Applying Enzymatic Cascades for ISCPR in ω-transaminase Systems

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PhD thesis

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Preface

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

The work presented in this thesis was conducted at the Center for Process Engineering and Technology (PROCESS), Department of Chemical and Biochemical Engineering, Technical University of Denmark (DTU), from December 2010 to February 2014. The main supervisor of the project was Professor Krist Gernaey (DTU, PROCESS) and the co-supervisors were Professor John Woodley (DTU, PROCESS) and Dr. Pär Tufvesson (DTU, PROCESS).

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Na kraju bih se htio zahvaliti cijeloj svojoj obitelji, pogotovo svojim roditeljima bez čije potpore sve ovo ne bi bilo moguće. Hvala vam što ste vjerovali u mene i što ste me poticali da dosegnem svoj potencijal. Hvala na riječima podrške u vrijeme kada mi je najviše trebala.

Abstract

Biocatalysis complements the classical organic synthesis, and in many cases the superior selectivity of a biocatalyst is a strong driver explaining why there are an increasing number of processes where traditional organic synthesis has been replaced or combined with biocatalytic industrial process steps. An important fact is also that different types of selectivity make biocatalysis an excellent tool for overcoming difficulties typically associated with organic synthesis. Regioselectivity of the biocatalysts offers potential process simplification compared to the organic synthesis routes (reduction of the number of protective/deprotective steps), and stereoselectivity of the biocatalyst enables production of the desired chiral compounds, which often are building blocks of APIs. Currently there are many established processes in the industry using biocatalysis (≈ 300), e.g the usage of lipases, esterases, ketoreductases and proteases and many more emerging biocatalysts such are monoamine oxidases, transaminases and P450 monooxygenases to name a few.

The focus of this thesis is the biocatalytic synthesis of small molecule pharmaceuticals (Mw<1000), and in particular the production of optically pure amines via ω-transaminases, which is an interesting class of reactions for the pharmaceutical industry. There are many challenges related to the realization and implementation of these technologies, and attempts of tackling them have been numerous. In some cases ω-transaminase catalyzed reactions are thermodynamically challenged and equilibrium shifting strategies are required. The proposed equilibrium shifting strategies are selection of an amino donor, excess of an amino donor, *in-situ* product removal (ISPR) and *in-situ* co-product removal. (ISCPR). For severely thermodynamically challenged reactions ISCPR by enzymatic cascades often provides the only viable option as equilibrium shifting strategy. In the literature several enzymatic cascades have been reported as an ISCPR for the ω-transaminase systems, however in most cases no process considerations have been made and the consequences of using a givens cascade in an industrial process context have thus not been considered properly.

In this research lactate dehydrogenase (LDH) (E.C. 1.1.1.27), alanine dehydrogenase (E.C. 1.4.1.1) (AlaDH) and yeast alcohol dehydrogenase (E.C. 1.1.1.1) (YADH) have been researched as co-product degrading enzymes and glucose dehydrogenase (GDH) (E.C. 1.1.1.47) and formate dehydrogenase (E.C. 1.2.1.2) (FDH) as co-factor regeneration enzymes. Additionally pyruvate decarboxylase (E.C. 4.1.1.1) (PDC) and acetolactate synthase (E.C. 2.2.1.6) (ALS) have been considered as co-product degrading options.

This work presents a procedure for cascade selection based on process considerations: thermodynamics, selectivity and operational stability while the final selection is further supported by the use of kinetic models. From the above presented cascade system options, the selection procedure identified the LDH/FDH cascade

system as the system that is most promising for future industrial implementation. Furthermore, the required improvements of the ω-transaminase have been identified as a function of the added cascade enzymes and for the case γ_{LDH} = 11 g L^{-1}, γ_{FDH} = 11 g L^{-1} and c_{NADH} = 0.1 mmol L^{-1}, it was found that the ω-transaminase activity expressed as $V_{max\_f,r}$ is required to be 55.33 mmol min^{-1} L^{-1} to achieve 95 % conversion within 24 h. Further investigation concluded that a significant LDH concentration reduction is possible if inhibition by lactate is alleviated (preferably by protein engineering). This thesis identified the UFMR (UltraFiltration Membrane Reactor) as a viable process design option and charge analysis showed that ISPR is possible via ion exchange resins or electrodialysis. An ISPR example showed that process intensification could yield significant reductions in the required ω-transaminase activity improvement (up to five fold improvement) needed to achieve a viable industrial process, as well as reduction of required tolerance toward product inhibition.

Although this thesis has been based on a specific case of a severely thermodynamically challenged ω-transaminase reaction (K_{eq} = $4.03 \cdot 10^{-5}$), the selection framework can be transferred to any thermodynamically challenged reaction where the use of ISCPR by enzymes is considered to shift the equilibrium. Therefore, this work delivers: a) a method for initial investigation of thermodynamic limitations and viability of one or more equilibrium shifting strategies; b) a method for selecting a viable cascade option for ISCPR based on industrial conditions; c) information on the required enzyme performance e.g. the activity of the ω-transaminase, and potentially required compromises using process intensification tools and methods.

Résumé på dansk

Det ses i stigende grad at processer der traditionelt er baseret på organisk syntese udskiftes eller kombineres med biokatalytiske procestrin. Dette skyldes i høj grad biokatalysatorers generelt fortræffelige selektivitet. Derudover giver de forskellige former for selektivitet, et enzym kan have (f.eks. regio-selektivitet, stereo-selektivitet og substrat selektivitet), fremragende muligheder til at overvinde vanskeligheder der typisk er forbundet med selektiv organisk syntese. Biokatalysatorers regio-selektivitet kan potentielt forenkle organisk syntese baserede processer, da der i mindre grad vil være behov produkt beskyttende og ikke beskyttende proces trin. Biokatalysatorers stereo-selektivitet gør det muligt at producere de ønskede chirale molekyler i højere grad. Chirale molekyler er meget ofte brugt som byggesten til aktive farmaceutiske komponenter (APIs), hvor det er vigtigt at have det chirale molekyle i så ren form som muligt. Nu til dags er der relativt mange industrielle processer der benytter biokatalyse (~300). Disse processer benytter typisk biokatalysatorer som lipaser, esteraser, ketoreductaser og proteaser. Ydermere, er der udsigt til industriel brug af mange flere nye typer af biokatalysatorer, f.eks. monoaminooxidaser, transaminaser og P450 monooxygenaser.

Denne afhandling fokuserer på syntesen af lægemidler baseret på små molekyler (MW<1000) ved brug af biokatalysatorer. Der fokuseres især på syntesen af optisk rene chirale aminer ved brugen af ω-transaminaser, der har stor interesse for den farmaceutiske industri. Der er mange udfordringer forbundet med brugen af ω-transaminase katalyserede reaktioner, og antallet af forsøg på overkomme disse udfordringer har også været talrige. En udfordring er bland andet at ω-transaminase reaktioner i nogle tilfælde er termodynamisk udfordrede, hvilket gør det nødvendigt at benytte diverse strategier for at skifte reaktionsligevægten. Oftest for at skifte reaktionsligevægten benyttes strategier, som at udskifte amindonoren, tilføre et overskud af amindonoren, samt fjerne produktet og/eller biproduktet *in-situ* (ISPR / IScPR). I de tilfælde hvor reaktionerne er ekstremt termodynamisk udfordret, er det oftest kun muligt at benytte IScPR med enzymatiske kaskadereaktioner til at skifte reaktionsligevægten. I den videnskabelige litteratur findes der flere eksempler på ω-transaminase processer, der benytter flere forskellige enzymatiske kaskadereaktioner som IScPR strategi. Dog i mange af disse rapporterede tilfælde mangler der at blive taget højde for hvordan implementeringen af kaskadereaktioner influerer de industrielleprocesser.

Det forskningsmæssige fokus for denne afhandling er baseret på enzymatiske kasakadereaktioner med laktatdehydrogenase (LDH) (EF 1.1.1.27), alanindehydrogenase (AlaDH) (EF 1.4.1.1) og gæralkoholdehydrogenase (YADH) (EF 1.1.1.1) til nedbrydning af biprodukter, IScPR strategi. Ydermere, er brugen af glukosedehydrogenase (GDH) (EF 1.1.1.47) og formiatdehydrogenase (FDH) (EF 1.2.1.2) blevet undersøgt for deres potentiale til at regenerere enzym co-faktorer. Derudover, er brugen af

pyruvatdecarboxylase (PDC) (EF 4.1.1.1) og acetolactatsyntase (ALS) (EF 2.2.1.6) som IScPR strategier også undersøgt.

I denne afhandling præsenteres en procedure der er yderst nyttig til at udvælge kaskadesystemer, til at skifte reaktionsligevægten, baseret på procesovervejelser. Procesovervejelserne inkluderer termodynamikken for den givne reaktion, selektiviteten og stabilitet ved drift. Den endelige udvælgelse er yderligere understøttet af brugen af kinetiske modeller. Fra de foroven præsenterede kaskade systemer indikerede den foreslåede procedure, at LDH/FDH kaskadesystemet er det mest lovende system, for potentiel fremtidig industriel implementering. Derudover, blev det observeret at forbedringerne i ω-transaminase synteseruten kunne beskrives som en funktion af mængden af tilsat kaskade enzym. I et tilfælde med γ_{LDH} = 11 g L^{-1}, γ_{FDH} = 11 g L^{-1} and c_{NADH} = 0.1 mmol L^{-1}, blev det fundet at ω-transaminase aktiviteten udtrykt ved $V_{max\_f,r}$ er nød til at være 55.33 mmol min^{-1} L^{-1}, for at opnå 95% omdannelse indenfor 24 timer. Yderligere undersøgelser indikerede, at der er muligt væsentligt at reducere LDH koncentrationen, hvis inhiberende effekter fra laktat på LDH mindskes, f.eks. ved protein engineering. Denne afhandling fandt frem til at en ultrafilteringsmembranreaktor (UFMR) er en lovende procesløsning. Analyse af polariteten af komponenterne i de benyttede reaktionssystemer indikerede at det potentielt vil være muligt at foretage ISPR fjernelse ved brug af enten ionbytnings partikler eller elektrodialyse. Et ISPR casestudy viste at denne form for procesintensivering, betydeligt kan mindske industrille proceskrav for ω-transaminase aktivitet, op til fem gange mindre, samt markant reducere produkt inhiberende effekter.

På trods af denne afhandling er baseret på et konkret casestudy for en termodynamisk udfordret ω-transaminase reaktion (K_{eq} = $4.03 \cdot 10^{-5}$), så kan den foreslåede kaskadeudvælgelsesprocedure ligeledes benyttes til andre termodynamiske udfordrede reaktioner. Det kræver dog at der overvejes IScPR implementering ved brugen af kaskadeenzymer til at forskydereaktionsligevægten. Gennem dette arbejde leveres der derfor følgende: a) en metode til at lave indledende undersøgelser af termodynamiske begrænsninger, samt brugbarheden af en eller flere strategier til at forskyde reaktionsligevægten; b) en metode til at udvælge brugbare IScPR kaskadereaktioner baseret på industrielt relevante procesforhold; c) og oplysninger om påkrævet ydeevne for enzymer, f.eks. aktiviteten af ω-transaminase, og potentielt mulige proceskompromiser der kan foretages ved brugen af procesintensiveringsværktøjer og metoder.

Abreviations

ABREVIATIONS	DESCRIPTION
ADP	Adenosine diphosphate
AHAS	Acetohydroxyacid synthase
AlaDH	Alanine dehydrogenase
ALS	Acetolactate synthase
API	Active pharmaceutical ingredient
ATA	ω-transaminase
CCS-IONP	Catechol-chitosan iron oxide nanoparticles
CDW	Cell dry weight
CS-IONP	Chitosan iron oxide nanoparticles
DMSO	Dimethyl sulfoxide
DSP	Downstream processing
e.e.	Enantiomeric excess
E-PLP	Enzyme – pyridoxal-5-phosphate complex
E-PMP	Enzyme – pyridoxaamine-5-phosphate complex
EtOH	Ethanol
FAD	Flavin adenine dinucleotide
FDH	Formate dehydrogenase
FMN	Flavin mononucleotide
GDH	Glucose dehydrogenase
IONP	Iron oxide nanoparticles
***i*-PrNH$_2$**	*i*-Propylamine
ISCPR	*in-situ* co-product removal
ISPR	*in-situ* product removal
ISSS	*in-situ* substrate supply
LDH	Lactate dehydrogenase
MBA	Methylbenzylamine
MTBE	Methyl *tert*-butyl ether
NAD$^+$	Nicotinamide adenine dinucleotide
NADP$^+$	Nicotinamide adenine dinucleotide phosphate

ABREVIATIONS	DESCRIPTION
PAT	Process analytical technology
PDC	Pyruvate decarboxylase
PLP	Pyridoxal-5-phosphate
PMP	Pyridoxaamine-5-phosphate
PSE	Process system engineering
Ribo	Ribose ring
ThDP	Thiamine diphosphate
ThPP	Thiamine pyrophosphate
UFMR	Ultrafiltration membrane reactor
YADH	Yeast alcohol dehydrogenase

Symbols

ABREVIATIONS	DESCRIPTION	UNIT
[A], c_A	Concentration of component A	[mmol L^{-1}]
e	Natural logarithm	[-]
F	Flow rate	[L min^{-1}]
I	Inhibitor concentration	[mmol L^{-1}]
k_d	Dissociation constant	[h^{-1}]
K_{eq}	Thermodynamic equilibrium constant	[-]
k	Reaction rate constant	[min^{-1}]
K_i	Inhibition constant	[mmol L^{-1}]
K_m	Michaelis-Menten constant	[mmol L^{-1}]
logP	Partitioning coefficient	[-]
M_w	Molar weight	[g mol^{-1}]
n_a	Amount of component a	[mmol L^{-1}]
pK_a	Acid dissociation constant	[-]
p_{vap}	Vapor pressure	[mm Hg]
R	Gas constant	[J mol^{-1} K^{-1}]
r_0	Initial reaction rate	[mmol min^{-1} g^{-1}]
r_t	Reaction rate at time t	[mmol min^{-1} g^{-1}]
S_{aq}	Aqueous solubility	[g L^{-1}]
t	Time	[min, h]
T	Temperature	[K, °C]
T_m	Melting temperature	[°C]
$t_{1/2}$	Half-life time	[h]
V	Volume	[L]
V_{max}	Maximum velocity	[mmol min^{-1} g^{-1}]
X	Amino donor excess	[-]
α	Stoichiometric coefficient	[-]
γ	Mass concentration	[g L^{-1}]
ΔG°	Gibbs free energy change	[J mol^{-1}]
τ	Residence time	[min]

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1 Introduction

1.1 Background

Indeed, biocatalysis complements the classical organic synthesis in many ways and its regio- stereo- and substrate selectivity led to the combination with or replacement of more classical organic synthesis approaches. The usage of the biocatalysis is continuously increasing in the pharmaceutical industry due to the greater availability of the enzymes and the diversity of the reactions enzymes can catalyze[1]. The new potentials for the pharmaceutical industry now lies in the stereoselectivity and application of biocatalysis of valuable components, e.g. optically pure amines. In some cases these reactions are severely thermodynamically challenged and a strategy for shifting of the equilibrium is imperative, e.g. excess substrate, *in-situ* product removal (ISPR) or *in-situ* co-product removal (ISCPR). For the cases where the co-product causes inhibition or it is preferable that the product remains in the reaction mixture, a possible strategy is *in-situ* co-product removal (ISCPR). There are two major requirements for ISCPR of severely thermodynamically limited reactions: to reduce selectively the concentration of co-product to very low concentration and to maintain it low over the course of the reaction. In such cases the use of enzymes for ISCPR purposes offers a clear advantage over other techniques. Enzymes are selective towards the desired molecule (co-product) which causes the thermodynamic shift only to the product side of the reaction and are extremely successful at removing the co-product at already low concentrations.

1.2 Motivation

In the particular case of biocatalytic synthesis of chiral components a synthesis of optically pure amines as pharmaceutical building blocks catalyzed by ω-transaminase is of high interest to the pharmaceutical industry. As the necessity for this process is increasing, it is extremely important for overcoming different challenges to develop a toolbox, e.g. expanding ω-transaminase libraries, increasing the library of enzymes capable of removing different co-products or other process related tools.

In some cases ω-transaminase catalyzed reactions are thermodynamically challenged and it is believed that reactions with low K_{eq} values (< 0.1) are too severely thermodynamically challenged and, as a consequence, that the process development is impractical. The hypothesis that is tested in this thesis is to show that reactions with K_{eq} values lower than 0.1 can be successfully implemented into a process, and that the cascades are a viable solution for this challenge. A case study was chosen where acetophenone and alanine are used as

substrates in reactions where a severe thermodynamic challenge is present (K_{eq} = 4.03·10^{-5}). The severity of the thermodynamic equilibrium of this particular system makes it a very interesting model case for an investigation of methods that are suitable for overcoming the thermodynamically challenged reactions. Additionally, it has been found that the co-product pyruvate can be removed by several different cascade options. Although these cascades have been reported in the literature no systematic comparison or guidelines for selection of specific cascades has been developed. Therefore, it is of high importance and the main focus of this thesis to develop methods for comparison and selection of different cascade systems with the final aim of achieving industrial implementation.

1.3 Objectives

The main objectives of this thesis was to develop a strategy for comparison of possible cascade enzymes and to select a specific process setup best suited to the characteristics of the selected cascade system. While pursuing the main objectives, following points were also addressed:

- The selection of cascade candidates was based on industrial conditions
- Final cascade selection was supported by developing and using kinetic models
- Further debottlenecking of selected cascade was possible investigating kinetic behavior identifying product inhibition of co-product consuming enzyme as possible challenge of process development
- While discussing a process setup several major topics were addressed
    - Formulation of all biocatalytically active components (enzymes and co-factors) plays an important role in process development and a part of the discussion is dedicated to this strategy.
    - Batch vs continuous, one pot vs two pots, feeding strategies, process control and downstream processing are some of the process considerations that are discussed.
    - The decision for choosing one process implementation option over another is motivated on the basis of simulations with the developed models or literature suggestions of similar process implementations.
    - The use of process intensification techniques, e.g. ISPR can successfully overcome some of the process challenges, e.g. ω-transaminase with product inhibition can be used or lower activity of ω-transaminase is required for successful process implementation or the combination of those two enzyme characteristic.

1.4 Structure of the PhD Thesis

The thesis has been divided into 6 main chapters:

- **Chapter 2 (Literature Overview)** provides an overview of the current developments in the field of synthesis of optically pure amines by ω-transaminases. The overview focuses on the reported cascade systems used for removing the co-product in ω-transaminase system, process implementations and biocatalyst formulation, to name a few. The objective of this overview is to summarize the current knowledge and state of the art of the research in the field of cascade systems, but also to indicate clearly that no decision making tools for cascade selection has been developed.
- **Chapter 3 (Considerations for a Cascade Selection)** is a framework designed to select a cascade system for thermodynamically challenged ω-transaminase system and ISCPR by enzymes is equilibrium shifting strategy of choice. The selection process is focused on guidelines for successful implementation of biocatalytic processes focusing mainly on final product concentration (> 50 g L^{-1}) and it is based on three important process considerations: thermodynamics of the system, substrate selectivity and operational stability of cascade enzymes. The objective of this chapter is to investigate the viability of proposed cascade candidates and select feasible candidates for further investigation.
- **Chapter 4** (**Developing Kinetic Models of LDH, GDH and FDH Enzymes to Assist the Selection Process**) discusses further selection of feasible cascade candidates. Kinetic models describing enzymes behavior in a very complex reaction media re develop to facilitate decision and to optimize enzyme concentrations. Detailed kinetic study also predicts the required ω-transaminase activity for a 95 % conversion within 24 h while debottlenecking of the overall cascade system identifies product inhibition of co-product removing enzyme (LDH) one of the mayor hurdles for rational enzyme consumption.
- **Chapter 5** (**Industrial Perspectives and Process Strategies for Implementation of ω-transaminase/LDH/FDH System**) discusses the process implementation of the selected cascade candidate. Several process options are proposed based on process considerations and requirements, such as pH regulation, biocatalyst formation, one vs two pot system to name a few. Every process configuration is discussed and either backed by process simulations or examples from the literature. Finally, downstream processing strategies are discussed and the benefits of possible ISPR techniques are highlighted. The objective of this chapter is to give a better overview of possible process implementation options and to discuss choices made based on the process conditions.
- **Chapter 6 and 7** conclude the thesis, identify open challenges and discuss future perspectives.

2 Literature Overview

2.1 Introduction

The production of optically pure amines is an interesting topic for the pharmaceutical industry, particularly via biocatalysis when using e.g. ω-transaminases, an emerging technology. There are many challenges following the realization and implementation of these technologies and attempts of tackling them have been numerous. It is therefore the aim of this overview to give a comprehensive overview of the work that has been published in the literature by describing the challenges which can be placed into two groups: biocatalytic and thermodynamic challenges. The focus of this thesis is primarily on the latter challenge, and in this literature overview several relevant equilibrium shifting strategies have been discussed. The focus has been placed on the ISCPR by enzymes which have been extensively documented in the literature. However, no guidelines have been reported on how to choose a cascade for shifting the thermodynamic equilibrium, and how to perform its implementation to an industrial process. Another question that arises is what are the consequences of using a particular cascade in the industrial process? This literature overview lays the foundation for understanding the current state of research on the production of optically pure chiral amines by ω-transaminase in general, and more specifically the cascade options for shifting the equilibrium. The literature overview is a prelude for more detailed analysis described in the following chapters.

The case study of asymmetric synthesis of optically pure amines by ω-transaminase places this work in the domain of the pharmaceutical industry. However the intent was that the conclusions made from observation of this system can be generic and implemented for a wide range of processes facing thermodynamic limitations. Currently, the enzymatic strategies for shifting the equilibrium are still not well established in the industry, and all investigated cases are so-called 1st generation processes, meaning all processes are still not industrially established. However, in the future these equilibrium shifting technologies will be part of a common process toolbox, and for achieving the well-defined and established industrial implementation as 2nd generation processes further research has to be conducted and an improved understanding of the cascade processes has to be obtained.

2.2 Biocatalysis in the Pharmaceutical Industry

Biocatalysis is already an established technology for some processes in the pharmaceutical industry, especially for the production of small-molecule pharmaceuticals. However continuous development of the biocatalysts and increased documentation on the broader substrate range they can catalyze makes biocatalysis applicable

for an increasing number of processes, not only in the pharmaceutical industry, but also for the production of fine and bulk chemicals. Biocatalysis is an interesting alternative to the current traditional metalo- and organocatalysis methods for several reasons. The biggest advantage is the outstanding selectivity of the biocatalyst, both with respect to regio- and stereoselectivity. This in turn decreases the complexity of classical synthetic routes by removing the need for multiple protection and deprotection steps. High selectivity also leads to higher yields. Reactions are usually performed under mild conditions, neutral pH, at room temperatures and in water medium without the need for organic solvents which in many cases can be carcinogenic and/or flammable.

Enzymes have been developing in nature for millions of years and are very well adapted to their natural surroundings: the conditions inside the cell. The natural enzymes are somewhat selective towards their natural substrate[2], tolerate small concentrations of substrates and products and operate under neutral pH and mild temperatures. However, these conditions are not suitable for the industrial processes. Traditionally, in the synthetic route a biocatalytic step was defined and a biocatalyst identified. The design of the process and process solutions were tailored to suit the biocatalyst properties and to overcome its shortcomings. In addition to the reaction engineering, attempts were made to stabilize and reuse enzymes by immobilization[3]. Nowadays however, it is possible to tailor the biocatalyst with specific properties (the ideal biocatalyst)[4] to the needs of the process. The protein engineering methods e.g. directed evolution enabled the development of enzymes suited to the specific industrial process. This, so-called third wave of biocatalysis[5] together with the development of other supporting technologies such as miniaturization technologies for high-speed screening and mathematical modelling, makes biocatalysis a rapidly expanding technology in the pharmaceutical industry and beyond.

The industrial biocatalytic process has to be economically viable, and should therefore be comparable to the values and economic parameters of a competing chemical process for producing the same product. Depending on the complexity of the biocatalytic process, there are many factors which determine the economic viability, and the published data on the economics of biocatalytic processes is scarce. However, the literature identifies two very important parameters for every biocatalytic process[6-8]. The first parameter is the added value of the biocatalyst and can be linked to the biocatalytic productivity [$g_{product}/g_{enzyme}$]. The higher the biocatalyst productivity, the lower the quantities that have to be used to achieve the desired amount of the product. The second parameter is the concentration of the product which determines the type of downstream equipment and the effectiveness of the product recovery. A more detailed and comprehensive list of process metrics which can be used for the comparison of the biocatalytic processes is given in the Table 1 which was presented by Tufvesson et al.[8] The authors point out that these metrics should be considered more as general

recommendations and that factor listing and evaluation for economic feasibility is determined on a case-to-case basis.

Table 1 Guidelines for successful biocatalytic processes adapted from [8]

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

There has been much effort in making the pharmaceutical processes greener. Several guidelines for achieving greener processes have been published, and biocatalysis provides certain opportunities to address some of these guidelines. Unlike chemocatalysis where catalysts are often heterogeneous and the catalytically active components are usually transition metals, biocatalysts are completely made from renewable materials. Furthermore, certain pharmaceutical processes involve long synthetic routes which result in a high E-factor [$kg_{waste}/kg_{product}$][9, 10]. Due to the exquisite selectivity of the biocatalysis and the ability to skip protection and deprotection steps (stereoselectivity), the E-factor can be significantly lowered, thus implying a greener process. The biocatalytic reactions are usually performed at ambient temperatures, at mild pH values in aqueous systems and other environmental benefits can also be achieved, e.g. the replacement of hazardous reagents or organic solvents in reactions[11].

2.3 Synthesis of Optically Pure Amines

The reactions in the human body are based on chirality, and therefore one stereoisomer of a certain compound is usually much more active than another. Moreover, the undesired stereoisomer can lead to reactions generating unwanted side effects. This is, therefore, one of the motivations of the pharmaceutical industry to produce optically pure compounds or building blocks. Out of many functional groups, synthesis of optically pure amines represents one important segment in the production of optically pure compounds.

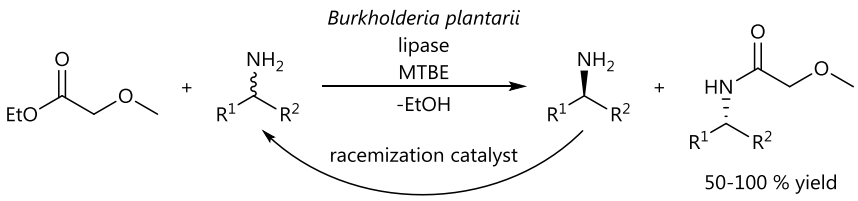
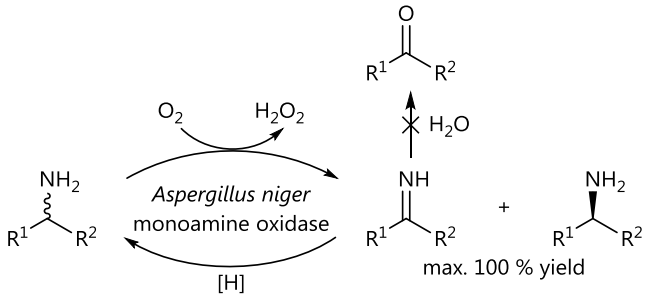
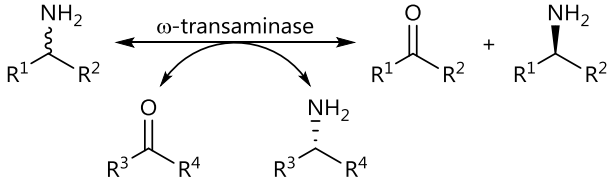
One of the most widely used chemocatalytic approaches for production of chiral amines is crystallization of racemic amines with chiral carboxylic acids[12]. This method starts from the racemic mixture, and therefore the maximum yield is only 50 %. Another chemocatalytic method relies on the reduction of the C=N double bond

from the prochiral precursor[13]. However, as described previously in the chapter 2.2 the biocatalytic approach gives an interesting alternative to the traditional methods of preparing chiral amines and it is the focus of this thesis. There are two main strategies of biocatalytic synthesis of chiral amines: (dynamic) kinetic resolution and asymmetric synthesis.

2.3.1 Enzymatic Kinetic Resolution of Racemic Amines

Kinetic resolution of racemic amines is a method that differentiates between the two enantiomers in the racemic mixture. The undesired enantiomer is reacted away thus increasing the optical purity of the racemic mixture. The major drawback of this method is the maximum conversion of only 50 % in a single kinetic resolution step. To improve this method and achieve higher yields, a racemization step was introduced to return the undesired product to the starting racemic mixture. This method is then called dynamic kinetic resolution. Either kinetic resolution or dynamic kinetic resolution of racemic amines can be achieved by using several enzymes. The overview of these technologies and applications is shown in the Table 2.

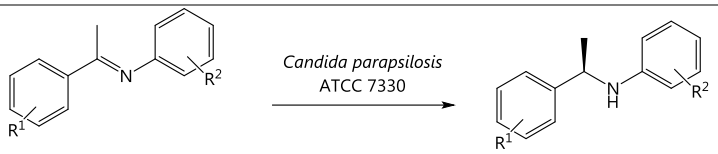
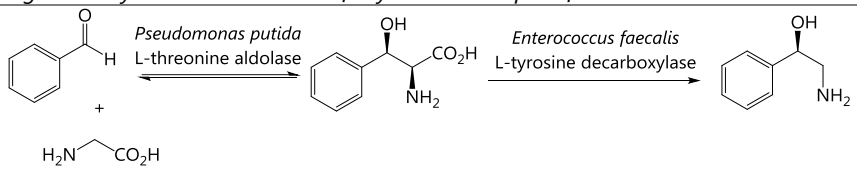
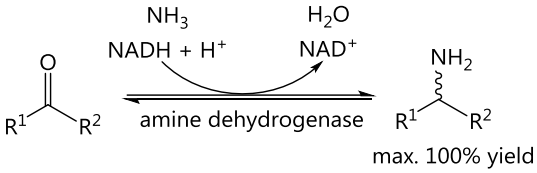
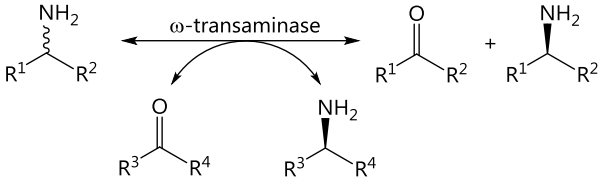
Table 2 The overview of kinetic resolution methods for production of chiral amines

enzyme	reaction scheme	literature
hydrolyases (E.C. 3.x.x.x)		15-18
	Figure 1 (Dynamic) kinetic resolution with hydrolases adapted from Höhne et. al.[14]	
monoamine oxidases (E.C. 1.4.3.4)		14, 19-21
	Figure 2 Deracemization with monoamine oxidase adapted from Höhne et. al.[14]	
ω-transaminases (E.C. 2.6.1.18)		22-35
	Figure 3 Kinetic resolution by ω-transaminase adopted from Höhne et. al.[14]	

2.3.2 Asymmetric Synthesis of Optically Pure Amines

Asymmetric synthesis, also called enantiomeric or chiral synthesis, is defined as a method that favors the formation of a specific enantiomer. The starting material is non-chiral and the enzyme is (R) or (S) selective, resulting in (R) or (S) chiral product respectively. The theoretical yield is 100 % which makes this method very interesting. The overview of possible biocatalytic methods is given in the Table 3.

Table 3 The overview of asymmetric synthesis methods for the production of chiral amines

enzyme	Reaction scheme	literature
ketimine reductases (E.C. 1.5.1.25)		36
	Figure 4 Asymmetric reduction of aryl imines adopted from Höhne et. al.[14]	
decarboxylases (E.C. 4.1.x.x)		38
	Figure 5 Combined use of L-threonine aldolase and L-tyrosine decarboxylase adapted from Turner et. al.[37]	
amine dehydrogenases (E.C. 1.4.99.3)		39, 40
	Figure 6 Asymmetric synthesis with amine dehydrogenase adapted from Höhne et. al.[14]	
ω-transaminases (E.C. 2.6.1.18)		41, 42
	Figure 7 Asymmetric synthesis with ω-transaminase adopted from Höhne et. al.[14]	

2.3.2.1 Asymmetric Synthesis of Chiral Amines by ω-transaminase

The first work regarding the production of optically pure amines by ω-transaminase was done by Stirling et. al[24] describing a kinetic resolution of a racemic mixture of amines. This research continued until 1999, favoring the (dynamic) kinetic resolution due to the faster reaction rates and favorable equilibrium. In 1999, the groundbreaking work by Shin and Kim[41] was published, involving the asymmetric synthesis of chiral amines by

the ω-transaminase. The asymmetric synthesis approach started to become a hot topic as the need for cheaper and simpler methods for production of chiral amines arose. Compared to the kinetic resolution technologies which require prior synthesis of racemic amines[14], asymmetric synthesis is usually favored due to the theoretic yield of 100 % and the fact that non chiral substrates are much more affordable than racemic mixtures[43]. Although the asymmetric synthesis approach promises a simpler method that achieves higher yields, there are challenges which are still being addressed today and will be discussed extensively in this thesis.

Much work has been done to solve the crystal structure of the ω-transaminases[44-49]. The reaction catalyzed by the ω-transaminases follows the Ping Pong Bi Bi mechanism which will be described in more detail in chapter 2.3.2.3.1.1. However, ω-transaminase requires pyridoxyl-5-phosphate co-factor (PLP) to transfer the amino group from an amino donor to acceptor[50]. The overall reaction can be divided into two half reactions where the first half reaction is the oxidative deamination of an amino donor. As shown in Figure 8 the PLP binds to the enzyme, forming an enzyme-PLP complex. The amino group from the amino donor is transferred to the E-PLP complex forming an enzyme - pyridoxamine-5-phosphate (E-PMP) complex while the residual amino donor is transferred to a corresponding ketone. The second half reaction is the reductive amination of the amino acceptor. The amino group from the E-PMP complex is transferred to the ketone substrate and chiral amine is produced, whilst the E-PMP complex is regenerated to the E-PLP. Such co-factor recycling system within the same reaction and using a single enzyme gives an outstanding advantage compared to the co-factor recycling systems of oxidation and reduction reactions, e.g. using dehydrogenases[51].

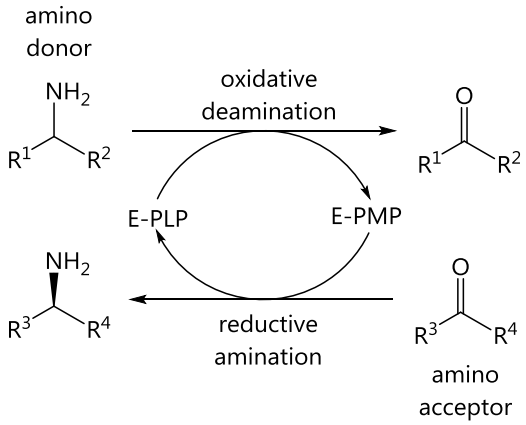


Figure 8 The mechanism of ω-transaminase reaction: oxidative deamination and reductive amination adapted from [52]

A diversity of primary amines show reactivity towards the ω-transaminases as amino donors. Both alkyl and arylalkyl amines are accepted by the ω-transaminase whilst the latter amines show surprisingly higher reactivities[53]. In the literature, α-MBA has been identified as an amino donor with high reactivity and several cases where other amino donors showed higher reactivity were reported, e.g. 1-aminoindan[53, 54], 1-aminotetralin[53], 1-methyl-3-phenylpropylamine[54], benzylamine[54] and L-α-alanine[54]. Reactivity of the ketones

varies depending on the type of the ketone used, e.g. the highest reactivities have been found for the α-keto acids (pyruvate) and aldehydes (propanal, benzaldehyde)[52]. However, the reactivity of prochiral ketones, which are the substrates for the production of the highly desires chiral amines, have been found to be lower than the pyruvate. This is the reason why the asymmetric synthesis of arylalkyl amines, e.g. (S)-α-MBA when using alanine as amino donor is challenging at best.

The potential usage of the ω-transaminases in the production of industrially valuable chiral components is significant. Components containing a chiral amine moiety are used as building blocks in a wide range of pharmaceutical or bioactive components and one such example is the production of (R)-amphetamine[55, 56]. (R)-4-phenylbutan-2-amine is the precursor for the antihypertensive dilevalol[57] and (R)-*p*-metoxyamthetamine is a building block for the bronchodilator (R,R)-formoterol[58]. 1-phenyl-1-propylamine is a precursor for a potent antidepressant[59] and (S)-2-amino-1-methoxypropane is a building block for the herbicides metolachor or Outlook®[23]. (S)-Rivastigmine {(S)-3-[1-(dimethylamino)-ethyl]phenyl ethyl-(methyl)carbamate} is one of the most potent drugs for treatment of Alzheimer's desease[60]. The possibility of the synthesis of β-amino acids by the ω-TA has also been reported in the literature[61-64]. These amino acids are the building blocks of many natural and synthetic drugs like antibiotics, enzyme inhibitors or peptide mimicking compounds with pharmaceutical properties, e.g. taxol[65].

As seen from these examples both enantiomers of chiral amines do have significant use, and it is therefore important for the pharmaceutical industry to find and develop both (R)-selective and (S)-selective ω-transaminases. The majority of the transaminases reported are (S)-selective[23], however a few were reported to be (R)-selective[66, 67] including the excellent example of enhanced ω-transaminase by directed evolution resulting in an enzyme capable of taking a bulky substrate in the case of Sitagliptin production[68].

2.3.2.2 Process Overview

One of the most extensive reviews regarding the process considerations of ω-transaminase and its implementation was reported by Tufvesson et al.[42] The authors show that the process of production of optically pure amines by ω-transaminases consists of four major steps as shown in Figure 9: fermentation, biocatalyst formulation, reaction and product recovery.

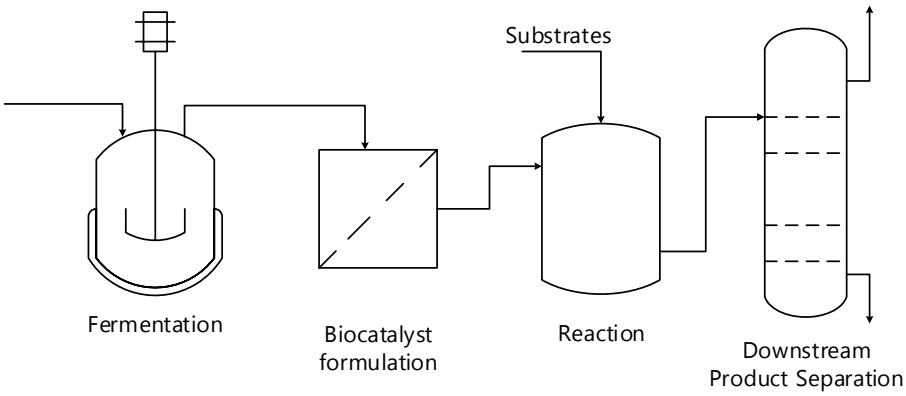


Figure 9 Overview of the process of production of optically pure amines by ω-transaminase adapted from Tufvesson et. al.[42]

In the fermentation process the biocatalyst is expressed in the host organism which produces the desired enzyme activity. The enzyme is then transferred into the required biocatalytic form. This form can be either whole-cell form or crude extract, depending on the process requirements, while the stability and reusability of the biocatalyst can be enhanced by various immobilization techniques[69]. However, the fermentation process and the biocatalyst formulation are optional processes and can be circumvented by purchasing the biocatalyst in the desired form from an external supplier. After the biocatalytic reaction is carried out under the desired conditions the biocatalyst is removed. In the last step the product is recovered and purified from the reaction mixture.

2.3.2.3 Process Challenges and Strategies

There are many challenges described in the literature concerning the implementation of an ω-transaminase catalyzed processes. These challenges can be divided into two major groups: challenges arising from the biocatalyst characteristics and thermodynamic equilibrium related challenges. The overview of those challenges and possible strategies to overcome them is given in the Table 4.

Table 4 The overview of process challenges and strategies in an ω-transaminase catalyzed reactions

group	challenge	Strategy
BIOCATALYST	Substrate inhibition	ISSS, protein engineering
	Product inhibition	ISPR, protein engineering
	Stability	Immobilization, protein engineering
THERMODYNAMICS	Unfavorable K_{eq} value	Excess substrate, choice of amino donor, ISPR, ISCPR

2.3.2.3.1.1 Biocatalyst Challenges

As mentioned before, wild-type enzymes are often not applicable to the industrial bioconversions due to their inability to cope with high substrate or product concentrations and unnatural substrates. To overcome these challenges, biocatalytic improvements have to be made and, while improving the biocatalyst, multiple improvement targets are often set. However, it is difficult to screen for multiple desired properties simultaneously[4] and a stepwise improvement strategy may be required. One of the most important properties that usually has to be improved is the enzyme activity which in turn decreases enzyme load and the added cost of the enzyme. Such attempts have been made for the ω-transaminase and one excellent example is the work by Martin et al.[70] where the authors managed to increase the activity of the enzyme by a factor of almost 300 while at the same time improving the stability of the enzyme towards the reaction conditions.

The ω-transaminase follows a Ping-Pong Bi Bi mechanism described in the literature[71, 72] and shown in the Figure 10. The enzyme exists in the E form and binds to the PLP forming the E-PLP complex followed by the bonding of the substrate A. Product P is then released and the enzyme form is changed from the E form to the F form which represents the E-PMP complex. The product B binds to the F form of the enzyme releasing the co-product Q and the enzyme reverts back to the E form. However, the E and the F form of the enzyme are not significantly different and it is not surprising that the substrate A could have some affinity towards the F form of the enzyme, as well as the substrate B might have some affinity towards the E form. These undesired affinities will result in a lower reaction rate at certain substrate concentrations. A similar effect can be observed with the products, e.g. Q can bind to the F form of the enzyme forming the dead-end complex.

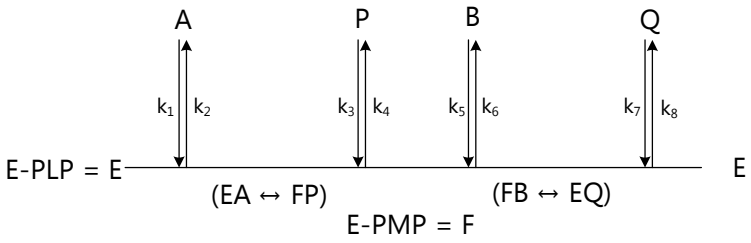


Figure 10 Ping-Pong Bi Bi mechanism adopted from the literature[72]

Product and substrate inhibition were reported in the literature as a major challenge in the kinetic resolution approach where both amine enantiomers can act as inhibitors forming a dead-end complex[73]. The ketone produced from the amine also showed high product inhibition[26, 74] and to overcome this challenge a significant research effort was done and numerous process solutions have been proposed. One of the proposed process solutions for alleviating the product inhibition in the kinetic resolution approach was applied by Shin and Kim by utilizing an auxiliary phase (organic solvent) for the extraction of the ketone[25]. There were other instances

where the separation of the ketone was performed using a hollow-fiber membrane[26], an enzyme membrane reactor[26] or packed-bed reactor[31]. However, these examples show product inhibition of the ketone, e.g. for the acetophenone product for the kinetic resolution approach. In the asymmetric synthesis the acetophenone is usually the prochiral substrate and substrate inhibition in this case can be alleviated by substrate feeding[75-78] or by other *in-situ* substrate supply (ISSS) techniques from an auxiliary phase, liquid or solid. Product inhibition also poses a significant challenge for the asymmetric synthesis approach and there have been several proposed *in-situ* product removal (ISPR) strategies. One strategy by Yun and Kim proposed an organic solvent bridge (isooctanol) for continuous removal of the product (S)-α-methylbenzylamine[79]. Another strategy by Truppo et al. involved selective adsorption of the same product to the ion-exchange resin[80]. The authors also suggested another approach involving a change of the overall chemistry of the process in such a way that the product of the ω-transaminase reaction would auto cyclize, and therefore would no longer fit into an active site of the enzyme and cause product inhibition[80]. All of these strategies are examples of process/chemistry manipulations to avoid substrate or product inhibition. However, another strategy that holds much promise and could overcome inhibition problems elegantly is protein engineering. One such example involved the production of Sitagliptin by ω-transaminase ATA-117[68]. Numerous mutations of the enzyme enabled higher substrate (*i*-$PrNH_2$) concentrations, but also higher stability in organic solvent and higher temperature with a wider range of substrate specificity, for example also accepting bulky substrates. The reports from Rothman et al.[81] and Yun et al.[74] also demonstrated that directed evolution of enzymes can alleviate product inhibition. Another, more recent study by Park and Shin identified ω-transaminase from *Ochrobactrum anthropi* which showed no product or substrate inhibition in the case of kinetic resolution[82]. According to the authors, this new ω-transaminase should be beneficial for the asymmetric synthesis approach as well.

2.3.2.3.1.2 Stability

The reaction conditions in the industrial process are usually far away from the conditions found in a cell. The concentrations of chemicals are much higher in the process and pH may be different from neutral. Non-natural substrates or products often have low water solubility and in many cases reactions are run in slurry conditions. In some cases, it is required to avoid emulsions, and co-solvents are used to increase solubility of reaction components. All these reaction conditions often decrease the stability of the enzyme, and to maintain the desired rate of the reaction additional quantities of enzyme must be added. However, if the stability of the enzyme can be improved sufficiently such that the required rate remains adequate throughout the reaction, the biocatalyst loading could be reduced. The deactivation of enzymes is often caused by unfolding or chemical modification and can be described as a first order kinetic reaction[83] as shown in Equation 2.1.

$$r_t = r_0 e^{-k_d \cdot t} \quad (2.1)$$

r_t – reaction rate at given time; r_0 – initial reaction; k_d – deactivation coefficient; t - time

Several strategies for increasing the stability have been described advocating the economic benefits based on the reusability of the biocatalyst. One of the most commonly used strategies for enzyme stabilization is the immobilization. This method mimics nature in a way that native intracellular enzymes seem to be bound to the cell membrane or localized in the gel-like surroundings of a cellular organelle[84]. The first industrial application of the method was continuous optical resolution of D,L-amino acids by immobilized aminoacylase[85] in 1969. The advantages of an immobilized compared to the free enzyme are improved operational and storage stability, easy recovery and reuse, possibility of continuous operation in a packed bed reactor and the potential of minimizing the protein impurities in the product[86]. However, known issues of enzyme immobilization are the loss of activity due to mass transfer limitations and the loss of the active enzyme[69].

There have been several reports on immobilization of the ω-transaminase in the soluble enzyme form. One of the first reports was made by Yi et al. where the ω-transaminase was covalently linked to different solid support materials[87]. The chitosan beads showed improved stability and retained 77 % activity after 5 cycles, however a severe substrate and product inhibition was present. The entrapment by sol-gel matrices was reported by Koszelewski et al.[88] and Lee et al.[89] where immobilization yields of 20-50 % and less than 20 % activity were reported. Immobilized enzyme showed improved activity at higher pH values and temperatures[88]. Another, more recent example of immobilization of free enzyme by covalent linkage to the catechol-chitosan (CCS) – iron oxide nanoparticles (IONPs) was reported by Ni et al.[90] The retained activity was dependent on the carrier and was 93, 95.7 and 87.5 % for the IONPs, CS-IONPs and CCS-IONPs respectively. The authors reported ~ 90 % activity after 5 cycles and improved activity for wider pH and temperature value ranges. The use of magnetic nanoparticles for immobilization purposes makes this method highly desirable due to the easy biocatalyst recovery and reuse.

Immobilization of whole cells or permeabilized cells containing ω-transaminase attracted some attention as well. Shin and Kim performed the entrapment of the whole cells in calcium alginate beads used in the kinetic resolution of racemic amines in a packed bed reactor[31]. This approach showed diffusion limitations and substrate and product inhibition, and it was later demonstrated by Martin et al. that V_{max} and K_m changed after immobilization[70]. Research by Cardenas-Fernandez et al. showed an easy and robust method of immobilization of whole and permeabilized cells with LentiKats®[91]. The authors showed that no diffusional limitations were found as well as no activity loss. Stability of the immobilized whole and permeabilized cells was increased under process relevant conditions and the residual initial rate was ~ 80 % after 5 cycles.

Although the immobilization strategies present certain advantages among which recovery, reuse and increased stability can be put forward, the loss of activity by immobilization and the immobilization process itself increase the cost of the enzyme for a given process[42]. To follow certain economic guidelines a high specific activity of a biocatalyst is required as long as possible[92] and that can also be achieved by protein engineering[93]. Currently the only literature report of a protein engineering approach to increase the stability of the ω-transaminase, among other improvements made, is the production of Sitagliptin by Savile et al.[68], where the authors showed impressive increase of the DMSO tolerance from 5 to 50 %.

2.3.2.3.2 Thermodynamic Limitations

One of the most challenging limitations concerning the asymmetric synthesis of optically pure amines by ω-transaminase are thermodynamic challenges. These challenges have been extensively documented and several approaches have been developed. This challenge arises from the low K_{eq} values which in turn result in low thermodynamic yields. K_{eq} or the thermodynamic equilibrium constant is the ratio between the product and the substrate equilibrium concentration quotients. For a general chemical reaction:

$$\alpha A + \beta B \rightleftharpoons \gamma C + \delta D \tag{2.2}$$

K_{eq} can be defined as:

$$K_{eq} = \frac{[C]^{\gamma} \cdot [D]^{\delta}}{[A]^{\alpha} \cdot [B]^{\beta}} \tag{2.3}$$

[A] – concentration of the component A; α – stoichiometric coefficient of the component A

By knowing the K_{eq} value, calculation of thermodynamic yield is fairly simple for given starting concentrations of the substrates. Comparing the calculated yield with the desired yield, one can decide whether an equilibrium shifting strategy is required and how effective it has to be. For the production of optically pure amines by ω-transaminase many cases exhibit unfavorable K_{eq} values. The K_{eq} is determined by the change of standard Gibbs free energy of reaction which is defined as the difference of standard Gibbs free energy of formation between products and substrates as shown in the Equation 2.4.

$$\Delta G^0 = \sum \Delta G^0_{f\ products} - \sum \Delta G^0_{f\ reactants} \tag{2.4}$$

The change of Gibbs free energy for the production of α-keto acids from amino acids is small and therefore the K_{eq} is small. A similar change can be observed for the production of chiral amines starting from prochiral ketones. In one such example Shin and Kim reported the production of α-MBA starting from acetophenone yielding only 0.5 % α-MBA, even with a tenfold excess of alanine[41]. They reported the K_{eq} value based on a

kinetic parameter estimation to be around 10^{-3}. Truppo et al. reported that the equilibrium becomes more favorable when 2-propylamine is used as amino donor instead of alanine, , however the equilibrium still favors the substrates[29]. It was clear that the K_{eq} of production of chiral amines from prochiral ketones is highly unfavorable, and the need for knowing the K_{eq} values for specific cases of interest was therefore emerging. Jankowski et al. developed a group contribution method for estimating the Gibbs free energy of formation for biochemical reactions in aqueous solutions at pH 7 and 25°C[94]. This methodology was applied in the work of Seo et al. for the comparison of the transamination potential of different amine donors[95]. Another possible method of determining the K_{eq} value is the experimental method which allows reactants to reach the equilibrium from both directions of the reaction[96, 97]. Due to the slow reactions rates and low stability and/or volatility of reactants, a modified method was proposed by Tufvesson et al which in turn provided the first experimental values for different pairs of keto acceptors and amino donors for different ω-transaminase systems[98].

The majority of the research on ω-transaminase systems reported thermodynamic challenges and identified them as top challenge for the asymmetric synthesis approach. This was one of the reasons why the kinetic resolution approach gained much popularity in the earlier days of research in transaminase systems. However, the advantages of the asymmetric synthesis approach, e.g. higher theoretical yields and cheaper starting material, were soon recognized as well as the need for a powerful equilibrium strategy in order to fully utilize this approach.

2.4 Strategies for Shifting the Thermodynamic Equilibrium

To achieve desired process-relevant yields in thermodynamically challenged systems, strategies for overcoming thermodynamic limitations must be used. These strategies are based on process solutions and cannot be achieved by enzyme improvement. The reason for this is the fact that the enzyme catalyzes the forward reaction, but also the reverse reaction, meaning that the reaction will reach the equilibrium state faster using the enzymes. However the value of the equilibrium constant itself will not be shifted. There are two types of equilibrium shifting strategies: the first one is based on changing the K_{eq} value by changing the chemistry of the reaction and therefore changing the Gibbs free energy of formation of components. This means that by selecting a more favorable amine donor a more favorable K_{eq} value can be achieved. The second type of strategies do not change the K_{eq} of the reaction but favor the formation of the product by manipulating the concentrations of reaction components. The latter can be achieved by excess substrate, *in-situ* product removal (ISPR) or *in-situ* co-product removal (ISCPR). The purpose of all equilibrium shifting strategies is to shift the thermodynamic equilibrium to achieve economically viable yields. Based on the desired yield and given K_{eq}

value, it is possible to calculate the "workload" that the equilibrium shifting strategy has to achieve, and, depending on the investigated case, what type or combinations of several different strategies is necessary.

2.4.1 Amino Donor Selection

As mentioned before, amino donor selection is an equilibrium shifting strategy that actually changes the K_{eq} value of the reaction system by choosing a different amino donor resulting in a more favorable K_{eq} value. An amino donor which is more energy stable will have a lower Gibbs free energy of formation, and will according to Equation 2.4 increase the change of Gibbs free energy of the system and thus the value of K_{eq}. However, it should be clear that the K_{eq} value must be significantly increased to achieve industrially relevant yields. Taking the concentration ratio of substrates to be 1:1 and assuming a required thermodynamical yield of 95 %, the K_{eq} value necessary to achieve such yields can be calculated using the Equation 2.3 and should be > 360. In the work by Tufvesson et al. K_{eq} values were determined for two different substrates, acetophenone and 4-phenyl-2-butanone, using several different amino donors[98]. The selection of a different amino donor indeed showed the change of K_{eq}, e.g. when using acetophenone for the amino donors alanine and 2-propylamine Keq values have been found to be $4.03 \cdot 10^{-5}$ and $3.33 \cdot 10^{-2}$ respectively. Although the K_{eq} was almost increased by a factor of 1000 in this example by the selection of a different amino donor, the yield was only increased from 0.6 to 15.4 % as calculated on the basis of Equation 2.3. This example shows that more stable amino donors still have to be found. However, it should be kept in mind that they also have to be biocompatible and accepted by the enzyme. In the combination with other equilibrium shifting strategies the amino donor selection strategy may prove to be helpful in shifting the K_{eq} value of thermodynamically challenged reactions.

2.4.2 Excess of Substrate (Amino Donor)

This equilibrium shifting strategy is based on Le Chatelier's principle where by increasing the substrate concentration, the equilibrium concentration of the product is increased. In the case of the ω-transaminase, keto-substrates in most cases have low solubility and are more expensive compared to the amino donors, and the excess of substrate is therefore typically applied by providing an excess of the amino donor. This equilibrium shifting strategy is one of the first to be considered when developing a process due to its simplicity. By knowing the K_{eq} and the maximum amount of the amino donor as a function of solubility, the equilibrium yield can be calculated using Equation 2.3. However, this method by itself can only be successfully when applied when the K_{eq} is slightly unfavorable or near 1, as was demonstrated by Savile et al. in the case of Sitagliptin production where a 10-fold excess of 2-propylmine was used[68]. Due to the simplicity of this shifting strategy the combination with other equilibrium shifting strategies can be potent and reduce their "workload". The possibilities for the implementation of this strategy, alone and in combination with other strategies, will be discussed in Section 3.4.1.

2.4.3 ISPR Technologies

Another equilibrium shifting strategy based on Le Chatelier's principle that manipulates the concentrations of reaction mixture components towards the product side is *in-situ* product removal or ISPR. This method is focused on the removal of the product as it is being produced by the reaction. The purpose of the ISPR can be either for shifting the equilibrium or alleviating the product inhibition. However, the focus of this thesis will be on the shifting of the equilibrium. The general approach of this technology is the removal of the product to a second phase (liquid, solid or gas) which then drives the reaction forward due to the equilibrium shift and facilitates the product recovery from the reaction mixture. The selection of the ISPR method in the case of ω-transaminases depends on the physical properties of the amino product and other components in the reaction mixture. The most common properties used for ISPR are differences in solubility, volatility, charge, hydrophobicity and molecular size[99]. In general, a favorable ISPR technology is the one which can selectively remove the product and generate a significant driving force for successful removal. In the following sections a brief overview of possible ISPR technologies will be given, and the discussion about individual challenges of application and implementation will be discussed in the Chapter 5.

2.4.3.1 Classical ISPR Technologies

One of the most frequently used ISPR methods in the ω-transaminase reaction is a liquid-liquid extraction. By shifting the reaction medium to acidic or basic conditions to overcome the protonation of the amine product, it is possible to remove the formed amine from the reaction mixture and alleviate product inhibition and shift the thermodynamics of the system. This approach was demonstrated by Kim and Shin[25] and Koszelewski et al.[100]. However, post-reaction recovery was only performed in the latter work. Integrated extractive recovery was applied by Yun and Kim[79] using a solvent bridge. The equilibrium shift was obtained by employing an *in-vivo* ALS cascade as an *in-situ* co-product (ISCPRR) removal strategy and the solvent bridge was used to alleviate product inhibition by (S)-α-MBA, as well as to achieve equilibrium shifting and as product recovery strategy. Another investigated method is the adsorption to a solid surface (resin) proposed by Woodley et al.[101], and later demonstrated by Truppo et al.[80] as an efficient strategy for overcoming both the product inhibition and thermodynamic constraints. Together with the LDH/GDH cascade system as an ISCPR strategy high yields were obtained starting from 50 g L^{-1} of substrate. The difference between the volatility of the compounds can also be used as a property that can be exploited for the removal of the product. One such example was reported by Hanson et al.[30] where (R)-*sec*-butylamine was recovered by distillation of the reaction mixture under basic conditions.

2.4.3.2 Cascades for ISPR

In the traditional organic synthesis route of the production of pharmaceutical compounds several production steps are present. The biocatalytic approach tries to reduce the number of these steps and the exact place of the substitution of a biocatalysis step in the synthesis route may vary. When the investigated biocatalytic reaction is not placed at the end of a synthesis route, the sequential reactions that follow can potentially be used as an ISPR technique. It is important to note that this approach has mainly been achieved by another sequential enzymatic reaction or reactions, and it is called multi-enzyme synthesis[102] or synthesis by cascades, and in general it means combined biocatalytic reactions without intermediate recovery steps[103]. The concept is taken from nature where successive biocatalytic reactions in cells form a metabolic pathway network. This concept is possible due to the high selectivity of the biocatalysts and has been exploited for manufacturing purposes as well. Although the application of multi-enzyme synthesis can be applied in the production of many valuable products [103, 104], its application in the case of the production of optically pure amines by ω-transaminase still has not been reported. Such approach could significantly decrease the thermodynamic challenges in the production of optically pure amines. However, the choice and viability of a sequential reaction will vary on a case-to-case basis.

Biocatalytic cascades as an ISPR in the ω-transaminase system may not yet have been fully researched. However, the non-catalytic cascade has been reported. The work was done by Truppo et al. using ethyl 4-acetylbutyrate as a substrate[80]. The resulting amine then spontaneously cyclized to 6-methyl-2-piperidone yielding > 99 % conversion starting from 50 g L^{-1} prochiral ketone.

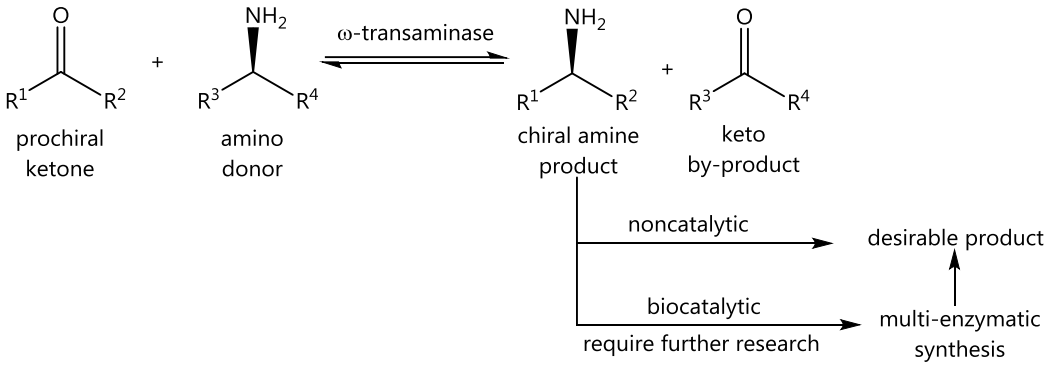


Figure 11 Possible ISPR strategies by implementing cascades

Using cascades as an ISPR strategy (Figure 11) has many advantages, and one of the most important ones is shifting the equilibrium value by a sequential reaction. Having this in mind, together with the fact that an intermediate step can be avoided, inhibition by the chiral amine intermediate can be alleviated and two or more (non)enzymatic reactions can be performed in one pot, and reducing the number of steps in the total

synthesis route. The ISPR by cascades thus sounds very promising for overcoming challenges such are unfavorable thermodynamic equilibrium or product inhibition. Unfortunately, challenges in the implementation still remain, and further research on this topic is needed.

2.4.4 ISCPR Technologies

The *in-situ* co-product removal (ISCPR) strategy is a procedure of removing the co-product of the reaction as it is being produced. ISCPR can be used when the co-product is inhibitory or toxic to the biocatalyst, when shifting the equilibrium of thermodynamically challenged reactions or for the combination of the aforementioned reasons. Similar to the ISPR strategy, the ISCPR is based on the Le Chatelier's principle, and, by removing the co-product, the formation of the product is favored. For achieving a certain thermodynamic yield, the usage of ISPR or ISCPR is theoretically indistinguishable meaning the same thermodynamic yield will be achieved if a given amount of product or co-product is removed from the reaction mixture. However, the major advantage of ISCPR over ISPR is that the co-product does not have to be recovered and it can therefore be reacted away into an inert compound. Several ISCPR approaches on the ω-transaminase system have been reported in the literature and will be mentioned. However, the applicability, challenges and compatibility with other equilibrium shifting technologies will be discussed in more detail in Chapter 5.

2.4.4.1 Classical ISCPR Technologies

As mentioned in the Section 2.4.4.1 on Classical ISCPR Technologies, there are several physical properties that can be exploited for successful separation of the co-product. However, only the difference in the volatility between the product and the co-product has been reported. When 2-propylamine or 2-butanamine is used as an amino donor, the resulting co-product, acetone or butanone respectively, is volatile and can be removed by evaporation as suggested by Yun et al.[28] Recently a model was developed by Tufvesson et al.[105] for assessing the feasibility of shifting the equilibrium by acetone removal for a given process. The authors discussed two removal models, sweeping and sparging. They defined operating windows for each approach and identified K_{eq} and the volatility of the ketone substrate as the key parameters. This approach can be very successful in the early stages of process development for the evaluation of the viability of evaporation as the ISCPR strategy that is most suitable for overcoming the equilibrium challenges.

2.4.4.2 Cascades

As opposed to the ISPR cascades, using cascades in ISCPR has the purpose to remove the co-product, in this case a keto co-product, in order to shift the equilibrium concentrations towards the product formation. The cascades in the ISCPR approach can be non-catalytic and biocatalytic (Figure 12). The example of non-catalytic cascades was demonstrated by Fotheringham who showed that the pyruvate can be efficiently decarboxylated

with hydrogen peroxide[106]. However, this method has a major drawback of resulting in decreased enzyme stability in the presence of hydrogen peroxide, or alternatively the pyruvate removal by hydrogen peroxide has to be performed in a separate vessel. Another example of non-catalytic cascades was presented by Wang et al.[107] In this work racemic 3-aminocyclohexa-1,5-dienecarboxylic acid was used as amino donor and the resulting ketone was effectively removed by spontaneous tautomerization to 3-hydroxybenzoic acid.

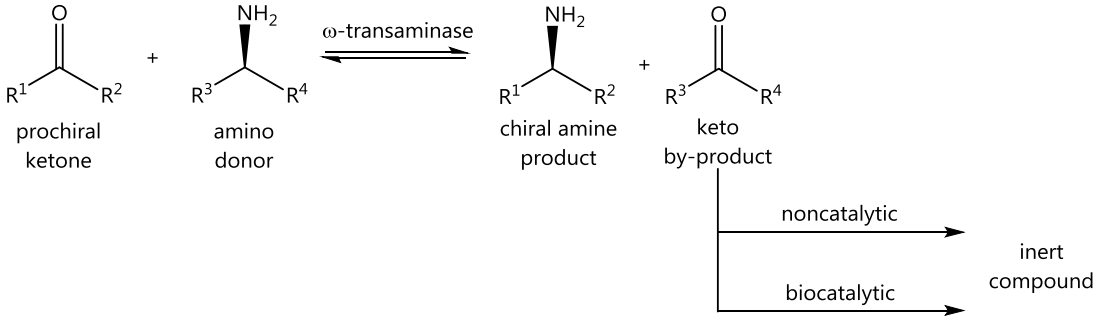


Figure 12 Possible ISCPR strategies by implementing cascades

Although the above-mentioned examples show the possibilities of non-enzymatic cascades for ISCPR purposes, the majority of the research for the implementation of ISCPR was done by biocatalytic cascades. The superb selectivity of the enzymes towards the keto co-product (pyruvate or acetone) together with the enzyme availability presented several interesting research opportunities which will be described in more detail in the following sections.

2.5 ISCPR by Enzymatic Cascade Systems in an ω-transaminase System

The metabolic pathway in living cells consists of a series of sequential enzymatic reactions leading to the production of life building blocks or energy. Since both acetone and pyruvate are part of this metabolic network, several natural enzymes exist capable of selective degradation. This biomimetic approach has been applied for an ISCPR in the ω-transaminase system and several cascade systems have been reported in the literature as a proof of concept of successfully overcoming thermodynamic limitations for the production of optically pure amines. There are two categories of the cascade systems: co-factor (NAD(P)H) recycling enzymes presented by the dehydrogenases and non co-factor recycling enzymes, e.g. thiamine pyrophosphate (ThPP) dependent enzymes.

2.5.1 Co-factor Recycling Enzymes

Dehydrogenases are enzymes which are involved in the removal of hydrogen from the substrate to an acceptor, most commonly NAD^+, in oxidation and reduction reactions. The majority of all redox enzymes use nicotinamide adenine dinucleotide (NAD^+ - NADH) as coenzyme while the other enzymes prefer the

corresponding phosphorylated co-factor ($NADP^+$ – NADPH). These are known enzymes which have been researched extensively and used in the industry. The availability of wild type and improved enzymes has led to the successful implementation of dehydrogenases in many industrial processes[108]. Another advantage is the high selectivity of dehydrogenases towards the co-product, meaning that no side reactions will occur.

2.5.1.1 Co-factor and co-factor regeneration methods

Nicotinamide adenine dinucleotide, NAD^+ is a coenzyme found in living cells and it consists of two nucleotides which are linked by two phosphate groups as depicted in Figure 13 A). In the reduction and oxidation reactions the co-factor acts as an electron carrier as shown in the Figure 13 B) and in contrast to the other co-factors e.g. FAD or FMN it is not covalently bound to the enzyme[109]. During the reaction the co-factor is chemically altered as well as stoichiometrically consumed by the reaction, and therefore it could also be called the co-substrate.

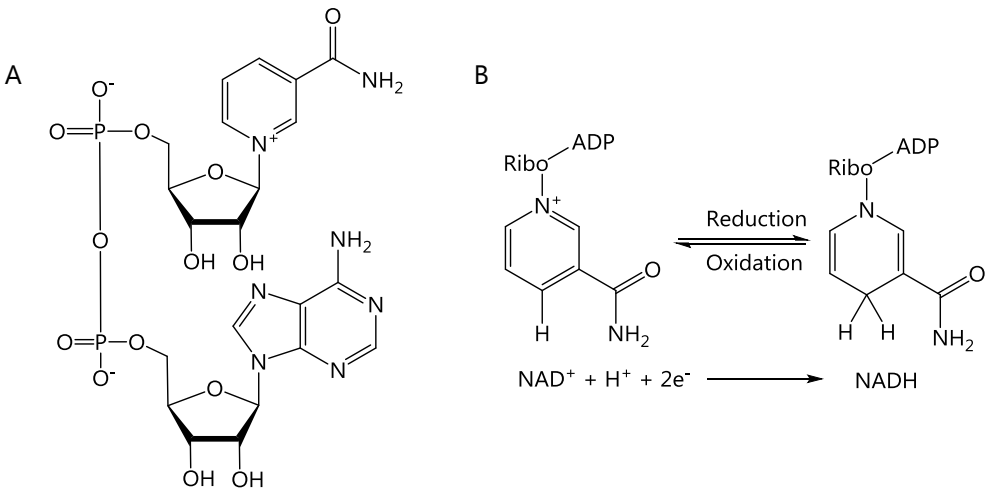


Figure 13 a) structure of NAD^+ and b) redox reactions of nicotinamide adenine dinucleotide

Due to the high cost of the co-factor and the stoichiometrical consumption, *in-situ* co-factor regeneration methods have been developed to overcome this important challenge[110-112]. Several regeneration methods have been reported: enzymatic (both isolated enzyme and whole cell), chemical, electrochemical and photochemical methods. Based on its several advantages including outstanding enzyme selectivity and enzyme availability, the enzymatic regeneration method has been researched more extensively. Compared to the whole cell approach, processes catalyzed by isolated enzymes result in a higher volumetric productivity and no side reactions are observed given the high selectivity of the enzymes[110]. The isolated enzyme approach of co-factor recycling can be described by coupling the two reactions catalyzed by the dehydrogenases, one consuming the co-factor and one regenerating it[111] as shown in Figure 14. In some cases only one dehydrogenase is used capable of accepting two different substrates, one in oxidized form and the other in its reduced form, with

obviously a clear advantage related to single enzyme usage. This approach is also called coupled enzyme approach.

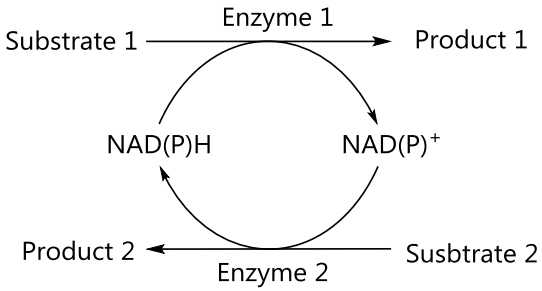


Figure 14 General scheme of enzymatic co-factor regeneration adapted from [111]

It is understandable that adding stoichiometric amounts of the co-factor is not economically feasible for an industrial process and that the recycling strategy must be put in place. The isolated enzyme approach has been given a lot of attention due to the advantages mentioned above and many successful examples have been implemented either as proof of concept or as a commercial process, e.g. the first commercial process of chiral synthesis of *tert*-L-leucine[113, 114]. More advantages of this approach can be seen when continuous ISCPR and ISSS via co-factor shuttling is achieved providing kinetic and thermodynamic advantages. However, this approach adds to the complexity of the overall reaction system, and is in many cases producing additional molecular species which have to be considered in DSP. The secondary or sacrificial reaction has to use cheap and available substrate, and both substrate and product have to be inert, non-toxic and biocompatible.

2.5.1.2 Co-factor Stability

When performing reactions using NADH dependent dehydrogenases, the stability of the co-factor plays a significant role. This important factor can influence the choice of reaction conditions and feasibility of the recycling methods. The reduced form is more stable in the basic solutions and the oxidized form is more stable in the acidic medium[115, 116]. However, both are quite unstable *in vitro* in aqueous solutions[117]. Degradation of the co-factor as well as enzymes can be described by a first order decay described by Equation 1. To achieve the desired reaction rates and to maintain them over the course of the reaction, the addition of the co-factor would be required. To avoid the replenishment of the co-factor, improving the stability has to be the most important objective. The first attempts of stabilizing nicotinamide co-factors began with the buffer selection and it was found that the Tris buffer improved stability considerably[118]. However, the application of the buffer in the industrial process is not common and other methods of increasing the stability of the co-factor stabilization e.g. immobilization or the use of co-factor analogues should be considered. Those and other methods will be discussed in detail in Section 5.3.3.2.

2.5.1.3 LDH/GDH system

The lactate dehydrogenase (LDH) (E.C. 1.1.1.27) /glucose dehydrogenase (GDH) (E.C. 1.1.1.47) is one of the most used systems for shifting the equilibrium in the ω-transaminase reaction. It was first described as an equilibrium shifting strategy by Shin and Kim[41]. The authors realized the need of a powerful equilibrium shifting strategy via pyruvate removal and used solely LDH enzyme or a whole cell system expressing LDH to remove the co-product resulting in a significant increase of the yield from 2 % to 90.2 % and 92.1 % respectively. The first usage of the complete LDH/GDH system was reported by Koszelewski et al. as a means to overcome thermodynamic challenges and achieve high yields[100]. Due to the successful proof of concept and the availability of these enzymes, a considerable number of research projects, e.g. Höhne et al.[119], Hwang et al.[120], Koszelewski et al.[88, 100, 121], Truppo et al.[29, 80, 122], Fuchs et al.[123], Mutti et al.[67, 124] and Pressnitz et al.[125] used this cascade system for pyruvate removal and shifting the unfavorable equilibrium toward the product formation.

Both LDH and GDH enzymes are dehydrogenases and follow the Ordered Bi Bi mechanism[72, 126] shown in the Figure 15. This mechanism can be explained on the example of the reaction catalyzed by LDH. The substrate A which is the co-factor NADH, binds to the active site of the enzyme forming an EA complex followed by the binding of the substrate B, in this example pyruvate. The formed product P (lactate) then leaves the active site followed by the product Q (co-factor NAD^+) leaving the enzyme. The overall ω-transaminase system coupled to the equilibrium shifting LDH/GDH system is depicted in the Figure 16. The equilibrium is shifted by selectively removing the pyruvate via an LDH catalyzed reaction forming lactate. The regeneration of the NAD^+ to NADH is performed by an auxiliary or sacrificial reaction catalyzed by the GDH. The auxiliary substrate glucose is transformed into glucono-1,5-lactone which then forms an equilibrium with glucono-1,4-lactone and gluconic acid via a hydrolysis reaction.

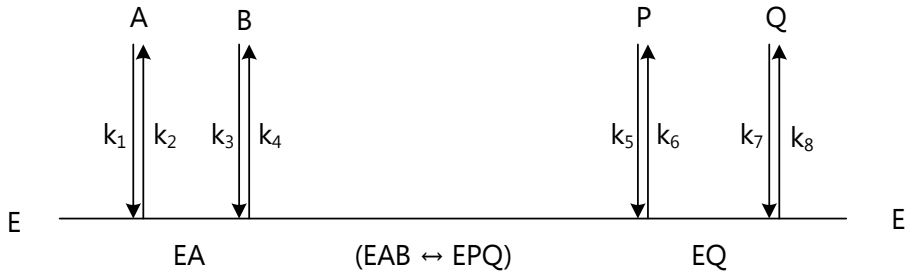


Figure 15 Ordered Bi Bi mechanism of dehydrogenases

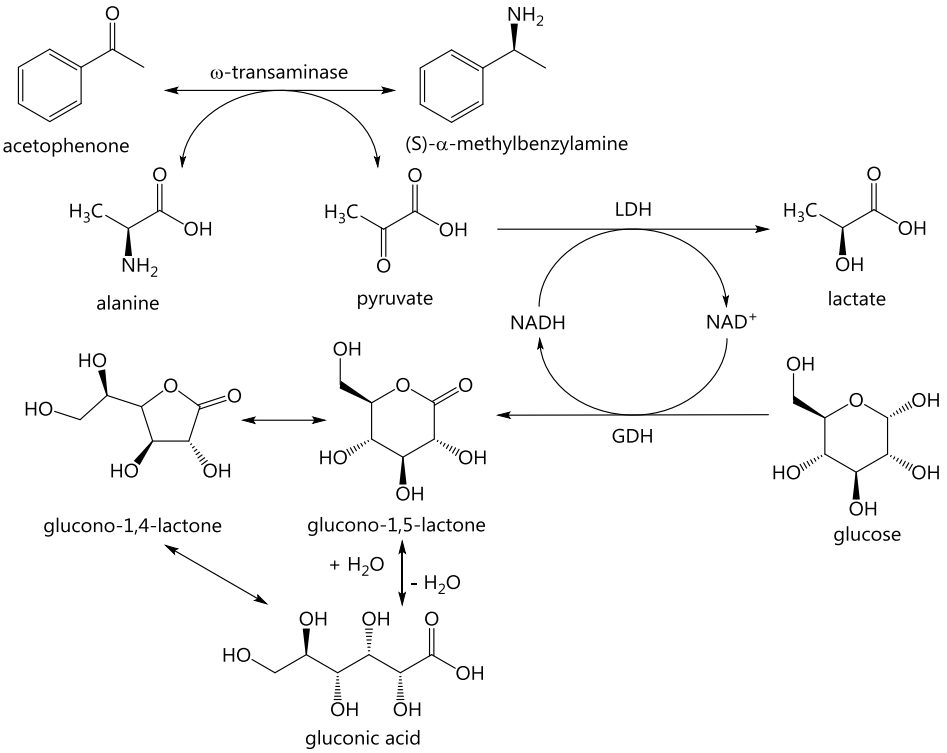


Figure 16 Proposed reaction scheme of the LDH/GDH system for shifting equilibrium of the ω-transaminase reaction

The biggest advantage of this equilibrium shifting system is the selectivity of all enzymes present towards the desired substrates within the reaction mixture. Products of this cascade system, lactic acid, glucono-1,5-lactone, glucono-1,4-lactone and gluconic acid, are non-reactive components and they remain in the reaction mixture. However, the accumulation of these molecular species raises the possibility of occurrence of inhibitory effects for both LDH and GDH enzymes. Another issue is the downstream processing challenge resulting from the presence of more species which needs to be considered when removing the desired component from the reaction mixture. The work by Kaswurum et al.[127] showed that the hydrolysis of glucono-1,5-lactone is not fast enough and significant quantities of this molecular species can be accumulated, thus inhibiting the reaction and making the downstream processing more complicated. Moreover, the buildup of gluconic acid causes a pH change which in turn requires pH control. Focusing on the concentration of media used for neutralization, two problems may occur. If the neutralization medium is highly concentrated, inadequate mixing could lead to high or low local pH values in the reaction medium leading to enzyme denaturation and co-factor degradation as well. If the neutralization medium is less concentrated, dilution of the reaction mixture can occur and concentration steps should then be applied which adds to the complexity of the overall process, and other challenges may occur, e.g. selective water removal.

2.5.1.4 LDH/FDH system

Lactate dehydrogenase/formate dehydrogenase (E.C. 1.2.1.2) (FDH) is a cofactor recycling system depicted in Figure 17 that is used for shifting the equilibrium of the D-amino acids production catalyzed by D-transaminase[128, 129]. This system has been reported by Koszelewski et al.[88, 100], Mutti et al.[124] and Pressnitz et al.[125] as a conceptual system. However, no experimental reports in the literature were found that would document the use of this cascade system in ω-transaminase systems.

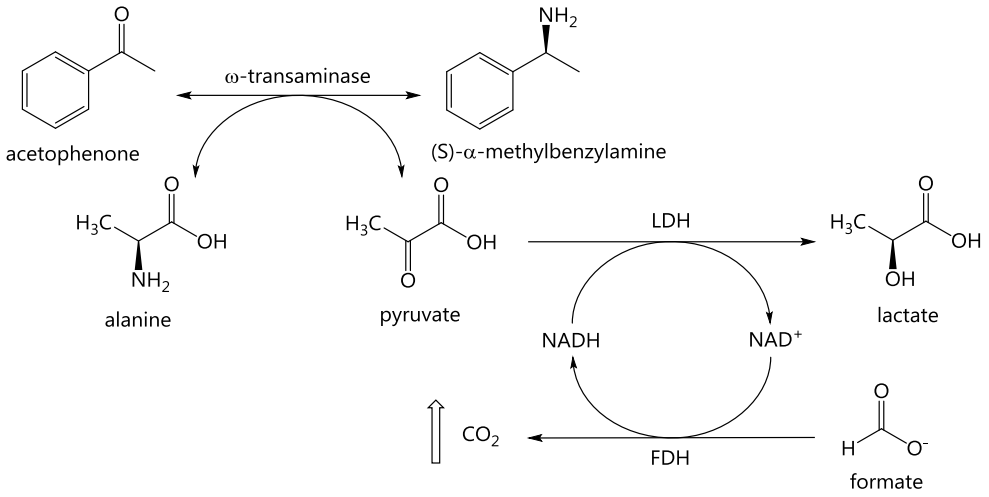


Figure 17 Proposed reaction scheme of the LDH/FDH system for shifting equilibrium of the ω-transaminase reaction

This system inherits all the advantages and disadvantages regarding the shared enzyme (LDH) from the previously described system. However, the co-factor recycling reaction has been substituted with FDH which should have several advantages. The product of this reaction is CO_2 which reduces the requirement for downstream processing and pH control. The product inhibition for this reaction is practically negligible and the thermodynamic equilibrium is further shifted to the product side due to the fact that CO_2 is leaving the system. The disadvantage of FDH is the lower enzyme activity compared to other enzymes, which means that more FDH should be added to achieve the same reaction rate, meaning a higher cost of the added enzyme. Dissolved CO_2 can also cause a slight pH shift and can form carbonates which in turn can cause downstream issues due to scaling.

2.5.1.5 AlaDH/GDH system

Alanine dehydrogenase (E.C. 1.4.1.1) (AlaDH) is an enzyme which follows an Ordered Ter Bi mechanism depicted in Figure 18. Depending on the host organism differences in the order of pyruvate and ammonia binding have been reported[130-132]. AlaDH/GDH is a system previously reported by Truppo et al. where three methods for overcoming the unfavorable thermodynamic equilibrium of the ω-transaminase system have been used: LDH/GHD and AlaDH/GDH as an ISCPR equilibrium shifting strategy and the usage of 2-propylamine as

an amino donor selection strategy[122]. The reported 96 % yield showed the thermodynamic ability of this system to overcome the undesired equilibrium for the given conditions. However, the reaction rates of the amine production were slower compared to the LDH/GDH system and the choice of 2-propylamine as amino donor.

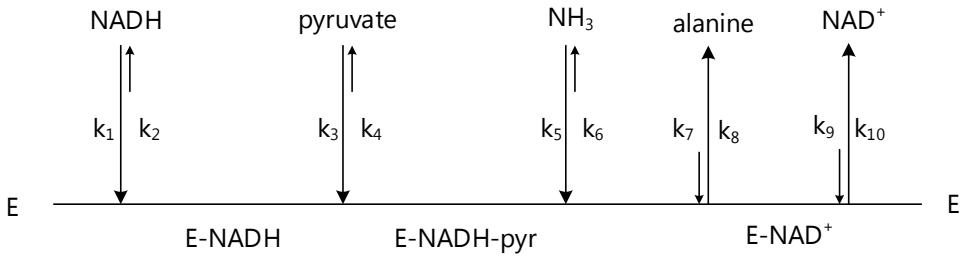


Figure 18 Ordered Ter Bi mechanism of AlaDH from Bacillus Subtilis adapted from Grimshaw and Cleland[131]

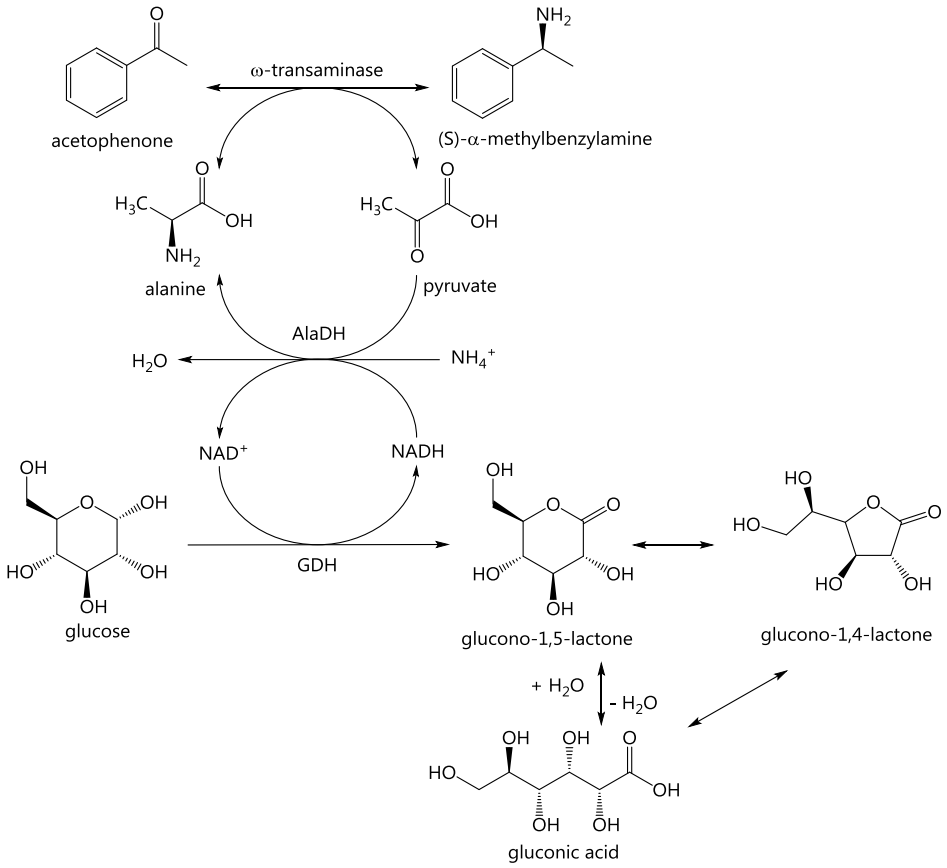


Figure 19 Proposed reaction scheme of the AlaDH/GDH system for shifting equilibrium of the ω-transaminase reaction

One of the biggest advantages and the point of interest of this system is the recycling of alanine, as depicted in Figure 19. This means that only catalytic amounts of alanine are required as amino donor, and that the inexpensive ammonia salts may be used as the primary amino donor choices. This approach makes the system extremely interesting from an economic point of view. However, several challenges have been observed regarding the use of a catalytic amount of alanine. If the ω-transaminase reaction starts with a minor amount

of alanine, the production of pyruvate will be slow due to the kinetic effects and thermodynamics of the reaction. It can be therefore be concluded that the regeneration of pyruvate back to the alanine will be a function of the reaction rate of the ω-transaminase reaction and will face the same kinetic and thermodynamic challenges. To overcome this bottleneck, more alanine has to be added to increase the reaction rate of the transamination. However, in this case a higher alanine concentration poses inhibition challenges for the AlaDH as well as unfavorable thermodynamic starting conditions meaning a high product concentration (alanine) and low substrate concentration (pyruvate). However, to fully utilize this enzyme and optimize the concentrations of the amino donors the properties of the given AlaDH and ω-transaminase have to be known, and the models including thermodynamic behavior have to be developed. In the reaction catalyzed by the AlaDH the production of additional molecular species is not an issue which makes DSP more practical. The advantages and disadvantages of the GDH reaction have been previously described in Section 2.5.1.3.

2.5.1.6 AlaDH/FDH system

This system has been reported in the literature by Koszelewski et al.[88, 133] and Mutti at al.[67, 124] As shown in Figure 20 pyruvate is recycled back to the alanine and co-factor by an auxiliary reaction catalyzed by the FDH. In all reported cases the amination was successful resulting in relative high yields showing that thermodynamic equilibrium can be shifted for the given conditions by recycling the alanine.

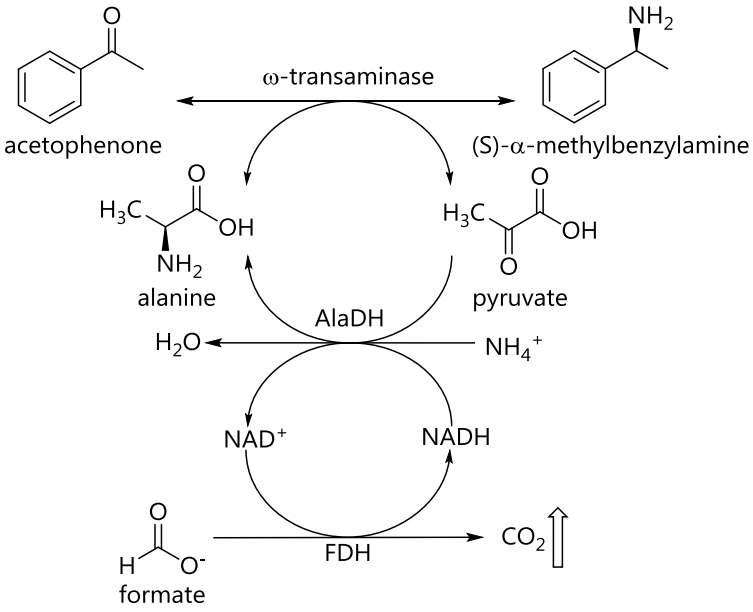


Figure 20 Proposed reaction scheme of the AlaDH/FDH system for shifting equilibrium of the ω-transaminase reaction

Earlier Sections 2.5.1.5 and 2.5.1.4 describe all the advantages and disadvantages of AlaDH and FDH catalyzed reactions respectively. However there are two unique advantages for this cascade system. The first is that only

one substrate can be used for both cascade reactions, the ammonium formate where the ammonium ion is used as one of the substrates for the AlaDH catalyzed reaction and formate serves as a substrate for the FDH catalyzed reaction. The second advantage is that the products of this cascade system are either utilized by the ω-transaminase reaction or leave the system in gaseous form facilitating the DSP significantly.

2.5.1.7 YADH/GDH system

All previously described cascade systems are based on the usage of alanine as an amino donor where the pyruvate was removed enzymatically. Previous investigations showed that pyruvate is the preferred substrate for the ω-transaminase, meaning that alanine is a rather thermodynamically poor amino donor. Another amino donor, 2-propylamine, was found to be more thermodynamically favorable[98], cheaper and the resulting co-factor acetone can be removed in several ways including an enzymatic cascade catalyzed by alcohol dehydrogenase (Figure 21). Possible selectivity issues of this alcohol dehydrogenase toward the two ketone substrates present in the reaction mixture, namely acetone and acetophenone, was pointed out. However, a selective alcohol dehydrogenase from *Saccharomyces cerevisiae* was found. This yeast alcohol dehydrogenase (E.C. 1.1.1.1) (YADH) possesses a narrow substrate range and the reactions with four carbon substrates or higher have very slow or no observable reaction rates[134]

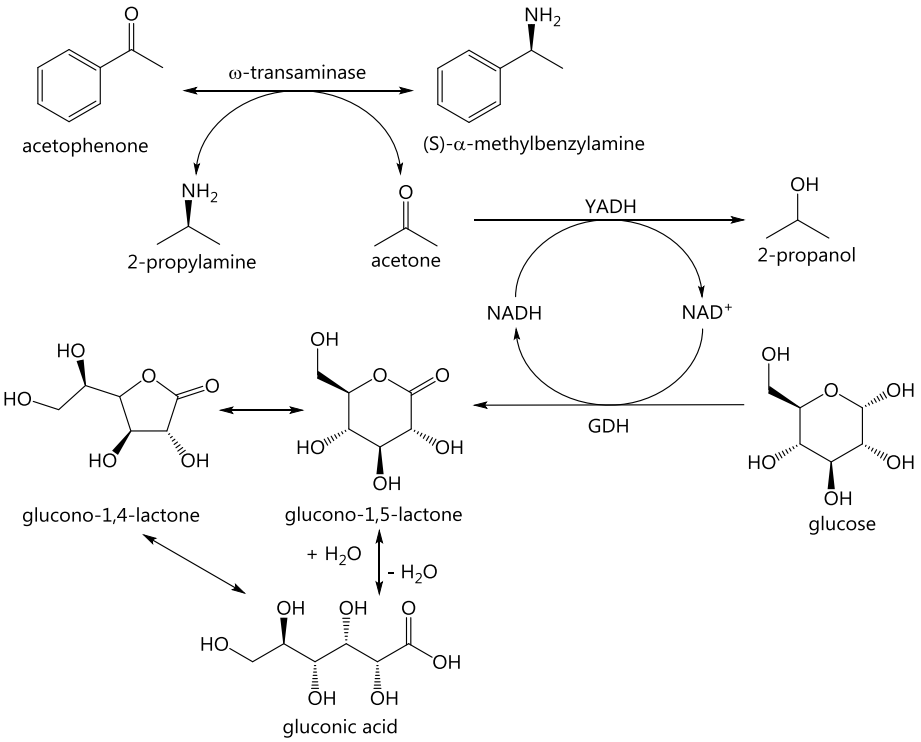


Figure 21 Proposed reaction scheme of the YADH/GDH system for shifting equilibrium of the ω-transaminase reaction

This cascade system can be used when other ISCPR methods, e.g. evaporation or pervaporation, cannot satisfy the requirements for the equilibrium shifting. Thus far, experiments on this particular cascade system have not yet been reported in the literature.

2.5.1.8 YADH/FDH system

This system was explored and reported in the work of Cassimjee et al. where the conversion of different ketones and 2-propylamine as an amino donor was compared[135]. The conversions without the cascade system were already high (60-90%) compared to the cases where alanine was used as amino donor. However when the YADH/FDH cascade was used complete conversion was observed. This cascade system is shown in Figure 22.

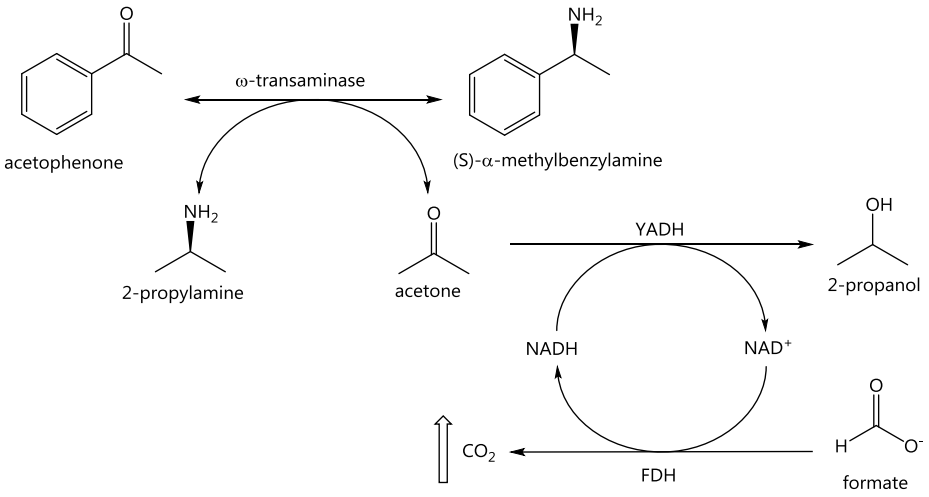


Figure 22 Proposed reaction scheme of the YADH/FDH system for shifting equilibrium of the ω-transaminase reaction

As shown in the work of Cassimjee et al. this cascade system can be applied to the systems using 2-propylamine as an amino donor where these systems are usually much more thermodynamically favorable compared to the ones where alanine is used as an amino donor. Sequentially, the required amount of the co-product removed to achieve a desired thermodynamic yield is less compared to the relating alanine systems, facilitating the application of potential ISCPR strategies.

2.5.2 Non Co-factor Recycling Enzymes

Previously described dehydrogenases used the co-factor as a co-substrate meaning that the co-factor was stoichiometrically consumed by the reaction and an additional recycling reaction was required. However, not all enzymes suitable for equilibrium shifting by ISCPR consume the co-factor stoichiometrically, e.g. thiamine diphosphate enzymes. Thiamine diphosphate (ThDP) or thiamine pyrophosphate (ThPP) co-factor is an active form of vitamin B_1. The purpose of this co-factor is versatile and in different metabolic pathways it assists in making and breaking bonds between carbon and sulfur, oxygen, hydrogen and nitrogen, as well as breaking

and making carbon-carbon bonds. ThDP is a true cofactor and remains on the protein site, where a divalent metal ion, usually Mg^{2+} is used to bind the cofactor to the enzyme.

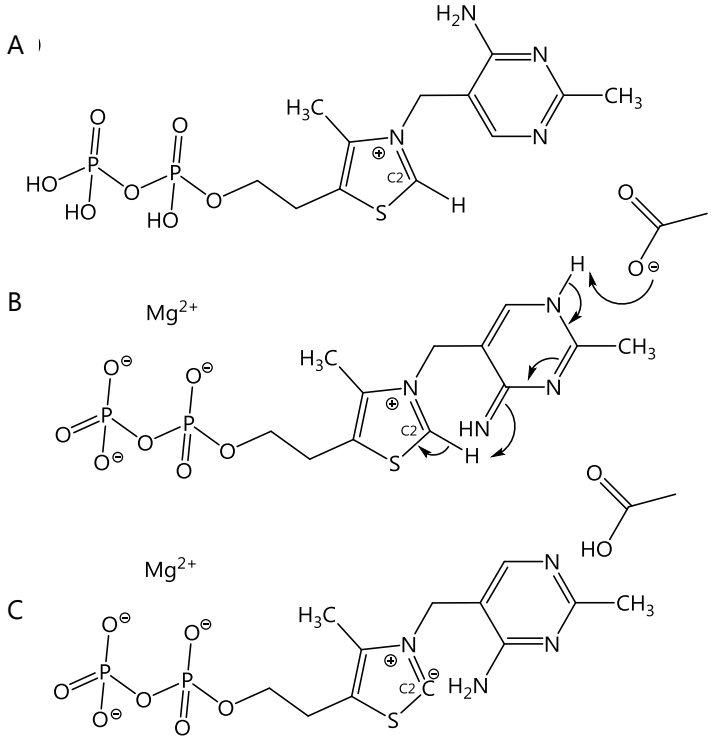


Figure 23 The structure of thiamine pyrophospate (TPP or ThPP) or thiamine diphosphate (ThDP) adapted from Frank et. al.[136] A) ThDP in a solution B) intermediate imine form of ThDP C) activated form of ThDP

The catalytic activity of thiamine diphosphate in ThDP dependent enzymes lies in the activation of a C2 carbon by deprotonation forming an activated form of ThDP shown in Figure 23 C). This is accomplished during the binding to the active site of the enzyme where the effects of conformation and electrostatics enable the removal of the proton from the C2 position. Tautomerization occurs in the intermediate step (Figure 23 B) and the nitrogen from the imine is responsible for proton removal. The activated form of ThDP then offers the activity of ThDP dependent enzymes and two such enzymes have been found capable of degrading pyruvate: pyruvate decarboxylase (PDC) and acetolactate synthase (ALS).

2.5.2.1 Pyruvate decarboxylase system

Pyruvate decarboxylase (E.C. 4.1.1.1) (PDC) is an enzyme that catalyzes the nonoxidative decarboxylation of pyruvate into acetaldehyde and CO_2 (Figure 24). It requires ThDP as a cofactor and Mg^{2+} for catalytic activity[137]. This cascade has been reported by Höhne et al. for production of 1-N-Boc-protected –aminopyrrolidine and –piperidine obtaining high yields.

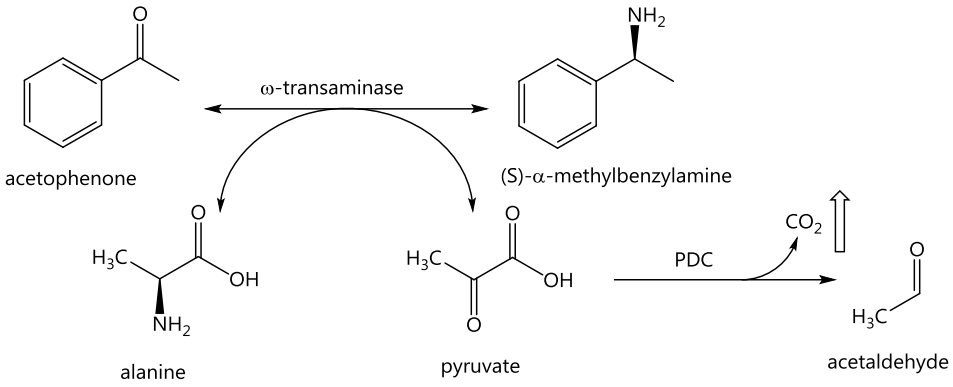


Figure 24 Proposed reaction scheme of the PDC system for shifting equilibrium of the ω-transaminase reaction

The obvious advantage of this pyruvate removal system compared to the dehydrogenases is in its monoenzymatic simplicity; only one enzyme is required and no co-factor recycling method is needed. Both products are volatile and facilitate downstream processing. Both product inhibition and unfavorable thermodynamics are also facilitated by the properties of the products[119]. The only possible disadvantage of this system is the selectivity of the ω-transaminase towards the acetaldehyde which is the preferred substrate. The by-product of this side reaction is ethylamine which is an explosive gas causing health problems.

2.5.2.2 Acetolactate synthase (ALS) system

Acetohydroxyacid synthase (AHAS) and more generally acetolactate synthase (E.C. 2.2.1.6) (ALS) is an enzyme found in plants and microorganisms and it is responsible for valine, leucine and isoleucine synthesis[138]. Acetohydroxyacid synthase (AHAS) naturally catalyzes the carboligation of a hydroxyl-ethyl group, obtained by the decarboxylation of the pyruvate. The second substrate can be either pyruvate or 2-ketobutyrate giving 2-acetolactate or 2-aceto-hydroxybutyrate. The active site is known to be promiscuous and accepts a wide range of substrates, It requires both divalent metal ions like Mg^{2+} providing linkage to the enzyme and a molecule of flavin adenine dinucleotide (FAD) which is not spent in the reaction[139]. However, some bacteria contain an FAD-independent form of the enzyme referred to as ALS. The ALS catalyzes the same reactions as the AHAS but with high preference to the pyruvate as a second substrate. As mentioned before, the ALS is one of the enzymes found in metabolic pathways of branched-chained amino acids and butanediol production. In *in-vivo* conditions one possible route is that the product (S)-2-acetolactate is decarboxylated by acetolactate decarboxylase (E.C. 4.1.1.5) to form acetoin. However, in in-vitro conditions (S)-2-acetolactate undergoes spontaneous non-enzymatic decarboxylation into acetoin and oxidative decarboxylation to diacetyl[140, 141]. The reaction mechanism in *in-vitro* conditions for ISCPR in the ω-transaminase reaction is depicted in Figure 25.

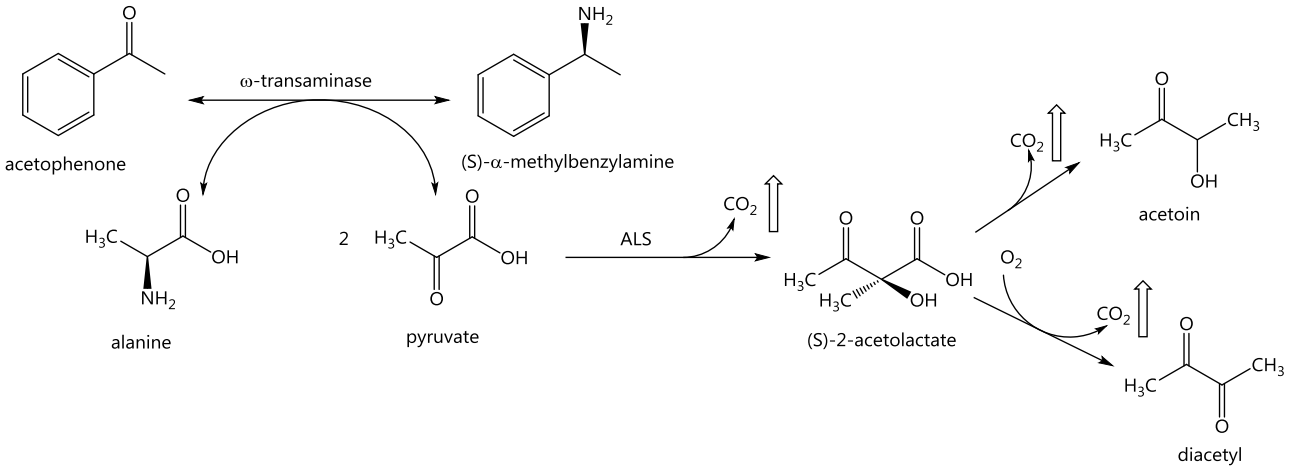


Figure 25 Proposed reaction scheme of the in vitro ALS system for shifting equilibrium adopted from [140]and [141] and applied to the ω-transaminase reaction

The ALS system was reported for shifting the thermodynamic equilibrium of the ω-transaminase system by Yun and Kim[79]. They co-expressed the ALS enzyme together with the ω-transaminase using recombinant *E. coli* cells and a 2.5 fold increase in the production of (S)-α-MBA was observed compared to the cells containing only ω-transaminase.

Similar to the pyruvate decarboxylase approach, this system looks promising due to its simplicity using only one single enzyme system with no cofactor recycling. Thermodynamic equilibrium is facilitated by the fact that one of the products is CO_2 and the sequential reactions of (S)-2-acetolactate follow. However, those reactions are also the main disadvantage of this approach. (S)-2-acetolactate, acetoin and diacetyl are ketones, and therefore they might potentially function as substrates for the ω-transaminase. The resulting mixture of amines would be very difficult to separate in order to recover the desired amino product. Another disadvantage is that this enzyme is currently not commercially available, therefore making this cascade option not very favorable.

2.6 Discussion and Conclusions

The use of biocatalysis in the pharmaceutical industry has already been established for some processes. However, still, more emerging biocatalytic processes are being developed due to the increasing availability of enzymes and reactions they can catalyze. The main advantage of biocatalysis as shown in this literature overview is result of its supreme stereo-, regio- and substrate selectivity which results in a simplification of the production process by reducing often complicated and demanding reaction steps. There are many examples of emerging technologies, and the focus of this thesis is the production of optically pure chiral amines by ω-transaminase.

Understanding the process starts from identifying the challenges and one very important group of challenges presented in this overview are biocatalytic challenges. Finding an enzyme with sufficient activities towards the desired substrates with sufficient stability and *e.e.* has become a major research undertaking pushing the screening (in micro-scale) and protein engineering technologies to their very limit when trying to develop an enzyme tailored to the specific reaction conditions. Other important biocatalytic challenges are substrate and product inhibitions which have been identified as a major hindrance for many ω-transaminases. Many process options, e.g. ISSS or ISPR, have been applied for alleviating the substrate or product inhibition respectively. However, developing enzymes without substrate or product inhibition might be a more elegant solution.

Another identified challenge for many asymmetric synthesis reactions catalyzed by ω-transaminase which are of industrial interest is the low K_{eq} value. To achieve economic feasibility and make a biocatalytic process comparable to a chemocatalytic one, several targets have to be met, one of them being sufficient product concentration (> 50 g L^{-1}). Achieving such high product concentrations requires high yields and in the case of thermodynamically challenged reactions, equilibrium shifting strategies are essential. Four equilibrium shifting strategies have been identified: Amino donor selection, Amino donor excess, ISPR and ISCPR. The selection of the equilibrium shifting strategy varies from case to case and it will partially depend on the K_{eq} of the reaction. For higher K_{eq} values, a less powerful equilibrium shifting strategy may be used, e.g. amino donor excess of 10 times for Keq ≈ 1 to achieve the desired thermodynamic yields[68]. Moderately challenged reaction conditions (K_{eq} < 1) require more efficient equilibrium shifting strategies, e.g. ISPR or ISCPR. A simple calculation based on Equation 2.3 can determine the allowed product or co-product concentration and determine how efficient ISPR or ISCPR, respectively, have to be. However, in the case of severely challenged reactions, e.g. an ω-transaminase reaction where acetophenone and alanine are used and K_{eq} is $4.03 \cdot 10^{-5}$, the choice of equilibrium shifting strategy narrows down to one extremely thermodynamically efficient strategy, namely ISCPR by enzymes. The choice of equilibrium shifting strategy also depends on the physical and chemical properties of the chemicals that need to be removed, as well as on other chemicals in the reaction mixture. For example, in the ω-transaminase reaction where acetophenone and 2-propylamine are used as a substrate, the resulting co-product is volatile acetone and ISCPR by evaporation might seem as a viable option for shifting the equilibrium. However, the study of Tufvesson et. al.[105] indicated that the volatility of the keto substrate and K_{eq} are important parameters in assessing the feasibility of ISCPR by acetone evaporation. Furthermore, this study showed that 30 % keto substrate loss may be expected for industrially relevant conditions making this equilibrium shifting strategy not selective enough towards the desired co-product. The thermodynamic yield is also a function of the selectivity of the equilibrium shifting strategy towards the desired component vs the similar substrates, and the lower the selectivity is, the lower the thermodynamic yield will be. The required selectivity towards the product (and not the substrate) increases with the severity of unfavorable

thermodynamic equilibrium. However, equilibrium shifting strategies can sometimes be utilized to overcome certain biocatalyst challenges as well. For example, when product inhibition is present, ISPR is sometimes possible to alleviate product inhibition and shift the equilibrium.

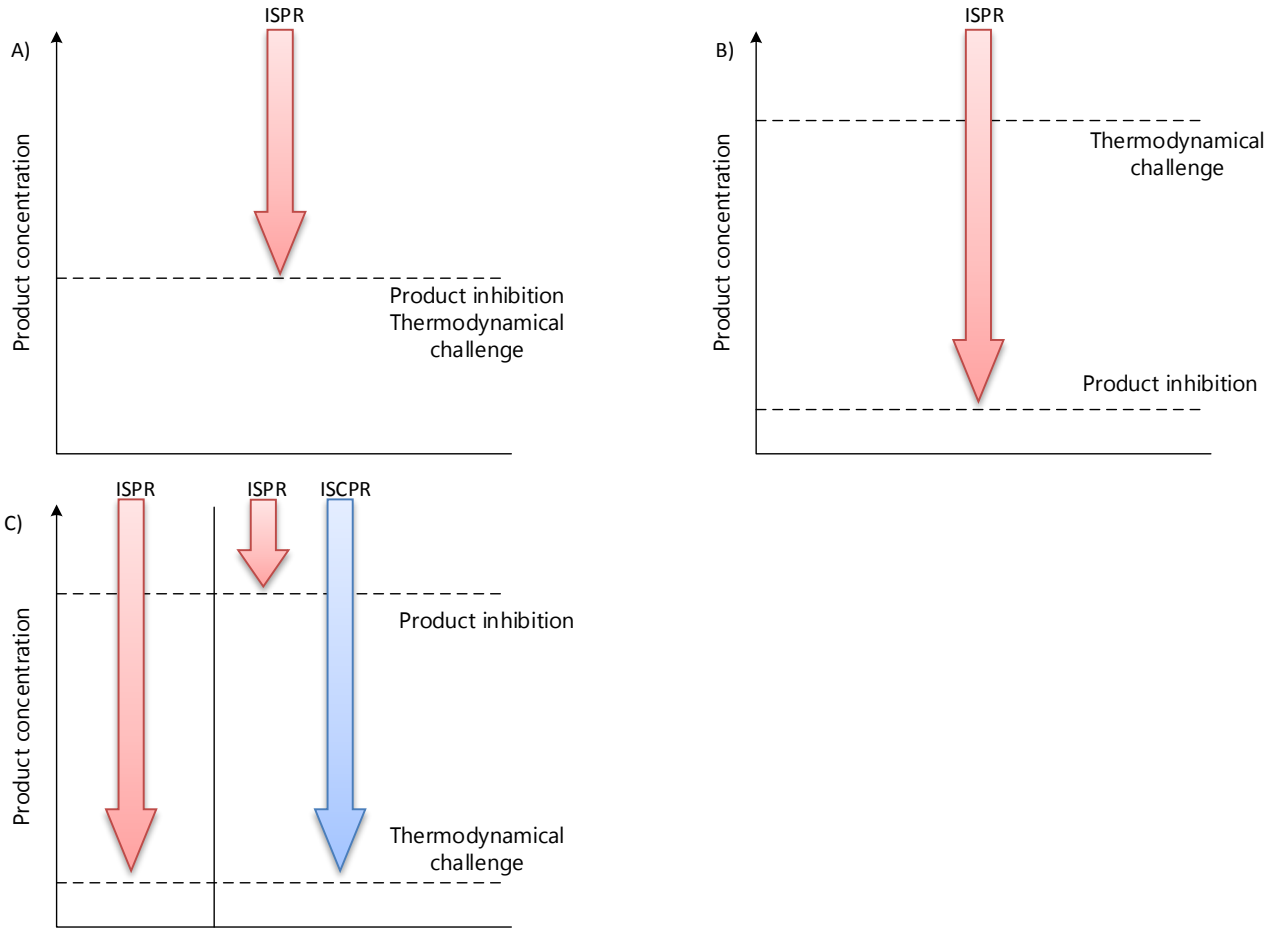


Figure 26 An example of use of ISPR for alleviating both product inhibition and shifting the thermodynamic equilibrium: A) product inhibition and thermodynamic challenge appear at same product concentration; B) product inhibition is more pronounced than equilibrium challenge; C) equilibrium challenge is more pronounced than product inhibition

Implementing ISPR or ISCPR technologies for tackling the challenges of product inhibition and/or thermodynamic challenge caused by unfavorable equilibrium will depend on which of these challenges are more expressed. If a reaction system is faced with both challenges, they can be expressed as following: A) product inhibition and thermodynamic challenge are expressed at similar product concentrations (low or high), B) product inhibition is much more severe than thermodynamic challenge and C) thermodynamic challenge is much more pronounced than product inhibition. Those three cases are displayed in the Figure 26 while showing how ISPR technology can be implemented in each case. Thermodynamic challenge is a term used here to describe the slowing down of the reaction due to the thermodynamic limitation. In the Figure 26 A) product inhibition and thermodynamic limitation are starting to slow down the reaction at similar product

concentration. In this case ISPR technique has to be powerful enough to reduce product concentration to the required levels which depend on the severity of product inhibition and thermodynamic challenge. Figure 26 B) describes a situation where product inhibition is much more pronounced than the thermodynamic challenge. For a successful implementation of this specific case an ISPR method must be powerful enough to alleviate product inhibition. The final example shown in the Figure 26 C) describes a case severely challenged by unfavorable thermodynamic equilibrium while product inhibition occurs at higher product concentrations. Two options can be used to overcome inhibition and thermodynamic challenges and the first one is using ISPR powerful enough to shift equilibrium to achieve the desired effect. If such ISPR technique is not applicable or nonexistent, second option may be considered. ISPR technique for alleviating product inhibition must be used, however the thermodynamic challenge can be overcome by powerful enough ISCPR technique. The best example of such case is the production of (S)-α-MBA by ω-transaminase using acetophenone and alanine as substrates. This reaction is highly thermodynamically challenged while most currently developed transaminases are product inhibited. Depending on the starting concentration of substrates and ω-transaminase used in the process, thermodynamic limitation is observed at μmol L^{-1} while product inhibition at mmol L^{-1} product concentrations, respectively. Powerful and selective ISPR technique is not known for this reaction, however, thermodynamic challenges can be successfully surpassed by implementing ISCPR by cascades. For a successful implementation of currently developed ω-transaminases, in addition, an ISPR method would be required. However, enzyme improvements by protein engineering might be an intelligent solution.

3 Considerations for a Cascade Selection

3.1 Introduction

In the previous chapter a description of potential cascade options has been presented. Moreover beneficial effects to the product yields have been documented on the basis of the available literature and positive as well as negative consequences of the use of the different cascade options have been discussed. However a major question has still not been answered: how to select a cascade for the process relevant implementation? The process of selecting the right cascade should be done by following as set of rules and guidelines based on industrially relevant conditions. However, no such guidelines have been proposed in the literature thus far. It is therefore the aim of this chapter to discuss such considerations and to develop a frame for selecting cascades for shifting the equilibrium in the ω-transaminase systems. Furthermore, the knowledge gathered by developing this selection process might be used in other reaction systems facing similar challenges.

The first step in any selection process is gathering the information and describing the potential candidates. In this work the order of obtaining the information plays a significant role, and the different information collection tasks have been placed in such an order as to minimize the time and money spent in a selection process. The first step is the preliminary literature data collection, and the purpose of this step is to achieve a fast and descriptive understanding of the cascade systems. What are the properties of an enzyme and what are the consequences of its implementation are two very important questions, and the literature information gathered has been divided into two major groups, here termed considerations: enzyme considerations and process considerations. Economic considerations are briefly discussed at the end of the section.

3.1.1 Enzyme Considerations

The first type that has been investigated are enzyme related considerations which describe properties of the cascade enzymes. The collected data, e.g. from the enzyme databases BRENDA, IntEnz or PDB, can describe enzymes in a general fashion, and serve the purpose of acquiring knowledge about the applicability to and interaction with the investigated ω-transaminase system. However, it is important to understand that the collected data will not represent the exact properties of the enzymes that will be investigated in this work for two reasons. The first reason is that the enzymes used in the literature and the enzymes that will be used in the industrial process are rarely from the same supplier and/or host organism, and therefore will have different properties to a certain degree. The second reason is that the experimental conditions under which the literature data have been obtained do not represent the conditions of the industrial process. Enzyme considerations are divided into a number of sections and are listed in Table 5.

Stability of enzymes in a cascade system is an important parameter for reasons described in the previous chapter. However, the literature does not provide information on the stability of cascade enzymes under process relevant conditions. Instead it is possible to identify cascade systems that potentially contain unstable components as well as solutions for improving their stability. In the case of dehydrogenases, three possibly unstable components have been identified: two enzymes that form a typical dehydrogenase co-factor recycling system as shown in Figure 14 and the co-factor itself. Possible solutions for increasing the stability of enzymes and co-factor will be discussed in Section 5.3.3.2.

Substrate selectivity is another important enzyme consideration that needs to be addressed when comparing different cascades. In this work two possible types of substrate selectivity issues have been identified. The first type of substrate selectivity is the selectivity of the co-product degrading enzyme towards the desired substrate. The desired substrate is the keto co-product pyruvate or acetone. However, if a cascade enzyme lacks selectivity, another ketone present in the reaction mixture can be converted in a parallel side reaction. The only possible ketone is the substrate of the ω-transaminase reaction, e.g. acetophenone. Such side reaction is highly undesirable and the depletion of the ketone substrate can have the consequence of a negative thermodynamic shift. However, such cases of cascade promiscuity have not been reported in the literature. Another type of insufficient selectivity is the substrate selectivity of the ω-transaminase towards the ketones or aldehydes produced by the cascade reactions. The products of PDC and ALS cascades are aldehydes and ketones respectively, which are possible substrates for ω-transaminase side reactions. A detailed overview of possible side reactions and their consequences will be given in the Section 3.4.2.

pH range is an important consideration in the process development and it is used for determining the optimal pH of multi-enzymatic systems. Enzymes may be very sensitive to the pH changes and the activity of an enzyme as a function of the pH is most often a bell-shaped curve[142]. Comparing and overlaying the pH curves of two or more enzymes under similar conditions will define a pH range under which all enzymes in a multi-enzymatic system can perform.

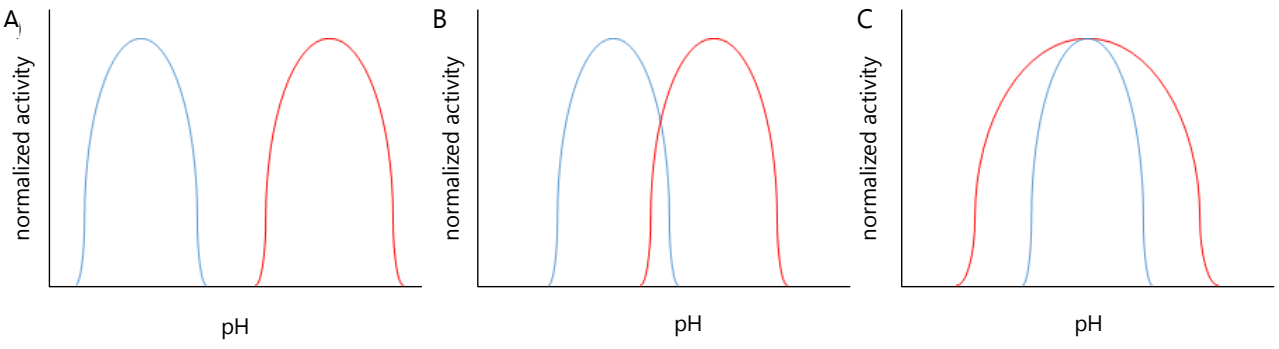


Figure 27 The possible positions of the pH profiles of two enzyme system

Table 5 Considerations for a cascade selection based on the enzyme properties

Enzyme Considerations							
	Stability	Substrate Selectivity		pH range		Kinetic parameters	
	Number of possibly unstable components	Selectivity of the co-product removal enzyme	Selectivity of the ω-transaminase towards the cascade products	pH range of the co-product removal enzyme	pH range of the co-factor recycling enzyme	"mild" conditions	
						V_{max} [mmol min^{-1} g^{-1}]	K_m [mmol L^{-1}]
LDH/GDH	3 (2 enzymes + co-factor)	No reaction observed	No ketones or aldehydes produced	**Figure 64** A) Appendix 3A	**Figure 64** B) Appendix 3A	LDH	
						4.43[143]	0.016[143]
						GDH	
						?	51[144]
LDH/FDH	3 (2 enzymes + co-factor)	No reaction observed	No ketones or aldehydes produced	**Figure 64** A) Appendix 3A	**Figure 64** C) Appendix 3A	LDH	
						4.43[143]	0.016[143]
						FDH	
						0.63[145]	0.47[146]
AlaDH/GDH	3 (2 enzymes + co-factor)	No reaction observed	No ketones or aldehydes produced	**Figure 64** D) Appendix 3A	**Figure 64** B) Appendix 3A	AlaDH	
						?	0.49[147]
						GDH	
						?	51[144]
AlaDH/FDH	3 (2 enzymes + co-factor)	No reaction observed	No ketones or aldehydes produced	**Figure 64** D) Appendix 3A	**Figure 64** C) Appendix 3A	AlaDH	
						?	0.49[147]
						FDH	
						0.63[145]	0.47[146]
YADH/GDH	3 (2 enzymes + co-factor)	No reaction observed	No ketones or aldehydes produced	**Figure 64** E) Appendix 3A	**Figure 64** B) Appendix 3A	YADH	
						?	?
						GDH	
						?	51[144]
YADH/FDH	3 (2 enzymes + co-factor)	No reaction observed	No ketones or aldehydes produced	**Figure 64** E) Appendix 3A	**Figure 64** C) Appendix 3A	YADH	
						?	?
						FDH	
						0.63[145]	0.47[146]
PDC	1	Theoretically no substrate promiscuity	Possible ω-transaminase substrate promiscuity towards acetaldehyde	**Figure 64** F) Appendix 3A	-	0.72[148]	2.29[148]
ALS	1	Theoretically no substrate promiscuity	Possible ω-transaminase substrate promiscuity towards (S)-2-acetolactate, acetoin and diacetyl	**Figure 64** G) Appendix 3A	-	?	1.1; 1.6; 8.7[149]

As shown in Figure 27 there are three possible positions of the pH profiles in a two-enzyme system. In the Figure 27 A) the pH profiles do not overlap and performing both reactions at the same pH is impossible while the choice of pH in the Figure 27 C) is obvious due to the fact that both enzymes have the highest relative activity at the same pH. Determining the optimal pH for the case shown in Figure 27 B) is more complicated and for the sequential reactions, e.g. ω-transaminase reaction → co-product removing reaction, the selection process is depicted in Figure 28.

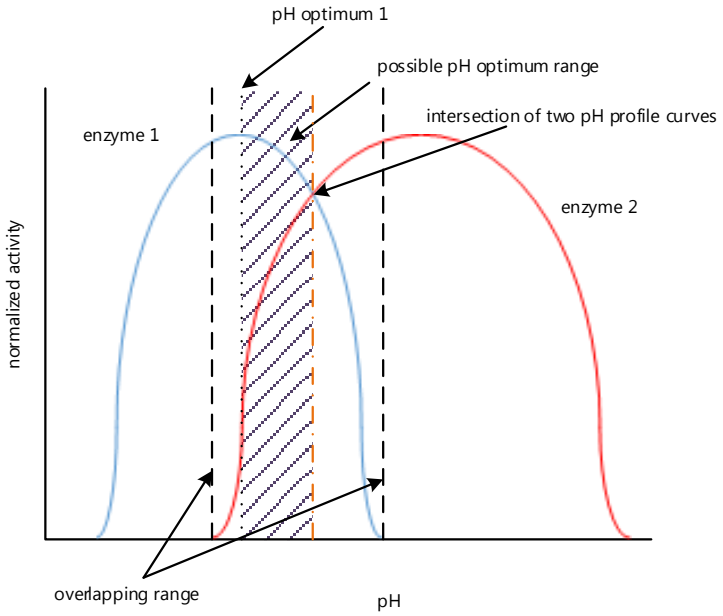


Figure 28 Narrowing the pH optimum of the two enzyme system

Narrowing the pH optimum of a two enzyme system can be done by taking the following steps:

Step 1: identify the bottleneck reaction and display its pH profile curve (enzyme 1)

Step 2: identify the pH optimum 1

Step 3: display the pH profile of an enzyme 2 and identify the overlapping pH range

Step 4: identify the pH where the two pH curves intersect

Step 5: the possible pH optimum range is between the line 'pH optimum 1' and the line 'intersection of two pH profiles'

The exact value of the pH optimum of the system is determined as a value where the reaction rate of the slowest enzyme (enzyme 1, e.g. ω-transaminase) will be at maximum while the reaction rate of the faster enzyme (enzyme 2, e.g. LDH) is equal to or higher than the reaction rate of enzyme 1. However, the latter requirement can also be met by adding an additional amount of enzyme.

Kinetic parameters like K_m and V_{max} can give a rough estimation on the behavior of the enzyme and simple kinetic models (Michaelis-Menten) can be made to estimate the differences between reaction rates of different cascade enzymes. As mentioned before, most of the time the reported kinetic parameters have been estimated under mild conditions and models derived from those parameters do not represent industrially relevant conditions.

3.1.2 Process Considerations

The second type of considerations which have been gathered in the comparison are the process considerations. They describe either the considerations that are not linked to the enzyme properties or the consequences of using a given cascade, e.g. products formed or pH shift.

Thermodynamic equilibrium is one of the most important considerations for cascade selection. The cascade selection will be based on how well a certain cascade can remove the co-product and what thermodynamic yield it will achieve. The thermodynamic consideration has been divided into three parts, of which the first is the K_{eq} of a reaction catalyzed by a given cascade. The second part is the determination of the allowed amount of remaining co-product in the reaction mixture to achieve 95 % conversion, which is a reasonable limit for the thermodynamic efficiency. The last part represents the cascade ability expressed in the form of the remaining co-product concentration and the resulting thermodynamic yield. A detailed explanation will be given in Section 3.4.1.

Downstream processing is an important consideration that describes and identifies all the products formed during the reaction as well as their quantities. The properties of the products and their concentrations will determine the type, effectiveness and cost of the DSP technologies. In some cases it is easy and straightforward to predict concentrations of all products. However, in some cases, e.g. in the case of ALS it is more difficult since side reactions take place and an unknown amount of byproduct is formed.

pH shift is a consideration which tries to identify the change of pH value during the reaction indicating the need for pH regulation. A higher pH change will result in stronger pH regulation and addition of a bigger volume of acid or base resulting in a higher dilution of the reaction mixture. The determination of the possible pH value at the end of the reaction is quite difficult due to the multitude of components in the reaction mixture, each with different effect on the pH. Some components shift pH more to the acidic pH region and some others more to the basic pH region which is the result of a change in pK_a value whilst some other components, e.g. alanine act as a buffer. The final pH value will be a function of changed pK_a values, concentrations of compounds in the reaction mixture and buffer effect. However, an indication of the pH change that is to be expected can be made via estimation of the ΔpK_a values of different reaction components. The ω-transaminase

reaction is typically a reaction that only slightly or not at all influences the pH: For alanine the pK_{a2} value is 9.69 while the pK_a of (S)-α-methylbenzymamine is 9.75 and the ΔpK_a value is +0.06 meaning this reaction has a negligible basic pH shift. The same procedure for calculating the ΔpK_a value of cascade components has been made for several systems and the values have been presented in Table 6.

3.1.3 Economic Considerations

The cost of cascade implementation will have an enormous impact on the selection process. When the viable cascade candidates are identified, the decision for an industrial implementation will most likely be based completely on the cost. For a given cascade candidate the cost will depend on the number of catalytically active components (enzymes and co-factors) and the cost of possible sacrificial (co-factor recycling) reactions. However, the goal of this chapter will be the identification of viable cascade options and in further chapters selection of cascade candidate will be driven by economic concepts e.g. optimization of enzyme (and co-factor) concentrations.

Table 6 Considerations for a cascade selection based on the process parameters

	Process parameters							
	Thermodynamic equilibrium					Downstream processing		
	Calculated K_{eq}		Allowed concentration of the co-product for 95 % thermodynamic yield [µM]	Cascade ability (remaining co-product [µM])	Thermodynamic yield	Cascade products		pH shift ΔpK_a
LDH/GDH	LDH	GDH	1.11	$1.46 \cdot 10^{-12}$	> 99.99 %	Lactate	[475 mM]	+ 1.36
	$2.12 \cdot 10^{4}$	$1.67 \cdot 10^{23}$				glucono-1,4-lactone	[475 mM]	-
						glucono-1,5-lactone		-
						gluconic acid		- 3.14
LDH/FDH	LDH	FDH		$1.76 \cdot 10^{-12}$	> 99.99 %	Lactate	[475 mM]	+ 1.36
	$2.12 \cdot 10^{4}$	$1.31 \cdot 10^{52}$				CO_2	[475 mM]	
AlaDH/GDH	AlaDH	GDH	2.11	$2.29 \cdot 10^{-8}$	> 99.99 %	H_2O	[475 mM]	-
	$1.73 \cdot 10^{6}$	$1.67 \cdot 10^{23}$				glucono-1,4-lactone	[475 mM]	-
						glucono-1,5-lactone		-
						gluconic acid		- 3.14
AlaDH/FDH	AlaDH	FDH		$2.37 \cdot 10^{-10}$	> 99.99 %	H_2O	[475 mM]	-
	$1.73 \cdot 10^{6}$	$1.31 \cdot 10^{52}$				CO_2	[475 mM]	
YADH/GDH	YADH	GDH	$9.21 \cdot 10^{2}$	$2.11 \cdot 10^{-8}$	> 99.99 %	2-propanol	[475 mM]	-
	10.3	$1.67 \cdot 10^{23}$				glucono-1,4-lactone	[475 mM]	-
						glucono-1,5-lactone		-
						gluconic acid		- 3.14
YADH/FDH	YADH	FDH		$2.12 \cdot 10^{-8}$	> 99.99 %	2-propanol	[475 mM]	-
	10.3	$1.31 \cdot 10_{52}$				CO_2	[475 mM]	
PDC	$1.80 \cdot 10^{30}$		1.11	$7.89 \cdot 10^{-24}$	> 99.99 %	Acetaldehyde	[475 mM]	-
						CO_2	[475 mM]	
ALS	N.A.			N.A.	N.A.	CO_2	> [475 mM]	
						(S)-2-acetolactate	[475 mM]	+ 9.90
						Acetoin		-
						Diacetyl		-

3.2 Feasibility Parameters – Base for Selection

The aim of the above described data collection was to create an overview of the cascade systems, a cascade database, and the impact they would have on a process. However, for a selection process such data is insufficient because it does not clearly indicate what the best cascade candidate for process implementation is. To identify the viable cascade option, the feasibility of process implementation of a given cascade must be challenged. Therefore, three feasibility parameters have been identified by which a comparison can be made, and a potential candidate for implementation can be identified. The three feasibility parameters are the thermodynamics of the system which is determined by the thermodynamic ability of a cascade system which represents the remaining equilibrium concentration of the co-product after a cascade has been implemented. The second parameter is substrate selectivity of the enzymes in the system indicating possible side reactions and issues associated with these phenomena, e.g. loss of product or difficult separation of product from a mixture of amines created by side reactions. The third parameter is operational stability of cascade enzymes which was experimentally determined. The order in which parameters have been placed also plays a significant role potentially reducing the workload, as well as the time and the cost needed for the selection process as presented by the scheme shown in the Figure 29.

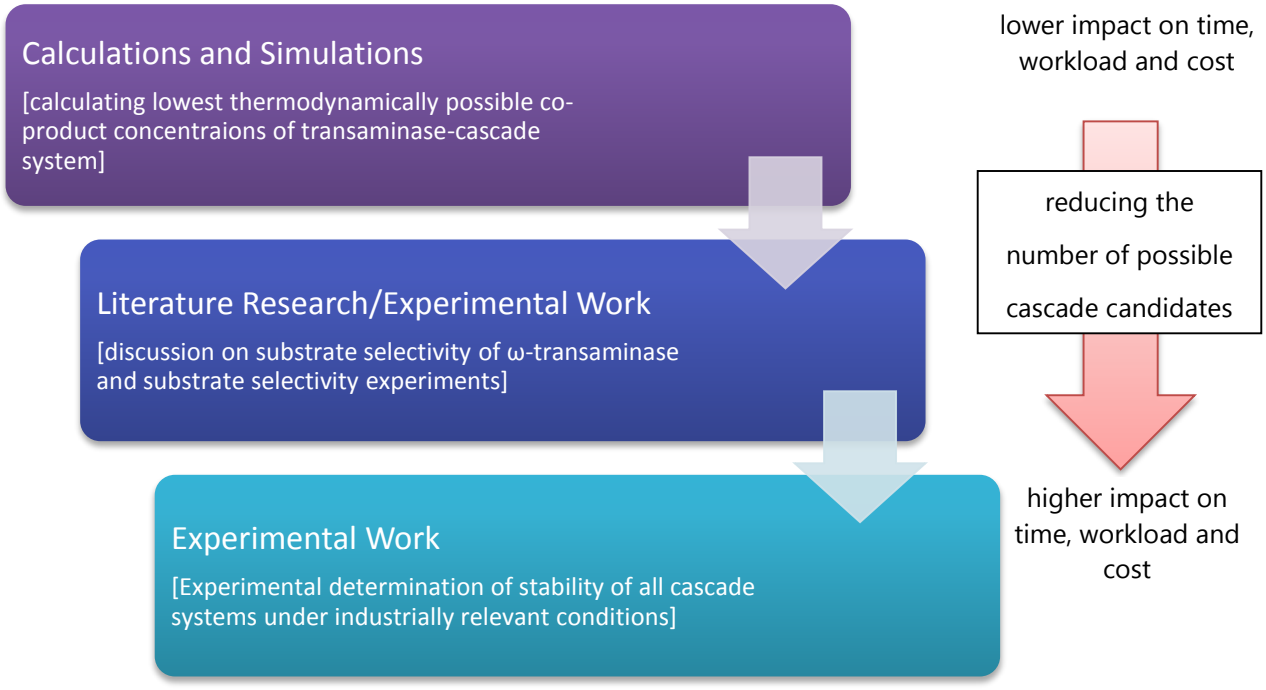


Figure 29. The justification of order in which the steps in the selection process are placed resulting in fewer candidates going through more time and workload consuming steps

3.3 Materials and Methods

3.3.1 Materials

The enzymes lactate dehydrogenase (LDH 1; EC 1.1.1.27), glucose dehydrogenase (GDH 1 and 2; EC 1.1.1.47) and formate dehydrogenase (FDH; EC 1.2.1.2) are obtained from Codexis (Redwood City, CA) in the form of crude extract. Another lactate dehydrogenase (LDH 2) and alanine dehydrogenase (AlaDH; EC 1.4.1.1.) were obtained from Evocatal (Düsseldorf, Germany). Yeast alcohol dehydrogenase (YADH; EC 1.1.1.1) was obtained from Sigma Aldrich (Brøndby, Denmark). Acetophenone, L-alanine, 2-propylamine, sodium pyruvate, acetone, ammonium formate, (D)-(+)-glucose, (D)-gluconic acid sodium, sodium lactate, β-nicotinamide adenine dinucleotide reduced dipotassium salt, β-nicotinamide adenine dinucleotide hydrate, 2-propanol Tris Sigma 7-9 and hydrochloric acid were obtained from Sigma Aldrich (Brøndby, Denmark). (S)-α-methylbenzylamine and potassium hydroxide were obtained from Merck KGaA (Darmstadt, Germany)

3.3.2 Simulations and Calculations

3.3.2.1 Thermodynamic Calculations

K_{eq} of individual cascade reactions. Gibbs free energies of the formation (ΔG_f°) of reaction components at pH 7 and 25 °C were taken from the literature[150] and the K_{eq} of individual cascade reactions was calculated by using Equation 2.4 and Equation 3.1.

$$K_{eq} = e^{-\frac{\Delta G^\circ}{RT}} \quad 3.1$$

Calculation of the allowed remaining co-product concentration for 95 % conversion was made using Equation 2.3, and K_{eq} values for the substrate pairs acetophenone-alanine and acetophenone-2-propylamine were obtained from the experimental work published by Tufvesson et al[98]. The initial concentration of acetone was 500 mmol L^{-1} and a 1X excess of amino donor (1 mol L^{-1}) was present, where excess is defined as:

$$c_1 = c_2(1 + X) \quad 3.2$$

c_1 – concentration of the component in excess (donor); c_2 – concentration of the comparing component (ketone substrate); X – excess

The allowed pyruvate concentration for 95 % conversion in the AlaDH case was calculated by modifying the Equation 2.3 by assuming the same alanine concentration for the initial value and the equilibrium value simulating the alanine recycle.

Cascade ability or how much of the co-product can a given cascade thermodynamically remove was calculated using an iterative procedure as suggested by the literature[151]. The thermodynamic equilibrium was postulated as described in Table 7.

Table 7 Initial thermodynamic concentrations of ω-transaminase/LDH/GDH system

ω-transaminase/LDH/GDH system							
ω-transaminase	ACP	+	alanine	↔	pyruvate	+	MBA
Initial conc. [mmol L^{-1}]	500		1000		0		0
Equilibrium conc. [mmol L^{-1}]	500 - x		1000 - x		x		x
LDH	NADH	+	pyruvate	↔	NAD^+	+	lactate
Initial conc. [mmol L^{-1}]	0.1		x		0		0
Equilibrium conc. [mmol L^{-1}]	0.1 - y		x - y		y		y
GDH	NAD+	+	glucose	↔	NADH	+	glucono-1,5-lactone
Initial conc. [mmol L^{-1}]	y		550		0		0
Equilibrium conc. [mmol L^{-1}]	y - z		550 - z		0.1 – y + z		z

Based on the Equation 2.3, K_{eq} values, initial concentrations and the assumptions stated at the end of this section, equations for calculating the equilibrium change in ω-transaminase (value x), LDH (value y) and GDH (value z), here were developed and are described by equations 3.3, 3.4 and 3.5:

$$x = \frac{c_{MBA} + c_{PYR} + K_{eq}^{TAm}(c_{ACP} + c_{Ala}) - \sqrt{\left(c_{MBA} + c_{PYR} + K_{eq}^{TAm}(c_{ACP} + c_{Ala})\right)^2 - 4\left(K_{eq}^{TAm} - 1\right)\left(K_{eq}^{TAm} c_{ACP} c_{Ala} - c_{PYR} c_{MBA}\right)}}{2\left(K_{eq}^{TAm} - 1\right)} \quad 3.3$$

$$y = \frac{c_{NAD^+} + c_{LAC} + K_{eq}^{LDH}(c_{PYR} + c_{NADH}) - \sqrt{\left(c_{NAD^+} + c_{LAC} + K_{eq}^{LDH}(c_{PYR} + c_{NADH})\right)^2 - 4\left(K_{eq}^{LDH} - 1\right)\left(K_{eq}^{LDH} c_{PYR} c_{NADH} - c_{LAC} c_{NAD^+}\right)}}{2\left(K_{eq}^{LDH} - 1\right)} \quad 3.4$$

$$z = \frac{c_{NADH} + c_{GLUC-1,5-LAC} + K_{eq}^{GDH}(c_{PYR} + c_{NAD^+}) - \sqrt{\left(c_{NADH} + c_{GLUC-1,5-LAC} + K_{eq}^{GDH}(c_{PYR} + c_{NAD^+})\right)^2 - 4\left(K_{eq}^{GDH} - 1\right) \cdot \left(K_{eq}^{GDH} c_{GLU} c_{NAD^+} - c_{GLUC-1,5-LAC} c_{NADH}\right)}}{2\left(K_{eq}^{GDH} - 1\right)} \quad 3.5$$

The equilibrium concentrations of reaction mixture components were calculated as shown in the Table 7 in the 'Equilibrium conc.' rows. Following the steps of calculation of equilibrium concentrations for each reaction, it can be observed that the equilibrium concentration of pyruvate in ω-transaminase was consumed by the LDH reaction which means that the equilibrium concentrations have to be recalculated. The next set of x, y and z values are calculated followed by calculations of new equilibrium concentrations of the reaction mixture. This

process continues iteratively until the whole cascade system reaches equilibrium. This equilibrium of the system can be defined as the case when the ω-transaminase reaction slows down and further MBA production is insignificant (iteration condition for stopping: $e = |c_{MBA,\,n} - c_{MBA,\,n-1}| < 10^{-15}$). Figure 30 shows an example of flow diagram for above described procedure for the program developed in Matlab®.

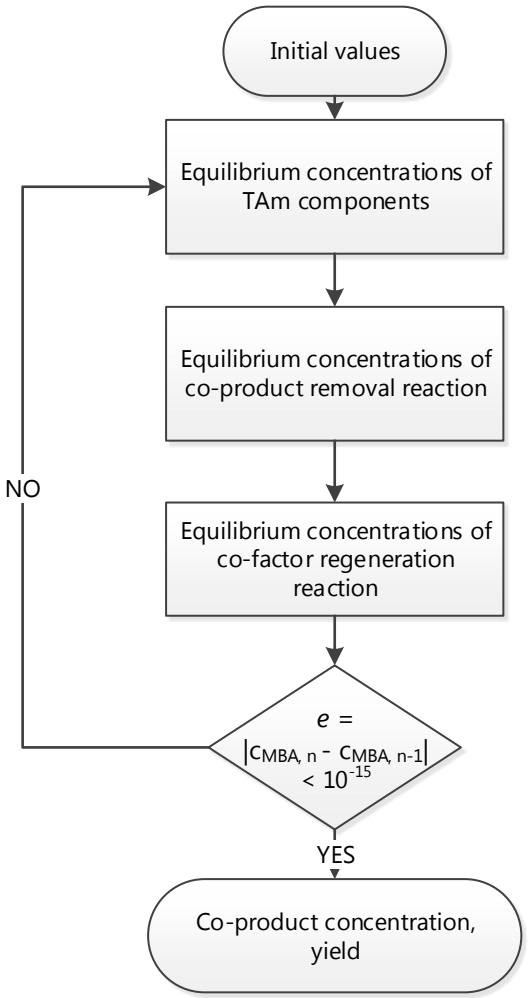


Figure 30 Flow diagram of calculating thermodynamic equilibrium concentrations of ω-transaminase coupled to a dehydrogenase cascade system

An example of Matlab® program for calculating equilibrium co-product concentration and thermodynamic yield for LDH/GDH system is given in Appendix 3B.

Several assumptions were made during the calculation: the concentrations of water and CO_2 were kept constant at $55.52 \cdot 10^3$ and 28.4 mmol L^{-1} respectively where these values represent the water concentration in aqueous solution and the equilibrium concentration of CO_2 in water under standard conditions. In the reactions catalyzed by GDH, only transformation from glucose to the glucono-1,5-lactone is considered.

Multiple equilibrium shifting strategies have been discussed and the effect of using ISPR, ISCPR and Amino donor excess was calculated using modified Equation 3.6 for two systems with different K_{eq} values shown in

the Figure 32 and Figure 36. The concentrations of product removed by ISPR [%] or co-product removed by ISCPR [%] are termed as c_{ISPR} and c_{ISCPR}, respectively.

$$K_{eq}^{TAm} = \frac{(c_{PYR} - c_{ISCPR})(c_{MBA} - c_{ISPR})}{c_{ACP}c_{Ala}(1 + X)} \quad 3.6$$

3.3.3 Experimental Studies

3.3.3.1 Stability Studies

Approximation of process relevant conditions. Three reaction mixtures representing three reaction systems, i.e. ω-transaminase-LDH/GDH, ω-transaminase-AlaDH/FDH and ω-transaminase-YADH/GDH, were prepared and termed reaction mixture 1, 2 and 3 respectively. The concentration of the components in the reaction mixtures was averaged between starting and ending concentrations of an industrial process (50 g L^{-1} of product ≈ 500 mmol L^{-1}). Reaction mixture 1 contained 30 mmol L^{-1} acetophenone, 1 mol L^{-1} L-alanine, 250 mmol L^{-1} (S)-α-methylbenzylamine, 250 mmol L^{-1} sodium lactate, 250 mmol L^{-1} (D)-(+)-glucose and 250 mmol L^{-1} (D)-gluconic acid sodium salt. Reaction mixture 2 contained 30 mmol L^{-1} acetophenone, 1 mol L^{-1} L-alanine, 250 mmol L^{-1} (S)-α-methylbenzylamine and 250 mmol L^{-1} ammonium formate. Reaction mixture 3 contained 30 mmol L^{-1} acetophenone, 1 mol L^{-1} 2-propylamine, 250 mmol L^{-1} (S)-α-methylbenzylamine, 250 mmol L^{-1} 2-propanol, 250 mmol L^{-1} (D)-(+)-glucose and 250 mmol L^{-1} (D)-gluconic acid sodium salt. The pH value of the reaction mixtures was adjusted to 7 with the addition of HCl or KOH.

Enzyme assay. For determination of the initial reaction rates of the co-product removal enzymes an assay was used containing 0.1 mmol L^{-1} of co-factor NADH and 1 mmol L^{-1} of pyruvate for LDH and AlaDH or acetone for YADH. The LDH, AlaDH or YADH assay contained reaction mixture 1, 2 or 3 respectively. The concentration of enzymes in the assay of AlaDH, LDH and YADH was 0.001, 0.01 and 0.2 g L^{-1} respectively. The assay for determining the initial reaction rates of co-factor recycling enzymes GDH and FDH contained reaction mixture 1 and 2 respectively and 0.1 mmol L^{-1} of NAD^+ while the concentration of both enzymes was 0.01 g L^{-1}. The assay mixtures lacking the co-factor NADH/NAD^+ were incubated at 30 °C in 4 mL glass vials in the thermoshaker (HLC, TG Instrument AB, 300 min^{-1}). Samples were taken at time 0, 4 and 24 h into 4 mL Quartz SUPRASIL® Precision cells and the reaction was started by addition of NADH or NAD^+ solution. The reaction rates of all enzymes were followed by the change of adsorption of NADH at 340 nm using a Shimadzu UV-1801 at 30 °C.

3.3.3.2 Selectivity studies

The assay contained 0.1 mmol L^{-1} NADH and 30 mmol L^{-1} acetophenone and the concentration of AlaDH, LDH and YADH was 1 g L^{-1}. The assay was carried out in 4 mL Quartz SUPRASIL® Precision cells by following the change of adsorption of NADH at 340 nm using a Shimadzu UV-1801 at 30 °C. The reaction was started by addition of the enzyme solution. The blank cuvette contained reaction mixture without the enzyme solution. An additional 100 µL of NADH solution was added to each cuvette during the reaction to verify that no observable reaction is occurring.

3.4 Results and Discussion

3.4.1 Thermodynamic Ability

For thermodynamically challenged reactions an equilibrium shifting strategy is mandatory and possible strategies have been described in the Section 2.4. The usage of thermodynamic calculations described in section 3.3.2.1 enables the calculation of a desired yield as a function of the changed K_{eq} value (amine donor selection), added substrate (amino donor excess) or allowed residual product or co-product (ISPR or ISCPR, respectively). Taking into account physical limitations of the system, e.g. solubility of amino donor, this approach can identify the applicable equilibrium shifting strategies as well as provide an estimation of how effective a strategy has to be to achieve the desired thermodynamic yield.

One possible strategy for determining the requirement for achieving that desired thermodynamic yield is calculating the allowed co-product concentration that can remain in the reaction mixture as a function of thermodynamic yield as shown in Figure 31. This calculation is based on industrially relevant conditions (c_{ACP} = 500 mmol L^{-1} and one fold excess alanine $c_{alanine}$ = 1 mol L^{-1}). 95 % thermodynamic yield has been chosen as a relevant target resulting in a product concentration in the range defined in the guidelines for successful biocatalytic processes[8] c_{MBA} = 475 mmol L^{-1} or $\gamma_{MBA} \approx$ 50 g L^{-1}. Under these conditions the allowed co-product concentration remaining in the reaction mixture is 1.11 µmol L^{-1} which means that any successful equilibrium shifting strategy must be able to remove the co-product to this value or lower.

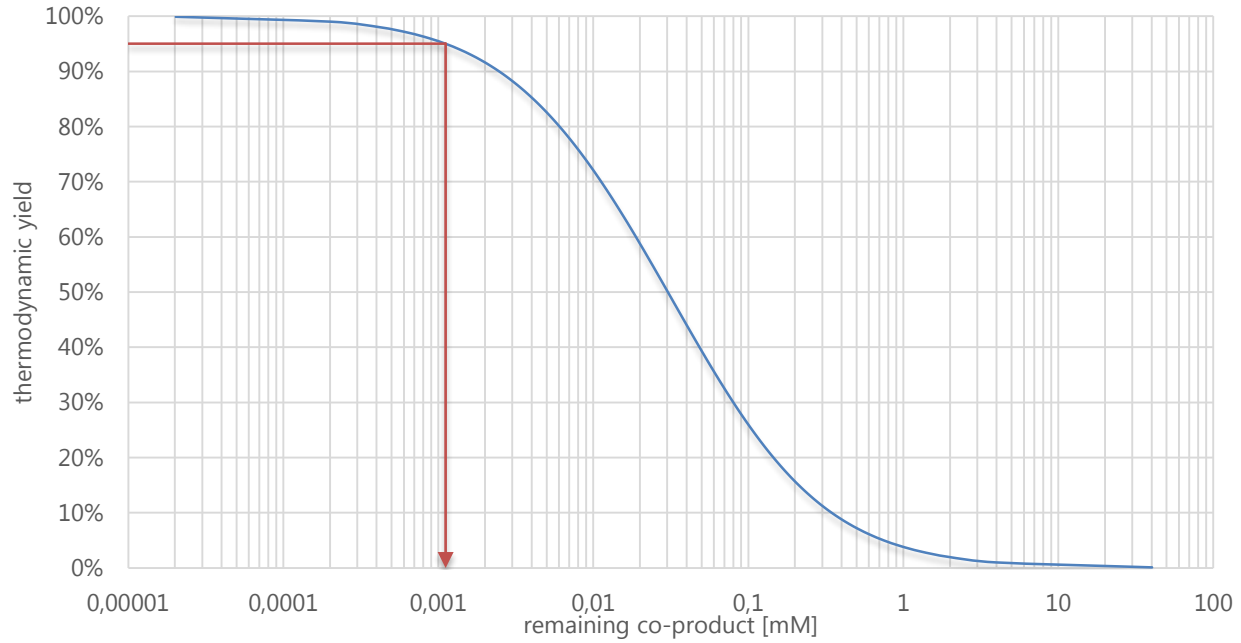


Figure 31 The allowed co-product concentration remaining in the reaction mixture as a function of the desired yield for an equilibrium value K_{eq}= 4.03·10^{-5}

The value of the allowed co-product concentration for the previously described case ($c_{pyruvate}$ = 1.11 µmol L^{-1}) shows how strong the thermodynamic challenge is. To achieve a desired yield of 95 %, 474.998 mmol L^{-1} of the co-product have to be removed by a single equilibrium shifting strategy which would require for this strategy to be extremely efficient. To overcome this challenge, it is possible to implement multiple equilibrium shifting strategies to assist the primary one. In the Figure 32 the yield has been calculated as a function of product removed by ISPR [%], co-product removed by ISCPR [%] and Amine donor excess to investigate the additive effect of those strategies (Equation 3.6).

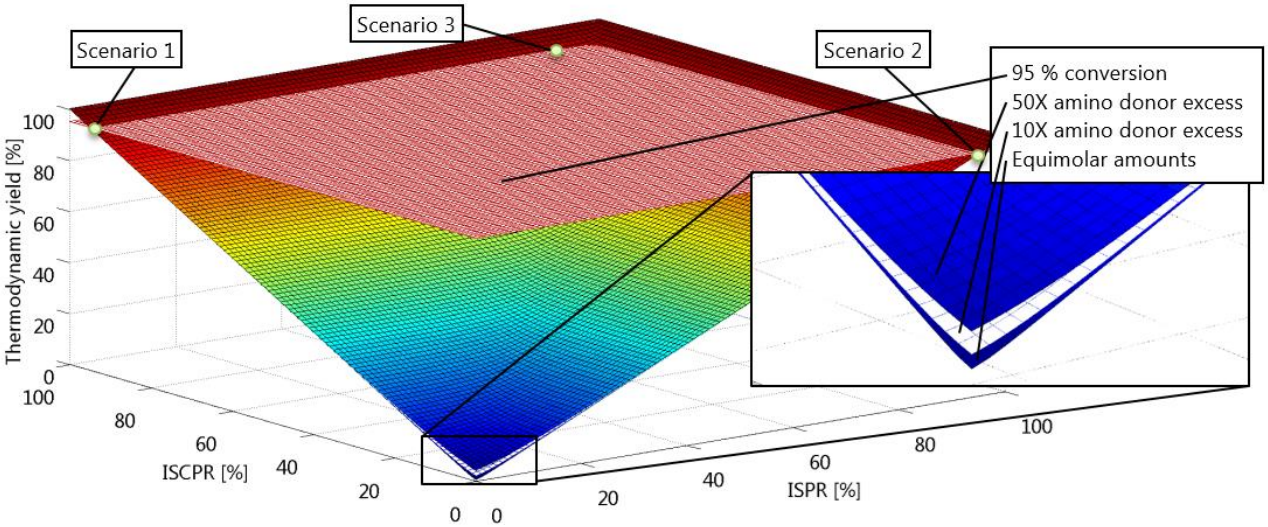


Figure 32 Thermodynamic yield as a function of co-product removed by ISCPR [%], product removed by ISPR [%] and excess of amino donor; intersecting horizontal plane indicates 95 % conversion (K_{eq} = 4.03·10^{-5})

The calculation showed that the use of ISPR (yz plane) or ISCPR (xz plane) applied individually result in the same effect meaning that the removed co-product required to achieve e.g. 95 % yield (Scenario 1) will correspond to the same amount of product removed to achieve 95 % yield (Scenario 2). However, if 95 % of possible co-product is removed by ISCPR together with an additional 80 % of the product removed by ISPR (Scenario 3), the resulting thermodynamic yield won't be significantly changed. This result shows in this case that secondary ISPR equilibrium shifting strategy does not contribute considerably to the yield. The addition of the amino donor excess strategy will also not improve the thermodynamic yield significantly, and will result in closely packed calculated surfaces representing yields as shown in the Figure 32 in the enlarged window. The lower dark blue surface represents equimolar amount of amino donor, the white the excess of 10 and the upper blue the excess of 50. This type of calculation can prove to be very beneficial in the early stage of process development indicating viable equilibrium shifting strategies. Taking e.g. ISCPR as a primary equilibrium shifting strategy, calculations have been made to show how much of the remaining co-product is allowed in the reaction mixture, and results are shown in the Figure 33. As predicted assisted ISPR and assisted donor excess allow more co-product concentrations in the reaction mixture for achieving 95 % yield, however this information does not show the percentage of the assistance of secondary and tertiary strategy. Figure 34 shows percentage-wise the assistance of secondary and tertiary equilibrium shifting strategy and for the case of 99 % assisted ISPR and 50X excess amino donor, the required co-product that needs to be removed was reduced only by 1 %. ISPR and excess amino donor in this case didn't provide significant assistance.

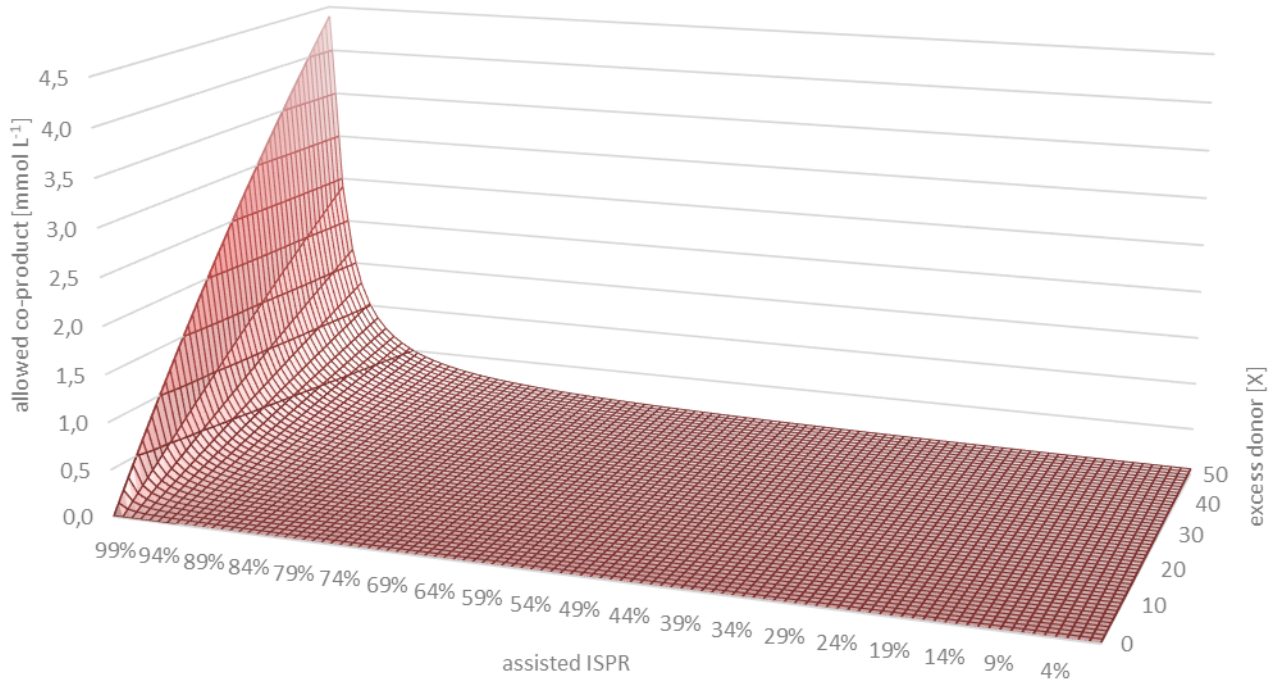


Figure 33 Allowed co-product concentration in the reaction mixture for achieving 95 % yield as a function of assisted ISPR and assisted excess donor (K_{eq} = 4.03·10^{-5})

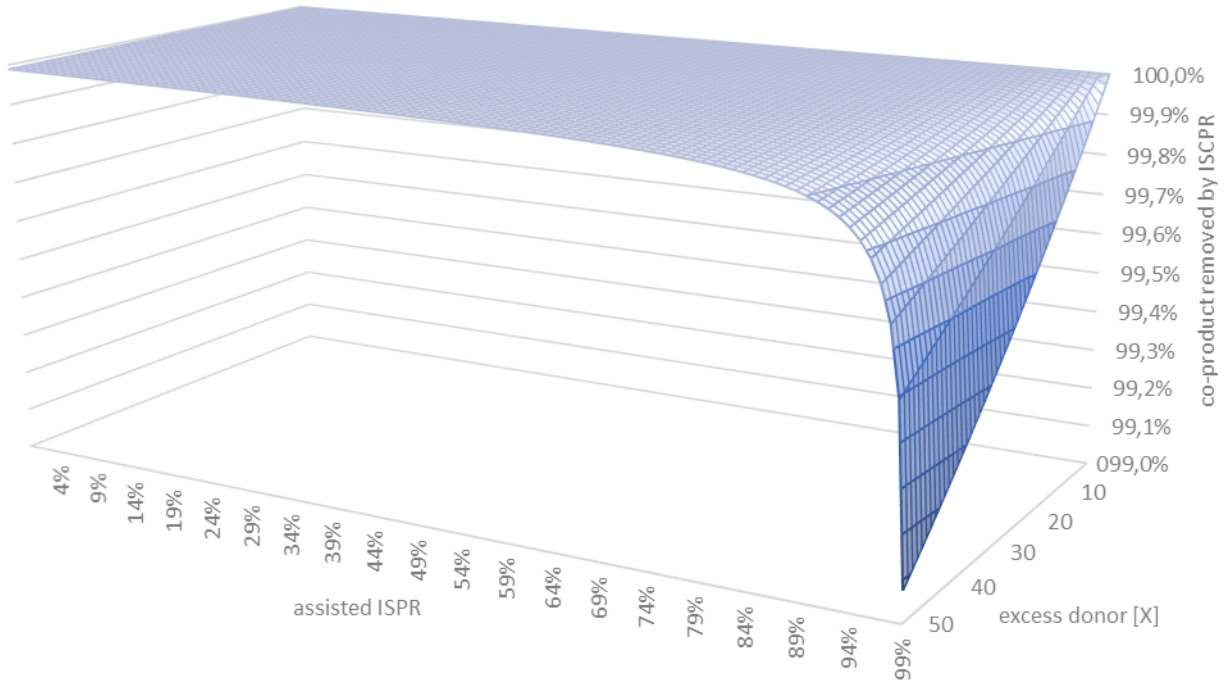


Figure 34 Percentage of the required co-product removed as a function of assisted ISPR and assisted excess donor (K_{eq} = 4.03·10^{-5})

Looking at the trends of ISPR and ISCPR at 95 % conversion (Figure 32) it is possible to plot a thermodynamic window of operation, i.e. a two-dimensional process map displaying regions of feasible operating ranges based on the boundaries of the system[152, 153]. In Figure 35 the drawn lines represent the intersection of yield surfaces with the 95 % plane. For the case of 94 % assisting ISPR and 50X donor excess (Scenario 4) the ISCPR requirement will be reduced from starting 95 % (no assisting strategies) to 94 % of removed co-product.

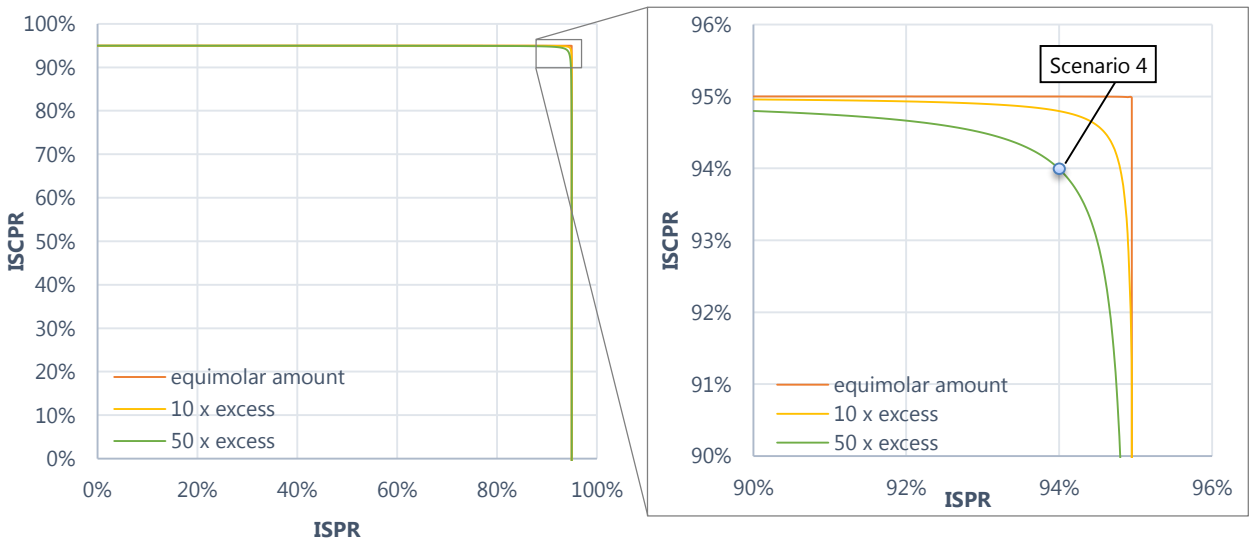


Figure 35 Window of operation for achieving 95 % conversion as a function of ISCPR, ISPR and amino donor excess; the area above the lines shows the window of operation (K_{eq} = 4.03·10^{-5})

The inability to reduce the requirements for achieving the desired yield by the assisting secondary and tertiary equilibrium shifting strategy is mainly a function of the K_{eq} value, which is extremely low in this example (K_{eq} = $4.03 \cdot 10^{-5}$). To prove this hypothesis, the same set of calculations have been made for a case where 2-propylamine is used as an amino donor (Figure 21) and the K_{eq} value is $3.33 \cdot 10^{-2}$.

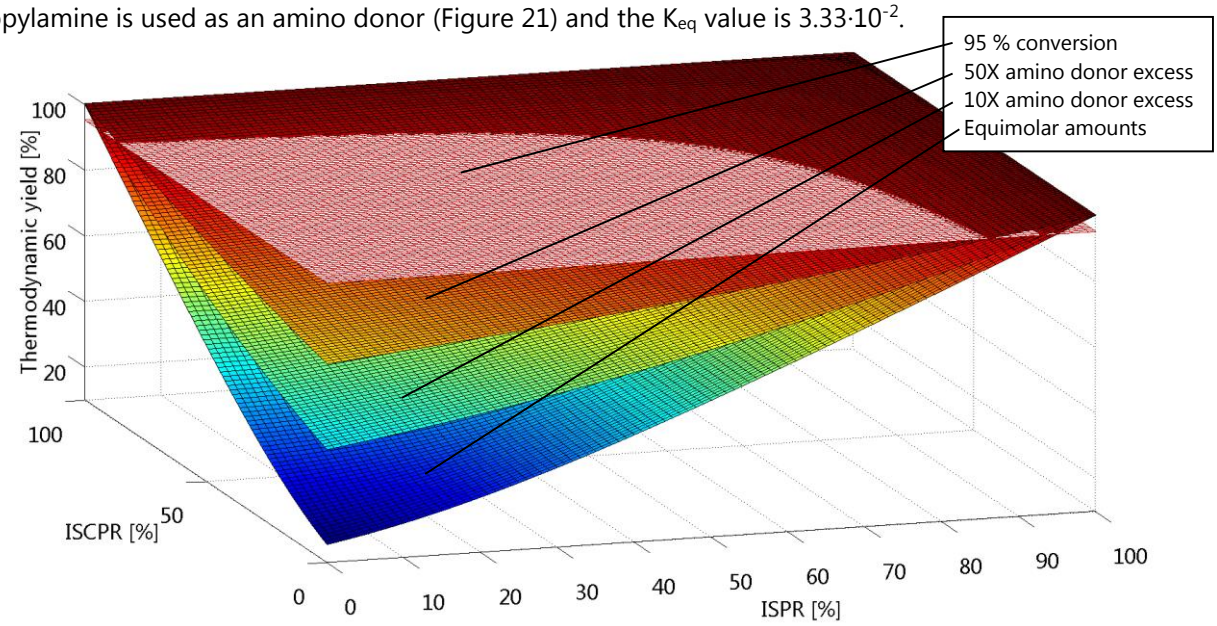


Figure 36 Thermodynamic yield as a function of co-product removed by ISCPR [%], product removed by ISPR [%] and excess of amino donor; intersecting horizontal plane indicates 95 % conversion (K_{eq} = $3.33 \cdot 10^{-2}$)

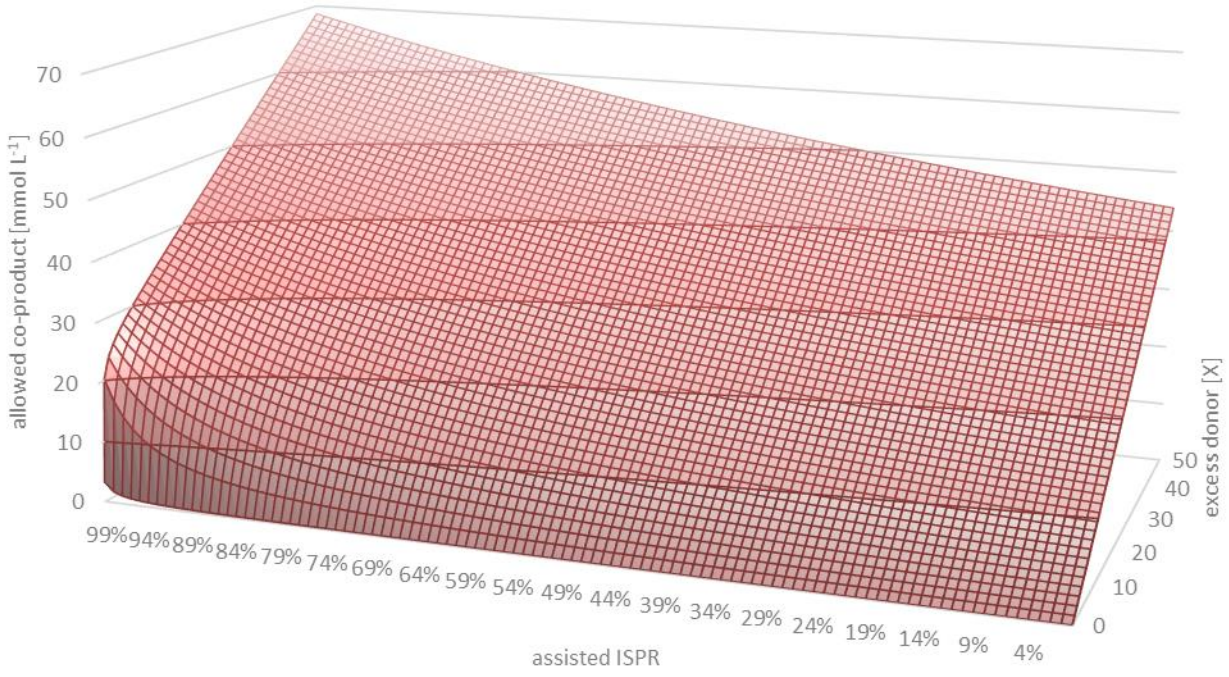


Figure 37 Allowed co-product in the reaction mixture for achieving 95 % yield as a function of assisted ISPR and assisted excess donor (K_{eq} = $3.33 \cdot 10^{-2}$)

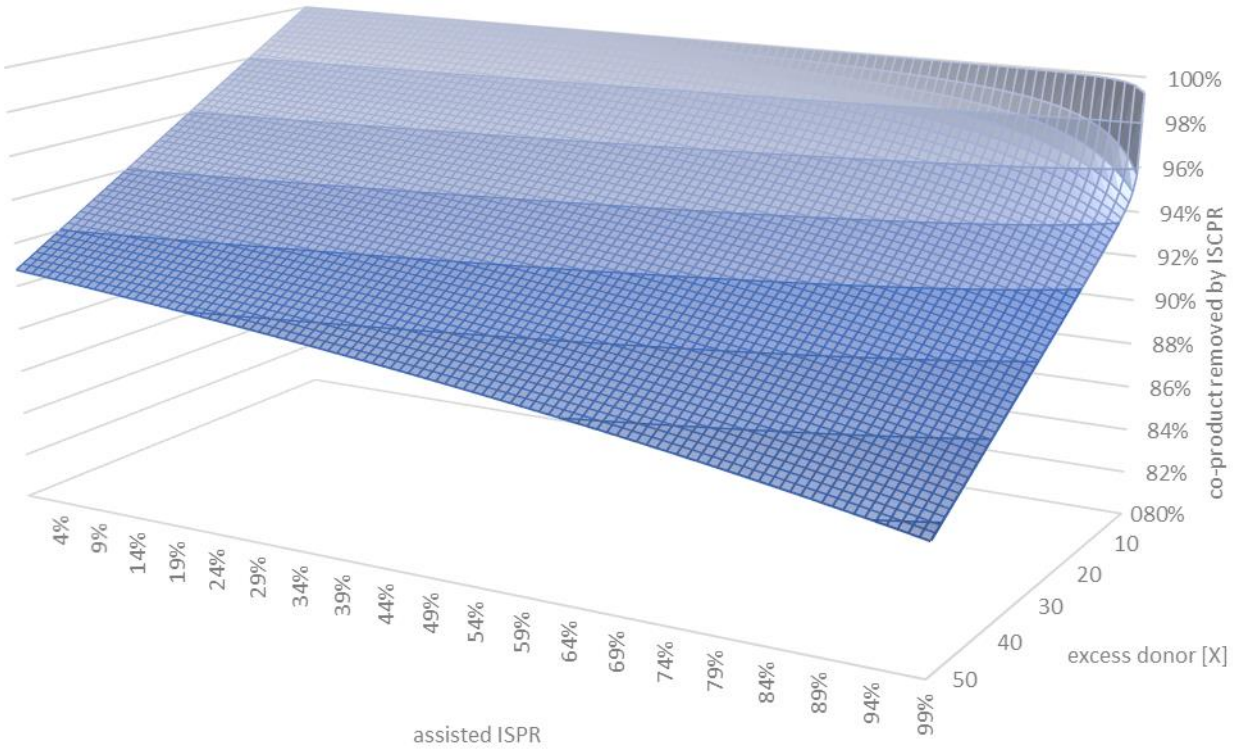


Figure 38 Percentage of the required co-product removed as a function of assisted ISPR and assisted excess donor (K_{eq} = 3.33·10^{-2})

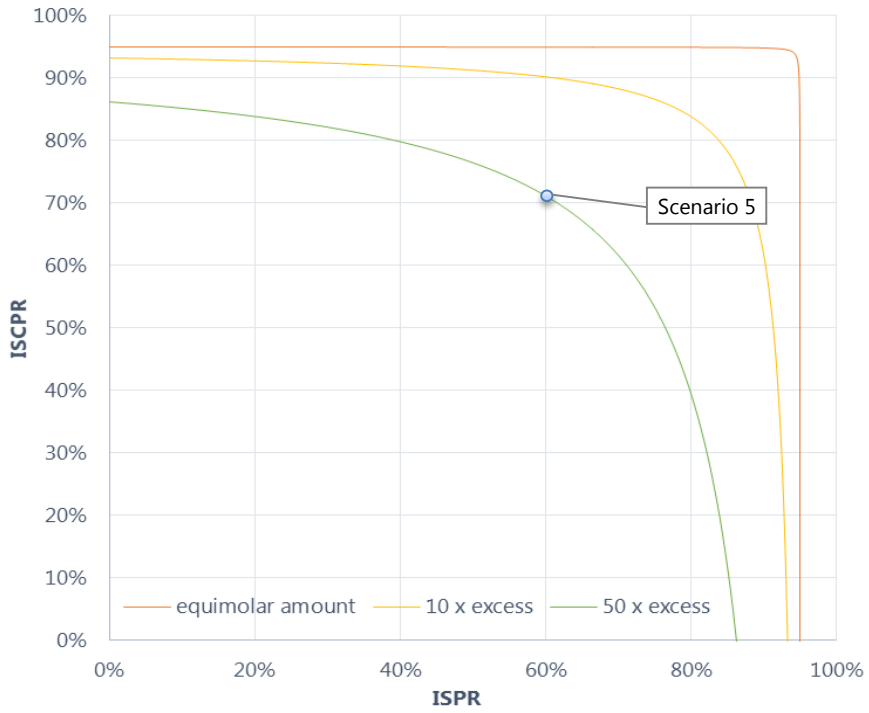


Figure 39 Operating window for achieving 95 % conversion as a function of ISCPR, ISPR and amino donor excess; the area above the lines shows possible window of operation (K_{eq} = 3.33·10^{-2})

The results for the two different cases (K_{eq} = 4.03·10^{-5} and K_{eq} = 3.33·10^{-2}) showed that the K_{eq} value plays a significant role in determining the extent of assistance provided by the secondary and tertiary equilibrium shifting strategy. As seen in the Figure 37 the allowed remaining co-product concentration for achieving 95 % conversion is much higher when secondary (ISPR) and tertiary (amino donor excess) strategies are applied. Figure 38 supports that statement by showing that when the secondary strategy (ISPR) is at 99 % and the tertiary (amine donor excess) at 50X, the primary equilibrium shifting strategy (ISCPR) has to remove 85 % of the required co-product to achieve 95 % thermodynamic yield. An improved assistance of the secondary and tertiary equilibrium shifting strategies is shown in the Figure 39 where e.g. for 60 % assisted ISPR and 50X of amino donor (Scenario 5) the required co-product removal by ISCPR is lowered from 95% to ≈ 70 %. Observing these results, the conclusion can be drawn that for highly thermodynamically challenged reactions, e.g. ω-transaminase reaction with acetophenone and alanine as substrates, only one equilibrium shifting strategy will have a dominant effect on the equilibrium shift and it will have to be very efficient and extremely selective. Since the amino donor excess strategy is usually limited by solubility of the donor, the choice of equilibrium shifting strategies will have to be made between ISPR and ISCPR. Taking into account the properties of the components in the reaction mixture, currently no ISPR technology satisfies the exclusive selectivity towards the product and thus lacks the ability to remove it to low enough values (1.1 µmol L^{-1}). However, due to the high substrate selectivity of enzymes and the ability to run the reactions at relatively high rates at low substrate concentrations (low K_m), ISCPR by enzymes provides currently the only viable equilibrium shifting strategy for severely thermodynamically challenged reactions. Compared to the more classical approaches e.g. ISCPR by chemocatalytic reaction, the obvious advantage of the enzymatic approach is its supreme substrate selectivity and the importance of this parameter will be discussed in the next section.

In the Figure 33 the allowed co-product concentration to obtain an industrially relevant 95 % thermodynamic yield was calculated. However, to choose a cascade capable of lowering the co-product to that concentration or even a lower concentration is a first step in a cascade selection process. This ability of achieving co-product removal is here termed the cascade ability and is represented in the system as the remaining co-product concentration [µmol L^{-1}] after the cascade system reaches equilibrium. If a cascade system is thermodynamically unable to remove the co-product to the desired concentration, either lower yields have to be considered or the cascade system has to be discarded from the selection process. The process of calculating the allowed co-product concentration and the cascade ability was described in the Section 3.3.2.1 and the results are shown in Table 8.

Table 8 Cascade ability vs. allowed co-product concentration with their respective thermodynamic yields of different cascade system candidates

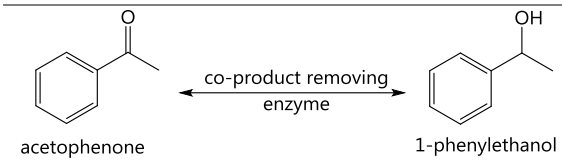
Enzymatic cascade	Allowed co-product [µmol L^{-1}]	Cascade ability (co-product [µmol L^{-1}])	Thermodynamic conversion
LDH/GDH	1.11	$1.10 \cdot 10^{-7}$	> 99.99 %
LDH/FDH	1.11	$1.25 \cdot 10^{-7}$	> 99.99 %
AlaDH/GDH	2.11	$9.28 \cdot 10^{-2}$	99.77 %
AlaDH/FDH	2.11	$1.66 \cdot 10^{-8}$	> 99.99 %
YADH/GDH	$9.21 \cdot 10^{2}$	$1.65 \cdot 10^{-1}$	99.12 %
YADH/FDH	$9.21 \cdot 10^{2}$	$1.81 \cdot 10^{-1}$	99.12 %
PDC	1.11	$7.89 \cdot 10^{-25}$	> 99.99 %
ALS	1.11	N.A.	N.A.

The calculations showed that all cascade systems have sufficient cascade ability and can reduce the co-product concentration to low enough levels to achieve thermodynamic yields > 99 %. The Gibbs free energy of formation for the (S)-2-acetolactate was unavailable in the literature so the calculation of K_{eq} value for the reaction catalyzed by ALS (Figure 25) as well as its cascade ability was not possible.

3.4.2 Substrate Selectivity

The second step of a cascade selection process is determining the substrate selectivity of the enzymes present in the reaction system, and two types of undesired substrate selectivity can be observed. The first type of undesired substrate selectivity relates to the co-product degrading enzyme. In all ω-transaminase cases the co-product is a ketone and if a co-product degrading enzyme does not possess sufficient selectivity, another ketone, e.g. acetophenone might be consumed instead as shown in Table 9.

Table 9 Possible reaction scheme of side reactions due to the insufficient substrate selectivity of the co-product removal enzyme

Side Reaction	**Consequence**
	• consumption of limiting substrate • undesired equilibrium shift

Insufficient substrate selectivity of the co-product removing enzymes was never reported in the literature. However, a quick experimental verification described in Section 3.3.3.2 was made to confirm these results (Appendix 3C).

The second type of undesired selectivity relates to the substrate selectivity of ω-transaminase towards the products of the cascade reactions. Two enzymes whose products might be substrate for the ω-transaminase reaction have been identified: PDC and ALS. These products are ketones and aldehydes and possible side reactions and their consequences for the PDC cascade have been listed in Table 10.

Table 10 Possible reaction schemes of side reactions due to insufficient substrate selectivity of the ω-transaminase towards the products of the PDC cascade

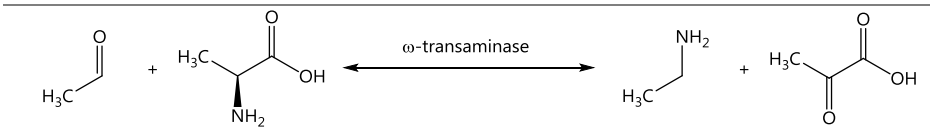
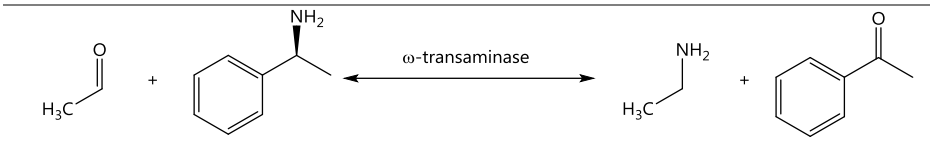
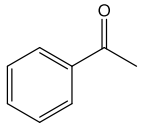
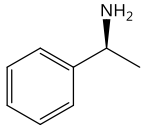
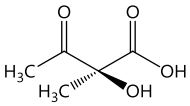
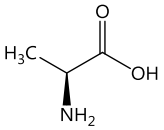
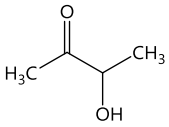
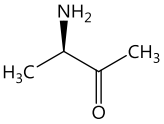
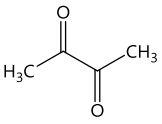
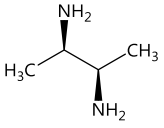
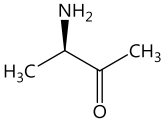
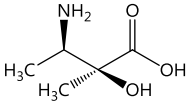
Side Reactions	Consequence
 acetaldehyde + alanine ⟷ ethylamine + pyruvate	• loss of amino donor • undesirable equilibrium shift • extra amine – harmful and flammable
 acetaldehyde + (S)-α-methylbenzylamine ⟷ ethylamine + acetophenone	• loss of amino product • extra amine - harmful and flammable

Table 11 Keto substrates and amino donors resulting in 18 possible side reactions in the ALS cascade system

Possible Keto Substrates	Possible Amino Donors
 acetophenone	 (S)-α-methylbenzylamine
 (S)-2-acetolactate	 alanine
 acetoin	 (*R*)-3-aminobutan-2-one
 diacetyl	 (2*R*,3*R*)-butane-2,3-diamine
 (*R*)-3-aminobutan-2-one	 (2*S*,3*R*)-3-amino-2-hydroxy-2-methylbutanoic acid

In the ALS cascade system overall 18 possible side reactions catalyzed by ω-transaminase may occur (Table 11) resulting in production of numerous unwanted amines, an undesirable equilibrium shift and, most importantly, a loss of the main amino product. The extent of each side reaction for both cascade systems will depend on its own specific K_{eq} and kinetic parameters. However, certain predictions can be made after studying the literature. Aldehydes have been identified in the literature as a preferred substrate for the ω-transaminase[52], and as a result the reactions shown in Table 10 are likely to take place. Regarding the promiscuity of

ω-transaminase towards the products of the ALS cascade, acetoin, diacetyl and 3-aminobutan-2-one show high structural resemblance to pyruvate, and are also very favorable substrates of the ω-transaminase[52, 154], and side reactions involving those molecules might be very likely to occur, however not yet experimentally confirmed. Due to the high probability of loss of product, the production of undesired amines which complicates DSP and the undesired equilibrium shift, the PDC and ALS cascade systems will be discarded from the selection process until an ω-transaminase with desired substrate specificity is developed.

3.4.3 Enzyme Stability

In the section 2.3.2.3.1.2 the reasons for requiring a high stability of the enzymes have been explained and justified and the same rules must be applied when selecting the cascade options for industrial implementation. The literature suggests the investigation of the operational stability of enzymes for industrial purposes[155]. This approach determines the stability of an enzyme throughout the course of the reaction. However, in this work industrially relevant conditions could not be met by application of the available ω-transaminase due to the lack of enzyme tolerance towards high concentrations of substrates and products. A compromise has been made and stability has been determined at simulated conditions. Knowing the concentrations of the reaction mixture at the beginning and the end of the reaction, averaged concentrations which do not change over time were chosen to represent process conditions of several processes with different cascades (reaction mixtures 1, 2 and 3; see section 3.3.3.1).

The stability results are usually presented in relative values, and are often fitted to first order kinetics (Equation 2.1) given a certain half-life time. However, if the values are given as reaction rates, comparing those values with experiments done in neat conditions (substrates, buffer) for the same substrate concentrations, valuable information can be retrieved, e.g. the degree of inhibition by components in the reaction mixture. If this simple experiment can show that inhibition is too high, e.g. no reaction can be observed under industrial conditions, then this enzyme can be excluded from the selection process until it is improved to tolerate a higher concentration of inhibitory components. In this work this was the case with YADH which showed no observable reaction under process relevant conditions (reaction mixture 3).

As shown in Figure 40 a certain fraction of the potentially realizable reaction rate was lost due to inhibition. However, a more detailed investigation and kinetic models are required to identify the inhibiting components and the strength of inhibition (Chapter 4).

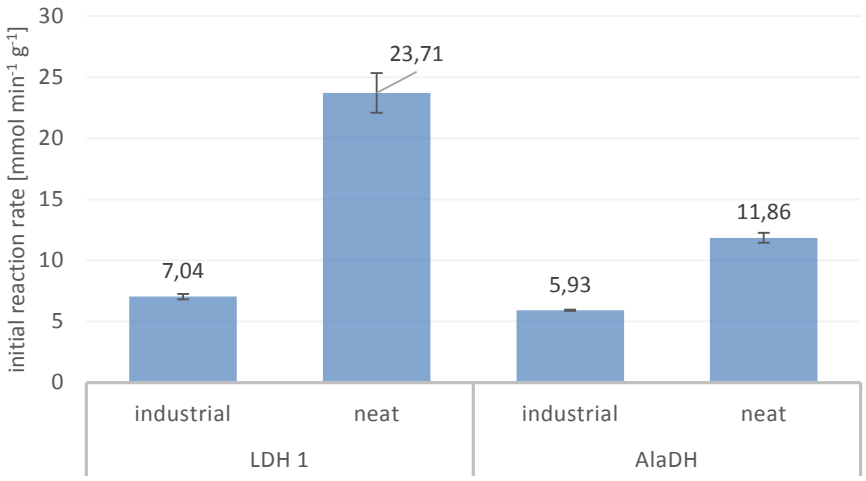


Figure 40 Difference in the initial reaction rates between neat and industrially relevant conditions for LDH 1 and AlaDH enzymes

Results of the investigated cascade enzymes represented as reaction rates are shown in Figure 41 and resulting half-time values in Table 12. Stabilities of individual enzymes fitted to a first-order decay model (Equation 2.1) are shown in Appendix 3D. This method does not only distinguish possible candidates between different types of enzymes, but also between the same enzymes from different sources and/or suppliers. For this reason, all cascade enzymes available for this project were tested for stability and were therefore also named for this purpose LDH 1, LDH 2, GDH 1 and GDH 2.

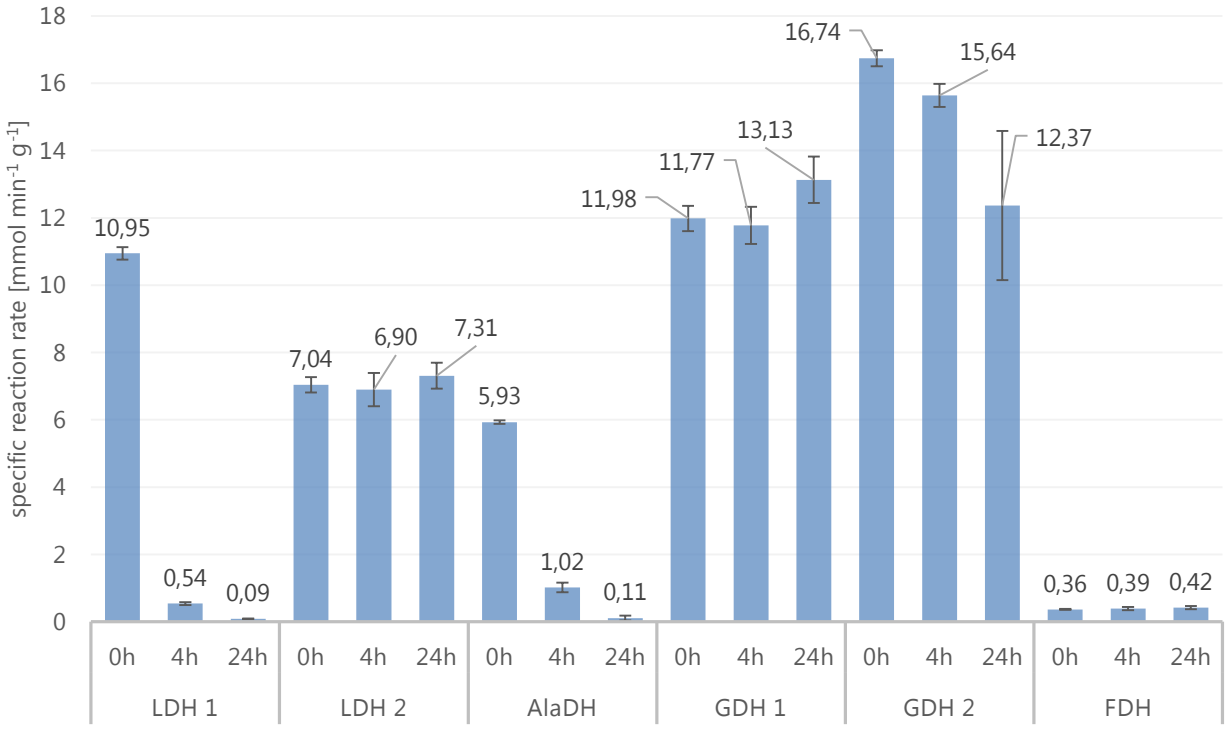


Figure 41 Cascade enzyme stability under corresponding process relevant conditions

Table 12 Results of k_d value and half-time for different cascade enzymes

	LDH 1	LDH 2	AlaDH	GDH 1	GDH 2	FDH
k_d [h^{-1}]	0.98	0	0.44	0	$1.28 \cdot 10^{-2}$	0
$t_{1/2}$ [h]	0.71	-	1.57	-	54.09	-

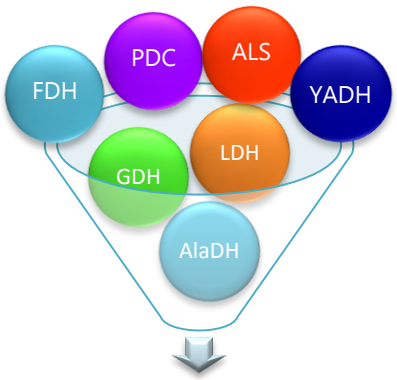
The exclusion criterion was set to a minimum reaction rate corresponding to 80 % of the initial reaction rate. However, in the future development of this method an exclusion criteria will be a function of the economic evaluation and the reaction rates. As shown in the Figure 41 LDH 1 and AlaDH have shown insufficient stability towards the process conditions. Their half-life time of 42 and 94 minutes respectively makes them very unstable and undesirable in a process. GDH 2 with a half-life time of 54 hours is showing a certain degree of instability with remaining activity ≈ 74 % after 24 hours. The last experimental point shows a higher degree of uncertainty meaning that this value could be higher than the exclusion criteria. The experiments with YADH showed no observable activity at time 0 hours, leaving only two possible cascade systems for industrial implementation: LDH 2/GDH 1 and LDH 2/FDH. Those enzymes showed no decrease of stability over the investigated period of time.

3.5 Conclusions

A novel strategy for selection of cascades for shifting the equilibrium of ω-transaminase systems was developed and successfully implemented on eight cascade systems. The selection process was based on three important feasibility considerations: thermodynamics, substrate selectivity and enzyme stability. The order of the different steps was carefully considered with the aim of decreasing the amount of time-consuming lab work. Therefore, calculation steps were scheduled first in the selection process, and experimental steps were put at the end. As shown in Figure 42 the first selection step is based on calculating the thermodynamic ability of the cascades. The exclusion criterion was ≥ 95 % thermodynamic yield and all cascade systems satisfied this criterion. The second step included considerations about substrate selectivity. The first element in these considerations was concerned with the substrate selectivity of the cascades, and it was experimentally confirmed that no observable reaction occurs with the acetophenone as an alternative keto substrate. The second element to be considered was related to the selectivity of ω-transaminase towards the products of the cascades. The aldehyde product of the PDC cascade is a preferable substrate of the ω-transaminase, and based on the structural similarity of the ALS products acetoin, diacetyl and 3-aminobutan-2-one with pyruvate, those side reactions have been identified as highly probable. Such reactions would result in an unfavorable equilibrium shift, the consumption of the main product and a more complicated DSP, and therefore the PDA and ALS cascade systems have been excluded from the selection process. The final selection step was the most laborious and time consuming, and therefore it was important to reduce the number of potentially feasible cascade options in the previous steps. This selection step was based on the enzyme stability under process

relevant conditions where the exclusion criterion was set to 80 % of the initial reaction rate for the duration of the process envisioned here for 24 h. LDH 1, AlaDH, GDH 2 and YADH showed insufficient stability.

Finally, the selection process resulted in two cascade systems as potential candidates for industrial implementation. Every cascade candidate offers certain advantages and disadvantages and to select one over another further investigation is required.



Cascade candidates

Step:	Requirement	Exclusions
1. **Thermodynamics**	Thermodynamic yield of 95 %	None
2. **Substrate selectivity**	Sufficient substrate selectivity	PDC, ALS
3. **Enzyme stability**	Enzyme stability under process relevant conditions	LDH 1, AlaDH, GDH 2 and YADH
Implementation options	LDH 2/GDH 1 ; LDH 2/FDH	

Figure 42 An overview of the selection process of the most promising cascades in ω-transaminase systems

4 Developing Kinetic Models of LDH, GDH and FDH Enzymes to Assist the Selection Process

4.1 Introduction

In the previous chapter, a selection framework was developed and presented, identifying LDH/GDH and LDH/FDH cascade systems as the best candidates for potential industrial implementation. The aim of this chapter is to provide a more detailed description of both cascade systems via development of kinetic models. Such models can then assist the selection process since they allow optimizing the amount of required enzymes. Another goal of kinetic model development is identifying the necessary improvement of ω-transaminase activity to reach desired goal of final product concentration of 50 g L^{-1} within 24 h. The activity of the ω-transaminase required for an industrial process will depend on the efficiency of the pyruvate removal, which is depending on the kinetic parameters of cascade enzymes, the concentrations of cascade enzymes and the concentration of co-factor. The investigation of cascade efficiency and its effect on the required ω-transaminase activity is an important aspect of process development, and defining this correlation can be a crucial part of the decision making process.

4.1.1 Multi-enzyme processes – one pot processes

Traditionally the implementation of enzymatic reactions to pilot or industrial scale has been performed mostly in a single reaction step and following that concept, e.g. a three enzyme process would be carried out in three sequential reactors in series with a single step performed in each reactor. However, due to the high substrate selectivity of enzymes, it is theoretically possible to perform the process studied here in a single reactor. Instead, an approach known as a multi-enzyme one-pot process is a necessity for this type of multi-enzyme processes, and represents an innovative way of carrying out multi-enzymatic reactions.

The implementation of a multi-enzyme one-pot process provides several advantages compared to the traditional one-step one-reaction approach. Provided that conditions of individual reactions (e.g. concentrations of reaction mixture components, temperature, pH) are well matched so that all enzymes can tolerate those conditions it is possible to exploit the cooperation between the enzymes in the one-pot process. The substrate consumed by the first reaction becomes the intermediate product, and then it can be consumed immediately by the second reaction and so on. Furthermore, the required reaction volume is reduced from multiple vessels to one reactor, and moreover the separation and isolation of intermediate products is eliminated[103]. This leads to a simplification of downstream processing and a reduction of operating costs.

4.1.2 Objectives of Model Development

Development of mathematical models is increasingly important in the process of investigating enzymatically catalyzed reactions. A number of parameters influence the reaction rate, e.g. chemical and physical properties of substrates, products and external components at different concentrations, properties and concentrations of the biocatalyst, thermodynamic equilibrium, pH and temperature to name a few.

There are several objectives for developing the kinetic model. One of the objectives is to analyze the dynamic process behavior by simulating the model while varying the input variables. This procedure allows better interpretation of the model, and will result in an improved understanding on how input variables, e.g. substrate concentrations, influence the process outputs. Another objective of such simulations is to provide a good plan for design of experiments when, e.g. the model needs to be validated. Furthermore, it is possible to optimize the process by adjusting the inputs and parameters in the model, e.g. optimal substrate concentrations, temperature, pH or process set-up. To achieve the desired objective, e.g. maximal yields, finding optimal conditions can be done utilizing either statistical or stochastic methods [156]. Finally, well defined and reliable models can be used for prediction of the process output and for process control. All the above-mentioned objectives are valid and applicable for both single and multi-enzyme processes. However, multi-enzyme processes benefit highly from model development due to the complexity of the system to be studied. Development of multi-enzyme kinetic model can be a powerful tool to achieve all of the abovementioned model development objectives.

4.1.3 Enzyme kinetics

4.1.3.1 Michaelis-Menten kinetics

The Michaelis-Menten equation (Equation 4.1) is the fundamental equation of enzyme kinetics and it was first derived for the simplest case of an irreversible enzyme reaction converting a single substrate into a product[157]. The rate of reaction as a function substrate concentration for the Michaelis-Menten kinetics is given in Figure 43.

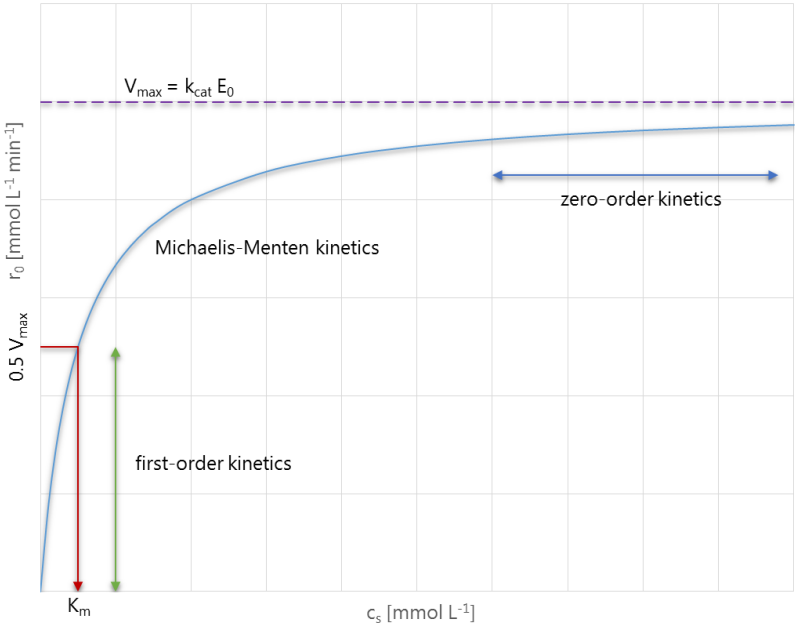


Figure 43 Initial reaction rates, r_0, plotted against increasing substrate concentrations for a reaction obeying Michaelis-Menten kinetics

$$r_S = \frac{V_{max} c_S}{K_m^S + c_S} \tag{4.1}$$

The kinetic model describes a rate of reaction as a function of a single substrate (Equation. 4.2). However, expressions for more complicated e.g. two, three or more substrate reactions have been developed. The two substrate Michaelis-Menten equation is shown in Equation 4.3 and similar equations can be obtained for e.g. three substrate reactions.

$$r_S = \frac{V_{max} c_{S1} c_{S2}}{(K_m^{S1} + c_{S1})(K_m^{S2} + c_{S2})} \tag{4.3}$$

The Equation 4.3 can be expanded further by observing the kinetic mechanism, including for example inhibition expressions caused either by substrates or products. It is quite often the case that enzymatically catalyzed reactions are inhibited by bonding of an undesired molecular species to the active site of the enzyme and thus blocking it. Three types of inhibition may occur: competitive, uncompetitive and noncompetitive inhibition described in Equation 4.4, 4.5 and 4.6 respectively.

$$r_S = \frac{V_{max} c_S}{K_m^S \left(1 + \frac{I}{K_i}\right) + c_S} \tag{4.4}$$

$$r_S = \frac{V_{max} c_S}{K_m^S + c_S \left(1 + \frac{I}{K_i}\right)} \tag{4.5}$$

$$r_S = \frac{V_{max} c_S}{(K_m^S + c_S)\left(1 + \frac{I}{K_i}\right)} \tag{4.6}$$

4.2 Materials and Methods

4.2.1 Materials

The enzymes lactate dehydrogenase (LDH; EC 1.1.1.27), glucose dehydrogenase (GDH; EC 1.1.1.47) and formate dehydrogenase (FDH; EC 1.2.1.2) were obtained from Codexis (Redwood City, CA) in the form of crude extract and all chemicals used in this chapter are stated in Chapter 3.3.1. D-(+)-gluconic acid δ-lactone was obtained from Sigma Aldrich (Brøndby, Denmark).

4.2.2 Activity Measurements

The activity measurements for determining the initial reaction rates in this chapter were based on spectrophotometric methods following either consumption or production of the co-factor NADH at 340 nm at pH 7 and 30°C. The calculation from measured absorbance to NADH concentration was made via a previously prepared calibration curve containing known concentrations of NADH. All enzymes were pre-incubated at 30°C 15 minutes prior to the experiments. The activity measurements were performed in a way that the concentration of the investigated component was varied over the desired range while concentrations of other components were fixed as shown by an example in Table 1. The final reaction medium was adjusted to pH 7 by addition of HCl or KOH solutions. The experiments were performed in disposable cuvettes. However, when acetophenone or (S)-α-methylbenzylamine were used 4 mL Quartz SUPRASIL® Precision cells were used. All experiments were done in triplicate.

Table 13 An example of the experimental recipe with concentrations and volumes in the case of investigating the effect of lactate on the initial reaction rates of the LDH reaction

lactate						
	Concentrations [mmol L^{-1}]					
lactictate	50	100	200	300	400	500
pyruvate	0.5	0.5	0.5	0.5	0.5	0.5
NADH	0.1	0.1	0.1	0.1	0.1	0.1
LDH [g L^{-1}]	0.01	0.01	0.01	0.01	0.01	0.01
Volumes [µL]						
lactate	250	500	1000	1500	2000	2500
pyruvate	500	500	500	500	500	500
NADH	100	100	100	100	100	100
enzyme	25	25	25	25	25	25
water	4125	3875	3375	2875	2375	1875

4.2.3 Model Development

LDH kinetic expression

The model is comprised of kinetic and mass balance equations. The kinetic equation for the LDH enzyme was developed from the two substrate Michaelis-Menten expression described by Equation 4.3, and was expanded by adding competitive inhibition by pyruvate, NAD^+, lactate and alanine observed from the results of initial reaction rate experiments. It was assumed that the reaction is irreversible due to the calculated $K_{eq} = 2.12 \cdot 10^4$, and therefore no expression containing K_{eq} was added. No observable loss in activity over a period of 24 h was observed in the experiments reported in Chapter 3, and therefore no stability expression was introduced in the equation. The final kinetic expression is given by Equation 4.7.

$$r_{LDH} = \frac{V_{max}^{LDH} c_{NADH} c_{PYR} \gamma_{LDH}}{\left(K_m^{NADH}\left(1 + \frac{c_{NAD^+}}{K_i^{NAD^+}}\right) + c_{NADH}\right)\left(K_m^{PYR}\left(1 + \frac{c_{LAC}}{K_i^{LAC}} + \frac{c_{ALA}}{K_{i,LDH}^{ALA}}\right) + c_{PYR} + \frac{c_{PYR}^2}{K_i^{PYR}}\right)} \quad 4.7$$

GDH kinetic expression

The GDH reaction and its simplified sequential reactions can be presented by Figure 44:

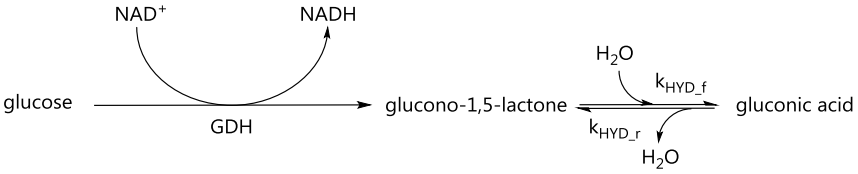


Figure 44 Simplified reaction scheme of GDH and its sequential hydrolysis reactions

GDH, as well as LDH, follows ordered bi bi enzyme kinetics and a kinetic expression can be developed from the two substrate Michaelis-Menten equation expanded to include competitive inhibition by products and external components. The K_{eq} for this reaction has been calculated ($K_{eq}=1.63 \cdot 10^{23}$) as relatively high, and taking into account that the equilibrium is even more facilitated by the sequential hydrolysis reaction, the assumption has been made that this reaction is irreversible. Stability experiments in Chapter 3 also showed no decrease in activity over 24 hours and the final kinetic expression is summarized in Equation 4.8.

$$r_{GDH} = \frac{V_{max}^{GDH} c_{NAD^+}\, c_{GLU} \gamma_{GDH}}{\left(K_{m,GDH}^{NAD^+}\left(1 + \frac{c_{NADH}}{K_{i,GDH}^{NADH}}\right) + c_{NAD^+}\right)\left(K_m^{GLU}\left(1 + \frac{c_{GLUC-1,5-LAC}}{K_i^{GLUC-1,5-LAC}} + \frac{c_{MBA}}{K_{i,GDH}^{MBA}}\right) + c_{GLU}\right)} \quad 4.8$$

The following reaction, the hydrolysis of glucono-1,5-lactone to gluconate, is described as pseudo-first order and it can be described by Equation 4.9. T The pseudo first-order rate constant as a function of pH is given by Equation 4.10 found in the literature[158]:

$$r_{HYD\_f} = k_{HYD\_f} c_{GLUC-1,5-LAC} \quad 4.9$$

$$k_{HYD\_f} = \{[H^+] \cdot 4.7 \cdot 10^{-2} + [OH^-] \cdot 4 \cdot 10^3 + 2.5 \cdot 10^{-4}\} s^{-1} \quad 4.10$$

Hydrolysis of glucono-1,5-lactone is a reversible reaction and the rate constant for the reverse reaction is a function of K_{eq_h} = 5.04, found in the literature[158] and the rate constant of hydrolysis as shown by Equation 4.11:

$$k_{HYD\_r} = \frac{k_{HYD\_f}}{K_{eq\_h}} \quad 4.11$$

Finally, the rate equation for reverse reaction can be summarized by Equation 4.12:

$$r_{HYD\_r} = k_{HYD\_r} c_{GLUC} \quad 4.12$$

FDH kinetic expression

FDH kinetics, similar to GDH and LDH, follow an ordered bi bi mechanism. The reaction has been assumed to be irreversible due to the high calculated K_{eq}=1.31·10[52], and due to the sequential reactions that follow $CO_{2(aq)} \rightarrow CO_{2(g)}$ and $CO_{2(aq)} + H_2O \rightarrow H_2CO_3$. The stability of FDH has been determined by experiments in the previous chapter showing no decrease in activity over 24 h. The final kinetic expression is given by the Equation 4.13.

$$r_{FDH} = \frac{V_{max}^{FDH} c_{NAD^+}\, c_{AMF} \gamma_{FDH}}{\left(K_{m,GDH}^{NAD^+}\left(1 + \frac{c_{NADH}}{K_{i,FDH}^{NADH}}\right) + c_{NAD^+}\right)\left(K_m^{AMF}\left(1 + \frac{c_{ACP}}{K_i^{ACP}}\right) + c_{AMF}\right)} \quad 4.13$$

ω-transaminase kinetic expression

The ω-transaminase follows the ping pong bi bi kinetic mechanism and the equation defining the rate of reaction was taken from the literature[72] and is shown in Equation 4.14.

$$r_{TAm} = \frac{V_{max,f}^{TAm} V_{max,r}^{TAm}\left(c_{ALA} c_{ACP} - \frac{c_{PYR} c_{MBA}}{K_{eq}}\right)\gamma_{TAm}}{V_{max,r}^{TAm} K_m^{ACP} c_{ALA} + V_{max,r}^{TAm} K_m^{ALA} c_{ACP} + \frac{V_{max,f}^{TAm} K_m^{MBA}}{K_{eq}} c_{PYR} + \frac{V_{max,f}^{TAm} K_m^{PYR}}{K_{eq}} c_{MBA} + \cdots} \quad 4.14$$

$$\cdots + V_{max,r}^{TAm} c_{ALA} c_{ACP} + \frac{V_{max,f}^{TAm} K_m^{MBA}}{K_i^{ALA} K_{eq}} c_{ALA} c_{PYR} + \frac{V_{max,f}^{TAm}}{K_{eq}} c_{PYR} c_{MBA} + \frac{V_{max,r}^{TAm} K_m^{ALA}}{K_i^{MBA}} c_{ACP} c_{MBA}$$

Mass balance equations

The ω-transaminase/LDH/GDH model is comprised of the following mass balance equations:

$$\frac{dc_{ALA}}{dt} = -r_{TAm} \quad 4.15$$

$$\frac{dc_{ACP}}{dt} = -r_{TAm} \quad 4.16$$

$$\frac{dc_{PYR}}{dt} = r_{TAm} - r_{LDH} \quad 4.17$$

$$\frac{dc_{MBA}}{dt} = r_{TAm} \quad 4.18$$

$$\frac{dc_{NADH}}{dt} = r_{GDH} - r_{LDH} \quad 4.19$$

$$\frac{dc_{NAD^+}}{dt} = r_{LDH} - r_{GDH} \quad 4.20$$

$$\frac{dc_{LAC}}{dt} = r_{LDH} \quad 4.21$$

$$\frac{dc_{GLU}}{dt} = -r_{GDH} \quad 4.22$$

$$\frac{dc_{GLUC-1,5-LAC}}{dt} = r_{GDH} - r_{HYD\_f} + r_{HYD\_r} \quad 4.23$$

$$\frac{dc_{GLUC}}{dt} = r_{HYD\_f} - r_{HYD\_r} \quad 4.24$$

In the ω-transaminase/LDH/FDH model Equations 4.19, 4.20 and 4.22 are replaced by Equations 4.25, 4.26 and 4.27 respectively. Furthermore, the product formation of FDH (CO_2) is not considered by the kinetic model and Equation 4.23 and 4.24 are therefore excluded from the model.

$$\frac{dc_{NADH}}{dt} = r_{FDH} - r_{LDH} \quad 4.25$$

$$\frac{dc_{NAD^+}}{dt} = r_{LDH} - r_{FDH} \quad 4.26$$

$$\frac{dc_{AMF}}{dt} = -r_{FDH} \quad 4.27$$

4.2.4 Estimation of Kinetic Parameters

4.2.4.1 Linear Regression

The initial estimation of the kinetic parameters was done by plotting Hanes plots (Figure 45 A) of the experimental results ($c_{component}$/reaction rate vs. $c_{component}$) and for the estimation of the inhibition constant of pyruvate a Dixon plot (Figure 45 B) was used ($c_{component}$ vs. 1/reaction rate). The fitting of experimental data was done by the least squares method in Excel. The linear regression plots with experimental data are shown in Appendix 4A, 4B and 4C.

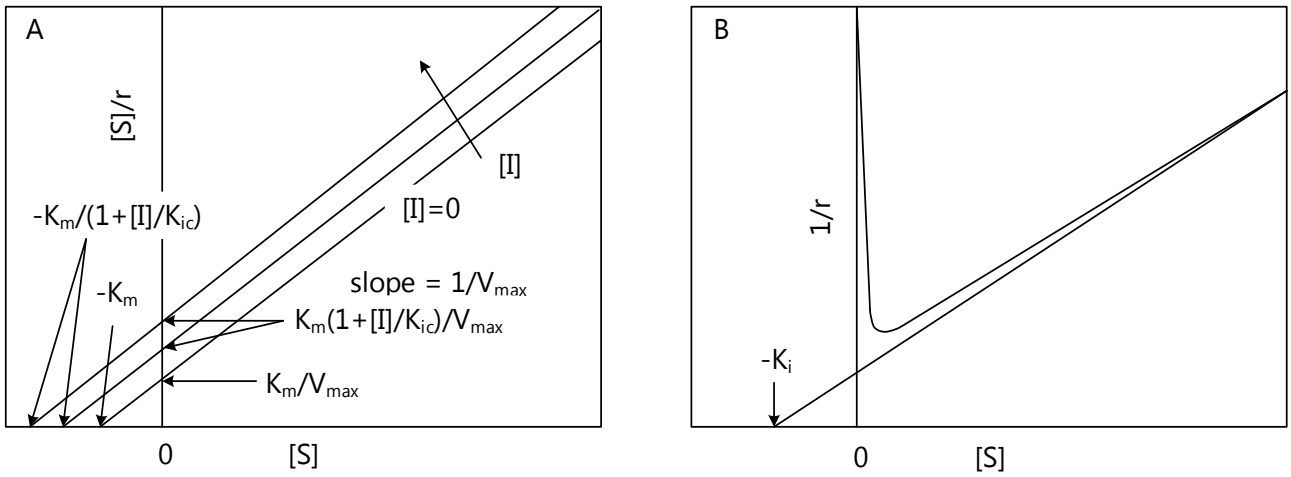


Figure 45 A) Hanes plot for determining kinetic parameters; B) Dixon plot for determining substrate inhibition

4.2.4.2 Nonlinear Regression

The estimated kinetic parameters obtained by linear regression have been used as initial values for a nonlinear regression. A global fit of experimental data to expressions described by Equations 4.7, 4.8 and 4.13 has been made in the OriginPro software package which uses the Levenberg-Marquardt algorithm to adjust parameter values in an iterative model-fitting procedure.

4.2.5 Calculating Required Improvement

Kinetic models of ω-transaminase/LDH/GDH and ω-transaminase/LDH/FDH containing Equations 4.7 - 4.27 have been simulated in Matlab®. Kinetic parameters of cascade enzyme models were estimated by nonlinear regression and kinetic parameters of ω-transaminase were taken from the literature[71]. However, V_{max_r} was a derived parameter calculated by Equation 4.28 known as the Haldane relationship. The simulation of acetophenone feeding was designed to maintain 30 mmol L^{-1} fed concentration until 470 mmol L^{-1} has reacted away. Then the feeding stops and total acetophenone concentration added to the process is 500 mmol L^{-1}The program was set to search for a $V_{max\_f,r}$ value that will result in 95 % conversion for given concentrations of cascade enzymes where $V_{max\_f,r}$ [mmol min^{-1} L^{-1}] represents V_{max_f} [mmol min^{-1} g^{-1}] multiplied by the concentration of ω-transaminase [g L^{-1}]. This procedure was repeated for desired range of concentrations of both cascade enzymes. An example of the program calculating $V_{max\_f,r}$ as a function of cascade enzyme concentration, and representing the ω-transaminase/LDH/GDH model is given in Appendix 4D.

$$V_{\max\_r} = \frac{V_{\max\_f}}{\sqrt{K_{eq}}} \sqrt{\frac{K_m^{PYR} K_m^{MBA}}{K_m^{ALA} K_m^{ACP}}} \qquad 4.28$$

4.3 Results and Discussion

4.3.1 Estimation of kinetic parameters

The initial rate experiments were performed as described in the section 4.2.2 where one investigated component was varied over a concentration range expected in an industrial process while concentrations of other components were fixed. In the industrial pharmaceutical processes buffers are usually not used and therefore the decision was made to adjust the pH by addition of acid or base. Furthermore, the pH change during the reaction is a function of the concentration of pH changing species, and in this particular case the limiting substrate (NADH or NAD^+) was 0.1 mmol L^{-1}. Therefore possible pH changes were disregarded as marginal.

A first estimation of kinetic parameters was done by linear regression and the purpose of this step was to provide initial values of these parameters for later estimation by nonlinear regression which was performed in OriginPro as described in section 4.2.4.2. Results for kinetic parameters estimated by both methods are given in Table 14.

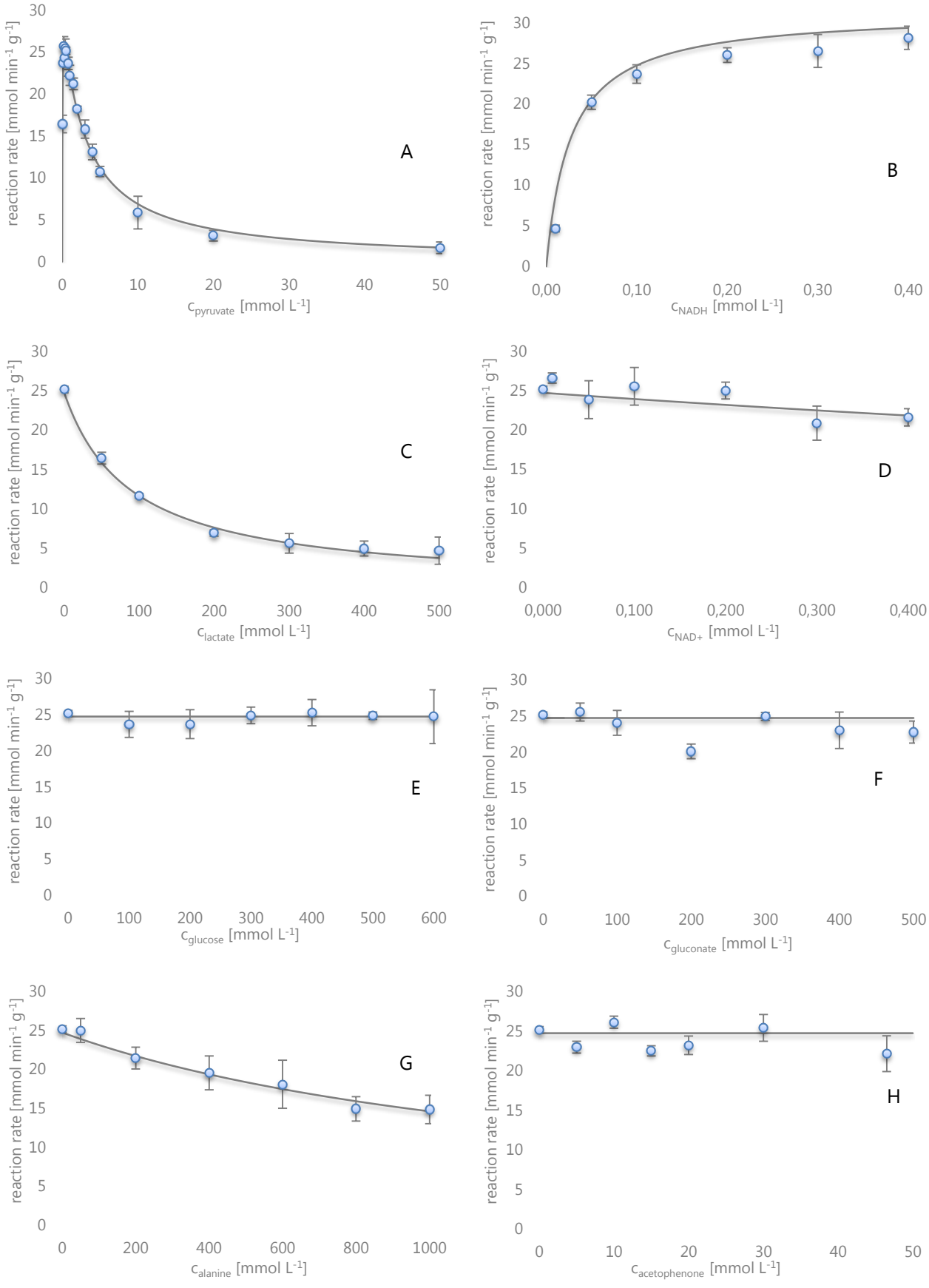
Table 14 Kinetic parameters of LDH, GDH and FDH reaction estimated by linear and nonlinear regression

Parameters	*Units*	*Linear regression*	*Nonlinear regression*
	LDH		
V_{max}^{LDH}	[mmol min^{-1} g^{-1}]	30.6908	39.0251 ± 1.5645
K_m^{NADH}	[mmol L^{-1}]	0.0386	0.0266 ± 0.0041
K_m^{PYR}	[mmol L^{-1}]	0.0203	0.0365 ± 0.0066
K_i^{PYR}	[mmol L^{-1}]	3.1649	2.8728 ± 0.3161
K_i^{LAC}	[mmol L^{-1}]	17.4114	5.1987 ± 0.9669
$K_i^{NAD^+}$	[mmol L^{-1}]	1.4052	0.6288 ± 0.2470
K_i^{ALA}	[mmol L^{-1}]	233.3150	83.9268 ± 15.8071
	GDH		
V_{max}^{GDH}	[mmol min^{-1} g^{-1}]	42.1053	53.4958 ± 1.9571
$K_{m,GDH}^{NAD^+}$	[mmol L^{-1}]	0.1380	0.1453 ± 0.0082
K_m^{GLU}	[mmol L^{-1}]	76.4760	75.7494 ± 9.4221
$K_i^{GLUC-1,5-LAC}$	[mmol L^{-1}]	4.6691	32.2336 ±5.2846
$K_{i,GDH}^{MBA}$	[mmol L^{-1}]	8.1615	13.9023 ± 1.7142
$K_{i,GDH}^{NADH}$	[mmol L^{-1}]	0.0309	0.3443 ± 0.0350
	FDH		
V_{max}^{FDH}	[mmol min^{-1} g^{-1}]	8.8106	15.5813 ± 0.9562
K_m^{AMF}	[mmol L^{-1}]	88.9427	78.6683 ± 7.1699
$K_{m,FDH}^{NAD^+}$	[mmol L^{-1}]	0.0671	0.0812 ± 0.0086
$K_{i,FDH}^{NADH}$	[mmol L^{-1}]	0.9126	0.7795 ± 0.2223
K_i^{ACP}	[mmol L^{-1}]	50.2556	32.9580 ± 5.6376

The kinetic parameters obtained by the nonlinear regression were reported together with the confidence intervals to indicate the reliability of the estimated values. For the LDH model, V_{max} and K_m values were

estimated accurately with very low confidence intervals meaning they can be trusted. However, the inhibition constants showed less reliability, especially K_i^{ALA} (83.9268 ± 15.8071 mmol L^{-1}). Similar behavior in the reliability of estimated data can be noticed for GDH and FDH.

The fitting of the estimated parameters obtained by nonlinear regression for the LDH, GDH and FDH kinetic expressions is shown in Figure 46, Figure 48 and Figure 50 respectively.


A
reaction rate [mmol min-1 g-1]
cpyruvate [mmol L-1]
B
cNADH [mmol L-1]
C
clactate [mmol L-1]
D
cNAD+ [mmol L-1]
E
cglucose [mmol L-1]
F
cgluconate [mmol L-1]
G
calanine [mmol L-1]
H
cacetophenone [mmol L-1]

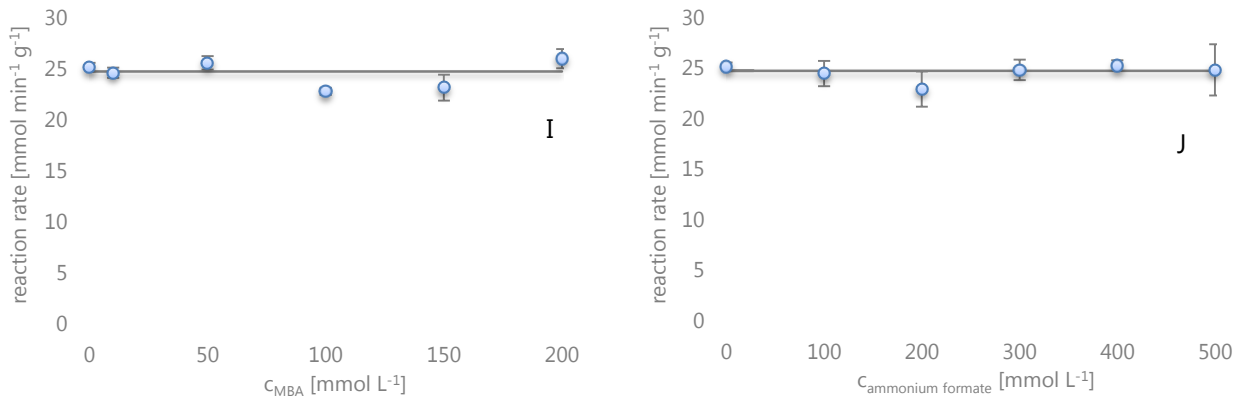


Figure 46 Investigation of the reduction of pyruvate catalyzed by LDH (30°C, pH 7 adjusted by HCl or KOH; γ_{LDH} = 0.01 g L^{-1}, $c_{pyruvate}$ = 0.5 mmol L^{-1} and c_{NADH} = 0.1 mmol L^{-1} if not varied). Initial reaction rates as a function of A) pyruvate, B) NADH, C) lactate, D) NAD^+ E) glucose, F) gluconate, G) alanine, H) acetophenone, I) MBA, J) ammonium formate

From Figure 46 A) it can be seen that LDH is highly inhibited by its substrate pyruvate, however this inhibition poses no challenge for the process. Experiments show that inhibition occurs from a substrate concentration $c_{pyruvate}$ = 0.2 mmol L^{-1} and upwards, but the model simulation shows that the maximum pyruvate concentrations during the reaction are equal to 37.71 µmol L^{-1} as shown in Figure 47. Therefore, the inhibition by substrate does not occur in practice. The conditions for this pyruvate concentration check have been selected to represent the highest accumulation of pyruvate for the process that still achieves 95 % conversion, meaning conditions for a low rate of pyruvate removal (γ_{LDH} = 9 g L^{-1}, γ_{GDH} = 1 g L^{-1}, c_{NADH} = 0.1 mmol L^{-1}) and a high rate of pyruvate production ($V_{max\_f,r}$ = 597.94 mmol min^{-1} L^{-1}). The model and its assumptions are described in section 4.3.2.

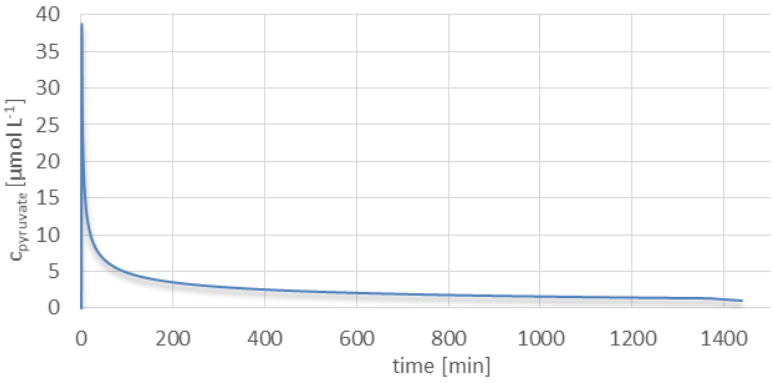
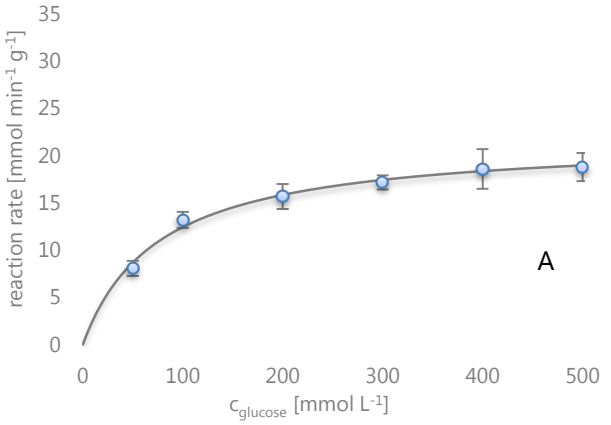
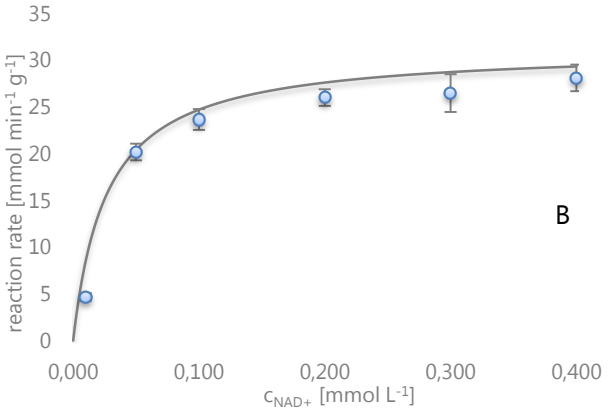
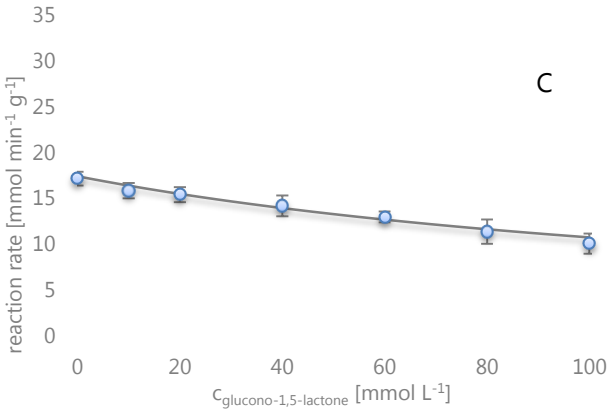


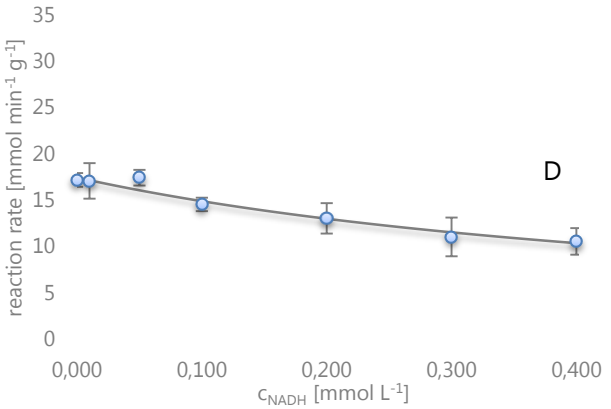
Figure 47 The progress curve of pyruvate simulated by the ω-transaminase/LDH/GDH model in MatLab®; the kinetic parameters are shown in the Table 14 and initial concentrations are the same as in Section 3.3.2.1; $V_{max\_f,r}$ = 597.94 mmol min^{-1} L^{-1}, γ_{LDH} = 9 g L^{-1}, γ_{GDH} = 1 g L^{-1}, c_{NADH} = 0.1 mmol L^{-1}

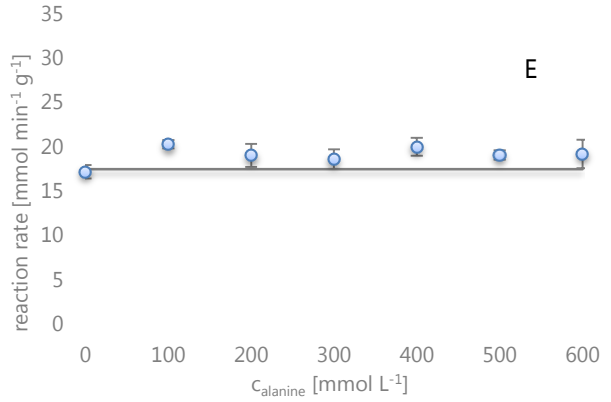
In the Figure 46 C) strong inhibition by the product lactate is observed meaning that the pyruvate removal will be more and more affected when approaching the end of reaction and inhibition by alanine shown in Figure 46 G) shows that pyruvate removal will also be affected at the beginning of the reaction by high alanine concentrations. The inhibition by pyruvate, lactate and alanine can be explained by structural similarities of these molecules which vary only in the keto, hydroxyl and amino group on the α-carbon atom, respectively.

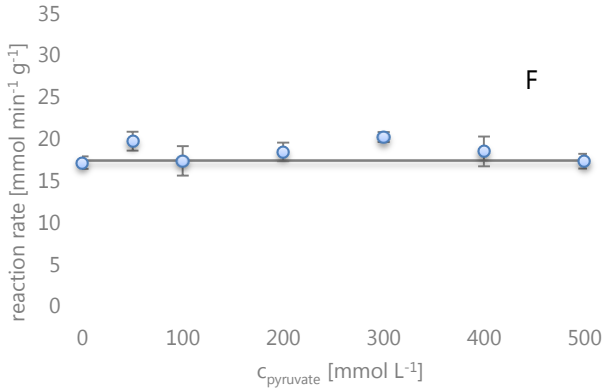

reaction rate [mmol min-1 g-1]
35
30
25
20
15
10
5
0
0
100
200
300
400
500
cglucose [mmol L-1]
A

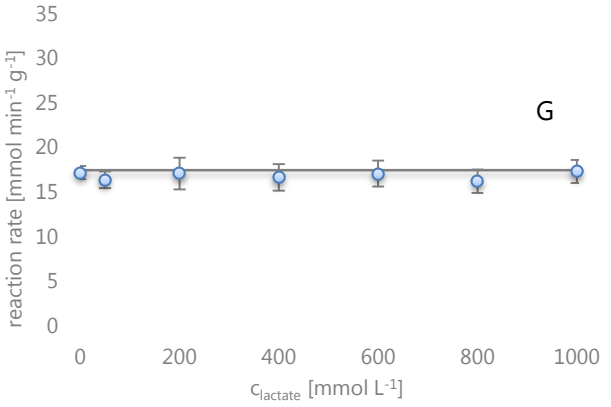

reaction rate [mmol min-1 g-1]
35
30
25
20
15
10
5
0
0,000
0,100
0,200
0,300
0,400
cNAD+ [mmol L-1]
B

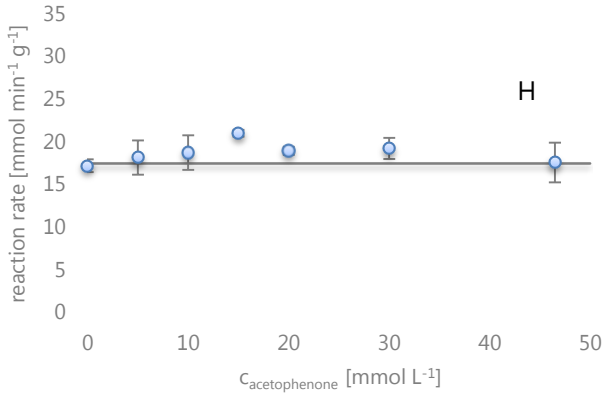

reaction rate [mmol min-1 g-1]
35
30
25
20
15
10
5
0
0
20
40
60
80
100
cglucono-1,5-lactone [mmol L-1]
C


reaction rate [mmol min-1 g-1]
35
30
25
20
15
10
5
0
0,000
0,100
0,200
0,300
0,400
cNADH [mmol L-1]
D


reaction rate [mmol min-1 g-1]
35
30
25
20
15
10
5
0
0
100
200
300
400
500
600
calanine [mmol L-1]
E


reaction rate [mmol min-1 g-1]
35
30
25
20
15
10
5
0
0
100
200
300
400
500
cpyruvate [mmol L-1]
F


reaction rate [mmol min-1 g-1]
35
30
25
20
15
10
5
0
0
200
400
600
800
1000
clactate [mmol L-1]
G


reaction rate [mmol min-1 g-1]
35
30
25
20
15
10
5
0
0
10
20
30
40
50
cacetophenone [mmol L-1]
H

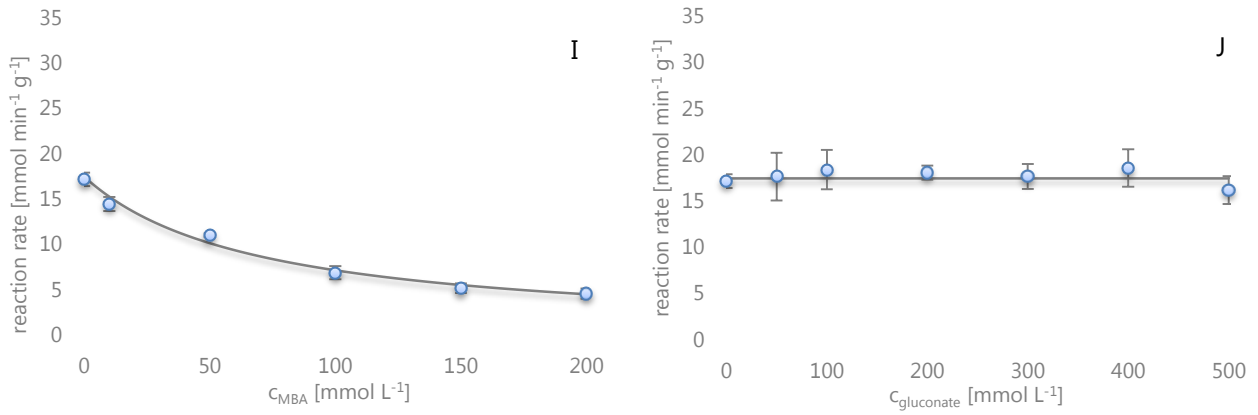


Figure 48 Investigation of the oxidation of glucose catalyzed by GDH (30°C, pH 7 adjusted by HCl or KOH; γ_{GDH} = 0.01 g L^{-1}, $c_{glucose}$ = 300 mmol L^{-1} and c_{NAD+} = 0.1 mmol L^{-1} if not varied). Initial reaction rates as a function of A) glucose, B) NAD^+, C) glucono-1,5-lactone, D) NADH E) alanine, F) pyruvate, G) lactate, H) acetophenone, I) MBA, J) gluconate

The initial rate experiments showed no observable substrate inhibition of the oxidation of glucose by GDH and a slight inhibition by product NADH was observed. It has been found in the literature that the GDH reaction can also be inhibited by glucono-1,5-lactone[127]. To simplify the system the formation of glucono-1,4-lactone has been excluded from this research due to the literature report stating that it forms in very small concentrations [159]. Based on the rates of the forward and backward hydrolysis of glucono-1,5-lactone, kinetic simulations showed that significant concentrations of glucono-1,5-lactone may accumulate in the system (Figure 49) and the decision was made to investigate the influence of this component on the reaction kinetics depicted by Figure 48 C). The inhibition by MBA was also observed as shown by Figure 48 I).

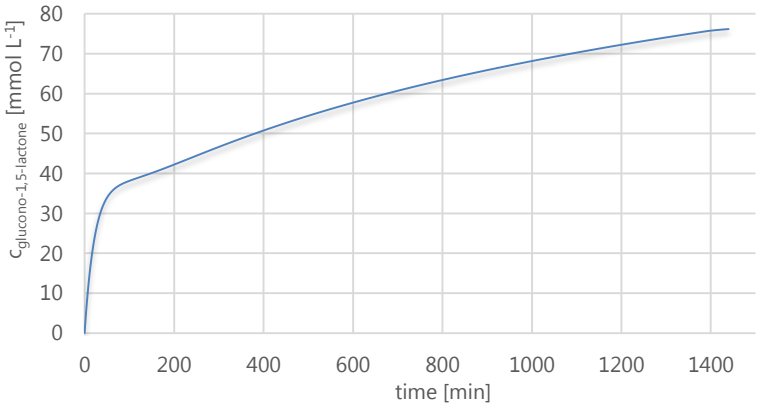


Figure 49 Simulated progress curve of glucono-1,5-lactone with the ω-transaminase/LDH/GDH system: $V_{max\_f,r}$ = 46.31 mmol min^{-1} L^{-1}, γ_{LDH} = 12 g L^{-1}, γ_{GDH} = 1 g L^{-1}, c_{NADH} = 0.1 mmol L^{-1}; initial concentrations same as in the Figure 51

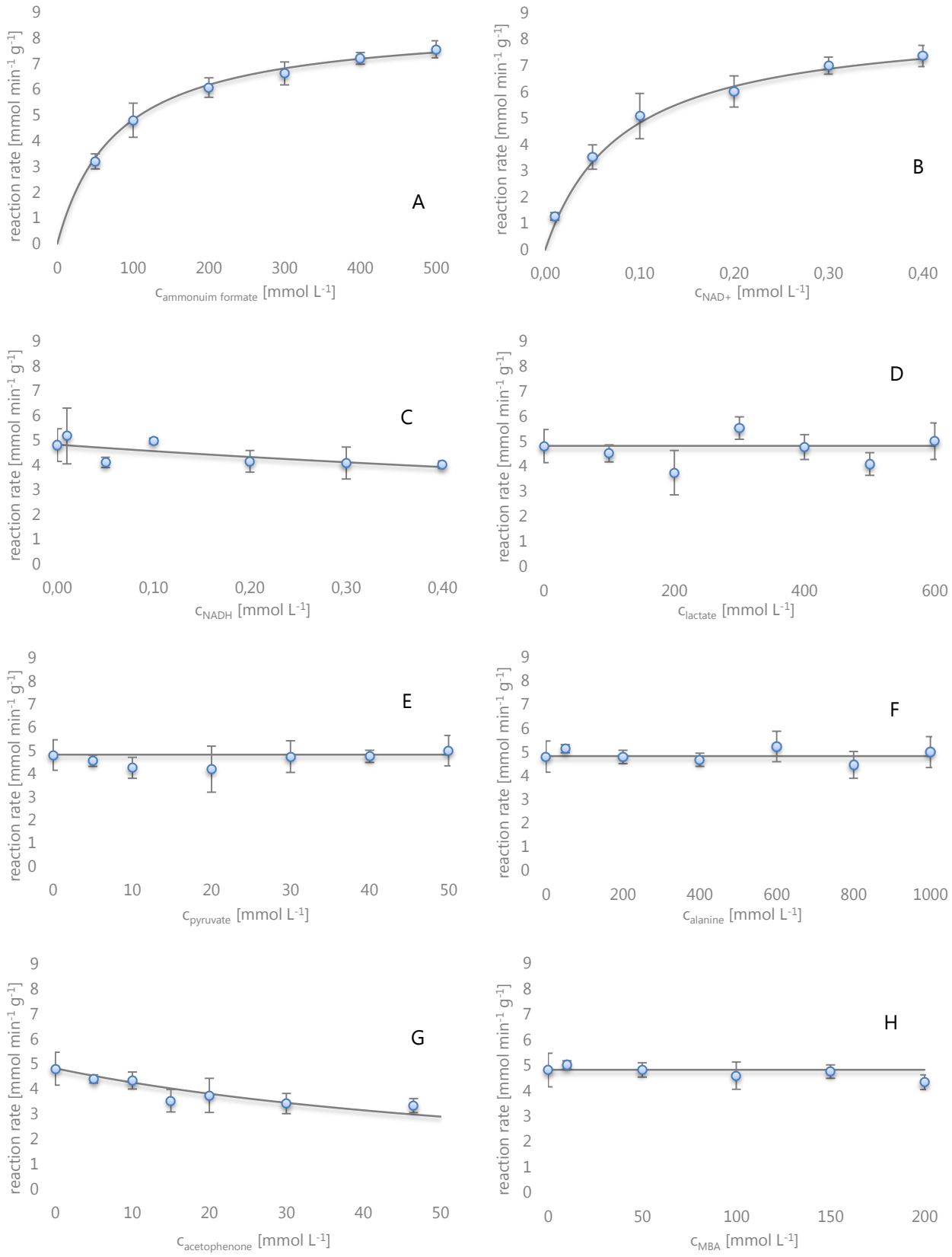


Figure 50 Investigation of oxidation of ammonium formate catalyzed by GDH (30°C, pH 7 adjusted by HCl or KOH; γ_{FDH} = 0.2 g L^{-1}, $c_{ammonium\ formate}$ = 100 mmol L^{-1} and c_{NAD+} = 0.1 mmol L^{-1} if not varied). Initial reaction rates as a function of A) ammonium formate, B) NAD^+, C) NADH, D) lactate E) pyruvate, F) alanine, G) acetophenone, H) MBA

The kinetic investigation of the oxidation of ammonium formate is shown in Figure 50 and a slight inhibition by the product NADH has been observed (Figure 50 C). A slight inhibition by acetophenone has been observed as well, as shown in Figure 50 G). Inhibition by NADH has been reported in many cases of kinetic investigation. However, the inhibition by acetophenone has never been published and structurally the closest occurrences of inhibition were reported in the literature for the *o*-nitrobenzaldehyde[160] and diacetyl[161].

4.3.2 Transaminase of tomorrow

The objective of this chapter is to develop kinetic models describing cascade enzymes and then to use the resulting model to assist the selection process. It is not only essential to estimate the feasibility and efficiency of the cascade enzymes applied to the currently developed ω-transaminase, but it is also important to evaluate the efficiency of cascade enzymes combined with the 'transaminase of tomorrow' – ω-transaminase developed to satisfy industrial requirements to produce 50 g L^{-1} of product. Currently, ω-transaminases are faced by several challenges described in Chapter 2, where product inhibition and activity are the most challenging bottlenecks. Even at the current activity of ω-transaminase and with the equilibrium shift provided by a cascade system, the reaction is usually challenged by product inhibition which in some cases occurs at concentrations as low as about 5 – 10 mmol L^{-1} of MBA. Such product inhibition requires an ISPR method to alleviate it. However, extremely low K_{eq} also requires exclusive selectivity of this ISPR method towards the MBA. Such high selectivity ISPR method has not yet been described or tested. However another possibility for overcoming this challenge might be explored by developing new enzymes via protein engineering or discovering new enzymes from new host organisms. One such example was published by Park and Shin[82] using ω-transaminase from *Ochrobactrum anthropi* which is devoid of both substrate and product inhibition for acetophenone concentrations up to 20 mmol L^{-1} and MBA concentration up to 500 mmol L^{-1}, which covers the majority of the concentration ranges in an industrial process. Such ω-transaminase would then be faced by only two challenges: the thermodynamics of the system which can be facilitated by cascade systems and sufficient enzyme activity to ensure 50 g L^{-1} of product in e.g. 24 h assuming sufficient stability is achieved either via protein engineering or immobilization techniques.

Inspired by these recent developments a 'transaminase of tomorrow' was envisioned to represent an enzyme suited for industrial conditions. The kinetic expression describing the reaction rate following the ping pong bi bi mechanism was taken from the literature (Equation 4.14). Taking into consideration the assumption that the Michaelis constants (K_m) have not been improved by protein engineering from the current state of development, those parameters were taken from the work of Shin and Kim[71]. Based on the previously discussed example, inhibition constants were set to a high number (1000 mmol L^{-1}) simulating no inhibition. V_{max_r} was calculated by Equation 4.28 and kinetic parameters of the 'transaminase of tomorrow' are shown in Table 15.

Table 15 Kinetic parameters of the 'transaminase of tomorrow' required by the kinetic expression described by Equation 4.14

Parameter	*Value*	*Unit*
K_{eq}	$4.03 \cdot 10^{-5}$	[-]
$V_{max\_f}^{TAm}$	Calculated by Matlab program	[mmol min^{-1} g^{-1}]
K_m^{ACP}	0.54	[mmol L^{-1}]
K_m^{ALA}	1.07	[mmol L^{-1}]
K_m^{MBA}	53.03	[mmol L^{-1}]
K_m^{PYR}	9.58	[mmol L^{-1}]
K_i^{ALA}	1000	[mmol L^{-1}]
K_i^{MBA}	1000	[mmol L^{-1}]
$V_{max\_r}^{TAm}$	Eq. 4.28	[mmol min^{-1} g^{-1}]

4.3.2.1 Required ω-transaminase Improvement

To develop a valid industrial process one of the most important questions that has to be answered is: What is the activity of ω-transaminase required for the production of 50 g L^{-1} of optically pure amines? This required activity will depend on the required time period that is available to achieve the goal, e.g. 24 h, and on the kinetic parameters of the ω-transaminase itself. Furthermore, the required activity of ω-transaminase will depend on the kinetic parameters of the cascade enzymes as well as on the amount of added enzymes. Models for ω-transaminase/LDH/GDH and ω-transaminase/LDH/FDH have been developed using equations 4.7 - 4.27. It was attempted to simulate the process at conditions based on the reality. The acetophenone solubility in water (45.8 mmol L^{-1} at 25°C) is the limiting factor for the possible substrate concentrations. It has been also observed in the experiments that high concentrations of alanine (1 mol L^{-1}) decrease the solubility of other components and that at $c_{acetophenone}$ = 40 mmol L^{-1} and $c_{alanine}$ = 1 mol L^{-1} the maximum solubility of acetophenone was reached and a second phase was observed. Due to these observations a feeding strategy was assumed keeping the concentration of acetophenone constant at 30 mmol L^{-1}. The amount of fed acetophenone was limited to the 500 mmol L^{-1}, meaning that the feeding stopped when the total amount of acetophenone in the system reached this concentration (30 mmol L^{-1} dissolved in the reaction mixture and 470 mmol L^{-1} expressed as produced MBA). When the concentration of the product MBA reached 470 mmol L^{-1} the feeding stopped. The feeding mechanism is described by the following reactions:

$$\frac{dc_{\mathrm{ACE}}}{dt} = 0$$

c_{MBA} < 470 mmol L^{-1} $\qquad$ c_{MBA} ≥ 470 mmol L^{-1}

Equation 4.16

For the feeding of pure acetophenone the overall change of volume was ≈ 6 %, and therefore the volume change was neglected in the model simulation. The program described in section 4.2.5 has been developed to

calculate the required activity of ω-transaminase expressed as $V_{max\_f,r}$ as a function of co-product removing enzyme concentration (LDH) and co-factor recycling enzyme concentration (GDH or FDH) for achieving 95 % conversion. The results of the simulations are shown in the Figure 51.

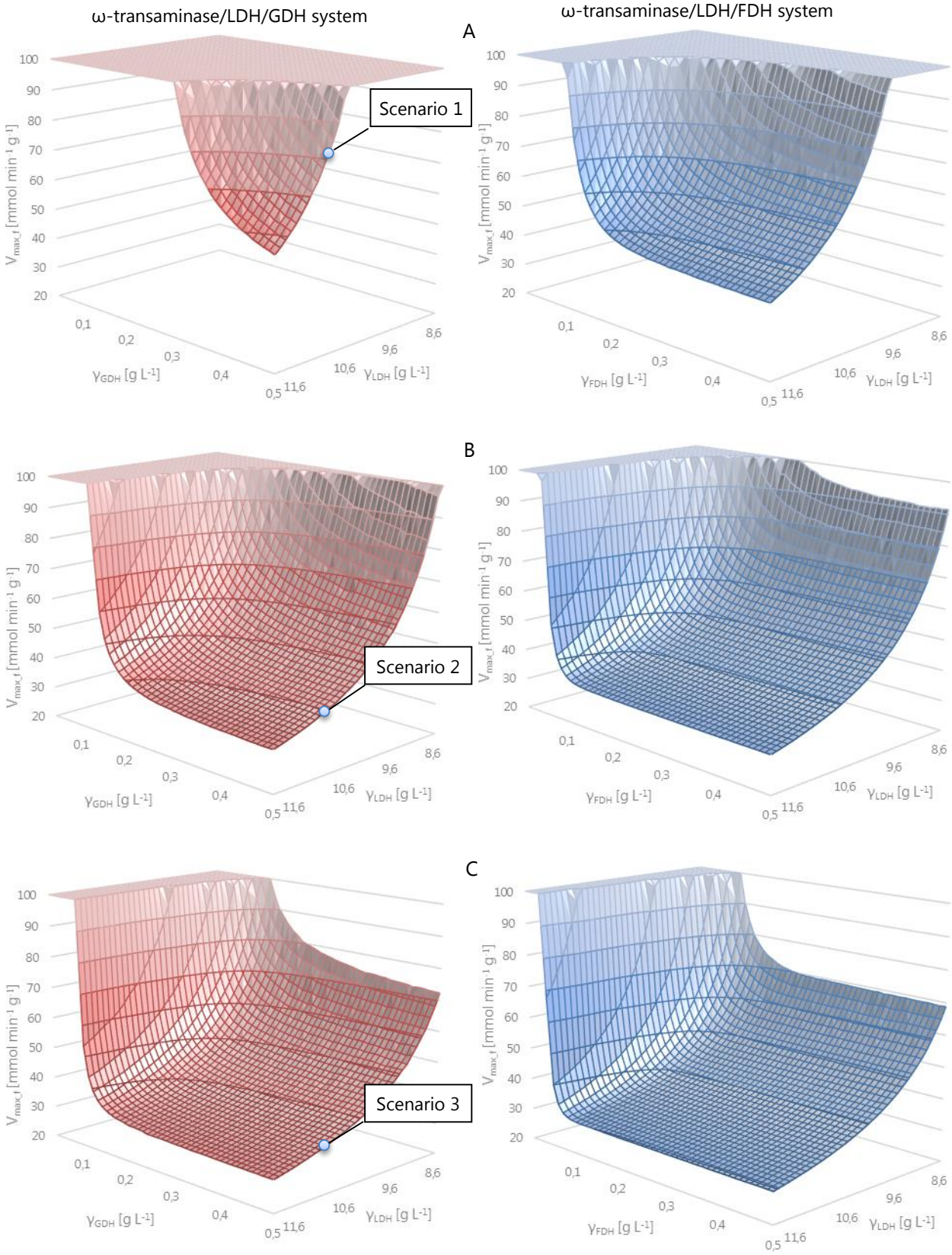


Figure 51 Required $V_{max\_f,r}$ of ω-transaminase for achieving 95 % conversion as a function of LDH/GDH and LDH/FDH concentrations based on initial concentrations: $c_{alanine}$ = 1 mol L^{-1}, c_{ACP} = 500 mmol L^{-1}, (fed at 30 mmol L^{-1}), $c_{pyruvate}$ = 0 mmol L^{-1}, c_{MBA} = 0 mmol L^{-1}, c_{NAD+} = 0 mmol L^{-1}, $c_{lactate}$ = 0 mmol L^{-1}, $c_{glucose}$ = 550 mmol L^{-1}, $c_{glucono\text{-}1,5\text{-}lactate}$ = 0 mmol L^{-1}, $c_{gluconate}$ = 0 mmol L^{-1}, $c_{ammonium\ formate}$ = 550 mmol L^{-1} and A) c_{NADH} = 0.1 mmol L^{-1} B) c_{NADH} = 0.25 mmol L^{-1} C) c_{NADH} = 0.5 mmol L^{-1}

The above presented results show the required ω-transaminase improvement in $V_{max\_f,r}$ [mmol min^{-1} L^{-1}] as a function of the concentrations of added cascade enzymes. As seen from Figure 51 the investigated concentration ranges of cascade enzymes vary for LDH from 8.6 – 12 g L^{-1} and for GDH and FDH from 0.01 – 0.5 g L^{-1}. The $V_{max\_f,r}$ value was investigated up to 100 mmol min^{-1} L^{-1} and this value has been as a reasonable limit for an enzyme development. It was also found that for a small further reduction in cascade enzyme concentrations $V_{max\_f,r}$ values increased exponentially.

The effect of all catalytically active components of a cascade system to the required ω-transaminase activity has to be explored to obtain complete information about system behavior. Therefore the influence of the co-factor concentration was investigated. In the Figure 51 for both cascade options for the case A), B) and C) the co-factor concentration was increasing 0.1, 0.25 and 0.5 mmol L^{-1} respectively. It can be noticed that higher co-factor concentrations reduce required $V_{max\_f,r}$ values for achieving 95 % conversion.

To compare the effect of the concentration of the co-factor between the LDH/GDH and LDH/FDH system a scenarios have been chosen with fixed cascade enzyme concentrations, e.g. for LDH/GDH system LDH (γ_{LDH} = 11 g L^{-1}) and GDH (γ_{GDH} = 0.5 g L^{-1}). For these enzyme concentrations and abovementioned co-factor concentrations (c_{NADH} = 0.1, 0.25 and 0.5 mmol L^{-1}) three scenarios were highlighted shown as Scenario 1, 2 and 3 in the Figure 51. The same was done for the LDH/FDH system and the results as $V_{max\_f,r}$ values at concentrations of LDH (γ_{LDH} = 11 g L^{-1}) and GDH or FDH (γ_{GDH} = γ_{FDH} =0.5 g L^{-1}) ac a function of co-factor concentration are plotted in the Figure 52. Comparing those to cascade systems indicates that the usage of FDH system requires less ω-transaminase improvement to achieve 95 % conversion at previously mentioned conditions. This difference is more expressed at lower co-factor concentrations and decreases at higher concentrations of the co-factor.

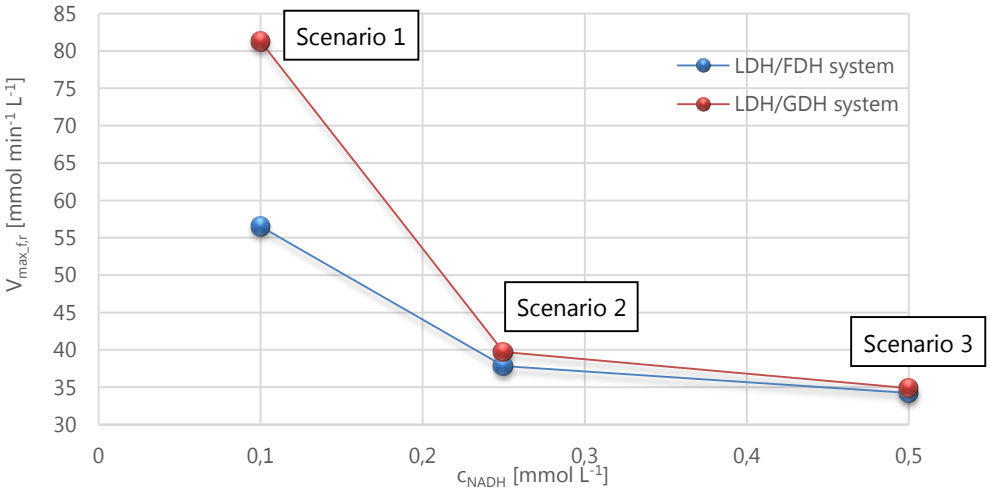


Figure 52 $V_{max\_f,r}$ as a function of added co-factor NADH at cascade enzyme concentrations for LDH/GDH system: γ_{LDH} = 11 g L^{-1} and γ_{GDH} = 0.5 g L^{-1} and LDH/FDH system: γ_{LDH} = 11 g L^{-1} and γ_{FDH} = 0.5 g L^{-1}

4.3.3 Debottlenecking the Process

Observing the results shown in Figure 51 it can be seen that acceptable enzyme improvement can be achieved at relatively low concentrations of GDH or FDH, i.e. 0.1 – 0.4 g L^{-1} depending on the enzyme used and the co-factor concentrations. However, in both cases the concentration of LDH is considerably higher ranging from 9 – 12 g L^{-1} which is a concentration that is more than one order of magnitude higher than for the co-factor recycling enzymes. This discrepancy in enzyme concentrations may hint towards the fact that the bottleneck of the process is the reaction catalyzed by LDH. Observing the kinetic behavior of LDH shown in Figure 46, inhibitions by pyruvate, lactate and alanine have been observed. It has been concluded in the previous discussion that inhibition by pyruvate does not occur under the investigated conditions, and therefore the inhibition by alanine and lactate must be responsible for the high concentrations of LDH required for achieving the industrial target. In order to observe the effects of those inhibition effects a slice from Figure 51 A) at γ_{GDH} = 0.5 g L^{-1} has been selected. The slice taken from Figure 51 is represented with the blue line showing the required $V_{max\_f,r}$ at current inhibition levels of LDH. During the reaction lactate is accumulated in the reaction media and thus more and more inhibiting the removal of pyruvate when approaching the end of reaction. To overcome the increasing inhibition and achieve functional pyruvate removal, high LDH concentrations are required. When the inhibition by lactate was excluded from the model, a huge improvement in the reduced LDH concentration was observed (red line). Inhibition by alanine is not so severe and the effect on the rate of pyruvate removal is decreasing over time as the concentration of alanine is decreasing. When inhibition by alanine was removed from the model, a slight improvement (green line) was observed. The enzyme that would benefit from the lack of both inhibitions showed the largest improvement both in concentration of LDH and $V_{max\_f,r}$ required for achieving the industrial target (purple line).

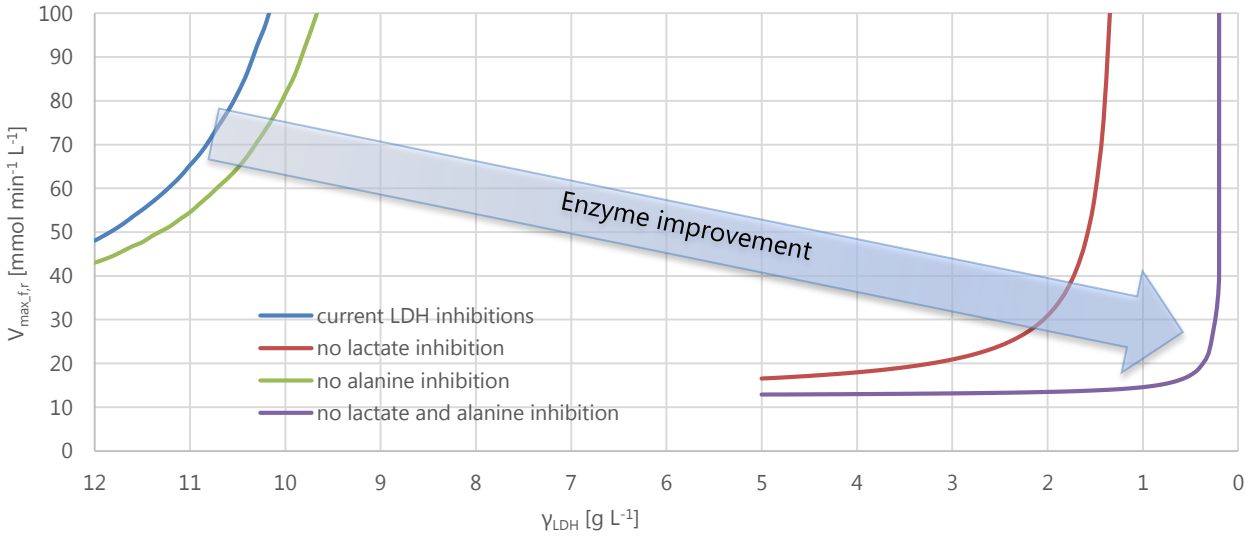


Figure 53 Reducing the LDH concentration required for achieving the industrial target (50 g product L^{-1} in 24 h) by removing the inhibition by alanine and/or lactate; γ_{GDH} = 0.5 g L^{-1} and c_{NADH} = 0.1 mmol L^{-1}

4.4 Conclusions

The goal of this chapter was to prove a set of tools to assist a cascade selection process via model development. By developing a model, a comparison can be made between viable cascade options for industrial implementation (LDH/GDH and LDH/FDH), e.g. based on the optimized concentrations of individual enzymes. The current state of ω-transaminase was not taken into consideration in model development due to the strong substrate and product inhibition, as well as the low activity. However, a 'transaminase of tomorrow' devoid of all inhibitions was envisioned as a possible industrially applicable enzyme. The required activity of the 'transaminase of tomorrow', expressed as $V_{max\_f,r}$ [mmol min^{-1} L^{-1}] was calculated as a function of cascade enzyme concentrations, demonstrating how much of activity improvement is required to achieve 50 g L^{-1} of product within 24 h. Simulations have shown that the improved activity is a function of how well the cascade system removes the co-product pyruvate. This ability to remove pyruvate was evaluated here via three parameters: the concentration of pyruvate removing enzyme (LDH), the concentration of co-factor recycling enzyme (GDH or FDH) and the concentration of the co-factor. Significant improvements in reducing the LDH and GDH/FDH concentration required to achieve 95 % conversion in 24 h while having $V_{max\_f,r}$ < 100 mmol min^{-1} L^{-1} were achieved by adding more co-factor. However, the final concentration of the co-factor will depend on the economic analysis considering the cost of co-factor vs. the cost of ω-transaminase improvement and the cost of the cascade enzymes.

Another outcome of the model development was that the model allowed identifying current bottlenecks of the process, which in this case were the unreasonably high LDH concentrations required for the conditions corresponding to an industrial process. Further investigation revealed that inhibition caused by alanine and lactate requires high concentrations of LDH for the establishment of a successful process. Alleviating those inhibitions resulted in a huge decrease in LDH concentrations required for an industrial process. An important conclusion that can be drawn from investigating the model simulation results is that cascade enzymes must be devoid of inhibitions mainly caused by cascade products. As the reaction progresses, the accumulation of cascade products will reduce the activity and as a consequence significant amounts of that enzyme will be required to achieve the desired product concentrations. Therefore, the 'cascade enzymes of tomorrow' must also be devoid of all inhibitions, especially by cascade products or other components whose concentration builds up over the duration of the process.

Based on the calculated GDH and FDH concentration required for successful industrial implementation the results vary at low and high co-factor concentrations. At low NADH concentrations the results clearly favor FDH as a choice of co-factor recycling. At higher NADH concentrations the differences between GDH and FDH concentrations are becoming smaller. However, taking into account the high price of the co-factor and the

cost of ω-transaminase improvement, the process optimization will favor processes aiming towards lower co-factor concentrations where FDH shows significant benefits over GDH. As described in the previous paragraph, this result can be partially explained by inhibition of GDH caused by glucono-1,5-lactone and also by MBA (accumulating component) while FDH is devoid of such inhibitions. Due to the lower requirements for ω-transaminase improvement in the presence of lower concentrations of NADH, the LDH/FDH system has been chosen as a best candidate for a potential industrial implementation.

5 Industrial Perspectives and Process Strategies for Implementation of ω-transaminase/LDH/FDH System

5.1 Introduction

Developing an industrial process is a complex and challenging procedure involving many features defined by many non-technical aspects, e.g. market, supply chain, safety, patents and regulatory aspects to name a few. The resulting overall process has to satisfy also economic, environmental and social impacts[162]. In the case of production of optically pure amines by ω-transaminase, this enzymatic process is usually not a standalone process but rather one step in the synthesis of valuable pharmaceutical products. This means that the ω-transaminase process has to be integrated into an existing or a new synthesis route as shown in Figure 54.

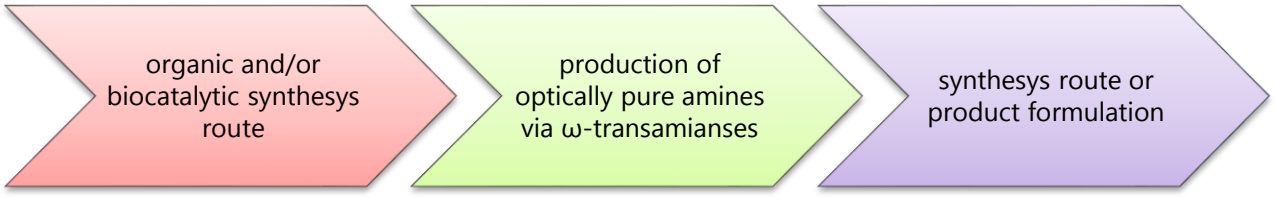


Figure 54 the integration of the ω-transaminase process in the overall synthesis route for API production

Process System Engineering (PSE) tools and methods will play a core role in the development of an industrial process for production of optically pure amines by ω-transaminase, where the resulting solution for each process is strongly case dependent. Several major influences will dominate the process design and will have to be continuously reevaluated. As shown in Figure 55 the framework for process design of this particular process is focused on several important considerations. Physical and chemical properties of chemicals will e.g. determine feeding strategies for low soluble substrates or determine the type of DSP. The thermodynamic calculation will indicate the applicability of different equilibrium shifting strategies; however the viability implementation strategies have to be tackled via process design. To ensure optimal conditions for successful biocatalytic conversions, e.g. optimal temperature, pH, substrate and product concentrations a Process Analytical Technology (PAT) strategy, including suitable control and monitoring tools, will have to be applied. Finally, the biocatalyst performance will affect the potential biocatalyst formulation options, and therefore the

process implementation. Process intensification via e.g. ISPR may also reduce the required biocatalyst performance, which is important to take into account during process design.

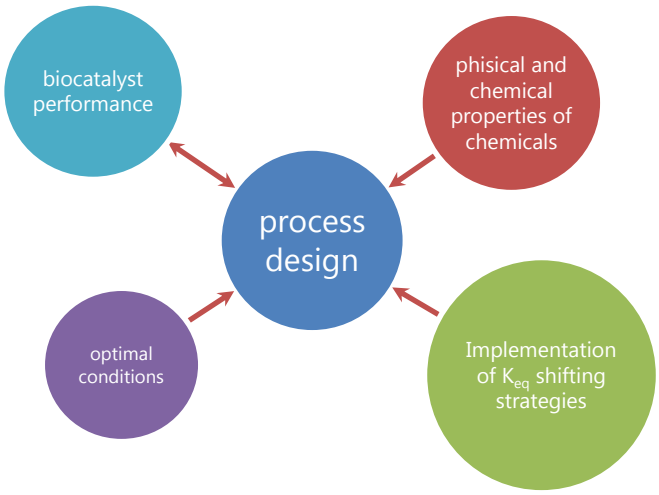


Figure 55 Considerations for development of an industrial process for production of optically pure amines, specifically for thermodynamically challenged cases utilizing cascade systems

There are several processes for production of optically pure chiral amines via ω-transaminases mentioned in the literature, and one of the most cited ones is the example of successful Sitagliptin production by Savile et al.[68]. However processes severely challenged by unfavorable equilibrium are not at all mentioned in the literature, and the same goes for processes utilizing cascade systems to overcome those challenges. It is therefore the objective of this chapter to discuss possible process solutions for such thermodynamically challenged processes. The potential process setup options are compared and a selection is made based on previous work, literature suggestions and in some cases supported by simulation of the previously developed models (Section 4.2.3).

5.2 Materials and Methods

5.2.1 Two Pot Strategy

A kinetic model described in section 4.2.3 was implemented, and mass balance equations were modified to describe a two pot system. Each individual reactor was considered as a constant volume CSTR connected in a loop as shown in Figure 56 and mass balance equations of all components were established for both reactors. A general mass balance equation for component A in a reactor can be expressed by Equations 5.1 and 5.2:

$$\frac{dn_A}{dt} = F_{inlet}c_{A,inlet} - F_{outlet}c_{A,outlet} + r_A V \quad 5.1$$

$$\frac{dc_A}{dt} = \frac{c_{A,inlet}}{\tau} - \frac{c_{A,outlet}}{\tau} + r_A \quad 5.2$$

The volume ratio was defined as shown in Equation 5.3 where V_1 is the working volume of the ω-transaminase reactor, and V_2 is the working volume of the external cascade reactor. V_1 is fixed at 1 dm^3 while V_r is varied over the investigated range.

$$V_r = \frac{V_1}{V_2} \quad 5.3$$

Mass balance equations used in the model are shown below:

ω-transaminase reactor	External cascade reactor	
$\tau_1 = \frac{V_1}{F}$	$\tau_2 = \frac{V_2}{F}$	5.4
$\frac{dC_{ALA,1}}{dt} = \frac{c_{ALA,2}}{\tau_1} - \frac{c_{ALA,1}}{\tau_1} - r_{TAm}$	$\frac{dC_{ALA,2}}{dt} = \frac{c_{ALA,1}}{\tau_2} - \frac{c_{ALA,2}}{\tau_2}$	5.5
$\frac{dc_{ACP,1}}{dt} = \frac{c_{ACP,2}}{\tau_1} - \frac{c_{ACP,1}}{\tau_1}$ [1]	$\frac{dc_{ACP,2}}{dt} = \frac{c_{ACP,1}}{\tau_2} - \frac{c_{ACP,2}}{\tau_2}$	5.6
$\frac{dc_{ACP,1}}{dt} = \frac{c_{ACP,2}}{\tau_1} - \frac{c_{ACP,1}}{\tau_1} - r_{TAm}$ [2]		
$\frac{dc_{PYR,1}}{dt} = \frac{c_{PYR,2}}{\tau_1} - \frac{c_{PYR,1}}{\tau_1} + r_{TAm}$	$\frac{dc_{PYR,2}}{dt} = \frac{c_{PYR,1}}{\tau_2} - \frac{c_{PYR,2}}{\tau_2} - r_{LDH}$	5.7
$\frac{dc_{MBA,1}}{dt} = \frac{c_{MBA,2}}{\tau_1} - \frac{c_{MBA,1}}{\tau_1} + r_{TAm}$	$\frac{dc_{MBA,2}}{dt} = \frac{c_{MBA,1}}{\tau_2} - \frac{c_{MBA,2}}{\tau_2}$	5.8
$\frac{dc_{NADH,1}}{dt} = \frac{c_{NADH,2}}{\tau_1} - \frac{c_{NADH,1}}{\tau_1}$	$\frac{dc_{NADH,2}}{dt} = \frac{c_{NADH,1}}{\tau_2} - \frac{c_{NADH,2}}{\tau_2} - r_{LDH} + r_{FDH}$	5.9
$\frac{dc_{NAD^+,1}}{dt} = \frac{c_{NAD^+,2}}{\tau_1} - \frac{c_{NAD^+,1}}{\tau_1}$	$\frac{dc_{NAD^+,2}}{dt} = \frac{c_{NAD^+,1}}{\tau_2} - \frac{c_{NAD^+,2}}{\tau_2} + r_{LDH} - r_{FDH}$	5.10
$\frac{dc_{LAC,1}}{dt} = \frac{c_{LAC,2}}{\tau_1} - \frac{c_{LAC,1}}{\tau_1}$	$\frac{dc_{LAC,2}}{dt} = \frac{c_{LAC,1}}{\tau_2} - \frac{c_{LAC,2}}{\tau_2} + r_{LDH}$	5.11
$\frac{dc_{AMF,1}}{dt} = \frac{c_{AMF,2}}{\tau_1} - \frac{c_{AMF,1}}{\tau_1}$	$\frac{dc_{AMF,2}}{dt} = \frac{c_{AMF,1}}{\tau_2} - \frac{c_{AMF,2}}{\tau_2} - r_{FDH}$	5.12

[1] for $c_{MBA,1} < 470$ mmol L^{-1}

[2] for $c_{MBA,1} \geq 470$ mmol L^{-1}

Reaction rates r_{LDH}, r_{FDH} and r_{Tam} are described by Equations 4.7, 4.13 and 4.14 respectively. The model assumes perfect mixing of both reactors, acetophenone feeding strategy described in Section 4.2.5 and the length of the connecting pipes is not taken into consideration. Initial concentrations were set: c_{ACP} = 30 mmol L^{-1}, $c_{alanine}$ = 1 mol L^{-1}, $c_{pyruvate}$ = 0 mmol L^{-1}, c_{MBA} = 0 mmol L^{-1}, $c_{lactate}$ = 0 mmol L^{-1}, c_{NADH} = 0.1 mmol L^{-1}, c_{NAD+} = 0 mmol L^{-1}, $c_{ammonium\ formate}$ = 550 mmol L^{-1}. Enzyme concentrations were set to achieve 95 % conversion for the above-mentioned conditions: $V_{max\_f,r}$ = 55.33 mmol min^{-1} L-1, γ_{LDH} = 11 g L^{-1} and γ_{FDH} = 0.3 g L^{-1}.

5.2.2 Stability of the Co-factor

Stability of the co-factor NADH has been investigated under conditions mentioned as reaction mixture 1 described in the section 3.3.3.1 and for 100 mmol L^{-1} concentrations of pH 7 buffers (phosphate buffer, Tris, Hepes and wide range buffer[163]) while the initial NADH concentration was 0.5 mmol L^{-1}. The experiment was performed at pH 7 and 30°C by following the adsorption of NADH at 340 nm in 4 mL Quartz SUPRASIL® Precision cells in a Shimadzu UV-1801 Spectrophotometer. The experiments were done in triplicate. The obtained values were fitted to the second-order decay kinetics described by Equation 5.13 with the OriginPro program which uses the Levenberg-Marquardt algorithm to adjust parameter values in the iterative parameter estimation / curve fitting procedure.

$$c_t = \frac{c_0}{1 + c_0 k_d t} \qquad 5.13$$

c_t – concentration at time t; c_0 – initial concentration; k_d – deactivation coefficient; t – time

5.2.3 Downstream Processing

The charge analysis was performed and plotted in the Excel CurTiPot program (pK_a database and distribution diagram generation).

The model developed in section 5.2.1 was modified to simulate the external ISPR unit. All kinetic expressions were previously developed and explained in the section 4.2.3 and the mass balances were modified in such a way that all enzymes and co-factors are present in UFMR (UltraFiltration Membrane Reactor) where all biocatalytic reactions take place. The external ISPR unit is modelled in such a way that 50 % of the inlet MBA concentration is removed and the corresponding mass balance equation 5.2 becomes now:

$$\frac{dc_{MBA}}{dt} = \frac{c_{MBA,inlet}}{\tau} - \frac{c_{MBA,outlet}}{\tau} - 0.5\frac{c_{MBA,inlet}}{\tau} = 0.5\frac{c_{MBA,inlet}}{\tau} - \frac{c_{MBA,outlet}}{\tau} \qquad 5.14$$

Mass balance equations for the model are shown below:

UFMR	External ISPR unit	
$\tau_1 = \frac{V_1}{F}$	$\tau_2 = \frac{V_2}{F}$	5.15
$\frac{dC_{ALA,1}}{dt} = \frac{c_{ALA,2}}{\tau_1} - \frac{c_{ALA,1}}{\tau_1} - r_{TAm}$	$\frac{dC_{ALA,2}}{dt} = \frac{c_{ALA,1}}{\tau_2} - \frac{c_{ALA,2}}{\tau_2}$	5.16
$\frac{dc_{ACP,1}}{dt} = \frac{c_{ACP,2}}{\tau_1} - \frac{c_{ACP,1}}{\tau_1}$ [1] $\frac{dc_{ACP,1}}{dt} = \frac{c_{ACP,2}}{\tau_1} - \frac{c_{ACP,1}}{\tau_1} - r_{TAm}$ [2]	$\frac{dc_{ACP,2}}{dt} = \frac{c_{ACP,1}}{\tau_2} - \frac{c_{ACP,2}}{\tau_2}$	5.17
$\frac{dc_{PYR,1}}{dt} = \frac{c_{PYR,2}}{\tau_1} - \frac{c_{PYR,1}}{\tau_1} + r_{TAm} - r_{LDH}$	$\frac{dc_{PYR,2}}{dt} = \frac{c_{PYR,1}}{\tau_2} - \frac{c_{PYR,2}}{\tau_2}$	5.18
$\frac{dc_{MBA,1}}{dt} = \frac{c_{MBA,2}}{\tau_1} - \frac{c_{MBA,1}}{\tau_1} + r_{TAm}$	$\frac{dc_{MBA,2}}{dt} = 0.5\frac{c_{MBA,1}}{\tau_2} - \frac{c_{MBA,2}}{\tau_2}$	5.19
$\frac{dc_{NADH,1}}{dt} = -r_{LDH} + r_{FDH}$	Not present in ISPR unit	5.20
$\frac{dc_{NAD^+,1}}{dt} = r_{LDH} - r_{FDH}$	Not present in ISPR unit	5.21
$\frac{dc_{LAC,1}}{dt} = \frac{c_{LAC,2}}{\tau_1} - \frac{c_{LAC,1}}{\tau_1} + r_{LDH}$	$\frac{dc_{LAC,2}}{dt} = \frac{c_{LAC,1}}{\tau_2} - \frac{c_{LAC,2}}{\tau_2}$	5.22
$\frac{dc_{AMF,1}}{dt} = \frac{c_{AMF,2}}{\tau_1} - \frac{c_{AMF,1}}{\tau_1} - r_{FDH}$	$\frac{dc_{AMF,2}}{dt} = \frac{c_{AMF,1}}{\tau_2} - \frac{c_{AMF,2}}{\tau_2}$	5.23

[1] for $c_{MBA,1}$ < 470 mmol L^{-1}

[2] for $c_{MBA,1}$ ≥ 470 mmol L^{-1}

Initial concentrations of components are the same as in the section 5.2.1, as well as the enzyme concentrations. The volume of the reactor was 1 L and the volume of the ISPR unit was 0.2 L while the flow was 0.1 L min^{-1}. $V_{max\_f,r}$ was in the range 15 – 55 mmol min^{-1} L^{-1} and for each value the corresponding K_i^{MBA} was determined such that the conversion reached 95 %. Perfect mixing was assumed in the UFMR and the length of connecting pipes was disregarded.

5.3 Results and Discussion

5.3.1 Type of Process: Batch or Continuous?

Indeed, one of the most important decisions while designing a process is to decide whether the process should be run in batch or continuous mode. The use of decision making tools for this particular problem has been described in the literature; however the authors of that study focused on lab scale processes [164]. For an industrial scale case, a more detailed approach is required including reuse of all possible process streams, e.g. recycling of nonconverted substrate, recycling of possible solvents, heat integration, monitoring, control and automation and complete economic and environmental assessment.

Traditionally, the use of batch and fed-batch processes in the pharmaceutical industry has been predominant despite the higher costs[165]. However, a continuous mode of operation has been emerging within the pharmaceutical industry as a new field with benefits including a significant reduction in cost, energy consumption, use of solvents and leading to the production of purer products to name a few[165, 166]. Taking into consideration recent trends and developments in continuous processing using e.g. microreactors[167, 168], microseparators[169, 170] and continuous product formulation[165, 171, 172] the change of established batch processes into continuous processes is slowly reshaping the pharmaceutical industry. However, switching from batch to continuous mode of operation is not applicable to all pharmaceutical processes. This decision will largely depend on the implementation of continuous processing throughout the stages of drug production as well as supply and demand of the final product.

For the investigated case of production of MBA using the ω-transaminase/LDH/FDH system the choice of batch or continuous favors the batch, and more precisely the fed batch mode of operation. This choice is mainly justified by the fact that his type of reactions is still a 1st generation process, meaning that the industrial implementation has not been established, and transaminases are still under development. As the ω-transaminase with required activity has not been engineered yet (see section 4.3.20), developing a processes might require an even longer reaction time than previously assumed (24 h) making a batch process an excellent choice due to its suitability for slowprocesses[173].

Temperature and pH monitoring and control are both required for this process. A suitable pH regulation can be achieved with a reliable pH meter and the addition of acid or base while a temperature regulation can be achieved via a standard thermostat. In both cases efficient mixing is required to avoid temperature and pH gradients. Due to the low solubility of the substrate acetophenone (45.8 mmol L^{-1} at 25°C) a suitable feeding strategy must be devised, and therefore the fed batch mode is considered as a viable option. In this work simulations were performed assuming the continuous presence of an acetophenone concentration of 30 mmol

L^{-1}, meaning that the acetophenone dosing compensated for consumption by the reaction. The use of analytical methods for acetophenone detection is unfortunately limited to at-line HPLC. Taking into consideration the residence time of the HPLC method, this type of process monitoring and regulation might have a slow response. Another option are model-driven soft sensors which are based on mass balances, however they have to be developed specifically for a given case and often result in a long development period [174]. However, the effort and time required to develop a suitable soft sensor might be acceptable, especially when considering how long time the overall ω-transaminase/LDH/FDH process development will take.

5.3.2 One Pot vs. Two Pot Process

One of the challenges facing the multi-enzyme processes can be the different operational stability of individual enzymes. In the particular case of the production of optically pure amines by ω-transaminases where the reaction is thermodynamically challenged and equilibrium shift is achieved by cascade enzymes, it could be a favorable operating strategy to separate, e.g. via two-pot process, enzymes with different stabilities. In this case it is possible to selectively remove unstable enzyme (e.g. ω-transaminases) from the stable enzymes (cascade enzymes). Applying this operating strategy could result in prolonging the reusability of stable enzymes while more unstable enzymes are replaced after the activity has dropped. One such example is external co-product removal shown in the Figure 56 B) while Figure 56 A) shows a one pot system.

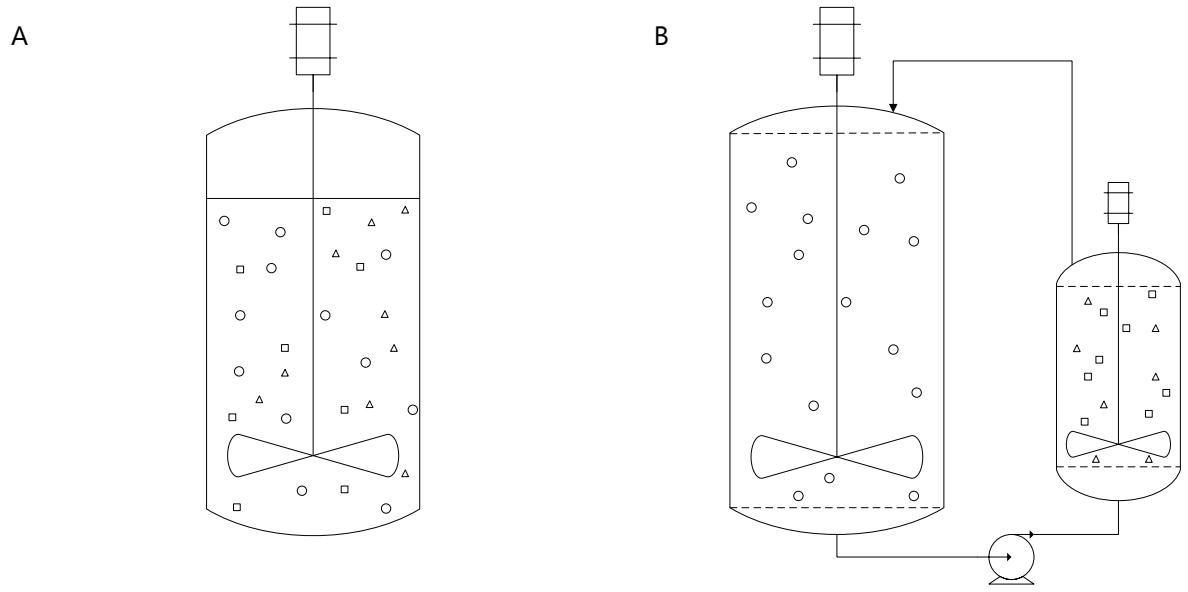


Figure 56 Process design options for a reactor: A) one pot system, B) external co-product removal; (○) – ω-transaminase, (□) – LDH, (Δ) – FDH

A model representing two pot system was developed as described in section 5.2.1. The kinetic model is developed for the enzyme in the soluble formulation, and thus the system represented in the Figure 56 B) is envisioned as two UFMRs with perfect mixing while the biocatalyst loading of the ω-transaminase was

expressed as $V_{max\_f,r}$ = 55.33 mmol min^{-1} L^{-1}, and the LDH and FDH loading was 11 and 0.3 g L^{-1} respectively, representing conditions under which the yield of 95 % is achieved in 24 h in a one pot system.

For a fixed V_1 volume of 1 L the yield was calculated as a function of the volume ratio range, where the latter was allowed to vary from 0.2 to 5, and considering a flow rate range from 0.5 to 10 L min^{-1} as depicted in Figure 57.

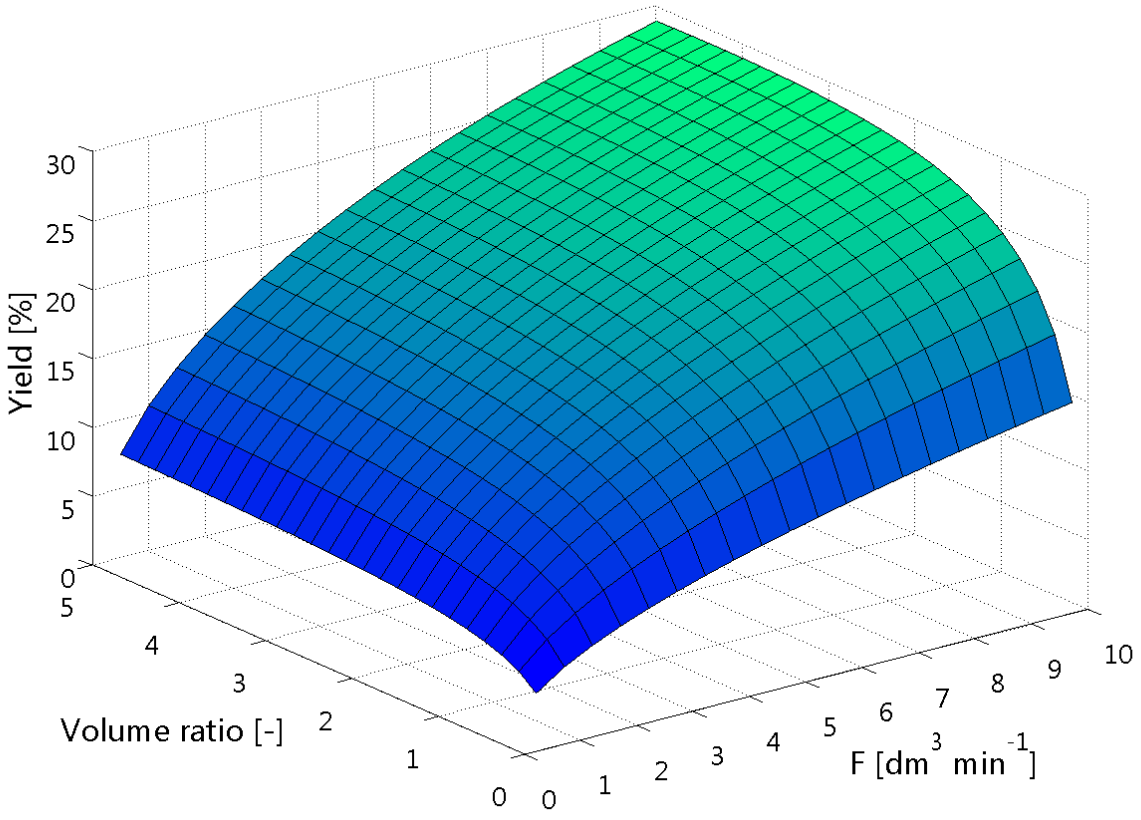


Figure 57 Simulated yield of the ω-transaminase/LDH/FDH system in the two pot process as a function of volume ratio and flow rate; simulation conditions are stated in section 5.2.1

Simulated results showed that an external co-product removal significantly reduces the yield, and it is not a viable option for the operation of the investigated ω-transaminase/LDH/FDH case. As shown in Figure 57 the yield is a function of the volume ratio up until value of approximately 3 and a further increase of the volume ratio does not influence the yield significantly. This can be explained by the fact that at higher volume ratios the external cascade reactor will have a larger volume and a higher residence time which will result in a more efficient pyruvate removal. Another investigated operating variable is the recycle flow rate of the system. At lower flow rates the pyruvate buildup in the ω-transaminase reactor will cause the reaction to slow down due to the highly unfavorable K_{eq}. For present initial conditions ($c_{acetophenone}$ = 30 mmol L^{-1} and $c_{alanine}$ = 1 mol L^{-1}) the reaction can proceed at best to an equilibrium value of c_{MBA} = 1.08 mmol L^{-1} without pyruvate removal.

Reducing the residence time by increasing the recycle flow rate the buildup of pyruvate in the ω-transaminase reactor is decreased and the yield that can be achieved is improved. One should indeed remember that a very high recycle flow rate yields a two pot system that will have a behavior that is close the behavior of the one pot system. However, those flow rates are impractical in reality and simulations have been performed for recycle flow rates up to 10 L min^{-1} which represents a flow rate corresponding to 10 working volumes of the ω-transaminase reactor that are exchanged per minute.

Based on these simulations, it is concluded that the external co-product removal for thermodynamically challenged reactions is undesirable. To prevent slowing down the reaction rate due to the thermodynamic challenge, *in-situ* co-product removal (ISCPR) is absolutely necessary for successful industrial implementation. However, the two pot system may be considered for other cases, e.g. systems which are much more thermodynamically favorable, in which case ISCPR can be used for both shifting the thermodynamic equilibrium and alleviating possible co-product inhibition.

5.3.3 Biocatalyst Formulation

Biocatalyst formulation plays an important role in the process development and for the case of production of (S)-α-methylbenzylamine different possibilities of formulation of three biocatalysts and one co-factor are discussed in the following sections.

5.3.3.1 Soluble Enzymes vs Immobilized Enzymes

In recent years, the impact of protein engineering has played a significant role in achieving significantly increased enzyme yields while further reducing enzyme production cost and increasing performance via improved stability and productivity[162]. To enable reuse and stabilization of enzymes immobilization techniques are often introduced. However, there are still industries where soluble enzymes are dominant, e.g. detergents, food manufacturing, biofuel production from starch and synthesis of chiral compounds e.g. Sitagliptin. The reason for using soluble enzymes in some of the above mentioned cases is that achieving a high efficiency of hydrolysis processes for high molecular weight, or insoluble, or adsorbed substrates is only possible with soluble enzymes. In the case of production of Sitagliptin a novel ω-transaminase has been tailored for the process conditions where among other improvements a significant effort has been placed in increasing the activity, and eventual immobilization would probably not bring further improvements. The general advantages and disadvantages of application of soluble and immobilized enzymes are shown in Table 16 and the main principles of enzyme immobilization are summarized in the Figure 58.

Table 16 Advantages and disadvantages of application of soluble and immobilized enzymes, adopted from Buchholz et. al.[162]

Advantages of soluble enzymes	**Disadvantages of soluble enzymes**	**Advantages of immobilized enzymes**	**Disadvantages of immobilized enzymes**
Low enzyme price	High enzyme price	High enzyme price requires immobilization	High cost of immobilization
Singular application	Limited productivity	Continuous processes, high productivity	
Low amounts (analytical, therapeutic application)			
Efficiency with insoluble, or adsorbed, or high molecular weight substrates)[a]		Fewer by-products[b]	More by-products[c]
Complex systems with coupled reaction path			Low efficiency with complex systems
Co-factor dependent reactions easy to implement			No co-factor retention
Application of membrane reactor systems (retention or recycling of enzymes)		Easy reuse	

a – enzyme remains in the product
b – example: less trisaccharides due to decreasing lactose gradient in the carrier in lactose hydrolysis
c – example: more reversion products due to increasing product gradient in the carrier with amyloglucosidase (dextrin hydrolysis)

The overview given in the Table 16 may hint towards a favorable enzyme formulation for both the ω-transaminase and the cascade system. As mentioned before, the ω-transaminase processes are still 1st generation processes and are not widely established as industrial processes. Still, much effort is put into developing transaminases with sufficient activity, and immobilization techniques usually do not contribute to the desired enzyme productivity. Therefore, to exploit the ω-transaminase activity optimally, a soluble form of the enzyme is preferred. The implementation of such processes requires an UFMR (UlraFiltration Enzyme Membrane Reactor) whose advantages and drawbacks are given in Table 17.

Table 17 Advantages and drawbacks of UFMR adopted from [175]

Advantages	Drawbacks
Continuous mode (substrate feeding)	Decrease of enzyme activity (loss of catalyst, shear stress…)
Free enzyme	Membrane fouling
Retention and reuse of catalyst	Pressure drop
Reduction of substrate/product inhibition	
Enzyme free product	
Control of product properties by enzyme and/or membrane choice	
Integrated process (single step reaction/enzyme separation)	

Another issue that needs to be discussed is the formulation of the cascade system. The previously mentioned conclusions in section 5.3.2 defined a one pot system as the only viable mode of operation, and therefore the formulation of the cascade system has to be compatible with the ω-transaminase formulation. The options for cascade immobilization are presented in Figure 58.

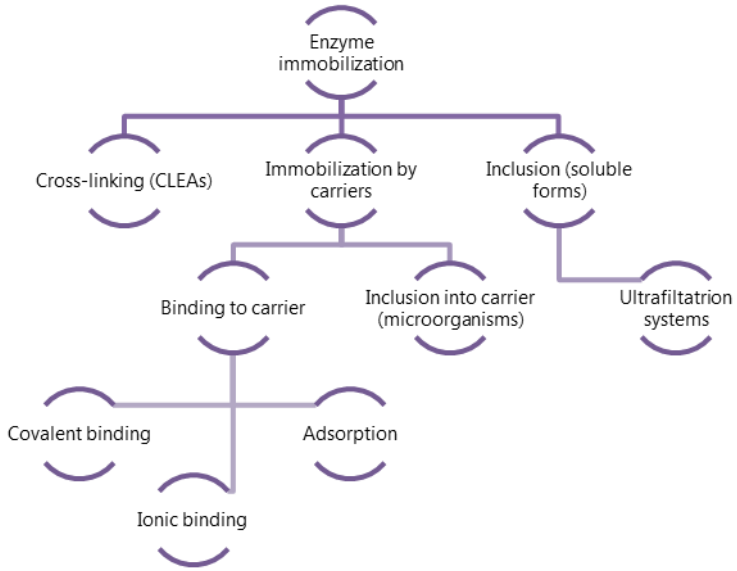


Figure 58 Main principles of enzyme immobilization, adopted from Buchholz et. al.[162]

The use of CLEAs and binding to the carrier as an immobilization method could cause the soluble ω-transaminase to adsorb to the carrier surface which would result in decreased transaminase activity. Previous attempts of covalently binding the co-factor and co-immobilizing two dehydrogenases has indeed proved to increase reusability and stability of the system[176-178]. However the mobility was severely hindered as well, resulting in reduced activity. The main objective of the cascade system in the production of optically pure amines via ω-transaminase is the fast and *in-situ* removal of the co-factor, and therefore the reduced activity of the immobilized form as well as the possible formation of concentration gradients make immobilized carriers

an impractical choice. The inclusion of the cascade in soluble form in separate compartments is not practical as a consequence of the earlier conclusion that one pot is the necessary mode of operation. The inclusion into carrier (microorganisms) could prove to be a viable option if all three enzymes (ω-transaminase, LDH and FDH) are co-expressed to avoid mass transfer limitations through the cell wall. However, due to the relatively low activity of the still developing ω-transaminase, the process would be limited by cell loading within the reactor and the amount of possible protein expression within the cell, while risking the formation of inclusion bodies at higher protein expressions[179]. The literature suggest that the method of choice for co-factor dependent two or multi-enzyme systems is the application of membrane reactors which retain or recycle all catalytically active components[162]. To retain the co-factor within the membrane usually immobilization on to polymers is used, e.g. PEG with sufficiently high molecular weight[180-182].

5.3.3.2 Co-factor stability

When utilizing dehydrogenase cascades for shifting the equilibrium, the stability of the co-factor NADH is in most cases not discussed or neglected. However, it has been found that the co-factor NAD(P)H is very unstable in *in-vitro* conditions[117] and its stability under working conditions has to be investigated. Experiments showed that the kinetics of degradation vary with changing the pH environment, and that in pH regulated media, e.g. buffers, the degradation follows first-order kinetics (Equation 2.1). In media without pH regulation, e.g. water or reaction mixture 1 (see section 3.3.3.1), degradation followed second order kinetics (Equation 5.13). The stability of the co-factor in different buffers is shown in the Appendix 5A, and the stability in the reaction mixture 1 is shown in Figure 59.

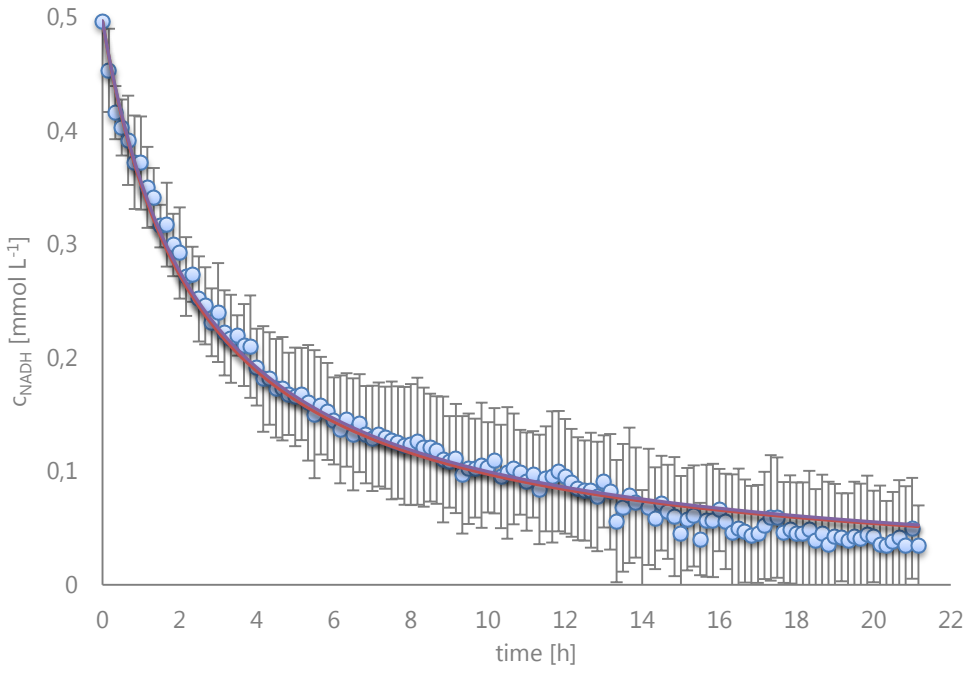


Figure 59 Stability of NADH in the reaction mixture 1 (see section 3.3.3.1) at 30°C and starting pH 7; $t_{1/2}$ = 2 h 28 min

It is clear that the above shown poor stability of the co-factor represents a major challenge for successful industrial implementation. One way to tackle this challenge is the use of nicotinamide analogues which has recently resurfaced as a very interesting topic in biocatalytic redox chemistry. A recent extensive overview of the use of synthetic co-factors by Paul et. al.[183] demonstrated a versatile role of co-factor analogues in biocatalytic and organic synthesis. A study by Ma et. al.[184] showed increased activity of LDH when 8-(6-aminohexyl)-amino-NADH was used compared to the native co-factor.

5.3.4 Downstream Processing

5.3.5 Introduction

Usually, in the reactions catalyzed by ω-transaminases substrates and products are very similar due to the reaction mechanism where keto- and amino- groups are exchanged. Additionally, the substrates and products of the cascade system have to be taken into consideration when defining a suitable product separation technique. This similarity in size, hydrophobicity and volatility makes the product separation challenging, as shown in Table 18.

Table 18 Physical and chemical properties of compounds in the investigated ω-transaminase/LDH/FDH system

	Mw [g mol^{-1}]	**T_m [°C]**	**pK_a**	**logP**	**p_{vap} [mm Hg]**	**S_{aq} [g L^{-1}]**
acetophenone	120.15	-9.86	-	1.58	$3.97 \cdot 10^{-1}$	6.1
alanine	89.09	292.0	2.35; 9.87	-2.96	$2.68 \cdot 10^{-8}$	165.0
MBA	121.18	-65.0	9.75	1.49	$5.00 \cdot 10^{-1}$	43.0
pyruvate	88.06	11.8	2.3	-1.24	$9.68 \cdot 10^{-1}$	soluble
lactate	90.08	16.8	3.86	-0.72	$8.13 \cdot 10^{-2}$	soluble
ammonium formate	63.06	116.0	3.77[1]; 9.25[2]	-0.27	-	soluble

1 pKa of formic acid
2 pKa of ammonia

However, it is important to distinguish product recovery from ISPR and recognize possibilities, advantages and challenges of both approaches. Product recovery describes a procedure where both reaction and downstream operation are performed in batch mode. When a biocatalytic process has reached the desired yield, the biocatalyst has been removed, preferably by techniques which will allow reuse of the biocatalyst, and the reaction mixture is manipulated to allow selective recovery of a product. Although no ω-transaminase process utilizing cascades has been found in the literature, for the purposes of demonstration, an industrial process has been envisioned based on the work of Koszelwsk et. al.[185] and the process flow diagram is shown in Figure 60.

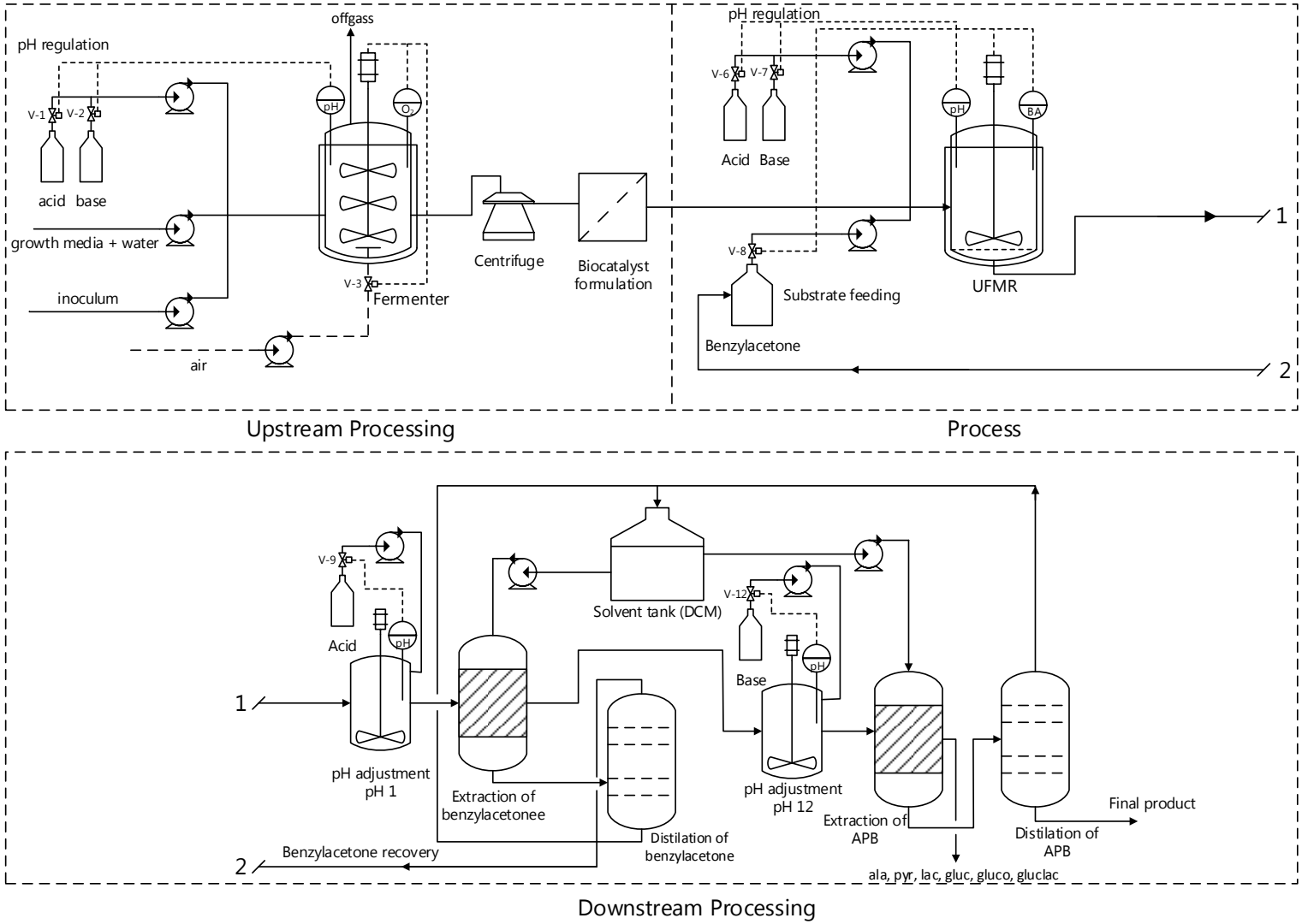


Figure 60 Envisioned industrial process setup for production of optically pure amines fitted to the product recovery method developed by Koszelewski et.al.[185]; For substrates BA (benzylacetone) and ala (alanine), the K_{eq} is $6.07 \cdot 10^{-4}$ (Tufvesson et al.[98]), and the LDH/GDH cascade system is deployed to shift the equilibrium; APB – 3-amino-1-phenylbutane; pyr – pyruvate; gluc – glucose; gluco – gluconate; gluclac – glucono-1,5-lactone; DCM – dichloromethane; UFMR – UlfraFiltration Membrane Reactor

The product recovery procedure developed by Koszelewski et. al.[185] was applied to the ω-transaminase/LDH/GDH system at laboratory scale with benzylacetone and alanine as substrates, and it involved pH regulation of the reaction medium after the completion of the reaction. First, the pH was adjusted to a pH value of 1 causing the protonation of alanine and the product APB. The extraction of acetophenone with dichloromethane (DCM) as a solvent was carried out, after which the pH was again adjusted to a value of 12. Under those conditions the product is neutrally charged and extraction with DCM is possible. In this process demonstration the final product is obtained by distillation of the DCM-APB mixture and the solvent is recycled back to the process. Also, the distillation of the DCM-BA mixture has been considered where both the solvent and the substrate benzylacetone are recycled back to the process.

However, applying ISPR to the process may bring many advantages to the given process, but first numerous challenges have to be tackled. ISPR techniques are based on *in-situ* removal of product during the reaction, either by removing the product directly within the reactor or by means of an external unit where the removal occurs. In the latter case a recirculation is established and a reaction medium containing less product is returned back to the reactor. In the investigated ω-transaminase/LDH/FDH case where acetophenone and alanine are substrates a severe thermodynamic limitation (K_{eq} = 4.03·10^{-5}) requires exquisite selectivity of the ISPR technique, since otherwise even trace quantities of the removed substrates would shift the thermodynamics in the opposite direction and make the process unfeasible.

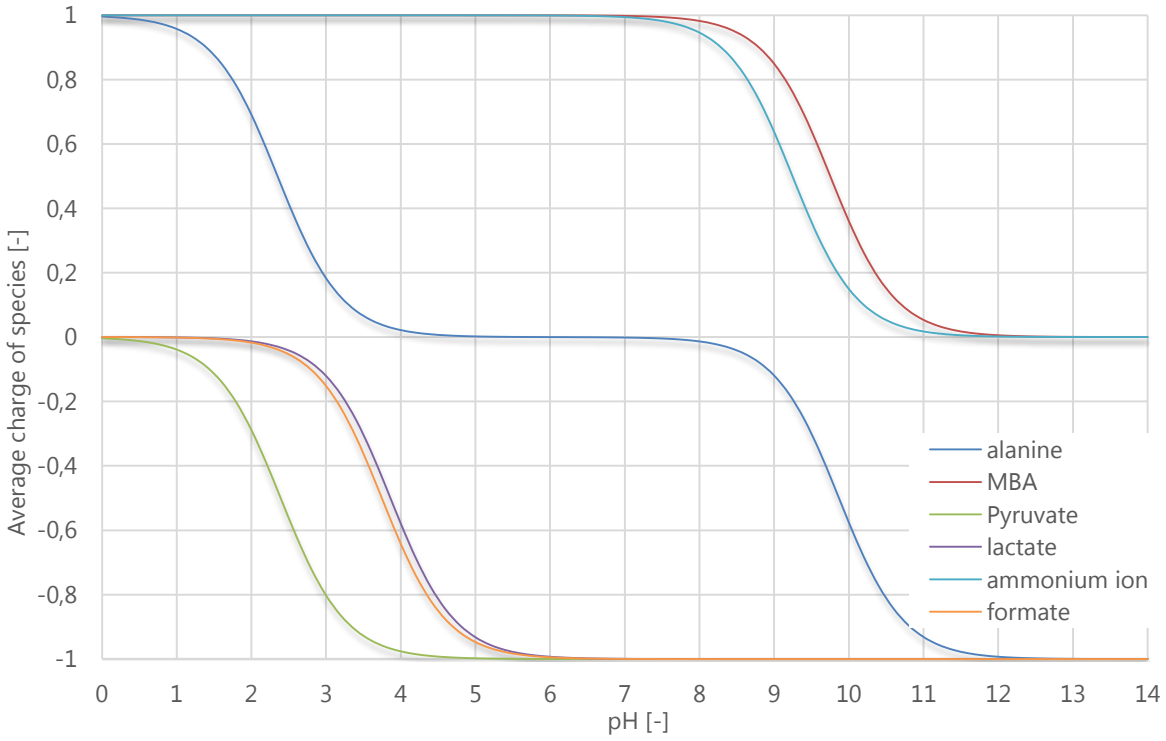


Figure 61 Charge analysis of the components of the ω-transaminase/LDH/FDH system as a function of pH

Based on the charge analysis shown in Figure 61, it is possible at operating pH 7 to selectively remove the product based on the differences in charge, meaning that separation either by ion exchange or electrodialysis should be possible. To avoid adsorption of enzymes to the ion exchange resins or fouling of the membranes in the electrodialysis module the application of ISPR techniques will have to be external as a consequence of the soluble form of the enzymes. Possible process implementations are shown in Figure 62.

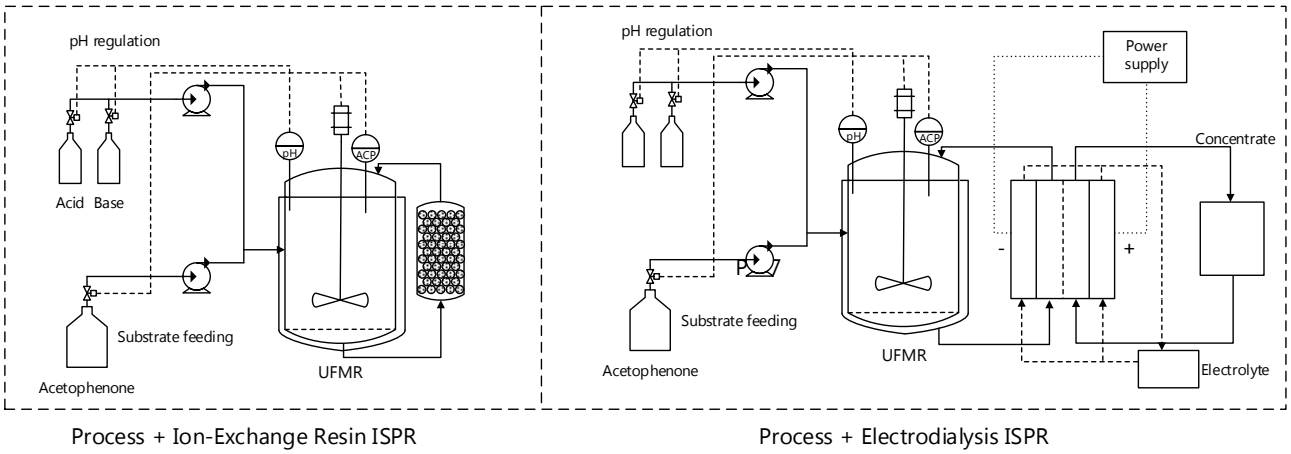


Figure 62 Possible process configurations based on separation on the basis of charge of individual compounds in the reaction medium

Both ion exchange and electrodialysis can be performed in batch and continuous mode. The batch mode of operation of both techniques would consist of several steps where the first step would be running the reaction the reaction until the desired yield is achieved. The second step is then the product recovery by ion exchange or electrodialysis while the final steps include e.g. washing the resins, extraction and purification of the product. However, the continuous mode of product removal or ISPR can prove to be very beneficial.

To illustrate the benefits of ISPR a theoretical case has been envisioned where the process of production of MBA via the ω-transaminase/LDH/FDH system is established and the ISPR method (either ion exchange resin or electrodialysis) is successful in selectively removing 50 % of the inlet MBA concentration. The volume of the UFMR is 1 L and the volume of the ISPR unit is 0.2 L, while the flow rate is 0.1 L min^{-1}. The concentrations of catalytically active components were selected such that they represent 95 % conversion under fed batch conditions ($V_{max\_f,r}$ = 55.33 mol min^{-1} L^{-1}, γ_{LDH} = 11 g L^{-1}, γ_{FDH} = 0.3 g L^{-1} and c_{NADH} = 0.1 mmol L^{-1}). Simulations showed that a 100 % conversion is achieved after approximately 8 hours when above described ISPR techniques is applied. This illustrative example shows that significant improvements of the yield can be achieved by process intensification. The outcomes of using an ISPR will result in either decreased required value of $V_{max\_f,r}$ for achieving a desired 95 % conversion within 24 h or higher allowed product inhibition, or combination of both.

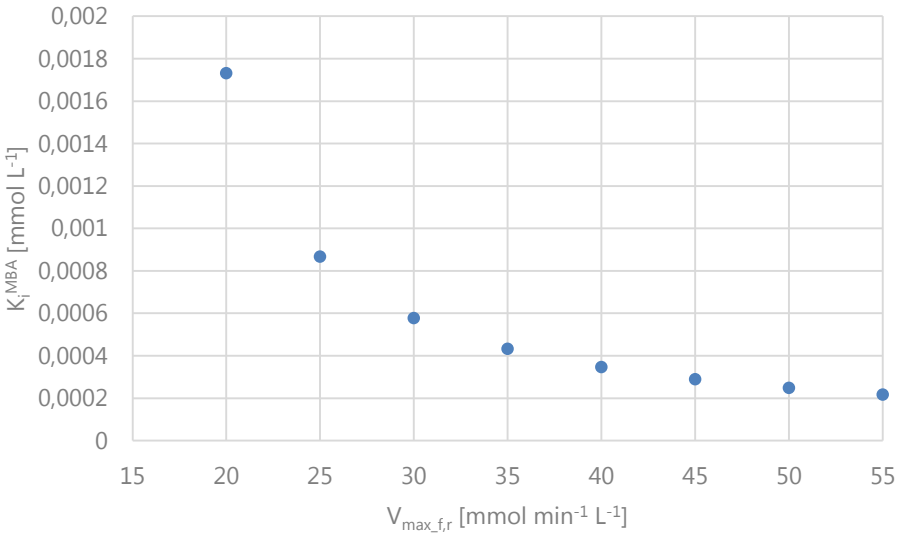


Figure 63 Possible values of K_i^{MBA} as a function of $V_{max\_f,r}$ in the case of 50 % ISPR for 95 % conversion; conditions for the simulation are stated in the section 5.2.3.

In the Figure 63 the combination of the required ω-transaminase activity, expressed as $V_{max\_f,r}$, and the inhibition by MBA, expressed as K_i^{MBA} are shown for the above described illustrative example of ISPR. It is for example possible to achieve 95 % conversion when $V_{max\_f,r}$ = 30 mmol min^{-1} L^{-1} and the allowed MBA inhibition is approximately K_i^{MBA} = 0.6 mol L^{-1}. This example clearly shows how process design and clever use of selective ISPR technologies can yield a significant reduction of the requirements for ω-transaminase development (activity, product inhibition).

5.4 Discussion

In this chapter the industrial implementation of processes of optically pure amine synthesis facing severe thermodynamic challenges was discussed, focusing on a specific case of MBA production from acetophenone and alanine with the use of the LDH/FDH cascade system.

It was concluded that the fed batch operating mode fits the best to the process, due to the fact that this is a relatively slow reaction and the activity of the ω-transaminase still has to be improved further. To exploit the developed activity of the ω-transaminase, the few industrially established processes, e.g. the Sitagliptin process, use the soluble enzyme. The literature suggests that the co-factor and multi-enzyme processes should be in the soluble form, enabling mobility of catalyst molecules. An UltraFiltration Membrane Reactor (UFMR) is currently an option for the ω-transaminase/LDH/FDH system. The possibility of external ISCPR was dismissed due to the significantly decreased yields when applying external ISCPR, which only confirmed the fact that ISCPR has to be performed internally. Another very important issue was the stability of the co-factor, which at

in-vitro conditions, especially at conditions predicted for the investigated case, has a very low stability and therefore stabilization measures e.g. immobilization will be required.

Two types of DSP – product recovery and ISPR, were also discussed in this chapter showing that product recovery is easier to perform by changing the reaction mixture conditions once the reaction is finished. However this procedure puts more demands to the performance of the ω-transaminase in terms of activity and requirements for reduced product inhibition. Using ISPR is extremely challenging in the investigated case and requires exquisite selectivity of product removal.

Charge analysis showed that selective removal of the product MBA is possible via charge separation techniques, e.g. by means of ion exchange resins or electrodialysis. Indeed, each technology will have to pass rigorous selectivity testing before it can be implemented as ISPR technique. A showcase was developed to indicate possible benefits of such an integrated approach where 50% of the inlet product was removed. The results for that particular case showed that both the required ω-transaminase activity expressed as $V_{max\_f,r}$ and the resistance towards product inhibition can be significantly reduced by means of process intensification.

6 Thesis Discussion and Conclusions

The focus of this thesis was the biocatalytic synthesis of optically pure chiral amines, small molecule building blocks for many important pharmaceutical compounds. Furthermore, equilibrium shifting strategies for the thermodynamic "worst case scenario" with K_{eq} = $4.03 \cdot 10^{-5}$ were investigated, concluding that only ISCPR by enzymes is a possible solution due to the selectivity of this method. Although literature suggested several options of ISCPR by enzymes, no attempts on industrial scale have been reported, and information on how to select between proposed cascades is also not available in the literature. The developed selection procedure was focused on the feasibility of the implementation of cascade enzymes into an industrial process. A first consideration was described as the thermodynamic ability of a cascade to remove the co-product to low enough concentrations to allow achieving 95 % conversion. The justification for this selection step lies in the fact that thermodynamic equilibrium of a cascade reaction cannot be improved by protein engineering, and if a cascade is thermodynamically unable to achieve the desired yield, it should be disregarded. The second step included a discussion about substrate selectivity of either cascades or ω-transaminase and identified outcomes of possible side reactions. The final step was based on experimental determination of the operational stability of cascade enzymes identifying viable options. This selection process yielded two promising implementation candidates: LDH/FDH and LDH/FDH.

Model development took the investigation another step forward, predicting for the two cascade candidates and under the given conditions – e.g. starting concentration of reaction mixture compounds, concentrations of catalytically active components and reaction time to name a few – the required future development of the ω-transaminase. Since process design, e.g. ISPR, was not taken into consideration at that point, the envisioned ω-transaminase had to be devoid of product inhibition. A second crucial development of the ω-transaminase was the required activity expressed as $V_{max\_f,r}$ [mmol min^{-1} L^{-1}] as a function of cascade efficiency to remove pyruvate expressed in mass concentration of cascade enzymes and molar concentration of the co-factor. Based on its higher performance for lower co-factor concentrations, the LDH/FDH system was chosen.

For long lasting reactions and due to the low solubility of the substrate acetophenone, the most suitable mode of operation proved to be fed batch. Simulations showed that external co-product removal is not a viable option, and therefore a one pot process was selected where all catalytically active components are present. The biocatalyst formulation was discussed at length and it was concluded that the soluble form of the ω-transaminase is required provided that its stability is satisfactory. The soluble form of the cascades was chosen based on previous similar process implementations, and immobilizing only the co-factor to retain it with the membrane of the UFMR.

A DSP technique has been proposed, based on a lab scale procedure obtained from the literature[185], where the final reaction mixture is modified via pH change to enable relatively selective extractions and final product separation and recovery. However, when ISPR is applied it was demonstrated that this comes along with several potential advantages, e.g. the required ω-transaminase activity for achieving 95 % conversion within 24 h can be reduced or an enzyme with product inhibition may be used. Additional charge analysis of the species indicated that at operating pH value a charge separation via ion exchange or electrodialysis is possible.

Based on the investigations done in this thesis, the key findings, the main novelties and conclusions are highlighted below:

- Shifting the equilibrium of a thermodynamically challenged reaction is possible and industrially applicable. Thermodynamically challenged extremes require powerful and more important selective strategies making ISCPR by enzymes the only solution. However a major challenge, especially for the investigated case, is not the low K_{eq} value but the inadequate activity of the enzyme e.g. the ω-transaminase. As a general rule that can be applied: lower K_{eq} requires higher enzyme activity.
- The cascade systems have to fulfill the following requirements:
    - Thermodynamic ability of a cascade system (ability of a cascade to remove thermodynamically the product to such concentrations that the required yield can be achieved)
    - Substrate selectivity: a) of cascades b) of the "main" enzyme, here ω-transaminase, towards the products of cascade reaction(s). For possible outcomes, see section 3.4.2
    - Operational stability of cascade enzymes and co-factors has to be investigated, and eventually the operational stability of the "main" enzyme needs to be evaluated as well
    - All cascade enzymes must be devoid of inhibition by accumulating components i.e. products of all present reactions
    - Co-product removing enzymes require low K_m which enables fast removal at low concentrations using reasonable enzyme concentrations
- If the "main" enzyme has a sufficiently high activity and possibly a low stability, immobilization is encouraged to achieve enzyme recycle and reuse
- Immobilization of cascade enzymes should be considered only when the immobilization technique does not create significant concentration gradients and mass diffusion issues
- Immobilization of co-factor recycling systems should involve only the immobilization of the cofactor to ensure retention within the membrane(s)
- Immobilization efforts must aim at compatibility between the "main" enzyme formulation and the cascade enzyme formulation, e.g. the soluble form of the enzyme might adsorb to the carrier of the immobilized enzyme if soluble and immobilized enzyme forms are mixed in a reactor.

- External ISCPR is not an option for severely thermodynamically challenged reactions, and therefore internal methods must be applied meaning that the place of co-product removal has to be in the micro-environment where its production takes place.
- The developed procedure for cascade selection is a generic procedure and can be applied not only to other systems catalyzed by an ω-transaminase, but also to any thermodynamically challenged reaction using ISCPR by enzymes as an equilibrium shifting strategy.

7 Future Work and Final Remarks

There are several challenges that still have to be addressed in the future.

Open challenges remaining for the current work:

A lot of time and energy was put into LDH, GDH and FDH characterization and model development. However, a validation of these models in a coupled mode, e.g. LDH/GDH and LDH/FDH and under process relevant conditions was not performed, and analytical methods need to be developed. Model validation would significantly increase the reliability and potential future use of the model in the development of a model based soft sensor for establishing a reliable acetophenone feeding.

Immobilization of the co-factor has been identified as one of the required steps for process implementation, followed by experimental re-estimation of kinetic parameters of the cascade enzymes. This is also work that needs to be done in the future.

Immobilization of cascade enzymes when there are no significant concentration gradients. Such aspects could be investigated in more detail using techniques such as computational fluid dynamics.

Ion exchange resins or electrodialysis were identified as the most feasible DSP options. One possible bottleneck, as seen in Figure 61, could be that two positively charged species are present at pH 7, i.e. MBA and a cation of a formate salt (NH_4^+). At the beginning of the reaction the ammonium concentration is approximately 550 mmol L^{-1} while the concentration of the MBA is 0. The ion exchange resin would quickly be saturated with ammonium ions, thus rendering the ISPR inoperable and pointing towards electrodialysis as the most suitable ISPR technique.

Running the proposed system in an UFMR together with characterization of the electrodialysis unit in a lab scale setup, and demonstrating the production of > 50 g L^{-1} of product still remains to be done in the future, as well as further process optimization and implementation to pilot and industrial scale.

Final remarks

This thesis is focused on the possibilities of ISCPR by currently developed enzymes and possible implementation options represent the currently viable options. However, the future enzyme development will surely allow other cascade options to become an interesting alternative to the current LDH/FDH. On that list of cascade options considered here, the AlaDH/FDH system will surely emerge offering alanine recycling and possibly also cheap amine sources to be used. However, the stability has to be improved and inhibition by

alanine has to be alleviated (Appendix 7A). Other processes / companies might go for “less is more” by eliminating the use and recycling of the expensive co-factor by using PDC or ALS cascades if more substrate selective transaminases are developed in the future.

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Appendix

Appendix 3A – pH profiles of cascade enzymes found in the literature

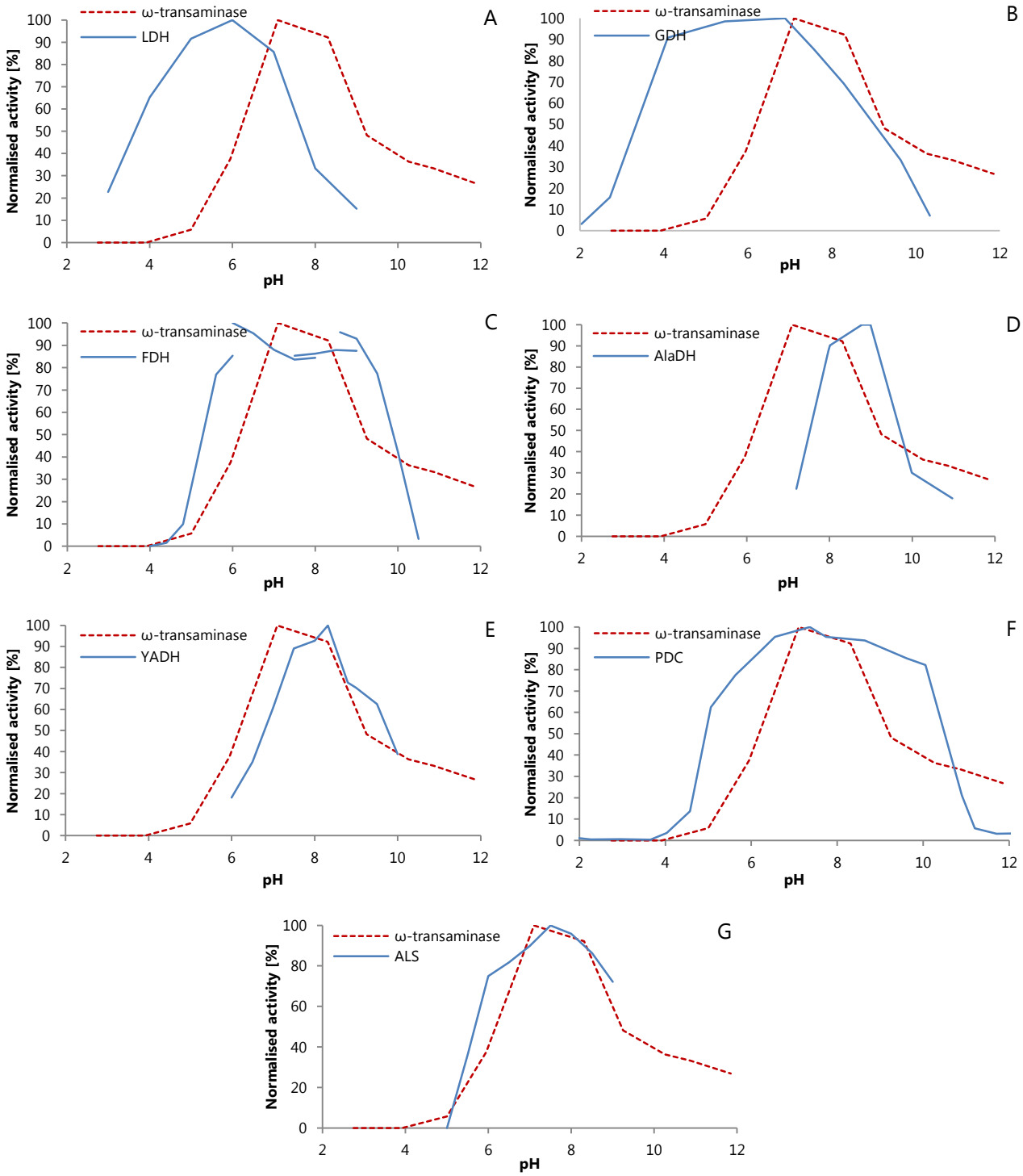

Figure 64 pH profile of ω-transaminase ATA-040 at 30°C, $c_{acetophenone}$ = 10 mmol L^{-1},$c_{2\text{-}propylamine}$ = 100 mmol L^{-1}, γ_{TAm} = 3 g L^{-1} at wide range buffer[163] pH 2-12 compared to pH profile of: A) LDH adopted from Li et. al.[186], B) GDH adopted from Kobayashi et. al.[187], C) FDH adopted from Ding et. al.[188], D) AlaDH adopted from Yoshida et. al.[189], E) YADH adopted from Drewke et. al.[190], F) PDC adopted from Hiromasa et. al.[191] and G) ALS adopted from Yoon et. al.[192]

Appendix 3B – An example of Matlab® code for calculating equilibrium concentrations of reaction components and the corresponding yield for the LDH/GDH system

```
clc
clear all

% Tolerances
tol      = 1e-15;
diff     = 1;

% Initial values
ACP          = 500;  % mM
Alanine      = 1000; % mM
MBA          = 0;    % mM
Pyruvate     = 0;    % mM
NADH         = 0.1;  % mM
lac          = 0;    % mM
NAD          = 0;    % mM
Keq1         = 1/24800;
Keq2         = 2.12e4;
Glucose      = 550;  % mM
Gluc15lac    = 0;    % mM
Keq3         = 1.67e23;
x = 1; y = 1; z = 1;
while (diff > tol)
prev = MBA;

% transaminase reaction
x = (MBA + Pyruvate + Keq1 * (ACP + Alanine) - sqrt((MBA + Pyruvate + Keq1 *
(ACP + Alanine))^2 - 4 * (Keq1 - 1) * (Keq1 * ACP * Alanine - Pyruvate *
MBA)))/(2 * (Keq1 - 1));

ACP          = ACP - x;
Alanine      = Alanine - x;
Pyruvate     = Pyruvate + x;
MBA          = MBA + x;

%LDH reaction
y = (NAD + lac + Keq2 * (Pyruvate + NADH) - sqrt((NAD + lac + Keq2 * (Pyruvate +
NADH))^2 - 4 * (Keq2 - 1) * (Keq2 * Pyruvate * NADH - lac * NAD)))/(2 * (Keq2 -
1));

Pyruvate     = Pyruvate - y;
NADH         = NADH - y;
lac          = lac + y;
NAD          = NAD + y;

% GDH reaction
z = (NADH + Gluc15lac + Keq3 * (Glucose + NAD) - sqrt((NADH + Gluc15lac + Keq3 *
(Glucose + NAD))^2 - 4 * (Keq3 - 1) * (Keq3 * Glucose * NAD - Gluc15lac *
NADH)))/(2 * (Keq3 - 1));

Glucose      = Glucose - z;
```

```
NAD          = NAD - z;
Gluc15lac    = Gluc15lac + z;
NADH         = NADH + z;

diff = abs(prev - MBA)
end

Pyruvate
Conversion = MBA / 500 * 100
```

Appendix 3C – Substrate selectivity of dehydrogenases towards the acetophenone

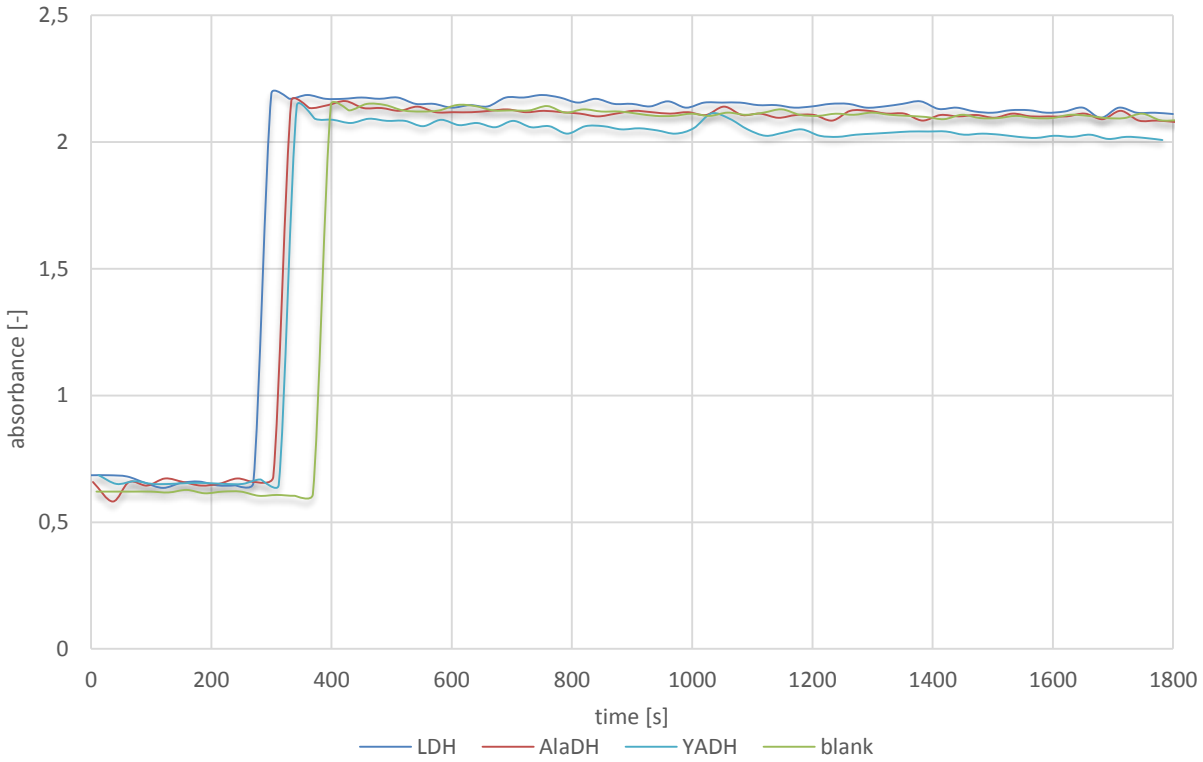

Figure 65 Progress curves for reduction of acetophenone catalyzed by LDH, AlaDH and YADH at pH 7 and 30°C at initial concentrations of substrates $c_{acetophenone}$ = 30 mmol L^{-1} and c_{NADH} = 0.1 mmol L^{-1} and with the concentration of all enzymes at 1 g L^{-1}. Additional 100 µL of NADH standard solution was added in the period from about 250 to 400 s to speed up the reaction if it is occurring slowly or to observe consumption of the cofactor if the reaction is too fast and any observable change in adsorption happened before the measurement in the spectrophotometer started

Appendix 3D – Relative stability of investigated cascade enzymes

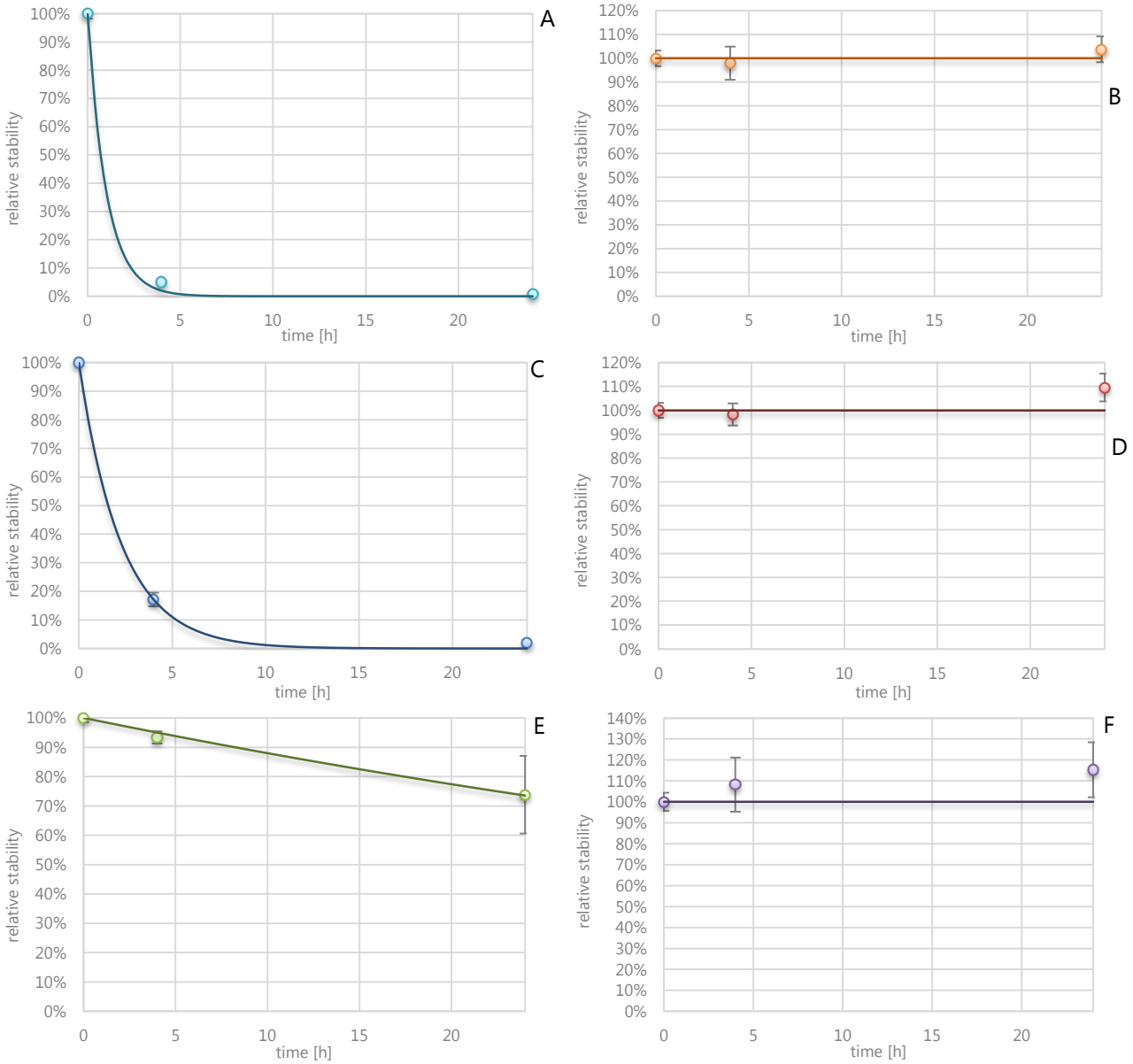

Figure 66 Relative stabilities of the investigated enzymes at pH 7 and 30°C and in the reaction mixture 1 and 2 defined in section 3.3.3.1 over a period of 24 h: A) stability of LDH 1 in reaction mixture 1; B) stability of LDH 2 in reaction mixture 1; C) stability of AlaDH in reaction mixture 2; D) stability of GDH 1 in reaction mixture 1; E) stability of GDH 2 in reaction mixture 1; F) stability of FDH in reaction mixture 2

Appendix 4A – Linear regression plots for LDH catalyzed reaction

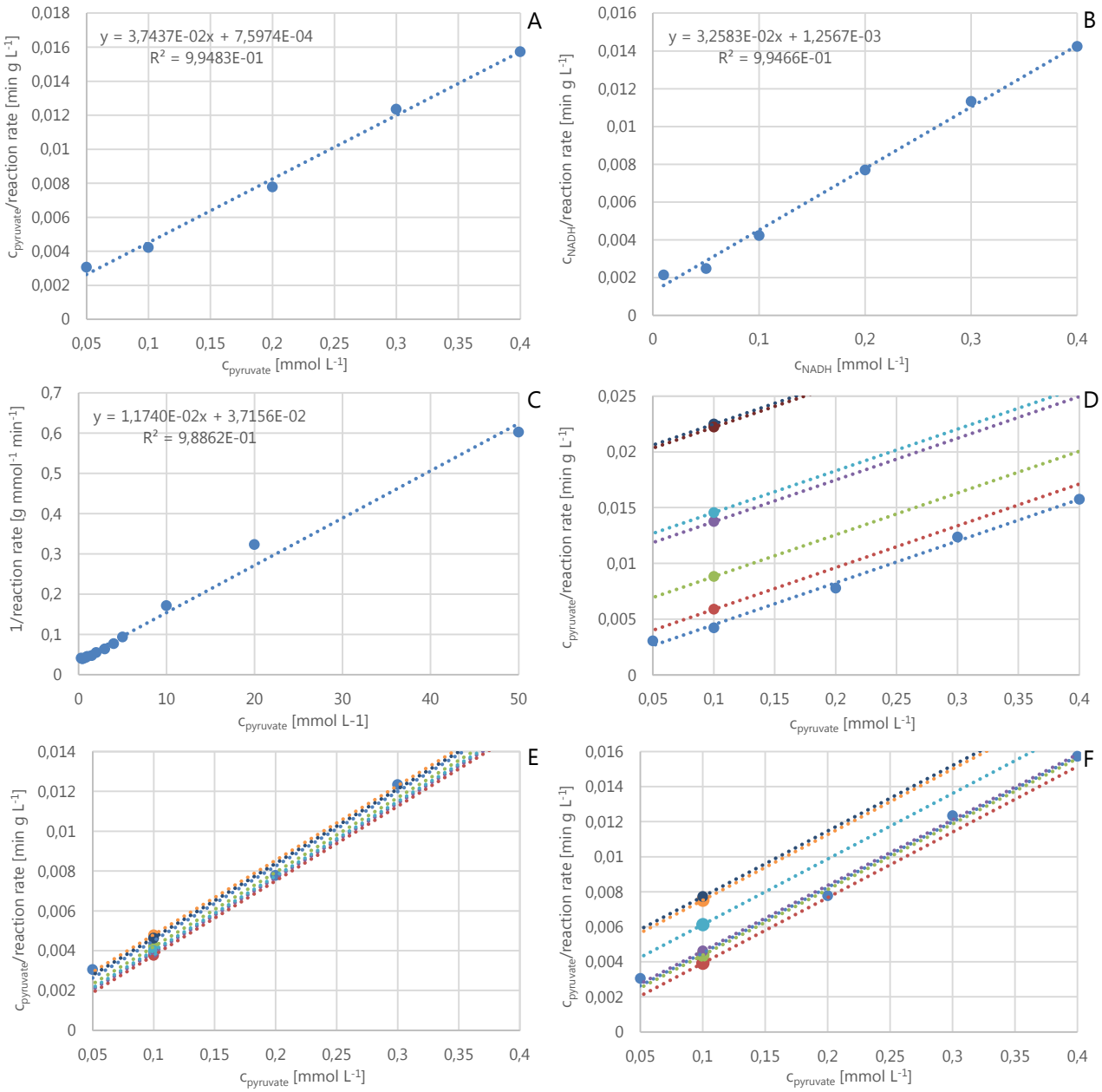

Figure 67 Linear regression plots of initial rate experiments shown in Figure 46 for estimating kinetic parameters of reaction catalyzed by LDH: A) Hanes plot over pyruvate range 0.05 – 0.4 estimating K_m^{PYR}, B) Hanes plot estimating V_{max}^{LDH} and K_m^{NADH}, C) Dixon plot estimating K_i^{PYR}; D) Hanes plots of increasing lactate concentrations estimating K_i^{LAC}; E) Hanes plots of increasing NAD⁺ concentrations estimating $K_i^{NAD^+}$; F) Hanes plots of increasing alanine concentrations estimating K_i^{ALA}

Appendix 4B – Linear regression plots for GDH catalyzed reaction

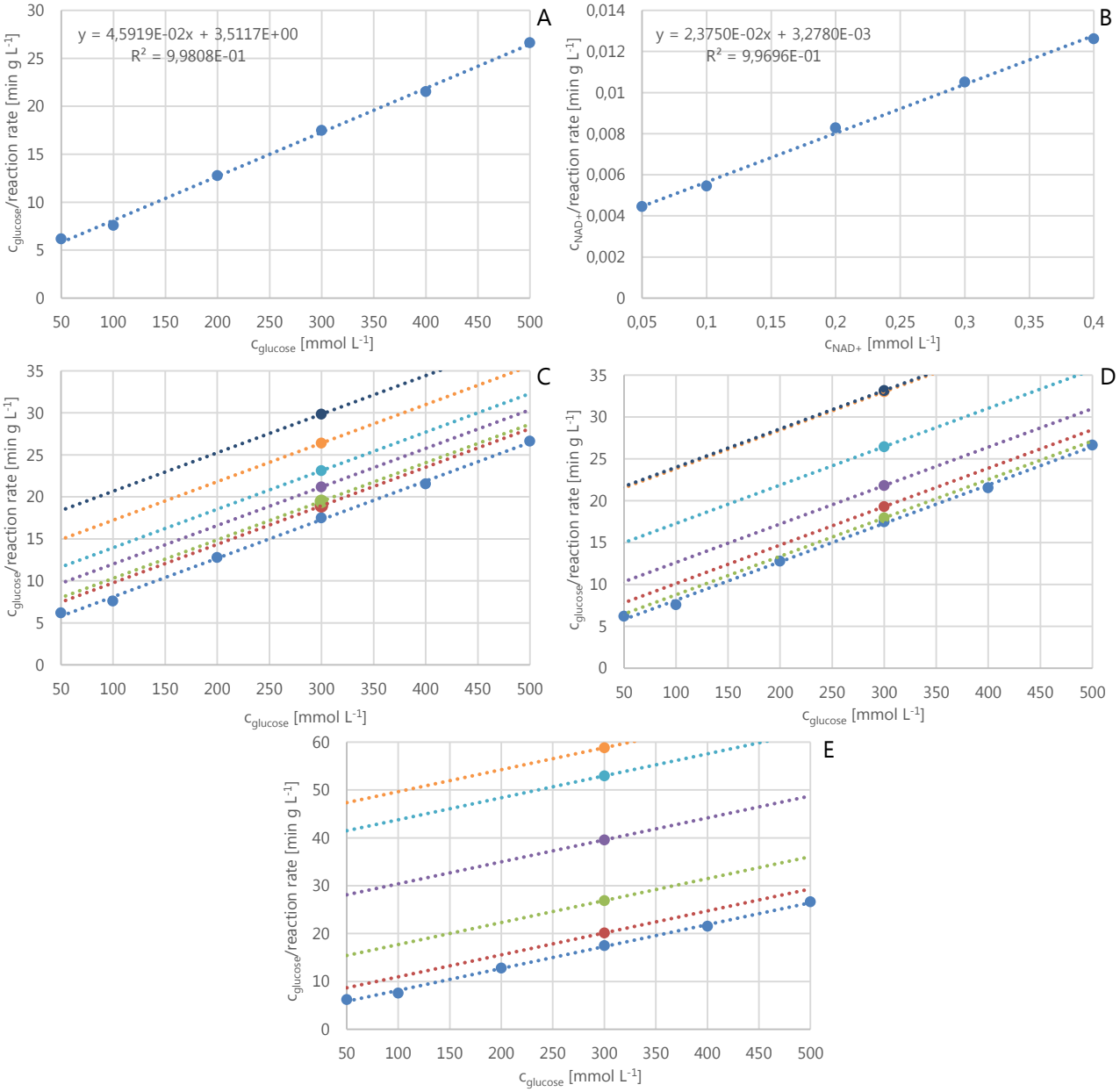

Figure 68 Linear regression plots of initial rate experiments shown in Figure 48 for estimating kinetic parameters of reaction catalyzed by GDH: A) Hanes plot over estimating $K_{m,GDH}^{GLU}$, B) Hanes plot estimating V_{max}^{GDH} and $K_m^{NAD^+}$, C) Hanes plots of increasing glucono-1,5-lactone concentrations estimating $K_i^{GLUC-1,5-LAC}$; D) Hanes plots of increasing NADH concentrations estimating $K_{i,GDH}^{NADH}$; E) Hanes plots of increasing MBA concentrations estimating $K_{i,GDH}^{MBA}$

Appendix 4C – Linear regression plots for FDH catalyzed reaction

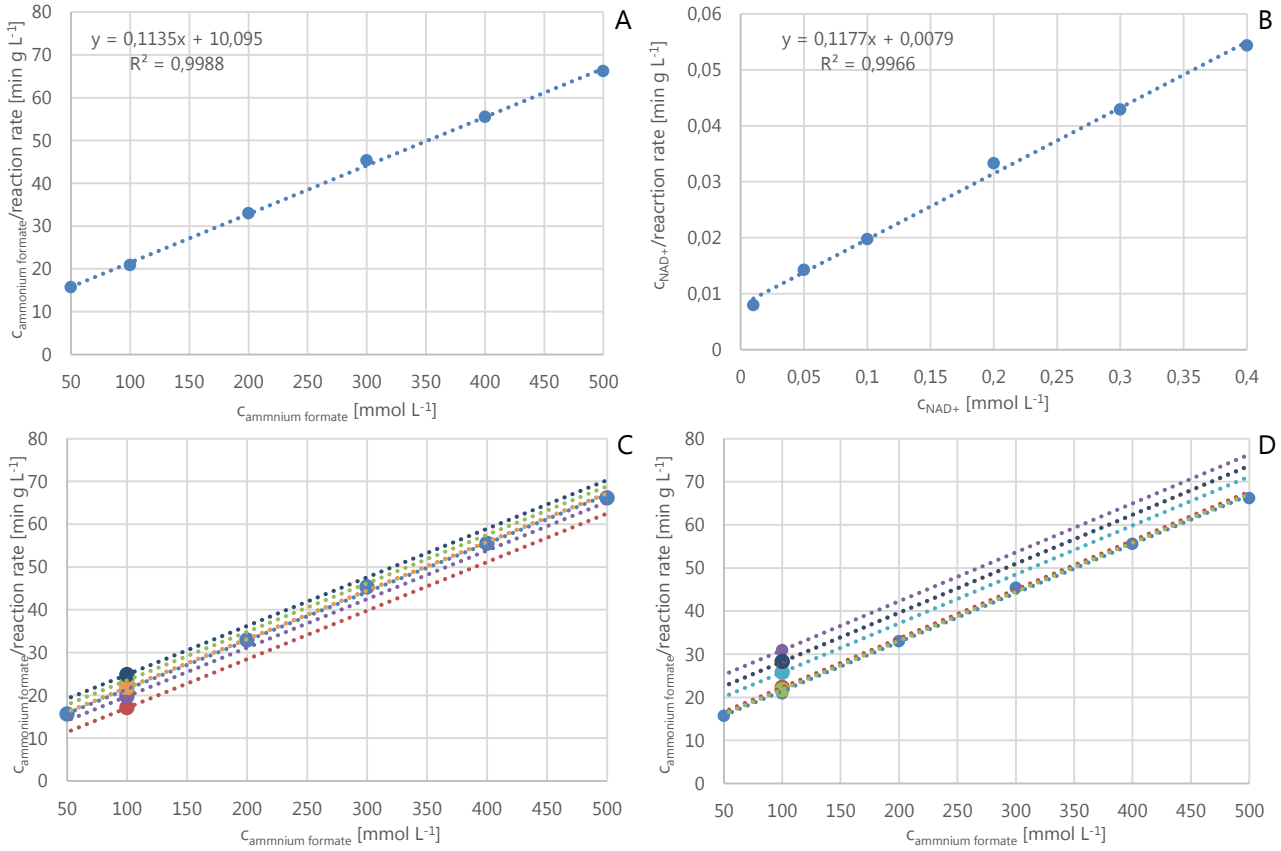

Figure 69 Linear regression plots of initial rate experiments shown in Figure 50 for estimating kinetic parameters of reaction catalyzed by GDH: A) Hanes plot over estimating K_m^{AMF}, B) Hanes plot estimating V_{max}^{FDH} and $K_m^{NAD^+}$, C) Hanes plots of increasing NADH concentrations estimating $K_{i,FDH}^{NADH}$; D) Hanes plots of increasing ACP concentrations estimating K_i^{ACP}

Appendix 4D – An example of Matlab® code calculating $V_{max\_f,r}$ of ω-transaminase to achieve 95 % conversion within 24 h and as a function of cascade enzyme concentrations

```
clear all
close all
clc

Vmax = zeros(100,35);
for i = 1:35
    for j = 1:100

% Transaminse parameters
K       = 1/24800;  % [-]
Enz     = 1;        % [g L-1]
Vmax_f  = 10;       % [mmol min-1 L-1]
KmACP   = 0.54;     % [mmol L-1]
KmAla   = 1.07;     % [mmol L-1]
KmMBA   = 53.03;    % [mmol L-1]
KmPy    = 9.58;     % [mmol L-1]
KiMBA   = 1000;     % [mmol L-1]  no inhibition
KiAla   = 1000;     % [mmol L-1]  no inhibition
Vmax_r = Vmax_f / sqrt(K) * sqrt(KmMBA * KmPy / KmACP / KmAla);%[mmol min-1 L-1]

% Cascade parameters (LDH & GDH)
Enz1    = [8.6 8.7 8.8 8.9 9 9.1 9.2 9.3 9.4 9.5 9.6 9.7 9.8 9.9 10 10.1 10.2
10.3 10.4 10.5 10.6 10.7 10.8 10.9 11 11.1 11.2 11.3 11.4 11.5 11.6 11.7 11.8
11.9 12];       % [g L-1]
Vmax1   = 39.0251;  % [mmol min-1 g-1]
KmNADH  = 0.0266;   % [mmol L-1]
KmPyr   = 0.0365;   % [mmol L-1]
KiPyr   = 2.8728;   % [mmol L-1]
Kilac   = 5.1967;   % [mmol L-1]
KiNAD   = 0.6288;   % [mmol L-1]
KiAlan  = 83.9268;  % [mmol L-1]
Enz21   = [10 9.9 9.8 9.7 9.6 9.5 9.4 9.3 9.2 9.1 9 8.9 8.8 8.7 8.6 8.5 8.4 8.3
8.2 8.1 8 7.9 7.8 7.7 7.6 7.5 7.4 7.3 7.2 7.1 7 6.9 6.8 6.7 6.6 6.5 6.4 6.3 6.2
6.1 6 5.9 5.8 5.7 5.6 5.5 5.4 5.3 5.2 5.1 5 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1
4 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3 2.9 2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2
1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 1 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1];
% [g L-1]
Enz2    = Enz21/10;
Vmax2   = 53.4958;  % [mmol min-1 g-1]
KmNAD   = 0.1453;   % [mmol L-1]
Kmgluc  = 75.7494;  % [mmol L-1]
Kiglucl = 32.2336;  % [mmol L-1]
KiMBAc  = 13.9023;  % [mmol L-1]
KiNADH  = 0.3443;   % [mmol L-1]
Khy     = (10^-7*4.7*10^-2+10^-7*4*10^3+2.5*10^-4)*60; %[min-1]
Keqhy   = 1/0.185;
Khyr    = Khy/Keqhy; %[min-1]

par  = [K Enz Vmax_f Vmax_r KmACP KmAla KmMBA KmPy KiAla KiMBA Enz1(i) Vmax1
KmNADH KmPyr KiPyr Kilac KiNAD KiAlan Enz2(j) Vmax2 KmNAD Kmgluc Kiglucl KiMBAc
KiNADH Khy Keqhy Khyr];
```

```
init = [1000 30 0 0 0.1 0 0 550 0 0];
Vmax(j,i) = fminsearch(@cost,Vmax_f,[],par,init);
    end
end

function j = cost (Vmax_f, par, C)

par(3) = Vmax_f ;
[t,C] = ode15s(@F_enzyme,[0:10:1440],C,[], par);
yield = C(end,4) / 500 * 100;

r = (95 - yield);
j = r^2;

function dC = F_enzyme(t,C,par)

K       = par(1);
Enz     = par(2);
Vmax_f  = par(3);
Vmax_r  = par(4);
KmACP   = par(5);
KmAla   = par(6);
KmMBA   = par(7);
KmPy    = par(8);
KiMBA   = par(9);
KiAla   = par(10);
Enz1    = par(11);
Vmax1   = par(12);
KmNADH  = par(13);
KmPyr   = par(14);
KiPyr   = par(15);
Kilac   = par(16);
KiNAD   = par(17);
KiAlan  = par(18);
Enz2    = par(19);
Vmax2   = par(20);
KmNAD   = par(21);
Kmgluc  = par(22);
Kiglucl = par(23);
KiMBAc  = par(24);
KiNADH  = par(25);
Khy     = par(26);
Khyr    = par(27);

% Transaminase
r = (Enz  * Vmax_f * Vmax_r * (C(1,1) * C(2,1) - C(3,1) * C(4,1) / K)) / (Vmax_r
* KmACP * C(1,1) + Vmax_r * KmAla / K * C(2,1) + Vmax_f * KmMBA / K * C(3,1) +
Vmax_f * KmPy / K * C(4,1) + Vmax_r * C(1,1) * C(2,1) + Vmax_f * KmMBA / KiAla /
K * C(1,1) * C(3,1) + Vmax_f / K * C(3,1) * C(4,1) + Vmax_r * KmAla / KiMBA *
C(2,1) * C(4,1));

% Cascades
r1 = Enz1 * Vmax1 * C(5,1) * C (3,1) / ((KmPyr * (1 + C(7,1) / Kilac + C(1,1) /
KiAlan) + C(3,1) + C(3,1)^2 / KiPyr)*(KmNADH * (1 + C(6,1) / KiNAD) + C(5,1)));
r2 = Enz2 * Vmax2 * C(8,1) * C (6,1) / ((Kmgluc * (1 + C(9,1)/Kiglucl + C(4,1) /
KiMBAc) + C(8,1)) * (KmNAD * (1 + C(5,1) / KiNADH) + C(6,1)));
r3 = Khy * C(9,1);
```

```
r4 = Khyr * C(10,1);

if C(4,1) <= 470

% differential equations
dC(1,1) = -r;           % alanine
dC(2,1) = 0;            % ACP
dC(3,1) = r - r1;       % pyruvate
dC(4,1) = r;            % MBA
dC(5,1) = r2 - r1;      % NADH
dC(6,1) = r1 - r2;      % NAD
dC(7,1) = r1;           % lactate
dC(8,1) = -r2;          % glucose
dC(9,1) = r2 - r3 + r4; % glucono-1,5-lactone
dC(10,1)= r3 - r4;      % gluconate

else

% differential equations
dC(1,1) = -r;           % alanine
dC(2,1) = -r;           % ACP
dC(3,1) = r - r1;       % pyruvate
dC(4,1) = r;            % MBA
dC(5,1) = r2 - r1;      % NADH
dC(6,1) = r1 - r2;      % NAD
dC(7,1) = r1;           % lactate
dC(8,1) = -r2;          % glucose
dC(9,1) = r2 - r3 + r4; % glucono-1,5-lactone
dC(10,1)= r3 - r4;      % gluconate

end
```

Appendix 5A – Stability of the co-factor NADH in different buffers

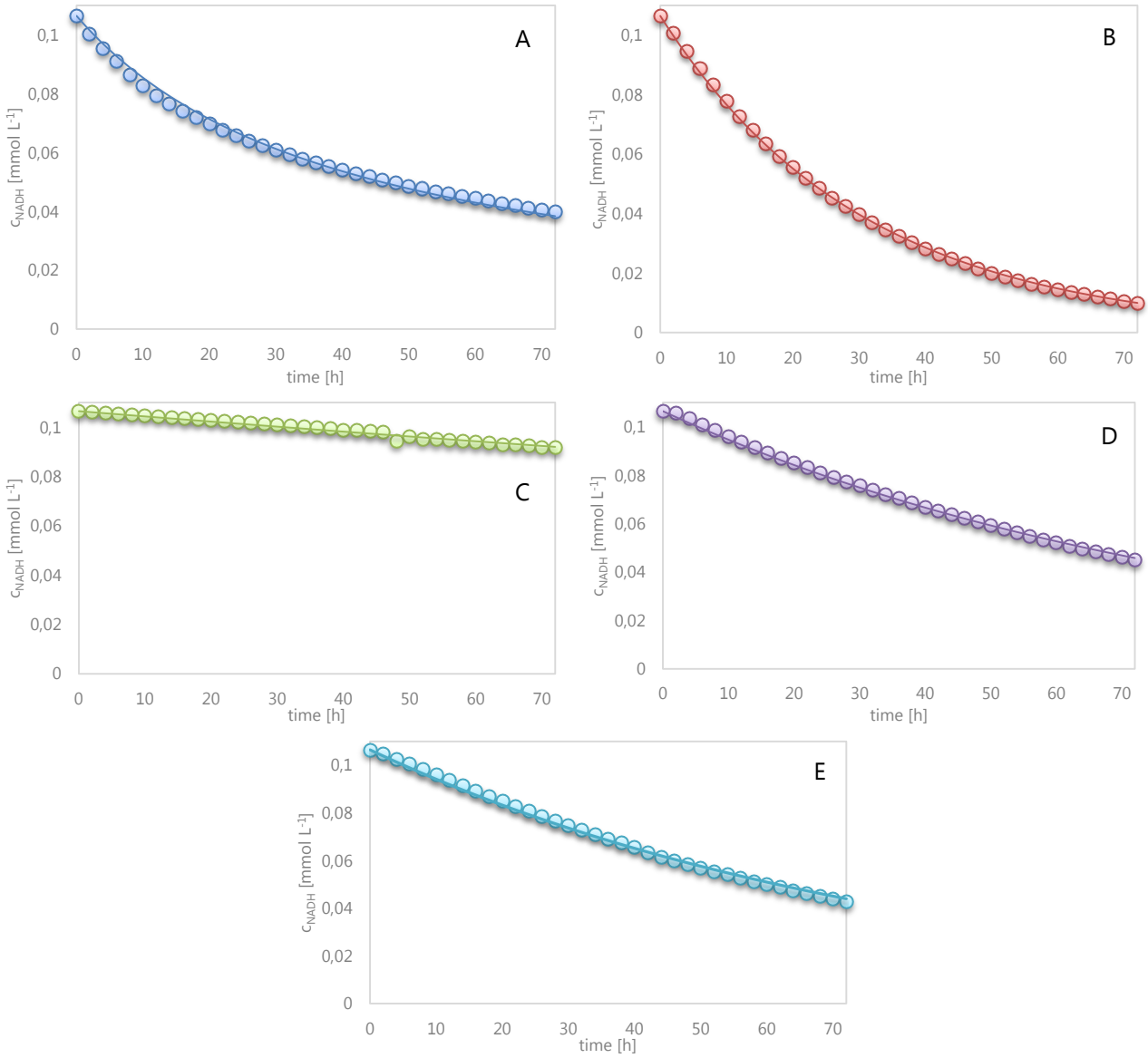

Figure 70 Stability of the co-factor NADH in different media (c_{buffer} = 100 mmol L^{-1}) at pH 7 and 30°C (○) – experiment, (-) – model: A) in water, fitted to second order kinetics $t_{1/2}$ = 40.67 h; in buffers following first order kinetics B) in phosphate buffer, $t_{1/2}$ = 21.09 h; C) in TRIS buffer, $t_{1/2}$ = 343.16 h; D) in hepes buffer, $t_{1/2}$ = 59.18 h; E) in wide-range[163] buffer, $t_{1/2}$ = 56.34 h

Appendix 7A – Inhibition profile of AlaDH by alanine

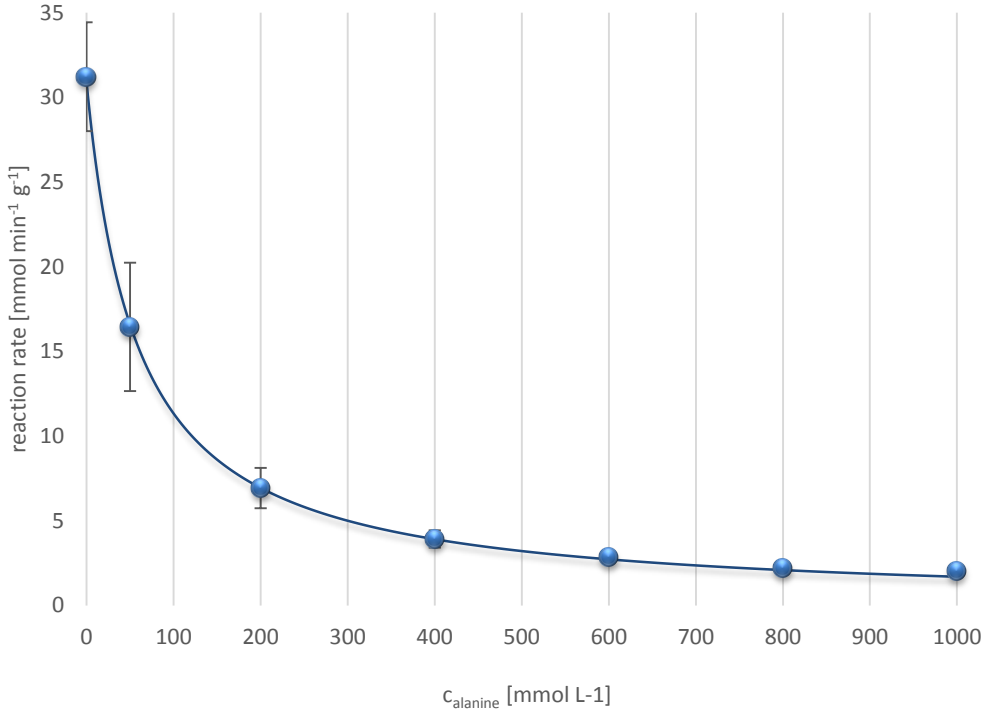

Figure 71 Inhibition profile of AlaDH by alanine at 30°C and pH 7 at $c_{pyruvate}$ = 1 mmol L^{-1}, c_{NADH} = 0.1 mmol L^{-1} and $c_{ammonium\ formate}$ = 250 mmol L^{-1}; (○) – experiment; (-) – model described by equation below:

$$r_{AlaDH} = \frac{V_{max}^{AlaDH} c_{NADH} c_{PYR} c_{AMF} \gamma_{LDH}}{\left(K_m^{NADH}\left(1 + \frac{c_{NAD^+}}{K_i^{NAD^+}}\right) + c_{NADH}\right)\left(K_m^{PYR}\left(1 + \frac{c_{ALA}}{K_i^{ALA}}\right) + c_{PYR} + \frac{c_{PYR}^2}{K_i^{PYR}}\right)\left(K_m^{PYR} + c_{AMF} + \frac{c_{AMF}^2}{K_i^{AMF}}\right)} \quad 1$$