the ideal biocatalyst requires extensive efforts (Wenda et al., 2011) and needless to say the thermodynamic limitations cannot be overcome with this strategy. For pharmaceutical industries, where the life of the product is governed by the patent lifetime, this results in a narrow window of action to define and develop a suitable process (Pollard and Woodley, 2007). It would therefore require a synergy between the protein and process engineers to reduce the time for a product to reach market (Wohlgemuth, 2011). Protein engineering is a field by itself and is not within the scope of this project. For further details please refer to the following publications (Bommarius et al., 2011; Bornscheuer et al., 2012; Dalby, 2011; Lutz, 2010; Strohmeier et al., 2011; Turner, 2009).

1.3.4 Process engineering for equilibrium displacement

Equilibrium displacement for reactions with a K_{EQ} value lower than 81 (in the synthetic direction) is required in order to achieve 90% yield (assuming the reaction is two substrate – two product system with a stoichiometry of 1 for all components). Process strategies therefore have to be adopted to overcome these reactions limited by equilibrium constraints. The effectiveness of the strategy required depends on the K_{EQ} value. For example, for the same scenario mentioned above, if the K_{EQ} value is close to 1 then adding a 10 fold excess of the substrate or co-substrate would be sufficient to shift the equilibrium. Moreover for reactions with unfavourable K_{EQ} value of below 0.5, other strategies of selective removal of product or co-product have to be introduced (Truppo et al., 2010; Yun and Kim, 2008). In many cases multiple strategies are required including adding an excess of substrate and *in-situ* product or co-product removal (Savile et al., 2010). Alternatively the choice of substrate could be modified such that the product formed goes through a rapid chemical modification (hydrolysis or cyclisation). Table 1.8 lists the various process strategies that can be used for shifting the equilibrium towards the synthetic direction

Table 1.8 Process strategies to shift the equilibrium towards product synthesis

Basis	Comments	References
Excess of substrate	Effective for reactions with K_{EQ} greater than 0.5	(Savile et al., 2010)
In-situ product removal physicochemical based	Needs to be highly selective. Applicable for reactions with K_{EQ} greater than 0.04	(Woodley et al., 2008b)
Enzymatic	K_{EQ} of second enzyme has to be greater than the first one	(Cassimjee et al., 2010; Truppo et al., 2009)
Chemical	Second reaction has to be thermodynamically favourable	(Li et al., 2012)
Spontaneous hydrolysis or cyclisation or complexation	Depends on the choice of substrate	(Schroen et al., 2002; Truppo et al., 2010)
Altering reaction condition		
Temperature	K_{EQ} increases with increasing temperature for endothermic reactions	(Daniel et al., 2008)
pH	The redox potential is lower at higher pH	(Hollmann et al., 2010)

1.4 Integrating porous resins in enzymatic processes

From the previous section, it could be seen that substrate feeding and *in-situ* product removal are two strategies which when incorporated in biocatalytic reactions can overcome substrate and product inhibition respectively. An interesting option to integrate these two strategies is to introduce an auxiliary phase into the bioreactor. Generally an organic solvent has been considered as an auxiliary phase in the bioreactor to overcome kinetic limitations (Ni and Xu, 2012; Schewe et al.,

2009; Yang et al., 2009). However, organic solvent exhibits certain operational challenges such as biocompatibility (Prpich and Daugulis, 2004), emulsification (Guieysse et al., 2005) and phase toxicity (Baldascini and Janssen, 2005). Hydrophobic ionic liquids have also been considered as a replacement for organic solvents. However the major drawback being high cost of ionic liquids limits its use in industry. Similarly, amorphous polymers have also been applied as an option as it is cheap and at the same time the chemistry of the polymer can be modified to fit the requirement of the process (Craig and Daugulis, 2013; Dafoe and Daugulis, 2012). Recently, an alternative auxiliary phase being porous resins has gained considerable recognition (Brenna et al., 2012; Hilker et al., 2008; Lee et al., 2011). Resins are generally inert in the bioconversion, offers simpler operational handling and product isolation (filtration).In this thesis the focus therefore has been directed towards porous resins as an auxiliary phase.

Resins have previously been used in combinatorial synthesis for supplying covalently attached substrate for catalysis on the surface of the resin (Basso et al., 2006; Humphrey et al., 2003; Laurent et al., 2008). Alternatively, the use of resins has also been focused on as a method for *in-situ* product removal (Guo et al., 2010; Liu et al., 2011; Lye and Woodley, 1999; Nielsen and Prather, 2009). However, selection of a suitable product selective support is difficult and although a few processes operate in industry (Burns and Wong, 2002; Johnson et al., 2000), non-selective resins have wider potential application (Brenna et al., 2012; Schmoelzer et al., 2012; Shorrock et al., 2004). Indeed the implementation of this strategy of integrating resins has extended to different types of biocatalytic reactions including oxidation (Hilker et al., 2008; Houng and Liau, 2003), reduction (Brenna et al., 2012; Schmoelzer et al., 2012) and hydrolysis (Lee et al., 2011). The objective of the resin is to act as a carrier for the inhibitory substrate and moreover to achieve high substrate loading. An initial equilibrium between the resin and the aqueous substrate concentration is displaced with biocatalytic consumption of substrate from the aqueous phase. Additional

substrate diffuses from the resin to accomplish *in-situ* substrate supply (ISSS). Similarly, as the product is formed, it partitions back into the resin to accomplish *in-situ* product removal (ISPR). In this way the full potential of the biocatalyst can be harnessed by enabling the biocatalyst to experience concentrations beneath the critical concentration, C_{crit} (Hilker et al., 2004; Kim et al., 2007; Vermue et al., 1993). Following synthesis, the resin will be separated by filtration prior to elution of the product (Hilker et al., 2005; Rojanarata et al., 2004; Vicenzi et al., 1997). Figure 1.4 illustrates the general scheme.

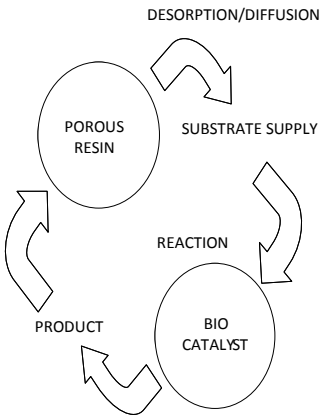


Figure 1.4 Principal of substrate supply and product removal using resin

The rate of supply is a key issue as the substrate supplied should complement the rate of product formation. Figure 1.5 illustrates the required rate of supply for a hypothetical case. The rate of supply (rate 1) moderated by the transfer constant, k_LA, needs to intersect the enzymatic reaction rate (rate 2) at a point where the rate is not limited by substrate availability or compromised by inhibition. During the time course, the driving force will reduce thus limiting substrate availability. Measures have to be taken to increase the mass transfer rate by either replenishing the resin with more substrate or alternatively increasing the mechanical stirring speed.

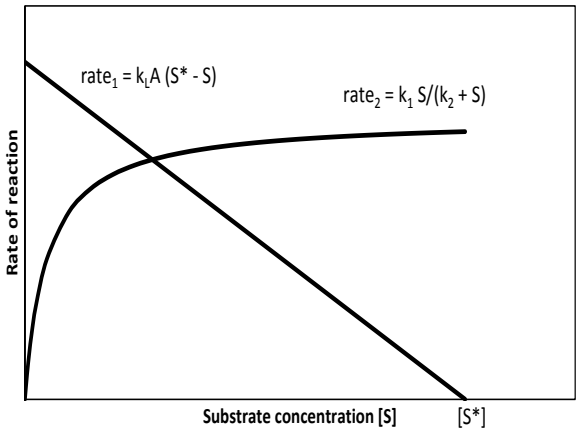


Figure 1.5 Substrate supply corresponding to rate to reaction

In many cases this has been applied effectively for overcoming inhibition by both substrate and product. An industrial example of such systems can be seen in the Lilly process for the production of LY300164 (a drug for improving Parkinson disease symptoms) using resin as the auxiliary phase (Vicenzi et al., 1997). The reactor was set up as shown in Figure 1.6

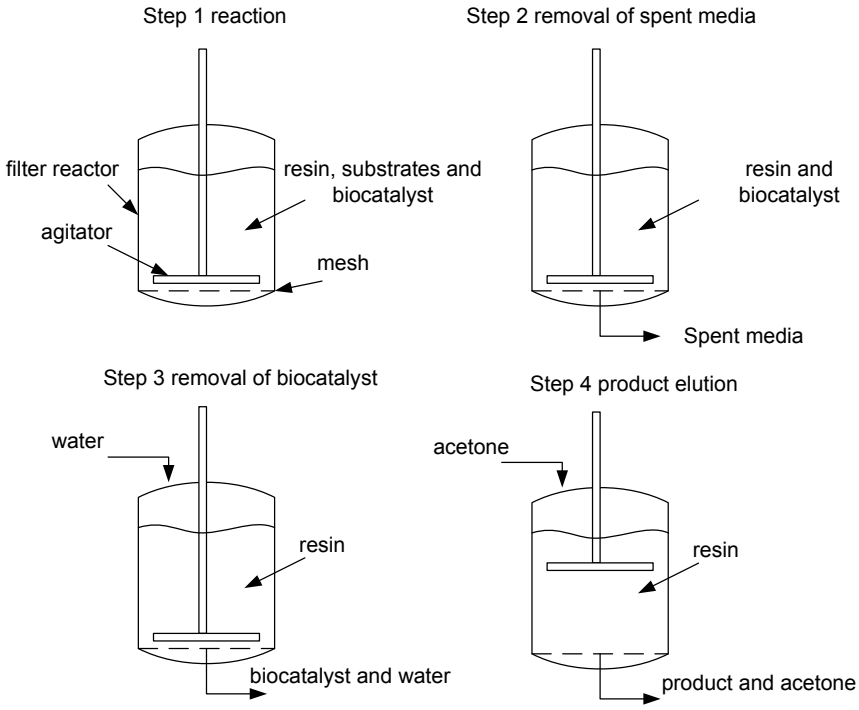


Figure 1.6 Operation of agitated filter for resin-based bioreactor

In the Lily process, a moderately non polar resin (XAD -7) with roughly equal affinity for both substrate and product was selected for the process. The entire process takes place in a single vessel. A mesh is used in order to retain the resins in the vessel for re-using. An agitator with low speed is used for mixing during the reaction. For separation of the resin from the micro-organism post reaction, water is added and the torque of the agitator is increased for washing the resins. The agitator is then raised allowing the resin to settle like a packed bed. Acetone is then used for eluting the product from the resin.

1.5 Overall framework for integrating resins in an enzymatic process

When, how and why? The three basic questions that arise when considering implementing any process strategy. Introducing resins is a very interesting option to overcome kinetic limitations, but perhaps it is not applicable in all cases. If a resin is required then how can one choose the resin in a logical way? Some guidelines need to be in place in order to guide the selection procedure. Furthermore the critical question is does integrating resins prove to be beneficial in terms of yield? These three fundamental questions need to be addressed in a systematic way. It would be desirable to have logical framework which would incorporate a heuristic method of making rule of thumb and thus assist in making an educated decision. The framework should be able to state when resins would be required based on a quantitative value. An example can be seen in the reduction of o-chloroacetophenone using whole cells expressed with xylose reductase and formate dehydrogenase. The half-life of the enzymes in the cells drastically dropped at concentrations of substrate and product exceeding 3 mM (Schmoelzer et al., 2012). In such cases, the presence of 30 % V/V resin Amberlite XAD7HP kept the aqueous concentration below toxicity limits and moreover improved the bio-reduction yield about 6 folds. In another study of the Baeyer-Villiger oxidation of bicyclo[3.3.0]hept-2-en-6-one similar to the previous example was limited by substrate inhibition at substrate concentrations above 3 g/L and product inhibition at product concentrations above 4 g/L. Integrating the reactor with 100 g/L of resin Optipore-L493 kept the ketone concentration below 1 g/L and moreover improved the yield from negligible to 83% (Simpson et al., 2001). Both these examples provide evidence that a resin was required to improve the synthetic yields. The framework should be able to heuristically guide the selection of the resin. Prior research has focused on selecting resin based on affinity (Vicenzi et al., 1997; Rojanarata et al., 2004) and working load ($g_{substrate}/g_{resin}$) (Hilker et al., 2004; Bechtold et al., 2012). These kinds of screening

worked effectively for single substrate and single product system (reduction and oxidation reactions), but for other industrially relevant enzymes such as transferases where it deals with two substrate and two product systems (Bornscheuer et al., 2012; Faber and Kroutil, 2005; Woodley, 2008), selecting a resin based on a single parameter alone would not be adequate. The problem becomes multi-objective function where other practical consideration of selectivity and cost has to be included into the objective function. Finally the question of why should this strategy be selected? The process has to be evaluated in terms of either improved yield ($g_{product}/g_{substrate}$) or ($g_{product}/g_{enzyme}$). A predictive model can provide such information and moreover also help in making "what if" analyses i.e. predict output for different process scenarios. "What if" analysis can help in identifying the bottlenecks of the strategy (e.g. low capacity of resins) or to help in optimizing the process (e.g. improved substrate mass transfer by using polymers with larger surface area (Rehmann and Daugulis, 2007). In order to address these questions a methodological framework has been developed. It follows a hierarchical approach where the output of each step becomes the input of the subsequent step. Figure 1.7 illustrates the work flow of the framework for integrating resins in an enzymatic process.

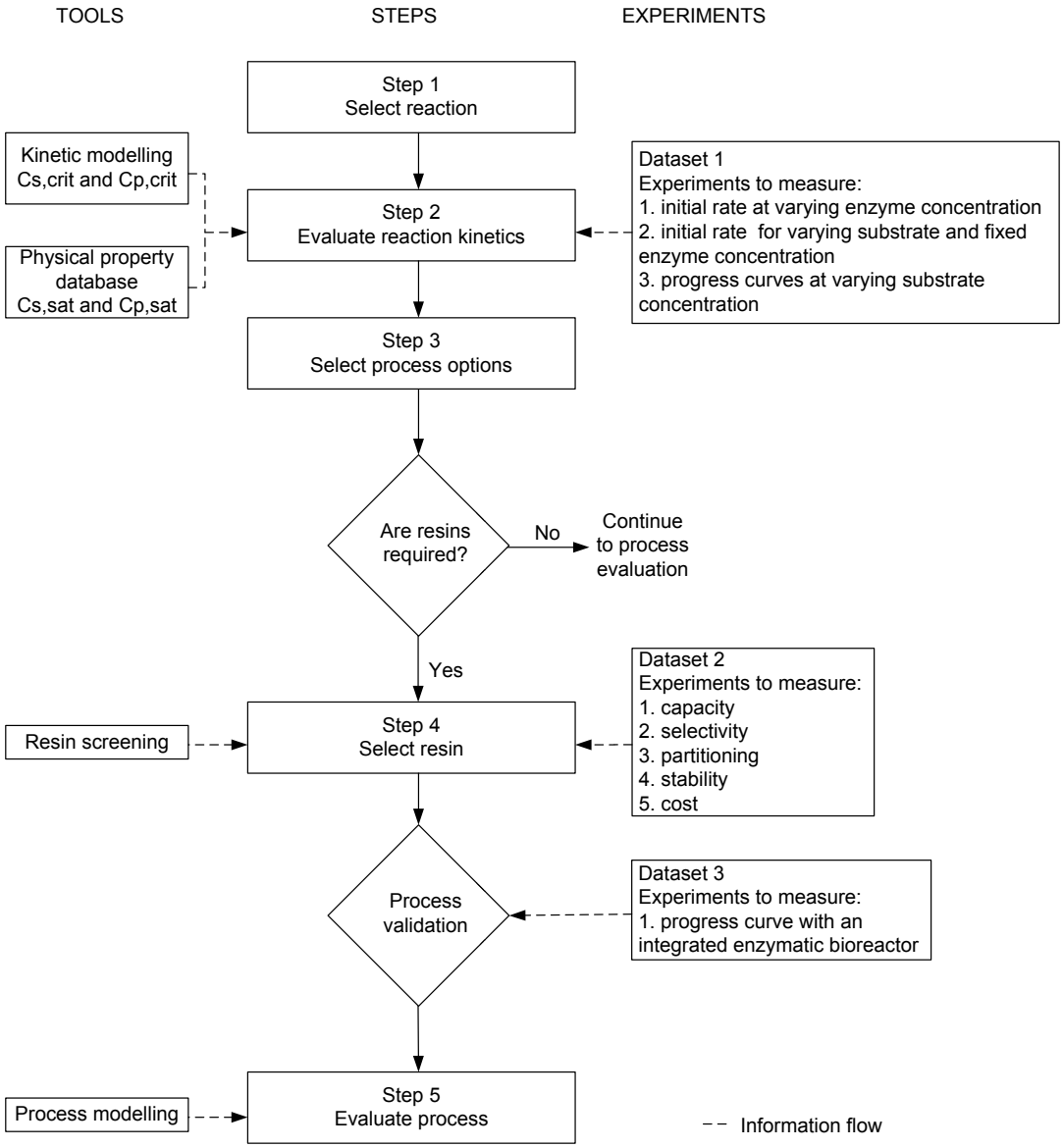


Figure 1.7 Methodological framework for integrating resins in a bioreactor

1.6 Description of proposed framework

1.6.1 Step 1. Select reaction

A reaction scheme has to be selected which will form the starting point for developing the process. The substrates and corresponding products need to be identified. In most cases however, the reaction of interest is given based on the project description. The selection can be guided by economics, commercial importance, or environmental considerations, substrate and product replacements. For example the production of high fructose corn syrup for making sweeteners from glucose using immobilized glucose isomerase has completely replaced the chemo-catalytic process because of its cost and environmental considerations (Jensen and Rugh, 1987).

1.6.2 Step 2. Evaluate reaction kinetics

The key characteristics of the reaction in terms of substrate (Cs,crit) and product (Cp,crit) inhibition and the aqueous solubility of substrate (Cs,sat) and product (Cp,sat) can define the process constraints. Figure 1.8 illustrates the operability area for operating a reaction. Knowledge of the critical concentrations are important information that can set targets for which parameter to optimize for protein engineers (Bornscheuer et al., 2012; Martin et al., 2007). Similarly the knowledge of the saturation concentration of the substrate and product coupled with the critical concentration of the substrate and product can set targets for reactor selection. In most cases the search for the ideal process has to be done in synergy between protein and process engineers to combat the process constraints (Savile et al., 2010; Schmoelzer et al., 2012). In order to gather information of substrate and product inhibition experimental investigation can be performed. Exhaustive numbers of experiments are required to gather this information, alternatively a kinetic model can be used not only to optimise the experimental efforts but also be able to predict outputs for new conditions (Al-Haque et al., 2012).

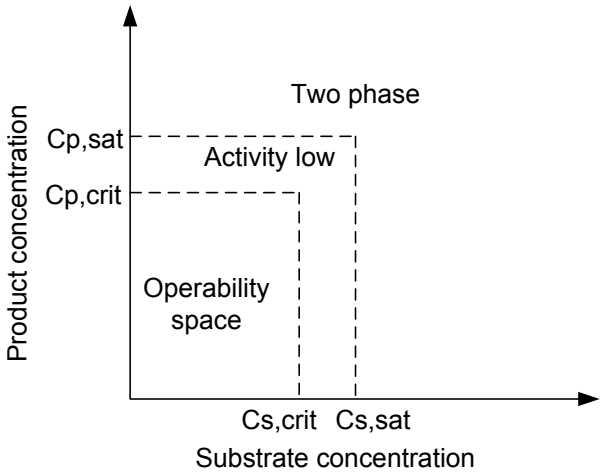


Figure 1.8 Operability space for substrate and product concentration. (Dashed line indicate the limits of substrate and product concentration)

Tool 1: Kinetic modelling

Kinetic models, either mechanistic or empirical, are a powerful tool readily applied in process systems engineering for multiple purposes such as process control, design and analysis (Sin et al., 2009). A validated model is useful for predicting reaction performance under different scenarios. Some likely example can be seen in the reactor selection for L-erythrulose synthesis (Vasic-Racki et al., 2003a), process optimization for the synthesis of neuraminic acid (Zimmermann et al., 2007). Kinetic modelling of a biocatalytic reaction can range from simple Michaelis-Menten kinetic model to much more complex model such as ping-pong bi bi. These kinetic models contain a significant number of parameters which are often highly correlated leading to issues with parameter identifiability (Santacoloma et al., 2010). Nonetheless a number of different methodologies have been proposed in order to minimize the experimental effort for estimating kinetic parameters (Vasic-Racki et al., 2011). Traditional approaches of using Levenspiel or Hanes plots can be considered obsolete because of the large degree of error that is incorporated in the linearization

(Nielsen et al., 2003). Methodologies based on non-linear optimization have been applied based on sensitivity analysis (Alcazar and Ancheyta, 2007) and assuming negligible inhibition region (Chen et al., 2008). Recently, a new and robust methodology has been proposed for estimating kinetic model parameters by model decomposition and multiple parameter initializations in a step-wise approach. The approach was validated using a ω-transaminase catalysed reaction to prove its reliability (Al-Haque et al., 2012).

Tool 2: Physical property database

The saturation concentration of the substrate and product are important data that have to be collected. Substrate fed beyond this concentration will operate in a two phase (dispersion for liquid substrates and slurry for solid substrates). If desired to achieve final product concentration beyond saturation point, it would require a substrate feeding and product removal strategies need to be implemented. These data can be collected from different physical property databases (e.g. NIST and ICAS) or estimated using property modelling (O'Connell, 2009).

1.6.3 Step 3. Select process options

Based on the inputs from step 2, different decisions have to be made to select the appropriate process configuration. A decision tree as shown in Figure 1.9 has been constructed based on the critical concentration for substrates and products coupled with their respective aqueous solubility. The goal of this tree is to provide guidance in the type of process that can be selected. When the critical concentration of the substrate lies below the solubility limit, substrate feeding strategies would be suggested (Schmoelzer et al., 2012). Likewise, a product removal strategy has to be considered if the critical concentration of the product is below the solubility limit of the product (Stark and von Stockar, 2003). In certain regions where the desired operating point is above the critical concentration for both the substrates and products then an integrated substrate feeding and

product removal strategy could be considered (Brenna et al., 2012; Hilker et al., 2005). Moreover, if it is required to operate in a region beyond the solubility limit then the reactor can be operated in two phase system i.e. either as a slurry (if substrate is solid) or dispersion (if substrate is liquid). However, in some cases working in slurry system might pose substrate availability limitations (Wick et al., 2002) and similarly dispersion might denature the biocatalyst (Kohlmann et al., 2011), in such situations a substrate feeding strategy should be considered as well to operate the reactor in a single phase. For formulation Figure 1.9, the following assumptions were made:

- The catalyst is not denatured in a multiphasic system
- Reaction equilibrium is not considered here

Based on Figure 1.9, it can be seen that in some cases substrate feeding is required, while in other cases ISPR is required and for few cases an integrated approach of substrate feeding and product removal would be suggested. In some cases it can be seen that a two phase reactor where either the substrate or product concentration is beyond saturation point. In order to design the process, certain type of data re required. In cases where a combination of the processes are to be integrated (e.g. continuous substrate feed coupled with product precipitation), data is required not only for the substrate feeding but as well as product precipitation. Table 1.9 suggests the data that have to be collected for the different processes options.

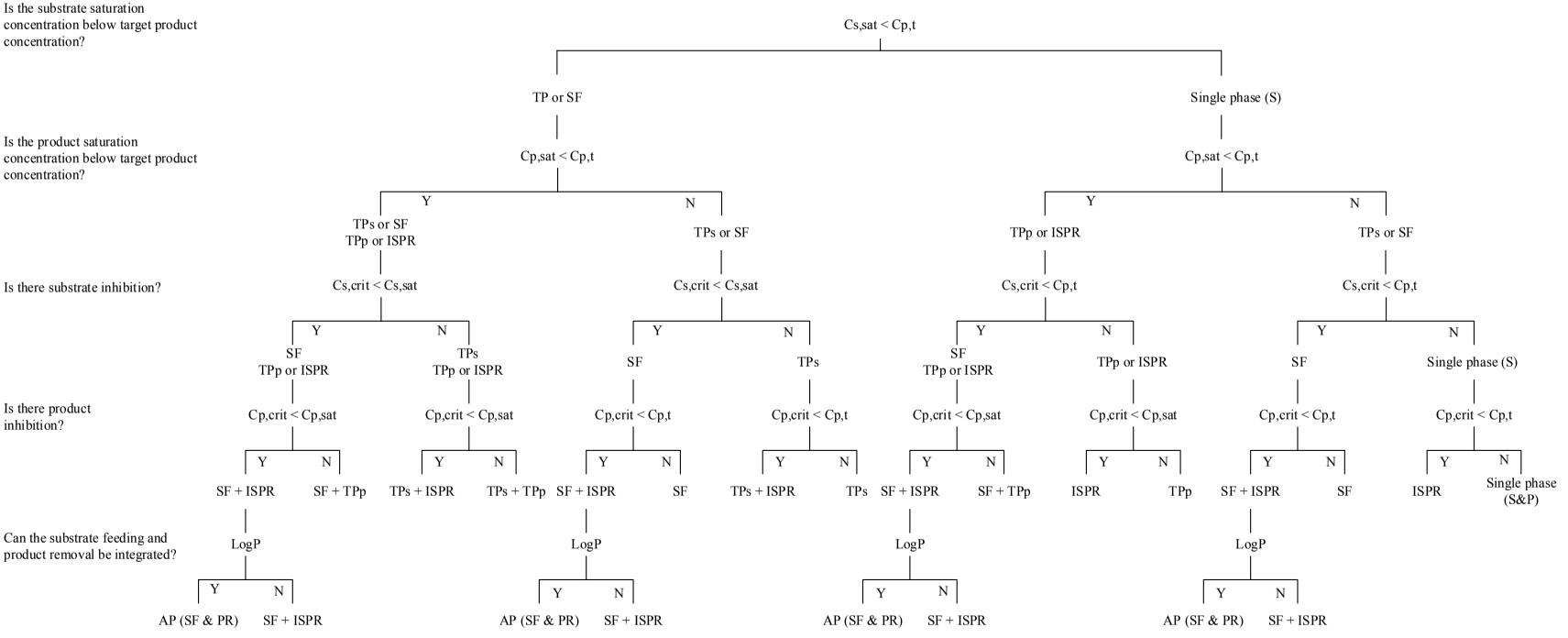


Figure 1.9 Decision tree for selecting process options. (Abbreviations: Cp,t – concentration of target product, Cs,sat – saturation concentration of substrate, Cp,sat - saturation concentration of product, Cs,crit – critical concentration of substrate, Cp,crit – critical concentration of product, TP – two phase, SF – substrate feeding, ISPR – in-situ product removal, AP – auxiliary phase, s – substrate, p – product, Y – yes and N – no)

Table 1.9 Data collection for different process options

Process options	**Data requirements**	**Reference**
Batch reactor	1. Critical concentration of substrate 2. Critical concentration of product	(Chin-Joe et al., 2002; Dahl et al., 1999)
Substrate feeding (Fed batch reactor)	1. Feed concentration 2. Rate of reaction (mM/min) at desired aqueous substrate concentration to adjust feeding rate 3. Critical concentration of product	(Amanullah et al., 2002; d'Anjou and Daugulis, 2001; Doig et al., 2002)
Packed bed reactor	1. Critical concentration of product to adjust length of reactor 2. Catalyst deactivation profile to adjust substrate flow rate	(Bhattacharya et al., 2004)
Continuous stirred tank reactor (CSTR)	1. Feed concentration 2. Critical concentration of substrate 3. Critical concentration of product 4. Catalyst deactivation profile to adjust substrate flow rate	(Visser et al., 2010)
In-situ product removal	1. Volatility (distillation, gas stripping) 2. Molecular weight (membrane, pervaporation, centrifugation) 3. Solubility (extraction, bi-phasic system, precipitation, crystallization) 4. Charge and hydrophobicity (ion exchange, adsorption)	(Dafoe and Daugulis, 2011; Gao and Daugulis, 2009; Liting et al., 2009; Schmoelzer et al., 2012; Steenkamp and Brady, 2008; Valadez-Blanco and Livingston, 2009)
Integrated substrate feeding and product removal	1. Capacity of product 2. Selectivity of product 3. Partitioning of components 4. Mass transfer rate of substrate supply and product removal	(Hagstrom et al., 2011; Heerema et al., 2011; Hilker et al., 2004; Houng and Liau, 2003)
Two phase (substrate)	1. Phase toxicity 2. Rate of dissolution 3. Substrate solubility	(Ulijn et al., 2003; Yang et al., 2009)
Two phase (product)	1. Phase toxicity 2. Product solubility	(Kumar and Bhalla, 2005; Schroen et al., 2002)

1.6.4 Step 4. Select resin

The resin has to be loaded with concentration of substrate to match the minimal industrial requirements. Subsequently, the substrate must partition from the resin to the aqueous phase at concentration within the operability space. The information about the operability space is acquired from step 2. Furthermore, with biocatalytic conversion, the formed product must partition back to the resin to maintain aqueous product concentration within the operability space. The selected resin has to be biocompatible i.e. be inert to the biocatalyst and moreover be cost-effective. In order to select the appropriate resin, an experimental guideline for pre-screening of resins has been developed.

Tool 1: Screening resins

The screening of resin is done in a heuristic approach where the decisions about selecting the resin is made based on the fulfilment of certain criterion specific for substrate supply and product removal strategy. The criterions include resin capacity, mass transfer rate and cost. More details will be discussed in the following chapter.

In order to compare resins, quantifiable values are required. The objective of this tool is to provide guidelines of the type of experiments required and what to expect from each set of experiments. For example, the substrate capacity of the resin can be measured by designing an experiment where the amount of resin is fixed while a certain concentration of substrate (solubility limit) is added to the vial containing the resins. The temperature and pH has to be fixed in accordance with the optimal biocatalytic reaction conditions. The concentration the substrate in the resin can be determined by mass balance.

1.6.5 Step 5. Evaluate process

With a reactor integrated with resins, it is required to evaluate the process. Key parameters such as mass transfer rate and improvement in product yield needs to be evaluated. Furthermore, greater understanding about the integration can be made and the bottlenecks can be identified, for example the required partitioning coefficient of the product might not be adequate for maintaining product concentration within the operability space. Recommendations can be made on how to optimize the process by for example predicting optimal resin amount (V/V). These kinds of analyses can be made using a model based approach. The validated kinetic model along with an appropriate isotherm model and mass transfer model can provide the knowledge required for evaluating an integrated resin bioreactor. On the other hand for processes that do not require resins, the process model can be used for optimizing the reaction or guiding reactor selection.

1.7 Objective and motivation

There is a clear motivation to incorporate a biocatalytic step within many organic synthesis. The high enantioselectivity of biocatalysts makes it extremely valuable in the fine chemical sector, and in particular in the production of pharmaceutical intermediates. However, there are certain limitations to some biocatalysts as was outlined earlier. Henceforth possible solutions have been suggested to exploit the full potential of the biocatalyst to aid in achieving the required process intensity. Integrating resins is inevitably a strong tool that can be applied to processes to overcome the kinetic limitations and enable high substrate loading of poor water soluble substrates. Resins have clear advantage over other auxiliary phases such as organic solvents. This strategy has been applied effectively to single substrate – single product systems (oxidation, degradation and hydrolysis). However, this concept has not been extended to other industrially relevant reactions which are two substrate – two product systems.

The objective of this Ph.D. thesis is to explore resin based auxiliary phases for multi-component systems, both conceptually and practically. In this manner, a generic platform can be established for biocatalytic reactions that require the integration of this strategy. It would be desirable to create a framework which can identify the key information about the reaction and the process in a systematic manner. Specific tools to assist the framework should be developed for characterizing the reaction kinetics, screening resins and evaluating the process. In this way greater knowledge about the enzymatic process with integrated porous resins can be gained and thus contribute with increased efficiency of process development with respect to time and resources. The framework should be validated with relevant case studies.

1.8 Rationale for case studies

In order to test the methodology three case studies have been selected. The case studies deal with the production of chiral molecules which in 2010 accounted for more than 40% of the world drug market of 856 bn. USD (Kaspereit et al., 2012). Each of the case studies contribute in gaining further understanding of the process and refinement of the framework in making the methodology a generic platform for all biocatalytic reactions and not specific for a single biocatalytic process.

The three case studies are:

Case study 1: Asymmetric synthesis of 1-phenylethyl amine using ω-transaminase

Case study 2: Asymmetric synthesis of 4-phenyl -2- butylamine using ω-transaminase

Case study 3: Enantioselective synthesis of 2-octanol using alcohol dehydrogenase

The first two case studies deal with the asymmetric synthesis of chiral amines using an isolated enzyme transaminase. Many of the challenges 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). Case studies 1 and 3 were selected for testing kinetic modelling methodology. Furthermore case studies 1 and 2 present a case in which the aqueous solubility of the substrate was low and displayed substrate and product inhibition. Consequently, the opportunity of introducing resins to achieve high substrate loading and overcome substrate and product inhibition was tested. Finally case study 1 and 3 were used for assessing and optimizing the process.

In order to give an overview of the contribution of the case studies in this thesis, Table 1.10 summarizes all the case studies, including the type of information that was found in the scientific literature and in which steps of the framework did it contribute.

Table 1.10 Compilation of the case studies

	1-phenylethylamine	**1-methyl-3-phenylpropylamine**	**2-octanol**
Components	4	4	6
Substrate	Acetophenone 2-propylamine	4-phenyl-2-butanone 2-propylamine	2-octanone Glucose
Co-factor	PLP	PLP	$NADP^+$ & NADP(H)
Product	1-phenylethylamine Acetone	1-methyl-3-phenylpropylamine Acetone	2-octanol gluconic acid
Co-solvent	N/A	N/A	TEGO IL K5
Enzyme and mechanism	TAm (EC 2.6.1.X): Ping-pong bi-bi	TAm (EC 2.6.1.18): Ping-pong bi-bi	ADH (EC 1.1.1.2): Ordered bi-bi GDH (EC 1.1.1.47): Ordered bi-bi
Contributions to the thesis:			
Kinetic modelling	Performed (Section 4.5)	N/A	Performed (Section 4.6)
Resin screening	Performed (Section 5.5)	Performed (Section 5.6)	N/A
Process evaluation	Performed (Section 6.3)	N/A	Performed (Section 6.4)

Abbreviations: PLP – Pyridoxial 5' phosphate; $NADP^+$ – Nicotinamide adenine dinucleotide phosphate; TAm – ω-transaminase; ADH – alcohol dehydrogenase; GDH – glucose dehydrogenase

1.9 Thesis outline

Chapter 1 provided an overview of biocatalysis including its advantages and limitations. For successful biocatalysis, some of these limitations or hurdles which are mainly related to process intensity have to be overcome. Many solutions were proposed to overcome these challenges. One of the solutions includes introducing porous resins to operate the reaction in a bi-phasic system. In order to systematically decide when and how to choose a resin, a methodological framework was developed. Consequently the different steps were described to provide understanding of the

backbone of this framework. In the same manner, the motivation and objective and rationale for selecting the case studies were stated. Chapter 2 elaborates more on auxiliary phases for bioreactors presenting the rationale and process considerations of introducing an auxiliary phase in a bioreactor.

The methodology is validated using three cases studies as mentioned in the previous section. The case studies are introduced in chapter 3 to present the different routes for producing chiral amines and chiral alcohols. Chapter 4 presents the results of case studies 1 and 3 for testing the kinetic modelling methodology. The kinetic model is further used in a batch reactor model to set targets for substrate and product concentration. Chapter 5 presents the results of case studies 1 and 2 for testing the resin screening methodology. In chapter 6, the results of chapter 4 are used to build process model. The aim of this chapter is to gain further understanding about the process itself and moreover use of the model to predict the process performance with new conditions. Chapter 7 contains an overall discussion about the thesis in general outlining the contribution of the different case studies to help to refine and validate the methodology. In chapter 8 a conclusion about the thesis in general and specific to the framework developed in this thesis and the case studies are outlined. Finally in chapter 9 some future work is proposed to continue the research of integrated auxiliary phases in bioreactors. Chapter 10 contains a list of references followed by Appendix 1 which discusses some of the key concepts about reaction kinetics and the different types of inhibition and Appendix 2 containing the publications that have been made through the efforts of this PhD.

Chapter 2

Integration of auxiliary phases in enzymatic processes

2.1 Introduction

There are several types of auxiliary phase that can be integrated into a bioprocess (Stark and von Stockar, 2003). Specific for substrate feed and product removal strategy, three kinds of auxiliary phase have received much attention recently. An overview of the different forms is given below:

1. Liquid – solid: adsorption (auxiliary phase is porous resins)
2. Liquid – liquid: extraction (auxiliary phases are water-immiscible organic solvent, amorphous polymer and ionic liquid)
3. Liquid – vapour: stripping (auxiliary phase is gas)

In this chapter the focus will be made on the first two types. Gas has not been considered as an auxiliary phase for further discussion as the application is limited only to volatile substrates such as flavours or pheromones catalysed by thermo stable enzymes (Ferloni et al., 2004; Lamare and Legoy, 1995).

Searching for an ideal auxiliary phase is in many cases limited to highly hydrophobic or charged compounds (Straathof, 2003). Alternative technologies such as fed-batch followed by product

recovery based on phase or charge change in some cases would be able to suffice the required specific catalyst productivity. Careful consideration has to be made to justify the requirement of an auxiliary phase. The selection of the process strategy has to be made based on the reaction characteristics, aqueous solubility and level of inhibition. In order for visualization a hypothetical case is considered where the catalyst is inhibited by both substrate and product as shown in Figure 2.1.

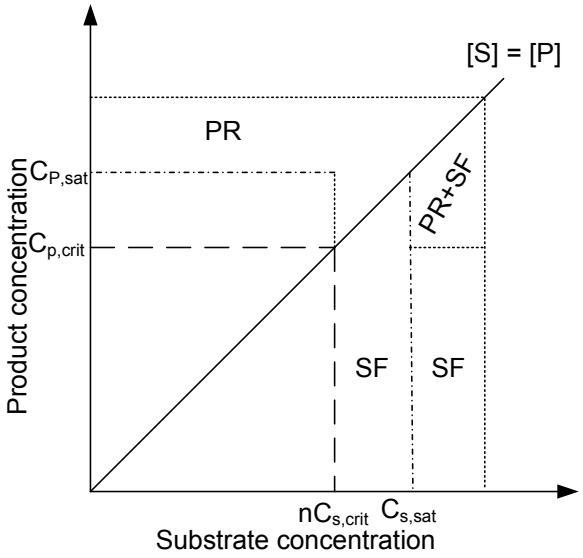


Figure 2.1 Reaction scenarios based on critical concentration and solubility of substrate and product (crit is critical concentration; sat is saturation of substrate (s) or product (p); n is >1 or <1; SF is substrate feeding and PR is product removal)

From Figure 2.1 it can be seen that for a feed concentration beyond the substrate critical concentration (Cs,crit) would require a substrate feeding strategy to be adopted. Similarly, to achieve product concentration beyond the product critical concentration (Cp,crit) a product removal technique has to be integrated. To overcome this limitation, an integrated substrate feeding and product removal technique can be adapted by means of introducing water-immiscible auxiliary phase (Braeutigam et al., 2009; Hilker et al., 2005; Prpich and Daugulis, 2007a; Straathof, 2003;

Sun et al., 2012; Ueatrongchit et al., 2009). However, further process considerations have to be taken into account when considering using auxiliary phase. Their details will be explained in the following section.

2.2 Process considerations for integrating auxiliary phases in enzymatic processes

2.2.1 Load and selectivity

Selectivity of the auxiliary phase is a key issue for the process consideration. Generally the auxiliary phase is selected based on the physical properties of the components i.e. hydrophobicity or ionic form at a specific pH or partition coefficient of the compounds of interest (Lye and Woodley, 1999). In some cases the properties of the substrate and product of interest have similar chemical and physical properties (eg. transaminase reactions) which raise the issue of effectiveness of the auxiliary phase (Tufvesson et al., 2011). If the auxiliary phase is overly selective for the substrate then it might be too tightly bound to the phase and would not partition into the aqueous phase in a desired rate causing the reaction to be substrate limited. On the other hand if the auxiliary phase is minimal selective for the substrate then the desired load ($g_{substrate}/g_{resin}$) would be low, thus requiring larger ratio of auxiliary phase to substrate ($X_{AP/S}$). Thus the economics of introducing the auxiliary phase has to be evaluated. Alternatively auxiliary phases such as amorphous polymers whose architechture can be modified would be highly beneficial for designing a tailored polymer to fit the process (Parent et al., 2012). An overview of some of the studies done for different types of auxiliary phases is presented in Table 2.1. A clear tendency can be seen in many cases where the substrate load is inversely proportional to the selectivity. A general trade-off has to be done between selectivity and capacity of the auxiliary phase.

Table 2.1 Selectivity for different target compound by auxiliary phases

Auxiliary phase	Target compound	Auxiliary phase load (V/V)	Substrate load (g/g)	Selectivity of product	Yield increased (fold)	Reference
Hyrtel	Cis (1S-2R) indandiol	18%	0.05	0.25	5	(Dafoe and Daugulis, 2011)
Amerlite XAD7HP	(S)-1-(2-chlorophenyl)ethanol	30%	0.07	1.50	2	(Schmoelzer et al., 2012)
NKA – II	(R)-methyl mandelate	3%	0.46	0.26	2	(Guo et al., 2010)
Lewatit VPOC1064	3-phenyl-2-butanone	20%	0.30	1.00	30	(Geitner et al., 2010)
Di-butyl ether	(S)-phenyl Ethanol	10%	0.24	0.42	5	(Valadez-Blanco and Livingston, 2009)
Cyclohexanone	Acetophenone	20%	0.01	1.26	15	(Shin and Kim, 1997)
[BMIM][NTF]	2-octanol	20%	0.47	0.70	12	(Braeutigam et al., 2009)
[BMIM][PF6]	*n*-butyl acetate	50%	0.26	0.71	2	(Park et al., 2005)

2.2.2 Product separation from auxiliary phase

It is of key importance to be able to select the appropriate separation method in a timely and systematic manner (Alvarado-Morales et al., 2010; Jaksland et al., 1995; Siirola, 1996). The auxiliary phase can be integrated directly into the reactor thereby allowing reaction and separation to take place in the same vessel. However, sometime the auxiliary phase may pose an adverse effect to the biocatalyst for which the auxiliary phase can be linked via a recycle loop to the reactor (Woodley et al., 2008b). In either case the auxiliary phase has to be regenerated and the complexity of the product separation increases with increasing number of components in the reaction media. Table 2.2 tabulates the separation scheme from the auxiliary phase for the different reactions in biocatalysis.

Table 2.2 Separation scheme for different types of reaction

Reaction #	Reaction	Down stream	Type of reaction
1	A ↔ Q	Q/A	Isomerases
2	A ↔ P + Q	Q/(A+P)	Lyase
3	A + B ↔ Q	Q/(A+B)	Lyase*
4	A + B ↔ P + Q	Q/(A+B+P)	Transferase

*reverse direction

In Table 2.2, A is the limiting substrate, Q the target compound while B and P are the co-substrate and co-product respectively. It can be seen as the chemistry becomes a little complex, i.e. the number of substrates and products increases from one to two, then the separation scheme in the downstream process requires high selectivity to generate high separation yields. Moreover, with increasing interest in multi-enzymatic processes, the total number of components in the reaction media can be greater than four (Santacoloma et al., 2011), thus requiring the separation process to

be highly selective otherwise a multi-step separation will be required. Some bioprocesses require certain kind of buffer to maintain a certain pH. The salts from the buffer can as well pose an extra complication not only to the auxiliary phase but as well as to the separation strategies. Furthermore in some bioprocesses it is essential to have co-factors such as nicotine amide to improve the viability of the biocatalyst, thus adding more constraints to the separation process (Khan and Daugulis, 2011b). For processes (especially the pharmaceutical industry) there is a requirement to meet high product specifications of very high product purity. This would result in multiple downstream steps to meet the specifications. Novel separation techniques such as chromatographic (Bechtold et al., 2006), membrane (Heerema et al., 2011), crystallization (Buque-Taboada et al., 2006) and tailored solvent (Lek-utaiwan et al., 2008) and polymers methods (Zhang et al., 2006) may need to be applied.

2.2.3 Bio-compatibility

The compatibility between the auxiliary phase and the biocatalyst is an important issue that has to be experimentally determined before introducing it to the process. Resin has an advantage over liquid auxiliary phase as it is completely inert to the biocatalyst (Prpich and Daugulis, 2004). In the case of liquid auxiliary phases, organic solvent and ionic liquids, a loss in activity is frequently noticed for the enzyme. Table 2.3 lists some of the results of different studies done with liquid – liquid biocatalysis. A general strategy for selecting solvents for whole cell catalysis is based on the log P values of the solvent (Bruce and Daugulis, 1991). The Log P value of the solvent should be higher than the Log P_{crit} of the microorganism to be biocompatible (Straathof, 2003). A study done by Lane et al., (1987) on free and immobilized lipases revealed that solvents with Log P value greater than 4 would not strip away the water associated within the enzyme, and hence retain the necessary water to remain catalytically active (Laane et al., 1987). However, this is not the case for isolated enzymes which are more susceptible to deactivation as they do not have any cellular

protection (Shin and Kim, 1997). Therefore the selection process is rather a trial and error in many cases.

Table 2.3 Stability of the different biocatalyst in liquid auxiliary phases

Auxiliary phase	Biocatalyst	Residual activity (%)	Reference
Cyclohexanone	ω-transaminase	65	(Shin and Kim, 1997)
Hexane	Carbonyl reductase	59	(Kansal and Banerjee, 2009)
Toluene	Carbonyl reductase	66	(Wang et al., 2011)
Bis(2-ethylhexyl) sebecate	Pseudomonas putida MC2 (whole cell)	100	(Prpich and Daugulis, 2007b)
BMIM – PF6	Laccase	8	(Zhou et al., 2008)
IEMIM – EtSO4	Mycobacterium sp. NRRL B-3805 (whole cell)	120	(Marques et al., 2010a)
C4MIM – PF6	Rhodotorula Sp. AS2. 2241 (whole cell)	98	(Wang et al., 2009)
BMIM – PF6	Alcohol dehydrogenase	15	(Zhang et al., 2008)

2.2.4 Reactor design

Once an ideal auxiliary phase is identified, it is necessary to determine the type of reactor that is suitable. Selection of a reactor is often determined by economics, reliability, or availability of a proven system. The choice of the reactor governs the application of the different auxiliary phases. The different types of reactors with different reactor configuration that can be used in biocatalytic reactions for batch reactions are shown in Figure 2.2.

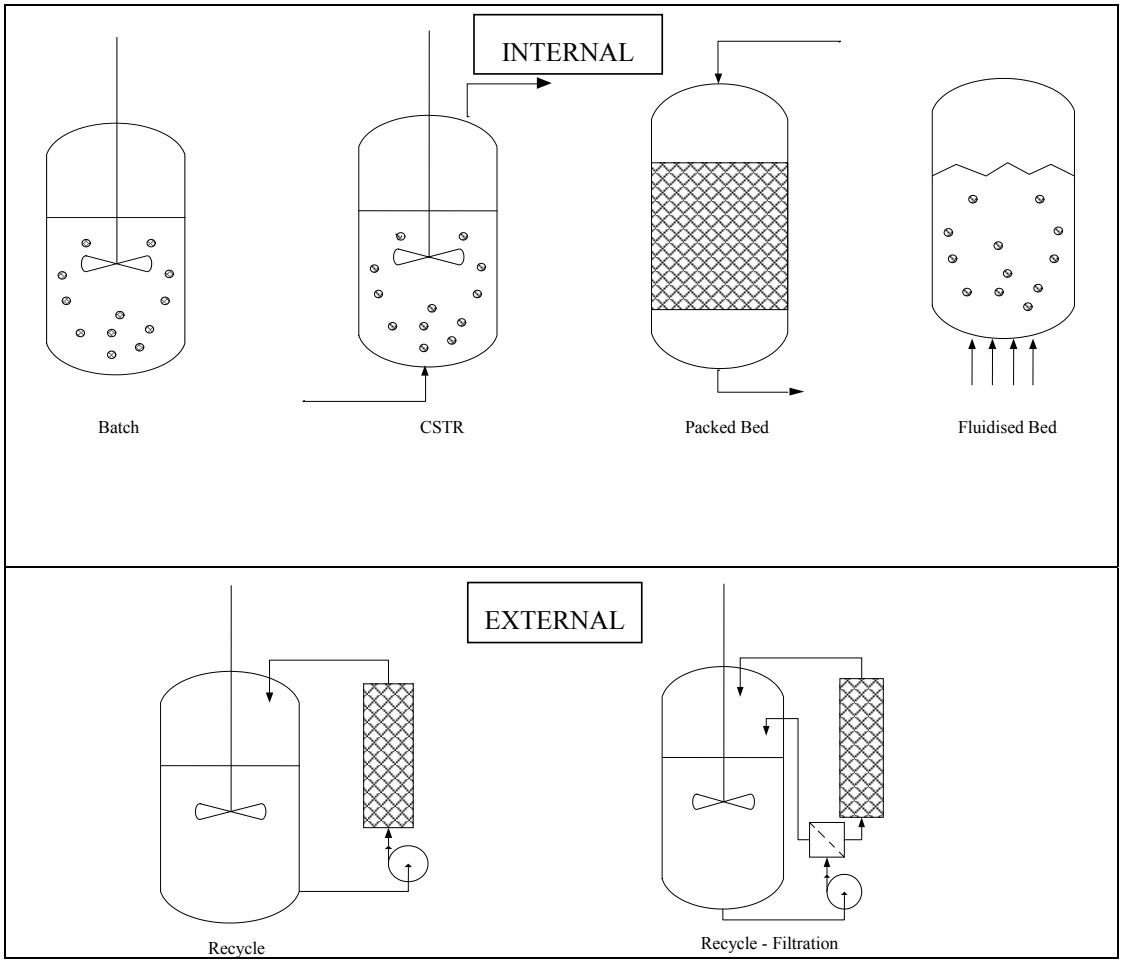


Figure 2.2 Reactor types with different modes of configuration (shaded area indicates auxiliary phase)

To identify the appropriate reactor, the first step is to determine the key characteristics of the reaction (Lilly and Woodley, 1996). This requires information, which can be obtained either from published literature or by specifically designed experimental work, on: (i) the form of the biocatalyst (solubilised, whole cell and immobilized) (Woodley, 2006), (ii) the interaction between the biocatalyst with the substrate(s) (the kinetic reaction model used to describe the reaction) (Vasic-Racki et al., 2003a) and (iii), the interaction of the biocatalyst with the resin (adverse effect of resin on contact with biocatalyst) (Shorrock et al., 2004). The reaction kinetics of the substrate

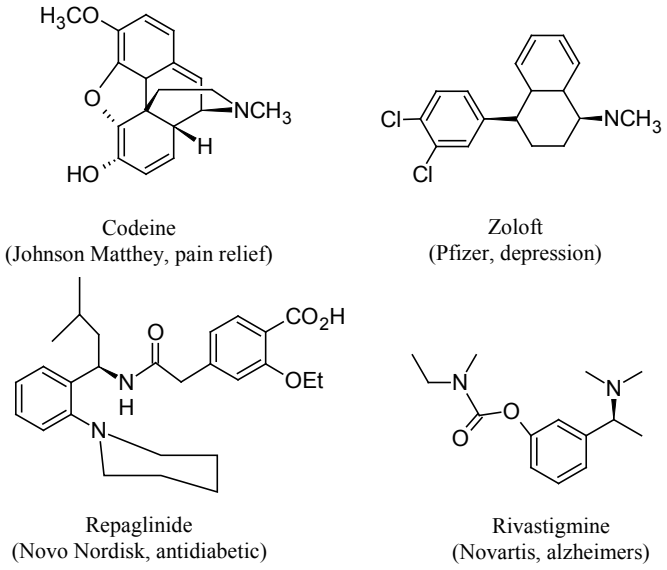
with the biocatalyst plays a major role in the selection of the reactor (Vasic-Racki et al., 2003b). A preliminary guidelines based on the reaction kinetics have been presented in Table 1.9. Specifically for auxiliary phase reactors the general mode of operation would be an internal configuration using a stirred tank reactor (Schügerl and Hubbuch, 2005). However in some cases the auxiliary phase might have an adverse effect on the catalyst and thus can be linked to the reactor by a recycle loop (Kim et al., 2007). These precautions should be considered when there is a necessity of pH/phase shifting or perhaps the mechanical agitation with resins or emulsions with solvents denatures the biocatalyst. Packed bed reactor would generally be used with resins as auxiliary phase for ease of handling. However, if the dimensions of the support are too small, it might result in a high pressure drop, in such cases a fluidized bed or expanded bed reactors would be the desired option. A further point that has to be evaluated is the volumetric requirements of the auxiliary phase (V/V). As a rule of thumb a stirred tank reactor incorporates 80 – 90% voidage while a fluidized bed reactor is around 70% and a packed bed reactor of 34% (Burton et al., 2002).

Chapter 3

Introduction to case studies

3.1 Synthesis of optically pure chiral amines

Enantiomerically pure chiral amines are of increasing commercial value in the fine chemical and pharmaceutical areas in view of their application as resolving agents for chiral acids (Palovics et al., 2007; Schindler et al., 2007), chiral auxiliaries/bases (Liu et al., 2007), catalysts for asymmetric synthesis (France et al., 2003; Nagano et al., 2012), intermediates for agrochemicals (Jackson, 2011) and especially as pharmaceutical intermediates (Patel, 2011). About 80% of the active compounds that pharmaceutical companies have in the pipeline are chiral, and it is estimated that this fraction will increase, as the development of active compounds continues to be improved (Breuer et al., 2004; Fernandez et al., 2008). Some examples of pharmaceutical drugs containing chiral amines can be seen in Figure 3.1.



Codeine
(Johnson Matthey, pain relief)

Zoloft
(Pfizer, depression)

Repaglinide
(Novo Nordisk, antidiabetic)

Rivastigmine
(Novartis, alzheimers)

Figure 3.1 Pharmaceutical drugs containing chiral amines

The importance of enantiopure active substances has been driven primarily by the strict regulations of the US Food and Drug Administration (FDA) and the European Committee for propriety medicinal products to stipulate the physiological action of each enantiomer of a pharmaceutical product. The current methods to prepare enantiomerically pure chiral amines are based upon resolution of racemates by crystallization of diastereoisomeric salts or by enzyme catalysed kinetic resolution of racemic substrates using hydrolases (lipase and accylase) and aminotransferases (ω-transaminase) (Turner and Truppo, 2010; Turner and Carr, 2007). These strategies have an inherent disadvantage of having a theoretical yield of 50%. Apart from resolution, deracemisation of racemic mixtures with monoamine oxidase is an attractive alternative, which when coupled with a reducing agent can yield close to 100% product in a single pass (Alexeeva et al., 2003). Asymmetric syntheses have received much attention as it can theoretically have a yield of 100%. These strategies include asymmetric hydrogenation of acetamides and imines, asymmetric addition of carbanions, conversion of ketones to amines using transaminases (Breuer et al., 2004). In contrast to

deracemisation with monoamine oxidase, which requires the prior synthesis of racemic amines, ω-transaminases can directly use the more readily available ketone. An overview of the different ways to produce chiral amines are shown in Figure 3.2

From Figure 3.2, it can be seen that in total there are 9 different methods for producing chiral amines. These methods can be classified into two categories:

1. Chemical synthesis processes

2. Biocatalytic processes

The limitations and challenges of the different routes will be discussed briefly in the following sections.

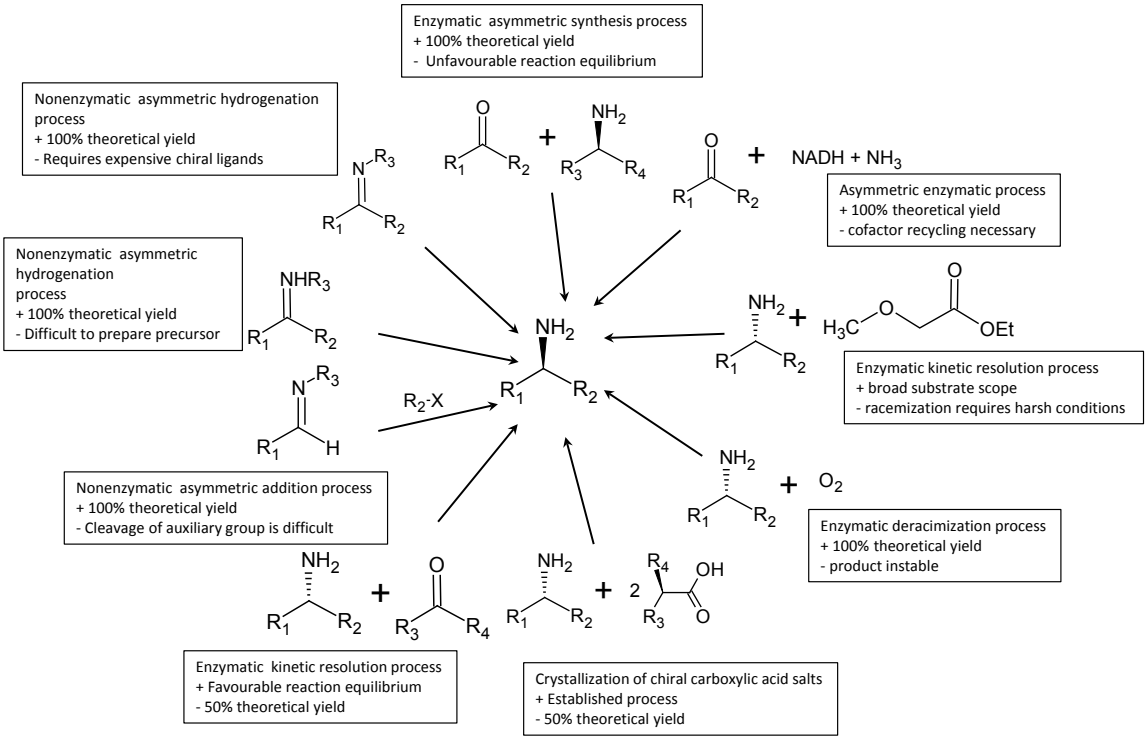


Figure 3.2 Different strategies to produce chiral amines

3.1.1 Chemical synthesis of chiral amines

The classic chemical approach for the production of optically pure chiral amines has been done by the crystallization of diastereomic salts of chiral carboxylic acids with racemic amines (Jacques et al., 1994). (R) or (S)-1-phenylethylamine can be prepared at industrial scale from the salts of (S)-malic acid (Loven, 1905) or (R)-mandelic acid (Ingersoll et al., 1933) or (R)-tartaric acid (Theilacker and Winkler, 1954) with very high optical purity. This process has in some cases been considered inefficient (maximum 50% yield) and may be viewed as uneconomic. Figure 3.3 illustrates the scheme for the production of enantiomerically pure chiral amines.

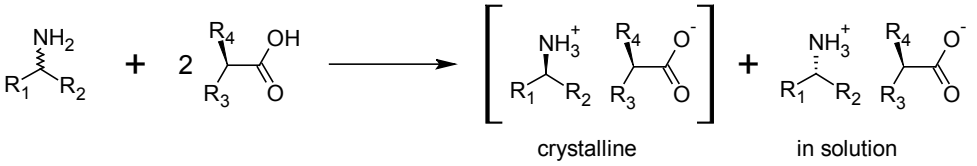


Figure 3.3 Racemate resolution through fractional crystallization of salts of racemic amines and enantiomerically pure carboxylic acids

This has led to increased efforts towards asymmetric chemical synthesis of chiral amines by catalytic asymmetric hydrogenation of imines and amides. These approaches require chiral auxiliaries or metal complexes with chiral ligands (Boezio et al., 2003; James, 1997). An extensive research effort has resulted in diverse sets of chiral ligands. The use of rhodium, ruthenium, and iridium in combination with certain chiral ligands are reported to catalyse the enantioselective hydrogenation of amides and imine (Li and Zhang, 2000; Tang and Zhang, 2002; Xiao and Zhang, 2001). The difficulty in this approach lies with the synthesis of the prochiral imine precursor to give the free amine (Breuer et al., 2004). The imine or amide precursor can be made by condensation of ketones with appropriate amines. This therefore presents a multistep process (Tararov and Börner, 2005). Moreover the chiral ligands and metal precursors are usually expensive and not easily available (Li and Zhang, 2000). Figure 3.4 illustrates the asymmetric hydrogenation processes.

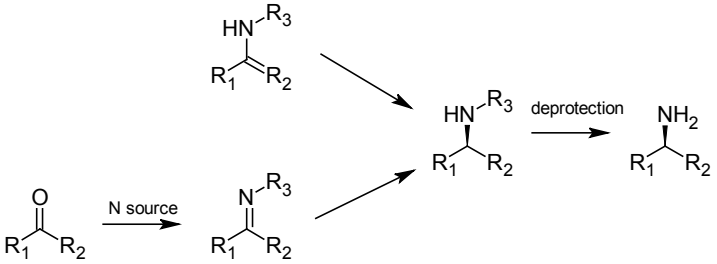


Figure 3.4 Asymmetric hydrogenation for the synthesis of chiral amines

An alternative approach is the asymmetric addition of carbanions to aldimines. Figure 3.5 illustrates the scheme for asymmetric addition process. Similar to the previous approach, it is a multi-step approach where the cleavage of the auxiliary group X can be difficult (Breuer et al., 2004).

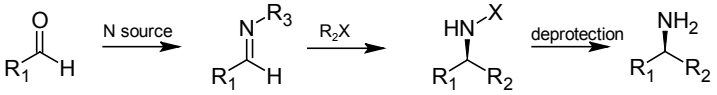


Figure 3.5 Asymmetric addition for the synthesis of chiral amines

3.1.2 Biocatalytic processes

Kinetic resolution using hydrolytic enzymes

The kinetic resolution of racemic amines can be accomplished by hydrolytic enzymes such as the subtilisin Carlsberg (protease), lipases (EC 3.1.1.3) and penicillin acylase (Breuer et al., 2004). A likely example can be seen in the patented BASF process at a multiton scale for the production of chiral amines using *Burkholderia plantarii* lipase in combination with an acylating agent to yield enantiomerically pure (S)-1-phenylethylamine and the corresponding amide (Funke et al., 2003). The versatility of the process with regard to possible substrates, the ability to recycle undesired

enantiomers by racemization and the recovery of the acylating agent are the main advantages of the process. Figure 3.6 illustrates the scheme used by BASF.

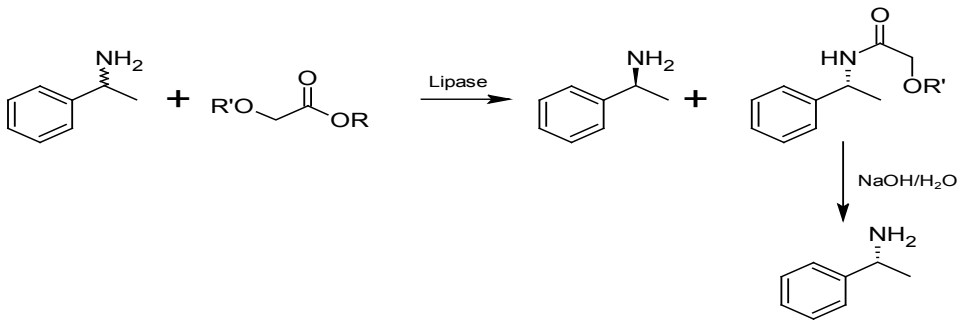


Figure 3.6 Lipase catalysed enantioselective amide formation (BASF).

Kinetic resolution using ω-transaminases

ω-Transaminases (TAm, EC 2.6.1.18) are capable of selectively producing chiral amines. The enzyme transfers an amino group from an amino donor to a ketone utilizing the cofactor pyridoxyl-5-phosphate (PLP) (Cho et al., 2008). This process has been industrially scaled up to 2.5 cubic meter scale by Celgene technology (Matcham and Bowen, 1996). In the Celgene process, a racemic mixture of amines can be resolved to form a ketone. Figure 3.7 illustrates the scheme.

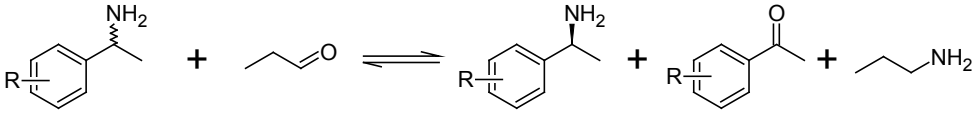


Figure 3.7 Kinetic resolution using ω-transaminase (Celgene)

However a general challenge to the kinetic resolution strategy using ω-transaminase has been the inhibition caused by both substrate and product leading to low product concentrations. Moreover the product mixtures can only be separated by cumbersome methods.

Deracemisation

Turner and collaborators at GlaxoSmithKline developed an alternative to kinetic resolution using an (S)-selective monoamine oxidase (MAO, EC 1.4.3.4) from *Aspergillus niger*. The enzyme can deracemise a racemic mixture of amines with a theoretical yield of 100% in the presence of a reducing agent for the (R)-enantiomer (Victorovna et al., 2003). The (S) selective monoamine oxidase selectively oxidises the (S)-amine enantiomer from a racemic (R, S) amine mixtures to the corresponding imine while leaving the other enantiomer intact (Alexeeva et al., 2003). The formed imine in the presence of a borane based reducing agents subsequently converts the imine to a (R)-amine. In this way the final product left in solution would be pure (R)-amine in high yield (Dunsmore et al., 2006). The major challenge for this reaction is in the selection of the reducing agent, which determines the efficiency of the process and furthermore has to be compatible with the enzyme and aqueous reaction media (Alexandre et al., 2002). Moreover, in some cases the imine formed can hydrolyse to ketones resulting in an undesired reaction, especially if the substrate is a primary amine (Alexeeva et al., 2012). Figure 3.8 illustrates the reaction scheme for the deracemisation process.

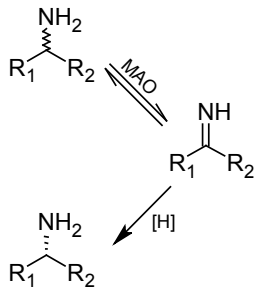


Figure 3.8 Deracemisation of primary amines using MAO and a non-specific imine reductant

Asymmetric synthesis with amine dehydrogenase

Amine dehydrogenases (EC 1.4.99.3) oxidises amines to ketones and ammonia. The enzyme requires redox cofactors such as NADH which has to be regenerated. Unfortunately very few highly enantioselective variants have been created of which only one enzyme extracted from *Streptomyces virginiae* can be found in the literature (Itoh et al., 2000). The reaction scheme is illustrated in Figure 3.9.

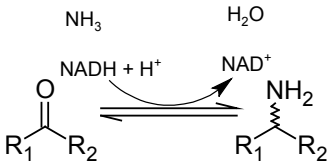


Figure 3.9 Asymmetric synthesis with amine dehydrogenase

Asymmetric synthesis using ω-transaminases

In a recent publication it was stated the benefits of the asymmetric synthesis of chiral amines namely the potential to achieve a 100% yield at mild operating conditions by the enzyme transaminases (TAm, EC. 2.6.1.18) (Tufvesson et al., 2011). The published review is focused 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. Transaminases catalyse the transfer of an amine (-NH2) 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 (Figure 3.10). Transaminase requires cofactor pyridoxal phosphate (PLP) to act as a shuttle to transfer the amine group. This cofactor is fully regenerated within the same two substrate reactions on the same enzyme, and hence does not pose the cofactor regeneration problems encountered in

oxidation/reduction reactions (Hwang et al., 2005). The general scheme for asymmetric synthesis of chiral amines is illustrated in Figure 3.10.

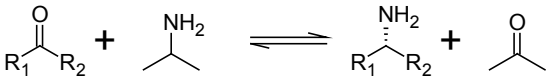


Figure 3.10 Asymmetric synthesis of chiral amines using transaminase

In spite of the many attractive features of asymmetric synthesis using transaminase catalysed 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.

The major challenges to this approach are:

1. The thermodynamic equilibrium favours the ketone starting material
2. Transaminases typically challenged with substrate and product inhibition at industrially relevant concentrations
3. Hydrophobic substrates at high concentration denature the enzyme

Many of the challenges 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). Therefore the objective of selecting these model reactions would be the ability to assess the feasibility of using these kinds of enzyme to systematically make process evaluations for scale-up considerations and also to further validate the usefulness of using resin as an auxiliary phase. At the same time many conclusions can be drawn from the analyses which will help gain further understanding and develop process strategies for the asymmetric

synthesis of chiral molecules. The study of this section about transaminases formed the basis for a peer reviewed journal publication as seen on Appendix 2.

3.2 Synthesis of optically pure chiral alcohols

The stereoselective reduction of inexpensive prochiral ketones to their corresponding alcohols is one of the most useful ways of introducing chirality in a molecule. Chiral alcohols can then be transformed into various functional groups (halides, thiols, amines, azides etc.) without racemization and concurrent loss of chirality of the product (Nakamura et al., 2003).It is supposed that 40% of all chiral centres in drugs are secondary or tertiary hydroxyl groups (Meyer et al., 1997).

Chiral alcohols can be synthesized by three approaches as listed below:

1. Chemical synthesis
2. Kinetic resolution of racemic alcohols
3. Asymmetric synthesis by biocatalysis

The limitations and challenges of the different approaches will be discussed briefly in the following sections of this chapter.

3.2.1 Chemical synthesis of chiral alcohols

The common techniques that have been applied for producing chiral alcohols are asymmetric hydrogenation and reduction using enantioselective chemical catalysts (Breuer et al., 2004). The asymmetric transfer hydrogenation of ketones to chiral alcohols has been demonstrated using a variety of chiral catalysts generally including phosphine ligands (Akutagawa, 1995; Blaser et al., 2003; Ikariya and Blacker, 2007; Noyori et al., 1995; Saito et al., 2001). In contrast,

enantioselective ketone reductions have been demonstrated successfully using CBS borane catalysts (Hett et al., 1998; Hirao et al., 1981). While these asymmetric chemical synthesis of chiral alcohols have been extremely useful in the production of pharmaceutically important intermediates (Blaser and Studer, 1999; O'Shea et al., 2005), the challenges set forth by the chemo-catalytic route are the demand for transition metals, high pressure and temperature and moreover the use of hazardous substances resulting in their application getting limited. Figure 3.11 illustrates the production of chiral alcohol by hydrogenation of reduction systems.

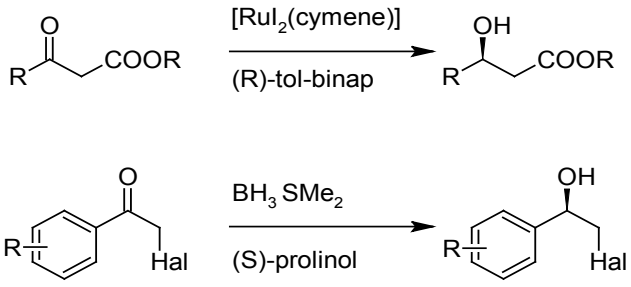


Figure 3.11 Asymmetric hydrogenation or CBS Borane reductions for alcohol production

3.2.2 Kinetic resolution

Enantioselective hydrolysis of racemic epoxides using cobalt complexes to catalyse the reaction can result in highly selective ring opening reactions. Water is generally used as the nucleophile but alternatively phenols can be used to provide the high selectivity required. The catalysis requires a complex metal catalyst which can be considered expensive if not recycled. While there have been chemo-catalytic methods developed for the enantioselective acylation of alcohols, industrial examples of these reactions are rare (Birman and Jiang, 2005). Figure 3.12 illustrates the scheme.

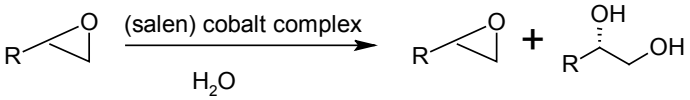


Figure 3.12 Enantioselective hydrolysis of racemic epoxides

Alternatively lipase mediated resolution can be carried out in the presence of an acylating agent. This concept has been applied by BASF for the synthesis of styrene oxides. Racemic chloroalcohols in the presence of succinic anhydride produce styrene oxide in two steps. The unconverted enantiomer is separated from the acylated enantiomer by means of distillation. Figure 3.13 illustrates the reaction scheme.

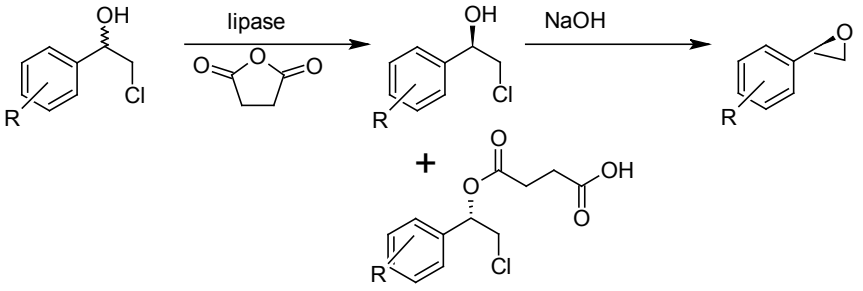
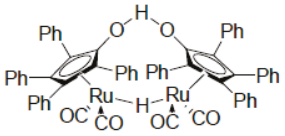


Figure 3.13 The enantioselective synthesis of styrene oxides through enzymatic acylation

A major drawback of resolutions is a maximum theoretical yield of 50 %. Typically the undesired product is either disposed or different strategies have to be taken to deracemise the undesired enantiomer back to racemic starting materials for another round of resolution.

Dynamic kinetic resolution allows conversion greater than the theoretical 50% conversion. However, additional steps have to be taken for such method. The majority of these processes use a combination enzyme/metal-catalysis (Kamal et al., 2008). Typically, a lipase is used to acylate one

enantiomer of the alcohol, while a metal catalyst continuously racemises the remaining enantiomer. Figure 3.14 illustrates the racemization catalysts. The key in making this system successful was the development of efficient racemization catalysts that were capable of working under the mild reaction conditions necessary for the lipase (Ahn et al., 2008). Most racemization catalysts are ruthenium based (Martin-Matute et al., 2005; Turner, 2003), and capable of operating at room temperature to facilitate the dynamic kinetic resolution.



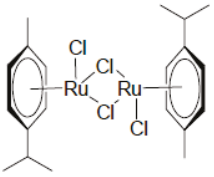


Figure 3.14 Alcohol racemization catalysts

3.2.3 Asymmetric synthesis of chiral alcohols

The use of enzymes for the reduction of ketones contributes with good yields and excellent enantioselectivity (> 99 % ee). Biocatalytic reductions can be run using whole cell systems, but these systems are frequently hampered by by-product formation from endogenous enzymes inside the cell (Pollard et al., 2006). Isolated ketoreductase on the other hand are simpler to handle and any undesired side reaction can be avoided. Moreover, with the use of genetic and protein engineering, a large range of ketoreductases are available with a broad spectrum of substrates with corresponding high enantioselectivity and activity (Kaluzna et al., 2005; Leuchs and Greiner, 2011; Zhu et al., 2005). Figure 3.15 illustrates this reaction.

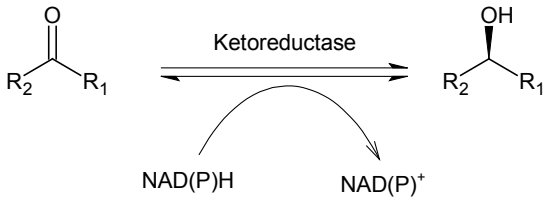


Figure 3.15 Ketone reduction using ketoreductase enzymes

A limitation of using isolated ketoreductase is the requirement of expensive cofactors. Therefore, co-factor recycling options have to be considered. Several methods are available including enzymatic coupling, chemical addition and substrate coupling. A recent publication from Huisman et al., 2010 summarized the different options with the advantages and disadvantages of each option (Huisman et al., 2010). The scalability of the strategy is also mentioned. Table 3.1 displays a summary of the different cofactor recycling options that are applicable for NADP dependent ketoreductases. The major challenges for asymmetric synthesis of chiral alcohols using isolated enzymes are:

i) Expensive co-factor NADP is required

ii) Hydrophobic substrates reduces the catalytic activity of the enzyme

Many of the challenges encountered with ketoreductase are common to other biocatalytic processes that require co-factors. Therefore the objective of selecting these model reactions would be to evaluate the possibility of determining the optimal reaction concentrations and at the same time to evaluate process feasibility for scale up considerations.

Table 3.1 Considerations for cofactor recycling (Huisman et al., 2010)

Technology	**Reductant**	**Product**	**Advantages**	**Disadvantages**
Whole cells	Glucose	Gluconic acid	Irreversible reaction	Potential for by-products from endogenous enzymes
Glucose dehydrogenase	Glucose	Gluconic acid	Irreversible reaction Highly active	Causes pH of reaction to drop, requiring pH control system
Ketoreductase	Isopropanol	Acetone	Same enzyme can be used for desired conversion and co-factor recycling	May require acetone removal to shift equilibrium towards desired product
Phosphite dehydrogenase	Sodium phosphite	Sodium phosphate	Irreversible reaction	High ionic strength of reaction medium Phosphate waste stream Phosphate destabilizes cofactor at low pH
Hydrogenase	H_2		Irreversible reaction	Hazardous
Electro chemical	Rhodium complex		Clean and sustainable	Low productivity
Photochemical	Photosynthetic microorganism		Sustainable	Low productivity

Chapter 4

Kinetic modelling of enzymatic reactions

4.1 Introduction

Detailed knowledge of the reaction kinetics is essential for effective implementation and operation of enzymatic processes. Furthermore, the kinetic parameters provide an insight into the biocatalytic reaction mechanisms (such as inhibition) (Straathof et al., 1992). Often, the ratio of $E_0K_{cat}^f/K_M^A$ is used to describe the biocatalyst performance (Koshland, 2002). 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 (Fox and Clay, 2009). The kinetic model 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) (Sin et al., 2009), process control and operational optimization (Findrik et al., 2005). Furthermore, the reaction equilibrium can be derived from the kinetic parameters, which is essential in selecting suitable process operating strategies for biocatalytic processes (Tufvesson et al., 2011).

Biocatalytic reactions obey mixed order (between 0 and 1) kinetics similar to the Langmuir-Hinshelwood model denoted as the Michaelis-Menten kinetics (Michaelis and Menten, 1913). The principals of the Michaelis-Menten kinetics have been further expanded to describe multi-substrate

reactions with complex reaction behaviour. For example, a generic equilibrium controlled bi-substrate reaction can be formulated as follows:

$$A + B \rightleftharpoons P + Q \qquad [4.1]$$

The kinetic model (full) for reaction 4.1 which follows a ping pong bi-bi mechanism in which substrate (A) is bound first, while the co-product (P) is released before the second substrate (B) and the final product (Q) leaves the enzyme last (Walsh, 1998), can be formulated as (Leskovac, 2003):

$$r_Q = -r_A = \frac{E_0 K_{cat}^f K_{cat}^r \left(\gamma[A][B] - \lambda \frac{[P][Q]}{K_{EQ}}\right)\left(1 + \frac{[I]}{K_{Si}^I}\right)}{K_{cat}^r K_M^B \gamma[A] + K_{cat}^r K_M^A \gamma[B] + \frac{K_{cat}^f K_M^Q}{K_{EQ}} \lambda[P] + \frac{K_{cat}^f K_M^P}{K_{EQ}} \lambda[Q] + K_{cat}^r \gamma[A][B] + \frac{K_{cat}^f K_M^Q}{K_i^A K_{EQ}} \gamma\lambda[A][P] + \frac{K_{cat}^f}{K_{EQ}} \lambda[P][Q] + \frac{K_{cat}^r K_M^A}{K_i^Q} \gamma\lambda[B][Q]} \qquad [4.2]$$

The dynamic equation consists of 9 parameters including terms such as the catalytic turnover of the reaction $\left(K_{cat}^f, K_{cat}^r\right)$, Michaelis parameters $\left(K_M^A, K_M^B, K_M^P, K_M^Q\right)$, inhibition parameters $\left(K_i^A, K_i^Q\right)$ that are derived from the core mechanism, un-competitive substrate inhibition parameter $\left(K_{Si}^I\right)$ due to formation of non-productive 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 Equation 4.3:

$$K_{EQ} = \left(\frac{K_{cat}^f}{K_{cat}^r}\right)^2 \cdot \frac{K_M^P K_M^Q}{K_M^A K_M^B} = \left(\frac{K_{cat}^f}{K_{cat}^r}\right) \cdot \frac{K_M^Q K_i^P}{K_M^B K_i^A} = \left(\frac{K_{cat}^f}{K_{cat}^r}\right) \cdot \frac{K_M^P K_i^Q}{K_M^A K_i^B} = \frac{K_i^P K_i^Q}{K_i^A K_i^B} \qquad [4.3]$$

While 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 the 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 $\left(1+\frac{[I]}{K_{Si}^{I}}\right)$ factored into Equation 4.2 either in the velocity term or the Michaelis constant term or both, depending upon whether the reaction exhibits uncompetitive, competitive or non-competitive inhibition, respectively (for details please refer to Appendix 1). For example, a reaction exhibiting uncompetitive inhibition with compound I, would result in the inhibition relationship factored into the numerator as shown in Equation 4.2. When attempting to estimate the parameters in Equation 4.2 it is often realized that the parameters may be strongly correlated, thereby compromising the uniqueness of the parameter values (Santacoloma et al., 2010). 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, Equation 4.2 can be decomposed into Equations 4.4a and 4.4b

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:

$$r_Q = -r_A = \frac{E_0 K_{cat}^{f}[A][B]}{K_M^A[B] + K_M^B[A] + [A][B]}\left(1 + \frac{[I]}{K_{Si}^I}\right) \quad [4.4a]$$

Reverse direction:

$$r_A = -r_Q = \frac{E_0 K_{cat}^{r}[P][Q]}{K_M^P[Q] + K_M^Q[P] + [P][Q]}\left(1 + \frac{[I]}{K_{Si}^I}\right) \quad [4.4b]$$

Where Equation 4.4a represents the consumption of substrate A for the forward direction, while Equation 4.4b 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 non-linear regression (NLR) method. The former requires a linearization of the kinetic expression at different initial rate conditions in order 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 (Ranaldi et al., 1999). Although simple to use, these methods introduce inaccuracy in the parameter estimation as the errors increase significantly at low concentrations of the substrate (Nielsen et al., 2003). 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 non-linear regression 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 optimisation routine such as the least squares method. This is clearly an improvement on the GP method since no model linearization is required, although mathematical software with curve fitting or an optimisation 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 (Zavrel et al., 2010).

An alternative approach developed by Chen and co-workers (Chen et al., 2008) 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 co-product was completely removed *in-situ*. On the other hand, many industrially relevant biocatalytic reactions (e.g. reactions catalysed by transaminases, transketolases and transaldolases) are equilibrium controlled reactions involving two substrates being converted into its corresponding products for which the optimization of the parameters have to be treated differently.

In this chapter 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 and co-workers (Chen et al., 2008) 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 chapter, the methodology is exemplified stepwise using the ω-transaminase catalysed reaction between acetophenone and 2-propylamine for the asymmetric synthesis of (S)-1-phenylethylamine and a multi-enzymatic process using alcohol dehydrogenase for the synthesis of 2-octanol.

4.2 Proposed Methodology for kinetic model parameter estimation

In order to deal with the problems discussed earlier, a robust systematic methodology for kinetic parameter estimation has been developed. This is built upon the previously documented methodology of Chen and co-workers (Chen et al., 2008). 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 6 sub-sets to match the different steps of the estimation. Figure 4.1 illustrates the proposed methodology.

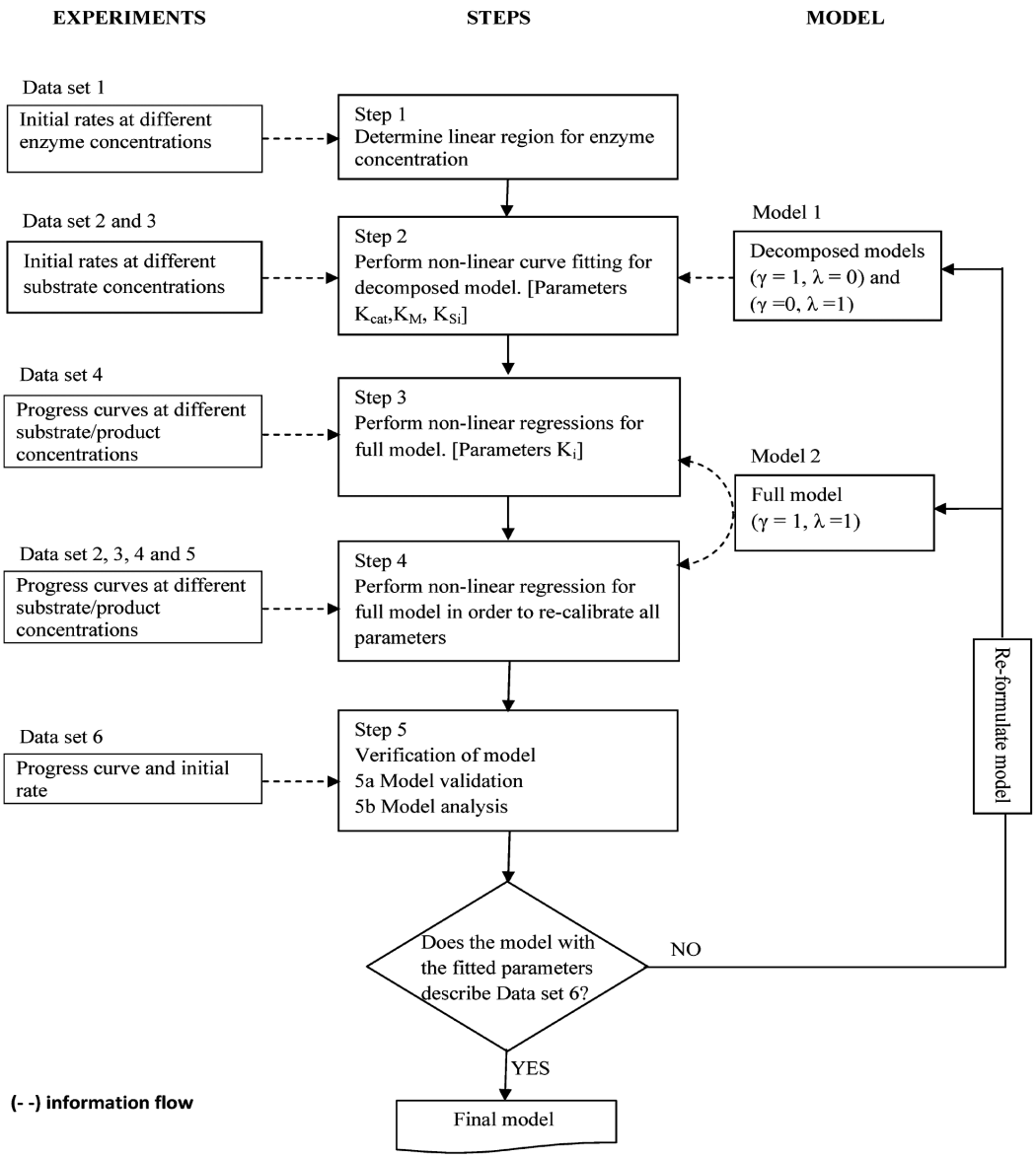


Figure 4.1 Proposed methodology for kinetic model parameter estimation

4.3 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 non-linear 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 non-linear effect of the enzyme concentration is not considered in this study.

In step 2 the kinetic model (full model) (Equation 4.2) is decomposed into two initial rate models for the forward and reverse reactions (Equation 4.4a and 4.4b) 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 $\left(K_{cat}^{f}, K_{cat}^{r}, K_{M}^{A}, K_{M}^{B}, K_{M}^{P}, K_{M}^{Q}, K_{Si}\right)$ in the decomposed models (Equation 4.4a and 4.4b). The objective function (Equation 4.5a and 4.5b) 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).

$$J_1(\theta) = \tfrac{1}{2}\sum_{j=1}^{N}\left(r_{A_{\exp},j} - r_{A,j}(\theta)\right)^2 \qquad [4.5a]$$

$$J_2(\theta) = \tfrac{1}{2}\sum_{j=1}^{N}\left(r_{Q_{\exp},j} - r_{Q,j}(\theta)\right)^2 \qquad [4.5b]$$

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 in order 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 $\left(K_i^A, K_i^Q\right)$ from the full kinetic model (Equation 4.2) 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 4.2 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 in order 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 (Equation 4.6) 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_n. Furthermore, the optimization is subjected to the constraint that parameter values are greater than 0 ($\theta > 0$, θ now represents the additional parameters).

$$J(\theta) = \tfrac{1}{2}\sum_{j=1}^{N}\left([y_n]_{j,\text{exp}} - [y_n]_{j,\text{model}}(\theta)\right)^2 \qquad [4.6]$$

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 (Equation 4.2). 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 (Tewari et al., 2000; Tufvesson et al., 2012) or by using group contribution methods (Jankowski et al., 2008). 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 Equation 4.3. Two parameters $\left(K_i^B, K_i^P\right)$ which are not included in the full model (Equation 4.2) are calculated using the relationship shown in Equation 4.3, the estimated parameters and the value of the equilibrium constant, K_{EQ}.

Once the final model with the corresponding parameters is obtained, it is verified with new data and analysed 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 Equation 4.7(Omlin and Reichert, 1999):

$$Cov(\theta) = \frac{\min J(\theta)}{N-P}\left[\left(\frac{d[y]}{d\theta}\right)^T \frac{1}{\sigma^2}\left(\frac{d[y]}{d\theta}\right)\right]^{-1} \qquad [4.7]$$

where min *J(θ)* corresponds to the minimum value obtained from the objective function (Equation 4.6), *N* is the number of data points and *P* is the number of estimated parameters, and $\left(\frac{d[y]}{d\theta}\right)$

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 Equation 4.8.

$$\theta_{1-\alpha} = \theta \pm \sqrt{diag(Cov(\theta))} \cdot t\left(N - p, \frac{\alpha}{2}\right) \quad [4.8]$$

In Equation 4.8, the diag(Cov(θ)) takes into account only the diagonal values of the covariance matrix of parameters (see Equation 4.7) for this calculation.

The linear relationship between the parameters is analysed with the correlation matrix COR(θ_i, θ_j) as shown in Equation 4.9 (Sin et al., 2008).

$$Cor\left(\theta_i, \theta_j\right) = \frac{Cov\left(\theta_i, \theta_j\right)}{\sqrt{Cov\left(\theta_i, \theta_i\right) Cov\left(\theta_j, \theta_j\right)}} \quad [4.9]$$

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 multi-substrate enzymatic reactions are generally correlated with each other. At the end of step 5, the model is ready for use in different applications.

4.4 Data collection

The experiments required to collect the six data sets can be summarized in Table 4.1 as follows:

Table 4.1 Collected data sets for the proposed methodology

	Initial rate[a]	**Initial rate[b]**	**Progress curve[c]**
Variables	Enzyme concentration	Substrate concentration	Substrate or product concentration over time
Data set 1	✓		
Data sets 2 and 3		✓	
Data sets 4 and 5			✓
Data set 6*		✓	✓

[a]Initial rate of reaction is measured for varying enzyme concentration at a fixed substrate concentration

[b]Initial rate of reaction is measured for varying substrate concentration for forward and reverse direction at a fixed enzyme concentration

[c]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

4.5 Case study: asymmetric synthesis of 1-phenylethylamine

In order to illustrate the application of the systematic methodology for kinetic model parameter estimation, the synthesis of 1-phenylethylamine (PEA) and co-product acetone (ACE) from acetophenone (APH) and 2-propylamine (IPA) in the presence of the ω-transaminase (EC 2.6.1.X) (TAm, ATA – 040, C-Lecta) has been studied. In the synthesis of optically pure chiral amines using TAm, the reaction is catalysed 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 co-product ketone (Leskovac, 2003) (see Figure 4.2). The enzyme requires pyridoxal phosphate (PLP) as a cofactor to act as a shuttle to transfer the amine moiety between the molecules (Hwang et al., 2005).

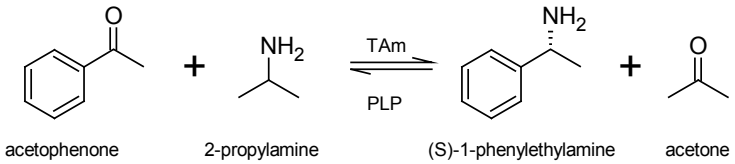


Figure 4.2 Transamination catalysed by ω-transaminase illustrating the synthesis of 1-phenylethylamine (PEA) and co-product acetone (ACE) from the substrates acetophenone (APH) and 2-propylamine (IPA) in the presence of the enzyme TAm

4.5.1 Reaction kinetic modelling

The ω-transaminase-catalyzed reaction is known to follow the so-called "ping-pong bi-bi" mechanism (Höhne and Bornscheuer, 2009; Segel, 1975). For details about the mechanism please refer to Appendix 1. The reaction is heavily influenced by (competitive) inhibition of the substrate acetophenone (APH) (Shin and Kim, 2002) and product 1-phenylethylamine (PEA) (Shin and Kim, 1999a; Shin et al., 2001), as well as having unfavourable reaction equilibrium in the synthetic direction (Shin and Kim, 1998; Tufvesson et al., 2012). Consequently, there is a strong motivation to go forward with the formulation and analysis of a kinetic model in order to predict reaction behaviour under different process conditions.

The kinetic model to describe the reaction mechanism includes the formation of four non-productive complexes, which are characterised by a substrate surplus inhibition constant $\left(K_{Si}^{APH}, K_{Si}^{IPA}\right)$ in the forward direction and $\left(K_{Si}^{PEA}, K_{Si}^{ACE}\right)$ in the reverse direction. The derived rate equation is as follows (Equation 4.10) ($\lambda = \gamma = 1$):

$$r_{[PEA]}=-r_{[APH]}=\frac{\left[E_0\right]K_{cat}^{f}K_{cat}^{r}\left([IPA][APH]-\frac{[ACE][PEA]}{K_{EQ}}\right)}{K_{cat}^{r}K_{M}^{APH}[IPA]\left(1+\frac{[PEA]}{K_{Si}^{PEA}}+\frac{[IPA]}{K_{Si}^{IPA}}\right)+K_{cat}^{r}K_{M}^{IPA}[APH]\left(1+\frac{[APH]}{K_{Si}^{APH}}+\frac{[ACE]}{K_{Si}^{ACE}}\right)+K_{cat}^{f}\frac{K_{M}^{PEA}[ACE]}{K_{EQ}}\left(1+\frac{[APH]}{K_{Si}^{APH}}+\frac{[ACE]}{K_{Si}^{ACE}}\right)+K_{cat}^{f}\frac{K_{M}^{ACE}[PEA]}{K_{EQ}}\left(1+\frac{[PEA]}{K_{Si}^{PEA}}+\frac{[IPA]}{K_{Si}^{IPA}}\right)+K_{cat}^{r}[IPA][APH]+K_{cat}^{f}\frac{K_{M}^{PEA}[IPA][ACE]}{K_{EQ}K_{i}^{IPA}}+K_{cat}^{f}\frac{[ACE][PEA]}{K_{EQ}}+K_{cat}^{r}\frac{K_{M}^{IPA}[APH][PEA]}{K_{i}^{PEA}}}$$

[4.10]

The constitutive equation of the chemical equilibrium is formulated using the Haldane relationship in Equation 4.11:

$$K_{EQ}=\left(\frac{K_{cat}^{f}}{K_{cat}^{r}}\right)^{2}\frac{K_{M}^{PEA}K_{M}^{ACE}}{K_{M}^{APH}K_{M}^{IPA}}=\left(\frac{K_{cat}^{f}}{K_{cat}^{r}}\right)\frac{K_{M}^{ACE}K_{i}^{PEA}}{K_{M}^{IPA}K_{i}^{APH}}=\left(\frac{K_{cat}^{f}}{K_{cat}^{r}}\right)\frac{K_{M}^{PEA}K_{i}^{ACE}}{K_{M}^{APH}K_{i}^{IPA}}=\frac{K_{i}^{PEA}K_{i}^{ACE}}{K_{i}^{APH}K_{i}^{IPA}} \quad [4.11]$$

Equation 4.10 is further decomposed into Equation 4.12a and 4.12b by considering the initial period of the experiment. In the present case study, the time interval for initial rate was considered to be the first 5 minutes of reaction time. During this period, it was assumed that the concentration of product was extremely small and thus the terms related to product and equilibrium in Equation 4.10 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 Equation 4.12a and 4.12b.

Forward direction:

$$r_{[PEA]}=-r_{[APH]}=\frac{\left[E_0\right]K_{cat}^{f}\left([APH][IPA]\right)}{K_{M}^{APH}[IPA]\left(1+\frac{[IPA]}{K_{Si}^{IPA}}\right)+K_{IPA}[APH]\left(1+\frac{[APH]}{K_{Si}^{APH}}\right)+[IPA][APH]} \quad [4.12a]$$

Reverse direction:

$$r_{[APH]} = -r_{[PEA]} = \frac{[E_0] K_{cat}^{r} ([PEA][ACE])}{K_M^{PEA}[ACE]\left(1+\frac{[ACE]}{K_{Si}^{ACE}}\right)+K_M^{ACE}[PEA]\left(1+\frac{[PEA]}{K_{Si}^{PEA}}\right)+[PEA][ACE]} \quad [4.12b]$$

Unlike the methodology of Chen and co-workers (Chen et al., 2008), 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.

4.5.2 Experimentation

Analytical methods

Samples were measured on an Ultimate 3000 HPLC (Dionex, Sunnyvale, CA, USA), equipped with a UV detector and a photodiode array detector. 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 trifluoruacetic acid and acetonitrile, with the following gradient 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 minutes respectively.

Experimental methods

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 approximately 400 rpm. The pH was maintained at 7 by addition of 100 mM of potassium phosphate buffer (pH 7). The concentration of co-factor 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 in order to stop the reaction. The samples were centrifuged for 5 minutes at 14100 rpm (MiniSpin plus, Eppendorf AG, Germany) and analysed by

HPLC with a final dilution of 50 fold. The experiments required to collect the six data sets are summarized in Table 4.2 as follows:

Table 4.2 Data collection for the application of the proposed methodology

Data set	**[Enzyme] (g*/L)**	**[IPA] (mM)**	**[APH] (mM)**	**[MBA] (mM)**	**[ACE] (mM)**	**Sampling time (minutes)**
1	0 – 10	1000	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	1000	1.8	0.8	-	Intervals of 30 minutes until equilibrium
5	1.8	1000	1.7	0.5	-	Intervals of 30 minutes until equilibrium
6a	3.6	-	-	5	1000	Intervals of 30 minutes until equilibrium
6b	1.8	-	-	1 – 30	1000	1, 3, 5
Parity plot	1.8	1000	2	-	-	Intervals of 30 minutes until equilibrium

*g represents lyophilized powder

4.5.3 Application of the proposed methodology

Step 1: Determine linear region for increasing enzyme concentration

Plotting the initial rate of reaction versus the concentration of the enzyme from data set 1 (see Figure 4.3), 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.

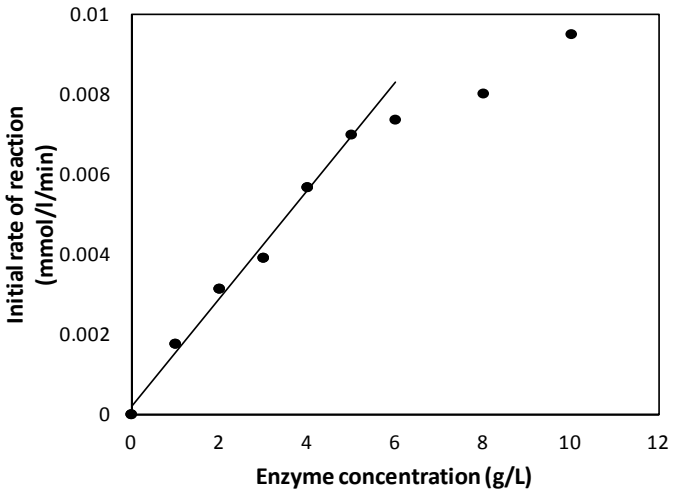


Figure 4.3 Enzyme linear region
Reaction condition: 1000 m M IPA, 5 mM APH, 2 mM PLP, 100 mM phosphate buffer

Step 2: Perform non-linear curve fitting for decomposed model

The kinetic model (Equation 4.10) was decomposed into two initial rate models as shown in Equation 4.12a and 4.12b. The objective function was formulated as shown in Equation 4.5a and 4.5b. The objective function was evaluated for different initial parameter values within the range of 0 – 1000. The new estimated parameters are selected as established in the methodology and listed in

Table 4.3 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 4.4 displays the performance of the model predictions with the estimated parameters compared to the experimental data (data sets 2 and 3). From Table 4.3 it could be seen that the parameters $\left(K_{Si}^{IPA}, K_{Si}^{ACE}\right)$, which lie in the denominator of the fraction in Equation 4.10, 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 (Equation 4.10). It can be seen visually that a very good fit of the experimental data has been achieved.

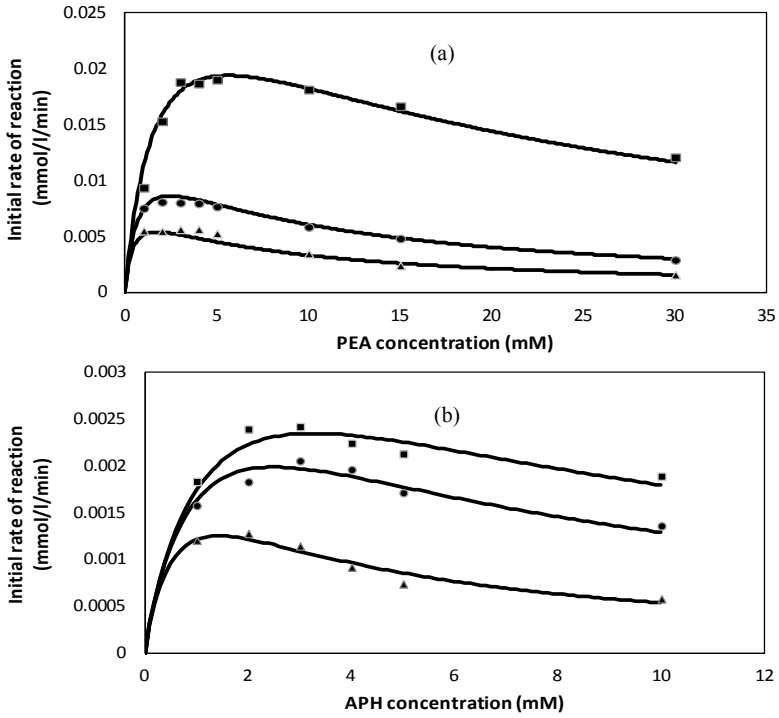


Figure 4.4 Experimental data (symbol) and model prediction (solid line) using estimated parameters from step 2.
Reaction conditions: (a) Forward direction – E_0 is 1.8 g/L, concentration of IPA is fixed at 500 mM [square], 300 mM [circle] and 100 mM [triangle], 2 mM PLP, 100 mM phosphate buffer (b) Reverse direction – E_0 is 1.8 g/L, concentration of ACE is fixed at 500 mM [square], 100 mM [circle] and 50 mM [triangle], 2 mM PLP, 100 mM phosphate buffer

Table 4.3 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}^{PEA}	2.41	±0.14
Substrate Surplus Inhibition Constants [mM]		
K_{Si}^{APH}	1.23	±0.16
K_{Si}^{PEA}	6.01	±0.21
K_{Si}^{IPA}	7.2E4	±7E5
K_{Si}^{ACE}	1.1E4	±1E4

Step 3: Perform non-linear regression for full model

The previously determined parameters $\left(K_{cat}^{f},\ K_{cat}^{r},\ K_{M}^{APH},K_{M}^{IPA},\ K_{M}^{PEA},K_{M}^{ACE},\ K_{Si}^{APH},K_{Si}^{PEA}\right)$ were fixed and the additional parameters $\left(K_{i}^{IPA},K_{i}^{PEA}\right)$ were regressed with data set 4 using the full kinetic model (Equation 4.10). The objective function was formulated as shown in Equation 4.6. The full dynamic process model represented by the set of ordinary differential equations was solved in Matlab® (The Mathworks, Natick, MA) using the ODE45 solver which was based on the Runge-Kutta algorithm for solving ordinary differential equations. Additionally, integration accuracy was set to 1.0E-06 in order to maintain accuracy in the application of the solver.

Table 4.4 gives the parameter values obtained by minimization of Equation 4.6. 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 (Shin and Kim, 1998), 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 analysed, 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 (Tufvesson et al., 2012).

Table 4.4 Estimated parameters values from steps 3 and 4

Parameters	Estimated Values	95% CI	Estimated Values	95% CI
	Step 3		Step 4	
Rate Constants [min^{-1}]				
K_{cat}^{f}	0.0025	±7E-5	0.0078	±7E-5
K_{cat}^{r}	0.023	±4E-4	0.013	±0.007
Michaelis Constants [mM]				
K_{M}^{APH}	1.50	±0.10	1.85	±4.78
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	±0.14	0.12	±0.01
Substrate Surplus Inhibition Constants [mM]				
K_{Si}^{APH}	1.23	±0.16	4.15	±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}	4281	±0.03	4281	±0.63
K_{i}^{PEA}	1.55	±0.01	0.11	±0.01
K_{i}^{ACE}	-	-	1E5	-
Equilibrium Constant				
K_{EQ}	-		0.033	

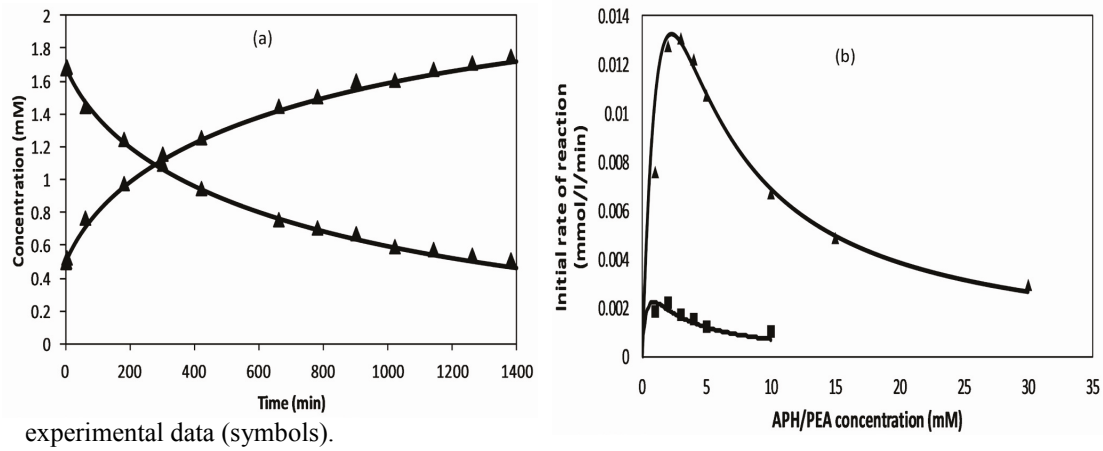
Step 4: Perform non-linear regression for full model for re-calibrating all parameters

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 (Tufvesson et al., 2012). The remaining parameters $\left(K_i^{APH}, K_i^{ACE}\right)$ which were not included in the model (Equation 4.10) were then additionally calculated using Equation 4.11.

The final estimates of the parameters are listed in Table 4.4 and Figure 4.5 illustrates the model predictions using the estimated parameters. From Figure 4.5 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 4.4, it can be seen that the parameter value K_{cat}^{f} which is the catalytic turnover of the reaction in the forward direction is much smaller than the parameter value K_{cat}^{r} 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 K_{cat}^{f} and K_{cat}^{r} values. This is also consistent with a study reported by Shin and Kim (Shin and Kim, 1998). The Michaelis constant of the co-substrates $\left(K_M^{IPA}, K_M^{ACE}\right)$ is higher than the corresponding reactants, which reflects the need to add an excess of the co-substrate in the reaction media in order to drive the reaction forward, which is consistent with other studies reported on transaminases (Kuramitsu et al., 1990). 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 $\left(K_i^{IPA}, K_i^{ACE}\right)$ are extremely high which was expected since 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 (Shin and Kim, 1997; Truppo et al., 2010). 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.

Figure 4.5 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 [square] and C_{APH} is varied from 1 – 10 mM, 2 mM PLP and buffer 100 mM. C_{ACE} is fixed at 100 mM (triangle) and C_{PEA} is varied from 1 – 30 mM, 2 mM PLP and buffer 100 mM

Step 5a: Model validation

In order 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 4.6.

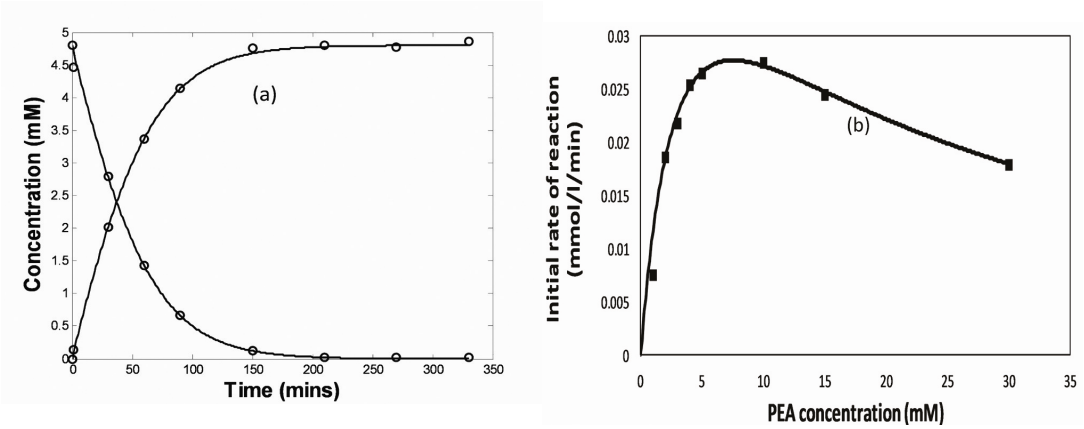


Figure 4.6 Model prediction using the estimated parameters (solid line) and experimental data (symbols).
Reaction conditions: (a) E_0 = 3.6 g/L, C_{APH} = 0 mM, C_{PEA} = 5 mM , C_{ACE}=1000 mM, 2 mM PLP, 100 mM phosphate buffer. (b) E_0 = 1.8 g/L, concentration of ACE is fixed at 1000 mM, 2 mM PLP, 100 mM phosphate buffer

Figure 4.6 shows the comparison of model predictions and experimental data for a different set of reaction conditions. As it can be seen, the agreement between the simulated data (solid line) and experimental data (symbols) is very good.

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 4.7. As expected (for these type of systems), parameters are significantly correlated. It can

be especially seen in the parameters K_M^{IPA} and K_M^{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 the reason why the proposed methodology reduces the possibility of accumulating errors in the parameter estimation and at the same time provides accurate estimates.

	K_{cat}^{f}	K_M^{IPA}	K_M^{APH}	K_{Si}^{APH}	K_{cat}^{r}	K_M^{PEA}	K_M^{ACE}	K_{Si}^{PEA}	K_i^{IPA}	K_i^{PEA}	
K_{cat}^{f}	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_{cat}^{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_{PPEA}	-0.29	0.62	-0.99	0.05	0.43	0.01	0.87	0.12	0.62	1	

Figure 4.7 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

4.5.4 Simulations of asymmetric synthesis of 1-phenylethylamine

The objective of performing simulations is to understand the effect of the concentrations of each of the components towards the enzyme productivity. From the results of the parameter estimation it was found out that the inhibition by the product 1-phenylethylamine and substrate acetophenone was contributing to the reduced activity of the catalyst. Moreover, it was estimated that the reaction is extremely unfavourable for asymmetric synthesis.

Inhibition by acetophenone and 1-phenylethylamine

The kinetic model was therefore used to identify an operability space to illustrate the influence of the substrate and product as shown in Figure 4.8. Figure 4.8A shows the effect of product and substrate concentration on the catalyst activity. The reaction is inhibited at rather low substrate concentrations (approximately 5 mM) as reflected on the kinetic parameters, namely the substrate inhibition constant of APH. The figure motivates *in-situ* substrate supply as at high concentration of acetophenone the catalyst is strongly inhibited, while at low substrate concentration the reaction rate is affected due to low substrate feeding. Therefore it is necessary to accurately compensate substrate feeding flow with the desired reaction rate (i.e. the rate of substrate consumption).

On Figure 4.8B the effect of co-product (acetone) and product (PEA) concentration in solution on the catalyst activity can be observed. As observed before, catalyst activity is highly decreased even at rather low PEA concentration due to product inhibition. Further, the catalyst activity is also affected by the concentration in solution of acetone and therefore acetone removal should be put in place, not only to enable equilibrium shifting, but also to increase catalyst activity, as at acetone concentrations higher than 30 mM the catalyst activity drops significantly.

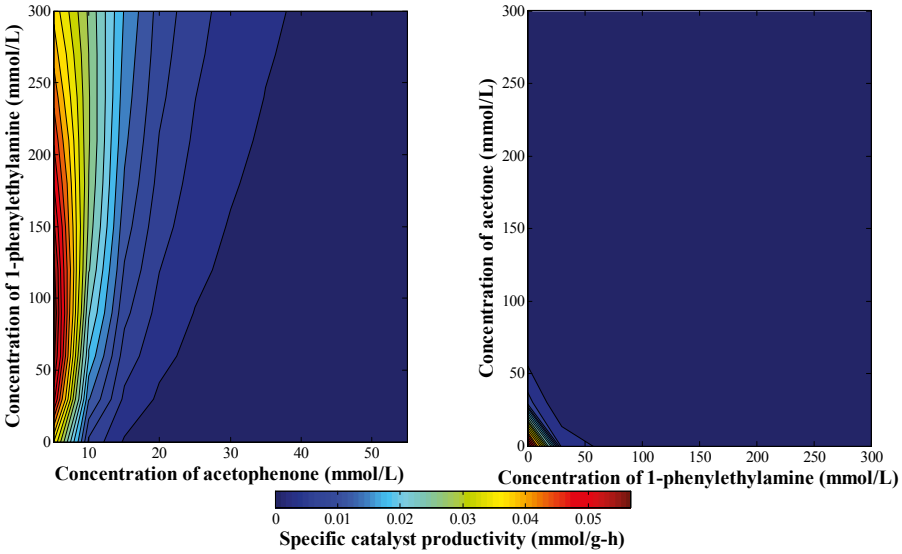


Figure 4.8 Catalyst activity synthesis of PEA using IPA as amine donor. A) Modelled catalyst activity at different product and substrate concentration. B) Modelled catalyst activity at different product and co-product concentration (acetophenone concentration is kept constant at 5 mM applying a substrate feeding strategy)

In-situ product removal of products

The product inhibition constant of 1-phenylethylamine, PEA, is very low suggesting that the reaction is strongly inhibited by PEA. This was also the conclusion reached from Figure 4.8. The removal of PEA would therefore greatly enhance the reaction rate. The addition of resin as an auxiliary phase would help to overcome the inhibition caused by PEA and would thus improve the productivity of PEA. 50% (V/V) resin Lewatit AF5 can keep the product concentration of PEA below 30 mM and at the same time maintaining a substrate concentration of around 5 mM. Though the resin is not highly selective for PEA but it can still help overcome the inhibition problem. A second strategy must be applied to remove the co-product acetone *in-situ*. Physical methods such as vacuum evaporation or enzymatic routes of adding an acetone selective alcohol dehydrogenase can be couple to remove the co-product very effectively. Figure 4.9 illustrates the impact on conversion the introduction of such process strategies.

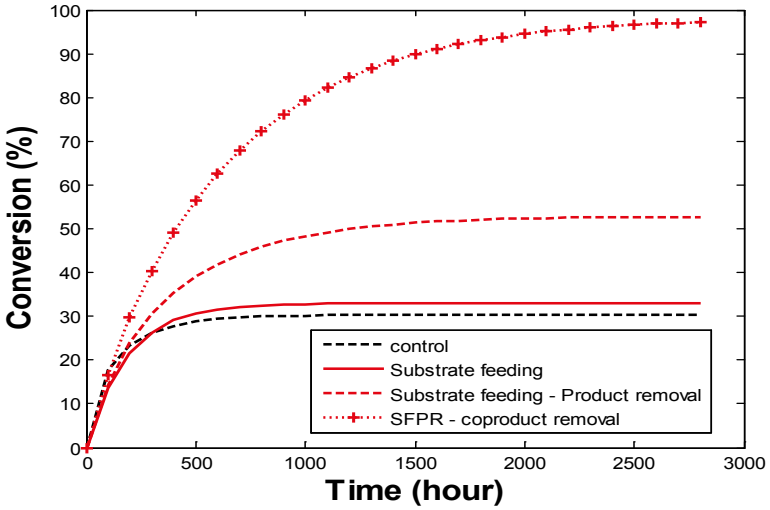


Figure 4.9 Improvement in process with different process strategies shown by means of simulation Initial conditions: E_0 = 3.6 g/L, C_{APH} = 300 mM, C_{IPA}=1000 mM. Substrate feeding: F = 0.4 mL/h, Co = 1.6 M, Vreactor = 1 L; Substrate feeding – Product removal: Resin = 50% (V/V); SFPR – coproduct removal: Acetone removal = 99% of r_{rxn}

4.6 Case study: enantioselective synthesis of 2-octanol

Enantiomerically pure 2-octanol (OL) is synthesised from 2-octanone (ON) using the enzyme alcohol dehydrogenase from the organism *Lactobacillus brevis* (LbADH, EC 1.1.1.2). The enzyme catalyses the reduction of the ketone to its corresponding alcohol. The enzyme requires stoichiometric amounts of co-factor nicotinamide adenine dinucleotide phosphate (NADPH) (Leuchs and Greiner, 2011). The co-factor is expensive and therefore recycling it has to be considered (Huisman et al., 2010). An enzyme coupled approach was selected based on the high selectivity and catalytic efficiency using the enzyme glucose dehydrogenase (GDH, EC 1.1.1.47). Co-substrate glucose (Glu) is used as a substrate for the GDH enzyme to yield gluconolactone

(GDL) which spontaneously hydrolyses to gluconic acid (Glu). The schematic of the reaction is shown in Figure 4.10.

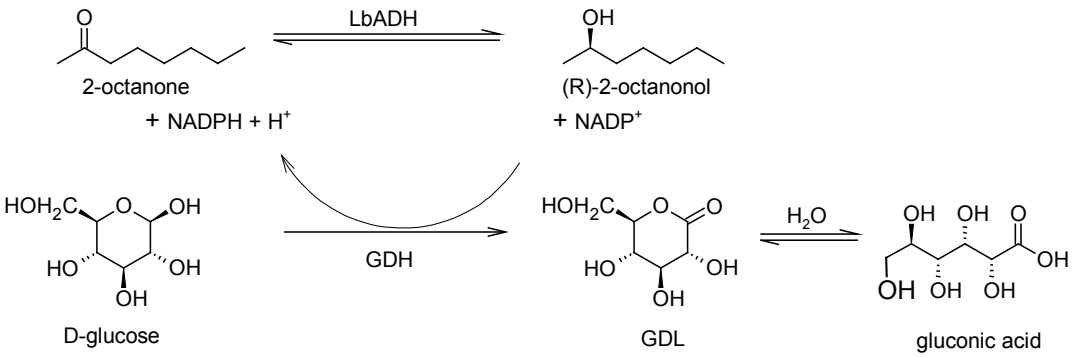


Figure 4.10 Reaction system for the enantioselective reduction of 2-octanone

4.6.1 Reaction kinetic modelling

The kinetic model to describe the reaction was acquired from a recent publication by Leuchs et al., 2013. Surplus inhibition by glucose and gluconolactone was included. The kinetic model is an ordered bi-bi equation as shown below in Equation 4.13 and 4.14. For details about the mechanism please refer to Appendix 1.

Forward direction:

$$r_{LbDH,f} = V_f \frac{[ON]}{K_M^{ON}\left(1+\frac{[OL]}{K_P^{OL}}\right)+[ON]\left(1+\frac{[ON]}{K_i^{ON}}\right)\left(1+\frac{[Glu]}{K_i^{Glu}}\right)\left(1+\frac{[Gdl]}{K_i^{Gdl}}\right)} \cdot \frac{[NADPH]}{K_M^{NADPH}\left(1+\frac{[NADP]}{K_P^{NADP}}\right)+[NADPH]} \quad [4.13]$$

Reverse direction:

$$r_{LbDH,r} = V_r \frac{[OL]}{K_M^{OL}\left(1+\frac{[ON]}{K_P^{ON}}\right)+[OL]\left(1+\frac{[OL]}{K_i^{OL}}\right)\left(1+\frac{[Glu]}{K_i^{Glu}}\right)\left(1+\frac{[Gdl]}{K_i^{Gdl}}\right)} \cdot \frac{[NADP]}{K_M^{NADP}\left(1+\frac{[NADPH]}{K_P^{NADPH}}\right)+[NADP]} \quad [4.14]$$

A similar model was used for the GDH reaction. However, for this reaction the co-product gluconolactone formed spontaneously hydrolyses to gluconic acid and therefore can be considered irreversible. Equation 4.15 presents the kinetic model to describe the GDH reaction. A substrate surplus inhibition of glucose was also considered in the model.

$$r_{GDH} = V \frac{[Glu]}{K_M^{Glu}+[Glu]\left(1+\frac{[Glu]}{K_i^{Glu}}\right)} \cdot \frac{[NADP]}{K_M^{NADP}\left(1+\frac{[NADPH]}{K_P^{NADPH}}\right)+[NADP]} \quad [4.15]$$

4.6.2 Experimentation

Kinetic measurements were carried out in a Biotek Powerwave HT multiplate fluorescence spectrometer in black 96-well-plates (nunc immuno modules). The total reaction volume was 200 µL. The pH was maintained at 7 by the addition of 100 mM of ADA (pH 7) buffer while the temperature was controlled at 30^{o}C. Ionic liquid, TEGO IL K5 of 0 or 100 g/L was used to increase solubility of the substrate. The experimental types and conditions are shown in Table 4.5.

Table 4.5 List of experiments for estimating kinetic model parameters

Data set	Type of experiment	[Enzyme] (mg/L)	[NADPH] (mM)	[2-on] (mM)	[$NADP^+$] (mM)	[2-ol] (mM)	[Glu] (mM)	[Gdl] (mM)
1*	Initial rate	1.25	0 – 0.20	0 – 54	0 – 0.05	0 – 10	0 - 675	0 – 450
2**	Initial rate	10	0 – 0.05	0	0.002 – 0.05	0	0.5 - 1350	0 – 450
3*	Progress curve	0.31	0.05	3.4 - 54	0	0	0	0
4**	Progress curve	1.25	0	0	0.08	0	84 - 675	0
5	Progress curve	50 (ADH) 280 (GDH)	0	60	0.10	0	200	0

*LbADH measurements
**GDH measurements

4.6.3 Application of proposed methodology

The kinetic models to describe the LbADH and GDH reaction were initial rate models and therefore there was no possibility to decompose the models further. The estimation routine therefore commenced from step 4.

Step 4: Perform non-linear regression for full model for re-calibrating all parameters

The calibration of the parameters was performed by using both the initial rate and progress curve data sets (data sets 1 and 3 or 2 and 4) simultaneously. Table 4.6 displays the estimated parameters for LbADH system while Table 4.7 tabulates the results of the estimation for the GDH system. The 95% confidence intervals of the estimates are calculated for the estimation as well.

Table 4.6 Parameter estimates for LbADH model

Parameters	Estimated value	95% CI
Velocity (mM/s)		
V^f	0.0003	2E-6
V^r	0.0002	3E-6
Michaelis Constant (mM)		
K_M^{ON}	0.21	0.02
K_M^{OL}	0.03	0.001
K_M^{NADPH}	0.04	0.001
K_M^{NADP}	0.83	0.05
Core Inhibition constant (mM)		
K_P^{ON}	0.01	0.002
K_P^{OL}	0.21	0.02
K_P^{NADPH}	0.36	0.03
K_P^{NADP}	0.21	0.02
Substrate Surplus Inhibition constant (mM)		
K_i^{ON}	163.05	10
K_i^{OL}	6065	4040
K_i^{Glu}	2985	739
K_i^{Gdl}	3818	1563

From Table 4.6 it can be seen that the parameter value V^f which is the velocity of the reaction in the forward direction is larger than the parameter value V^r which is the velocity of the reaction in the reverse direction, thereby indicating that the forward reaction is more favourable. This is also

consistent with a study reported by Leuchs et al., 2012. The Michaelis constant of the co-substrate, NADPH, K_M^{NADPH} is much smaller than for the co-product, NADP, K_M^{NADP} providing indication that the enzyme is more selective for NADPH than NADP. Further it can be seen from the substrate surplus inhibition constants, K_i^{OL}, K_i^{Glu} and K_i^{Gdl} of the components 2-octanol, glucose and gluconolactone respectively are extremely large. These parameters therefore do not significantly affect the rate of reaction and could be eliminated from the model.

Table 4.7 Parameter estimated for GDH model

Parameters	**Estimated value**	**95% CI**
Velocity (mM/s)		
V	0.0001	9E-7
Michaelis Constant (mM)		
K_M^{Glu}	2.72	0.22
K_M^{NADP}	0.02	0.001
Core Inhibition constant (mM)		
K_P^{NADPH}	0.03	0.02
Surplus Inhibition constant (mM)		
K_i^{Glu}	3783	278

From Table 4.7 it can be seen that the velocity parameter, V, is relatively smaller as compared to V^f for the LbADH reaction thus indicating the necessity of adding the GDH enzyme in excess to match the LbADH rate of reaction. Furthermore, it can be seen that the Michaelis constant, K_M^{NADP}, is much smaller as compared to K_M^{NADPH} for the LbADH reaction. This therefore indicates that the GDH enzyme is quite selective for NADP used and thus would be effective in regenerating the co-

factor. The substrate surplus inhibition of glucose, K_i^{Glu} is extremely large as compared to the operating concentration of glucose in the experimental condition. It could therefore be suggested that this parameter could be eliminated from the model.

Step 5a: Model validation

An independent data set, data set 5, was used for validating the model predictions. The models for LbADH and GDH are combined in sequence and are used in a batch reactor model.

In order to further confirm the validity of the models, the estimated parameters used in the model were checked against new data (data set 5) of progress curve carried out using different initial conditions as shown in Figure 4.11. The experiment was carried out in a batch reactor where both enzymes were introduced simultaneously. The models were also formulated to mimic the experimental conditions. Figure 4.11 illustrates the model predictions.

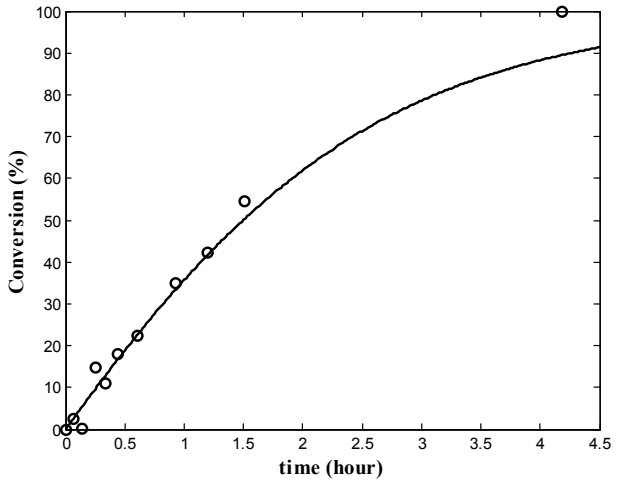


Figure 4.11 Model prediction using the estimated parameters (solid line) and experimental data (symbols). Reaction conditions are shown in Table 4.5, data set 5.

Figure 4.11 shows the comparison of model predictions and experimental data using data set 5 which was not used in parameter estimation. As can be seen, the agreement between the simulated data (solid line) and experimental data (symbols) is very good. However, the model tends to underestimate at higher conversions. Nevertheless, the model can predict the overall tendency of the progress of the reaction.

Step 5b: Model analysis

From the parameter estimation, it was found that there were many parameters for each model. However, some of the parameters resulted in extremely large values with a big confidence interval. In order to minimize the simulation time, it would be desirable to eliminate redundant parameters that do not influence the model predictions. Based on the estimates of Table 4.6, it was envisaged that removing the three substrate surplus inhibition constants of 2-octanol, glucose and gluconolactone would be appropriate. Furthermore from Table 4.7, it was envisaged to eliminate the substrate surplus inhibition constant of glucose. Moreover, the parameter K_P^{NADPH} also displayed a large confidence interval relative to the estimated value. Consequently, this parameter could also be eliminated. The model structures were modified by eliminating the parameters. In order to identify each model modification, the model structures are named as presented in Table 4.8.

Table 4.8 Parameters that are eliminated for each model structure

Model #	Eliminated parameter
Model 1	K_i^{OL} K_i^{Glu} K_i^{Gdl} (from ADH model)
Model 2	K_i^{OL} K_i^{Glu} K_i^{Gdl} (from ADH model) K_i^{Glu} (from GDH model)
Model 3	K_i^{OL} K_i^{Glu} K_i^{Gdl} (from ADH model) K_i^{Glu} K_P^{NADPH} (from GDH model)

The modified models were then implemented in Matlab® in order to analyse the effects of the model simplification both graphically and numerically in comparison with the original model.

Table 4.9 Output model comparison between original model and simplified model

Model	Eliminated parameters	Deviation (%)
Model 1	3	0.001
Model 2	4	0.5
Model 3	5	7.5

The deviation is calculated as the average value of the errors between the modified and the original models, presented as a percentage. Table 4.9 shows that even though maximum five parameters are removed from the original model, the simplified models show differences of less than 10%. It demonstrates that the insignificant parameters in this case can be removed from the model without producing big differences in the model prediction accuracy.

For the graphical comparison, the concentration of the desired product 2-octanol is taken as model output. Figure 4.12 shows the plots obtained from the dynamic simulation of each modified model.

It shows that all simplified models can well follow the dynamics of the original process. Furthermore the deviation between different model predictions is again really small.

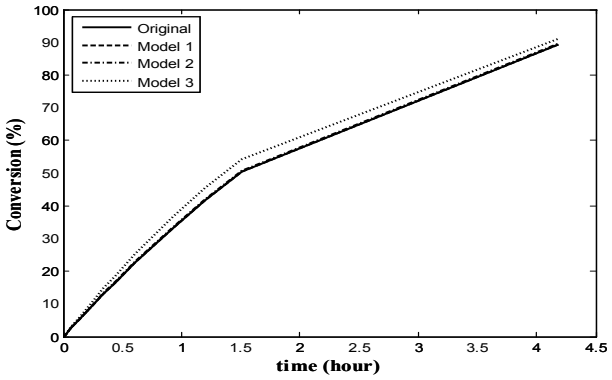


Figure 4.12 Comparison between dynamic simulations of the modified models

4.6.4 Simulations for the production of 2-octanol

Inhibition by 2-octanone and 2-octanol

The kinetic model was used to identify an operability space to illustrate the influence of the substrate and product as shown in Figure 4.13. Figure 4.13 shows the effect of product and substrate concentration on the catalyst activity. The reaction is very inhibited at rather low substrate concentrations as reflected on the kinetic parameters, namely the substrate surplus inhibition constant of 2-octanone. However, the reaction was not inhibited by the product. The figure therefore motivates a substrate feeding strategy. Alternatively a continuous stirred tank reactor would be suggested which would maintain low substrate concentration in the reactor and at the same time achieve high catalyst yield ($g_{product}/g_{enzyme}$).

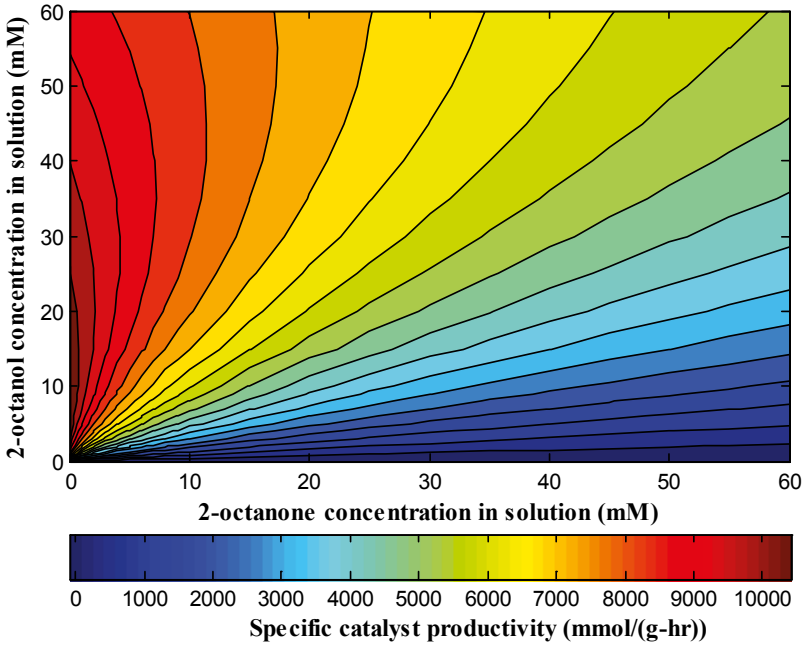


Figure 4.13 Operability space for 2-octanol production

4.7 Discussions about kinetic modelling

In the proposed methodology for kinetic model parameter estimation, model decomposition along with non-linear regression of both initial rate data and progress curves has been incorporated. Although, non-linear regression 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 final model with the fitted parameters is able to describe both types 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 in order to minimize the estimation errors.

The kinetic model was further useful for outlining the operability space of each of the case studies. It was evident from the first case study that the substrate (APH) and product (PEA) reduced the activity of the catalyst. It would therefore suggest that using an auxiliary phase such as porous resins can greatly enhance the productivity of the catalyst. However, this strategy alone cannot overcome the challenges of the reaction. The unfavourable thermodynamic challenge has to be met by by incorporating a strategy to remove the co-product, acetone. Different strategies such as evaporation (Martin et al., 2007; Savile et al., 2010), gas stripping (Zanfir et al., 2008) and enzymatic cascade (Ricca et al., 2011) are possible options which have to be evaluated before its applications in the process.

The kinetic study of synthesis of 2-octanol using a cascade of enzymes LbADH and GDH revealed that the kinetic model contained several parameters which were redundant for accurate model predictions. The structure of the model was simplified based on a step by step model analysis. The original model was over parameterized and a feasible simplification could be performed without losing reliability of the model. It was also found that the simplified models still showed a good performance with a deviation of less than 10% from the original model. The results of simulations using the kinetic model suggested that a continuous stirred tank reactor (CSTR) would be beneficial to overcome substrate inhibition (Leuchs et al., 2013; Vasic-Racki et al., 2003a). However, for a CSTR the conversion is measured based on the leaving concentration and therefore more than one CSTR would be required to achieve a high final conversion.

4.8 Conclusions

A methodology to estimate the kinetic model parameters of complex biocatalytic reactions has been developed. The methodology is exemplified with the case study of an ω-transaminase-catalysed reaction which displays severe substrate and product inhibition and enantioselective production of

2-octanol which is limited by substrate 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 in order to reduce the number of parameters to be estimated simultaneously. 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 non-linear regression 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. The usefulness of building the kinetic models was shown by building operability spaces for the different reactions. The model simulations suggested different process strategies based on the characteristics of the reactions. Moreover, quantitative values were able to be calculated which would be extremely important when selecting process strategies.

Chapter 5

Screening porous resins for integrating in enzymatic processes

5.1 Introduction

Of the different types of auxiliary phases discussed in Chapter 3, porous resins are the selected type used for this thesis. The resins are comprised of porous polymeric matrix varying in particle size, pore size, hydrophobicity and functional groups. In this study, the resins that are considered are neutral (non-ionic) that work primarily on hydrophobicity based on adsorption. Generally separation based on hydrophobicity has low capacity (Woodley, 2012). It is therefore of particular importance to identify a resin that has high capacity for the inhibitory substrate and product to reduce resin loading. Such an example can be seen in the screening of resins for a microbial Baeyer-Villiger oxidation (Hilker et al., 2004). The selected resin had the highest capacity ($g_{substrate}/g_{resin}$) of 0.3 (10 g substrate on 30 g of resin) among the tested resins thus enabling the opportunity to integrate with an internal configuration in a batch reactor (Hilker et al., 2005). Similar studies where the resin loading was the primary selection criteria can be seen in the synthesis of Ethyl (S)-2-Ethoxy-3-(p-methoxyphenyl) propanoatethe (Bechtold et al., 2011) and in the synthesis of (S)-1-(2-chlorophenyl)ethanol (Schmolzer et al., 2012). In other cases, the affinity of the resin for the either the substrate or product was the key parameter for selecting the resin. Such a study was done in the synthesis of D-Phenylglycine (Rojanarata et al., 2004). The resin Amberlite IRA-400 could

maintain the substrate concentration at a level where the reaction rate was not impeded and simultaneously had an affinity for the co-product enabling to shift the reaction towards the synthetic direction. In the study done by Vicenzi et al., 1997 resin Amberlite XAD-7 was selected as it had intermediate resin polarity and at the same time moderate affinity for the substrate and product thus enabling to overcome substrate and product inhibition simultaneously.

Prior studies using porous resins as an auxiliary phase as a means for *in-situ* substrate feeding and product removal were done for single substrate – single product system. For multi substrate/product systems such as transferases or aldolases, selectivity (affinity) and capacity are key parameters which have to be evaluated simultaneously in the screening procedure. The selected resin should preferably display high affinity for the "inhibitory" compounds and simultaneously hav low affinity for the "non-inhibitory" compounds as it would compromise the resin capacity. Similarly the resin should preferably have a higher partitioning for the inhibitory product over the inhibitory substrate in order to drive the reaction in the synthetic direction. When considering reactions which display substrate and product inhibition such as the ω-transaminase catalysed reactions with components having similar chemical properties, a compromise has to be made between capacity and selectivity.

The substrate to resin ratio (load) plays an essential role in determining the equilibrium aqueous concentration of the substrate in the reactor. On the other hand, the rate of *in-situ* product removal increases with increasing amount of resin. Therefore, an optimal amount of resin has to be experimentally evaluated which would facilitate the required mass transfer of the substrate from the resin to the aqueous phase and product from aqueous phase to the resin (Woodley, 2012). Moreover the amount of resin plays a role in determining the type of reactor that can be used (Burton et al.,

2002). Finally the cost of the resin has to be evaluated. For the process to be economic, the resin has to be recyclable and easily regenerated. Table 5.1 tabulates the essential criteria for the resins.

Table 5.1 Criteria for selection of resin

High resin capacity for product (≥ 0.12)
Low selectivity for non-inhibitory substrate and product to bind with the resin ($S \approx 0$)
Moderate selectivity for inhibitory substrate and product to bind with the resin ($S \geq 1$)
Greater partitioning for inhibitory product over inhibitory substrate ($P_P/P_S \geq 1$)
Negligible affinity to enzyme (if soluble) ($S \approx 0$)
Re-usable (Multiple times)
Durable in reactor (non-brittle)
Biocompatible (no effect on enzyme – denaturation)

5.2 Proposed methodology for screening resins

A rational methodology for screening resins based on the criteria mentioned in Table 5.1 is shown below in Figure 5.1. The screening is decomposed into three hierarchical steps where the result of each of the steps becomes the input for the subsequent step. The experimental data is broken down into four sub sets to match the different steps of the screening. Two types of database are used here, the first provides physical properties of the reactants and the second is a database containing information about commercially available resins.

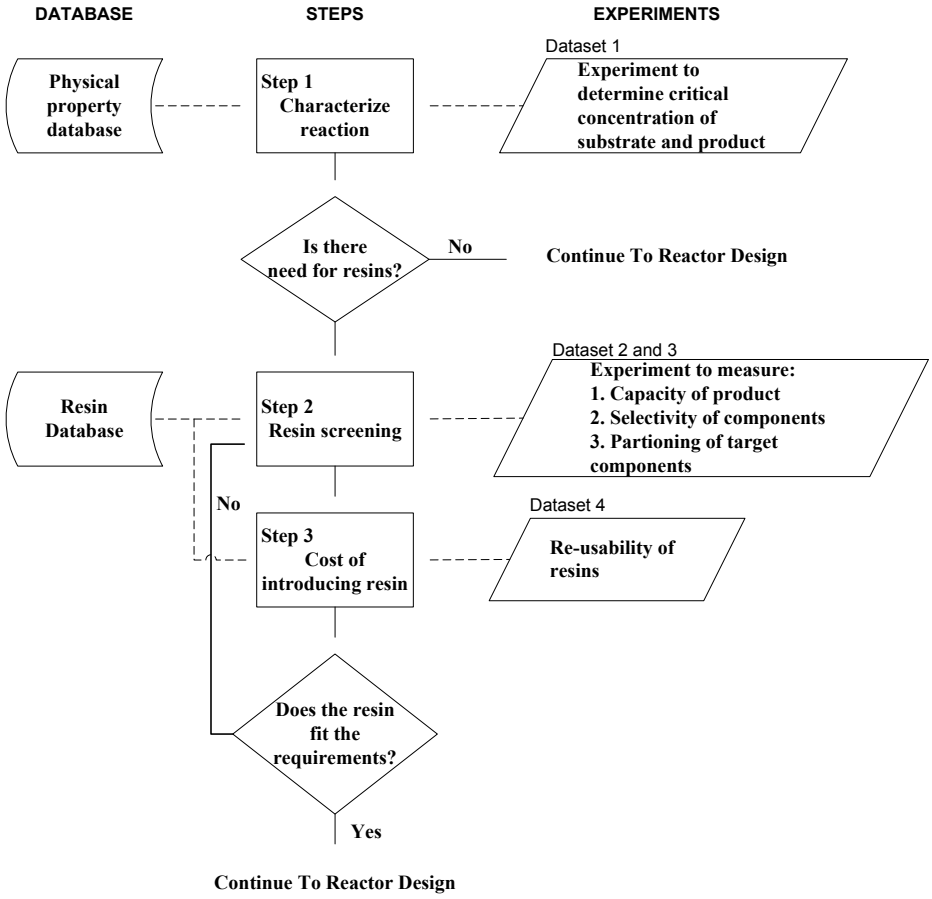


Figure 5.1 Proposed methodology for selecting resin

5.3 Description of proposed methodology

Step 1: Characterize reaction

Step 1 is performed to characterize the reaction using dataset 1 and a physical property database. Dataset 1 is used to determine the critical concentration of the different components. The components which display inhibition will be considered for adsorption on the porous resin. To adequately explore the type of resin required for the process, the important parameters of the different components, Log P and water solubility values, are identified from commercially available physical property databases. These parameters provide a measure of the hydrophobicity of the components (Jaksland et al., 1995).

Step 2: Screen resin

In step 2 different types of resins were selected from a resin catalouge obtained from the different company sites (www.sigmaaldrich.com). The screening of these resins was first done using data set 2 where the capacity of the target compound was measured experimentally. The resins which displayed the highest capacity (mol/kg_{resin}) for target compound were selected for further screening.

The second screening is done based on the selectivity of the target compound over the other reactants. This parameter provides an indication of which compound it has a higher affinity for. The information is obtained from data set 2 where the adsorption capacity for all the reactants is measured. The selectivity is calculated using Equation 5.1.

$$\text{Selectivity}, S = \frac{\%C_i \text{ adsorbed}}{\%\text{product adsorbed}} \qquad [5.1]$$

where C_i are the other components

The resin which displays selectivity lower than 1 indicate the resin has less affinity for the compound with respect to target product. A selectivity of higher than 1 indicates the resin has

higher affinity for the compound rather than target product while 1 indicates the resin displays equal selectivity for both compound and target product. Resins displaying selectivity of lower or equal to 1 is selected for further screening.

The third screening is done using data set 3 where the partitioning of the inhibitory product and inhibitory substrate are experimentally measured by fixing the amount of resin and varying the concentration of those components. For the resin to be the best candidate, it has to display favourable adsorption isotherm to provide evidence of its affinity for binding to the given reactant.

Step 3: Evaluate cost of introducing resin

The added value of introducing the resin as the second phase is evaluated based on the cost of the resin. The resin should be relatively cheap. Otherwise it has to be able to be re-used several times to minimize this cost effect.

5.4 Data collection

The type of data that has to be collected for the proposed approach is summarized as follows:

Data set 1: initial rate of reaction was measured for varying substrate concentration at a fixed enzyme concentration.

Data set 2: the corresponding equilibrium concentration of the different reactants at fixed initial concentrations was measured in the reactor in the presence of fixed amount of resin. The concentration in the resin is calculated based on mass balance. The resins were always used as wet.

Data set 3: the adsorption isotherms are measured with varying concentration of the target components at a fixed amount of resin.

Data set 4: the repeated performance of the resin was measured by evaluating the capacity of the resin for the target product. The resin was packed in a column with product flowing top-down at a flow rate of 2 bed volumes per hour. After the product was loaded on the resin, acetonitrile was used to elute the product in a reverse flow at 2 bed volume per hour. This process was repeated several times to measure the resins ability to be re-used.

Physical property database: commercially available databases such as EPISuite , can be used for obtaining properties of the different components

Resin database: databases from resin distributor companies such as Sigma-Aldrich can be used for getting information about the different types of resins (surface area, bead size, pore diameter and chemistry) and the cost of the resin.

5.5 Case study: asymmetric synthesis of 1-phenylethylamine

The synthesis of optically pure 1-phenylethylamine using ω-transaminase (EC 2.6.1.X, ATA - 040, C-Lecta) (TAm) was selected for illustration of the methodology. The reaction is catalysed 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 co-product ketone (Koszelewski et al., 2010). These enzymes require pyridoxal phosphate (PLP) as a cofactor to act as a shuttle to transfer the amine moiety between the molecules (Hwang et al., 2005). The following reaction was selected for the synthesis of 1-phenylethylamine (PEA) as shown in Figure 5.2.

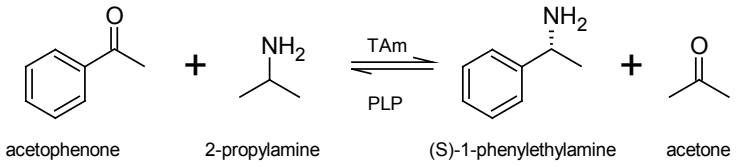


Figure 5.2 Reaction pathway for the synthesis of 1-phenylethylamine

The specific challenges that have to be addressed in this system include substrate and product inhibition and low aqueous solubility of the substrate (Chapter 4.5). Consequently, there is a strong motivation to introduce resins to overcome these challenges.

5.5.1 Experimentation

Material

Reagents and solvents of highest purity were purchased from Sigma-Aldrich (Buchs, Switzerland) and used without further purification. The unpurified enzyme ω-transaminase (ATA-044) 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. All resins were kindly provided by Sigma Aldrich (Buchs, Switzerland). The moisture content of the resins was measured by calculating the loss of weight after drying for 12 hours at 100^{o}C.

Methods

Analytical methods:

HPLC

Samples were measured on an Ultimate 3000 HPLC (Dionex, Sunnyvale, CA, USA), equipped with a UV detector and a photodiode array detector. 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 trifluoruacetic acid and acetonitrile, with the following gradient 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 minutes respectively.

Spectrophotometer

The concentration of 2-propylamine was measured *ex-situ* on an UV 1800 Spectrophotometer (Shimdzu, Kyoto, Japan) quantified at wavelength of 590 nm with fixed temperature of 30^{o}C using protocols developed by Rahman and co-workers (Rahman et al., 2003). The concentration of acetone was measured *ex-situ* on an UV 1800 Spectrophotometer (Shimdzu, Kyoto, Japan) quantified at wavelength of 280 nm with fixed temperature of 30^{o}C using an internal standard. Note that these compounds can be only measured independently and not in a mixture.

GC

The concentration of APH and 1-phenylethylamine were determined using an internal standard (20 mM 1-octanol in ethanol) with an autosampler TriPlus, Thermo Scientific, Dreieich, Germany automatically from a flow cell in the product stream with 0.5 µL sample mixed with 1 µL of standard solution (20 mM of 1-octanol in ethanol) and injected into a Focus or Trace GC from Thermo Scientific, Dreieich, Germany (carrier gas: H_2, 80 kPa) with a temperature setting of 100°C (5 min), 10°C/min to 140°C (0 min) and 40°C/min to 180°C (1 min) to measure APH (5.3 min), PEA (5.8 min) and 1-octanol (7.1 min).

Experimental methods:

Data set 1: Inhibition studies

Initial rate experiment was performed with fixed concentration of IPA at 1000 mM while the concentration of the co-substrate was varied from 0 – 30 mM. To observe the product inhibition of PEA, initial rate experiment was performed with fixed concentration of IPA at 1000 mM and APH at 10 mM. PEA was added at a range varying from 0 – 10 mM. The pH was maintained at 7 by addition of 100 mM of potassium phosphate buffer (pH 7) to each vial. To a final reaction volume

of 3 mL, concentration of crude enzyme was fixed at 2.1 g/L. Samples were withdrawn at 1, 3 and 5 minutes and diluted 5 times with 6 N HCl to end the reaction. The concentration of APH and PEA were measured using the HPLC.

Data set 2: Adsorption capacity of reactants

50 mg of each resin was added to four 3 mL vials. Each vial contained 50 mM of APH or 300 mM PEA or 2 M IPA or 2 M ACE. The vials were agitated at 300 rpm at 30^{o}C for 24 hours. A sample of the aqueous solution was drawn from the vial to determine the concentration of the reactants in the vial. The concentration in the resins was calculated based on mass balance. Samples were analysed by either HPLC (for APH and PEA) or spectrophotometer (for ACE and IPA). Note the resin was wetted with de-ionized water prior to use.

Data set 3: Adsorption isotherms between resin and components

50 mg of each resin was added to five 3 mL vials consisting of a range of component concentrations (0 till solubility limit of limiting substrate). The vials were agitated at 300 rpm at 30^{o}C for 24 hours. A sample of the aqueous solution was drawn from the vial to determine the aqueous concentration of the reactants in equilibrium in the vial. The concentration of the reactants in the resins was calculated by mass balance. Samples were analysed by using HPLC.

Data set 4: Re-usability of resins

The micro reactor which was constructed from a polymer of acrylonitrile butadiene styrene (ABS) was at first tested for adsorbing capacity for the reactant PEA. The polymer was cut into different sizes and incubated in 300 mM PEA solution. Samples were withdrawn after 24 hours to confirm that there was no significant evidence to support that PEA adsorbed on to ABS. The resin was packed in a column (1 mL volume) to a bed volume of 0.5 mL to test the performance of the resin (adsorption capacity). 300 mM of PEA was flowed through the column at a flow rate 0.05 mL/min

for 2 hours. Samples were taken every 10 minutes at the outlet for analysis by HPLC. Afterwards, the PEA was extracted from the resin with acetonitrile flowed through the column at 0.05 mL/min. This procedure was performed for repeated batches.

Synthesis of 1-phenylethylamine

The process was set up in a batch mode where an external resin packed column was connected to a membrane reactor. Prior to the experiments, the reactor was flushed with ethanol and water. A 10 kDA cut-off polyethersulfone membrane from Sartorius Stedim (ø= 63 mm) was wetted in water and then carefully placed into the reactor avoiding air-inclusions. The reaction mixture was then pumped into the membrane reactor and through the substrate loaded column (210 mM APH) to initiate the reaction. After the column the flow went through a flow cell and back to the membrane reactor. The reaction mixture contained 1.8 g/L of crude ATA-44 enzyme, 1000 mM 2-propyl amine, 2 mM PLP. Conversion was determined by automatically taking samples of 0.5 µL with an autosampler from a flow cell and directly injecting the sample together with 1 mL of an internal standard (20 mM of 1-octano1 in ethanol) into a Focus GC (Thermo Scientific, Dreieich, Germany) for GC analysis. The samples were used for determining APH and PEA.

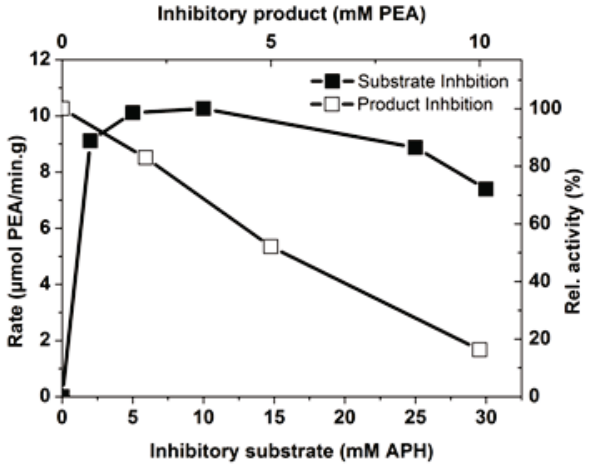
Product elution from the resin packed column

The product from the column was eluted using n-heptane with a flow rate of 30 mL/h. 200 µL of sample was collected every two hours at the outlet and diluted with 800 µL of standard solution (20 mM of 1-octanol in ethanol). The concentration of PEA and unreacted APH content was determined via GC.

5.5.2 Application of the proposed methodology

Step 1: Characterize reaction

Plotting the activity of the enzyme versus concentrations of the different components from data set 1 (see Figure 5.3) shows that APH and PEA were causing inhibition. Increasing concentration of these compounds in the reactor resulted in decreased activity of the enzyme. The other reactants, IPA and ACE, did not show any inhibitory effect within the experimental conditions (data not shown).



5.5.3

Figure 5.3 Inhibition by substrate acetophenone (open squares) and product 1-phenylethylamine (filled squares)
Reaction condition: E_0 = 2.1 g/L, C_{IPA} = 1000 mM, Phosphate buffer = 100 mM (pH 7)

To further explore the properties of the reactants, a database (EPI) was used to obtain information for selecting the type of resin. Table 5.2 tabulates the properties of the reactants involved in the desired reaction scheme (Figure 5.2). The Log P value along with the solubility of the reactant expresses the hydrophobicity or hydrophilicity of the components. Based on these parameters it can be seen that the reactants PEA and APH are both hydrophobic while the others are hydrophilic. It will be thus beneficial to carry out the reaction in a two phase reactor using neutral resins to exploit the hydrophobicity of the reactants by selectively supplying APH and removing PEA *in situ* in

order to optimize the productivity of the catalyst (mass of product per mass of catalyst) which is required in order for the process to be feasible (Tufvesson et al., 2011b).

Table 5.2 Physical properties of the reactants

Component	Molecular weight g/mol	Water solubility [g/L]	Log P
APH	120.15	6.13	1.58
IPA	57.09	1000	0.26
PEA	121.18	42	1.49
ACE	58.08	1000	-0.24

Step 2: Screen resin

In this study the resins were looked up in the Sigma Aldrich catalogue as listed in Table 5.3 and were tested for their potential to be the resin that fulfilled the selection criteria. The resins were selected on the basis that they are commercially available at relatively low cost and neutral charged. The resins differed in surface area, pore diameter and matrix of the resin. The moisture content was also measured to correlate the dry weight of the resins.

Table 5.3 Resin properties

Adsorbent	Type	Matrix	Surface Area [m^2/g]	Pore Diameter [Å]	Moisture content [%]
MCI gel	CHP20P	SDB	500	400-600	58.1
Diaion	HP-20SS	SDB	500	260	49.6
Amberchrom	CG300C	SDB	700	300	72.3
Lewatit	VPOC1064	SDB	800	50	68.4
Sepabeads	SP850	SDB	1000	38	65.8
Optipore	L493	SDB	1100	46	49.7
Amberlite	XAD7HP	SA	450	90	51.9
Lewatit	VPOC1600	SAM	130	150	64.2
Co-polymer	Poly ESDB	ESDB	650	100	52.6
Lewatit	AF5	Carbon	1200	80	46.4

Note: SDB – styrene divinyl benzene; SA – styrene acrylate; SAM – styrene methyl-acrylate; ESDB – ethylstyrene divinyl benzene

Capacity for 1-phenylethylamine

The capacity for PEA (mol/kg_{resin}) was measured experimentally for each of the resins using data set 2. The capacity is tabulated in Table 5.4. As it can be seen that the resins with the larger surface area displayed a higher capacity for PEA. This result indicates that the matrix of the resin plays little role for dictating the affinity of the components. It should be specifically noted that resins L493 and AF5 had the highest capacity for PEA.

Selectivity of 1-phenylethylamine

The selectivity of PEA over the other components was calculated using data set 3 and presented in Table 5.4. From Table 5.4, it can be seen that none of the resins were more selective for PEA with respect to APH. The selectivity ratio was always greater than 1 for APH indicating that the resins

were non-selective for PEA and thus could be effective for only overcoming the kinetic limitations (inhibition) and not for shifting the reaction towards product side. The resin L493 displayed the highest selectivity of APH/PEA. Moreover, the resin showed selectivity of almost unity or lower for the other reactants. On the other hand, resin AF5 displayed selectivity much lower than unity for IPA/PEA and co-product ACE/PEA. Resin CG300C displayed commendable selectivity for PEA over IPA and ACE but the capacity for PEA was nominal. Therefore, resins AF5 and L493 were selected for further investigation.

Table 5.4 Capacity and selectivity of target molecule

Adsorbent	**Capacity**		**Selectivity**		
	[mol/k_g]	**[g/g]**	**[APH/PEA]**	**[IPA/PEA]**	**[ACE/PEA]**
CHP20P	1.7	0.21	5.55	2.42	0.60
HP-20SS	1.8	0.22	4.34	1.14	0.27
CG300C	1.9	0.23	2.56	0.10	0.77
VPOC1064	1.8	0.22	4.76	6.25	0.07
SP850	2.0	0.24	5.00	2.85	0
L493	2.3	0.28	2.56	1.06	0.37
XAD7HP	1.7	0.20	4.00	1.72	0.29
VPOC1600	1.2	0.14	4.16	0.23	1.05
Poly ESDB	2.2	0.26	4.54	2.27	0.21
AF5	4.3	0.52	3.57	0.34	0.51

Adsorption Isotherms between resin and components

The distribution of the different compounds between the resin phase and the aqueous phase was tested with the two candidates selected from previous screening. The isotherms for the different resins are shown in Figure 5.4. It can be seen that the resins either follow L shaped or S shaped

curve which is in accordance with adsorptions studies done previously for ketones (Hilker et al., 2004) and amino acids (Grzegorczyk and Carta, 1996). In Figure 5.4 it can be seen that the resin AF5 has a much stronger affinity for both APH and PEA as compared to resin L493. The distribution coefficient of APH between the resin and the aqueous phase for AF5 and L493 was determined to be 5112 and 450 L/kg respectively. The distribution of PEA between the resin and aqueous phase for AF5 and L493 was determined to be 69 and 43 L/kg respectively. These values were calculated with the assumption that the isotherms follow Langmuir adsorption isotherm. These values will be determined in a reliable and systematic way in a following chapter. These values can be used to provide the partitioning tendency of the resins. The partitioning of acetophenone in various aqueous-organic phase systems was measured in a study done by Shin and Kim, 1997 (Shin and Kim, 1997). The highest partitioning coefficient of 191.9 was achieved using a 20% v/v mixture of toluene. In the same study, the partitioning of PEA in 20% v/v of cyclohexanone in phosphate buffer at pH 7 was measured to be 14.45. Consequently it can be suggested that resins have higher distribution coefficients than solvents (Straathof, 2003). Based on the attributes of the resin AF5, high capacity for PEA, relatively high selectivity of target compound PEA over IPA and possibility of high loading of substrate, it was selected to for the final screening of step 3.

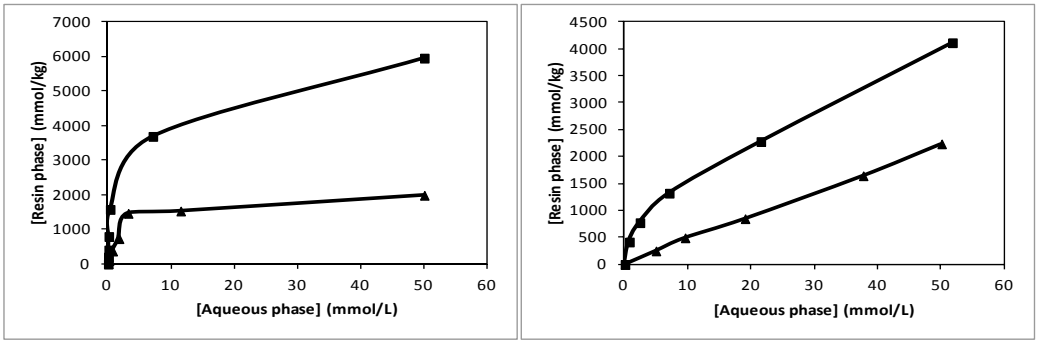


Figure 5.4 Adsorption isotherms of the different components in the reaction media. left: acetophenone, right: 1-phenylethylamine. (Legend – AF5 (square), L493 (triangle))

The cost of resins if assumed for bulk purchase, are relatively low. The cost of resin Optipore L493 can be assumed to cost around $2.2/kg based on the cost as quoted in Sigma-Aldrich website. Similarly, activated carbon costs around $0.60/kg (Sherman, 2012). The costs are relatively lower when compared to typical solvents used as a water-immiscible auxiliary phase such as toluene ($3/kg). However, to be more attractive than solvents as an immiscible auxiliary phase and as well as to reduce the impact on downstream operating cost, it is necessary to be able to re-cycle the resins.

The performance of the resin (adsorption capacity) was experimentally measured for five batches using data set 5 as shown in Figure 5.5. It can be noted that there is a slight deviation in the experimental results. The deviations could be explained through experimental errors and sample handling. However, the data provides evidence that the resin was quite durable and resistant to the different solvents and in principle can be used many times without compromising its performance.

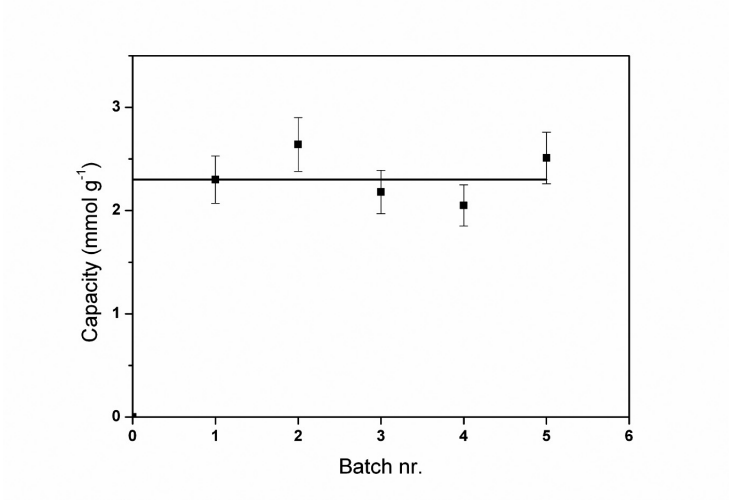


Figure 5.5 Recyclability and re-usability of the resin. Solid line represents the average capacity of the resin

5.5.4 Synthesis of 1-phenylethylamine: proof of concept

The resin AF5 was used for the asymmetric synthesis of PEA. The design of the appropriate reactor using solid resins will primarily depend upon the following criteria: first, the interaction of the biocatalyst with the resin (adverse effect on resin contact with biocatalyst), second, the interaction of the reactants with the resin (stability), third, the working volume of the resin. For this reaction, it was noticed that the enzyme was as well getting adsorbed to the resin. Therefore to overcome this bottleneck, it was envisaged using a membrane reactor with cut-off point less than the size of the enzyme to be used. A resin packed column would be externally connected thus operating as a recycle reactor (Figure 5.7). The stability of the resin in the presence of reactants and solvent was quite good as could be seen in the re-usability experiments. The working volume (phase ratio) of resins required was calculated using a kinetic model that was derived in a previous study (Al-Haque et al., 2012). A kinetic model was used for simulating different scenarios by varying the volume ratio of resin (calculated by using the grams of resin and the resin density) to reactor volume. It was assumed that the distribution of the substrate and product with the resin relies only on the distribution coefficient which was determined from data set 3. Further it was assumed that mass transfer rate of substrate release and product removal was instantaneous (Brenna et al., 2012). Figure 5.6 illustrates the process overview for the supply of substrate and removal of product in the presence of the resin AF5.

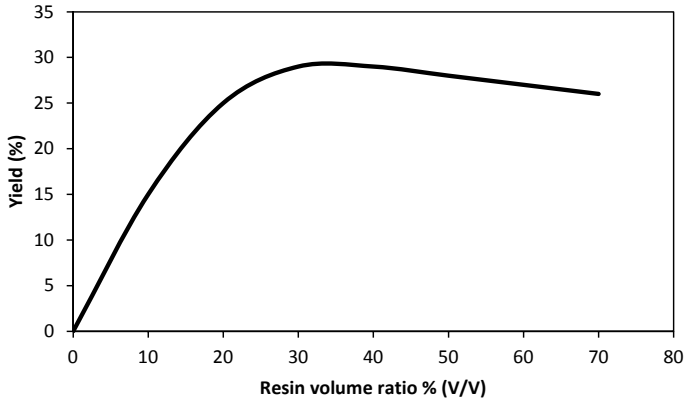


Figure 5.6 Optimal ratio of resins.
Reaction condition: Ei = 1.8 g/L, C_{IPA} = 1000 mM, C_{APH} = 210 mM

From Figure 5.6 it can be seen that with increasing resin volume ratio, the yield increased until it crossed a certain peak after which it dropped. This phenomenon can be explained by the rationale that with increasing resin volume ratio, the equilibrium concentration of acetophenone and 1-phenylethylamine is lower. Hence the kinetic limitation is overcome and the yield correlates to the maximum yield based on the equilibrium constant. Further increase in resin volume ratio decreases the substrate availability causing the reaction to be substrate limited and hence, the decrease in yield. Therefore the optimal resin volume in this case should correspond to around 30% (V/V), preferably in a packed bed reactor. It was then possible to carry out the reaction in the reactor configuration as shown in Figure 5.7. Two sets of experiments were carried out, the first was control (without any resin to act as a benchmark) and second with resin to illustrate the improvement in the system. The reaction conditions were identical to each other.

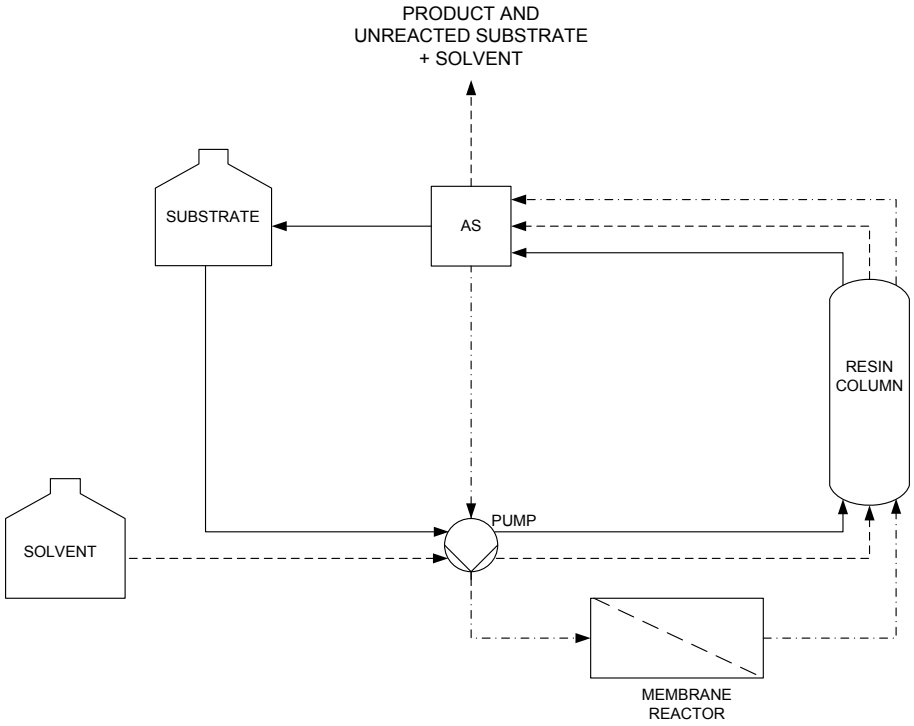


Figure 5.7 Process flow sheet for the synthesis of chiral amines using resin. Step 1) Substrate loading on resins (solid line) Step 2) Integrated substrate feeding – product removal with a membrane reactor (dash dot line Step 3) Elution of product (dash line). AS is auto sampler.

The resin column was loaded with the specified volume of resin. Substrate solution was pumped through the column for 24 hours at a flow rate of 2 bed volume per hour (Step 1). After which the pump was stopped and an enzyme membrane reactor was linked externally to the column (Step 2). The corresponding amount of enzyme, co-factor and co-substrate was added to the reactor and the membrane was enclosed on the top to restrain any enzyme from leaving the reactor. The reaction commenced by restarting the pump to allow the reaction media to circulate through the membrane, the resin column and the reactor. The reaction was monitored for 48 hours. Biocatalytic conversion of substrate from the aqueous phase caused additional substrate to partition from the carrier phase to accomplish in-situ substrate supply (ISSS). Similarly, as 1-phenylethyl amine was formed, it

partitioned back into the carrier phase to accomplish in-situ product removal (ISPR). After 48 hours, the pump was stopped. The membrane reactor was removed and replaced with a solvent tank (step 3). The results of the experiment are shown in Figure 5.8.

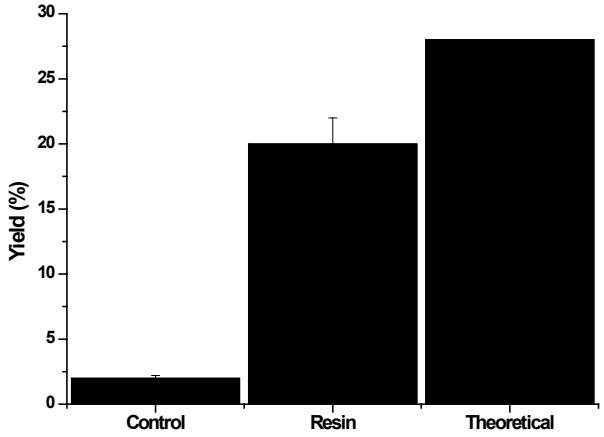


Figure 5.8 Yield achieved with the auxiliary phase reactor using the selected resin Reaction conditions: Eo = 1.8 g/L, C_{IPA} = 1000 mM, C_{APH} = 210 mM, C_{PLP} = 2 mM, phosphate buffer = 100 mM (P_H 7)

From Figure 5.8, it can be seen that with the introduction of resin as an auxiliary phase there was a substantial improvement in the yield as compared to the control column. To make a further comparison, the theoretical yield was calculated based on the knowledge on the equilibrium constant value (Tufvesson et al., 2012). It can be noted that the accomplished experimental yield is quite comparable to the theoretical yield.

5.5.5 Discussion

Integration of porous resins provided significant benefits to the reaction. However selecting resins for multi-component systems requires the screening protocol to take into account further considerations. Emphasis has to be made at the capacity of the resin, selectivity of the product and the partitioning ratio of the key components for a multi-component system. The screening protocol

developed is systematic and effective in selecting a resin from a pool of resins which fulfilled all the criteria mentioned in Table 5.1. Resin AF5 was found to be the best candidate from the available list of resins. The resin had relatively high capacity which allowed a working load of around 4 mmol/g of resin. The resin was more selective for the key product over the non-inhibitory compounds and the resin was cheap and moreover showed the tendency to be re-usable several times. However, the partitioning coefficient for acetophenone was much higher than 1-phenylethylamine, which resulted in an accumulation of the 1-phenylethylamine in the reactor and inhibited the enzyme. Therefore, efforts have to be placed in engineering the resin to have higher or similar partition of PEA/APH.

A further aspect that was not possible for testing was to evaluate if the reaction was substrate limited. Dynamic data that follows the progress of the reaction is required in order to evaluate that. Generally, enzymatic reactions are relatively slow and it can be assumed that the reaction was not limited by substrate transfer from the resin to the aqueous phase. Alternatively, measures can be taken to increase the mass transfer rate by increasing the surface area (using smaller particles) or by reducing the pump flow rate to increase the residence time of the reaction media in the column.

From Figure 5.8, it can be seen that the yield achieved did not exceed equilibrium constraints. Therefore, this indicates that this strategy would assist in increasing synthetic yield. Alternative, the formed acetone can be selectively removed. Different strategies such as stripping/sweeping with an inert gas like nitrogen (Zanfir et al., 2008) or adding an enzymatic cascade to selectively remove acetone (Cassimjee et al., 2010) can be applied.

5.6 Case study: asymmetric synthesis of 1-methyl-3-phenylpropylamine

For the second case study, the asymmetric synthesis of 4-phenyl-butylamine with 2-propylamine as amine donor using ω-transaminases (EC 2.6.1.18) has been selected. The product is of industrial relevance and is currently produced by companies such as Cambrex (Martin et al., 2007). The process is operated at high temperatures (above 60^{o}C) and amine donor concentration of 1500 mM to displace the unfavourable reaction equilibrium towards product synthesis (Tufvesson et al., 2011). Based on a simplified cost calculation, it was found that the process cost was relatively high and was suggested to be reduced by immobilizing and re-using the enzyme and implementing alternative unit operations for product separation. In order to address these issues, it was envisaged that using resins would be an effective way of separating the product from the reaction media *in-situ* (Shorrock et al., 2004). Moreover the resin introduced would enable a high load of substrate concentration and simultaneously maintain low aqueous substrate and product concentration to overcome kinetic limitations (Hilker et al., 2005). The cost of the enzymes can be reduced by re-using them. Such an approach can be implemented by immobilizing the enzymes to increase operational stability and simpler for recovery (Sheldon, 2007). Cross-linked enzyme aggregates (CLEAs) were considered applicable for this purpose because of its advantages of improved operational stability towards denaturation by heat and organic solvents and furthermore the minimal diffusional limitation (Sheldon, 2011). 2-propylamine was selected as amine donor as opposed to typically used alanine because the reaction is favoured with 10 fold higher rate of reaction (Cardenas-Fernandez et al., 2012). Moreover 2-propylamine is highly volatile meaning it can be easily distilled and re-used. However, the problem with unfavourable reaction equilibrium cannot be overcome with using resins. The resins are non-selective and will bind with both the substrate and product. Additional strategies have to be considered to remove the co-product in order to shift

the reaction towards product synthesis. A number of different approaches such as operating at high temperature (60^{o}C) (Savile et al., 2010), stripping with nitrogen (Zanfir et al., 2008) and selective enzymatic cascade reactions (Cassimjee et al., 2010) can be applied. The latter two approaches were considered for testing in this study. Operating the reaction at 60^{o}C was not opted as it would result in a significant loss in working media and furthermore be ineffective for evaporating off diluted acetone (Tufvesson et al., 2011). Figure 5.9 illustrates the overall reaction scheme was used for testing the integration of resin.

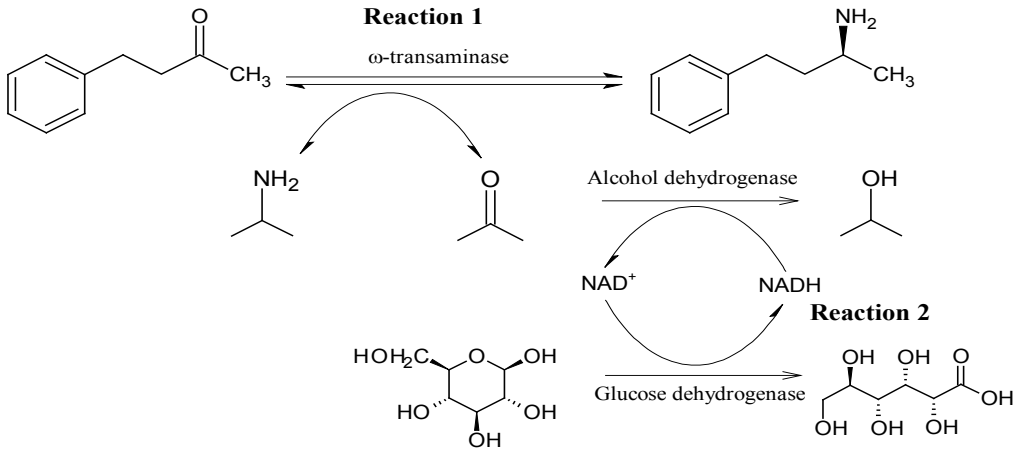


Figure 5.9 Overview of the reaction scheme

5.6.1 Experimentation

Materials

Reagents (4-phenyl-2-butanone (PB), 2-propylamine (IPA), 1-methyl-3-phenylpropylamine (MPP) and acetone (ACE) and solvent (acetonitrile) were purchased from Sigma-Aldrich at the highest available purity (Buchs, Switzerland) and used without further purification. CLEA preparation of Codexis ATA-117 (TAm, EC 2.6.1.18) was kindly supplied by CLEA Technologies (Delft, Netherlands). The enzyme alcohol dehydrogenase (ADH, EC 1.1.1.1) from *Saccharomyces*

cerevisiae which came as lyophilized powder was purchased from Sigma-Aldrich (Buchs, Switzerland). The enzyme glucose dehydrogenase (GDH, EC 1.1.1.47) was kindly supplied by Codexis (Redwood city, CA) came as lyophilized powder. In all experiments the enzyme amount refers to either grams of lyophilized powderof mL of CLEA. All the different resins were kindly supplied by Sigma-Aldrich (Buchs, Switzerland).

Methods

Analytical methods

Samples were measured on an Ultimate 3000 HPLC (Dionex, Sunnyvale, CA, USA), equipped with a UV detector and a photodiode array detector. 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 trifluoruacetic acid and acetonitrile, with the following gradient 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: 4-phenyl-2-butanone (254 nm, 5.7 minutes) and 1-methyl-3-phenylpropylamine (254 nm, 4.1 minutes).

Inhibition studies of the substrate

Initial rate experiments were performed with 1 M 2-propylamine while the concentration of 4-phenyl-2-butanone was varied between 0 and 10 mM in 2 mL volume. Reactions were run at 30^{o}C in 100 mM Tris buffer (pH 7) at 300 rpm. Reaction started with the addition of 0.20 mL of CLEA-transaminase. Samples of 200 µL were withdrawn after 72 hours and added to a centrifuge tube containing 800 µL of 6 N HCL in order to stop the reaction (data not shown). The samples were analysed by HPLC.

Adsorption capacity of components

50 mg of the resin was added to four 3 mL vials. Each vial contained 12 mM 4-phenyl-2-butanone or 42 mM 1-methyl-3-phenylpropylamine or 2 M 2-propyl amine or 2 M acetone. The vials were agitated at 300 rpm at 30^{o}C for 24 hours. A sample of the aqueous solution was withdrawn after 24 hours to determine the concentration of the components in equilibrium by either HPLC or spectrophotometer (method explained in previous case study).

Adsorption isotherm between resin and reactants

50 mg of the resin was added to five 3 mL vials consisting of a range of reactant concentrations (0 till solubility limit of of limiting substrate). The experiment was performed for both 4-phenyl-2-butanone and 1-methyl-3-phenylpropylamine. The vials were agitated at 300 rpm at 30^{o}C for 24 hours. A sample of the aqueous solution was withdrawn after 24 hours to determine the concentration of the reactants in equilibrium by HPLC. The concentration of the reactants in the resins was calculated by mass balance.

Re-usability of resins

The resin was packed in a stainless steel column (1 mL volume) with 60% (V/V) resin to test the performance of the resin. 10 mM of 1-methyl-3-phenylpropylamine was flowed through the column at a flow rate 0.5 mL/min for 2 hours. Samples were taken every 10 minutes for analysis by HPLC. Afterwards, 1-methyl-3-phenylpropylamine was extracted from the resin with acetonitrile flowed through the column at 0.05 mL/min for 6 hours. The column was then flushed with ethanol and water and was ready to be loaded again. This procedure was performed for repeated batches.

Optimal resin concentration

Batch adsorption experiments were performed using 4 mL vials containing 3 mL of 175 mM 4-phenyl-2-butanone, 500 mM 2-propylamine and 175 mM 1-methyl-3-phenylpropylamine, 175 mM acetone in 100 mM tris buffer with varying amount of resin. The vials were placed in a thermoshaker (Model 11, HLC Biotech, Bovenden, Germany) for 24 hours at 30 ˚C with orbital agitation of 300 rpm. At the end of the equilibrium period, 500 µL of sample was taken from the liquid phase to measure the concentration of the reagents by HPLC.

Transamination of 4-phenyl-2-butanone (Reaction 1)

Transamination of 4-phenyl-2-butanone was conducted at 2 mL scale with the following in the reaction media: 1 M 2-propylamine, 0.5 g/L pyridoxal-5-phosphate, 0.20 mL CLEA -ω-transaminase. 350 mM (50 g/L) of 4-phenyl-2-butanone and 20% (v/v) resin (AF 5). Reactions were run at 30^{o}C and 100 mM Tris buffer (pH 7) in a 4 mL glass vial at 300 rpm. Upon completion of the reaction (72 hours), the resins were filtered off and the product was eluted with acetonitrile and analysed by HPLC.

Nitrogen saturated with water was continuously swept over the reaction media at a flow rate of 90 mL/min.

Transamination of 4-phenyl-2-butanone (Reaction 2)

The procedure was conducted as explained in reaction 1 with the addition of 1 g/L NAD^{+}, 350 mM glucose, 20 g/L of crude ADH and 1 g/L of crude GDH in the reaction media.

5.6.2 Application of the proposed methodology

Step 1: Characterize reaction

An inhibition study has been performed of the ω-transaminase reaction in order to set targets of substrate and product concentration for the resin. From Table 5.5 effect of substrate concentration on the percent yield can be seen when the reaction is conducted by adding the substrate to a reaction media containing 10% (V/V) Clea-Tam and 1 M 2-propylamine. At very low concentration, the observed yield was compromised due to inhibition. Therefore, it would be desirable that the aqueous concentration of substrate and product in the reactor is less than 6 mM in order to achieve minimum yields of 70%.

Table 5.5 Effect of substrate concentration on yield after 72 hours (10% V/V Clea-Tam, 1 M 2-propylamine (pH 7), 2 mM PLP)

[4-phenyl-2-butanone] mM	Yield %
1	96
3	75.4
5	72.1
6	66.8

The properties of the components were investigated using a physical property database (EPI). Table 5.6 tabulates the properties of the reactants involved in the desired reaction scheme (Figure 5.9). The Log P value along with the aqueous solubility of the reactant expresses the hydrophobicity of these components. Based on these parameters it can be seen that the reactants MPP and PB are both hydrophobic while the others are hydrophilic. It would be desirable to select a porous resin which acts primarily on the hydrophobicity of the components.

Table 5.6 Physical properties of the reactants

Component	Molecular weight g/mol	Water solubility [g/L]	Log P
PB	148.21	1.62	1.96
IPA	57.09	1000	0.26
MPP	149.24	12	2.12
ACE	58.08	1000	-0.24

Step 2: Screen resin

Resins were selected from the Sigma Aldrich catalogue as listed in Table 5.7. Based on the experience from previous case study, resins with surface area greater than 1000 m^2/g resin were selected for investigation.

Table 5.7 Resin properties

Adsorbent	Type	Matrix	Surface Area [m^2/g]	Pore Diameter [Å]	Moisture content [%]
Sepabeads	SP850	SDB	1000	38	65.8
Optipore	L493	SDB	1100	46	49.7
Lewatit	AF5	Carbon	1200	80	46.4

Note: SDB – styrene divinyl benzene

Capacity for MPP

The capacity for MPP ($mmol/g_{resin}$) was measured experimentally for each of the resins using data set 2. The capacity is tabulated in Table 5.8. As can be seen, the resins with the largest surface area, AF5, displayed the highest capacity for MPP among the tested resins. This result is consistent with the previous case study.

Selectivity of MPP

The selectivity of MPP over the other compounds was calculated using data set 3 and presented in Table 5.8. From Table 5.8 it can be seen that all the resins were moderately selective for MPP with respect to PB. Resin AF5 was the least selective for co-substrate IPA. Similarly the resin did not have a strong affinity for acetone either. Therefore resin AF5 was selected for further investigation. Resin SP850 was not selected as it displayed resin capacity lower than AF5.

Table 5.8 Capacity and selectivity of target molecule

Adsorbent	Capacity		Selectivity		
	[mol/kg]	[g/g]	[PB/MPP]	[IPA/MPP]	[ACE/MPP]
SP850	1.47	0.22	0.65	0.56	0
L493	1.46	0.22	1.13	0.42	0.14
AF5	1.65	0.25	1.01	0.09	0.07

Adsorption Isotherms of PB and MPP

These components revealed to be indeed displaying favourable adsorption isotherms as can be seen in Figure 5.10. It can also be noted that the resin showed similar partitioning for both substrate and product. The selectivity for the product with respect to the substrate was not significantly high and thus cannot be used for selectively removing the product to assist in shifting the reaction towards product synthesis.

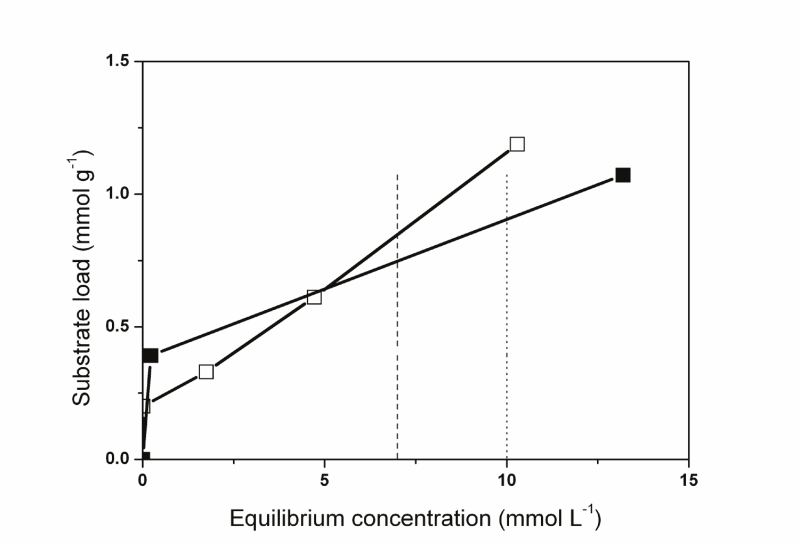


Figure 5.10 Adsorption isotherm of the limiting substrate and target product (open symbol is 4-phenyl-2-butanone and filled symbol is 1-methyl-3-phenylpropylamine. Dotted line indicate solubility limit of substrate and dashed line indicated critical concentration of product).

Step 3: Evaluate cost of introducing resin

The performance of the resin (adsorption capacity) was experimentally measured for five batches using data set 5 as shown in Figure 5.11. The result provided evidence that no significant loss in the performance of the resin was observed even after five batches, thus indicating its resistance towards the different components and solvents, hence, in principle the resin can be used several times.

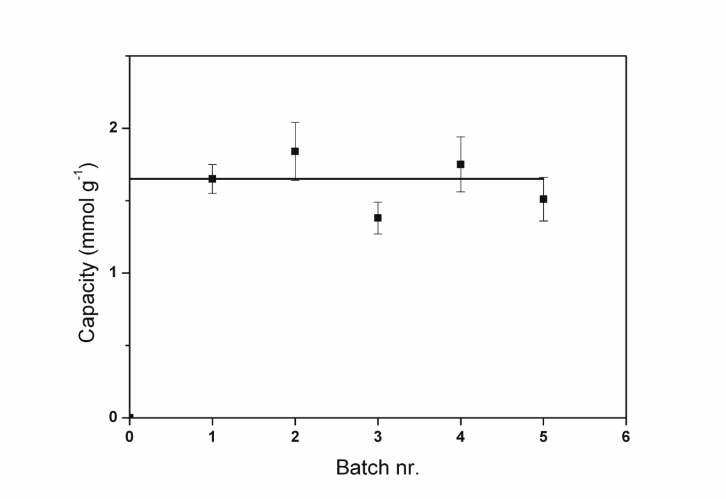


Figure 5.11 Recyclability and re-usability of the resin. (Solid line represents the average capacity of the resin)

5.6.3 Synthesis of 1-methyl-3-phenylpropylamine: validation

To evaluate the required amount of adsorbent, a reaction mixture was made to mimic 50% conversion i.e. 175 mM of 4-phenyl-2-butanone, 175 mM of 1-methyl-3-phenylpropylamine, 175 mM of acetone and 500 mM 2-propylamine was incubated in a vial containing varying amount of resins. The corresponding concentration of 4-phenyl-2-butanone and 1-methyl-3-phenylpropylamine in the aqueous phase was measured. The results of the corresponding concentrations are tabulated in Table 5.9.

Table 5.9 Equilibrium concentration of the reagents

Substrate load (g/g)	Resin load (v/v)	4-phenyl-2-butanone (mmol/L)	1-methyl-3-phenylpropylamine (mmol/L)
0.80	10%	2.97	53.06
0.60	15%	1.58	27.86
0.40	20%	0.18	2.67
0.30	30%	0.01	0.02
0.20	50%	Not detectable	Not detectable
0.10	70%	Not detectable	Not detectable

From Table 5.9, it can be seen that with a resin loading of 0.40 g/g (40 gram substrate per 10 gram of resin) corresponding to 20% (v/v) was required to maintain concentrations of the substrate and product within the critical concentration of the ω-transaminase reaction. A higher resin loading such as 30% (v/v) would result in the reaction being kinetically limited by substrate availability.

Prior to selecting the reactor configuration, some experiments were carried out to measure any deviation of specific catalyst activity of the enzymes in the presence of resins. The results indicated that the resins were indeed inert and did not affect the catalysis (data not shown).

Two modes of operation were carried out: single phase to act as a benchmark and bi-phasic system with the inclusion of resins suspended in the reactor. The single phase mode resulted in a nominal yield of 1 %. To overcome this limitation four strategies were suggested: strategy1) Addition of resin to reaction 1, strategy 2) Coupling of reaction 1 and reaction 2, strategy 3) Coupling the second strategy with water saturated nitrogen sweeping over the reaction media and strategy 4) Coupling the second strategy with reaction 2 to integrate the resin and cascade reaction in one pot. The results of these strategies are tabulated in Table 5.10. From Table 5.10 it can be clearly seen that employing the different process strategies had a positive impact on the yield of the ω-

transaminase reaction. As expected, the product concentration increased significantly with strategy 1 and even further with strategy 2, as compared to the benchmark reaction.

Table 5.10 Process improvements with each strategy

Scenario	**1-methyl-3-phenylpropylamine (mM)**	**Yield (%)**
Single phase (benchmark)	3.68 ± 0.01	1.02
Strategy 1 (reaction 1+ reaction 2)	37.27 ± 0.34	10.23
Strategy 2 (reaction 1+ resin)	143.72 ± 2.41	40.21
Strategy 3 (strategy 2 + N_2 sweeping)	169.87 ± 2.34	44.67
Strategy 4 (strategy 2 + reaction 2)	236.68 ± 3.96	69.61

5.6.4 Discussion

Each strategy added to the single phase reaction improved the product yield as compared to the benchmark. Even though the starting amine donor concentration was 3 folds greater, it did not help in significantly contributing to higher yields. With that in mind, a selective cascade enzyme, ADH, was added to the reaction media containing 1 M IPA. This greatly enhanced the yield contributing to a 10 fold higher yield. Unfortunately, the yield achieved did not manage to even reach equilibrium yields, which was calculated to be around 67%. The yield was calculated based on theoretical reaction equilibrium data acquired from other studies (Tufvesson et al., 2012). The result provided evidence that indeed the reaction was challenged with inhibition problem as equilibrium yields could not be achieved with an excess of amine donor and a selective cascade reaction. Bearing that in mind, porous resins were added to minimize the aqueous substrate and product concentration experienced by the enzyme. In strategy 2, an excess of amine donor coupled with the resins proved to be beneficial for the reaction. The yield achieved of around 40% was significantly

higher than the benchmark reaction. Higher yields could not be achieved because at the end of the reaction, the concentration of MPP in the aqueous phase was measured to be around 8 mM causing the enzyme to be inhibited. Attempt was made to improve the yield further by sweeping saturated nitrogen over the reaction media to remove formed acetone (strategy 3). This strategy proved to be unsuccessful as the yields did not improve significantly. Furthermore, it was observed that the volume of the reaction media reduced on account of nitrogen sweeping. It would be suggested to include a strategy to recycle the lost reaction media. With strategy 4, around 70% yield could be attained. It was noticed that the activity of the enzyme ADH was quite poor for which 20 g/L of the enzyme was added. Higher concentration could not be added as it was precipitating in the reactor.

The results of Table 5.10 points out that multiple strategies are required in order to achieve yields higher than equilibrium. The problem set forth from this case study indicates the necessity of evolving the enzyme ADH to improve the specific activity in order to minimize the enzyme loading. It would be also desirable to increase the operability space of the transaminase enzyme in order to be more tolerant to higher substrate and product concentration. Increasing the phase ratio of resin would decrease total accumulated concentration of MPP in the reactor, though it would simultaneously reduce the available substrate concentration. Alternatively, the resin could be engineered to be equally selective for both PB and MPP.

5.7 Conclusions

A method to select resin to be used as an auxiliary phase has been developed. The methodology is exemplified with two case studies of ω-transaminase-catalysed reactions. The methodology systematically approaches the problem where the output of each step becomes an input for the following step. The screening is done based on the capacity for the target molecule, selectivity for the target molecule over the other components, adsorption isotherms of the different reactants to

measure the partitioning and finally the cost of the resin coupled with re-usability studies to identify a final candidate. Porous resins present to be an interesting alternative to overcome kinetic limitations which is evident from both the case studies. The resins were non-selective, making them suitable for the multi-purpose task of an integrated substrate supply and product removal. However, the requirement of yields higher than equilibrium restrictions imposes the need for coupling porous resins with a selective product removal technique, as it was shown in the second case study. The outcome of the above being that multiple strategies are required, in order to facilitate high productivity of the enzyme.

Chapter 6

Model based approach for evaluating processes

6.1 Introduction

Process modelling presents a very powerful tool that can translate existing information and data to useful predictions for new conditions (Al-Haque et al., 2012; Vasic-Racki et al., 2003b). Process models are multi-objective functions that predict the behaviour of the real system to gain insight into the reaction kinetics and in turn be able to tune the reaction conditions in order to harness the full potential of biocatalysis (Brass et al., 1997; Sin et al., 2009). Such an example can be seen in the kinetic resolution of α-methylbenzylamine where the kinetic model guided the optimal ratio of enzyme and co-substrate concentration to provide the highest yield (Shin and Kim, 1998). While in other cases it can be used for optimizing the enzyme ratio for bi-enzymatic processes (Chen et al., 2006; Van Hecke et al., 2009) or for evaluating *in-situ* product removal opportunities and different reactor configurations (Frykman et al., 2005; Sun et al., 1999; Takors, 2004). Consequently, the sensitivity of the kinetic parameters can be investigated to see what effect it has on the output of the process in order to guide catalyst improvements (Sayar et al., 2009). Furthermore kinetic models can be used to simulate different process scenarios for different reactor configurations to make "what-if" analysis. In that context analysis can be made whether it would be beneficial to run a reaction in a batch reactor or packed bed reactor or in a continuous stirred tank reactor.

Furthermore, mathematical modelling can be used to generate windows of operation to graphically visualize the operating space bounded by both process and system constraints (Chhatre et al., 2011; Law et al., 2008; Woodley and TitchenerHooker, 1996). In the case of integrated resin bioreactors it is important to formulate mathematical models in order to evaluate the process feasibility, e.g. analysing the required partition coefficients and alternative reactor configurations (internal or external) necessary to alleviate substrate and product inhibition. Consequently, the models can be used conceptually to assess the process feasibility and as well to guide the type of experiments that need to be carried out

6.2 Modelling needs

Mathematical modelling of bioprocesses is an important tool in both academia (Gernaey et al., 2010; Glassey et al., 2011; Morales-Rodriguez et al., 2012) and industry (Brass et al., 1997; Fox and Clay, 2009; Jimenez-Gonzalez and Woodley, 2010) for evaluating a process. Methodologies can be seen for modelling reaction kinetics for both single (Al-Haque et al., 2012) and multi-enzyme processes (Santacoloma et al., 2011) based on first principles, reactor modelling for various types of reactor (Fogler, 2005) and mass transfer models (Quijano et al., 2010) to describe the substrate transfer rate from resin to aqueous phase.

To model a process, the different phenomena that take place in the process have to be included in the mathematical model. These phenomena can range from mode of substrate transport (diffusion), reaction (kinetic), separation (partitioning) and the type of reactor (flow reactor or batch reactor). The assumptions made have to be stated beforehand as it influences the type of model required for the process. For example, a reactor operating in a plug flow reactor has to include the flow term as compared to a batch reactor where no flow term is required. Similarly, introducing a resin into the

reactor requires partitioning terms to be included in the model. Table 6.1 lists the different types of mathematical models required to describe the different reactor types.

Table 6.1 Mass balances for different reactor types

Reactor type	**Type of model**	**Example**
Stirred tank batch reactor	Batch reactor	$dC/dt = r_1$
Substrate feeding	Semi-batch reactor	$d(CV)/dt = r_1V$
Plug flow	Tubular reactor	$dC/dV = r_1/ v_0$
Continuous flow in and out	CSTR	$V = v_0(C_0 – C)/ r_1$

Different constitutive models have to be included in the mass balance to describe the different phenomena taking place in the reactor. Table 6.2 lists the different type of models that are used in this thesis.

Table 6.2 Types of models required to describe the process

Phenomena	**Type of model**	**Example**
Reaction	Rate model	$r_1 = k_1C/(K_2 + C)$
Partitioning between resin and reactants	Adsorption model	$q = q_{max}C^*/(P + C^*)$
Flux of substrate supply and product removal	Mass transfer model	$Q = k_LA(C – C^*)$

The candidate models from Table 6.2 will then be inserted into the mass balance of the desired reactor (Table 6.1). The mass balance of a batch reactor, where only reaction is taking place, will have the structure of Equation 6.1.

$$\frac{dC}{dt} = k_1C / (K_2 + C) \qquad [6.1]$$

The different models are combined to describe the process of interest in order to evaluate its feasibility and moreover to gain further process understanding. In this way one will be able to assess the bottlenecks of the process and thus to be able to propose solutions to overcome them.

6.3 Case study: asymmetric synthesis of 1-phenylethylamine

The process of interest is the synthesis of 1-phenylethylamine (PEA) and co-product acetone (ACE) from acetophenone (APH) and 2-propylamine (IPA) in the presence of the ω-transaminase (EC 2.6.1.X) (transaminase, ATA - 040). From previous chapters it has been assessed that the reaction is limited by substrate and product inhibition and therefore it would be desirable to introduce an integrated approach of substrate feeding and product removal, using porous resins as an auxiliary phase in a batch reactor. There are three phenomena that take place in this process. First, the interaction of the different compounds with the enzyme (reaction) and second the interaction of the different compounds with the resin (partitioning) and third the rate of substrate supply and product removal (flux). Figure 6.1 displays the general scheme for the production of 1-phenylethylamine with porous resins as the auxiliary phase.

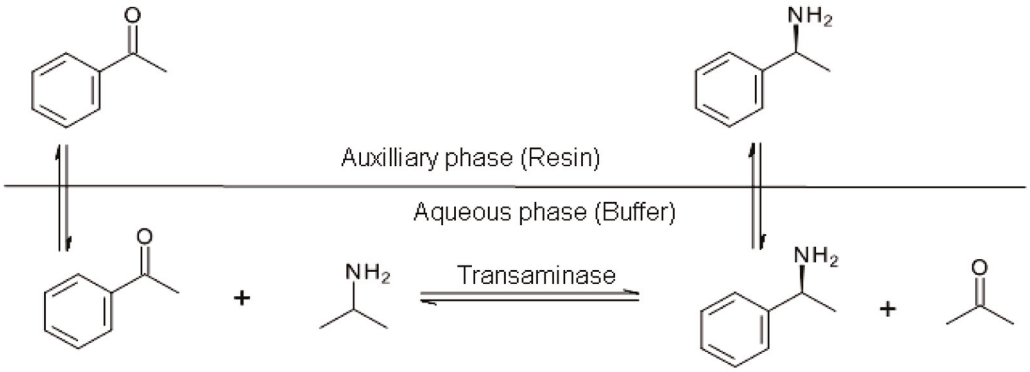


Figure 6.1 Synthesis of 1-phenylethylamine (PEA) and co-product acetone (ACE) from the substrates acetophenone (APH) and 2-propylamine (IPA) in the presence of the enzyme transaminase in an integrated resin-batch reactor

The objective of the process model is to describe the dynamic process behaviour of the reactor and to gain further understanding of the process parameters and in turn identify the process bottlenecks.

6.3.1 Model formulation

Mole balance in aqueous phase

Samples are measured only in the aqueous phase for simplicity and therefore the mole balance is made only in the aqueous phase.

$$dC_{APH}/dt = -Q_{ss}/V - r_{PEA} \quad [6.2]$$

$$dC_{PEA}/dt = -Q_{PR}/V + r_{PEA} \quad [6.3]$$

Kinetic model

The kinetic model for this reaction includes surplus inhibition terms of acetophenone and 1-phenylethylamine. The inhibition has been identified as competitive (data not shown). The kinetic model has been derived as follows (Equation 6.4, $\lambda = \gamma = 1$), while the parameters have been reported in a previous study (Al-Haque et al., 2012):

$$r_{[PEA]} = -r_{[APH]} = \frac{\left[E_0\right]K_{cat}^{f}K_{cat}^{r}\left([IPA][APH] - \frac{[ACE][PEA]}{K_{EQ}}\right)}{K_{cat}^{r}K_{M}^{APH}[IPA]\left(1+\frac{[PEA]}{K_{Si}^{PEA}}+\frac{[IPA]}{K_{Si}^{IPA}}\right)+K_{cat}^{r}K_{M}^{IPA}[APH]\left(1+\frac{[APH]}{K_{Si}^{APH}}+\frac{[ACE]}{K_{Si}^{ACE}}\right)+ K_{cat}^{f}\frac{K_{M}^{PEA}[ACE]}{K_{EQ}}\left(1+\frac{[APH]}{K_{Si}^{APH}}+\frac{[ACE]}{K_{Si}^{ACE}}\right)+K_{cat}^{f}\frac{K_{M}^{ACE}[PEA]}{K_{EQ}}\left(1+\frac{[PEA]}{K_{Si}^{PEA}}+\frac{[IPA]}{K_{Si}^{IPA}}\right)+ K_{cat}^{r}[IPA][APH]+K_{cat}^{f}\frac{K_{M}^{PEA}[IPA][ACE]}{K_{EQ}K_{i}^{IPA}}+K_{cat}^{f}\frac{[ACE][PEA]}{K_{EQ}}+ K_{cat}^{r}\frac{K_{M}^{IPA}[APH][PEA]}{K_{i}^{PEA}}} \quad [6.4]$$

Partitioning model

The compounds APH and PEA are hydrophobic while the other two compounds IPA and ACE are hydrophilic. The selected resin acts on the hydrophobicity of the compounds to partition the substrate APH from the resin phase to the aqueous phase and the product formed, PEA, partitions from the aqueous phase to the resin phase. Adsorption models as shown in Equation 6.5 to 6.7 are generally considered to describe this phenomenon (Likozar et al., 2012).

1. Sips

$$q^* = \frac{q_{\max} C_{eq}{}^{n}}{P + C_{eq}{}^{n}} \qquad [6.5]$$

2. Langmuir

$$q^* = \frac{q_{\max} C_{eq}}{P + C_{eq}} \qquad [6.6]$$

3. Freundlich

$$q^* = q_{\max} C_{eq}{}^{n} \qquad [6.7]$$

It is assumed that all points within the resin remain in local thermodynamic equilibrium and as a result the models shown in Equation 6.5 to 6.7 can be applied to this process (Pedersen et al., 1985). It would be therefore pertinent to discriminate the different models in order to assess which model describes the experimental data best.

Mass transfer model:

The flux for APH from the resin to the aqueous phase can be formulated as Equation 6.8.

$$Q_{SS} = k_{SS}*A*mass*(C_{APH,\ resin} - P_{APH}C_{APH,\ aqueous}) \quad [6.8]$$

The flux for PEA removal from the aqueous phase to the resin phase can be formulated as Equation 6.9:

$$Q_{PR} = k_{PR}*A*mass*(P_{PEA}C_{PEA,\ aqueous} - C_{PEA,\ resin}) \quad [6.9]$$

For both Equation 6.8 and 6.9, the value of the surface area, A, can be acquired from the supplier of the resin as shown in Table 6.3. The value of P can be calculated from the appropriate adsorption model.

6.3.2 Experimentation

Reagents and enzyme

Reagents and solvents of the highest purity available 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 amounts refer to grams of lyophilized powder. The resin Lewatit AF 5 which is carbon based was kindly supplied by Sigma-Aldrich (Buchs, Switzerland). The properties of the resin are listed in Table 6.3.

Table 6.3 Properties of Lewatit AF 5 resin

Matrix structure	Carbon
Ionic form	Neutral
Particle size (mm)	0.4 – 0.8
Pore volume (cm^3/g)	0.15
Surface area (m^2/g)	1200
Density (g/L)	600
Moisture (%)	46.4

Analytical methods

HPLC

Samples were measured on an Ultimate 3000 HPLC (Dionex, Sunnyvale, CA, USA), equipped with a UV detector and a photodiode array detector using a method described elsewhere (See Chapter 4).

GC

Samples were measured online using an internal standard with an autosampler TriPlus, Thermo Scientific, Dreieich, Germany automatically from a flow cell in the product stream. See previous chapter for details on the method.

Experimental methods

Data set 1 for estimating kinetic model parameters

This data set was not required for this case study as the kinetic model with estimated parameters has been acquired from a previous study (See Chapter 4).

Data set 2 for estimating partition coefficients

This data set was acquired from Chapter 5.

Data set 3 for estimating mass transfer coefficients.

150 mg of the resin loaded with saturated concentration of 50 g/L of APH was released into a 3 mL vial containing water. The vials were agitated at 300 rpm at 30^{o}C. Samples were withdrawn at time intervals of minutes 1, 5, 10, 30, 60 and 1440 to determine concentration of APH by HPLC.

50 g/L of PEA was added into a 3 mL vial containing 150 mg of the resin. The vials were agitated at 300 rpm at 30^{o}C. Samples were withdrawn at time intervals of minutes 1, 5, 10, 30 and 60 to determine concentration of PEA by HPLC.

Data set 4 for validation

Two sets of experiments were performed for this data set. Experiment 1) 100 mM of acetophenone was pre-adsorbed with 50% V/V resin. The resin was loaded in a column which was linked to a membrane reactor by an external loop. The membrane had a cut-off point of 10 kDa thus restricting the enzyme to leave the reactor. A reaction media containing 1 M 2-propylamine, 2 mM cofactor (PLP) and 3 g/L ω-transaminase (ATA-40, C-LEcta) was introduced into the reactor. A magnetic bar rotating at 300 rev/min was used to maintain a well-mixed reactor. The reaction started by turning on a pump with the flow rate adjusted to provide a residence time of 1 hour. APH and PEA content was determined via GC. Experiment 2) the conditions were identical to experiment 1 except 300 mM of acetophenone was pre-adsorbed.

6.3.3 Parameter estimation

Estimating partition coefficients

Data set 2 was used to estimate the parameters from the different adsorption models. The model prediction, as can be seen in Figure 6.2, was used further to compare the reliability of the model

prediction to the experimental value by calculating the goodness of fit R^2. From Table 6.4, it can be seen that the Sips model describes the experimental data the best. This was unexpected as the model includes three parameters which were optimized as compared to Langmuir and Freundlich isotherms which consist of two parameters. The Langmuir and Freundlich isotherm correspond similarly with the measured data, as it may be observed from R^2 values in Table 6.2. The results of the partitioning coefficient are in accordance with studies done in an organic solvent such as cyclohexanone (Shin and Kim, 1999b). At pH 7, the partition coefficient of PEA and buffer was 0.21 while for APH was 95.64.

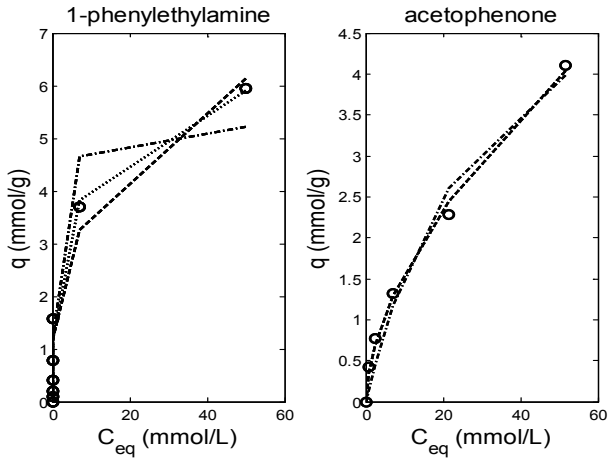


Figure 6.2 Equilibrium data for the adsorption of 1-phenylethylamine and acetophenone on resin, (symbols represent measured data; solid lines represent model prediction using estimated parameters; dotted line (Sip), dash-dot line (Langmuir) and dash-dash line (Freundlich)).

Table 6.4 Estimated adsorption parameters for different models

Parameter	1-phenylethylamine	acetophenone
Sips isotherms		
q_{max} (mmol/g)	8.63	16.36
P ($mmol^n/L^n$)	3.45	38.57
N	0.51	0.57
R^2	0.98	0.99
Langmuir isotherm		
q_{max} (mmol/g)	5.31	6.41
P (mmol/L)	1.02	31.42
R^2	0.95	0.97
Freundlich isotherm		
q_{max} (mmol/g)	1.74	0.42
N	0.32	0.57
R^2	0.97	0.99

From the results of Table 6.4, it was decided to use the estimated parameters of the Sips isotherm in the process model based on the goodness of the fit.

Estimating mass transfer coefficients

Data set 3 was used to calculate the mass transfer coefficient values. The partition coefficients of the Langmuir model from Table 6.4 were used in the mass transfer models. The estimated parameters are shown in Table 6.5, from where it can be seen that the mass transfer coefficient for substrate supply was smaller than for product removal. This is in agreement with other studies using cyclohexanone as a solvent (Shin and Kim, 1999b) and moreover the substrate acetophenone is more hydrophobic than 1-phenylethylamine and thus is bound firmer to the resin. Furthermore it can be seen from Figure 6.3 that the concentration of 1-phenylethylamine in the resin increases like a first-order process until it reaches equilibrium conversion. It can be stated that the reaction is not

limited by mass transfer rate of the substrate release and product uptake. However in cases where the reaction rate is faster than the substrate release rate from the resin, it would be required to improve the process by increasing the available surface area of resin or varying the flow rate through the resin column.

Table 6.5 Estimated mass transfer co-efficient

Parameter	**Value* [m/min]**	**R^2**
k_{SS}	1.19E-5	0.97
k_{PR}	5.43E-4	0.99

*Note: The agitation was fixed at 300 rpm

The estimated parameters were used as input to the process model to provide predictions for different substrate loading. The equations were solved using an ordinary differential equation solver of MatLab™ (ODE15s). Figure 6.3 illustrates the asymmetric synthesis of 1-phenylethylamine using ω-transaminase in an auxiliary resin phase reactor. Two different experimental conditions were simulated with substrate loading of 100 mM and 300 mM. The solid and dashed line indicates the model predictions while the symbols, both round and square, are experimental measurements. It is evident that the model describes the data very well. Initially the substrate concentration in the aqueous phase rises, as substrate is released from the resin until it reaches a pseudo equilibrium. Biocatalytic consumption of the substrate causes more substrate to be released from the resin to compensate the displaced equilibrium. The concentration of the substrate in the aqueous phases is maintained beneath the inhibitory level. The product formed partitions back onto the resin. The concentration is always maintained low to alleviate product inhibition. In this way the reaction proceeds until equilibrium is reached.

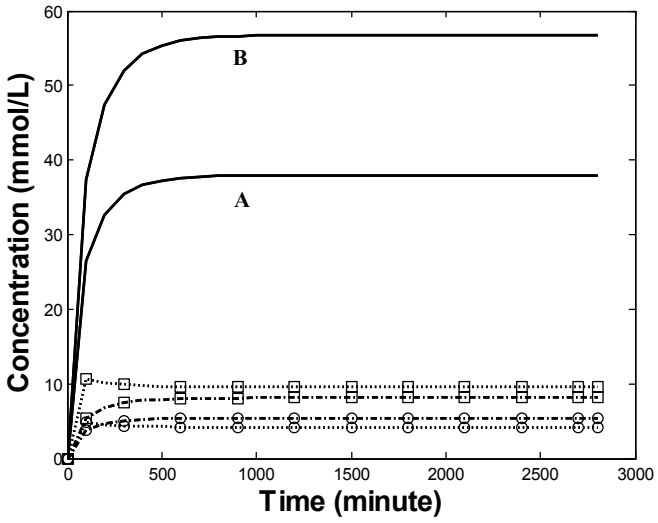


Figure 6.3 Asymmetric synthesis of 1-phenylethylamine using resin as an auxiliary phase. Solid and dashed lines are model prediction. (Solid line) 1-phenylethylamine in resin, (solid-dash line) 1-phenylethylamine in aqueous, (dashed line) acetophenone in aqueous. Square symbol is experimental measurements for condition A and round symbol is experimental measurements for condition B. Experimental conditions: (A) Enzyme = 3 g/L, IPA = 1000 mM, APH (resin) = 200 mM, resin = 50% V/V (B) Enzyme = 3 g/L, IPA = 1000 mM, APH (resin) = 300 mM, resin = 50% V/V

6.3.4 Simulations

Phase ratio of the two phases

Figure 6.4 shows the simulation results to predict the effect of the phase ratio between phases on the reaction rate. In this simulation, the amount of the substrate was fixed irrespective of the volume ratio. Therefore, initial concentration of the substrate in each phase is different with respect to the volume ratio. As the proportion of the solid phase increases, the conversion increases, suggesting that the reaction rate increases rapidly according to the increase in the proportion of solid phase. However, after a certain volume ratio (exceeding 30% V/V), the conversion decreases. This phenomenon occurs because of the decrease in the equilibrium concentration of acetophenone in the

aqueous phase, resulting in the reaction rate to be substrate limited. Such a phenonemna was also observed in the kinetic resolution of α-methylbenzylamine in a bi-phasic mixture of cyclohexanone and phosphate buffer. With increasing phase ratio of cyclohexnone the aqueous concentration of α-methylbenzylamine decreased thus reducing the rate of reaction (Shin and Kim, 1997).

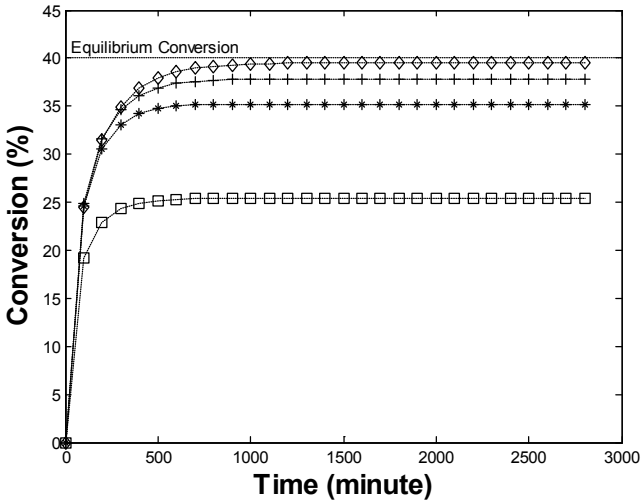


Figure 6.4 Asymmetric synthesis of 1-phenylethylamine using resin as an auxiliary phase at different volume ratio square symbols (10% V/V), diamond (30% V/V), plus (50% V/V), star (70% V/V)

Partitioning of 1-phenylethylamine and acetophenone in the resin

The influence of the partitioning coefficient plays a vital role in the case of substrate supply and product removal. Figure 6.5 illustrates the impact of varying the ratio of the partition coefficient. From Figure 6.5 it can be seen that in cases, where the partition coefficient of acetophenone is higher than the product 1-phenylethylamine, the reaction is substrate limited resulting in lower conversion. However, in cases where the partition coefficient equals each other than the maximum conversion that can be attained would be the equilibrium conversion. However, if the resin is more

selective for the product, then then resin can be used for driving the reaction towards product synthesis to achieve conversions that is not limited by equilibrium.

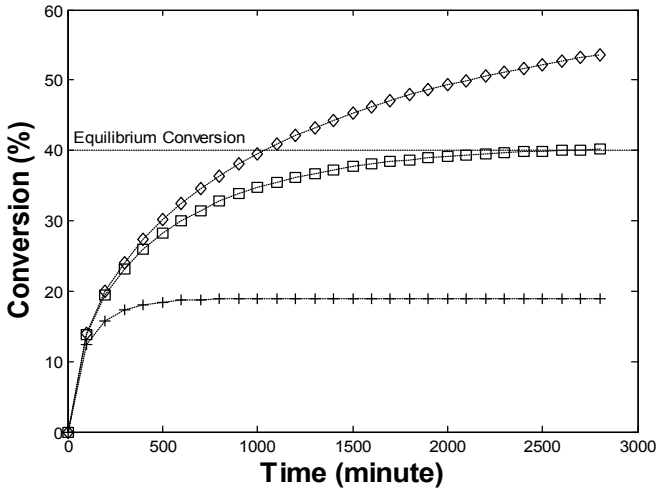


Figure 6.5 Asymmetric synthesis of 1-phenylethylamine using resin as an auxiliary phase at different distribution coefficient ratio. Plus symbol (P_{APH}/P_{PEA} = 10), square symbol (P_{APH}/P_{PEA} = 1) and diamond symbol (P_{APH}/P_{PEA} = 1/10)

For substrates and products that are moderately polar, it is difficult to find a suitable auxiliary phase that can selectively remove the target product (Straathof, 2003). An experimental methodology to identify a suitable resin has been demonstrated in Chapter 5. Selection based on the properties of the polymer such as the degree of crystallization and thermodynamic affinity has also been exploited (Parent et al., 2012). However, efforts need be placed in designing auxiliary phases that are compatible with this strategy. From Figure 6.5 it could be seen that the selective removal of the product is essential to achieve high yields. Efforts need to be placed at tailoring resins to effectively distinguish between substrates and products. Such an approach has advanced in the field of molecular imprinted polymers for selective extraction (Lasakova and Jandera, 2009; Masqué et al., 2001). Molecular imprinting in polymers is a process in of polymerising functional and cross-linked monomers around a template molecule in order to generate specific recognition sites for specifically

rebinding the target molecule (Turiel and Martin-Esteban, 2010). Such a technology has advanced in different industrial sectors among which pharmaceutical is one (Lasakova and Jandera, 2009). However, low capacity and slow mass transfer are the bottlenecks that have yet to be overcome (Chen et al., 2011).

6.4 Case study: Enantioselective synthesis of 2-octanol

Enantiomerically pure (R)-2-octanol is produced from 2-octanone using the enzyme LbADH (EC 1.1.1.2). The enzyme is co-factor dependent and thus the co-factor has to be recycled to make the process economically feasible. An enzyme coupled approach was selected based on the high selectivity and catalytic efficiency using the enzyme GDH (EC 1.1.1.47). Co-substrate glucose is used as a substrate for GDH. From previous chapters it has been assessed that the reaction is limited by substrate inhibition and therefore one proposal was to use a continuous stirred tank membrane-reactor to overcome the kinetic limitation. Resin is not required in this case study as the reaction is not compromised by product inhibition. Therefore there is only one phenomena taking place in this process i.e. the reaction. The schematic of the reaction is shown in Figure 6.6:

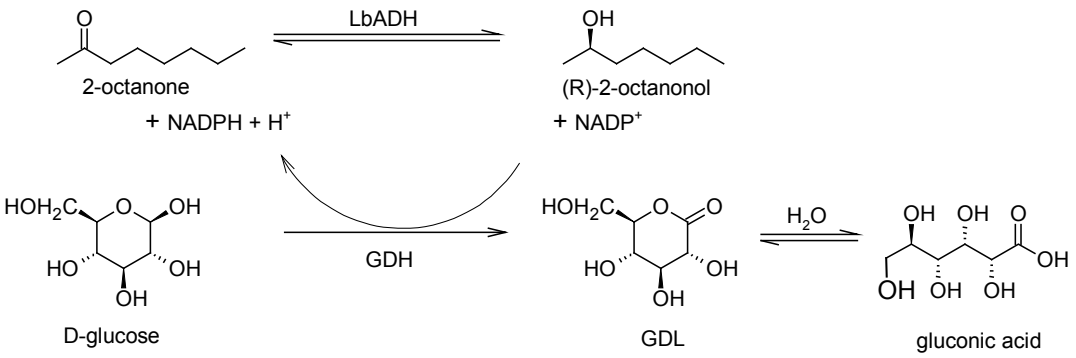


Figure 6.6 Reaction system for the enantioselective reduction of 2-octanone

6.4.1 Model formulation

Mass balance

A CSTR model is used for the mass balance.

$C_0 - C + r_1 V = 0$

Kinetic model

The kinetic model to describe the reaction has already been described in section 4.6. The final modified Michaelis-Menten equation is as shown below in Equation 6.10 and 6.11.

Forward direction:

$$r_{LDH,f} = V_f \frac{[ON]}{K_M^{ON}\left(1+\frac{[OL]}{K_P^{OL}}\right)+[ON]\left(1+\frac{[ON]}{K_i^{ON}}\right)} \cdot \frac{[NADPH]}{K_M^{NADPH}\left(1+\frac{[NADP]}{K_P^{NADP}}\right)+[NADPH]} \quad [6.10]$$

Reverse direction:

$$r_{LDH,r} = V_r \frac{[OL]}{K_M^{OL}\left(1+\frac{[ON]}{K_P^{ON}}\right)+[OL]} \cdot \frac{[NADP]}{K_M^{NADP}\left(1+\frac{[NADPH]}{K_P^{NADPH}}\right)+[NADP]} \quad [6.11]$$

A similar model was used for the GDH reaction.

$$r_{GDH} = V \frac{[Glu]}{K_M^{Glu}+[Glu]} \cdot \frac{[NADP]}{K_M^{NADP}+[NADP]} \quad [6.12]$$

The model parameters have been estimated previously as presented in Section 4.6.

6.4.2 Simulations

In the simulation of the synthesis of 2-octanol, cofactor NADPH degradation of 4.1 x 10^{-6} h^{-1} and enzyme deactivation of 1.92 x 10^{-5} h^{-1} was determined (data not shown).

Effect of the concentrations of enzyme and co-factor NADP

The kinetic model developed can be used in determining the optimal initial concentrations of the enzymes and co-factor. At a given 2-octanone concentration, the use of excess of enzymes or expensive cofactor should be avoided. The optimal concentrations of cofactor NADP and enzyme ratio of LbADH/GDH were evaluated to identify which combination can provide the highest conversion. Figure 6.7 shows the effect of varying the enzyme ratio and NADP on conversion at a fixed concentration of 2-octanone. The ratio of enzyme concentration displays a higher sensitivity to conversion than with NADP. At sufficient concentrations of NADP approximately above 0.025 mM and enzyme ratio above 1, in the case of 60 mM of 2-octanone, the increase in the concentration of NADP nor enzyme ratio increases conversion.

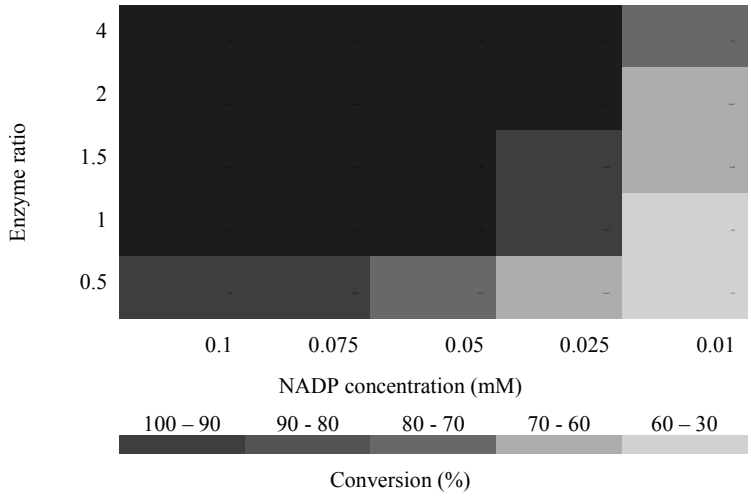


Figure 6.7 Optimization of enzyme ratio and co-factor concentration as a function of conversion Reaction conditions: 60 mM 2-octanone and 675 mM glucose

Effect of reaction engineering

The modelling of the reaction kinetics as a tool for reaction engineering plays an important role in assessing the feasibility of processes (Al-Haque et al., 2012). The modelling also facilitates identification of the most effective reactor mode (Vasic-Racki et al., 2003b). From the estimated parameters of Table 4.6, it was seen that the substrate surplus inhibition of 2-octanone was evident. In order to overcome this limitation it was envisaged that operating in a continuous stirred tank reactor (CSTR) would be beneficial. CSTR are a favourable reactor configuration in view of enzyme deactivation that is introduced by one of the substrates, since operating at high conversion in the steady state yields a constant low substrate concentration. Consequently, the rate of deactivation should be very small. However, a general limitation of using CSTR is that a full conversion cannot be achieved unless the residence time is extremely large or more than one CSTR is used in series to mimic a packed bed reactor. The results of the optimized reaction conditions from section 6.5.1 were used to simulate the reaction in CSTRs. Figure 6.8 illustrate the model simulations of conversion as a function of residence time. The simulation is evaluated for both a single CSTR and two CSTR in series.

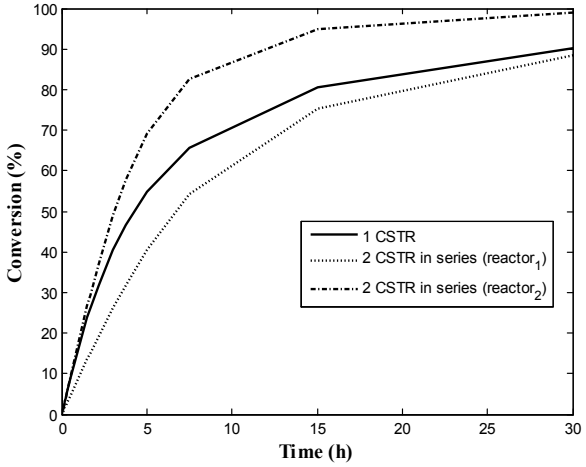


Figure 6.8 Conversion as a function of residence time in continuously stirred tank reactors (CSTR). Black line is one CSTR and black line is two CSTR in series (dotted is first CSTR and dash-dot is second CSTR)

From Figure 6.8 it can be seen that with a single CSTR the maximum conversion that can be reached is less than 90%. However, the time required was more than 24 hours. With the help of simulation it could be seen that adding another CSTR in series would enable reaching almost a 100% conversion within 24 hours.

The cascade of two membrane reactors in series can be proposed for the substrate inhibition problems. The system consists of two equally sized continuously operated enzyme membrane reactors with enforced flow where the retention of soluble enzyme in the reaction vessel is achieved by means of an ultrafiltration membrane (Yuryev et al., 2011). An advantage of this system is that the reactor operates under sterile conditions without diffusion limitations enabling continuous homogeneous biocatalysis with a very good exploitation of the biocatalyst.

6.5 Discussion

Process modelling plays a key role in selecting the most effective process design. Process models can be used to simulate for different reaction conditions or to study the implications of different process designs. These kinds of simulations can reduce the number of experiments for optimizing reaction conditions and predict experimental results in ranges where experimental design is difficult or cumbersome. The process model can be broken down into sub problems, to reduce the complexity of the mathematical formulation and also to help design experiments more effectively. Each sub-problem can be solved individually to provide a greater insight into the process. The result of each core is added to the subsequent core to include the observed phenomenon in the process model. Such an approach can be used to identify the bottlenecks of of each step of the process and propose solutions to overcome it.

The process modelling of the ω-transaminase case study helped in analysing critical parameters such as mass transfer rate, solid-liquid volume ratio and partitioning coefficients. In this case study, there was no observed mass transfer limitation as the enzymatic reaction is relatively slower. Substrate and product inhibition could be mitigated using resins, however the partitioning coefficient of the resin was not effective enough to overcome the equilibrium constraint. Therefore more than one process strategy or more selective product removal technologies such as molecularly imprinted polymers or a sequential cascade reaction can be applied to displace the equilibrium towards the synthesis direction.

The case of the bi-enzymatic synthesis of 2-octanol serves as an interesting case study, in which process modelling has helped in meeting the challenges to facilitate large scale production. In redox reactions, the cost of the co-factor is a typical barrier to implementing this kind of chemistry at large scale. Through process modelling, it was possible to optimise both the concentration of the co-factor and the enzyme ratio in order to generate the highest conversion. An enzyme ratio of 1.5

[LbADH] per [GDH] in the presence of 0.05 mM of NADP can yield conversions of higher than 90%. The results of this simulation were directly inputted to the process model in order to simulate strategies to overcome substrate inhibition. By means of simulation, it could be seen that using a CSTR would be beneficial for reactions which suffer from enzyme deactivation through one of its substrate (2-octanone). Furthermore, it was evaluated that to reach an almost 100% conversion two CSTRs have to be used in series. It would be therefore proposed to use two membrane reactors in series with soluble enzymes. In this way the overall productivity can be maximised.

6.6 Conclusions

Process modelling was an effective tool for analysis and gaining process knowledge for the two cases studies. In the first case study, the process model helped to evaluate the trade-offs between the thermodynamic partitioning and the three rate equations (substrate supply, product removal and rate of reaction). The predictions provided the necessary information required to optimize the process and set the required partitioning coefficients and phase ratio required for the process. The rate of supply and removal were not a limitation for this relatively slow reacting enzyme. The limitation for the process was the low partitioning of PEA over APH to the resin. Further efforts have to be made to design resins to display similar or higher partitioning for PEA with respect to APH. Process modelling in the second case study helped in optimizing the process in terms of co-factor concentration and enzyme ratio. Two membrane reactors in series were proposed in order to overcome the substrate inhibition problem and achieve almost a 100% conversion within 24 hours.

Chapter 7

General discussion

The main goal of this thesis has been to explore the possibilities of integrating resins in enzymatic processes. A systematic framework has been developed in characterizing the reaction, selecting a resin and evaluating the process as a means of justifying the integration of resin in enzymatic processes. Three case studies were used to illustrate the applicability of the methodology to fulfil different objectives. The case studies were selected not only for being industrially relevant but as well having certain challenges, through which process tools and strategies could be developed to overcome these challenges. Every aspect of the framework was tested: characterising the reaction by kinetic modelling, selecting the resin and evaluating the process using a model based approach.

Biocatalysis in many cases presents as an excellent alternative to chemo catalysis, especially when the key concern is stereo- or regio- selectivity. Several challenges pertaining to low specific catalyst activity for some enzymes (transferases), in the presence of industrially relevant concentration and yield limited to equilibrium conversion, can discourage industries to consider this option. However several solutions can be suggested to overcome these challenges to harness the full potential of biocatalysis. Some solutions can only be met by protein engineering while others can be alleviated by reaction engineering options. In many cases, a combination of both solutions has to be implemented to tackle difficult synthesis. A decision tree, as presented in Chapter 1 (Figure 1.9),

can help to ease the selection of the appropriate process options, guide data collection and identify key parameters that have to be considered (e.g. phase toxicity, mass transfer, inhibition).

Using a non-selective auxiliary phase is an interesting option to improve the viability of the enzymatic processes, which are limited by low catalyst productivity due to substrate and product inhibition. It is an integrated approach of substrate feeding and product removal. However, attempts to introduce any technology require some considerations to be taken into account. Several parameters, such as resin capacity, product selectivity, enzyme biocompatibility and cost effectiveness have to be considered. In some cases, using resin would be advantageous while in other cases organic solvent or ionic liquid could be the better option. The choice of the auxiliary phase has to be done on a case by case basis. For example, in the enantioselective microbial reduction of 3,4-methylene-dioxyphenylacetate to the corresponding (S)-alcohol using *Zygosaccharomyces rouxii,* Cp,crit was experimentally measured to be 2 g/L indicating the need for ISPR (Zmijewski et al., 1997). A water-immiscible organic solvent, hexane, provided a distribution coefficient of 1, thus was unsuitable for extraction. On the other hand, porous resin XAD-7 gave distribution of around 30 – 40 (Vicenzi et al., 1997) which resulted in many fold increased volumetric productivity of the process.

In order to choose a process option, the reaction has to be at first characterized. The critical concentration and aqueous solubility of the different components have to be evaluated. Kinetic modelling, which takes into account inhibition and equilibrium relation, is an effective method for determining the operability space of the reaction. When trying to estimate the kinetic model parameters, it was realized that estimating them is not straight forward. In fact, the global minimum can be rarely attained for such complex models and thus often leading to identifiability issues of the parameters. Different strategies are required, including model decomposition and multiple parameter initializations and consequently both initial rate and progress curve data are required to

increase the probability of attaining a global minimum. In order to evaluate the reliability of all parameters in the model, different types of analysis have been suggested. In that manner, results are used to prioritize further analysis of significant parameters or to consider model simplifications of insignificant parameters, all with a statistical support, as illustrated in Chapter 4. The methodology developed was indeed found to be more robust and provided reliable predictions in comparison to other approaches. A parity plot comparing the different approaches can be seen in Figure 7.1.

Figure 7.1 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 developed approach which displays a better fit to describe the experiment. In the Chen methodology, the prediction fits quite well in the initial time period, but with increasing time the reversibility of the reaction, combined with the substrate and product inhibition, contributes to deviations from the prediction.

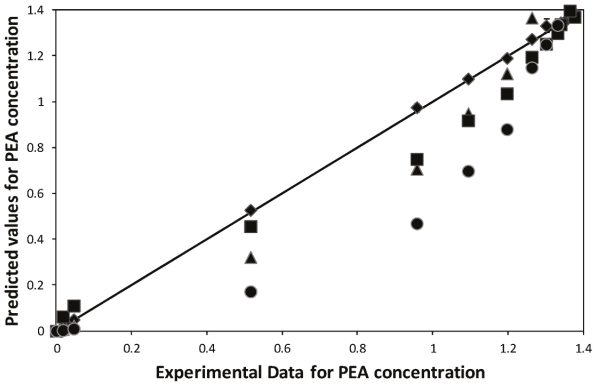


Figure 7.1 Parity plot of different estimation methodologies.
Proposed methodology (diamond), graphical approach (triangle), non-linear regression (square) and Chen and co-worker (circle). Reaction conditions: E_0 = 1.8 g/L, C_{IPA}=1000 mM , C_{APH} = 2 mM, 2 mM PLP, 100 mM phosphate buffer (pH 7)

Designing smart experiments is a key part of developing any kinetic model. Much of the effort is required for designing the experiments, performing the experiments as well as analysing the data.

Type of experiments (initial rate or time course), what to measure (which components) and how often to measure (every minute or every 30 minutes) are important considerations for data collection. Consequently, it would be suggested to decompose the complex enzymatic kinetic model to initial rate model, to minimise the number of parameters to be estimated simultaneously and moreover to be able to design experiments tailored for the model. In this way the number of experiments required can be optimized. Table 7.1 depicts the number of experiments that were required for estimating the kinetic model parameters of case study 1.

Table 7.1 Different methodologies to estimate kinetic model parameters for equilibrium reactions

Type of experiment	Methodology	Number of experiments	Limitations
Initial rates	Graphical plotting	High (130)[a]	Applicable only for reactions that follow simple Michaelis-Menten kinetics. High estimation errors
	Non-linear regression	High (125)[b]	Low accuracy when parameters are correlated
Progress curves	Non-linear regression	Low (20)[c]	Reliability is dependent on the need for good initial guess of parameters
Initial rates and progress curves	Chen and co-workers	Intermediate (43)[d]	Unreliable for equilibrium reactions including regions of negligible inhibitory region
	Proposed approach	Intermediate (52)[e]	The prediction of K_{EQ} is subjected to greater uncertainty as compared to obtaining it by experimentation

[a](Gyamerah and Willetts, 1997)
[b](Shin and Kim, 1998)
[c] The minimum number of data points corresponds to the number of parameters
[d](Cheng et al., 2012)
[e](Al-Haque et al., 2012)

Initial rate models are not sufficient in large scale processes where high conversion of the substrate is desired. An initial reaction rate model cannot be used to accurately predict neither the overall reaction rate nor the equilibrium of the reaction. It could be seen that for the third case study, the model prediction towards the end of the reaction was deviated from the experimental data (Figure 4.11). It would therefore be suggested to use a dynamic model which can preferably describe the

rate of reaction as a function of all relevant components at all relevant concentrations, taking into account the effect of the equilibrium relation in the equation.

Using porous resin based on the chemistry for binding the substrate and product is a non-selective route. Therefore, for equilibrium limited reactions such as transaminase, more than one strategy is required to drive difficult reactions, such as transaminase catalysed reaction, to completion. Selecting the appropriate option has to be done in a systematic manner. Adding an excess of the substrate and simultaneous removal of co-product by enzymatic or physical means have to be evaluated on a case by case basis. A key parameter being the reaction equilibrium, K_{EQ}, can in many instances provide the headspace of the process flow sheet. Figure 7.2 shows the relationship between K_{EQ} and maximum allowed co-product concentration to achieve 90% yield when using an initial concentration of 1M ketone and a 10-fold excess of amine donor for the synthesis of a chiral amine.

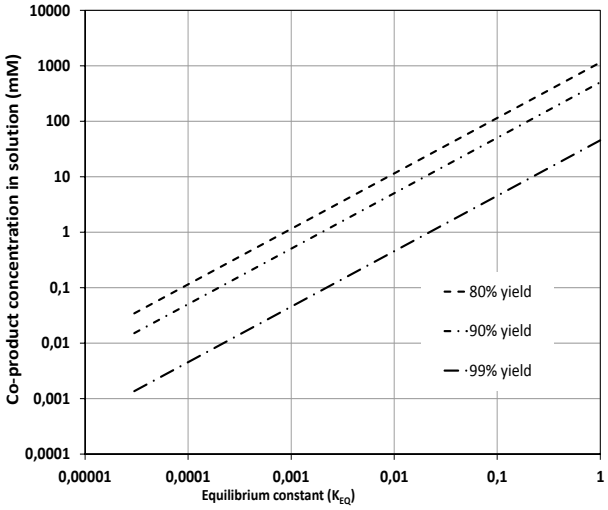


Figure 7.2 Concentration of allowed co-product to reach 90% yield when using an initial concentration of 0.5 M ketone and a tenfold excess of amine donor for the synthesis of chiral amine

As it can be seen in Figure 7.2, at K_{EQ} values of 0.01, the allowed co-product concentration will need to be <10 mM. This information can be then used by process engineers to evaluate alternative processes for shifting the equilibrium.

Selecting resins for the transaminase case studies is quite challenging because of the similarity in the properties between the substrates and products. It was observed that the capacity of the resin and the selectivity for the product had an inverse relationship. Figure 7.3 illustrates the tendency of all the resins that were investigated for the transaminase case studies.

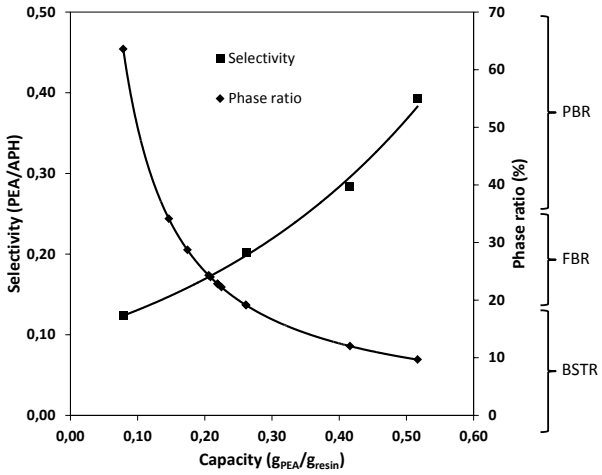


Figure 7.3 Selectivity of product and phase ratio as a function of resin capacity

As it can be seen in Figure 7.3, a trade-off has to be made between capacity and selectivity, which can be crucial from an economic point of view. Low resin capacity points towards the need for higher resin loading. The phase ratio in turn determines the equilibrium concentration of the substrate in the reactor and the type of reactor that can be used (stirred tank reactor or packed bed reactor). In the first case study, it was evident that the selectivity for the product was lower than for the substrate, leading to the requirement of a resin loading of 40% (V/V) in a packed bed reactor. On the other hand, for the second case study 20% (V/V) of resin loading was sufficient to maintain

a substrate and product concentration below the critical concentration. A stirred tank reactor would be suggested for such a case. A further key finding was that the resin had a relatively strong affinity for the enzyme studied in this thesis. A membrane separated reactor or immobilized enzymes would be suggested for ω-transaminase reactions. Alternatively, whole cell catalysis can be opted, which would not penetrate into the micro pores of the resin.

Process modelling of biocatalytic processes was the third aspect that was incorporated in the framework. Extremely valuable information can be acquired by means of modelling. In the case of integrated resin-enzyme processes, the rate of substrate supply and product removal can be a bottleneck of the system. However, what was realized from the evaluation is that the reaction was not mass transfer limited. The rate of reaction for this enzyme is much lower than the rate of substrate supply, and therefore for such systems using resins would not pose as a problem. Alternatively, if the enzyme was 10 times faster, the reaction would be mass transfer limited. For such cases, it is possible to increase the surface area by using resins with smaller pores, otherwise organic solvents would be a better choice. In the second case study of 2-octanol production, the reaction was challenged with substrate inhibition. In such cases, a classic reaction engineering approach can be applied, in which using a continuous stirred tank reactor (CSTR) would overcome the substrate inhibition problem. A CSTR operates at low substrate concentration, so the rate of enzyme deactivation on account of substrate inhibition would be minimised. Process modelling can allow investigating such scenarios without the need of practically doing the experiments. The results should be validated experimentally to confirm the simulations. Modelling should be a tool applied at all scales of the process to help decision making easier and also to facilitate the transition from lab scale to pilot scale experiments.

Chapter 8

Conclusions

8.1 Conclusions of the framework

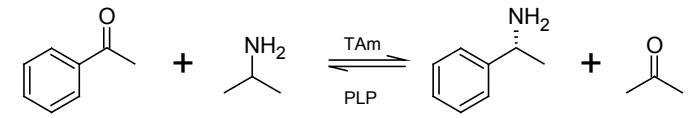
The main topic of interest of this thesis is the integration of porous resins in enzymatic processes. It is indeed an interesting option which however has been limited to only single substrate and single product systems (reduction, oxidation and microbial degradation). The concept has thus been expanded to multi-component systems in this thesis, in order to create a generic platform for using this technology in enzymatic processes. The first decision of selecting the appropriate process option among a pool of process options can be quite challenging. Furthermore, if porous resins are an option, then how can one choose the porous resin and moreover how to integrate into a process? With that context, a systematic framework has been developed in this thesis in order to address such issues of when, why and how to integrate resins in bioprocesses.

The framework provides guidance for the process development in a systematic way. The main problem is broken into five steps where the solution of each step provides an input to the subsequent step. The key parameters of a process can be identified by going through those steps and the suggested tools of each step can effectively guide data collection. Three tools have been developed for this framework: i) A robust methodology for kinetic model parameter estimation, ii) A systematic approach for selecting resins and iii) Process evaluation using a model based approach. For the first tool, the methodology developed can assist in estimating kinetic model

parameters which can predict initial rate and progress curve data sets. 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. Apart from the robustness of the methodology, the number of experiments required was optimized to be much lower than other approaches. The second tool of resin screening provides a heuristic approach to select resins for multi-component systems. The different criteria for selecting resins for this approach have been stated and proved to be adequate. With the third tool of process modelling it was possible to identify important parameters such as phase ratio, mass transfer coefficients for the integrated resin-enzyme approach and reactor configuration (internal or external) and type (batch or flow reactor). The framework has been validated with three case studies in this thesis. Although this framework has only been applied to two types of enzymatic classes (transferase and ketoreductase), it is possible to apply this framework for the evaluation and implementation of other biocatalytic reactions, which have similar characteristics as the studied cases.

8.2 Conclusions of case study 1: asymmetric synthesis of 1-phenylethylamine

This case deals with the synthesis of optically pure chiral 1-phylethylamine using the enzyme ω-transaminase (TAm). The enzyme requires co-factor pyridoxial phosphate (PLP) which is regenerated within the reaction. The overall reaction scheme for this case is:



8.2.1 Reaction characterization

The key reaction characteristics obtained for this case are the following:

First, the reaction is challenged with an unfavourable thermodynamic equilibrium towards the synthetic direction and has been estimated to be 0.03.

Second, the reaction is further challenged with both substrate and product inhibition limiting the productivity of the catalyst. Substrate concentration (APH) above 10 mM results in more than 50% loss in initial activity. Similarly, production concentration (PEA) above 30 mM inhibits the enzyme.

Third, the substrate APH has an aqueous solubility of 40 mM. Supplying substrate beyond the saturation point denatures the enzyme.

The reaction therefore presents an interesting case where the inclusion of a non-selective porous resin will help to achieve the high substrate loading required to be an economic process and simultaneously alleviate substrate and product concentration below the inhibitory concentration to improve the productivity of the catalyst.

8.2.2 Resin screening

The different components of the reaction were investigated to reveal that the components acetophenone and 1-phenylethylamine were moderately hydrophobic (Log P ≈ 1.50 while 2-propylamine and acetone were hydrophilic (Log P < 0).

Porous resins found from the Sigma Aldrich catalogue with differing surface areas, pore diameter and matrix types were selected for screening. Lewatit AF5 displayed the highest resin capacity (2.3 mmol/g) and moreover was minimal selective for co-substrate 2-propylamine and co-product acetone (S < 0.5). Furthermore, the resin was stable with no loss in performance after 5 batches.

30% phase ratio was required in order to maintain substrate and product concentration within limits. The resin showed affinity for the enzyme as well (20% protein content was adsorbed) and thus the

resin had to be compartmentalized in an external column separated with an ultrafiltration membrane for the process.

An experiment performed with 50 g/L (0.4 M) APH with the integration of the porous resins, resulted in yields comparable to equilibrium yields (26%) as opposed to the control experiment (no resins), where less than 2% yield was achieved.

8.2.3 Process evaluation

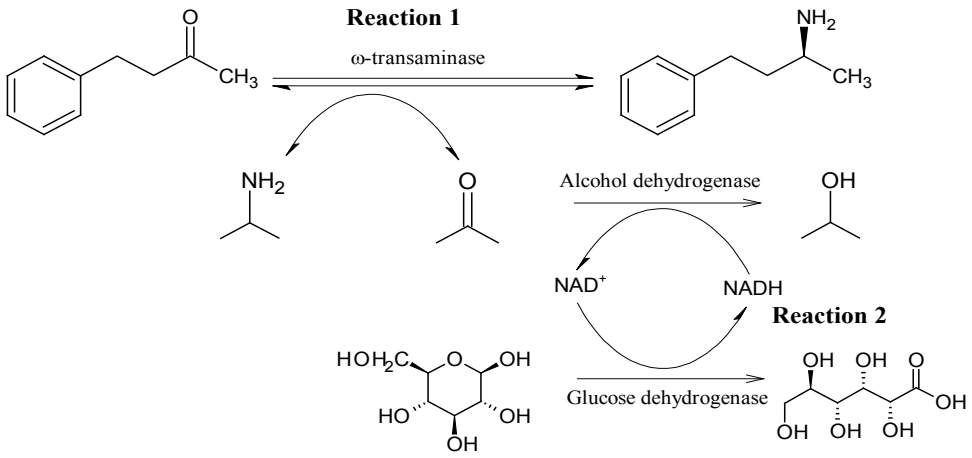
Three different models were tested to investigate which model best describes the adsorption isotherms of acetophenone and 1-phenylethylamine. Sips isotherm model presented the best fit of the experimental data ($R^2 \approx 1$). The distribution coefficient for PEA and APH was estimated to be 1.02 and 34.41 respectively. This is in agreement with other studies using cyclohexanone as a solvent (Shin and Kim, 1999b) and moreover the substrate acetophenone is more hydrophobic than 1-phenylethylamine and thus is bound more firmly to the resin.

The mass transfer coefficient for substrate supply was smaller than for product removal. This is in accordance with the distribution coefficient previously estimated. However, the reaction was not mass transfer limited as the rate of product formation steeped exponentially upwards.

The load ratio was investigated further and based on simulations it could be seen that 30% (V/V) was the optimal setting for this process. Similarly, the distribution coefficients were varied in simulations to predict the output. The very low distribution of PEA was indeed not adequate to reach equilibrium yields. Distribution coefficient for PEA is required to be similar to APH to reach equilibrium yields or higher to attain conversion beyond that.

8.3 Conclusions of case study 2: asymmetric synthesis of 1-methyl-3-phenylpropylamine

This case deals with the synthesis of optically pure chiral 1-methyl-3-phenylpropylamine, a product produced by Cambrex. The reaction was done using enzyme ω-transaminase (TAm) immobilized by CLEA. The overall reaction scheme for this case is:



8.3.1 Reaction characterization

The reaction characteristics obtained for this case are the following:

First, the reaction is challenged with an unfavourable thermodynamic equilibrium towards the synthetic direction and was found from literature to be 0.59.

Second, the reaction achieved less than 70% conversion with a starting substrate concentration of 6 mM on account of inhibition (equilibrium yield should be around 99%).

Third, the substrate 4-phenyl-2-butanone has an aqueous solubility of 12 mM. Supplying substrate beyond the saturation point denatures the protein of the enzyme.

The reaction therefore presents an interesting case where the inclusion of a non-selective porous resin will help to achieve the high substrate loading, required to be an economic process and simultaneously help to keep aqueous substrate and product concentration below 6 mM.

8.3.2 Resin screening

Similar to the previous case study, the key substrate and product were moderately hydrophobic (Log P > 1) while the other components were hydrophilic (Log P < 1).

Three different resins were selected having surface area greater than 1000 m^2/g, of which resin Lewatit AF5 proved to have the best capacity (1.65 mmol/g). The resin showed moderate selectivity for the key substrate and product ($S \approx 1$) while low for the other components ($S < 0.1$).

A reaction mixture of 50% conversion was simulated to test different phase ratio which would correspond the final aqueous substrate and product concentration in the reactor to be less than 6 mM. 20% phase ratio was determined to be optimal to keep substrate concentration to be 0.18 mM and product 1.67 mM.

8.3.3 Process evaluation

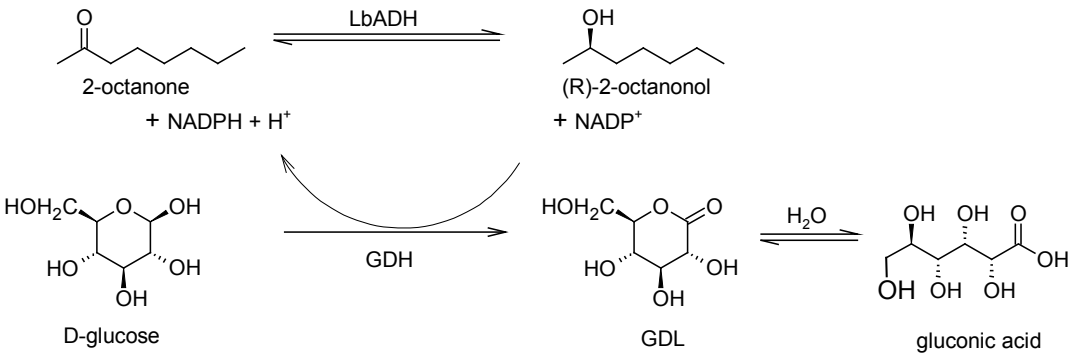
Integration of porous resins in the enzymatic process resulted in yields of around 40%. Higher yields could not be achieved because at the end of the reaction the concentration of 1-methyl-3-phenylpropylamine in the aqueous phase was measured to be around 8 mM causing the enzyme to be inhibited. Two strategies were attempted to improve the yield further, by removing acetone *in-situ*: sweeping saturated nitrogen over the reaction media and adding a selective enzyme (reaction 2) in sequence with reaction 1. The strategies proved to be successful to improve the yields to around 70%. However, it was observed that the volume of the reaction media reduced on account of nitrogen sweeping while the activity of the cascade enzyme was quite poor for which reason 20 g/L

of the enzyme was added. Higher concentration could not be added as it was precipitating in the reactor.

From the results it points out that multiple strategies are required in order to achieve yields higher than equilibrium. The problem set forth from this case study indicates the necessity of evolving the cascade enzyme to improve the specific activity in order to minimize the enzyme loading. On the other hand, efforts could be placed in engineering the resins to be more selective for the key product and thus help in shifting the equilibrium as well.

8.4 Conclusions of case study 3: enantioselective synthesis of 2-octanol

Optically pure 2-octanol is synthesized from 2-octanone using the enzyme LbADH (EC 1.1.1.2). The enzyme is co-factor dependent and thus the co-factor has to be recycled. An enzyme coupled approach was selected based on the high selectivity and catalytic efficiency using the enzyme GDH (EC 1.1.1.47). Co-substrate glucose is used as a substrate for the GDH enzyme. An overview of the reaction scheme is shown as follows:



8.4.1 Reaction characterization

The key reaction characteristics of this reaction have been identified as follows:

First, the substrate 2-octanone was inhibiting the enzyme. Concentrations above 10 mM strongly reduced the initial activity of the enzyme.

Second, the reaction was favourable in the synthetic direction, which was realized with a higher Vmax value in the forward direction than the reverse direction.

Finally the aqueous solubility of 2-octanone was poor (5 mM), for which a co-solvent (ionic liquid) was added to the reaction to increase the solubility to 60 mM.

8.4.2 Process evaluation

Effective utilization of the co-factor is a major drawback for these kinds of redox reactions. The co-factor concentration and enzyme ratio were optimized by using process modelling. The co-factor concentration could be reduced to 0.025 mM while maintaining enzymes ratio of 1:1.5.

By means of process modelling, it was revealed that substrate inhibition was a limitation to this process, thus using non-selective resins would not be suggested. Alternatively, continuous stirred tank reactors which work in low concentration of substrate were suggested. Coupling two CSTR's suggested the possibility of attaining almost a 100% conversion within 24 hours.

Chapter 9

Future work

Biocatalysis is now frequently considered as an option in the synthesis of complex molecules. Some of the major limitations that were once considered such as inhibition, sparingly water soluble substrates can be solved by introducing resins as an auxiliary phases as has been discussed in this thesis. Majority of the research has been conducted in whole cell catalysis where the reaction will not be mass transfer limited. However, soluble enzyme benefits of fast kinetics. It would be interesting to investigate the influence of bead size, geometry and type of resin on the reaction.

From the screening of resins it was found that a single resin could not be identified to be called the 'super' resin. It would be desirable that research efforts are placed in tailoring resins to fit the process requirements. This concept is being developed with solvent design and it would be also interesting to expand it towards designing a resin which can be applied not only based on hydrophobicity and ionization but also based on the chemistry. Screening kits for selecting resins could be devised.

Furthermore with the increased interest of using resins as an auxiliary phase, it would be very beneficial to create a standardized database. Generally the supplier of the resin can only provide specific details regarding the resin such as functional properties and physical properties. If a database could be made available with the type of resin, target molecule, partition coefficient for all reactants and capacity for substrate then it would help facilitate the efficient selection of resin.

Resins alone are not the only auxiliary phase that is used for processes. It would also be interesting to investigate organic solvent and ionic liquids as auxiliary phases. A clear limitation of liquid phase auxiliary phase is the compatibility issue. Even though the number of 'green' solvents is not so many but it still is a challenge in identifying a solvent which does not negatively impact the enzyme. Solvent selection methodology directed towards biocatalysis would be an important tool to develop.

With the development of different auxiliary phases, it would be interesting to investigate multiphasic system. A cascade of reactions could be carried out with the substrates immobilized in different auxiliary phases loaded in a packed bed reactor. This would give rise to interesting reactor designs and process options.

An ideology by protein engineers are that inhibition of an enzyme can be solved by altering the protein structure of the enzyme. This may be true within the next five years. However, the thermodynamic equilibrium is independent of the enzyme and depends on the reaction. *In-situ* product removal would prove to be a powerful tool for biocatalytic reactions. It would help to integrate the process, increase product concentration and thus make downstream processing easier. It could be envisaged to devise micro scaled reactors to test different configuration of *in-situ* product removal. In this way one would be able to assess the different options without spending expensive resources such as chemical and enzymes.

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Appendix 1

Reaction mechanisms

Monosubstrate enzyme kinetics

This appendix compiles the reaction rate expressions for some of the enzyme mechanisms that are used in this thesis. Specific definitions used are elaborated to make it easier for the reader to follow the text.

For a simple single component system where the substrate S is catalysed by enzyme E, the reaction mechansim can be illustrated as shown in Equation A.1

$$E + S \rightleftharpoons ES \rightarrow E + P \qquad [A.1]$$

It is assumed that the first step of the reaction is reversible and fast enough to reach equilibrium to form a complex ES. The second step in the reaction is asumed to be slower than the first treaction and considered to follow a first-order reaction which forms the product P. These two assumptions gave rise to the so called ”michaelis-menten rate” equation as shown in Equation A.2 (Michaelis and Menten, 1913).

$$r = \frac{V_m C_S}{K_M + C_S} \qquad [A.2]$$

Particular derivations of the reaction rate expressions are obtained when the enzyme isinfluenced by substrate (s) and product (s) (e.g. enzyme inhibition). In this thesis only linear inhibitions have been encountered. However, there are other types of non-linear inhibitions that can take place which have to be treated differently (Cornish-Bowden, 2004).

Linear inhibition

Competitive inhibiton: This form of inhibition occurs when the inhibitory compound, I, compete with substrate A for the same active site of the enzyme. Figure A.1 illustrates the reaction sequence.

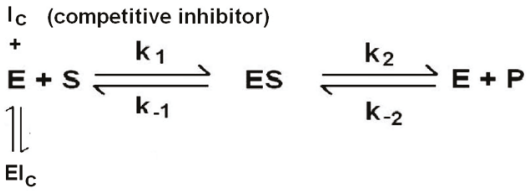

Figure A.0.1 Mechansim that produces competitive inhibition

In order to account for this inhibition, the rate expression of Equation A.2 is modified to Equation A.3 to include the inhibition term which affects only the Michaelis-Menten constant.

$$r = \frac{V_m C_S}{K_M\left(1+\frac{C_{I_c}}{K_{ic}}\right)+C_S} \qquad [A.3]$$

Non-competitive inhibition: This form of inhibition occurs when the inhibitory compound, I, bind reversibly with the acttive site of the enzyme and the enzyme complex. Figure A.2 illustrates the reaction sequence.

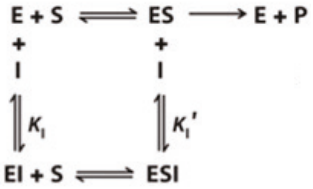

Figure A.0.2 Mechansim that produces non-competitive inhibition

This type of inhibition is quite rare and is generally used only in text books. To incorporate this effect on the kinetic model (Equation A.2) is modified to Equation A.4.

$$r = \frac{V_m C_S}{K_M\left(1+\frac{C_{I_{NC}}}{K_{NC_1}}\right)+C_S\left(1+\frac{C_{I_{NC}}}{K_{NC_2}}\right)} \qquad [A.4]$$

Un-competitive inhibition: This form of inhibition occurs when the inhibitory compound binds with the enzyme-substrate complex to reduce the net rate of reaction. Figure A.3 illustrates the reaction sequence.

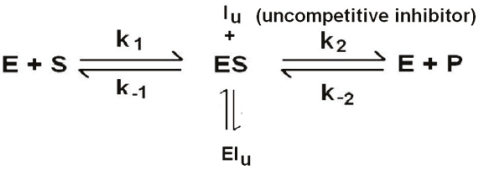

Figure A.0.3 Mechanism that produces un-competitive inhibition

In order to account for this inhibition, the rate expression of Equation A.2 is modified to Equation A.5 to include the inhibition term which affects only the Vm constant.

$$r = \frac{V_m C_S}{K_M + C_S}\left(1+\frac{C_{I_U}}{K_{I_U}}\right) \qquad [A.5]$$

Bisubstrate enzyme kinetics

In this thesis, two models have been used: 1) Ping pong bi bi model and 2) the modified Michaelis-Menten model.

The first model has been derived on the basis that the reaction follows the sequence ash shown in Figure A.4

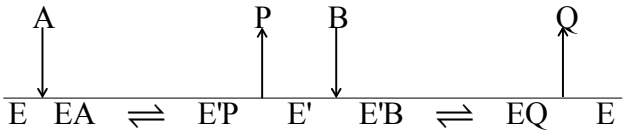

Figure A.0.4 Ping Pong Bi Bi mechanism

For this kind of reaction sequence, the kinetic model can be derived from steady-state kinetics using methods such as King-Altman or Wong-Hanes which are well explained in the literature (Cornish-Bowden, 2004;Leskovac, 2003).

The kinetic model to describe this reaction is shown in Equation A.6.

$$r = \frac{V_m^f V_m^r \left(C_A C_B - \frac{C_P C_Q}{K_{EQ}} \right)}{V_m^r K_M^B C_A + V_m^r K_M^A C_B + \frac{V_m^f K_M^Q}{K_{EQ}} C_P + \frac{V_m^f K_M^P}{K_{EQ}} C_Q + \ldots \\ V_m^r C_A C_B + \frac{V_m^f K_M^Q}{K_i^A K_{EQ}} C_A C_P + \frac{V_m^f}{K_{EQ}} C_P C_Q + \frac{V_m^r K_M^A}{K_i^Q} C_B C_Q} \qquad [A.6]$$

For the second model, the equation has been derived based on the reaction following the sequence shown in Figure A.5 which is called the compulsory-order ternary-complex mechanism.

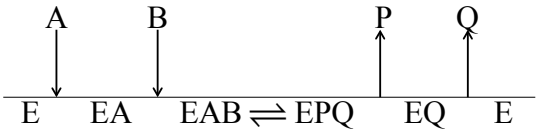

Figure A.0.5 Compulsory-order ternary-complex mechanism

The kinetic model to describe this mechanism has been derived simlarly as the previous one as shown in Equation A.7.

$$r = \frac{\frac{V_m^f C_A C_B}{K_i^A K_M^B} - \frac{V_m^r C_P C_Q}{K_i^Q K_M^P}}{1 + \frac{C_A}{K_i^A} + \frac{K_M^A C_B}{K_i^A K_M^B} + \frac{K_M^Q C_P}{K_M^P K_i^Q} + \frac{C_Q}{K_i^Q} + \frac{C_A C_B}{K_i^A K_M^B} + \frac{K_M^Q C_A C_P}{K_i^A K_M^P K_i^Q} + \ldots \frac{K_M^A C_B C_Q}{K_i^A K_M^B K_i^Q} + \frac{C_P C_Q}{K_M^P K_i^Q} + \frac{C_A C_B C_P}{K_i^A K_M^B K_i^P} + \frac{C_B C_P C_Q}{K_i^B K_M^P K_i^Q}} \quad [A.7]$$

To simplify the model, experiments can be designed at times very close to zero at which point the influence of product can be assumed to be negligible. The model is then called the initial rate model or modified Michaelis-Menten equation as shown in Equation A.8

$$r = \frac{V_m^f C_A C_B}{\left(K_M^A + C_B\right)\left(K_M^B + C_A\right)} \quad [A.8]$$

Nomenclature

R	Rate (mM/min)
V_m	Velocity parameter (mM/min)
K_M	Michaelis-Menten constant (mM)
K_I	Inhibition constant (mM)
A, B, P, Q, S	Components of the reaction (mM)
E	Enzyme (mM)

K_{Ic}	Competetive inhibition constant (mM)
K_{NC1} and K_{NC2}	Non-competetive inhibition constant (mM)
K_{Iu}	Uncompetetive inhibition constant (mM)

Appendix 2

Dissemination

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1. Poster presentations

Al-Haque N, Tufvesson P, Gani R, Woodley JM. (2012). Auxiliary phase reactors for ω-transaminase catalysed reaction. Biocat conference, Hamburg, Germany 2 – 6 September

Al-Haque N, Tufvesson P, Gani R, Woodley JM. (2011). Substrate Inhibition in ω-transaminase Catalyzed Reaction. Biotrans conference, Sicily, Italy 2 – 6 October

Al-Haque N, Tufvesson P, Gani R, Woodley JM. (2010). Application of solid resins for controlled substrate supply to biocatalytic reactions. BEST conference, Bologna, Italy 5 – 8 September

2. Publications

Paper 1. Process considerations for the asymetric synthesis of chiral amines using transaminases

Authors: Tufvesson, P ; Lima Ramos, J ; Jensen, JS ; Al-Haque, N ; Neto, W; Woodley, JM

Journal: Biotechnology and Bioengineering (2011) 108: 1479 – 1493

Paper 2. A robust methodology for kinetic model parameter estimation for biocatalytic reactions

Authors: Al-Haque N, Santacoloma PA, Neto W, Tufvesson P, Gani R, Woodley JM

Journal: Biotechnology Progress. (2012) 28: 1186 – 1196

REVIEW

BIOTECHNOLOGY and BIOENGINEERING

Process Considerations for the Asymmetric Synthesis of Chiral Amines Using Transaminases

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ABSTRACT: Biocatalytic transamination is being established as key tool for the production of chiral amine pharmaceuticals and precursors due to its excellent enantioselectivity as well as green credentials. Recent examples demonstrate the potential for developing economically competitive processes using a combination of modern biotechnological tools for improving the biocatalyst alongside using process engineering and integrated separation techniques for improving productivities. However, many challenges remain in order for the technology to be more widely applicable, such as technologies for obtaining high yields and productivities when the equilibrium of the desired reaction is unfavorable. This review summarizes both the process challenges and the strategies used to overcome them, and endeavors to describe these and explain their applicability based on physiochemical principles. This article also points to the interaction between the solutions and the need for a process development strategy based on fundamental principles.

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 pro-chiral ketones or by kinetic resolution of racemic amines. Transaminases catalyze the transfer of an amine ($-NH_2$) group from an amine donor, usually an amino acid or a simple amine such as 2-propyl amine, to a pro-chiral acceptor ketone, yielding a chiral amine as well as a co-product ketone or alpha-keto acid (Fig. 1). Transaminases require the cofactor pyridoxal phosphate (PLP) to act as a shuttle to transfer the amine group (Eliot and Kirsch, 2004). This cofactor is fully regenerated within the same two substrate reaction on the same enzyme, and hence does not pose the cofactor regeneration problems encountered in oxidation/reduction reactions (Hwang et al., 2005; Pannuri et al., 2003). Generally speaking, transaminases are suitable catalysts due to their high stereoselectivity, and ability to operate under environmentally mild reaction conditions. Transaminases and their function have been known for quite some time (Christen and Metzler, 1985) and the technology is already used in industry to produce selected chiral amines (Pannuri et al., 2003). Even so, in spite of the many attractive features of transaminase catalyzed reactions, there are still a number of challenges that need to be dealt with in order to make transaminase processes feasible for the production of a wider range of amines.

In the reaction step, two general strategies are used to obtain the target chiral amine; either direct asymmetric synthesis or kinetic resolution of a racemic amine. The latter alternative is the commonly used option in industry today although it is hampered by a 50% theoretical yield, unless a racemization step is included to enable a dynamic kinetic resolution (DKR). Nonetheless, using this strategy high enantiomeric excess (ee) values are easily attainable. However, in this report, the focus will be on direct asymmetric synthesis, since this is the state-of-the art of the

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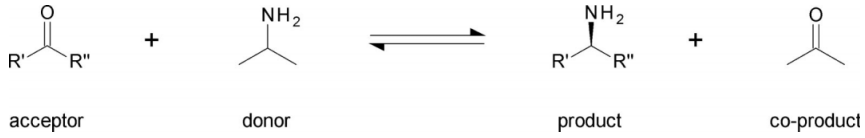

Figure 1. Overview of the biocatalytic transamination reaction.

technology and for the future the preferred reaction configuration, although more challenging than the resolution strategy.

Many of the problems encountered with transaminases are common to other biocatalytic processes and consequently many parallels can be drawn to other biocatalytic reactions for the production of chiral molecules (e.g., chiral alcohols). A number of reviews have been published describing the general features of transaminases, for instance by Taylor et al. (1998) and Stewart (2001), whereas Hwang et al. (2005) describes the different subgroups of transaminases and their substrate specificities related to the 3D structure, as well as protein engineering efforts to tailor the specificities. In a review by Koszelewski et al. (2010c) the recent developments in the field are described, with a focus on the different sources of ω-transaminases available.

The current review takes a process perspective and the focus is on the considerations for developing industrial transamination processes at large scale, summarizing the challenges and strategies to meet a number of proposed success criteria for an efficient and economic process. The article also reviews the different proposed solutions and analyzes these from a feasibility point of view supported by calculations and examples. We also suggest engineering tools to model and assess the process to move this technology towards a rational approach for developing large-scale processes.

Process Overview

The biocatalytic transaminase catalyzed production scheme consists of four major steps (Fig. 2); fermentation, biocatalyst formulation, reaction, and product recovery. Unless the biocatalyst is purchased from an external supplier (in which case the first two steps can be disregarded), the desired enzyme activity is expressed in a host microorganism to high product titer and thereafter prepared in a suitable form (biocatalyst formulation) for the reaction step (Fig. 3). To avoid unnecessary costs the biocatalyst is used in the crudest possible form; either as whole cells or cell-free extract (crude enzyme). Immobilization of the cells or enzymes can furthermore be used to facilitate recovery and improve the stability, thereby extending the use of the catalyst to multiple batches.

After the reaction is complete the biocatalyst is removed (biocatalyst separation) and the product is separated and purified. In each step of the process there are a number of factors that will determine the final economic viability of the process and the optimization of each step is essential in order to achieve a viable process.

There is little quantitative data published on the economics of biocatalytic processes and how the different performance metrics affect the cost of the total process. However, some of the metrics commonly used to benchmark the process (Bommarius et al., 2001; Straathof et al., 2002; Tufvesson et al., 2010) are summarized in Table I. In a previous article (Tufvesson et al., 2011) we have described the requirements for biocatalyst productivities for fine and pharmaceutical processes.

Many parameters work together to determine the success of an industrial biocatalytic process and clearly the limits in Table I are not absolute and should therefore be seen as general recommendations. Definitive requirements can only be determined on a case-by-case basis taking into account the added value of the process, competing technologies, and so on.

One of the most important factors is the added cost of the biocatalyst, which is why it is essential to maximize the biocatalyst productivity (g product/g biocatalyst). In addition, the product concentration is a key parameter as it determines the equipment cost and ease of downstream

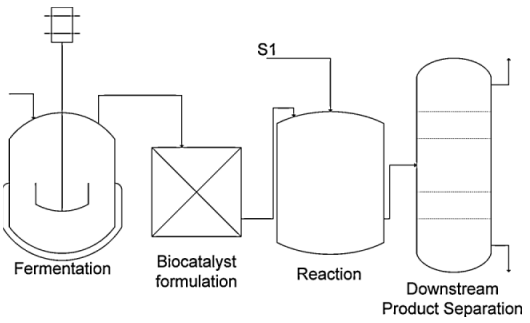

Figure 2. Overview of the biocatalytic transamination process.

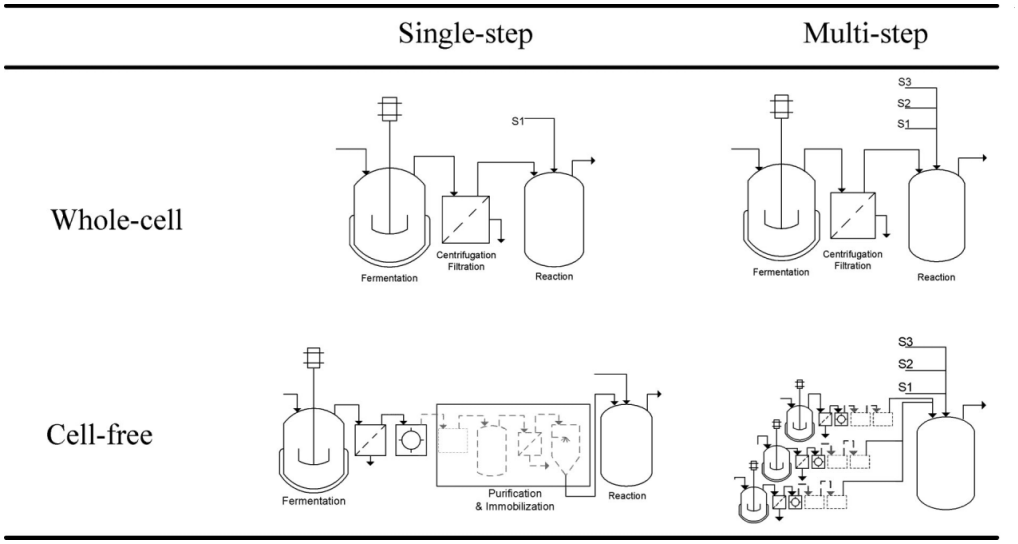

Figure 3. Detailed process design for the first three steps in the biocatalytic transamination process. S1—main feeding stream (amine donor, amine acceptor, PLP), S2—substrate for co-product removal, S3—co-factor regeneration.

separation and recovery. In order to compare how the state-of-the art transaminase technology relates to the above stated requirements, a summary of published reaction conditions and process metrics has been compiled in Tables II and III.

As can be seen from Figure 4, most studies are far from meeting the required industrial process requirements, with three notable exceptions; the work by Truppo et al. and the work by Savile et al., both reports from 2010 and the work by Martin et al. (2007). Even though none of these studies fulfill all of the guidelines in Table I, Martin et al. (2007) shows a very high catalyst productivity, especially considering that the catalyst is a whole cell. Savile et al. (2010) on the other hand demonstrate that the process can be run at substrate loadings significantly above the recommended minimum concentration. Ways for improving the reaction performance have included both biocatalyst improvements (e.g., by protein engineering or immobilization) and process improvements (e.g., by in situ product removal).

In the following sections of this article the main process challenges in biocatalytic transamination will be presented and solutions (and/or suggestions) for process improvement will be discussed.

Table I. Success factors for the economic feasibility of a biocatalytic process (Tufvesson et al., 2010).

Fermentation	
Cell titer	50–100 g CDW/L (if intra cell.)
Protein titer	1–10 g/L
Biocatalyst formulation	
Retention of activity	High
Stabilization	Improve catalyst productivity >5 times
Reaction	
Product concentration	>50 g/L
Catalyst productivity	10–35 g product/g whole cell (DCW) 100–250 g product/g free enzyme (crude) 50–100 g product/g immob. enzyme
Stereoselectivity	>98% ee
Yield	>90%

Process Challenges and Strategies

In order to meet the success criteria put forward above, a thorough knowledge of the reaction system is required relating both to the reaction thermodynamics, the physical characteristics of the reaction components and the possibilities and limitations of the given biocatalyst.

There are many challenges inherent to transaminase processes that need to be dealt with and numerous reports have been published that address one or more of these challenges. Frequently the suggested solutions, or technologies, solve more than one problem, for instance the use of an auxiliary phase may solve issues related to substrate and product inhibition as well as low water solubility, but on the other hand the solution might pose other problems such as lower biocatalyst stability. An overview of transaminase process challenges have been put together in Table IV, along with the suggested technologies or strategies used to

Table II. Summary of some the most important published transaminase reaction for the synthesis of amino acids.

Target compound	Amine donor	C amine acceptor (mM)	Ratio D/A	Temperature, pH, enzyme	Yield (%)	ee	Time (h)	Vol. prod (mM/h)	CatProd (gP/gwTAm) (gP/gCDW)	Refs.
Cell-free catalyst										
L-phosphinothricin	Glu + Asp	500	0.2 + 1.2	50, 8.0, column with immobilized phosphinothricin TAm and Glu: oxalacetate TAm	82.7	>99	24	17.2	—	Bartsch et al. (1996)
(3S)-amino-3-phenylpropionic acid	3-aminobutyrate	10	2	37, 7.5, *C. rugosa* lipase and TAm from *Mesorhizobium* sp.	20	>99	24	0.1	0.4	Kim et al. (2007b)
Ala	MBA	10	1	37, 7.0, cell extract containing ω-TA from *C. violaceum* expressed in *E. coli*	95	—	3	3.2	1.3	Kaulmann et al. (2007)
Gly		10	1		95	—	3	3.2	1.1	
L-phenylalanine	L-Asp	12.5	2	22, 7.0, free TAm and oxaloacetate decarboxylase	98.5	—	12	1.0	—	Rozzell (1985)
L-tyrosine	L-Asp	10	1	24, 7.0, TAm and oxaloacetate decarboxylase	99	—	1	9.9	0.0	
L-HPA	L-Lys	200	1.5	37, 9.0, aspartate aminotransferase (aspC from *E. coli*)	97	>99.9	24	8.1	0.9	Lo et al. (2005)
Cascades (cell-free)										
1-N-Boc-3-aminopyrrolidine	L-Ala	5	22	37, 7.0, TAm from *V. fluvialis* and PDC (Pyr decarboxylase)	80	99	1	4.0	—	Höhne et al. (2008)
2-aminobutyrate	L-Glu	50	1	37, 8.5, crude enzyme extract from *E. coli* (encoding branched-chain TAm and ornithine TAm)	91.92	—	5	9.2	7.9	Li et al. (2002)
L-*tert*-leucine	L-Glu	40	1		72.98	—	16	1.8	6.4	
Cascades (whole cell)										
Phenylalanine	Asp	100	1.5	37, 8.4, *E. coli* overexpressing Tam-AspC and phosphoenolpyr. carboxykinase PcK	93.0	—	2	46.5	3.4	Chao et al. (1999)
2-aminobutyrate	L-Asp	500	1.0	37, 7.5, *E. coli* (carrying wTAm and alsS from *B. subtilis*)	54.0	—	24	11.3	0.3	Fotheringham et al. (1999)
2-aminobutyrate	L-Asp	500	1.0	37, 7.5, *E. coli* (carrying tyrB (Tyr aminotransferase) from *E. coli* and alsS (acetolase synthase) from *B. subtilis* and ilvA (threonine deaminase) from *E. coli*)	53.7	—	24	11.2	0.3	Fotheringham (2001)
2-aminobutyrate	L-Asp	500	1.0	37, 8, *E. coli* (carrying tyrB from *E. coli*, alsS from *B. subtilis*)	58.3	—	24	12.1	0.3	
L-HPA	L-phenylalanine	40	2.5	37, 8.4, *E. coli* (AroAT from *Enterobacter* sp., carbonyl reductases, phenylpyruvate descarboxylase and GDH)	96.3	—	24	1.6	—	Hwang et al. (2009)
2-aminobutyrate	L-Asp	500	1.0	37, 7.5, *E. coli* (tyrB gene from *E. coli*, alsS gene from *B. subtilis*)	58.3	—	24	12.1	0.6	Ager et al. (2001)
2-aminobutyrate	L-Asp	500	1.0	37, 7.5, *E. coli* (ilvA gene from *E. coli*, tyrB gene from *E. coli*, alsS gene from *B. subtilis*)	53.7	—	24	11.2	0.6	
Whole cell										
beta-(2-Thienyl)-D-alanine	L-Asp	176	1.2	40, 8.0, *E. coli ATCC11303* (with tyrB), without atmospheric O2	82.0	>98	24	6.0	4.1	Meiwes et al. (1997)
L-HPA	Asp	10	1.0	37, 7.2, *E. coli* (tyrB + wtaA)	83.0	99.0	10	0.8	0.1	Cho et al. (2003)
2-aminobutyrate	Ala	10	1.0	37, 7.2, *E. coli* (avtA + wtaA)	90.0	95.0	5	1.8	0.1	
L-HPA	L-Glu	100	1.5	37, 8.5, Tyrosine-aminotransferase (glutamic-aromatic aminotransferase) from *E. coli*	95.0	>99	2	47.5	—	Chen et al. (2000)

D/A, donor to acceptor; MBA, methylbenzylamine; Ala, alanine; Asp, aspartate; Glu, glutamate; Gly, glycine; HPA, homophenylalanine IPA, 2-propyl amine; Lys, lysine; TAm, transaminase.

Table III. Summary of some the most important published transaminase reaction for the synthesis of chiral amines.

Target compound	Amine donor	C amine acceptor (mM)	Ratio D/A	Temperature, pH, enzyme	Yield (%)	ee	Time (h)	Vol. Prod (mM/h)	CatProd (gP/gwTAm) (gP/gCDW)	Refs.
Cell-free catalyst										
MBA	MPPA	5	10	30, 7, Cell-free extract from *V. fluvialis* JS17	21.4	—	24	0.04	—	Shin and Kim (1999)
MBA	3-aminoheptane	5	10		10.1	—	24	0.02	—	
MBA	*sec*-butylamine	5	10		7.7	—	24	0.02	—	
Sitagliptin	IPA	491	2	45, 7, mutant of ATA-117, 50% DMSO	92	>99	24	18.82	30.67	Savile et al. (2010)
MBA	IPA	20	50	30, 7.5, ATA-113/117	95	>99	24	0.79	0.46	Truppo et al. (2009a)
MBA	*sec*-butylamine	10	10	40, 6.0, purified TAm from *M. aurum*	19.9	>99	71	0.03	—	Takashima et al. (2004)
MBA	IPA	2	311	37, 7, *E. coli* (ω-TAm from *A. citreus*, YADH, FDH)	99.0	>99.9	24	0.07	1.35	Cassimjee et al. (2010)
Cascades (cell-free)										
MBA	L-Ala	30	10	30, 7, cell-free extract from *V. fluvialis* JS17, LDH	5.83	>99	12	0.15	—	Shin and Kim (1999)
2-butylamine	Ala	50	5	30, 7, ATA-113 + ATA-117, lactate dehydrogenase mix	99	>99	24	2.06	0.30	Koszelewski et al. (2009)
4-phenylbutylamine		50	5	30, 7, ATA-113 + ATA-117, lactate dehydrogenase mix	99	>99	24	2.06	0.62	
Mexiletine		50	5	30, 7, ATA-113 + ATA-117, lactate dehydrogenase mix	99	>99	24	2.06	0.74	
MBA	Ala	416	2	30, 7.5, ATA-113/117, GDH CDX-901, LDH-102, with ISPR (ion exchange resin)	99	>99	19	21.68	9.98	Truppo et al. (2010)
MBA	Ala	50	10	30, 7.5, ATA-103/117, LDG/GDH	96	99	10	4.80	1.16	Truppo et al. (2009a)
MBA	NH_4^+ via Ala	50	1	30, 7.5, ATA-103, LAADH-117/GDH	96	99	48	1.00	1.16	
2-aminopentane	L-Ala	76	4	30, 7, *E. coli* (BM-ω-TAm from *B. megaterium*), AADH	97.0	>99	24	3.07	0.64	Koszelewski et al. (2010b)
1-methoxy-2-propylamine		76	4		97.0	>99	24	3.07	0.66	
p-Methoxyamphetamine	L-Ala	76	4	30, 7, *E. coli* (ω-TAm from *C. violaceum*), AADH	94.0	>99	24	2.98	1.18	
2-aminopentane		76			94.0	>99	24	2.98	0.62	
1-Methoxy-2-propylamine		76			96.0	96.0	24	3.04	0.65	
2-aminopentane	L-Ala	39	4	30, 7, *E. coli* (BM-ω-TAm from *B. megaterium*), L-lactate from bovine heart	99.0	>99	24	1.61	0.67	
1-methoxy-2-propylamaine		39			94.0	>99	24	1.53	0.65	
p-Methoxyamphetamine	L-Ala	39	4	30, 7, *E. coli* (CV-ω-TAm from *C. violaceum*), L-lactate from bovine heart	94.0	>99	24	1.53	1.21	
2-aminopentane		39			99.0	>99	24	1.61	0.67	
Cascades (whole cell)										
MBA	Ala	10	10	37, 7, *E. coli* (ω-TAm from *V. fluvialis* JS17, ALS from *B. subtilis* 168)	34.0	—	20	0.17	0.21	Yun and Kim (2008)
2-amino-1,2,4-butanetriol	MBA	20	1	25, 7.0, *E. coli* (with transketolase from *E. coli* and TAm from *P. aeruginosa*)	21.0	—	62	0.07	0.85	Ingram et al. (2007)
Whole cell										
(R)-3,4-dimethoxyamphetamine	(R)-MBA	154	1	30, 8.5, *Anthrobacter* sp. KNK168	81.8	>99	40	3.15	—	Iwasaki et al. (2006)
MBA	L-Ala	30	10	30, 7, *V. fluvialis* JS17	90.2	>99	24	1.13	—	Shin and Kim (1999)
S-aminotetralin[a]	IPA	130[a]	11.5[a]	55, 7, *E. coli* containing mutant mesophilic TAm (from *A. citreus*)	88.5	>99.9	48	2.40	16.93	Martin et al. (2007)

D/A, donor to acceptor; MBA, methylbenzylamine; MPPA, 1-methyl-3-phenylpropylamine; Ala, alanine; IPA, 2-propyl amine; TAm, transaminase MPPA, 1-methyl-3-phenylpropylamine.
[a]Assumed—ketone: tetralone (MW: 147.22 g mol^{-1}) concentrations: ketone 130 mm and IPA 1,500 mm (due to lack of further information).

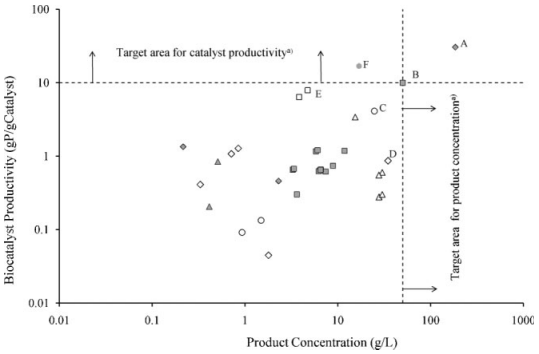

Figure 4. State of the art in transaminase reaction for chiral amines synthesis plotted AS Biocatalyst productivity versus product concentration. ◇: Cell-free catalyst; □: cascades (cell-free); Δ: cascades (whole cell); ○: whole cell; gray markers: synthesis of chiral amines; white markers: synthesis of amino acids. (A) Sitagliptin, Savile et al. (2010); (B) α-MBA, Truppo et al. (2010); (C) β-(2-thienyl)-D-alanine, Meiwes et al. (1997); (D) LHPA, Lo et al. (2005); (E) 2-aminobutyric acid, Li et al. (2002).

overcome these, as well as the further implications of using a specific technology.

Thermodynamic Limitations

A critical issue that needs to be addressed in a biocatalytic transamination reaction is the thermodynamic equilibrium of the reaction system since knowledge about the thermodynamics of the reaction will determine which process solutions are feasible on an industrial scale.

The transamination reaction is reversible and the maximum achievable conversion is thus determined by the initial concentrations and the thermodynamic equilibrium constant (K) of the reaction. K in turn is determined by the change in Gibbs free energy for the reaction, which is given by the difference in ΔG between the products and the reactants. For the amine transfer from an amino acid to an alpha keto acid to form another amino acid, the change in Gibbs free energy is small and thus the equilibrium constant is around one (Taylor et al., 1998). However, for the transfer of an amine group from an amino acid to acetophenone, a commonly investigated ketone, the equilibrium is strongly in the favor of the amino acid (amine donor). Kim and Shin

Table IV. Bottleneck analysis.

	Challenges							
	Process related			Biocatalyst related				
	Low thermodynamic equilibrium	Low substrate solubility	Substrate and product degradation	Inhibition	Stability	Activity	Separation of biocatalyst	Major limitations
Solutions								
Chemistry related								
Stronger amine donor	✓							Cost and availability of the donor
Excess amine donor	✓							Inhibition and stability of enzyme; Not applicable for low Keq; donor cost; downstream separation
Process related								
Solvent (co-solvent/2-phase)		✓						Enzyme stability; downstream separation; VOC
Separation of (co-)product by (a) distillation	✓							Co-distillation of water and/or other components
(b) Extraction (solvent, membrane, resin)	✓	✓	✓	✓				Selectivity between substrates and products
Controlled supply of substrate (fed-batch)			✓	✓				Capacity
Degradation of co-product (cascade)	✓							Compatibility; Added cost of biocatalysts; co-factor recycling
Biocatalyst related								
Whole cell	✓							Side-reactions; separation; GMO regulations
Immobilization					✓		✓	Deactivation; development cost; higher biocatalyst cost
Enzyme development					✓	✓		Development time and cost

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

(1998) report a K of about 10^{-3} (for the synthesis of α-methylbenzylamine) based on parameter estimation when performing a kinetic study. Still there are indications from other studies that it may be even more unfavorable than this, since transamination of acetophenone with 10 equivalents L-alanine was completed at 3% conversion, as opposed to the theoretical equilibrium conversion of 9% (Truppo et al., 2009a). When using 2-propyl amine instead of amino acid as the donor, the equilibrium becomes more favorable (Truppo et al., 2009a) but still the equilibrium lies strongly in favor of the reactants. Although Shin and Kim (1999) have compared the effect of different amine donors on the yield, they do not show that equilibrium has been reached. To our knowledge no other reports determining the equilibrium constants using different donors have been published. Even so, the relative amine donating potential of many compounds can be qualitatively extrapolated from literature, for instance the donor potential of α-MBA is order of magnitudes higher than for 2-propylamine, which in turn has much higher potential than alanine. It is clear that the availability of an even stronger amine donor could be very beneficial.

By knowing the reaction Gibbs free energy (ΔG), one can determine the process strategy needed to meet the requirements in terms of yield and product concentration. Different strategies inherently bring about different cost structures and therefore one can identify the reactions that are likely to be able to be scaled-up and applied in industry. Therefore knowledge of the reaction equilibrium constant and/or Gibbs free energy allows a more intelligent process design. Jankowski et al. (2008) have developed a group contribution method for estimating Gibbs free energies for biochemical reactions in aqueous solutions at pH 7 and 25°C, having a standard error of ±2 kcal/mol. This methodology was recently applied by Seo et al. (2011) in the comparison of the transamination potential of different amine donors, where 1-aminoindan was estimated to be thermodynamically favorable for the transamination of acetophenone. Considering the uncertainty in the group contribution method it was within the standard error, that an experimental yield of only 37% was obtained using four equivalents of amine donor, indicating a thermodynamically unfavorable reaction (Seo et al., 2011).

In order to overcome the thermodynamic limitations in transaminase reactions there are several solutions that have been shown to (at least) partly overcome these: addition of excess of amine donor, application of ISPR (in situ removal of product or co-product), auto-degradation of the product, use of enzymatic cascades or whole-cell catalysis.

Addition of Excess Amine Donor

The easiest option for shifting the equilibrium towards a high yield of the product would in principle be to use an excess of the amine donor. This strategy was applied by Savile et al. (2010) for the production of Sitagliptin at high substrate concentrations using approximately 10-fold excess of 2-propylamine. However, the use of this strategy is limited to those cases when the equilibrium is only slightly unfavorable. In fact, from the Savile article it can be extrapolated, that the K in this case is close to unity.

The reason for the limitation to this strategy is that if the substrate concentration is to be kept at a high level (>50 g/L), there will be an upper limitation of how large an excess of amine donor can be used, with stoichiometric equivalents in the range of 1–50 times approaching the limits of amine donor solubility. Figure 5 plots the necessary excess of amine donor required to achieve a yield of 90% at varying value of K. As can be seen from Figure 5, to achieve a yield of 90% an excess of 100-fold is required if the K value is 10^{-1}. Similarly, if K the value is 10^{-3}, an excess of 10,000-fold would be required, which for obvious reasons is unrealistic.

As a consequence of this, for transaminations where K is lower than 10^{-2} adding an excess of amine donor will not be sufficient to reach the process metrics and thus additional strategies are required.

Removal of Product or Co-Product

A second method to shift the equilibrium position in favor of the desired product is to remove the product or co-product from the media during the reaction itself, that is, in situ product removal (ISPR). Again, the equilibrium constant of the reaction determines how low a concentration of product or co-product is required to achieve the target

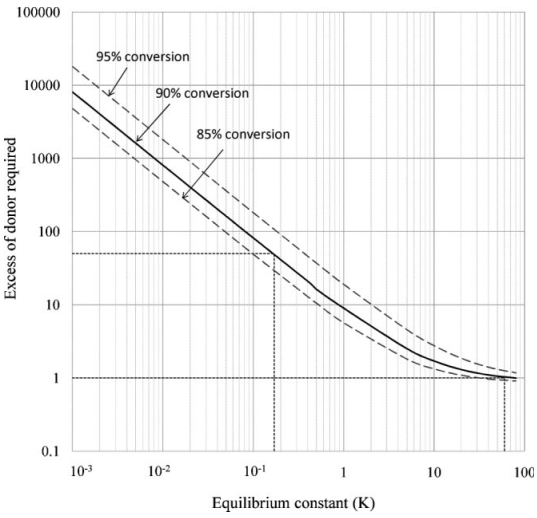

Figure 5. The equilibrium constant (K) determines the excess of amine donor required to reach a thermodynamic equilibrium of 90% (solid line). The broken lines are visual support for an excess of 10 and 50, which can be considered process boundaries.

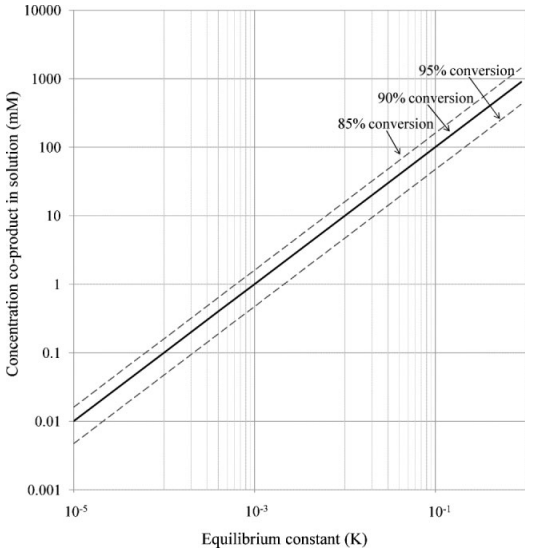

Figure 6. Concentration of co-product required to reach 90% yield when using an initial concentration of 1 mol/L ketone and a tenfold excess of amine donor for the synthesis of chiral amine.

yields. Figure 6 shows the relationship between K and co-product concentration to achieve 90% yield when using an initial concentration of 1 M ketone and a 10-fold excess of amine donor for the synthesis of a chiral amine. As can be seen in Figure 6, at K values $<10^{-3}$ the required co-product concentration will need to be <1 mM. This is important to keep in mind when considering which methods can be used to shift the equilibrium.

The best strategy for ISPR will depend on the properties of the product amine as well as the other components in the reaction mixture. In general, a strategy will be favorable when it produces a big driving force for separating the product from the other components. The physico-chemical properties that are most commonly exploited for ISPR are volatility, solubility, charge, hydrophobicity, and molecular size (Lye and Woodley, 1999).

ISPR strategies are particularly relevant when considering transamination reactions, as they enable a shift of the reaction equilibrium position as well as reducing product inhibition (as will be discussed later). There are many examples to illustrate the use of ISPR strategies in connection with transaminase catalyzed reactions. A summary of the different approaches for ISPR, including the improvement achieved and main drawbacks, can be found in Table V.

Liquid–liquid extraction is a common strategy for the downstream recovery that allows the recovery of a large range of different amines. Extraction under either acidic or basic conditions allows control of the amine product if

Table V. In situ recovery techniques applied for biocatalytic transamination.

Reaction system	Comparison of ISPR vs. without ISPR	ISPR method applied	Major drawback	Refs.
α-MBA ⟷ APH (*B. thuringiensis* JS64)	Ninefold higher reaction rate	Extraction with organic solvent	Decreased enzyme stability	Shin and Kim (1997)
α-MBA ⟷ APH (*B. thuringiensis* JS64)	99% vs. 54.7% (ee)	Membrane extraction (perstraction)	Demand for highly purified enzyme[a]	Shin et al. (2001a)
sec-But.A ⟷ 2-butanone (*E. coli* BL21)	98% vs. 32% (ee)	Evaporation of the volatile inhibitory product	Evaporation of the reaction media (e.g., water)	Yun et al. (2004)
sec-ButA ⟷ (R)*sec*-ButA (*B. megaterium* SC6394)	Enzymatic resolution of racemic mixture with 99% (ee)	Distillation of the volatile amine product	Limit number of amines can be recovered using distillation	Hanson et al. (2008)
4P2B ⟷ (R)4PB2A (ATA-117)	Achieved 92% conversion (99% ee)	Extraction with organic solvent combined pH setting[b]	Organic solvents used; potential decrease of enzyme stability	Koszelewski et al. (2008b)
APH ⟷ α-MBA (ATA-113 and ATA-117)	99% vs. 10% (*max conv.*)	Extraction with resins	Selectivity of resin	Truppo et al. (2010)

α-MBA, α-methylbenzylamine; APH, acetophenone; *sec*-But. A, *sec*-butylamine; 4P2B, 4-phenyl-2-butanone; 4PB2A, (R)-4-phenylbutan-2-amine.
[a]To reduce the residence time and consequently minimize product inhibition.
[b]Followed by evaporation under reduced pressure of the organic solvent in order to obtain the product.

protonated, and thus provides an efficient tool for separating the amine from other components in the product stream (in particular the remaining substrate ketone). For example, such an approach was applied in the post-reaction recovery of (*R*)-4-phenylbutan-2-amine by Koszelewski et al. (2008b). Integrating the extractive recovery with the reaction step would allow a shift of the equilibrium by extracting the product into the second phase, that is, in situ extraction. ISPR by employing adsorbing resins for extracting the product has also been suggested (Woodley et al., 2008). This strategy was shown by Truppo et al. (2010) to be an efficient method to also overcome product inhibition and shifting equilibrium in the production of both (*R*)- and (*S*)-methyl benzylamine. At substrate concentrations of 50 g/L (0.4 M), 200 g of ion-exchange resin was used for product adsorption, resulting in improved reaction rates and yields. This strategy further allowed the product to be easily recovered by filtration and washing of the resin. However, the added cost of using large amounts of resin needs to be considered. Multiple re-uses of the resin will be necessary for a reasonable cost contribution.

There are limitations with all separation strategies. A common limiting factor is related to the selectivity of the separation and the relative concentrations of the reaction components, including the solvent. For instance, an observed problem when using either solvent or resin extraction is that the ketones or amines have similar distribution behavior and therefore will co-extract into the solvent or resin unless another driving force is in place such as ionization. This is well illustrated in the report by Truppo et al. (2010) employing the use of resins to extract the product. The amine donor 2-propyl amine was seen to compete with the product (MBA) for binding to the resin. The similarity between the p*K*a value of the product and the amine donor (9.54 and 10.73, respectively) also excludes using ionization for separation, since at pH 7 more than 99% of both compounds are protonated. This problem was, however, alleviated in the report by changing the amine donor to alanine and implementing a cascade enzymatic system to degrade the pyruvate (as will be described below).

Evaporation of a volatile product (or co-product) may also be an option for shifting the equilibrium towards the product. This has been suggested as an option if 2-propanamine or 2-butanamine is used as the amine donor yielding acetone or butanone, respectively, as the co-substrate (Yun et al., 2004). For volatile amines, distillation could also be a possible route for product recovery in kinetic synthesis (see Table VI). For example (*R*)-*sec*-butylamine (boiling point 63°C) was recovered by distillation of the product mixture under basic conditions (Hanson et al., 2008). Also Savile et al. (2010) report a slight improvement in yield by sweeping the reactor with nitrogen gas to remove the formed acetone.

The selectivity problem is, however, also very problematic when using the evaporation strategy. Assuming ideal conditions, an estimate of the vapor composition can quickly be estimated based on Raoult's law (see Eq. 1, Table VI).

Table VI. Vapor pressure values of pure compounds at 25°C.

Compound	P_{vap} (mbar)
Acetophenone	0.53
α-methylbenzylamine	0.72
Alanine	Non volatile
Pyruvic acid	1.7
Acetone	309
2-propyl amine	773[a]
Acetaldehyde	1202
2-butyl amine	237[a]
2-butanone	121
Water	30.7

[a]At reaction conditions (pH 7) the vapor pressure of amines are negligible due to protonation of the amine.

$$x_i^{vap} = \frac{p_i^* x_i^l}{\sum p_i^* x_i^l} \quad (1)$$

x_i^{vap} molar fraction of compound i in vapor; p_i^* vapor pressure of pure compound; x_i^l molar fraction of compound i in liquid phase.

As an example, if 10 mM acetone is being removed from a water solution the relative amount of water ($C_{water} \sim 55$ M) evaporated will be over 500 times that of acetone. Hence, in a thermodynamically unfavorable system, the concentration of acetone will need to be reduced significantly beyond this point as shown previously, making the problem more difficult. Similarly, the volatility of any co-solvent and the donor amine need to be considered when using this approach.

Auto-Degradation of Co-Product

A very convenient, but not widely applicable approach is the use of a self-degrading co-product or products. Fotheringham and coworkers (Ager et al., 2001; Li et al., 2002) found that when using ornithine or lysine as amine donor, the formed amino-keto acid is cyclized spontaneously thus favoring the reaction in the direction of the amine (Ager et al., 2001; Li et al., 2002; Lo et al., 2005). Truppo et al. (2010) used a similar approach where the product cyclized, thereby shifting the equilibrium of the reaction.

Enzymatic Cascade Reactions

A much explored approach to obtain a high yield of the desired product is to couple the transamination reaction to other enzymatic steps (Fig. 3) that convert the co-product (e.g., pyruvate or acetone) into a non-reactive species or back to the original substrate. A multitude of different coupling reactions have been proposed and reported. These are summarized in Table VII and are reviewed beneath.

Table VII. Enzymatic cascades for shifting the equilibrium.

	Enzymes	Co-reactants[a]	Co-products	Refs.
Oxaloacetate degradation	PcK, PK	a-KG, ATP	Pyruvate, CO_2	Chao et al. (1999)
Pyruvate degradation	ALS	Alanine	CO_2, acetoine	Fotheringham et al. (1999)
	PDC	Alanine	CO_2, acetaldehyde	Höhne et al. (2008)
	PDC, ADH, FDH	Alanine, NADH, Formate	CO_2, ethanol	Not reported
	LDH, GDH	Alanine, Glucose, NADH	Lactic acid, gluconic acid	Shin and Kim (1999)
	LDH, FDH	Alanine, Formate, NH_4^+, NADH	Lactic acid, CO_2	Koszelewski et al. (2008b)
Co-product degradation	(Y)ADH, GDH	2-PA/BA, glucose, NADH	2-propyl/butyl alcohol, gluconic acid	Not reported
	(Y)ADH, FDH	2-PA/BA, formate, NADH	2-propyl/butyl alcohol, CO_2	Cassimjee et al. (2010)
Alanine recycling	AADH, GDH	Alanine, NH_4^+, NADPH	Pyruvate (low), H_2O	Truppo et al. (2009a,b)

ALS, acetolactate synthase; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase; FDH, formate dehydrogenase; GDH, glucose dehydrogenase; 2-PA/BA, 2-propyl amine or 2-butyl amine; PcK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase.

[a]Reactants that are required for the reaction additionally to the amine acceptor (ketone).

One early strategy, employed by Chao et al. (1999), was the combined use of phosphoenolpyruvate carboxykinase (EC 4.1.1.32; PcK) and pyruvate kinase (EC 2.7.1.40) to convert the formed oxaloacetate to pyruvate in a two-step reaction. A simpler strategy was reported by Fotheringham and co-workers (1999, 2001) in a process for making amino acids, where the transamination was coupled to acetolactate synthase (EC 2.2.1.6), which converted the formed pyruvate by-product to the non reactive acetoin. Significant yield and purity advantages over the process using the transaminase alone were reported, with an eight to tenfold increase in the ratio of product to the major impurity. Another common strategy to eliminate the pyruvate is through the addition of lactate dehydrogenase (LDH, EC 1.1.1.27), converting pyruvate to lactic acid while simultaneously oxidizing NADH to NAD^+ (Höhne et al., 2008; Hwang et al., 2009; Shin and Kim, 1999; Truppo et al., 2009a). Although the system has been shown to work effectively, the main drawback is the requirement of the co-factor NADH, which needs to be re-generated. When using cell-free transaminase, this can be achieved by adding glucose dehydrogenase (GDH, EC 1.1.99.10) or formate dehydrogenase (FDH, EC 1.2.1.2) together with glucose or formate. The same effect could also be achieved by using a whole-cell system as most organisms already have a system for pyruvate metabolism and NADH regeneration. In a report by Höhne et al. (2008) it was shown that the equilibrium can instead be shifted by the use of pyruvate decarboxylase (PDC, EC 4.1.1.1). The major argument for using this (in contrast to LDH) is that cofactor recycling is eliminated, and the reaction is practically irreversible as the products are very volatile (acetaldehyde and CO_2), and would be evaporated for the desired shift of equilibrium (Höhne et al., 2008). Truppo et al. (2009b) developed a novel system for the resolution of racemic amines using a transaminase coupled with an amino acid oxidase (AAO, EC 1.4.3.2). In contrast to previously reported approaches that use a stoichiometric amount of amine acceptor, the system described here employs a catalytic amount of amine acceptor (pyruvate) that is continuously recycled in situ by an AAO and molecular oxygen. Pyruvate can also be reconverted into L-alanine with L-alanine dehydrogenase (EC 1.4.1.5) coupled with FDH for NADH regeneration, which therefore in principle only consumes stoichiometric amounts of ammonium formate (Koszelewski et al., 2008b).

Regardless of the cascade system, the interactions and compatibility of each of the enzymes and their associated reagents need to be considered. For instance, the introduction of high concentrations of formate (for use with FDH) is likely to affect the activity and stability of the other enzymes. The interactions can be formalized in an interaction matrix table (Santacoloma et al., 2011). Characteristics related to the catalyst constrains, process modeling, and cascade or network interactions, reactor selection, monitoring and control are also described by Santacoloma et al. (2011).

Whole-Cell Biocatalysis

Despite the fact that the multi-enzyme cascade approach has the potential to be very successful (e.g., Koszelewski et al., 2008a; 2009; Truppo et al., 2010), the economical burden of using multiple enzymes is significant (Tufvesson et al., 2010). In particular the combination with the addition of co-factor (NAD(P)H) will increase the process cost, even when using low concentrations (Berenguer-Murcia and Fernandez-Lafuente, 2010). A suitable strategy to overcome this limitation is using a whole cell as the biocatalyst. Whole-cell strategies (Fig. 3) have become a very promising field especially for bioconversions which usually require a cofactor addition and/or regeneration (León et al., 1998). The wild-type microorganism containing the desired transaminase may be used, but the more common approach is to clone the desired transaminase into a host vector. For example the use of recombinant *E. coli* (Ingram et al., 2007; Koszelewski et al., 2009) or *Pichia pastoris* (Bea et al., 2010) expressing ω-transaminase, optionally following a similar approach as seen for cascades, creating so called cassettes over-expressing the production of the enzymes involved in the degradation or recycling of the co-product. Nevertheless, the number of available ω-transaminases with a known gene sequence is still rather limited (Clay et al., 2010; Koszelewski et al., 2010b).

In Tables II and III examples of the use of whole-cell systems are given. Reported yields are usually in the range of

80–99% with comparable enantioselectivity and similar reaction rates to enzyme cascades.

Several authors (Cassimjee et al., 2010; Fotheringham et al., 1999; Koszelewski et al., 2010b; Panke et al., 1999; Yun and Kim, 2008) have shown that chromosomal integration of genes under a suitable regulatory system to an *E. coli* or *P. pastoris* mutant is a very useful route for constructing a whole-cell biocatalyst that is able to synthesize chiral amines to high specific activities and that can maintain activity for extended periods under reaction conditions in the presence of an organic phase. However, the adequate expression level of each protein still remains a challenge (Kratzer et al., 2010). Other typical drawbacks found in whole-cell biocatalysis, such as uncontrolled side reaction (and consequently unwanted side products) and slower reaction rates (due to trans-membrane diffusion problems and higher metabolic burden), are also encountered in the transaminase reaction using whole cell. Consequently the lower cost of using whole cells has to be weighed against these drawbacks to find the most suitable catalyst form (Woodley, 2006).

Biocatalyst Limitations

Transaminases can be found with activity for a broad range of substrate ketones as has been recently reviewed by Kroutil et al. (Koszelewski et al., 2010c), although S-selective enzymes are more common. However, even if an enzyme with the desired specificity and selectivity can be found, the activity and stability must be high enough to allow a biocatalyst productivity that results in a feasible cost contribution from the biocatalyst (Tufvesson et al., 2010). Improvement of the biocatalyst is very often required for industrial application. For instance, poor stability of the enzyme could require it to be replenished throughout the course of reaction to maintain a sufficient rate. However, if the enzyme stability is improved to a point where it maintains a rate for a longer period of time the loading can be reduced significantly.

The cost of the biocatalyst is dependent on variables, such as expression level, efficiency of the fermentation protocol, enzyme specific activity and the form of the biocatalyst (e.g., whole cell, cell-free extract (crude enzyme), purified or immobilized enzyme). With an optimized production protocol the biocatalyst does not need to be excessively expensive, although the development of an optimized process takes time and requires many different skills (e.g., cloning, fermentation, purification/immobilization). Excluding development costs, a likely cost for an efficiently produced in house biocatalyst used for pharmaceutical production is calculated to be around 10–35 €/kg for whole cells (dry cell weight), 100–250 €/kg crude enzyme (cell-free extract) and 50–100 €/kg for an immobilized preparation (Tufvesson et al., 2011). This in turn puts requirements on the productivity of the biocatalyst in terms of product produced per amount of biocatalyst for an economical process.

A common problem is substrate and product inhibition of the enzyme. For instance, in the transamination of MBA from acetophenone, both substrates and products are known to inhibit the enzyme activity severely already at millimolar concentrations (Truppo et al., 2009a). This could be managed by multiphasic reactions, for example, using an auxiliary solvent or a resin, but it is also conceivable that this could be overcome by modifications to the enzyme itself.

Improvement of the Biocatalyst

Several recent examples illustrate very well the advances in biocatalyst improvement, such as the development of a process for the anti-diabetic drug Sitagliptin by Savile et al. (2010), and the work by Martin et al. (2007). The state-of-the-art methodology to develop the enzymes to fit process requirements is based both on random changes to the protein, combined with the addition of a selective pressure to find the improved mutants (Turner, 2009) and an understanding of the relationship between protein structure and its properties (Frushicheva et al., 2010). Approaches such as saturation mutagenesis (Reetz and Carballeira, 2007), and the use of multivariate statistical techniques, for example, ProSAR (Fox et al., 2007) has evolved into an extremely powerful tool to develop highly efficient tailor made catalyst with less effort than ever before. For instance Martin et al. (2007) managed to improve the activity of a transaminase by a factor of almost 300, while at the same time improving the stability of the enzyme toward the process conditions, yielding a much more economic process. Other examples are given in reports by Rothman et al. (2004) and Yun et al. (2005) who managed to overcome product inhibition by directed evolution, Cho et al. (2008) redesigned the substrate specificity of an ω-transaminase for the kinetic resolution of aliphatic chiral amines.

To obtain a biocatalyst with the desired properties it is important to screen under the preferred reaction process conditions. However, it is generally difficult to screen for all the desired properties simultaneously (Burton et al., 2002), why a gradual adaptation might be beneficial (Tracewell and Arnold, 2009). Also, due to the high costs associated with the techniques for biocatalyst improvement improvements in the biocatalyst should go together with process improvements.

Separation and Recycling of Biocatalyst

When the reaction is finished all detectable enzyme needs to be completely removed or eliminated to ensure product purity and also to avoid problems with emulsions being formed in the downstream processing. Fast and easy separation of the biocatalyst from the reaction medium can also be a key factor for enzymatic resolution reactions where the reaction has to be stopped at a given conversion to achieve an adequate ee of the product. In particular, when using whole cells and high concentration of organic compounds or mixing (resulting in cell lysis), the separation

can be problematic due to formation of emulsions or foaming. A simple method is to denature the enzyme to an insoluble precipitate by acidification, filtration is then sufficient to remove the majority of the enzyme precipitate (Savile et al., 2010). For high value products, discarding the enzyme after reaction can be economically feasible. However, in cases when the biocatalyst cost needs to be reduced recycling of the biocatalyst could be necessary.

Immobilization. Immobilization of enzymes can provide several advantages compared to free enzymes, including: easy recovery and reuse of enzyme, improved operational and storage stability of the enzyme, the possibility for continuous operation in packed bed reactors, and minimizing protein contamination in the product (Sheldon, 2007). Well-known problems of immobilization are loss in activity due to introduction of mass transfer limitation and by loss of active enzyme. A less discussed issue is that the required preparation step increases the cost of the enzyme. However, the cost contribution of the immobilized enzyme in the applied process has the potential to be lower than for free enzyme, since the immobilized enzyme can be reused for many reaction cycles.

Immobilization of whole cell ω-transaminase by entrapment in calcium alginate beads has been applied for the kinetic resolution of chiral amines in a packed bed reactor (Shin et al., 2001b). Entrapment of whole cells in calcium alginate beads was found to cause diffusion limitations and changes in substrate and product inhibition (Shin et al., 2001b). It was also reported that also both V_{max} and K_M changed when cells were immobilized in calcium alginate beads (Martin et al., 2007).

Immobilization of free ω-transaminases has been achieved both by covalent linkage to different solid support materials (Yi et al., 2007) and by entrapment in sol–gel matrices (Koszelewski et al., 2010a; Lee et al., 2006) with reported immobilization yields of ~20–50% protein and less than 20% activity. ω-transaminase immobilized on chitosan beads was reported to retain 77% activity after five reaction cycles, but was also susceptible to severe substrate and product inhibition (Yi et al., 2007). Immobilization of ω-transaminase in sol–gel matrices resulted in improved enzyme activity at higher pH and temperatures compared to free enzyme (Koszelewski et al., 2010a). Easy separation of product from sol–gel immobilized (*R*)-selective ω-transaminase allowed a two-step deracemization, consisting of kinetic resolution with the (*R*)-selective immobilized ω-transaminase and asymmetric synthesis with an (*S*)-selective ω-transaminase, to be carried out with a product yield of 89% (Koszelewski et al., 2010a).

When scaling up a reaction using immobilized biocatalyst the resistance of the particles to mechanical forces needs to be considered as this can limit their applicability. The use of a packed bed reactor would alleviate this problem but could be limited by the pressure drop over the bed or mass transfer (Lilly and Woodley, 1994).

Solubility Limitations and Use of Solvents

For the success of most biocatalytic routes, it is also critical to be able to supply substrates at a concentration above 50–100 g/L (Pollard and Woodley, 2007). A common characteristic inherent to aqueous biocatalytic processes is the low solubility of many substrates in water. Operating the process at too low a substrate concentration would lead to a low volumetric productivity and thereby high costs for equipment and downstream processing for product recovery. A list of some of the compounds used for transamination reactions is shown in Table VIII. From the table it is evident that for compounds such as acetophenone and homophenylalanine, a feeding strategy has to be employed to supply the substrate at a high concentration (Kim et al., 2007a). When a biocatalytic route is limited by substrate availability, whether due to low aqueous solubility, slow dissolution rate, or inhibition/toxicity, the controlled addition (feeding) of the substrate into the reaction medium is a common solution (D'Anjou and Daugulis, 2001; Doig et al., 2002; Lynch et al., 1997). This strategy can also help to minimize imine dimer formation (Savile et al., 2010, supplementary information).

Table VIII. Physical properties of different compounds at 25°C.[a]

		Aqueous solubility, S_{aq}		Critical conc. for cell, C^{aq}_{crit}	
Compound	Log *P*	g/L	mol/L	g/L	mol/L
Acetophenone	1.58	6.1	0.05	2.1	0.02
α-methylbenzylamine	1.49[b]	42	0.45	9.5	0.08
Alanine	−2.99	165	1.9	26.4	0.30
Pyruvic acid	−1.24[b]	Fully miscible	11.4	109	1.24
Acetone	−0.24	Fully miscible	17.2	100.1	1.72
2-propanamine	0.26	Fully miscible	16.9	100.5	1.70
Butanone	0.29	223	3.1	32	0.44
Butylamine	0.97	Fully miscible	1.53	105	1.44
Homophenylalanine	−1.20	5[a]	0.03	1.9	0.01
2-oxo-4-phenylbutanoic acid	0.96[b]	21.3[a]	0.12	6.1	0.03

[a]Data from EPI Suite—Estimation Software (http://www.epa.gov/oppt/exposure/pubs/episuitedl.htm).
[b]Estimated data using atom/fragment contribution method.

The substrate itself can be added beyond its solubility, thereby forming a second phase. However, this can cause toxicity and stability problems depending on the properties of the compound. The molecular toxicity, or the critical concentration (C_{crit}), is defined as the concentration at which the catalytic activity is lost (Osborne et al., 2010) or reduced by half (Vermue et al., 1993). Compounds with an aqueous solubility between 0.0003 and 1 M usually require an auxiliary phase for the purpose of in situ substrate addition (Straathof, 2003). As seen in Table VIII, this range includes for instance α-methylbenzyl amine, acetophenone, homophenylalanine and 2-oxo-4-phenylbutanoic acid which are known to have low solubility or inhibitory effects toward the catalytic activity (Kim and Shin, 1998; Lo et al., 2005).

To increase productivity, either a water miscible co-solvent (e.g., THF, iso-propanol, DMSO) that increases the solubility of the substrate in the aqueous phase can be added, or a water immiscible solvent (e.g., toluene, heptane, ethyl-acetate) can be added to act as a reservoir for the substrate. For instance Koszelewski et al. (2008b) investigated the effect of different water miscible solvents on the amination of 4-phenyl-2-butanone. It was seen that the addition of 15% DMSO gave the best activity for the enzyme. On the other hand, addition of solvents will decrease the stability of the biocatalyst and might also cause downstream problems, since the solvent needs to be separated from the product. DMSO for instance is known to be problematic to remove completely. Furthermore, water miscible solvent added up to 25% of volume generally only increases the solubility to a limited extent thereby limiting the usefulness of this approach.

An example of a 2-phase system was reported by Shin and Kim (1997), who used cyclohexanone in the resolution of MBA, which increased the reaction rate ninefold and allowed the resolution of 500 mM MBA with an ee of >95% at 51% conversion (Shin and Kim, 1997). A reported drawback was decreased stability of the enzyme due to the aqueous/organic interface. This problem was alleviated in another report by Shin and co-workers, using a reactor with the two liquid phases separated by a membrane (Shin et al., 2001a). Membrane extraction was also used in connection with a packed-bed reactor where whole cells were immobilized in calcium alginate beads (Shin et al., 2001b).

Many different solvents can be used for this purpose, although for industrial applications it is important that the solvents are generally regarded as safe (GRAS), which limits the number of available solvents. Also, the environmental impact of using solvents should be considered as solvent (volatile organic compound) emissions are one of the main contributors to the environmental impact of pharmaceutical processes (Jiménez-Gonzàlez et al., 2004). Further, the costs and efforts associated with wastewater treatment of side streams containing organic solvents are often complex and closely related with the solubility and toxicity of the solvents used (León et al., 1998).

Conclusions

Biocatalytic transamination is on the verge of taking-off as a tool for the production of chiral amines. Figure 4 points to the fact that the state-of-the art in transaminase processes has been insufficient for successful industrial application until very recently. The work by Truppo, Martin, Savile and respective co-workers could indeed indicate a breakthrough for transaminase technology. It is interesting to note that the first achieved process feasibility by reaction methods such as the use of enzymatic cascades and ISPR (Truppo et al., 2010), while the two others achieved improved process feasibility by protein engineering techniques to improve product catalyst productivity as well as tolerance to higher concentrations of the substrate (Martin et al., 2007; Savile et al., 2010). Further, the recent work on novel cascade reactions by Höhne et al. (2008) and by Koszelewski et al. (2008b) are significant contributions to the field that may in the future enable the asymmetric synthesis of products made by thermodynamically challenging reactions.

However, there is a need for the development of platform technologies to facilitate implementation and shortening of development times and uncertainties. Such technologies would include a broader availability of affordable transaminases, cascade systems or optimized whole-cell systems, preferably in an immobilized form. Also, protocols and kits for selecting the most appropriate separation procedure (e.g., resin selection for ISPR) could also simplify the development procedure. Further, the scientific community needs to be aware of the economic constraints present in industry to address the issues of biocatalyst productivity (g product/g biocatalyst), process intensity (g/L) and space time yield (g/L h). There is often a trade-off between the cost of the catalyst improvement and the benefits that can arise from such efforts. In an ideal situation, process and biocatalyst improvements should go side-by-side, in order to diminish the risk of improvement of one of the process metrics at the expense of another.

A rational process selection methodology, where the process set-up is given by the intrinsic properties of the system, for example, reaction thermodynamics, substrate solubility, enzyme kinetics (e.g., inhibition), would be desirable and would simplify and improve biocatalytic process design. However, as can be seen above, the choices made are highly interdependent and knowledge gaps still make such an approach out of reach. Even so, guidelines and rules of thumb are desirable to identify if a process is feasible allowing better choices to be made. For instance, knowledge of the thermodynamic properties of the reaction is crucial information in the early process development determining which process solutions are feasible. Still fundamental knowledge about the technologies to achieve high yields in thermodynamically unfavorable systems is lacking.

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A Robust Methodology for Kinetic Model Parameter Estimation for Biocatalytic Reactions

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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 $E_0K^f_{cat}/K^A_M$ is used to describe the biocatalyst performance.[4] 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.[5] 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:

$$A + B \leftrightarrow P + Q \quad (1)$$

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)

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and the final product (Q) leaves the enzyme last,[10] can be formulated as Eq. 2[11]:

$$r_Q = -r_A = \frac{E_0 K_{cat}^f K_{cat}^r \left(\gamma[A][B] - \lambda \frac{[P][Q]}{K_{EQ}}\right)\left(1 + \frac{[I]}{K_{Si}^I}\right)}{K_{cat}^r K_M^B \gamma[A] + K_{cat}^r K_M^A \gamma[B] + \frac{K_{cat}^f K_M^Q}{K_{EQ}}\lambda[P] + \frac{K_{cat}^f K_M^P}{K_{EQ}}\lambda[Q] + K_{cat}^r \gamma[A][B] + \frac{K_{cat}^f K_M^Q}{K_i^A K_{EQ}}\gamma\lambda[A][P] + \frac{K_{cat}^f}{K_{EQ}}\lambda[P][Q] + \frac{K_{cat}^r K_M^A}{K_i^Q}\gamma\lambda[B][Q]} \quad (2)$$

The dynamic equation consists of nine parameters including terms such as the catalytic turnover of the reaction (K_{cat}^f,K_{cat}^r), Michaelis parameters (K_M^A, K_M^B, K_M^P, K_M^Q), inhibition parameters (K_i^A,K_i^Q) that are derived from the core mechanism, uncompetitive substrate inhibition parameter (K_{Si}^I) 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:

$$K_{EQ} = \left(\frac{K_{cat}^f}{K_{cat}^r}\right)^2 \cdot \frac{K_M^P K_M^Q}{K_M^A K_M^B} = \left(\frac{K_{cat}^f}{K_{cat}^r}\right) \cdot \frac{K_M^Q K_i^P}{K_M^B K_i^A} = \left(\frac{K_{cat}^f}{K_{cat}^r}\right) \cdot \frac{K_M^P K_i^Q}{K_M^A K_i^B} = \frac{K_i^P K_i^Q}{K_i^A K_i^B} \quad (3)$$

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 $\left(1 + \frac{[I]}{K_{Si}^I}\right)$ 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):

$$r_Q = -r_A = \frac{E_0 K_{cat}^f [A][B]}{K_M^A [B] + K_M^B [A] + [A][B]}\left(1 + \frac{[I]}{K_{Si}^I}\right) \quad (4a)$$

Reverse direction (Eq. 4b):

$$r_A = -r_Q = \frac{E_0 K_{cat}^r [P][Q]}{K_M^P [Q] + K_M^Q [P] + [P][Q]}\left(1 + \frac{[I]}{K_{Si}^I}\right) \quad (4b)$$

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

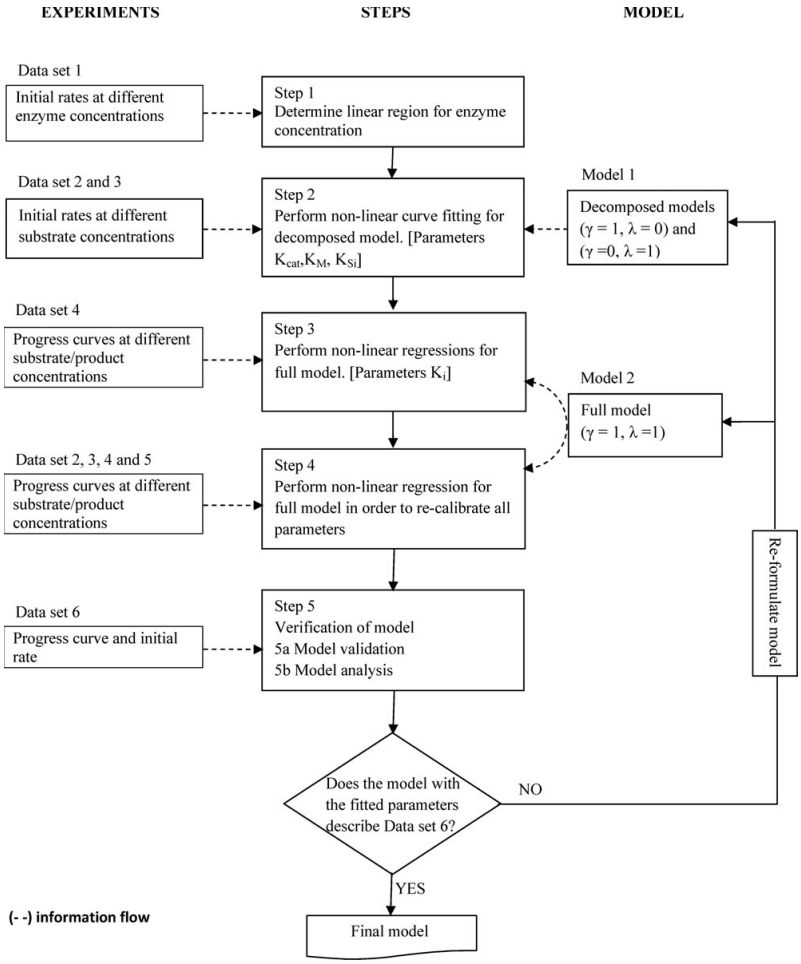

Figure 1. Proposed methodology for kinetic parameter estimation.

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}^I) 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).

$$J_1(\theta) = 1/2\sum_{j=1}^{N}\left(r_{A_{exp},j} - r_{A,j}(\theta)\right)^2 \tag{5a}$$

$$J_2(\theta) = 1/2\sum_{j=1}^{N}\left(r_{Q_{exp},j} - r_{Q,j}(\theta)\right)^2 \tag{5b}$$

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_n. Furthermore, the optimization is subjected to the constraint that parameter values are greater than 0 ($\theta > 0$, θ now represents the additional parameters).

$$J(\theta) = 1/2\sum_{j=1}^{N}\left([y_n]_{j,exp} - [y_n]_{j,model}(\theta)\right)^2 \tag{6}$$

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 $\left(\frac{d[y]}{d\theta}\right)$ 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 $(1 - \alpha)$ 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.

$$\theta_{1-\alpha} = \theta \pm \sqrt{\mathrm{diag}(\mathrm{Cov}(\theta))} \cdot t\left(N-p, \frac{\alpha}{2}\right) \tag{8}$$

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[23]:

$$\mathrm{Cor}(\theta_i, \theta_j) = \frac{\mathrm{Cov}(\theta_i, \theta_j)}{\sqrt{\mathrm{Cov}(\theta_i, \theta_i)\mathrm{Cov}(\theta_j, \theta_j)}} \quad (9)$$

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:

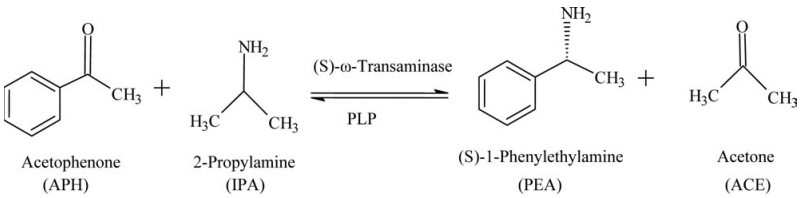

Figure 2. Transamination catalysed by ω-transaminase illustrating the synthesis of 1-phenylethylamine (PEA) and co-product acetone (ACE) from the substrates acetophenone (APH) and 2-propylamine (IPA) in the presence of the enzyme TAm.

Table 2. Data Collection for the Application of the Proposed Methodology

	Variable					
Data Set	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.

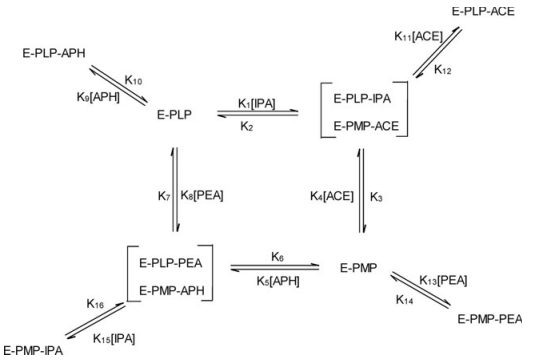

Figure 3. King-Altman representation of the ω-transaminase reaction mechanism.

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] ($\lambda = \gamma = 1$):

$$r_{[PEA]} = -r_{[APH]} = \frac{[E_0]K_{cat}^{f}K_{cat}^{r}\left(\lambda[IPA][APH] - \gamma\frac{[ACE][PEA]}{K_{EQ}}\right)}{K_{cat}^{r}K_{M}^{APH}\lambda[IPA]\left(1+\gamma\frac{[PEA]}{K_{Si}^{PEA}}+\frac{[IPA]}{K_{Si}^{IPA}}\right)+K_{cat}^{r}K_{M}^{IPA}[APH]\lambda\left(1+\frac{[APH]}{K_{Si}^{APH}}+\gamma\frac{[ACE]}{K_{Si}^{ACE}}\right)} + K_{cat}^{f}\frac{K_{M}^{PEA}[ACE]}{K_{EQ}}\gamma\left(1+\frac{[APH]}{K_{Si}^{APH}}+\lambda\frac{[ACE]}{K_{Si}^{ACE}}\right)+K_{cat}^{f}\frac{K_{M}^{ACE}[PEA]}{K_{EQ}}\gamma\left(1+\frac{[PEA]}{K_{Si}^{PEA}}+\lambda\frac{[IPA]}{K_{Si}^{IPA}}\right) + K_{cat}^{r}\lambda[IPA][APH]+K_{cat}^{f}\frac{K_{M}^{PEA}\lambda\gamma[IPA][ACE]}{K_{EQ}K_{i}^{IPA}}+K_{cat}^{f}\gamma\frac{[ACE][PEA]}{K_{EQ}}+K_{cat}^{r}\frac{K_{M}^{IPA}\lambda\gamma[APH][PEA]}{K_{i}^{PEA}} \quad (10)$$

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.

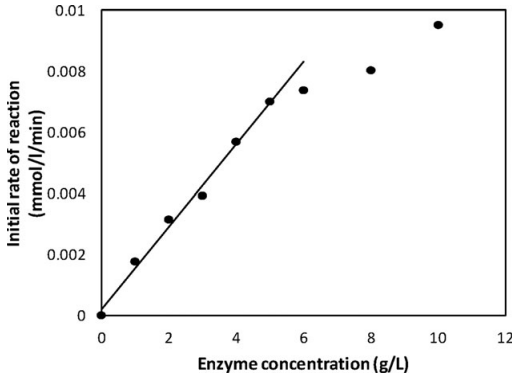

Figure 4. Enzyme linear region.

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}^{PEA}	2.41	±0.14
Substrate inhibition constants (mM)		
K_{Si}^{APH}	1.23	±0.16
K_{Si}^{PEA}	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}^{PEA}, K_{M}^{ACE}, K_{Si}^{APH}, K_{Si}^{PEA}) were fixed and the additional parameters (K_{i}^{IPA} and K_{i}^{PEA}) 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]

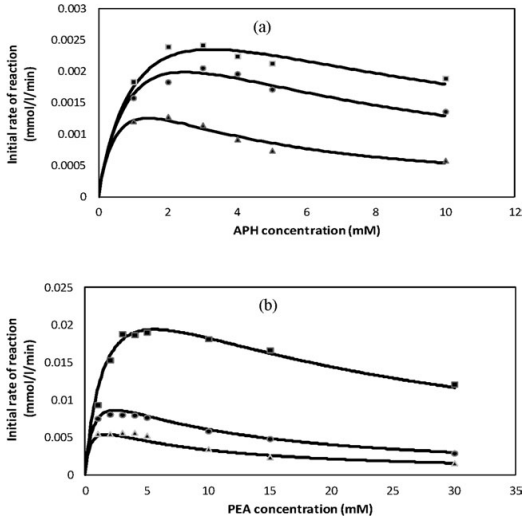

Figure 5. Experimental data (symbol) and model prediction (solid line) using estimated parameters from step 2.

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}^{f} which is the catalytic turnover of the reaction in the forward direction is much smaller than the parameter value K_{cat}^{r} 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}^{f} and K_{cat}^{r} 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}^{f}	0.0025	±7E-5	0.0078	±7E-5
K_{cat}^{r}	0.023	±4E-4	0.013	±0.007
Michaelis constants (mM)				
K_M^{APH}	1.50	±0.10	1.85	±4.78
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	±0.14	0.12	±0.01
Substrate inhibition constants (mM)				
K_{Si}^{APH}	1.23	±0.16	4.15	±3E-4
K_{Si}^{PEA}	6.01	±0.21	10.38	±3E-4
Core inhibition constants (mM)				
K_i^{APH}	–	–	0.09	–
K_M^{IPA}	4,281	±0.03	4,281	±0.63
K_M^{ACE}	1.55	±0.01	0.11	±0.01
K_M^{PEA}	–	–	1E5	–
Equilibrium constant				
K_{EQ}	–		0.033	

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.

Figure 7 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 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_M^{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,

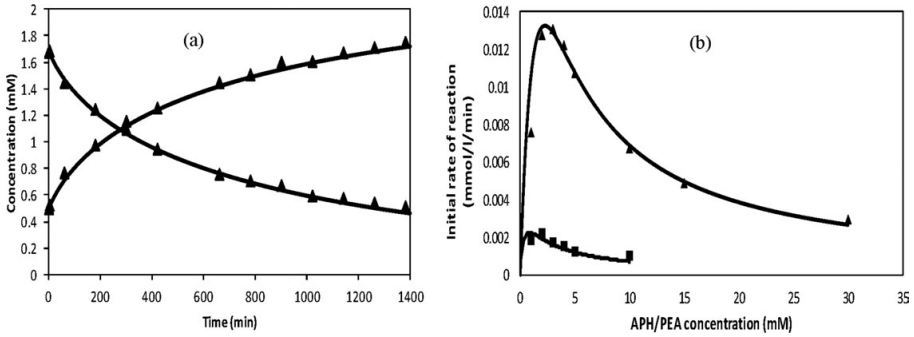

Figure 6. Model predictions using the estimated parameters of the model (solid line) and experimental data (symbols).

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.

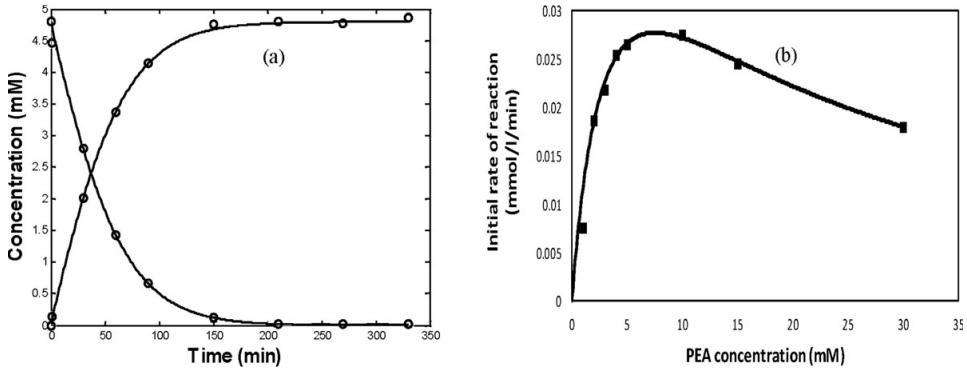

Figure 7. Model predictions using the estimated parameters of the model (solid line) and experimental data (symbols).

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_{cat}^{f}	K_M^{IPA}	K_M^{APH}	K_{Si}^{APH}	K_{cat}^{r}	K_M^{PEA}	K_M^{ACE}	K_{Si}^{PEA}	K_i^{IPA}	K_i^{PEA}
K_{cat}^{f}	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_{cat}^{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_{PPEA}	-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.

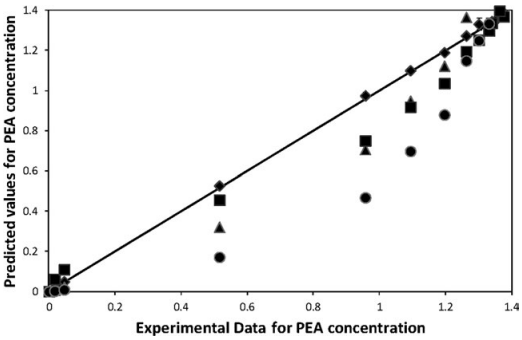

Figure 9. Parity plot of different estimation methodologies.

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_m and K_{cat}. 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 Willetts used 130 experiments to determine the parameters for a transketolase reaction.[32] †Shin and Kim used 125 experiments to determine the parameters for an ω-transaminase reaction.[29] ‡The minimum number of data points corresponds to the number of parameters. §Chen et al. used 43 experiments to determine the parameters for a transketolase reaction.[18] #52 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[33] 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_r^{cat}, K_M^{APH}, and K_M^{IPA} 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

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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_{Si} = substrate inhibition constants, mM
K_{EQ} = chemical equilibrium constant
γ, λ = reaction direction indicator
y = 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

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