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CONSIDERATIONS FOR IMPLEMENTATION OF NOVEL ENZYME-BASED PROCESSES

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DTU Chemical Engineering
Department of Chemical and Biochemical Engineering

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Abstract

Biocatalysis is the use of enzymes to catalyze chemical reactions. It is an established synthesis route in chemical synthesis, alongside conventional chemistry. Biocatalysis is often applied due to excellent regio and stereoselectivity, in addition to its environmentally benign properties. This thesis aims at increasing the potential use of industrial biocatalysis, both in terms of broadening its current use and expanding it to new applications. This academic study is carried out through two case studies. These two case studies were chosen because they represent each end of the spectra of biocatalytic applications. The first case study is expanding the use of an established biocatalyst. The second case study investigates the potential of a novel biocatalyst. In addition, the two case studies have very different implementation challenges, impeding current use. Therefore, arguably, the lessons learned from these two case studies justify general conclusions for biocatalysis, irrespective of their application. The work in this thesis therefore contributes, not only to industrial biocatalysis in these two areas, but also increases the understanding of biocatalysis as a whole.

The first section investigates the use of ω-transaminases (ω-TAs) in the pharmaceutical industry for new applications. ω-TAs are well-established biocatalysts in the synthesis of chiral amines. They catalyze an amino transfer reaction between an amino donor (donor) and amino acceptor (acceptor), which yields an amine product (product) and a keto co-product. Chapter 3 establishes the importance of donor selection. Firstly, by experimentally comparing the equilibrium constant(K_{eq}) of five reactions, where five common amine donors are reacted with the same acceptor. The K_{eq} varies greatly between the donors tested, from K_{eq} of 1 for the most favorable reaction to K_{eq} of 4 x 10^{-5} for the least favorable donor. Furthermore, a quantum mechanical method (QMM) is introduced as a tool to predict the thermodynamic yield of any acceptor/donor pair. This method successfully predicted the yield of ω-TA reactions. Finally, in Chapter 4, a methodology for rapid implementation of ω-TA reactions in small singular

batches is outlined and demonstrated. This method uses a three-step selection procedure to evaluate if a given reaction is suitable for simple scale-up. The 3 criteria evaluated is thermodynamics, enzyme activity and product inhibition. Other relevant properties such as down-stream processing and enzyme stability were accounted for through implementation of specific requirements in terms of biocatalyst loading and maximal reaction time, outlined in the method. The thermodynamic criterion is determined by K_{eq} and must be less than 0.02 (resolution reactions) or over 1 (synthetic direction). The activity criterion requires specific activity greater than 0.05 g/g/h (g product/g biocatalyst/hour). Finally, the inhibition criterion is met if less than 50% activity is lost when 5% of the target concentration product was present. This methodology is then successfully demonstrated by subjecting two target products 1-(4-bromophenyl)ethylamine and (*S*)-(+)-3-amino-1-Boc-piperidine to the methodology. One of the two products, (*S*)-(+)-3-amino-1-Boc-piperidine, passes the evaluation and is successfully run at 25 mL scale with initial acceptor concentrations of up to 75 g/L and up to 70% yield. It can be argued that the tools and results presented in this section could enable a more widespread use of ω-TAs, especially in applications where fast implementation is paramount, by reducing development time.

Section II examines the novel use of Carbonic Anhydrase (CA) in carbon capture and storage (CCS). CA is a highly efficient catalyst which hydrates CO_2 to yield bicarbonate. Current CCS methods often use primary amines as capture solvents due to high kinetic rates. A drawback of these solvents is that they suffer from high energy requirements during solvent regeneration. Therefore, it has been proposed to use different solvents, such as hindered/tertiary amines or inorganic salts, which does not carry the same energy penalty in solvent regeneration. However, the implementation of these solvents is impeded by slow kinetics. Therefore, the use of CA as a kinetic promoter can enable the use of such energy efficient solvents in CCS. A key challenge to implementation of CA in CCS is enzyme stability under process relevant conditions, especially during extended exposure. Therefore Chapter 7, investigates in detail the stability of one CA under such conditions. Three parameters, temperature, solvent type and pH is investigated both individually and cumulatively. It is concluded that temperature is the dominant factor in enzyme

deactivation under the conditions tested here. At 70 °C and over, enzyme deactivation occurred at a high rate. Furthermore, the CA used here is stable in the pH range of 7 - 11. However, extended exposure (150 days), reveals that a higher pH (pH 10) negatively affects enzyme stability. Finally, lower enzyme stability is correlated with a higher solvent pKa. In a capture facility, the highest temperatures are experienced desorption column. Therefore, Chapter 8 explores one strategy for preventing CA's from entering this area, namely ultrafiltration (UF). The potential impact of UF is investigated through a model. Three UF membranes with selectivity ranging from 90% to 99.9% and desorber temperatures ranging from 60 °C to >100 °C is modeled. The results show that UF is an efficient strategy to extend enzyme viability with respect to temperature. Higher temperatures in the stripper require more selective membranes. Furthermore, it is found that at temperatures above 100 °C, where instant deactivation is modeled, even the most selective membrane is not selective enough to retain high enzyme activity over time. UF may therefore not be a viable option for very high temperatures, or other conditions where instant deactivation is observed. The results in Section II indicate that CA's are technically feasible in CCS applications, with careful process design. However, a detailed economic assessment is needed in each case to evaluate if CA is an economically competitive alternative.

Although the two case studies here are very different, several underlying criteria are valid for both case studies, and arguably, can be extended beyond these case studies. Chapter 10 summarizes the lessons learned in this thesis, with a special focus on integrated process design. It is concluded that enzyme engineering, process engineering, and reaction engineering must be carried out simultaneously early in process development for optimal results.

Dansk Resumé

Biokatalyse er brugen af enzymer til at katalysere kemiske reaktioner. Biokatalyse er en etableret metode i kemisk syntese i tillæg til konventionel kemi. Biokatalyse anvendes tit, fordi det har en fremragende region og stereoselektivitet, samt miljøvenlige egenskaber. Denne afhandling har til formål at øge brugen af industriel biokatalyse, både ved at udvide den nuværende andvendelse og ved at udvikle biocatalyse til nye applikationer. Dette gennemføres gennem to casestudier. Disse to casestudier er valgt, fordi de repræsenterer hver sin ende af skalaen af biokatalytiske applikasjons områder. Det første casestudie udvider brugen af en etableret biokatalysator. Det andet casestudie undersøger potentialet ved en ny biokatalysator. Derudover har de to casestudier meget forskellige implementeringsudfordringer, som hindrer nuværende brug. Derfor kan erfaringerne fra disse også berettige generelle konklusioner for biokatalyse. Denne afhandling bidrager derfor ikke kun til øget industriel biokatalyse på disse to kerne-områder, men øger også forståelsen af biokatalyse generelt.

Det første casestudiet undersøger nye anvendelser af ω-transaminaser (ω-TA'er) i medicinalindustrien. ω-TA'er er veletableret biokatalysatorer i syntesen af chirale aminer, der katalyserer en aminotransferreaktion mellem en amindonor (donor) og aminaccepter (accepter), som giver et aminprodukt (produkt) og et keto-coprodukt. Kapitel 3 viser vigtigheden af donorvalg. Først ved, at eksperimentelt sammenligne ligevægtskonstanten (K_{eq}) ved fem reaktioner, hvor fem almindelige donorer reageres med den samme acceptor. K_{eq} varierer meget mellem de testede donorer, fra K_{eq} på 1 til den mest gunstige reaktion på K_{eq} på 4 x 10^{-5} for den mindst gunstige reaktion. Endvidere indføres en kvantemekanisk metode (QMM) som et værktøj til at forudsige termodynamikken af ethvert accepter/donorpar. Metoden beregnet med god nøjaktighed K_{eq} af et set ω-TA reaktioner. Til sidst vises og demonstreres en metode der tilrettelægger hurtig implementering af ω-TA reaktioner i små batcher i kapitel 4. Denne metode anvender en tre-trins udvælgelsesprocedure for at vurdere, om en given reaktion er egnet til simpel opskalering. De 3 kriterier, der er vurderet, er termodynamik, enzymaktivitet og

produktinhibering. Andre relevante egenskaber såsom oprensning og enzymstabilitet blev redegjort for gennem implementering af specifikke krav, med hensyn til biokatalysatoraktivitet og maksimal reaktionstid i fremgangsmåden. Det termodynamiske kriterium bestemmes af K_{eq}, og skal være er under 0.02 (resolveringsreaktioner) eller over 1 (syntetisk retning). Aktivitetskriteriet kræver specifik aktivitet over 0.05 g/g/h (g produkt/ g biokatalysator/time). Endelig indfries inhiberingskriteriet, hvis aktiviteten er reduceret med mindre end 50%, når 5% af koncentrationen af produktet var til stede. Denne metode demonstreres derefter ved at bruge to produkter 1-(4-bromphenyl)ethylamin og (S)-(+)-3-amino-1-Boc-piperidin. En af de to produkter, (S)-(+)-3-amino-1-Boc-piperidin, består evaluerings-kriterierne og skaleres så op til 25 mL med koncentrationer på op til 75 g/L og op til 70 % omdannelse. De værktøjer og resultater, der præsenteres i dette afsnit, kan muliggøre en mere udbredt anvendelse af ω-TA'er, især i applikationer, hvor hurtig implementering er afgørende.

Afsnit II undersøger den nye anvendelse af enzymet Carbonic Anhydrase (CA) i kulstofopsamling og -opbevaring (CCS). CA er en yderst effektiv katalysator, som hydrerer CO_2 til bicarbonat. Nuværende CCS-metoder bruger ofte primære aminer som opløsningsmidler, der har høje kinetiske hastigheder. En ulempe ved disse opløsningsmidler er, at de lider af et højt energibehov under regenereringen. Det er derfor blevet foreslået, at anvende andre opløsningsmidler, som hindrede/tertiære aminer eller uorganiske salte, der ikke har samme energibehov ved regenerering. Implementeringen af disse opløsningsmidler begrænses nu af langsom kinetik. Derfor kan brug af CA som en kinetisk promoter muliggøre brugen af disse energieffektive opløsningsmidler i CCS. En udfordring for brug af CA i CCS, er enzymstabilitet under procesrelevante betingelser, især under længere eksponering. Derfor undersøger kapitel 7 i detalje stabiliteten af CA under disse forhold. Tre parametre, temperatur, opløsningsmiddeltype og pH undersøges både individuelt og kumulativt. Det konkluderes, at temperatur er den dominerende faktor ved enzymdeaktivering under disse betingelser. Ved 70 °C og derover var enzymdeaktiveringen høj. Desuden er den CA, der anvendes her, stabil i pH-området på 7-11. Udvidet eksponering (150 dage) viser imidlertid, at en højere pH (pH 10) påvirker enzymstabiliteten negativt. Endelig vises det at der er sammenhæng mellem lavere

stabilitet og højere pKa for opløsningsmiddel. I et CCS anlæg er de højeste temperaturer i desorptionskolonnen. Kapitel 8 udforsker derfor en strategi der forhindrer CA at komme ind i dette område, nemlig ultrafiltrering (UF). Effekten af UF undersøges gennem en model. Tre UF membraner med selektivitet fra 90% til 99,9% og desorber temperaturer fra 60 ° C til> 100 ° C er modelleret. Resultaterne viser, at UF er en effektiv strategi til at forlænge enzymets levedygtighed med hensyn til temperatur. Højere temperaturer kræver mere selektive membraner. Endvidere konstateres det, at ved temperaturer over 100 ° C, hvor øjeblikkelig deaktivering er modelleret, er selv den mest selektive membran ikke selektiv nok til at opretholde høj enzymaktivitet. UF er måske derfor ikke en god løsning for meget høje temperaturer eller andre forhold hvor øjeblikkelig deaktivering observeres. Resultaterne i afsnit II viser, at brug af CA er teknisk muligt i CCS, med nøjaktigt procesdesign. Der er imidlertid behov for en detaljeret økonomisk vurdering, i hvert enkelte tilfælde, for at vurdere, om CA er et økonomisk konkurrencedygtigt alternativ.

Selvom de to casestudier her er meget forskellige, gælder flere underliggende kriterier for begge casestudier, hvilket også kan bruges ud over disse casestudier. Kapitel 10 opsummerer erfaringerne i denne afhandling med særlig fokus på integreret procesdesign. Det konkluderes med at enzymteknik, procesteknologi og reaktionsteknik bør udføres samtidig og tidligt i procesudviklingen for at opnå de bedste resultater.

Preface

The work presented in this thesis was mainly conducted in the PROSYS research center at the Department of Chemical and Biochemical Engineering, Technical University of Denmark as partial fulfillment of the requirements for a Ph.D. degree in Engineering. The Work was performed between September 2013 and October 2017 and was supervised by Professor John Woodley and co-supervised by Associate Professor Nicolas von Solms. The work has been in part been performed within the INTERACT European Union Seventh Framework Programme FP7/2007 2013 under grant agreement n° 608535, and the AMBIOCAS European Union 7th Framework Programme (Grant agreement no: 245144).

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Til Élise,

Liten?
Jeg?
Langtifra.
Jeg er akkurat stor nok.
Fyller meg selv helt
På langs og på tvers
fra øvert til nederst.
Er *du* større enn deg selv
kanskje?

-Inger Hagerup

List of papers

Published papers during the Ph.D. included in this thesis:

Gundersen, M. T., Abu, R., Schürmann, M. and Woodley, J. M. (2015) 'Amine donor and acceptor influence on the thermodynamics of ω-transaminase reactions', Tetrahedron: Asymmetr., 26(10–11), pp. 567–570.

Meier, R. J., Gundersen, M. T., Woodley, J. M. and Schürmann, M. (2015) 'A Practical and Fast Method to Predict the Thermodynamic Preference of ω-Transaminase-Based Transformations', ChemCatChem, 7(17), pp. 2594–2597.

Gundersen, M. T., Tufvesson, P., Rackham, E. J., Lloyd, R. C. and Woodley, J. M. (2016) 'A Rapid Selection Procedure for Simple Commercial Implementation of ω-Transaminase Reactions', Org. Process Res. Dev., 20(3), pp. 602–608.

Gundersen, M. T., von Solms, N. and Woodley, J. M. (2014) 'Enzymatically Assisted CO_2 Removal from Flue-gas', Energy Proced., 63, pp. 624–632.

Gundersen, M. T., Gladis, A., Loldrup Fosbøl, P., von Solms, N. and Woodley, J. M. (2017) 'Operating considerations of ultrafiltration in enzyme enhanced carbon capture', Energy Proced., 114, pp. 735–743.

Published papers during the Ph.D. not included in the thesis:

Grabner, B., Nazari, M., Gundersen, M. T., Loïs, S., Fantini, S., Bartsch, S., Woodley, J. M., and Gruber-Woelfler, H. 'Room-Temperature Solid Phase Ionic Liquid (RTSPIL) Coated ω-Transaminases in Organic Solvents', ChemSusChem, Submitted

Gladis, A., Gundersen, M. T., Fosbøl, P. L., Woodley, J. M. and von Solms, N. 'CO_2 Mass transfer model for Carbonic Anhydrase-enhanced aqueous MDEA solutions', Chem. Eng. J., 335, pp. 197-208

Gladis, A., Gundersen, M. T., Thomsen, K., Fosbøl, P. L., Woodley, J. M., von Solms, N. (2017) 'Comparison of the Kinetic Promoter Piperazine and Carbonic Anhydrase for CO_2 Absorption' Energy Proced., 114, pp. 719-725

Voges, M., Abu, R., Gundersen, M. T., Held, C., Woodley, J. M., and Sadowski, G. (2017) 'Reaction Equilibrium of the ω-Transamination of (S)-Phenylethylamine: Experiments and ePC-SAFT Modeling', Org. Process Res. Dev., 2017, 21 (7), pp. 976–986

Gladis, A., Gundersen, M. T., Fosbøl, P. L., Woodley, J. M. and Von Solms, N. (2017) 'Influence of temperature and solvent concentration on the kinetics of the enzyme carbonic anhydrase in carbon capture technology', Chem. Eng. J., 309, pp. 772–786.

Abu, R., Gundersen, M. T., Woodley, J. M. (2015) 'Thermodynamic Calculations for Systems Biocatalysis', Proceed. 25th Eur. Symp. Comp. Aid. Process Eng., pp. 233–238.

Kunze, A. K., Penner, V., Gundersen, M. T., Woodley, J. M., Górak, A., Lutze. P., 'Biocatalysts for enhanced CO_2 post-combustion capture efficiency in absorption columns', 10^{th} International Conference on Distillation & Absorption

Abbreviations and Nomenclature

Abbreviations

Acceptor	Amino Acceptor
ACP	Acetophenone
AIB	2-Aminoisobutyric Acid
Ala	Alanine
AMP	2-Amino-2-Methyl-1-Propanol
API	Active Pharmaceutical Ingredient
AS	Asymmetric Synthesis
CA	Carbonic Anhydrase
CCS	Carbon Capture and Storage
CDIAC	Carbon Dioxide Information Analysis Center
CHA	Cyclohexylamine
CLM	Contained Liquid Membranes
DE	Reaction Energy
DEA	Diethanolamine
DKR	Dynamic Kinetic Resolution
DMAE	Dimethylaminoethanolamine
DOE	Department of Energy
Donor	Amino Donor
e.e.	Enantiomeric Excess
EECCS	Enzyme Enhanced Carbon Capture and Storage
EOR	Enhanced Oil Recovery
GHG	Greenhouse Gas
IPA	Isopropylamine
IPCC	Intergovernmental Panel on Climate Change
ISPR	*In Situ* Product Removal
K_2CO_3	Potassium Carbonate
KR	Kinetic Resolution
KRED	Ketoreductase
LbL	Layer by Layer
MAPA	3-(Methylamino)Propylamine
MBA	α-Methybenzylamine

MDEA	N-Methyldiethanolamine
MEA	Monoethanolamine
NDA	3,5,7-Nonadiene-2-Amine
NETL	National Energy Technology Laboratory
PCCC	Post-Combustion Carbon Capture
PEA	1-Phenylethylamine
PLP	Pyridoxal 5'-Phosphate
PMP	Pyridoxamine 5'-Phosphate
PNP	Para-Nitro-Phenyl
QM	Quantum Mechanic
UF	Ultrafiltration
V0	Reaction Rate
Vmax	Reaction Rate at Saturation
WGIII	Working Group III
ω-TA	ω-Transaminase

Nomenclature

Nomenclature	Description	Units
e.e.	Enantiomeric Excess	%
E_0	Enzyme Concentration	mM
K	Predicted Yield	%
K_{cat}	Turnover Number	s^{-1}
K_{eq}	Thermodynamic Equilibrium Constant	Dimensionless
K_{eq}^{app}	Apparent Equilibrium Constant	Dimensionless
K_L	Liquid Side Mass Transfer Coefficient constant	Dimensionless
K_M	Michaelis-Menten Constant	mM
LCOE	Levelised Cost of Energy	$ (USD)
pKa	Acid Dissociation Constant	Dimensionless
[S]	Substrate Concentration	mM
V_0	Reaction Rate	mM/s
V_{max}	Reaction Rate at Saturation	mM/s
ΔG	Gibbs Free Energy	J
ΔH°	Heat of Reaction	J

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Chapter 3: Donor selection in ω-transaminase reactions

Section II

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Chapter 9: Conclusion and future work, Section II

Summary

Chapter 10: Lessons learned

Appendix A

Included Publications

Chapter 1:
Introduction to Thesis

This chapter presents the thesis as a whole and introduces relevant topics. Several of the topics covered in this chapter are reoccurring throughout the thesis, it is therefore important that the reader has a basic understanding of these topics.

1.1 Scope of the thesis

The objective of this thesis is to enable a more widespread use of biocatalysis by bridging the gap between academic research with industry. This is done by giving several hands-on tools for easy implementation of biocatalysis. This thesis covers biocatalysis in industry through two very different case studies, the use of ω-transaminases (ω-TAs) in pharmaceutics, and carbonic anhydrase (CA) in carbon capture. These two case studies were chosen because they represent each end of the spectra of biocatalytic processes. The hypothesis of this thesis is that investing these two diverse case studies does not only add the value of the conclusions drawn from the specific studies but furthermore, permits general conclusions about industrial biocatalysis to be drawn.

Section I, investigates the use of ω-TAs in the pharmaceutical industry. A well-established biocatalytic route to chiral amines for commercial pharmaceuticals. Here the implementation ω-transaminases (ω-TAs) beyond its current use is investigated, particularly at small singular batches in early stages of clinical trials, where long development time is limiting its implementation. Facilitating the use of ω-TAs in for such applications is here facilitated by developing substrate selection tools (Chapter 3) and guidelines for commercial implementation of transaminase based reactions (Chapter 4). The use of ω-TAs is a high value, small-scale

application, where fast implementation and high substrate purity is paramount (Gundersen *et al.*, 2015, 2016; Meier *et al.*, 2015).

Section II, covers the use of carbonic anhydrase in enzyme enhanced carbon capture and storage (EECCS). A novel technology, which is yet to be implemented on a commercial scale. EECCS is one possible solution to reducing global greenhouse gas (GHG) emissions. EECCS has the potential to enable the use of low energy solvents, and thereby reducing the carbon footprint of the process. Here, several challenging aspects of EECCS is evaluated and discussed. Both enzyme stability (Chapter 7) and process strategies to extend the lifetime of active enzymes (Chapter 8) were investigated. Due to the large scale and low economic value of carbon dioxide, the cost of enzymes and the process must be very low to justify the use of this technology. This requires simple solutions and very stable enzymes under operating conditions. The use of carbonic anhydrase in carbon capture is a low value, large-scale application of biocatalysis (Gundersen, von Solms and Woodley, 2014; Gundersen *et al.*, 2017).

Although these two case studies are different, they share some common underlying features. In both case studies, and arguably for industrial biocatalysis in general, it was found that integrated process design was paramount for success. It is concluded that enzyme engineering, process engineering, and reaction engineering should ideally be carried out simultaneously. A summary of common lessons learned from the two case studies is presented in Chapter 10.

1.2 Enzymes

Enzymes are biological catalysts. In Nature, enzymes catalyze a plethora of vital chemical reactions. Enzymes are optimized to the delicate environment of a cell. They are highly selective, and can often catalyze reactions at very low concentrations. The environment of a cell is maintained at a steady temperature and pH. Enzymes are however technically no different than any other catalyst. They are agents that speed up the reaction rate of a reaction, without being consumed in the process (Berg, Tymoczko and Stryer, 2006). This is done by stabilizing the transition state of the reaction (Golynskiy and Seelig, 2010). Biocatalysts do not alter the physical

properties of a reaction to any great extent, with a few notable exceptions. Biocatalysts do for example not alter the thermodynamics of a given reaction or the solubility of most reactants. However, proteins can act as surfactants and cause foaming, which is discussed in section II.

Biocatalysis is taking enzymes out of their natural environment and applying them in a controlled fashion (Bornscheuer *et al.*, 2012). In this thesis, biocatalysis is defined in its broadest context, where any non-natural applications of enzymes or other biocatalyst are defined as biocatalysis. Including, but not limited to, the use of whole cells in brewing, to engineered isolated enzymes in pharmaceuticals. Biocatalysis is a well-established method of catalysis in many fields, with a few hundred industrial examples (Woodley, 2013; Salmon and House, 2015). A broad range of available reactions are being used, which is continuously increasing (Clouthier and Pelletier, 2012; Truppo, 2017). The enzyme market is assumed to be around 10% of the catalysis market, about 3.4 Billion USD in 2012 (Milmo, 2012). Biocatalysis is often used due to its excellent regio- and stereoselectivity, mild reaction conditions, simpler chemistry or because it can by-pass several steps used in conventional chemistry, all of which contributes to the economic benefit of using enzymes over other catalysts in the selected processes (Bornscheuer *et al.*, 2012; Woodley, 2013; Lima-Ramos, Tufvesson and Woodley, 2014; Truppo, 2017). Furthermore, biocatalysis can offer an advantage when conventional chemistry methods causes hazardous conditions or safety concerns, and in replacing rare transition metal catalysts (Lima-Ramos, Tufvesson and Woodley, 2014; Woodley, 2017). Although the pharmaceutical industry is dominating the field of biocatalysis, it is also used extensively in other areas as well, such as the fine chemical and food industry (Truppo, 2017) a few selected applications are discussed below.

When applying enzymes outside of their natural environment, it should be kept in mind, that they are often applied in conditions that are far from their native environment. Enzymes used in industrial biocatalysis are subjected to physical conditions that are very different from the environment of a cell. Industrial biocatalysts are often exposed to conditions such as organic solvents, high substrate and product concentrations, high salt concentrations, heterogeneous temperatures and sub-optimal pH conditions (Wohlgemuth, 2010; Huisman and Collier, 2013; Woodley, 2013).

Enzymes used in biocatalysis can be applied as isolated enzymes, crude enzyme extract or as whole cells. The use of isolated enzymes is now dominating the biocatalysis field, especially in the food and pharmaceutical industry, in part due to reproducibility and easier implementation (Truppo, 2017). However, each formulation comes with its advantages and disadvantages, which is further discussed in section 1.4.2.2, below.

Industrial application of enzymes are often limited by low enzyme stability under process relevant conditions, low catalytic activity for a specific substrate, substrate and/or product inhibition at high substrate and/or product concentrations (Golynskiy and Seelig, 2010; Huisman and Collier, 2013). Furthermore, it is important to consider the economic requirements of industrial synthesis.

1.3 Examples of biocatalysis

1.3.1 Biocatalysis for commodity chemicals

Although enzymes may at first appear as highly technical, and therefore costly catalysts only suitable for specialized applications, this is not always the case (Woodley, Breuer and Mink, 2013). Numerous commodity chemicals are made using enzymes (Sheldon, 2014). Although the pharmaceutical industry often uses highly specialized and evolved enzymes, another sector of the enzyme industry, dominated by proteases and lipases, uses enzymes on a bulk scale. A well-known household example of biocatalysis is the use of proteases in laundry detergents, which along with food applications currently dominates the enzyme market by volume (Milmo, 2012). The use of industrial lipases was first enabled by stabilization through directed evolution (Estell, Graycar and Wells, 1985). Other well-known examples of biocatalysis in bulk is the use of glucose isomerase to convert glucose to fructose, to enhance flavor (Jensen and Rugh, 1987). Finally, penicillin-G acylase is used to make semi-synthetic antibiotics (Bruggink, Roos and de Vroom, 1998).

1.3.2 Enzymes in the pharmaceutical industry

In the pharmaceutical industry, enzymes are often used specifically because of their high regio and stereoselectivity, to produce chiral medications. A notable example of this is the synthesis of an intermediate of the drug Atorvastatin, with the use of a ketoreductase (KRED) (Ma *et al.*, 2010). Described here is one of several chemo enzymatic routes towards Atorvastatin (Ma *et al.*, 2010; Bornscheuer *et al.*, 2012). Atorvastatin is the active ingredient of the drug commercially known as Lipitor, a cholesterol-lowering drug, with annual sales of over 10 billion USD at its peak (Ma *et al.*, 2010; Bornscheuer *et al.*, 2012). The development of this biocatalytic process won the Presidential Green Chemistry Challenge Award in 2006 (Bornscheuer *et al.*, 2012). It is a multi-enzyme process that was marketed as 'green by design', it was deemed greener than the synthetic routes because it used milder conditions, had fewer by-products and less waste. (Ma *et al.*, 2010; Clouthier and Pelletier, 2012). A KRED was used to selectively reduce one of two neighboring ketones to a chiral alcohol, to regenerate the NADPH/NADP co-factor a glucose dehydrogenase was used, and finally, a halogen dehalogenase was used to substitute a chlorine with cyanide.

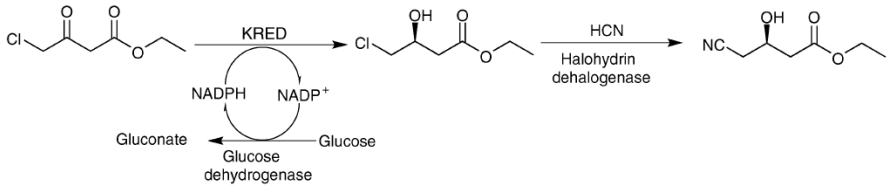

Figure 1.1: Overview of the Atorvastatin process, adapted from Clouthier and Pelletier, 2012.

Other examples of the use of biocatalysts in the pharmaceutical industry include ω-transaminase in the production of Sitagliptin, a diabetic drug (Savile *et al.*, 2010), covered in detail in Chapter 2. The reduction of a ketone to a chiral alcohol with a KRED to synthesize an intermediate of the asthma medicine Montelukast (Liang *et al.*, 2010). Recently FDA approved, Niraparib for treating ovarian cancer. The biocatalytic route to Niraparib uses a ω-transaminase to catalyzed the racemic resolution of a late stage intermediate of the drug (Chung *et al.*, 2014).

1.4 Tools in biocatalysis

To enable the use of enzymes in industry, whether in bulk for low-cost applications such as laundry detergents, or for blockbuster drugs as Atorvastatin, it is necessary to optimize the enzymes and their use for maximal benefit. This section describes some of the most important tools for enabling the widespread use of biocatalysis. This is not an exhaustive list, but contain the most important tools and tools that are particularly relevant to this thesis.

1.4.1 Optimizing enzymes

Any application of enzymes outside of their natural habitat requires enzymes that are both capable of catalyzing the reaction of interest and are stable under process relevant conditions. Two methods of obtaining such enzymes are outlined here: Enzyme engineering and genome mining.

1.4.1.1 Enzyme engineering

Enzyme engineering can enable a suitable enzyme for a particular process by altering the amino acids in the protein of interest (Bornscheuer *et al.*, 2012). It has been called the single most important enabler of biocatalysis (Truppo, 2017). Enzyme engineering starts by defining the target conditions and optimizing the enzymes towards that target. It enables us to modify the enzyme to fit production needs, rather than altering the process to fit the enzyme (Bornscheuer *et al.*, 2012; Lima-Ramos, Neto and Woodley, 2014; Truppo, 2017). Enzymes are often engineered towards increased thermostability, catalytic activity, substrate specificity or stereoselectivity (Golynskiy and Seelig, 2010; Clouthier and Pelletier, 2012). Enzyme engineering can take a long time, for example, the highlighted example of engineering a transaminase towards the synthesis of a Sitagliptin intermediate (see Chapter 2 for details) took over a year (Truppo, 2017). This enzyme was engineered by industry leaders in the field (Savile *et al.*, 2010).

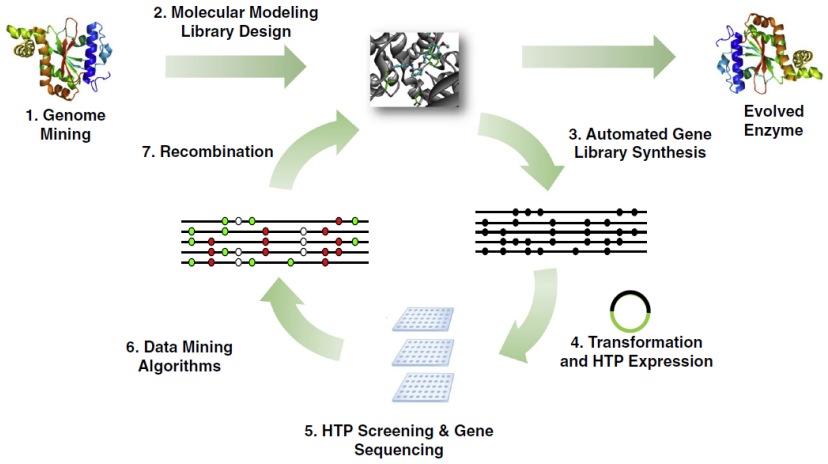

Figure 1.2: Integrated enzyme engineering methodology. Adapted from Lalonde, 2016.

Several methods of engineering an enzyme can be used. Directed evolution tries to optimize specific traits by using random mutagenesis, where non-specific amino acids are exchanged in a protein, using methods such as error-prone pCR and gene shuffling to increase genetic variation in the protein (Golynskiy and Seelig, 2010). This is a simple method of altering an enzyme. However, since it's a non-specific method, it requires very large screening capacity. For example, if 2 amino acids are to be exchanged anywhere on a 200 amino acid enzyme, there are over 7 million possibilities (Bornscheuer *et al.*, 2012). Of which many of the possibilities are either unchanged or inactive. If the number of mutations is increased to 3 amino acids, the possibilities are a staggering 9 billion options (Bornscheuer *et al.*, 2012). It is therefore clear that either very efficient methods for screening or more targeted methods are needed. There are two main screening methods used for enzyme engineering, the most efficient is 'selection' method which has a capacity of 10^9-10^{13} (Golynskiy and Seelig, 2010), where an organism's survival depends on the function of the enzyme, this depends on that only the variants that are viable can survive the selection. Although this is a very efficient method of screening, it is often not suitable for the target product. Therefore, 'screening' methods are most often employed, where depending on

the type of screening used the capacity can range from a few hundred variants to over 1 million, here each variant is investigated separately for a specific trait(Golynskiy and Seelig, 2010).

Since selection methods are not usually available, a more targeted approach: 'rational design' has been developed. It is a knowledge-based approach, based on a good understanding of the enzyme, by a 3-dimensional crystal structure and knowledge about the substrate binding site and the transition state (Golynskiy and Seelig, 2010; Bornscheuer *et al.*, 2012). This technology has been enabled by cheaper sequencing and increased computing power (Bornscheuer *et al.*, 2012). Selected amino acids may then be targeted and exchanged. For example, if 2 specific amino acids are targeted, and all possibilities are tested, only 400 variants are generated, compared to the 7 million with random insertion. Clearly, this becomes a less daunting task for screening. Furthermore, *in silico* methods can be used to model the effect of the mutations before they are tested. Enzyme engineering has also benefited from streamlined methods and automation, which is speeding up the process. However, due to the complexity of the task enzyme engineering remains a difficult challenge. Several of the enzymes used this thesis was engineered.

1.4.1.2 Genome mining

Genome mining is a method that can be used to find natural enzymes from a variety of organisms that either has a specific activity or that are stable under certain process conditions. Two main approaches are used. Firstly, sequencing methods can be used where, large amounts of DNA are sequenced, and bioinformatics methods are used to identify functionality. Alternatively, functionality screens can be used to find enzymes with a specific activity in a sample space. Genome mining is particularly useful when a suitable enzyme for the process conditions needed are difficult to obtain. For example, finding a thermostable enzyme with a known function, such as carbonic anhydrase in Section II, where either of the two methods above could be used to search for thermostable variants in thermophile organisms. Or in certain processes, for example in the food industry, where enzyme engineering may be prohibited. Finally, the bioinformatics data obtained in genome mining might also be used in enzyme engineering. For example, if a

specific amino acid is highly conserved among species in an enzyme, it is likely that that amino acid should not be modified. Methods for enzyme mining is rapidly improving, furthermore, large-scale sequencing efforts are also enabling a widespread use of bioinformatics to obtain new isozymes. In this thesis enzyme mining is particularly relevant in Section II, where Carbonic Anhydrases are mined from thermophiles to obtain thermostable enzymes.

1.4.2 Process engineering

This section will include some of the most important tools in process engineering and those most relevant to the thesis. This is not an extensive list of all possible process engineering tools. Process engineering can help in overcoming limitations, which cannot be solved with enzyme engineering, such as thermodynamics. Furthermore, integrated process engineering with enzyme engineering can aid in increasing the product concentration, a particularly difficult target to reach with biocatalysis (Lima-Ramos, Neto and Woodley, 2014; Woodley, 2017). Several other process metrics such as high biocatalyst yield (g product/g catalyst), and high space-time yield (g product/L/h), also known as productivity, is also often challenging to obtain with biocatalysis. Often these targets can only be reached through good process engineering (Lima-Ramos, Tufvesson and Woodley, 2014).

1.4.2.1 Enzyme Immobilization

Enzyme immobilization is one of the most important facilitators of biocatalysis after protein engineering (Truppo, 2017). Enzyme immobilization is the attachment of an enzyme onto a carrier or surface, in a matrix or self-cross-linked (Sheldon, 2007). Enzyme immobilization is used to separate enzyme from products, to recycle enzymes for cost benefits, increase stability, enable the use of organic solvents and to restrict enzymes to certain areas of the process (Tischer and Kasche, 1999; Sheldon, 2007). A drawback of immobilization is that it increases mass transfer resistance, which in turn reduces reaction rates. This phenomenon is most dominant with enzymes with high reaction rates and in settings that inhibits diffusion rates, such as enzyme immobilization in gel membranes (Tischer and Kasche, 1999). Furthermore, although enzyme

immobilization may reduce operating costs, due to the advantages listed above, enzyme immobilization will also increase the direct the cost of the biocatalyst. One study estimated a 4 fold increase in costs, by adding immobilization (Tufvesson *et al.*, 2011). However, this is highly dependent on the cost of the enzyme and the type of immobilization. Although enzyme immobilization is applied across a broad range of applications, a uniform general method for implementation and selection is not yet in place. It is often found that immobilization methods with good performance are often too technically specialized and costly to be uniformly applied. For example, in this project, the use of magnetic nanoparticles was used as a method of immobilization (data not shown). The beads are firstly too costly to be applied on a large scale, and furthermore requires a His-tagged protein for binding and is therefore not universally applicable. Improvements in immobilization are highly sought after and has been called for by industry (Truppo, 2017).

1.4.2.2 Enzyme formulation

Enzymes in biocatalysis are generally applied in 3 main formulations. Whole cells, crude enzyme extract, and isolated enzymes.

Whole cells are the simplest form of enzyme formulation, it is the least costly and most available form. Due to the protective environment of the cell, enzymes are typically more stable in this formulation than in other formulations (Lima-Ramos, Neto and Woodley, 2014). However, due to certain drawbacks, such as mass transfer limitations across cell membranes, the use of whole cells are often haunted by low product concentrations, often resulting in unfavorable economics. However, a notable exception, by Meadows and colleagues, found that whole cells gave comparable yields with a commercial liquid formulation, in the chiral synthesis of (*S*)-1-(5-fluorpyrimidin-2-yl)ethylamine, an intermediate of the JAK2 inhibitor AZD1480 (Meadows *et al.*, 2013). Furthermore, the in carbon capture whole cells are used with algae-based capture to produce fuels or fine chemicals (Klinthong *et al.*, 2015; Seth and Wangikar, 2015).

Crude enzyme extract is simply the cell lysate without further modification. This eliminates the cross-membrane barrier, but thereby also removes the protective environment of

the cell. Due to the presence of other enzymes in the cell lysate, it could mean that other unwanted side-reactions are also catalyzed. Added complexity in purification might be added if a pure product, such as a pharmaceutical is needed. Finally, as seen in Section II of this thesis, crude enzyme extract might have unwanted physical properties, such as foaming.

Finally, isolated enzymes can be used, this is purified, most often recombinant, enzymes. Isolated enzymes are advantageous because they are easier to remove and less enzyme is used due to their high activity/kg (Bornscheuer *et al.*, 2012). The high concentration also allows for easier shipment, and as with crude enzyme extract, no diffusion limitations due to cell membranes are found (Bornscheuer *et al.*, 2012). In pharmaceutical biocatalysis, isolated enzymes are dominating. It allows for easier and faster implementation and gives the option of using biocatalysis early in the development of a new drug (Truppo, 2017).

1.4.2.3 In Situ Product removal (ISPR)

ISPR is a technique where a product or co-product is removed simultaneously as the reaction is carried out. This may alleviate product or co-product inhibition of the enzyme and can improve the thermodynamics of a reaction. This is particularly important with thermodynamically challenged reactions, such as the ω-transaminase reaction discussed in Section I (Tufvesson *et al.*, 2015). ISPR techniques used in biocatalysis can, for example, be product removal of a volatile compound (Tufvesson, Bach and Woodley, 2014), precipitation driven synthesis (Ulijn *et al.*, 2001; Ulijn and Halling, 2004) and two-phase systems (Shin and Kim, 1997; Meadows *et al.*, 2013). Furthermore, in biocatalytic reactions, a cascade reaction, a reaction carried out by a second enzyme, can be used to convert a co-product to another chemical. Thereby relieving thermodynamic constraints, in addition to alleviating co-product inhibition (Fesko *et al.*, 2013; Fuchs, Farnberger and Kroutil, 2015). ISPR techniques need to be adapted to the reaction in question. Recently, the use of distillation based ISPR was used to increase yield of the chiral product of (*S*)-2-Pentanol, albeit with a lipase. This is the first time distillation has been used with biocatalysis, it the ISPR toolbox in biocatalysis is still advancing, and more such advances

can be expected in the future, alleviating challenges like thermodynamics and inhibition in biocatalysis (Kühn, 2017).

1.4.2.4 Fed-batch

Fed-batch is a simple technique where one or more substrates are added to the reactor as the reaction advances. This technique may aid in keeping the substrate soluble, limit substrate inhibition, and reduce substrate degradation in the case of an unstable substrate (Woodley, 2013, 2017). Due to the lower concentration of substrate in the reactor, fed-batch may negatively impact kinetics, however, this is not likely when it is applied to overcome substrate inhibition. In this Chapter 4, fed-batch was considered a simple method of overcoming substrate inhibition for the ω-TA reaction.

1.5 Conclusion

The availability of new and more stable enzymes is rapidly expanding. It is likely that with the expansion of *de novo* computational design of enzymes, enzymes will be able to catalyze many reactions not possible today. With good screening methods and early integration of process design, fast implementation of biocatalysis could enable a widespread use.

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Section I

Section I

Chapter 2: Introduction to ω-transaminases

2.1 Introduction

ω-Transaminases (ω-TAs, E.C. 2.6.1.18), also known as amino aminotransferases, reversibly catalyzes the amination of ketones, by transferring an amino group from an amino donor (donor) to an amino acceptor (acceptor). ω-TAs are in subgroup II, out of 4 subgroups of transaminases (Malik, Park and Shin, 2012). ω-TAs have over the last decade gained widespread use both in academia and industry, particularly in pharmaceuticals, due to exceptional regio and stereoselectivity, where few alternatives are available (Mathew and Yun, 2012; Fuchs, Farnberger and Kroutil, 2015; Tufvesson *et al.*, 2015; Guo and Berglund, 2017). ω-TAs are particularly useful because they have a broad substrate spectra, and can provide a single step amination of ketones, keto acids and aldehydes (Kaulmann *et al.*, 2007; Koszelewski *et al.*, 2010; Mathew and Yun, 2012; Paul *et al.*, 2013).

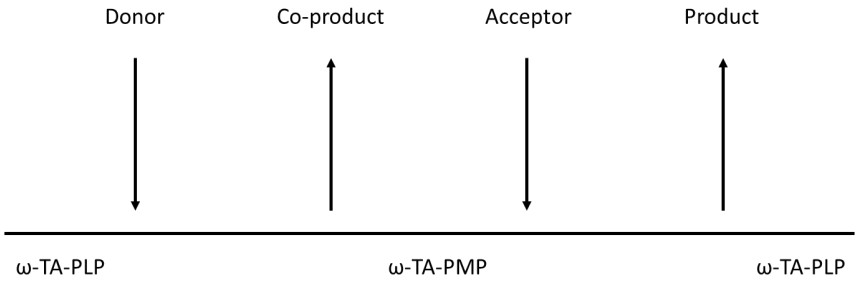
Transaminases were first discovered by Schoenheimer in 1942, 3 years after Braunstein in 1939 had confirmed the transfer of an amine moiety between a α-keto acid and an amino acid (Mathew and Yun, 2012). ω-TAs were off to a slow start and were only industrially applied more than 50 years later, in the early 1990's by Celgene. Industrial use of the enzyme was subsequently followed by academic interest (Shin and Kim, 1997). The early use of ω-TAs was mostly focused on kinetic resolution (Malik, Park and Shin, 2012).

2.1.1 Reaction mechanism

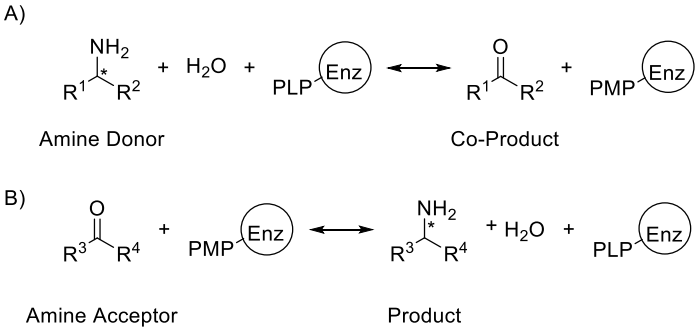
ω-TAs are co-factor dependent enzymes, where the co-factor pyridoxal 5'-phosphate (PLP), a vitamin B_6 based co-factor (Guo and Berglund, 2017), acts as an electron and nitrogen shuttle (Kroutil *et al.*, 2013). Here a brief description of the reaction mechanism follows, more details

can be found in several excellent reviews (Seo *et al.*, 2011; Malik, Park and Shin, 2012; Mathew and Yun, 2012; Kroutil *et al.*, 2013; Fuchs, Farnberger and Kroutil, 2015).

ω-TAs follows a ping-pong bi-bi reaction mechanism (Scheme 2.1), that consists of two half-reactions, outlined in Scheme 2.2. Initially, PLP forms a Schiff base with a covalently bound lysine. In the first half reaction, Scheme 2.2 A), oxidative deamination of the amine donor is carried out, where the amino group of the donor is transferred to PLP, thereby converting PLP to pyridoxamine 5'-phosphate (PMP), furthermore a water molecule in the active site is incorporated to convert the amino group of the amino donor to a keto group. The ketone co-product is finally released from the active site. In the second half reaction, the reductive amination of the amine acceptor is carried out, Scheme 2.2 B) the amine acceptor reacts with the enzyme bound PMP co-factor to convert the keto group into an amine moiety, regenerating the PLP co-factor. Finally, the amine product and a water molecule are released and the reaction can start anew (Malik, Park and Shin, 2012; Fuchs, Farnberger and Kroutil, 2015). The second half reaction, Scheme 2.1B), has been found to be the rate-limiting step (Seo *et al.*, 2011).



Scheme 2.1: Cleland diagram of ω-transaminase, ping-pong bi-bi mechanism.



Scheme 2.2: ω-transaminase reaction mechanism adapted from (Mathew and Yun, 2012; Fuchs, Farnberger and Kroutil, 2015).

Most ω-TAs are *S*-selective (Koszelewski *et al.*, 2010), due to the fact that many biomolecules including most amino acids are (*S*)-isomers, save cysteine (Berg, Tymoczko and Stryer, 2006). However, the abundance of (*R*)-selective ω-TAs are increasing, both by finding more natural (*R*) selective ω-TAs (Koszelewski *et al.*, 2010; Malik, Park and Shin, 2012), and by generating novel (*R*)-selective ω-TAs by enzyme engineering (Svedendahl *et al.*, 2010).

Stereo and regio-selectivity in ω-TAs are provided from the tertiary structure of the enzyme, in addition to some selectivity being offered from the PLP co-factor. The active site of the enzyme consists of a small and a large binding pocket, Figure 2.1 (Mathew and Yun, 2012; Fuchs, Farnberger and Kroutil, 2015). The binding of the substrate in a fixed orientation will thereby provide a specific stereoselectivity.

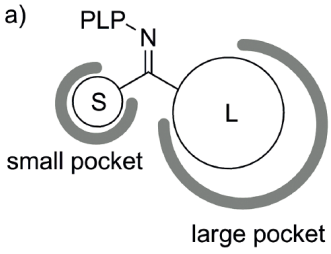


Figure 2.1: Binding pocket of a ω-transaminase (Fuchs, Farnberger and Kroutil, 2015).

2.1.2 Kinetic resolution vs asymmetric synthesis

2.1.2.1 Kinetic resolution (KR)

ω-TAs can be applied in two ways to produce chiral amines. Firstly, ω-TAs can be applied in kinetic resolution (KR), Figure 2.2. In KR a racemic mixture of *R*- and *S*-amines are combined with an amine acceptor, to resolve one chiral product. ω-TAs will then react with one of the selected amines, depending on the enzymes' selective preference. That stereoisomer will then be fully converted to the product, and the other isomer is left with 100% purity. This method has several advantages. Firstly, as mentioned above, most ω-TAs are *S*-selective, KR thereby gives access to R-isomers (Koszelewski *et al.*, 2010). Furthermore, it allows the use of pyruvate as an amino acceptor, a natural substrate of ω-TAs, which is an accepted acceptor for many ω-TAs, and furthermore has very good thermodynamic properties in the resolution direction, further discussed in Chapter 3. However, the drawback of KR is that it has a maximum theoretical yield of 50%. When the starting material is costly this is particularly problematic. However, the use of dynamic kinetic resolution (DKR), overcomes this challenge. In DKR another enzyme, such as alanine dehydrogenase, is used to convert the ketone product back to the racemic-amine starting material. Thereby increasing the theoretical product yield to 100% (Höhne and Bornscheuer, 2009; Koszelewski *et al.*, 2010). However, this adds cost and complexity by adding one or more enzymes.

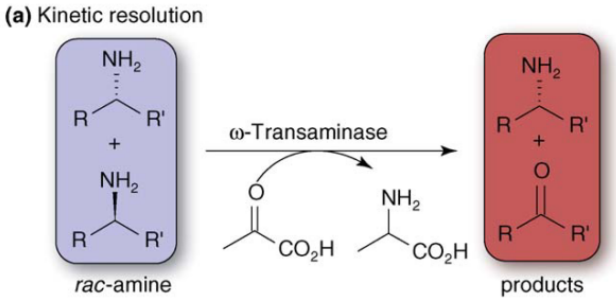


Figure 2.2: Kinetic resolution with transaminases (Koszelewski et al., 2010).

2.1.2.2 Asymmetric synthesis (AS)

A more direct route to chiral amines and the focus of this thesis is asymmetric synthesis (AS). Where a donor and acceptor is reacted according to Scheme 2.2 above, to produce a product and co-product. This method has a theoretical yield of 100% and does not require any additional enzymes, thereby providing a simpler and less costly method of synthesis than kinetic resolution. However, many target products are haunted by poor thermodynamics, thereby producing low yield with asymmetric synthesis, further discussed below (Koszelewski *et al.*, 2010). Furthermore, the use of AS requires 100% stereoselectivity to obtain an enantiomerically pure product, which is not a requirement for KR (Koszelewski *et al.*, 2010).

2.2 Chiral Amines

Chiral amines are a frequent and important functional group in pharmaceutics, agrochemicals and fine chemicals. They are used as starting points for API's and as resolving agents for obtaining carboxylic acids (Höhne and Bornscheuer, 2009). They are particularly attractive in pharmaceutics because of their high bioactivity (Koszelewski *et al.*, 2010; Paul *et al.*, 2013; Pressnitz *et al.*, 2013) The most common non-synthetic route to chiral amines is the resolution of racemates, using chiral carboxylic acid salts. The most common synthetic routes use chiral auxiliaries or metal complexes with chiral ligands to produce chiral amines (Höhne and Bornscheuer, 2009). Metal complexes often suffer from high cost and unstable pricing (Truppo, 2017). Furthermore, synthetic routes to chiral amines often requires protection of other functional groups due to harsh conditions and high temperatures during reaction (Fuchs, Farnberger and Kroutil, 2015)

In addition to ω-TAs, several other biocatalytic routes can be used to obtain chiral amines illustrated in Figure 2.3. Other notable examples include lipases, amine oxidases, imine reductases, amine dehydrogenases, ammonia lyases and pictet-spenglerases (Ghislieri and Turner, 2014; Kohls, Steffen-Munsberg and Höhne, 2014). ω-TAs are currently the only well-developed enzyme that provides a synthetic path to chiral amines. In addition, ω-TAs has the

advantage on not requiring co-factor recycling and a broad substrate spectra compared to alternative biocatalysts.

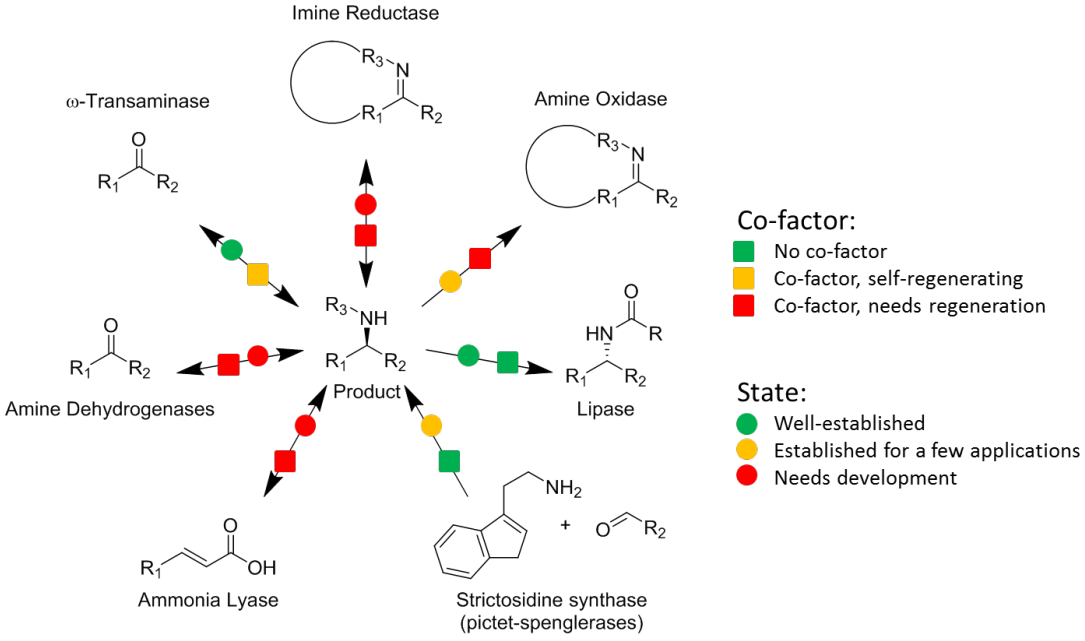


Figure 2.3: Biocatalytic paths to chiral amines.

2.3 Pharmaceutics

Pharmaceutics differs from other uses of biocatalysis due to high product cost, allowing for higher cost per kg than any other biocatalytic application. Active pharmaceutical ingredients (APIs) are priced in the excess of 100€/kg, whereas in comparison bulk chemicals are only valued to 1 €/kg (Tufvesson *et al.*, 2011). However, due to the nature of the product, APIs are also subject to stringent product requirements, in particular, high chiral purity is required. Furthermore, developing a drug is an expensive process, and patents have limited lifetime, it is therefore essential that process development is as fast as possible, to not limit a product's economic lifetime (Truppo, 2017). The pharmaceutical industry is currently under immense pressure, with patents

expiring, increased competition, slower innovation and longer implementation (Welch, Hawkins and Tom, 2014).

2.3.1 Implementation challenges

Although the use of ω-TAs is a well-established technology for the synthesis of chiral amines. Their application is still limited, especially in areas where ample processing time is not available. The focus of the next two chapters is on overcoming some of these challenges. A brief overview of the most important challenges are outlined below.

2.3.1.1 Thermodynamics

A major hindrance for the implementation of ω-TAs is unfavorable thermodynamics. Many target products such as α-methybenzylamine (MBA), favors substrate formation over product formation, with many commonly used donors. To overcome this, process engineering strategies outlined in Chapter 1, or careful donor selection described in detail in Chapter 3, can be employed. Alternatively, KR or DKR can be used to overcome unfavorable thermodynamics in AS.

2.3.1.2 Inhibition

Inhibition is a major concern with most biocatalytic reactions. Where the binding of one or more substrates and/or products limit the reaction rate. This is particularly challenging in biocatalysis because the substrate and product concentration required for an economically viable process are often much higher than what enzymes experience in Nature. Therefore, at high substrate and product concentrations inhibition can be problematic and hinder process intensification. In ω-TAs reactions, inhibition is further augmented. The ping-pong bi-bi mechanism (Scheme 2.1), requires two independent half-reactions (Scheme 2.2), inhibition can therefore occur on four separate occasions, in addition, the substrate/product may bind to the non-reactive form of the enzyme, forming an apoenzyme. Furthermore, the aromatic structure of many substrates/products can strongly interact with the active site of the enzyme, thereby increasing

inhibition. Inhibition of the amine acceptor and the co-product (both ketones) has been found to exhibit strong inhibition (Malik, Park and Shin, 2012).

2.3.1.3 Meeting economical process metrics

Many reported studies do not meet necessary process parameters such as productivity, product concentrations (Meadows *et al.*, 2013). Furthermore, high-cost substrates are often employed, in fact, on some occasions the substrates are more costly than the products, making an economically viable process impossible (Seo *et al.*, 2011). In order to simplify this, Chapter 4 outlines an easy to follow, step-by-step method with cut-off values for fast implementation of ω-TA reactions for small singular batches (Gundersen *et al.*, 2016).

2.4 Notable examples

A highly publicized commercial example of ω-TA is its use in synthesizing a late stage intermediate of Sitagliptin (commercially known as Januvia), an antidiabetic drug (Savile *et al.*, 2010). This example is highlighted both as a great achievement in enzyme engineering and in the integration of enzyme engineering and process engineering. Briefly, a native ω-TA was subject to 11 rounds of enzyme engineering, where substrate specificity towards the target acceptor was established and increased stability under process relevant conditions was obtained. The target acceptor, a prositagliptin ketone, was accepted after several rounds of enzyme engineering, where a smaller substrate was used in the first rounds. Subsequent rounds increased substrate concentration from 2 to 100 g/L, donor concentration from 0.5 M to 1 M, co-solvent (DMSO) tolerance from 5 to 50% and temperature tolerance from 22 °C to 45 °C. The final process resulted in a 92% reaction yield and over 99.95% *e.e.* (Savile *et al.*, 2010). High donor concentration overcomes thermodynamic limitations, and increased temperature stability, enabled the use of co-product (acetone) removal, to further alleviate thermodynamic constraints. This route provided a more cost-effective and greener alternative to a rhodium-based synthetic route (Savile *et al.*, 2010).

2.5 Conclusion

This chapter has given an introduction to the use of ω-TAs, especially in the synthesis of chiral amines for pharmaceutics. From the literature reviewed here, it is clear that ω-TAs are highly beneficial biocatalysts, especially for chiral pharmaceutical products. However, the enzyme still has the potential for a more widespread use. To facilitate a broader use of ω-TAs a better understanding of process options and donor selection is needed, which is covered in the next two chapters of this section.

2.6 References

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Section I

Chapter 3: Donor selection in ω-transaminase reactions

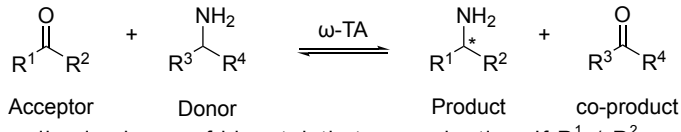
This chapter is based on the two published articles 'Amine donor and acceptor influence on the thermodynamics of ω-transaminase reactions' and 'A practical and fast method to predict the thermodynamic preference of ω-transaminase-based transformations'. The former article was published in the journal Tetrahedron: *Asymmetry* (2015), pages 567-570, by the authors Maria Gundersen, Rohana Abu, Martin Schürmann and John Woodley. The latter article was published in the journal ChemCatChem (2015), pages 2594-2597, by the authors Robert J. Meier, Maria T. Gundersen, John M. Woodley and Martin Schürmann. Not all data from the papers are included in this chapter and additional manipulation of the data has been carried out. Furthermore, this chapter discusses donor selection beyond that found in the two mentioned articles. Copies of the full articles and supporting information, as published, can be found in Annex A.

3.1 Abstract

ω-transaminases (ω-TAs) is an established route to chiral amines, especially in the pharmaceutical industry. Nevertheless, one limitation for successful implementation and scale-up of the reaction is unfavorable thermodynamics, which can give low yields and challenges in downstream processing. The ω-TA reaction requires two substrates, an amine acceptor (acceptor) and an amine donor (donor), both of which contribute to the thermodynamic equilibrium of the reaction. However, the amine donor does not affect the structure of the chiral amine. It is therefore possible to optimize the donor selection to obtain thermodynamically feasible reactions. This chapter discusses methods for donor selection to improve thermodynamics, both in terms of the two published papers above and with respect to relevant current literature. The results presented here showcases donor flexibility and impact of any given reaction, and the importance of carefully choosing a suitable donor early in the process development of any given synthetic route.

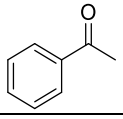
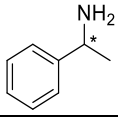
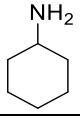
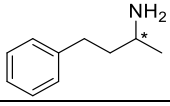
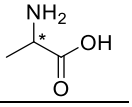
3.2 Introduction

As mentioned in the previous chapter, ω-TAs has over the last decade gained significant interest as biocatalysts in the synthesis of enantiopure chiral amines. The popularity of the enzyme stems in part from high selectivity and a broad substrate repertoire. The reaction is an aminotransferase reaction, where an amine moiety is transferred from an amino donor to an amino acceptor. Yielding a carbonyl co-product, and target product, an amine. The target product is a chiral amine if the following conditions are met: the two substituents of the amine are not identical, and neither of them is hydrogen. ω-TAs can either be applied in the synthetic direction, with asymmetric synthesis (AS), the main focus of this thesis, or in kinetic resolution (KR) both discussed in detail in the previous chapter (Koszelewski *et al.*, 2010). This provides a useful way to produce chiral amines for pharmaceuticals and fine chemicals, as demonstrated by several commercial applications (Savile *et al.*, 2010; Seo *et al.*, 2011). However, in practice several challenges are often encountered during process development such as enzyme inhibition, poor substrate binding, and/or unfavorable thermodynamics. To shift the equilibrium towards product formation, one has to, in practice, often to add an excess amount of the amino donor and/or remove the co-product, discussed in Chapter 1 and 2. Additionally, low yields can be the result of biocatalyst related issues, such as enzyme inhibition or instability of the enzyme. Such limitations can be overcome by enzyme modification through protein engineering (Bornscheuer *et al.*, 2012). However, the thermodynamics of the reaction, often found to be the primary limitation, is independent of the enzyme, therefore alternative solutions need to be used (Woodley, 2013).



Scheme 3.1: Generalized scheme of biocatalytic transamination. If $R^1 \neq R^2$ and R^1, $R^2 \neq H$, the product is a chiral amine.

Table 3.1: Structure of amines and ketones discussed Chapter 3. Acetophenone (ACP), MBA: α-methybenzylamine (MBA), Cyclohexylamine (CHA), isopropylamine (IPA), 1-phenylethylamine (PEA), alanine (ALA)

		
ACP	**MBA**	**CHA**
		
IPA	**PEA**	**Ala**

3.3 Thermodynamic solutions

One of the main hindrances to implementation of ω-TAs on a commercial scale is unfavorable thermodynamics. Here, strategies for overcoming unfavorable thermodynamics is discussed.

3.3.1 Excess amine donor

A common strategy to overcome thermodynamic constraints is to add an excess amount of the amine donor (Paul *et al.*, 2013). Adding excess donor will increase the concentration of the substrates, therefore following Le Chatelier's principle, this increases the conversion of the acceptor. The conversion of the donor will at the same time be lower. However, there are several complications with this solution. Depending on the structure of the donor, this might add complexity in product isolation in downstream processing. For example with donor compounds such as IPA (Table 3.1), an excess donor might not be problematic, since the donor compound has drastically different properties to most target compounds, demonstrated by the Sitagliptin synthesis (Savile *et al.*, 2010). However, in other cases, such as the use of MBA (Table 3.1), the donor compound may be structurally similar to the product, and causing separation to become more challenging.

Furthermore, adding excess amounts of donor amine can only overcome a reasonably small thermodynamic limitation. For a commercial product, the product concentration in the final product must be upwards of 50 g/L (Tufvesson *et al.*, 2011). It is

therefore limited how high the donor concentration can be, in both practical and economic terms. Practically, the molecular weight of the donor will determine the maximal concentration of the donor in the reaction, a higher molecular weight equals a lower maximal concentration. Economically, unless the donor is recycled, the cost of the donor versus the cost of the product will determine the maximal donor concentration for viable economics. This is further discussed in the next chapter, where the practical implementation of such reactions is discussed. In Chapter 4, the limitation set is maximum 20-fold donor excess. The increased yield from increasing the donor concentration from 1:1 to 1:20 (acceptor:donor), is calculated for 5 specific examples in Table 3.3 below.

3.3.2 Product/Co-product removal

Another possible solution is to remove the co-product with ISPR techniques discussed in Chapter 1. In ω-TA reactions, there are 3 main methods of co-product removal; physical removal, spontaneous chemical reactions and cascade reactions.

3.3.2.1 Physical co-product removal

Physical co-product removal depends only on the physical properties of the components (substrates and products), and the solvent of the reaction. A simple strategy used with a volatile co-product, such as IPA, is evaporation (Tufvesson, Bach and Woodley, 2014). For example, it has been demonstrated that IPA, in excess, under reduced pressure where the co-product acetone is evaporated can result in increased yield (Savile *et al.*, 2010; Tufvesson, Bach and Woodley, 2014). A drawback of this method is that the co-product must be volatile, and higher temperatures must be used to facilitate evaporation. Higher temperatures can reduce enzyme stability, discussed in detail in Section II. Another strategy that has been employed with ω-TAs is the use of an immiscible co-solvent, for product or co-product removal. In a two-phase reactor, the reaction is carried out in the water phase, after conversion the product or co-product partitions into the immiscible phase, due to a higher solubility in that solvent. It should be noted that the substrates may also partition into the immiscible solvent, and thereby lowers the thermodynamic advantage from the solvent. Nonetheless, it was demonstrated that two-phase

reactions had higher product yields than the same reaction conditions in a one-phase system (Meadows *et al.*, 2013; Gundersen *et al.*, 2016). However, it should be noted that co-solvent has been shown to impact stereochemistry, and thereby reducing *e.e.* (Koszelewski *et al.*, 2008). Finally, precipitation driven synthesis could be an alternative, with the use of the right reaction pair and solvents. Precipitation driven synthesis is often carried out as a solid to solid synthesis. Where a solid substrate is deposited in a small amount of liquid. It is then important that the solubility of the product is lower than the thermodynamic equilibrium (K_{eq}). The product will then precipitate out of the reaction before reaching equilibrium, and conversion continues until all the starting material is dissolved (Ulijn *et al.*, 2001). To the best of my knowledge, precipitation based synthesis has not to date been carried out with ω-TAs.

3.3.2.2 Spontaneous chemical reaction

An interesting option in the synthesis of chiral amines is the use of donor molecules which undergo a spontaneous chemical reaction after conversion. A spontaneous reaction is a reaction that proceeds in a reasonable time frame without the intervention of a catalyst. In the ω-TA reaction, the donor molecule will undergo a spontaneous reaction after it is converted into the co-product. The reaction should be irreversible, and will thereby drastically shift the equilibrium towards the product side, without any further intervention. A notable example is the use of the donor 3-aminocyclohexa-1,5-dienecarboxylic acid, which undergoes a spontaneous reaction to form an aromatic alcohol after the transfer reaction (Wang, Land and Berglund, 2013). Furthermore, O'Reilly has developed several so-called 'smart-donors' with spontaneous ring formation. The first generation of donor, o-xylylenediamine, suffered from high costs and polymerization of the co-products (Green, Turner and O'Reilly, 2014). The second generation smart donors, use affordable readily available di-amine donors, such as cadaverine and putrescine, and gave higher yields than IPA under similar conditions (Gomm *et al.*, 2016).

3.3.2.3 Cascade reactions

Finally, enzymatic or chemical cascade reactions may be used to convert the co-product into another chemical to shift thermodynamic equilibrium. Numerous examples have been used for

this, and several excellent reviews can be found (Simon *et al.*, 2014; Abu and Woodley, 2015; Fuchs, Farnberger and Kroutil, 2015). A frequently converted co-product is pyruvate the co-product found with the use of the donor Alanine (Ala). Pyruvate is then converted to either a corresponding alcohol or back to alanine through another enzyme. Unlike ω-TA, the second enzyme used in the cascade reaction may not self-regenerate the co-factor, and a third enzyme might be needed to regenerate the co-factor, this adds both cost and complexity to the process development. Although, the use of cascade reactions is highly successful under some circumstances, in many instances the extended development time and cost is too high to be justified, especially in pharmaceutics where rapid development time is paramount (Truppo, 2017).

One can also use conventional synthetic chemistry cascades, although this is used to a lesser extent. This may in part be due to the similarity of the starting materials and product/co-product of the ω-TA reaction, which requires high selectivity. Furthermore, as mentioned in previous chapters. ω-TAs are often employed in the synthesis of API intermediates, it should therefore be possible to couple the next reaction in the same reactor, and thereby convert the product of the ω-TA reaction to the next intermediate. However, this is also rarely done in practice, perhaps because of the high product purity required, side reactions which would complicate downstream processing, and reaction conditions which are incompatible with the enzyme.

Although the solutions outlined above may be successful, they are not always applicable on large scale, due to increased complexity and costs. A simpler method is therefore proposed, where careful selection of the donor may be employed.

Table 3.2: Overview of equilibrium shifting methods for ω-TA reactions

	Cost	Development Time	Improvement obtained	Availability
Excess amine donor	Depends on cost of donor	Short	Low	High
Physical co-product removal	Low	Short	Intermediate	Intermediate
Spontaneous chemical reaction	Depends on cost of donor	Short	High	Intermediate
Cascade reactions	High	Intermediate	High	Intermediate

3.4 Experimental determination of Donors.

Here, the apparent equilibrium constant ($K_{eq}{}^{app}$) was experimentally determined for five donors, with the same acceptor: ACP. Thereby, comparing their half-reactions. Experimental procedures can be found in Annex A, in the published paper (Gundersen *et al.*,2015). These experiments illustrate the effect of commonly used donors on the reaction yield, Table 3.3. When comparing these results, it must be kept in mind that any structural change to any given molecule occurs on both the reactant and product side. For example, in the Tetrahedron article by Gundersen and colleagues (2015) (full article in Annex A), compare the two half-reactions of two *ortho*-substituted MBA derivatives, one with a fluorine and one with an alcohol substituent. It was observed that the fluorine substitution had a lower thermodynamic yield. However, that does not indicate that the fluorine donor is more stable than the alcohol donor, it only indicates that that the donor/co-product pair of the fluorine-substituted MBA favors reactants more strongly than the alcohol-substituted MBA.

The results in Table 3.3 were extended beyond the results in the published paper by furthermore calculating thermodynamic yield with 1:1 and 1:20 initial acceptor: donor concentration. From these results, two main conclusions can be drawn. Firstly, the donor selection has a high impact on yield, the best donor has 50 % and 95 % yield, with 1:1 and 1:20 initial concentration, respectively. In comparison, the poorest donor only achieves 3% yield with a 20-fold donor excess. Furthermore, the results in Table 3.3 illustrate that donor excess may

only alleviate thermodynamic constraints when they are in an intermediate range. For example, with PEA as a donor, a 50% increase in yield is observed by increasing the donor concentration 20-fold. However, Ala under the same conditions only increases yield by 2%. These data indicate that careful donor selection is important to successfully implement ω-TA reactions commercially.

Table 3.3: Effect of donor selection. K_{eq}^{app} and thermodynamic yield (%) with 1:1 and 1:20 initial acceptor:donor concentration, with the acceptor **ACP**

donor	K_{eq}^{app}	1:1	1:20
MBA	1	50%	95%
PEA	0.18	30%	81%
IPA[a]	$3.3*10^{-2}$	15%	54%
CHA	$6.0*10^{-3}$	7%	29%
Ala[a]	$4.0*10^{-5}$	1%	3%

a) (Tufvesson *et al.*, 2012)

3.5 *In silico* experiments

The results above demonstrated the impact of a careful selection the amine donor in the ω-TA reaction, for optimal thermodynamic conditions. Because the thermodynamic yield of a reaction is influenced by the structural and energetic differences between the reactants and the products, and the biocatalyst does not affect the thermodynamics, it would be preferential to select the ideal reaction pair early in process development. This would enable any process and enzyme engineering to be targeted to the optimal reaction pair. However, since in practice determination of equilibrium requires a ω-TA with the appropriate selectivity, the donor is often chosen on availability and enzyme reactivity. Therefore, although in principle a plethora of possible compounds could be used as a donor, only a few are used, namely MBA, IPA and Ala (Table 3.1).

With this in mind, it was reasoned that it would be useful to evaluate a wider range of donors and in particular establish their effect on the reaction thermodynamic yield. Here, an *in silico* approach is used, that does just that. It predicts the thermodynamic equilibrium of any given reaction pair with sufficient accuracy to decide whether a particular substrate combination is suitable for subsequent experimentation.

Ab initio calculations are widely used to calculate reaction (free) energies and kinetic parameters of chemical reactions (Van Speybroeck, Gani and Meier, 2010). However, these types of calculations have not been applied to any great extent in enzymatic reactions, perhaps due to the limited substrate spectra of many enzymes. As mentioned in Chapter 1, enzymatic reactions are no different than other catalyzed reactions, there is therefore no practical restriction that limits the use of such calculations in biocatalysis. The approach used here is based on *ab initio* calculations of total energies of all the molecules involved. It gave good prediction of the equilibria of transaminase reactions. However, it should be noted that the key strength of this approach lies in predicting whether or not a reaction is thermodynamically favorable. To eliminate those reactions that will not give high conversion in AS or alternatively identify those same reactions as good reactions for KR. It should also be noted that although favorable thermodynamics is necessary for a high yield, it is not necessarily sufficient. Other factors such as enzyme reactivity and/or inhibition may still prevent high yield. However, arguably with early implementation of thermodynamically favorable reaction pairs, those challenges may be tackled with enzyme engineering, see Chapter 1 for details.

3.5.1 Computational procedures

A hybrid density function was used in combination with a basis set in the Spartan 10 program (Wavefunction Inc, 2014), to calculate minimal energies for each molecule. Since the ω-TA reaction involves two starting materials and two products, Scheme 3.1, the *ab initio* calculations are applied to all four structures (structure optimization or, equivalently, energy minimization). The energy difference between the left-hand side and the right-hand side of the reaction, that is, the reaction energy (DE), was calculated from these four individual energies. If the energy is negative, the reaction is favored in the synthetic direction, if positive the reaction is not favored. In addition, in the presented method entropy contribution is neglected the solvent effects, and the influence of the dynamic nature of the structures, due to the complexity of calculations if such effects were to be accounted for. However, as is often in quantum calculations, if the solvent and other experimental conditions (such as temperature and pH) are kept the same in all

experiments, a cancellation of the contributions might be expected, which would lead to a simple model involving molecular energies alone (Van Speybroeck, Gani and Meier, 2010). The results indicate that this applies to this approach.

Equation 3.1: The predicted yield (K). R is the gas constant and T is the absolute temperature

$$e^{\left(-\frac{\Delta G}{RT}\right)} = K = \frac{[A][B]}{[C][D]}$$

Equation 3.2: K expressed in terms of product formation (yield)

$$e^{\left(-\frac{\Delta G}{RT}\right)} = K = \frac{x^2}{(1-x)^2}$$

Equation 3.3: Yield (x) for a 1:1 (acceptor:donor) starting concentration

$$x = \frac{e^{\left(-\frac{\Delta G}{RT}\right)} + \left(\frac{\Delta G}{\Delta T}\right)}{1 - e^{\left(-\frac{\Delta G}{RT}\right)}} \times 100\%$$

3.5.2 Validation of the method

The accuracy of the method was evaluated for ω-TA reactions by comparing calculated yield values with experimental data, including the reactions in Table 3.3 and extracted from relevant experimental data from scientific literature (Tufvesson *et al.*, 2012; Fesko *et al.*, 2013; Meadows *et al.*, 2013; Gundersen *et al.*, 2015, 2016). The yield was calculated by using a 1:1 ratio of donor/acceptor starting material, according to equation 3.3 above. Calculated and experimentally obtained values are compared in Figure 3.1, see the Supporting Information in Annex A for raw data (Meier *et al.*, 2015). The data used were carefully selected based on stringent criteria, to ensure that the thermodynamic equilibrium was reached. All data chosen for the comparison were run at low concentrations, under 100 mM (to limit inhibition effects). The reported values were considered to be at equilibrium if either stated by the authors in the respective papers or confirmed by calculations before inclusion. These calculations compared ΔG of the reactions to

uncover any inconsistencies in the reported values. If for example the same acceptor was reacted with two separate donors, the ΔG difference between the two reactions was calculated and compared with the expected ΔΔG of the reaction from the results in Table 3.2 above. The stringent selection procedure was necessary to ensure that the experimental equilibrium was not skewed towards the reactants due to inhibition or other experimental challenges.

The method predicted with high accuracy (within ≈ 5 kJ mol^{-1}) low yield reactions. Indeed, it can be seen that all experiments with less than 25% yield are predicted to have yield below 40%. This highlights the key advantage of the method: to eliminate thermodynamically unfavorable reactions. The same trend was observed for very favorable reactions, Figure 3.1. However, it can be observed that the intermediate reaction pairs suffer from an underestimation of yield from the experimental results compared to the modeled results. Many of the reactant pairs where the experimental results are underestimated contain a reactant with an aromatic aryl group, often ACP or an ACP derivative. Aromatics such ACP has previously been shown to be inhibitors of ω-TAs (Shin and Kim, 1997). However, this trend is not observed for all reactions with these structures. It is therefore probable that the error lies with the experimental results rather than with the predictive method. Furthermore, the accuracy of the achieved *ab initio* molecular energies such as heat of formation, can under optimal conditions be accurate within 4 kJ/mol (Van Speybroeck, Gani and Meier, 2010). The reactants used here is of a simple nature, accuracy of the energy difference calculated here, for the reaction described in Scheme 3.1, is expected to be in the order of 4 kJ/mol. However, it should be noted that the uncertainty of the calculated reaction values is greatest in the intermediate yield area, rather than high or low yield. This is due to the fact that very high or low yield 4 kJ/mol difference in ΔG does not have a high impact. However, for reactions in the intermediate range (where ΔG is close to 0 kJ/mol), a 4 kJ/mol difference has a much greater impact. The maximum uncertainty, found at 50% yield, is approximately 20%.

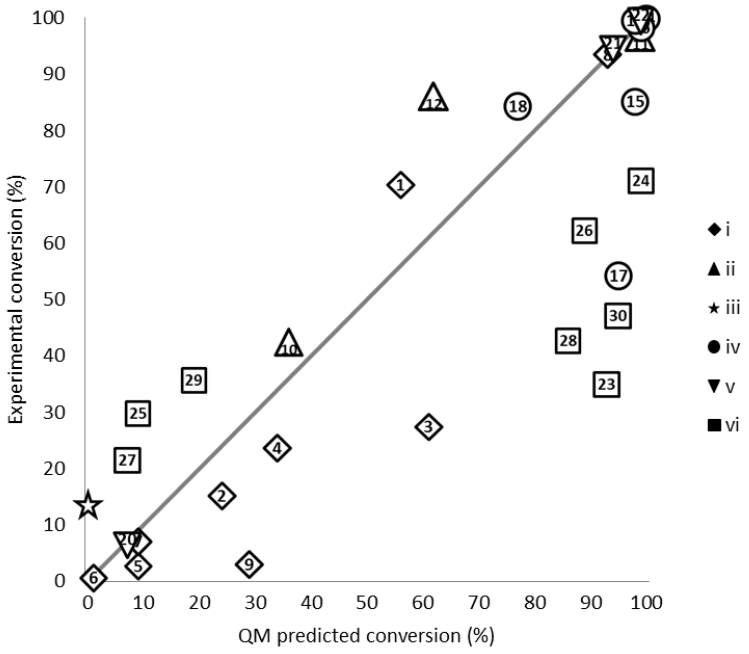


Figure 3.1: Comparison of predicted and measured yield with a 1:1 donor/acceptor initial concentration. Data were extracted from i) (Gundersen *et al.*, 2015) ii) (Gundersen *et al.*, 2016) iii) (Tufvesson *et al.*, 2012) iv) (Fesko *et al.*, 2013) v) (Meadows *et al.*, 2013). The line represents a match between experimental and predicted quantum mechanics (QM) values. The number in each point refers to the reaction number, as listed in the Supporting Information, found in Annex A.

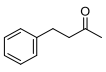
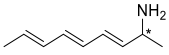
3.5.3 Application of method

As mentioned above, the key feature of the approach lies in eliminating unfavorable reaction pairs early in the development. If a broad screen of donors is used, this could further be cross-checked with availability, safety and economic consideration, early in process development. This may be particularly useful here, as all that is required is to calculate the energy for each compound once. The predicted yield of the reaction depends on the sum of the four individual energies from each compound in the reaction. In this way, a set of donors can be applied to any desired acceptor. For example, if a set of 50 donor/co-product pairs is already calculated, by just calculating the energies of one new acceptor/product pair, this can be matched with the donors and 50 possibilities with the new acceptor reactant are available. Which further can be used to

eliminate unfavorable reaction pairs. Upon going through these 50 energies those reactions that are thermodynamically unfavorable can immediately be eliminated and focus subsequent efforts on favorable reactions. The average processing time for a new calculation is in the range of a 2-4 hours depending on the size and complexity of the molecule.

To demonstrate the method a small comparison of common and uncommon acceptor/donor pairs has been calculated in Table 3.4. Where the commonly applied donors Ala, IPA and MBA is compared with a new amine donor which, to the best of my knowledge, has not previously been used in ω-TA reactions, 3,5,7-nonadiene-2-amine (NDA), Table 3.4. The calculations indicate that NDA is a more thermodynamically favorable donor than any of the 3 commonly used amine donors in the comparison. From this example, it can be stipulated that the method could be very useful as a screening method in an early stage of any ω-TA development.

Table 3.4: Predicted (QMM) reaction yield (%) for 16 selected reactions with 1:1 (acceptor:donor) initial concentration

Donors/acceptors				
	0.1	6.3	4.8	<0.1
	4.4	74.1	68.2	0.1
	79.5	99.6	99.5	6.5
	95.9	99.9	99.9	29.2

3.6 Conclusion

This chapter both discusses and demonstrate the importance and effect of the amine donor on the thermodynamic equilibrium. Careful donor selection, especially in early development of a given process, can determine the overall success of the project. It was demonstrated that with a

thermodynamically challenging acceptor such as ACP, the yield with a 1:1 (acceptor:donor) initial concentration can vary from 50% with the donor MBA to under 1% with Ala if no additional equilibrium shifting techniques are applied. Furthermore, an *ab initio* QMM method was proposed. It successfully demonstrated the ability to predict thermodynamically favorable reactions *in silico*. This is particularly important because ideally the reaction pair should be selected early in the process development. Because it would allow for the simultaneous development of both substrate specificity and reaction conditions.

The result in this chapter gives easy to use tools to select successful reaction pairs in the ω-TA reaction, which can be applied early in process development. Arguably, these tools can increase the probability of successfully implementing ω-TA reactions and reduce the development time. They can therefore increase the overall use of ω-TAs, and biocatalysis, in the pharmaceutical industry.

3.7 References

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Section I

Chapter 4: Fast Implementation of ω-Transaminase Reactions

This chapter contains the published article 'A Rapid Selection Procedure for Simple Commercial Implementation of ω-Transaminase Reactions'. This article was published in the journal 'Organic Process Research & Development' in 2016, pages 602-608, by the authors Maria T. Gundersen, Par Tufvesson, Emma J. Rackham, Richard C. Lloyd, and John M. Woodley. Unlike the other paper-based chapters in this thesis, this paper has not been modified beyond formatting and is included as published, with the exception of the materials and methods section, which can be found in the annex with the full copy of the paper as published.

4.1 Abstract

A stepwise selection procedure is presented to quickly evaluate whether a given ω-transaminase reaction is suitable for a so-called "simple" scale-up for fast industrial implementation. Here "simple" is defined as a system without the need for extensive process development or specialized equipment. The procedure may be used when investment in intensive process development cannot be justified or when rapid execution is paramount, for applications such as small singular batches. The three step evaluation procedure consists of: (1) thermodynamic assessment, (2) biocatalyst activity screening, and (3) determination of product inhibition. The method is exemplified with experimental work focused on two products: 1-(4-bromophenyl)ethylamine and (*S*)-(+)-3-amino-1-Boc-piperidine, synthesized from their corresponding pro-chiral ketones each with two alternative amine donors, propan-2-amine, and 1-phenylethylamine. Each step of the method has a threshold value, which must be surpassed to allow "simple" implementation, helping select suitable combinations of substrates, enzymes, and donors. One reaction pair, 1-Boc-3-piperidone with propan-2-amine, met the criteria of the three-step selection procedure

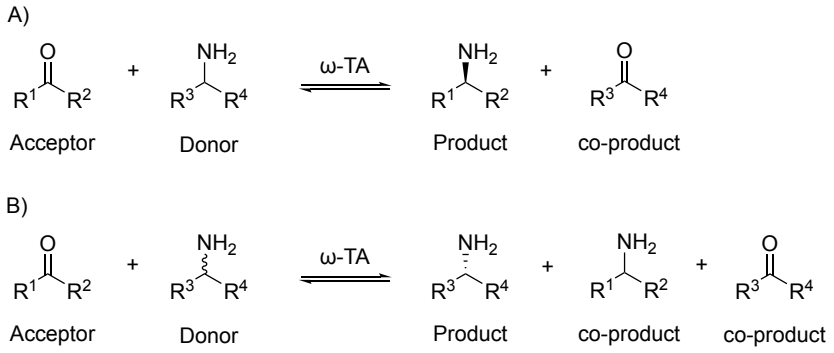
and was subsequently run at 25 mL scale synthesizing (*S*)-(+)-3-amino-1-Boc-piperidine at concentrations up to 75 g/L. However, the highest product yield (70%) was obtained at a lower substrate concentration of 50 g/L.

4.2 Introduction

Over the past decade, biocatalysis has become an established and useful complement to conventional chemical catalysis for the synthesis of fine chemicals. Most often, biocatalytic methods have been selected due to exceptional selectivity (regio-and/or enantioselectivity) (Pollard and Woodley, 2007). In fact, the majority of industrially applied biocatalytic reactions today yield optically pure chiral products that are used in the fine chemical industry as building blocks for agrochemicals and pharmaceuticals (Straathof, Panke and Schmid, 2002). In particular, biocatalytic transamination chemistry has been identified as one of the key emerging areas for the pharmaceutical industry (Pollard and Woodley, 2007; Clouthier and Pelletier, 2012) as a means of producing optically pure chiral amines. This paper focuses on the biocatalytic synthesis (and resolution) of chiral amines of high optical purity using ω-transaminase (ω-TA) (E.C. 2.6.1.18), which is a type of amino transferase. ω-TA was chosen as a catalyst for this work due to its outstanding stereoselectivity and broad ketone substrate repertoire. Two ω-TA-catalyzed paths are available toward optically pure chiral amines, using either asymmetric synthesis or kinetic resolution. Although the latter is challenged by a maximum 50% yield, (Koszelewski, Tauber, *et al.*, 2010; Tufvesson, Lima-Ramos, Jensen, *et al.*, 2011; Malik, Park and Shin, 2012) both are considered as potential options for the "simple" scale-up.

ω-TA catalyzes an amino transfer reaction, illustrated in Scheme 4.1. Briefly, in the synthetic direction (Scheme 4.1A) the amino donor (an amine), and the amino acceptor (a prochiral ketone), here referred to simply as the "donor" and "acceptor", respectively, react with the enzyme in a sequential fashion producing the desired target chiral amine product and a coproduct. Detailed descriptions of the sequential ping-pong bi-bi enzymatic reaction mechanism can be found elsewhere (Henson and Cleland, 1964; Steffen-Munsberg *et al.*, 2013). In the resolution reaction (Scheme 4.1B) the same reaction takes place, but now the amino donor

is added as a racemic mixture. Through reaction therefore, one isomer is left unreacted, which becomes the desired optically pure product.



Scheme 4.1: Examples of Potential ω-TA Reactions Using (A) a Synthetic Route and (B) a Resolution Route.

The amino moiety alone is transferred between the two starting substrates, and therefore in the synthetic direction, the molecular structure of the chiral product will be determined by the structure of the acceptor molecule. This means that the donor molecule can be freely chosen, since it neither affects the target product structure nor the stereoselectivity. In principle therefore, a plethora of possible donors could be chosen, although in the scientific and patent literature only a handful of amine donors have been reported. The authors have recently proposed a novel quantum mechanical method to determine the free energy of compounds and hence the thermodynamic feasibility of using novel amino donors for this reaction type, irrespective of kinetic considerations (Meier *et al.*, 2015). This along with a wider implementation of this technology in the future is likely to lead to a broader range of different amino donors.

Despite the interest in such reactions, they are often demanding to implement on an industrial scale due to frequent thermodynamic and kinetic challenges (Tufvesson, Lima-Ramos, Nordblad, *et al.*, 2011). While many technical solutions are available to overcome these challenges, the proposed solutions are frequently complex and often require significant process development time. Indeed, for some applications, a fast and simple process development is not

only desirable but may be essential for commercial success. In these cases, it will be more important to rapidly develop a simple process, than to obtain an economically optimal process. Such situations include pharmaceutical synthesis in the early phases of clinical testing and other cases such as small singular batches, where investment in extensive process development cannot be justified. Using this logic, and from a knowledge of the properties of a given ω-transaminase-catalyzed reaction and the available enzymes to catalyze the reaction, we reasoned that it should be possible to categorize a particular reaction as "complex" (requiring extensive development) or "simple" (with easy implementation and scale-up). We therefore suggest that an evaluation method allowing the identification and selection of "simple" reactions (and eliminating the "complex" ones) would prove a valuable tool for process chemists.

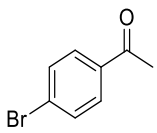
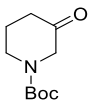
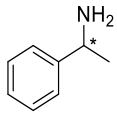
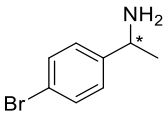
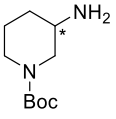
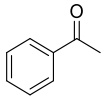
The scope of this manuscript is therefore to present a stepwise decision-making procedure to quickly identify if a "simple" scale-up is feasible for a given reaction. Hence, solutions such as biocatalyst modification by protein engineering (Tufvesson, Lima-Ramos, Nordblad, *et al.*, 2011), amino donor recycling (Höhne *et al.*, 2008; Höhne and Bornscheuer, 2009), and equilibrium shifting methods (Truppo, Rozzell and Turner, 2010; Tufvesson, Bach and Woodley, 2014), have not been considered here. The three-step decision-making procedure involves an evaluation of: (1) thermodynamics, (2) biocatalyst activity, and (3) product inhibition. Each step is evaluated against a threshold value, which must be met in order to identify a given case as suitable for "simple" implementation.

4.3 Results

In order to exemplify the method, experimental data on two chiral target products were evaluated, 1-(4-bromophenyl)-ethylamine (**5**) and (*S*)-(+)-3-amino-1-Boc-piperidine (**6**). These compounds were selected because both products are commercially attractive and additionally biocatalytic transaminations to synthesize both **5** (Shin and Kim, 2002) and **6** (Höhne et al., 2008; Höhne, Robins and Bornscheuer, 2008) have been reported previously. In these reactions, optical purity was necessary and evaluated, but a specific stereoisomer was not required. The prochiral ketone substrates, the amino acceptors, 4-bromoacetophenone (**1**) and 1-Boc-3-

piperidone (**2**), corresponding to the products above, were reacted with two possible donor molecules propan-2-amine (**3**) and 1-phenylethylamine (**4**) (Table 4.1). Both amino donors have frequently been used in a wide variety of biocatalytic transamination. Between them, they represent different classes of donor. For instance, donor **3** serves as an inexpensive achiral donor. In contrast amino donor **4** is a more costly chiral compound which has also been reported to be inhibitory (Al-Haque et al., 2012) with downstream processing complications due to separation issues when the product shares structural similarity. However, donor **4** also offers a significant thermodynamic advantage, since the carbonyl coproduct, acetophenone (**8**) formation is highly favorable (Table 4.1). Academically, a more common amino donor that has often been reported is the use of alanine (or pyruvate as a acceptor for the resolution reaction) (Koszelewski, Tauber, et al., 2010; Kroutil et al., 2013). We have previously shown that the thermodynamics using this donor very strongly favors the reverse resolution reaction (Gundersen et al., 2015), and therefore we have not considered this further in this work.

Table 4.1: Compounds used in this chapter. 4-bromoacetophenone (**1**), 1-Boc-3-piperidone (**2**), propan-2-amine (**3**), 1-phenylethylamine (**4**), 1-(4-bromophenyl)ethylamine (**5**), 3-amino-1-Boc-piperidine (**6**), acetone (**7**), acetophenone(**8**)

Reactants	Acceptor ketones		Amino donors	
				
	1	**2**	**3**	**4**
Products	**Target chiral products**		**Co-products**	
				
	5	**6**	**7**	**8**

4.3.1 Method Development

In order to enable rapid evaluation, the three selection criteria are each assigned a threshold value, which must be met to enable implementation of a simple scale-up. The proposed procedure is outlined in Figure 4.1. In the figure, full lines indicate that the reaction has met (green lines) or failed (red lines) the individual criteria. Likewise, dashed lines indicate an alternative strategy by adjusting one of the variable reaction components (the amino donor or the biocatalyst). The threshold values for each criterion are also indicated in the legend of Figure 4.1, the justification for which is given in the following section.

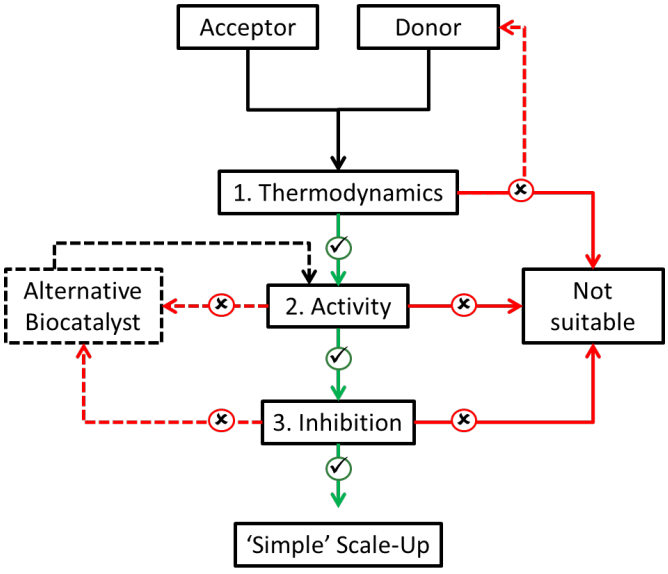


Figure 4.1: Decision making procedure for a simple scale-up. Green lines marked with a check mark or red lines marked with an X indicate if a given criterion is met or not met, respectively. Dashed lines and boxes indicate options for reassessment if a criterion is not met. Each criteria has cut off values for simple implementation. 1. The thermodynamic criteria is meet when K_{eq} is less than 0.02 (resolution reactions) or greater than 1 (synthetic direction). 2. The activity criterion requires a specific activity greater than 0.05 g/g/h. 3. The inhibition criteria is met at less than 50% activity loss, with 5% of target concentration product present. Possible remediation options, if a given criterion for a simple scale-up is not met, can be to consider an alternate amino donor or test an alternative biocatalyst (dashed lines).

4.3.1.1 Thermodynamic Assessment

Unfavorable thermodynamics presents one of the main barriers to the implementation of the transaminase catalyzed reactions on an industrial scale (Koszelewski, Tauber, *et al.*, 2010). The thermodynamic equilibrium constant (K_{eq}) of the reaction is important since it determines the maximum reaction yield for a given concentration of substrates. Thus, we reasoned it is one of the most important parameters for determining the optimal process configuration (Tufvesson, Lima-Ramos, Jensen, *et al.*, 2011; Tufvesson *et al.*, 2012; Tufvesson, Bach and Woodley, 2014). For this reason, we suggest the first step in the procedure should be to determine if a candidate reaction has a suitable thermodynamic equilibrium constant to make a "simple" scale-up feasible. In the synthetic mode, thermodynamic feasibility is here defined as a K_{eq} above 1.0, since lower values of K_{eq} would require a high excess (more than 20-fold) of the amino donor to obtain sufficient reaction yields (95% or higher), for eventual industrial implementation. Use of such an excess makes the reaction costly and practically difficult to carry out at high substrate concentrations. In a similar way, we reasoned that for reactions with a low K_{eq}, a kinetic resolution would be a better choice for the reaction. On the other hand, the resolution reaction requires more stringent conversion requirements since the separation of the amine product from the unreacted half of the racemic donor starting material is of course quite challenging. Hence we have chosen a K_{eq} threshold of 0.02 in the resolution direction, meaning only values lower than this are suitable for a simple scale-up.

In this work, the concentration-based equilibrium constant was experimentally determined using a previously described method (Tufvesson *et al.*, 2012). Since the value is obtained for comparative purposes, practical (rather than standard) conditions were used, meaning it is more accurate to describe the constant as "apparent", $K_{eq}^{\ app}$. In principle to save time as an alternative to experimental measurement, in silico methods could be used to estimate such values, although the accuracy is perhaps questionable. Here the $K_{eq}^{\ app}$ for the two chiral amine products **5** and **6** were measured experimentally using the two donors **3** and **4**, as described above. The $K_{eq}^{\ app}$ for the four reactions (Table 4.2) varied by a factor of 10^4 from the most challenging pair, **1** and **3**, at 0.025 to the most favorable pair, **2** and **4**, which had a $K_{eq}^{\ app}$ of 450,

in the synthetic direction. Thus, thermodynamics is indeed highly variable between the four selected reaction pairs. After applying the threshold criteria one of the two products, **5**, was eliminated from further investigation. This may indicate that highly conjugated aryl compounds are not suitable for simple scale up and should be assisted by other process technologies and strategies. For example, it has been reported that one of the compounds we have used as a donor here **4**, could also synthesized and successfully scaled in combination with *in situ* product removal, alleviating both the thermodynamic and inhibitory strains (Truppo, Rozzell and Turner, 2010). None of the reaction pairs evaluated here was found suitable for the resolution reaction, although alanine, the amine donor often found most suited for resolutions reactions was not tested as discussed previously (Gundersen *et al.*, 2015).

Table 4.2: Experimental Values for K_{eq}^{app}

	Donors	
Acceptors	**3**	**4**
1	0.025[a]	0.5
2	32	450

[a] Data previously reported in Tufvesson, Bach and Woodley, 2014.

Clearly it is possible to carry forward more than one amine donor to the subsequent evaluation steps, although this is not helpful for the procedure, which aims to focus effort on those cases with the biggest chance of simple scale-up success. In this case, due to the low cost and high water solubility, amine donor **3** was selected for further evaluation.

4.3.1.2 Biocatalyst Activity Screening

No matter how favorable the thermodynamics, without sufficient activity the reaction will not be completed in a reasonable time, and issues like enzyme inactivation may arise. Hence, the next step of the procedure is to find a suitable biocatalyst with sufficient activity. Candidates for biocatalyst screening can be obtained from commercial screening kits or in-house enzymes. For the "simple" scale-up, strategies such as protein engineering are not considered. Low activity of an enzyme preparation will negatively impact downstream processing, by adding extra proteinaceous material which impedes product recovery. Therefore, the maximum biocatalyst

loading was set to 10% v/v irrespective of the biocatalyst formulation. Additionally, product concentration should be in the range of ≥50 g/L (Tufvesson, Lima-Ramos, Jensen, *et al.*, 2011; Tufvesson, Lima-Ramos, Nordblad, *et al.*, 2011) to assist downstream product recovery. Finally, due to biocatalyst stability concerns, we reasoned it necessary to complete the reaction within 96 h. On this basis, we calculated a minimal biocatalyst specific activity (sometimes termed "biocatalyst productivity"), as a threshold value for the "simple" of 0.05 g/g/h (g product/g biocatalyst/hour).

For this case study a small screen with four enzymes was conducted, using the reactant pair **2** and **3** selected from the previous section. In this screen four selected enzymes were tested, two of which were known to be (*R*)-selective and two (*S*)-selective. We reasoned that for this case study the particular stereoselectivity of the enzyme did not influence the overall procedure. Additionally, since this screen was conducted with an achiral amine donor and the pro-chiral ketone, the selectivity of the enzyme would not affect the reactivity with these substrates. The screen showed a large variation between the least and most reactive candidates (Table 4.3). Details of the individual enzymes (ATA-47, Tar0, Tar1, and Ars-ωTA) are given in the experimental section of the paper. Tar0 was found to give a specific activity of 0.003 g/g/h, whereas the best candidate (ATA-47) gave a 20-fold higher value of 0.054 g/g/h. ATA-47 was therefore carried to the next step. Likewise the enzyme Ars-ωTA had a high specific activity of 0.048 g/g/h, close to the threshold value.

Table 4.3: Specific Activities Obtained with Reactant Pair **2** and **3**, with Selected Enzymes

Enzyme	Selectivity	g/g/hr
Ars-ωTA	*S*	0.048
Tar0	*R*	0.003
Tar1	*R*	0.012
ATA 47	*S*	0.054

4.3.1.3 Determination of Product Inhibition

The final step of the procedure considers product inhibition of the enzyme, which due to the requirement for high product concentrations (50 g/L) in industrial processes (Straathof, Panke and Schmid, 2002), is a frequent hurdle for process intensification of enzyme reactions in general, and ω- TAs in particular (Truppo, Rozzell and Turner, 2010). Hence, we set the threshold value here at a 50% reduction in reaction rate in the presence of 2.5 g/L product, under the assay conditions used here (see Experimental Section). Here only product inhibition is assessed, since substrate inhibition can relatively easily be overcome by substrate feeding.

In order to experimentally test for product inhibition, the initial reaction rate of ATA-47 was measured using 100 mM **3** and 10 mM **2**, in the presence of various concentrations of the product **6**, up to 10 mM. Importantly, the substrate concentrations were chosen to avoid limiting the reaction by thermodynamic constraints. Inhibition was observed with 10 mM product and amounted to a 10% initial rate reduction, compared to initial conversion rates in the absence of product. Initial conversion rates were assumed when less than 10% of limiting starting material was converted.

4.3.1.4 Discussion

First, with respect to thermodynamics, the procedure enables the elimination of unfavorable cases. Clearly each donor or acceptor molecule has an associated free energy which contributes to the net thermodynamics of a given reaction. In this way for instance a comparison of the equilibrium constants of two reactions (with different acceptors, but using the same donor) can be used to interpret the effect of changing acceptors. In an analogous way, one could determine the K_{eq} for a given acceptor with one donor and extrapolate the K_{eq} to other donors with the same acceptor, given one knows the difference in ΔG between the reactions, as discussed elsewhere (Gundersen *et al.*, 2015).

Second, the biocatalyst activity is assessed, since low activity will have drawbacks in the form of low space-time yields and may prevent the reaction from going to completion due to enzyme deactivation. One solution would be to apply high biocatalyst concentration, but these may negatively impact downstream processing by hindering product recovery. Thus, the

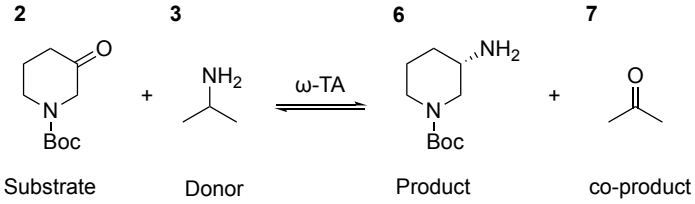
threshold for the enzyme is defined as minimum specific activity, which for the "simple scale-up" was set at 0.05 g/g/h. Biocatalyst recycle was not considered for the simple scale-up.

Finally, a determination of product inhibition is carried out. This is a frequent hurdle for process intensification of ω-TAs (Truppo, Rozzell and Turner, 2010), due to the high product concentrations (50 g/L) required to simplify the product recovery (Straathof, Panke and Schmid, 2002). In contrast to the high concentration intensity of commercial processes, enzymes are designed to work under physiological (dilute) conditions. This frequently leads to process intensification challenges with biocatalytic reactions. For example transaminases display a ping-pong bi-bi reaction mechanism, with two sequential half reactions (Henson and Cleland, 1964), and this type of reaction mechanism is often plagued by inhibition from competitive dead-end complexes of products bound to the apo-enzyme or the incorrect form of the holoenzyme. Hence, understanding the inhibition profile of a potential product is vital in evaluating the possibility of a simple scale-up. As such, we advocate that, if severe inhibitory effects are observed with low product concentrations, it implies a high risk of inhibition under process scale concentrations.

The three-step evaluation method has been successfully applied to a case study, and one reaction pair with one biocatalyst was deemed suitable for "simple" scale-up.

4.3.2 Intensification and Scale-Up.

In the previous sections, the selection procedure for a simple scale-up toward the synthesis of **6** identified acceptor **2** with donor **3** (Scheme 4.2) using ATA-47 as suitable. In the event ATA-47 was substituted by ArS-ωTA since the difference in activity was negligible and the latter enzyme has been reported to have excellent stereoselectivity (Koszelewski, Goritzer, *et al.*, 2010; Mutti and Kroutil, 2012).



Scheme 4.2: Synthetic Transaminase Reaction Carried Out. Compounds: 1-Boc-3-piperidone(**2**), propan-2-amine(**3**), (*S*)-(+)-3-amino-1-Boc-piperidine(**6**), acetone(**7**).

4.3.2.1 Reaction Optimization: pH and Donor Loading

Prior to scale-up, a small optimization study was undertaken to evaluate if reaction rates could be enhanced by simple optimization within the biocatalyst stability range. A range of pH and donor loadings was explored in an attempt to improve kinetics, with both short (0.5–2 h) and long (18 h) reaction times; the latter time point was chosen to investigate enzyme stability under the given conditions.

The rate dependency on pH was tested between pH 7 and 9, with 40 mM acceptor and 500 mM donor (Figure 4.2). Other studies have found up to 40% variation in yield in this pH range for similar reactions (Koszelewski *et al.*, 2008). Here the fastest reaction rates were identified at pH 9 for all time points. The greatest difference was found in the 18 h reaction times, where average reaction rates are 45% faster at pH 9 compared with pH 7, indicating that this is the best pH, within the pH range tested, with respect to kinetics, and that the enzyme is more stable under these conditions. Since the pKa of the amine donor **3** is 10.6 (Hall, 1957), meaning a higher pH would render a higher fraction of the substrate uncharged and thus reactive, in principle operating at a higher pH would therefore be beneficial from the perspective of the reaction rate. Nevertheless, in this study we limited the pH range to keep the study simple and manageable, consistent with the philosophy of this work, and therefore did not test the reaction at higher pH values than 9.

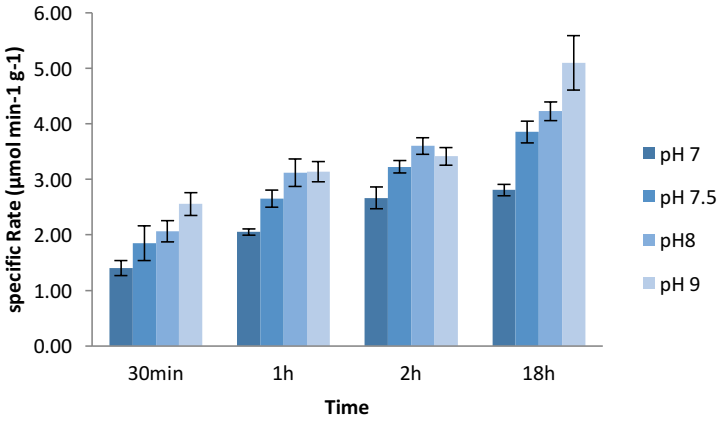


Figure 4.2: Specific rates measured at four reaction time points at four different reactions, each carried out at different pH values (pH 7.0, pH 7.5, pH 8, and pH 9 from left to right at each time point, respectively).

Furthermore, the same method was used to determine optimal donor loading. Donor concentrations could potentially be limiting, dependent upon K_M (Höhne and Bornscheuer, 2009). Clearly an excess concentration of the donor (over acceptor) could be used which might also drive the equilibrium (Shin and Kim, 1997; Koszelewski *et al.*, 2008). This was tested experimentally but at all concentrations tested, the rate was unaffected by donor concentration (Figure 4.3), suggesting a K_M beneath 100 mM. For subsequent experiments 1 M **3** was used.

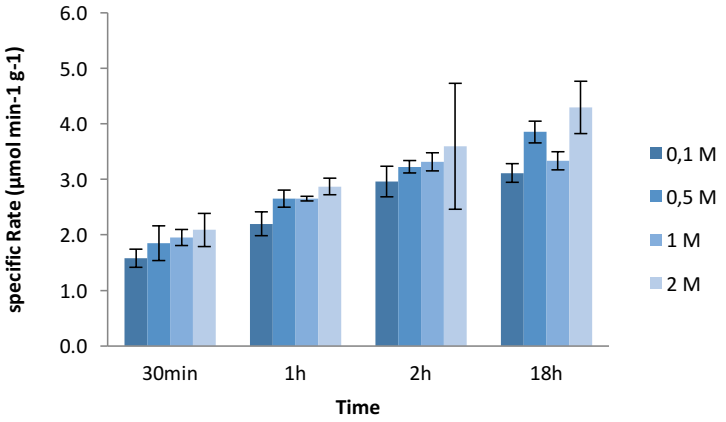


Figure 4.3: Specific rates found at four time intervals at four donor concentrations (0.1, 0.5, 1, and 2 M from left to right at each time point, respectively) used to investigate the optimal donor loading for the reaction

4.3.2.2 Reaction Intensification

As indicated above a viable scale-up depends on reaction intensification (i.e., the synthesis of high product concentrations) (Tufvesson, Lima-Ramos, Nordblad, *et al.*, 2011). This is important in the simple scale-up because too low a concentration will add volume to the reaction and thus complicate the process. The reaction of **2** and **3** using Ars-ωTA was therefore intensified by increasing the substrate concentration up to 75 g/L. Three reactions were done in scintillation vials at concentrations of 25, 50, and 75 g/L. The reactions proceeded smoothly (Figure 4.4) at both 25 g/L and 50 g/L but not at 75 g/L, the latter most likely due to mass transfer limitations from low solubility and decomposition of the starting material in aqueous conditions. The latter was further investigated and confirmed (data not shown). To the best of our knowledge no other study has investigated the stability of this compound in water, either for biocatalysis (Höhne *et al.*, 2008) or chemical catalysis. In the 25 and 50 g/L reactions final conversions of acceptor **2** to chiral amine target **6** of 70% were observed. Figure 4.5 shows that the initial reaction rates are similar at all substrate concentrations tested, indicating that the reaction is not kinetically controlled (above K_M).

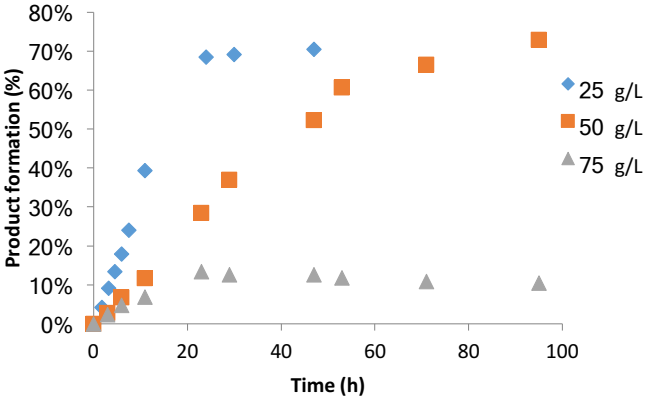


Figure 4.4: Reaction profile over 96 h with initial substrate concentrations of 25, 50, and 75 g/L.

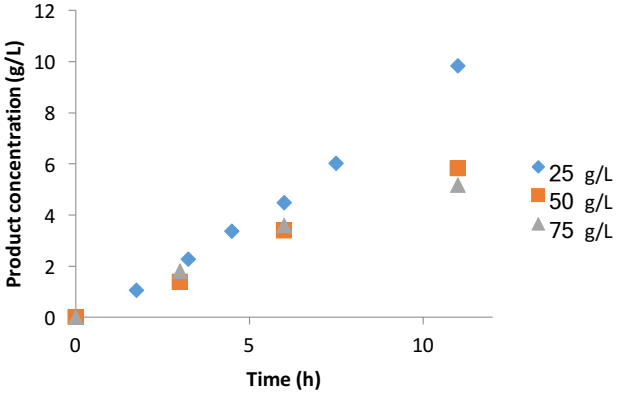


Figure 4.5: Initial product formation for the first 12 h of the reaction with initial substrate concentrations of 25, 50, and 75 g/L.

4.3.2.3 Product Identification

Finally, the reaction was run at 25 mL scale for 96 h to isolate product. At 50 g/L substrate concentration the final reaction composition was analyzed to contain 89% **6** and 11% **2** (with an isolated product yield of around 70%). This composition is in excellent agreement with that found in the 50 g/L 1 mL scintillation vial experiment, which gave 91% target chiral amine **6** and 9% ketone **3**, after 96 h.

4.4 Conclusion

A simple stepwise procedure has been described, to facilitate the selection of suitable substrate-donor-enzyme combinations to allow so-called "simple" scale-up. Each step in the procedure has a threshold value which must be met to allow simple implementation. We believe that this method will prove useful both to select good candidates for this technology and to eliminate those that may require further development. A simple case study was used to illustrate the power of the procedure, sequentially eliminating unsuitable substrates, donors, and enzymes. Beyond this case study, analogous procedures could be used for the evaluation of other "simple" biocatalytic processes.

4.5 References

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Section I

Chapter 5: Conclusion and future work, Section I

5.1 Conclusion

Section I, focused on the implementation of ω-TA reactions in new areas, particularly towards the pharmaceutical industry. The section had a practical focus, with easy to use tools and methods to enable a more widespread use of this reaction, especially towards small-scale singular batches.

Firstly, in Chapter 3 the benefits of carefully selecting the amine donor in the reaction pair is discussed, to provide the reaction with favorable reaction kinetics. It was shown that donor selection with the same acceptor ranged from less than 1% to 50%, when the acceptor:donor ACP:MBA/Ala, respectfully, was used in a 1:1 initial concentration (Gundersen *et al.*, 2015). Furthermore, this chapter provided a QMM tool to predict of any given reaction pair with *ab initio* calculations (Meier *et al.*, 2015). Which enables the possibility of selecting reaction pairs early in the development of a new process.

Furthermore in Chapter 4 of this thesis, outlines a selection procedure for fast implementation of ω-TA reactions (Gundersen *et al.*, 2016). The method has a 3 step easy to use protocol, which considers thermodynamics, enzyme activity and inhibition, each step has clear cut-off values. Each step must be met in order to ensure easy and fast implementation of the ω-TA reaction.

- Thermodynamics: K_{eq} is less than 0.02 (resolution reactions) or greater than 1 (synthetic direction).
- Biocatalyst activity: Specific activity greater than 0.05 g/g/h (g product/g biocatalyst/hour).

- Product inhibition: Less than 50% activity loss, with 5% of target concentration product present

If the criterion is not met, strategies can be used to overcome the challenge. Criterion 1 can be overcome by changing the donor, alternatively with the tools from Chapter 3. Criterion 2 and 3 can be overcome by changing the biocatalyst, or by enzyme engineering.

This methodology was then successfully demonstrated by subjecting two target products 1-(4-bromophenyl)ethylamine and (*S*)-(+)-3-amino-1-Boc-piperidine to the methodology. One of the two products, (*S*)-(+)-3-amino-1-Boc-piperidine, meet the criteria and was successfully run at 25 mL scale with initial acceptor concentrations of up to 75 g/L and up to 70% yield. The tools and methods in this section could enable a more widespread use of ω-TAs, especially in applications where fast implementation is paramount, by reducing development time.

Although ω-TAs is a well-established biocatalytic route in the pharmaceutical industry, an even more widespread use could be possible. In pharmaceuticals, a key aspect is early implementation. The results presented in this section allows for simultaneous process and enzyme development, allowing for faster development and implementation. In conclusion, the tools provided in this section can enable fast implementation of ω-TAs, particularly important in pharmaceutical applications. Due to the frequent thermodynamic challenges, the benefits of ω-TAs are best exploited in the synthesis of chiral molecules. For non-chiral amines, current chemical synthesis methods are highly efficient, and at the present time, biocatalytic methods do not compete economically.

5.2 Future work

The results in this section indicate that it is indeed possible to increase the use of biocatalysis in the pharmaceutical industry and that increased use could be facilitated by simultaneous process and enzyme development. It is therefore suggested that the results in this section could be used

as a starting point to further expand the use of biocatalysis as a whole. Specific suggestions are listed below.

- Firstly, the QMM method described in Chapter 3 can be further extended beyond ω-TAs. In essence, this method can be used to calculate the thermodynamic favorability of any given reaction, independent of the catalyst. In particular, KRED catalyzed reactions could be interesting to investigate, as the substrate is similar to that of ω-TA's.

- Furthermore, it is clear from the work carried out in this section that the use of transaminases is at times challenging. As mentioned in Chapter 2, ω-TAs is only one of several biocatalytic routes to chiral amines, in addition to conventional chemical methods. The use of retrosynthetic methods, such that are used in conventional synthetic chemistry, would therefore highly benefit reactions such as ω-TA. To early determine if ω-TAs are indeed the best route to a target molecule. Such a method has been outlined by Turner and O'Reilly (Turner and O'Reilly, 2013). The incorporation of a database covering available biocatalytic reactions would be an excellent starting point for this.

- Precipitation driven synthesis is an interesting option for driving thermodynamically unfavorable reactions. Although this method is not possible for all reactants, it could be applicable in certain cases (Ulijn *et al*., 2001).

- Finally, it is clear that there is a need for simpler guidelines for implementation of biocatalysis. Although the guideline in Chapter 4 is a good starting point. Similar guidelines could be developed for other enzymes and for different applications. Specific examples where such guidelines can be useful include: enzyme immobilization and enzymatic oxidation reactions.

5.3 References

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Section II

Section II

Chapter 6:
Introduction to carbonic anhydrase in carbon capture

6.1 Carbonic anhydrase

6.1.1 Introduction

The enzyme carbonic anhydrase (CA, EC 4.2.1.1) is found in almost all living organisms (Supuran, 2016). CA's are a superfamily of enzymes that encompasses six highly versatile classes of isozymes (Supuran, 2016). CA reversibly hydrates CO_2 into bicarbonate (Scheme 6.1). CA has recently gained further interest because it can be used as a catalyst in greenhouse gas (GHGs) mitigation in carbon capture projects, so-called enzyme enhanced carbon capture and storage (EECCS) (Gundersen, von Solms and Woodley, 2014; Gundersen *et al.*, 2017). EECCS is the focus of Section II and will be discussed in detail in the last section of this chapter.

$$CO_2 + H_2O \leftrightharpoons HCO_3^- H^+$$

Scheme 6.1: Reaction catalyzed by carbonic anhydrase.

6.1.2 Additional reactions

In addition to reversibly catalyzing the hydration of carbon dioxide, CA has been found to catalyze several other reactions. This is particularly relevant for EECCS because the gas which is to be purified may contain a number of contaminants. It is therefore important to know if CA's, and especially the particular CA in use, will react with other components of the flue gas. It is unknown if these additional reactions serve a physiological purpose. The reactive metal ion and the large active site pocket (discussed below) may invite to unintended side reactions. Due to the versatility of CA's, unlike the carbon dioxide to bicarbonate reaction, which is the defining

reaction of this class, it is highly varied which other reactions these enzymes catalyze and to which extent. Therefore, one cannot draw conclusions based on the study of one enzyme to the whole superfamily of CA's. They have been found to catalyze reactions with cyanamide, carbonyl sulfide, and carbon disulfide among other substrates. Furthermore, CA's has been found to have a broad esterase activity, including carboxylic esters, thioesters, and sulphonic acid esters among others (Supuran, 2016). It should be noted that ester bonds are particularly cleavable and are subject to autohydrolysis in water. Finally, CA's has been found to bind tightly certain metal ions and to cyanide, in fact, tighter than CO_2 (Salmon and House, 2015).

6.1.3 Applications

CA is subject to research in several areas. The majority of research on CA's is in medical research, in addition, CA's are used in climate mitigation and for specialized applications. Although these fields use the same enzyme (albeit different isozymes) there is very little interaction between the areas. Human CA's are a major target of investigation, as they have been found to be involved in neurological disorders, glaucoma, epilepsy, cancer and obesity, among other diseases (Hassan *et al.*, 2013; Supuran, 2016). Much of this research is focused towards inhibitors for pharmaceutical use. Human CA is also investigated for use in artificial organs, more specifically in artificial lungs and blood substitutes (Boone, Habibzadegan et al., 2013). Furthermore, because CA's are vital to survival and differ greatly between organisms, bacterial and fungal CA's are a target of antibiotic and anti-fungal research.

In addition to direct applications for humans or human pathogens, some research also focuses on technical applications. These include biosensors and scavengers for trace metals like Zn(II) and other 2^+ oxidized metals (Boone, Habibzadegan et al., 2013). Due to the tight binding of cyanide, it has also been proposed to use CA as a cyanide scavenger (Salmon and House, 2015). CA's are also being used to sequester CO_2 in small enclosed spaces like submarines and space crafts (Boone, Habibzadegan et al., 2013)

Since CA's are well understood and serve as a model enzyme, in particular, the enzyme hCAII, it has also been subject to *de novo* computational engineering. Heinisch and colleagues computationally engineered the scaffold of hCAII with the Rosetta software, where

the zinc ion was replaced in the active site with an artificial Iridium organometallic cofactor, which catalyzed the asymmetric reduction of a cyclic imine, with 96% *e.e.* (Heinisch *et al.*, 2015).

6.1.4 Historical background

The enzyme was identified from observations that CO_2 release from serum occurred faster than expected from un-catalyzed mass transfer rates (Meldrum and Roughton, 1933a, 1933b). The reaction is essential to living organisms as it provides a way of removing carbon dioxide from single cells, and in multi-cellular organisms is a mechanism of transporting CO_2 in and out of the organism and between organs. It has been suggested that CO_2 transport across membranes (trans-membrane transport) was the first function of the enzyme and other functions evolved later (Henry, 1996). In particular, the conversion of HCO_3^- to CO_2 is physiologically very important, because the un-catalyzed reaction is slow (Hassan *et al.*, 2013; Russo *et al.*, 2013). Furthermore, bicarbonate is more abundant (and soluble) in cells than dissolved CO_2, which increases thermodynamic strain (Henry, 1996; Russo *et al.*, 2013).

6.1.5 Reaction Mechanism

CA's follow Michaelis-Menten reaction kinetics, the reaction rate is described in equation 6.1. Where the rate of the reaction (V_0) is described by the substrate concentration [S], and the maximum rate at full substrate saturation (V_{max}) and the Michaelis-Menten constant (K_M) which is the substrate concentration at ½ of V_{max}. (Salmon and House, 2015). Equation 6.1 is valid under the condition that the enzyme and water are in excess compared to the substrate. In applications discussed here it is also useful to consider the reaction rate when enzyme availability is a limiting factor, and therefore must be accounted for in the equation. Rates under such conditions follow Equation 6.2. Where E_0 is the enzyme concentration and K_{cat} is the turnover number, which is the maximum reactions catalyzed per active site in a specified time interval, usually 1 second.

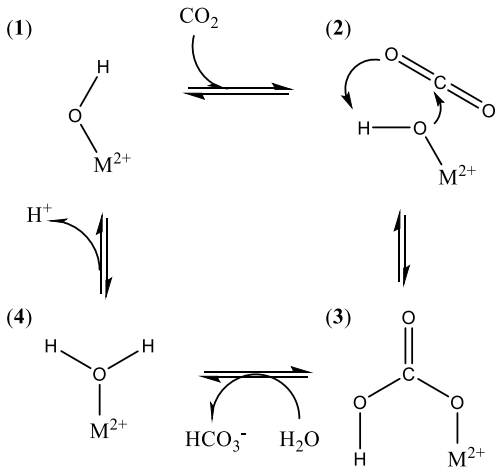
Equation 6.1:

$$v_0 = \frac{v_{max}[S]}{(K_M + [S])}$$

Equation 6.2:

$$v_0 = \frac{[S]}{(K_M + [S])} \times K_{cat} \times E_0$$

The most studied CA is the hCAII a human enzyme, which falls into the α-class, described below (Boone *et al.*, 2014; Salmon and House, 2015; Supuran, 2016). Hence, the most detailed reaction mechanism is described for this isozyme (Boone, Habibzadegan et al., 2013; Salmon & House, 2015; Tripp, Smith, & Ferry, 2001). hCAII follows a two-step ping-pong mechanism, which is described in detail in several excellent reviews (Boone, Habibzadegan et al., 2013; Salmon & House, 2015; Tripp et al., 2001). A generalized reaction scheme is outlined in Scheme 6.2, based on the reaction mechanism outlined for hCAII, but further generalized to encompass most other CA's. It should be noted that for at least one CA, a different mechanism has been suggested, that does not fit the mechanism outlined here (Tripp, Smith and Ferry, 2001; Supuran, 2016). The general mechanism is as follows: (**1**) A hydroxide bound to a metal in the 2^+ oxidation state M(II), usually this is Zn(II), but Co(II) and Ca(II) has also been reported. (**2**) M(II) nucleophilically attacks CO_2 (aq). (**3**) Which forms a metal-bound bicarbonate. (**4**) Which is further displaced by a water molecule, finally a proton is removed from the metal-bound water to regenerate the metal-bound hydroxide, and the cycle is started anew. It is found that the last step of regenerating the hydroxide by deprotonation is the rate-limiting step (Tripp, Smith and Ferry, 2001; Salmon and House, 2015). In fact, the CA's with the highest reaction rates, has a so-called 'proton shuttle', which consists of a number of coordinated amino acid residues, that efficiently transports the proton from the active site out to the bulk phase (Boone, Habibzadegan et al., 2013; Salmon & House, 2015; Supuran, 2016). The role of M(II) is to act as a Lewis acid, which lowers the pKa of water from 14 to 7, rendering the reaction possible under physiological conditions (Tripp, Smith and Ferry, 2001). The presence of the metal ion is therefore necessary for the reaction to take place and is therefore the only truly conserved aspect of CA's.



Scheme 6.2: Generalized reaction mechanism of carbonic anhydrase.

6.1.6 Overview of CA classes

CA is a superfamily of enzymes, with the six classes α-, β-, γ-, δ-, ζ-, and η –class, briefly described below. For the focus of this section, it is important to note the diversity between these classes. Often when specific aspects of CA's are studied, whether for medical or EECCS applications, general conclusions based either on a detailed study of the hCAII or another CA used in that particular study, is often drawn. However, this may not be true for all, or even most, CA's. The diversity within this superfamily is astonishing. The enzyme appears to have convergently evolved at least three times (Supuran, 2016). The γ-class have most likely evolved separately, and the 3 remaining classes δ-, ζ- and η- have evolved from the α- or β-class (Supuran, 2016). The overall superfamily of enzymes shares few sequence and structural similarities beyond the +2 oxidized metal ion in the active site, all CA's are therefore metalloenzymes (Supuran, 2016). The most common metal is Zn(II). Furthermore, the enzyme always has one hydrophilic pocket for binding water, and one hydrophobic pocket for binding CO_2 (Supuran, 2016). The active site pocket is large compared to the substrate, in the CA's that has been crystallized to date. The size of the active site ranges from 140 to 280 Å, whereas CO_2 is just over 1 Å long (Supuran, 2016). However, beyond these few common features, the classes vary greatly. In addition to large

structural and sequence differences, active CA's can be found as monomers, dimers, dimers of dimers (tetramers) and trimers (Supuran, 2016).

6.1.6.1 α-class

Due to its medical relevance, the α-class is the most studied class of CA's. All human CA's are in this class, in fact, all mammalian CA's belong to the α-class (Boone *et al.*, 2014). This class of CA's span over a broad spectrum of organisms from mammals to protozoa and bacteria. Co(II) can substitute Zn(II) for many enzymes in this class, maintaining similar activity (Supuran, 2016). In this class, the metal is coordinated by 3 histidines and one water molecule. Furthermore, the α-class predominantly monomeric, but homodimers have also been reported. The fastest CA's are found in this class, due to their efficient way of overcoming the rate-limiting step. They have a so-called proton shuttle, where a series of histidine residues, efficiently transport protons out of the active site (Salmon and House, 2015; Supuran, 2016)

6.1.6.2 β-class

This class of CA's is found in a broad specter of organisms, such as plants, bacteria and algae (Tripp, Smith and Ferry, 2001; Supuran, 2016). The β-class is structurally very different from the α-class. CA's in the β -class are mostly dimers or multiples of dimers in their active form. This class is further divided into two distinct subclasses; Type I, which has an open active site and Type II which has a closed active site. Type I has the Zn(II) bound with 2 cysteines, 1 histidine and water. Type II has a non-canonical active site, where the active site water is replaced by aspartate, which to the best of my knowledge is the only CA where this occurs. Furthermore, the ε-class, only containing the enzyme CsoSCA, was re-categorized and now falls under the β-class. This enzyme was categorized as its own class due to the lack of active site similarities compared to other CA's, however, after eluting the crystal structure, the initial authors discovered that the enzyme does indeed belong to the β-class. But not directly under Type I or II, as the active site is quite evolved, and it might be serving a different function altogether (Sawaya *et al.*, 2006).

6.1.6.3 γ-class

The γ-class is found in archaea, cyanobacteria and bacteria. It is noted by the presence of Fe(II), not replacing the zinc. As with the α-class Zn(II) is coordinated by 3 histidines and water. The active enzyme is a trimer with 3 active sites on each interface.

6.1.6.4 δ-class and ζ-class

Are only found in marine diatoms. In both these classes, Zn(II) can be replaced by Cd(II). The δ-class is structurally similar to the α -class, and γ-class where the Zn(II) is coordinated by 3 histidines and water. ζ-class is similar to that of the β class, type I, the ζ class is furthermore thought to be monomeric. No crystal structure of either of these two classes has been eluted to date.

6.1.6.5 η-class

η-class is a newly discovered, and not yet well-studied class of CA's. Until now it has only been found in protozoa. Although no crystal structure is eluted of this class yet, it has been suggested that Zn(II) is coordinated by histidine, glutamine and water (Del Prete *et al.*, 2016; Supuran, 2016).

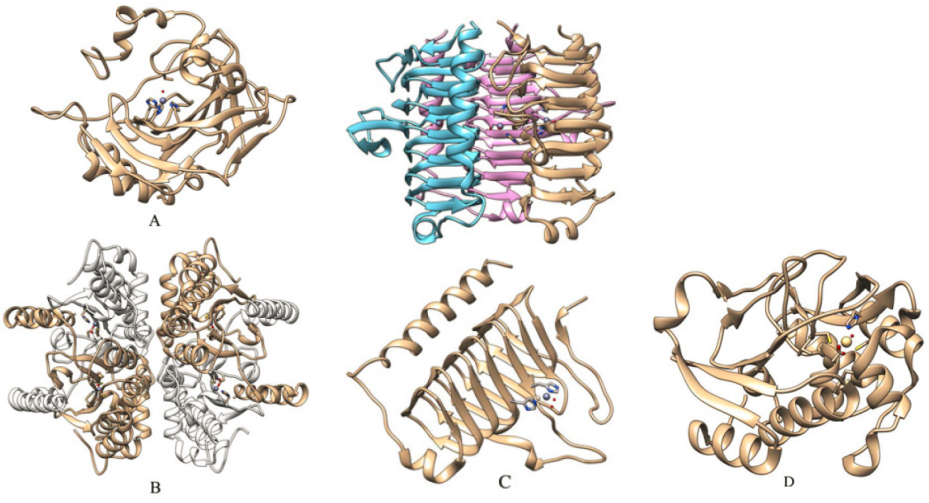


Figure 6.1: A) α-class, monomer (hCAII, PDB: 1F2W), B) β- class, tetramer (VchCA from *V. cholera*, PDB: 5CKX), C) γ-class upper: homotrimer, lower: monomer (Cam from *M. Thermophilia,* PDB: 1QRG), D) ζ-class, monomer (R3 domain of *T. weissflogii*, PDB:U3K8) (Supuran, 2016).

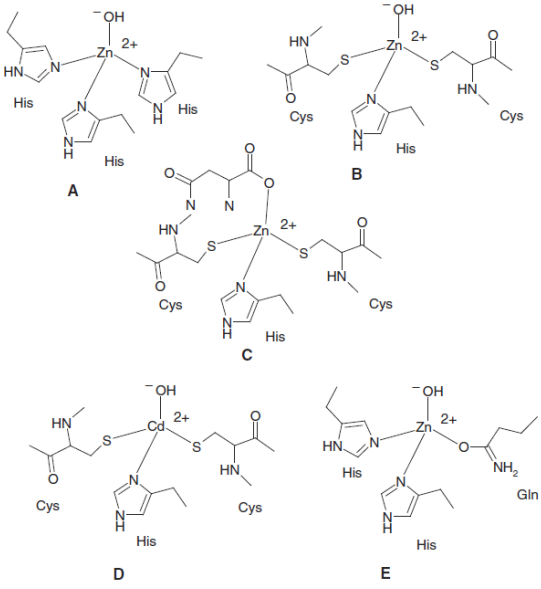


Figure 6.2: Example of the diverse metal ion coordination pattern in CA's (Supuran, 2016)**.** These can be found in the following classes of CA's: (**A**) *α*-, *γ* - and *δ*-class, (**B**) *β*-class (type I), (**C**) *β*-class (type II), (**D**) *ζ* -class (Note: Cd(II) replaces Zn(II)), (**E**) *η*-class (Supuran, 2016).

6.2 Carbon Capture and Storage

6.2.1 Overview

It is beyond reasonable doubt that greenhouse gas (GHG) emissions (save those from the natural carbon cycle) are an imminent threat to the global climate, and to our future existence on the planet (Rockström *et al.*, 2009). To overcome the increasing impact on the environment, it is vital that atmospheric concentrations of carbon dioxide and other GHGs are reduced. According to a recent report from the intergovernmental panel on climate change (IPCC), annual CO_2 emissions in 2010 were over 40 GT, and if current trends continue annual emissions will be over 100 GT by 2100 (IPCC, 2005). The same report indicates that a future scenario, without implementation of CCS in the near future, is unlikely to succeed in reducing annual CO_2 emissions to a safe level (IPCC, 2005). It should, however, be noted that CCS is not an ideal long-term solution. Obtaining energy without the combustion of fossil fuels, with alternatives such as wind energy and nuclear fusion, which does not produce vast amounts of CO_2 in the process, is a better long-term option. However, until such technology is ready to meet all energy needs, which is 30-50 years away, CCS should serve as an intermediate in managing carbon emissions. Without intervention, the annual emissions of CO_2 are going to continue to drastically increase. Furthermore, it should be noted that one of the main issues of CCS and greenhouse gas emission reduction, is the aspect of scale (Krebs, 2012). By 2050 about 80 $GtCO_2$ is expected to be emitted annually, and CCS should be responsible for a significant section of this reduction (20-40%) (IPCC, 2005). Currently, the biggest, and only commercial plant captures approximately 1 $MtCO_2$ per year (Idem et *al.*, 2015). At the end of September 2016, two other plants were under construction and eleven plants were in the planning phase (PCCCS-MIT, 2015).

Furthermore, CO_2 emissions are not only emitted from the power sector, the emitted CO_2 comes from a multitude of sources; this highlights the next issue of CCS: the distribution of sources of released CO_2 (Krebs, 2012). The carbon dioxide information analysis center (CDIAC) annually releases the previous year's carbon budget. Figure 6.3 illustrates the historic CO_2 flux, directly adapted from the report, clearly, fossil fuel use and industry is the major positive carbon

emitter (Le Quere *et al.*, 2016). Furthermore, this is divided into the five major categories in Figure 6.4, adapted from given report (Le Quere *et al.*, 2016).

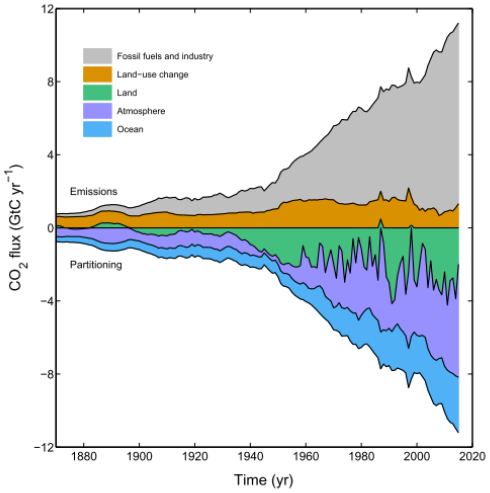


Figure 6.3: Historic CO_2 flux, from the CDIAC report 2016 (Le Quere *et al.*, 2016).

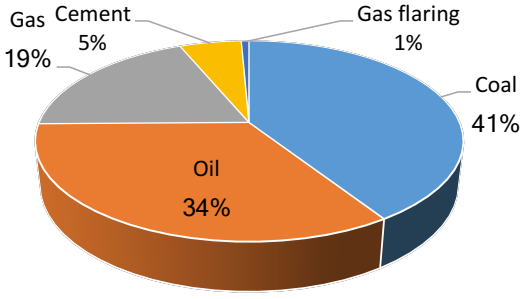


Figure 6.4: Distribution of the 9.9 Gt CO_2 emitted in 2016 adapted from data in Le Quere *et al.*, 2016.

Finally, it should not be overlooked that CO_2 is only one, although the major contributor, of several contributing GHG's. Methane gas, for example, is 25 times more potent as a GHG than

CO_2. Thus, global warming is a complex issue where numerous strategies must be employed in concert for a sustainable solution.

In CCS there are several options for capture, transport and storage. This thesis focuses solely on the capture aspect. Furthermore, this chapter is predominantly aimed towards the use of CA's in post-combustion carbon capture (PCCC) to capture CO_2 from pulverized coal (PC) plants. It has been reported that 41% of all CO_2 emissions in 2016 came from the coal sector (Le Quere *et al.*, 2016). A majority of the research with EECCS focuses on the PC application. Although it is true that this application has by far the biggest potential in terms of volume of CO_2 captured, it is neither the application closest to commercialization for EECCS nor the application where the use of CA's is most beneficial. The two other sources of CO_2 were EECCS is most promising is natural gas and biogas. In fact, it has been reported that the relative efficiency penalty of CCS in natural gas, is half that of PC, at only 15% (Haslbeck *et al.*, 2013). Flue gas from PC contains approximately 10-15% CO_2 after combustion. The remainder of the gas contains mostly inert SO_2, which is removed in a prewash, Nitrogen (N_2) and some minor contaminants. Furthermore, the flue-gas from PC plants is often targeted because of distribution. When defining a large source of CO_2 as an outlet of over 0.1 $MtCO_2$/year, a majority of the large sources are found in the PC sector. PC plants had almost 5000 sources with a total annual outlet of over 10,000 $MtCO_2$, accounting for over 60% of the sources and almost 80% of the CO_2 released from large sources (IPCC, 2005). Most of CCS has only tax benefits in the form of avoiding carbon taxes as an economic incentive, which currently is in the range of 50 USD per tonne CO_2 avoided. This is in stark contrast to the cost of API's covered in the previous section, at 1000 USD per kilogram. Furthermore, in select locations, the captured CO_2 can be used in enhanced oil recovery (EOR), which increases oil production and therefore may give a direct economic benefit.

However, natural gas and biogas are also good targets for CCS, especially EECCS. Flue gas from natural gas and biogas gas contains approximately 0-10% and 25-50% CO_2, respectively. The major component of this gas is methane, and the gas is used for combustion, either directly as fuel or for electricity production. Where CO_2 content reduces efficiency. A product specification of 2% CO_2 is often the upper limit of natural gas provided to consumers

(Rufford *et al.*, 2012). Therefore, the capture of CO_2 is not only environmentally beneficial but also commercially important.

Universal targets for post-combustion CCS is often set at 90% capture of a 15% CO_2 stream. These targets are obtained from the PC power plants, which has about 15% CO_2 stream (IPCC, 2005).

6.2.2 Reactive absorption

Reactive absorption is a gas/liquid absorption process. Where a gas containing CO_2 comes in contact with a solvent in an absorption device where the CO_2 dissolves into the solvent, where it further reacts to transform into a new chemical. This solvent is then transported to a desorbing device where pure CO_2 is desorbed/stripped from the solvent. A typical reactive absorption capture unit is described in Figure 6.5.

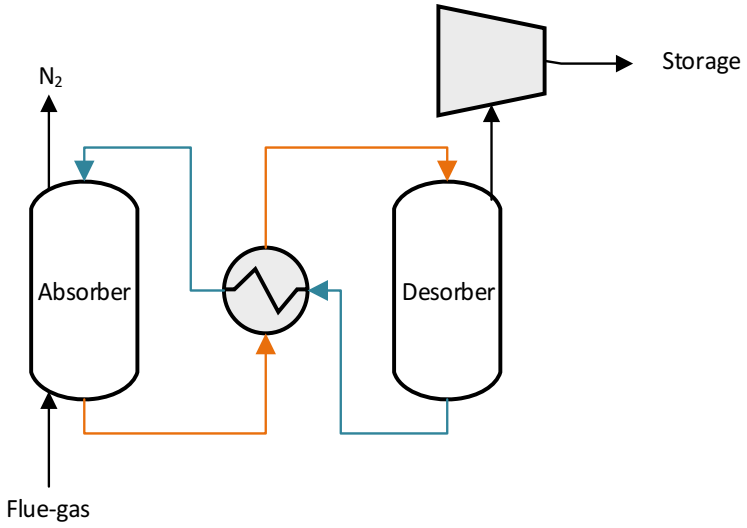


Figure 6.5: A simplified process scheme of reactive absorption. Flue gas (4-50% CO_2) and the lean solvent (low CO_2 concentration, **blue** line) enters the absorber, where about 90% of the CO_2 is absorbed into the rich solvent (high CO_2 concentration, **orange** line). The remaining of CO_2 escapes with the N_2 outlet into the atmosphere. In the desorber, the rich solvent is usually heated to 120 °C and the CO_2 is stripped from the solvent, regenerating the lean solvent sent back to the absorber and a pure CO_2 stream which is dried and sent for compression and storage.

Because the reaction occurs on the liquid side of the interface of a gas and a liquid, the driving force of the physical absorption of CO_2 from the gas into the solvent is the concentration difference between the gas (CO_2 partial pressure) and the liquid. Because CO_2 is further catalyzed to another product, the reaction rate directly impacts the absorption rate of CO_2 into the liquid. This relationship can be described with the liquid film model (Figure 6.6). Furthermore, physical properties of the solvent can affect the flux of CO_2 into the liquid (Salmon and House, 2015).

In the stripper the CO_2 is released by changing the driving force, by elevating the temperature, decreasing the pressure (partial vacuum) or lowering the pH (Salmon and House, 2015). The rate of desorption depends on the same factors as in absorption. Therefore, a catalyzed reaction in the dehydration direction which increases reaction rate will have a positive impact on desorption rates.

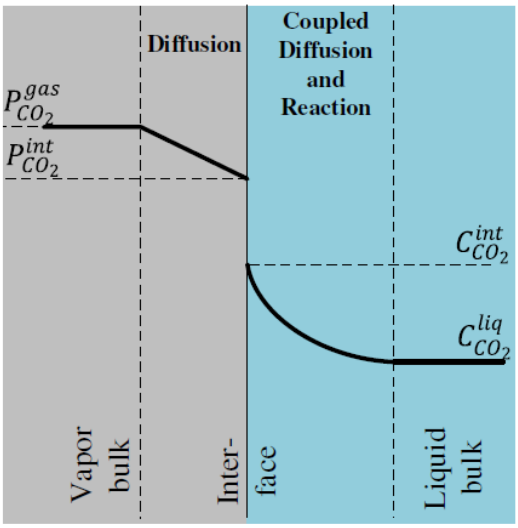


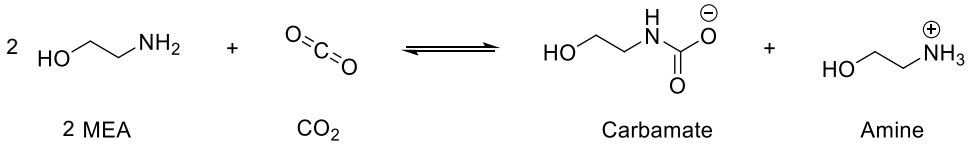
Figure 6.6: Liquid film model (Gladis *et al.*, 2017).

Reactive absorption can furthermore be roughly divided into two categories, carbamate forming reactions and bicarbonate forming reactions. Solvents such as primary and secondary amines fall into the first category. Where a covalent bond is formed between the dissolved carbon dioxide

and the solvent. Solvents like inorganic salts, ionic liquids, and tertiary amines fall into the second category, where the CO_2 is hydrated with water in the solvent to form bicarbonate.

6.2.2.1 Carbamate forming solvents

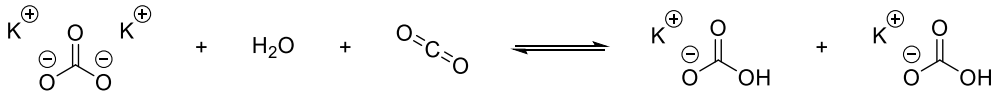
Primary and secondary amines react readily with dissolved CO_2 in the solvent to form carbamates (Salmon and House, 2015). Dissolved CO_2 reacts with two solvent molecules and forms a carbamate and a charged amine, Scheme 6.3 (MacDowell *et al.*, 2010; Salmon and House, 2015). This reaction is rapid and exothermic, thus the solvent will heat up as the reaction proceeds. Additionally, some of the carbamates are further converted to bicarbonate (MacDowell *et al.*, 2010; Penders-van Elk and Versteeg, 2016). However, at CO_2 loading relevant for CCS, the carbamate reaction is dominant (Gabrielsen, 2006). Monoethanolamine (MEA) is often used as a base case for CO_2 scrubbing and will therefore be described in detail here as an example of carbamate forming solvents. The main advantage of solvents such as MEA is fast kinetics, which in turn allows for a short contact time between the gas and the solvent. Practically, that means that the absorption tower in a capture facility can be smaller and/or that the packing can have less surface area (Penders-van Elk and Versteeg, 2016). A disadvantage of these solvents are the low capacity, with a maximum of 50% loading (mol CO_2/mol solvent), and most importantly is the energy requirement in solvent regeneration. Because CO_2 is covalently bound to the solvent, it requires significant amounts of energy in regeneration, to break the covalent bond. The heat of reaction ($\Delta H°$) for MEA is 84 KJ/mol CO_2 released. Due to the fast reaction rate of this solvent, adding enzyme is often not found to further enhance absorption rates. However, it was found that MEA with CA had a higher bicarbonate content then uncatalyzed MEA (Salmon and House, 2015). Thus, less energy would be required during desorption, since bicarbonate has a lower heat of reaction.



Scheme 6.3: Carbamate forming reaction of MEA and CO_2.

6.2.2.2 Bicarbonate forming solvents

The second class of solvents used in reactive absorption based CCS is the so-called bicarbonate forming solvents. These solvents will not form a covalent bond between the solvent and the carbon dioxide, Scheme 6.4. Tertiary amines and inorganic alkaline salts are examples of such solvents (Salmon and House, 2015). The solvent supports a higher concentration of bicarbonate, and thus a higher CO_2 loading than what otherwise would be possible in water. This reaction has potentially a higher loading capacity, as theoretically it is possible to load one mol of CO_2 per mol solvent, double the loading of carbamate forming solvents (MacDowell *et al.*, 2010).



Scheme 6.4: Bicarbonate forming reaction of Potassium carbonate, water and CO_2.

The physical loading of a solvent follows Henry's law (Equation 6.3), which is based on the concentration (molar fraction) of CO_2 in the liquid compared to the concentration (partial pressure) of CO_2 in the gas phase, divided by Henry's constant (Pierre, 2012). The reaction is largely pH dependent and will drive towards a pH equilibrium, therefore all bases are potential solvents in CO_2 capture because it makes the reaction CO_2 to HCO_3 favorable, which is more soluble in water (Pierre, 2012).

Equation 6.3: Henry's law:

$$x_i(CO_{2(aq)}) = \frac{p(CO_{2(g)})}{k_H}$$

This reaction is identical to Scheme 6.1 above and is ideally suited to the use of CA's to enhance reaction rates. One of the major hindrances with using such solvents is the inherently slow reaction kinetics in un-catalyzed solutions. An issue which can be overcome by the use of a catalyst such as CA. There are several advantages of these types of solvents, they are often benign solvents and have less of an environmental impact and safety issues. Also, perhaps more importantly, this type of solvent, because it does not form a covalent bond, generally requires less energy for desorption. Therefore, these solvents can often be stripped at lower temperatures compared to covalent forming solvents, which can reduce the energy requirement.

When comparing solvents, it is important to have the full picture. To illustrate the loading capacity in practical terms the loading capacity of 3 common solvents were calculated in terms of wt% and mol%, using the Thomsen UNIQUAC model. The UNIQUAC model is a simple thermodynamic model that determines the solute concentration in water, although the model is simplified it describes solute concentrations and states with high accuracy (Thomsen, 2005). The calculations were carried out for the following solvents and concentrations: 30% wt/wt MEA, 30% wt/wt N-methyldiethanolamine (MDEA) and 15% wt/wt Potassium carbonate (K_2CO_3). Figure 6.7 compares the loading capacity in terms of mol/mol loading (Figure 6.7A) and kg/kg loading (Figure 6.7B). Figure 6.7 illustrates that when molecular weight and usual concentrations are taken into account (Figure 6.7B), which is practical loading capacity of these solvents, the loading capacity greatly differs from the mol/mol loading often used in literature. In addition to this the water volume should be considered, and the heat capacity of the solvent, as a temperature increase will also require a significant amount of energy for heating the solvent and water. The heat of absorption for the 3 solvents used here are MEA 84 kJ/mol MDEA is 59 kJ/mol and K_2CO_3 is 27 kJ/mol (Salmon and House, 2015).

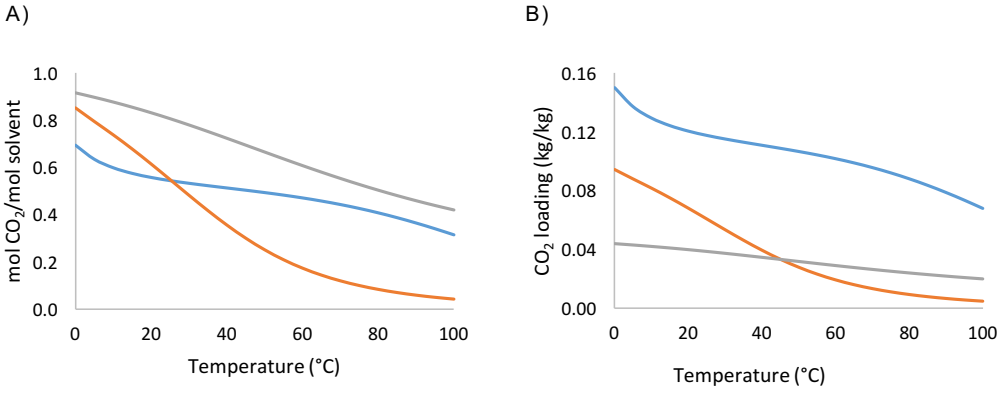


Figure 6.7: Comparing loading capacities for typical CCS solvents using the Thomsen UNIQUAC model (Thomsen, 2005). MEA 30% $^{wt}/_{wt}$ (**Blue**), MDEA 30% $^{wt}/_{wt}$ (**Orange**) and K_2CO_3 15% $^{wt}/_{wt}$ (**Grey**). A) mol CO_2/mol solvent is used to compare molar solvent capacity. B) kg CO_2/kg solvent, is used to compare actual solvent capacity.

6.3 Enzyme Enhanced Carbon Capture and Storage

6.3.1 Introduction

As mentioned above, carbonic anhydrase is a potential catalyst for CCS. In this section, the practical application of CA in CCS will be discussed. It should be noted that CA's in CCS differs from other areas of biocatalysis. It is not an enzyme commonly used in biocatalysis, due to the inexpensive product and starting material of the reaction. It is for example not mentioned in key review articles in biocatalysis (Bornscheuer *et al.*, 2012; Clouthier and Pelletier, 2012; Tao and Kazlauskas, 2013).

One of the main advantages to CA's are that they can enable reactive absorption technology at lower desorber temperatures (Salmon and House, 2015). CA's have much higher reaction rates, by several orders of magnitude compared to other catalysts used in CCS, but do carry a stability penalty (Savile and Lalonde, 2011). Furthermore, the addition of CA reaches a plateau, several studies have shown that adding more than 3g/L enzyme provided little additional enhancement in capture rates (Thee *et al.*, 2015; Qi *et al.*, 2016). In addition, due to the low cost

of the product, the costs of adding additional enzymes must be carefully considered. It should be noted that both these studies employed a microbial enzyme provided by the same company; Novozymes. Due to the diversity of this superclass of enzymes, outlined above, these results are likely not uniform across all CA's (Salmon and House, 2015)

Furthermore, the addition of an enzyme yields the question if the effect of the enzyme is only due to the activity of the enzyme, or if in part the effect comes from changing the physical properties of the solvents. This was investigated by testing the physical solubility of N_2O and liquid side mass transfer coefficient (K_L) value with the addition of CA in a 2 kmol/kg MDEA. The solubility slightly decreased in the unloaded solution, and increasing CA concentration decreased the K_L. However, when the solution was slightly loaded no effect from CA was found (Penders-van Elk *et al.*, 2012). Therefore, the addition of an enzyme is not likely to have an effect on CO_2 solubility or K_L, during a capture set-up, as the solvent will never be fully unloaded. However, proteins can act as emulsifiers, and cause foaming. This physical effect of the enzymes can be problematic in a large setup and will have a profound effect on the absorption rates as it increases the surface area of the solvent.

The cost of various EECCS processes has been evaluated but is not covered in detail here as it is case specific. Briefly, several evaluations found EECCS to be economically comparable to other reactive absorption CCS options (Reardon *et al.*, 2014; Penders-van Elk, Fradette and Versteeg, 2015). It was found that in a catalyzed MDEA process, the cost of the enzyme would likely be acceptable (Penders-van Elk, Fradette and Versteeg, 2015).

Furthermore, the use of whole cells is not considered, see Chapter 1 for a discussion of enzyme formulations. However it should be noted that one area of EECCS is using algae to capture CO_2 with the use of sunlight, often to produce fine chemicals or fuel (Klinthong *et al.*, 2015; Seth and Wangikar, 2015). Although this is a very elegant solution it is not considered in detail in this thesis. Primarily, because it is far from implementation and because the method of capture is very different from the method used in with free enzymes.

6.3.2 Sources of CO_2

As mentioned above, CO_2 can come from a number of sources. Most of the EECCS research was targeted towards CCS from PC, currently, 41% of all released CO_2 comes from coal sector (Le Quere et al., 2016). The main focus here is towards using CA to capture CO_2 from PC flue gas because that is the main focus of the literature studies cited in this section.

However, several other sources are applicable for the use of CA's. The key aspect of any source is the initial CO_2 concentration and the pressure of the gas, accounting for the CO_2 partial pressure. Furthermore, the impurities of the gas must be evaluated, as some impurities may be inhibiting CA activity. Finally, the ease of application and the economic potential must be investigated. Furthermore, CA has the advantage of high selectivity towards CO_2, which is not maximized with CCS (Migliardini *et al.*, 2014). Ye and Lu found that the CA in their experiments did not react with common CCS contaminants (Ye and Lu, 2014). Primary amines, by comparison, are much more reactive, and by far less selective to contaminants. At the moment a few applications are standing out, namely natural gas and biogas upgrade (Rufford *et al.*, 2012). A few specialized applications of EECCS are also considered economically viable. For example, the company CO2 Solutions has provided CO_2 for the carbonated soda industry (CO2 Solutions inc., 2017*)*. Other applications such as purifying air in small spaces such as spaceships and submarines are other examples of such specialized applications (Boone, Habibzadegan et al., 2013). Furthermore, EECCS has been proposed in aluminum production and concrete production (Salmon and House, 2015).

Table 6.1: Typical concentration ranges of CO_2 from various sources

Source of CO_2	Typical CO_2 concentration (%)
PC	10-15%
Natural gas	0-10%
biogas	25-50%

6.3.3 Enzyme stability

Enzyme stability, particularly at higher temperatures which may be required for solvent regeneration, is a hindrance for the longevity of CA in CCS applications (Savile and Lalonde, 2011;

Salmon and House, 2015). Targeted efforts are carried out to obtain thermostable CA's. Researchers are continuously searching for more stable CA's, especially in thermophile organisms (Savile and Lalonde, 2011). Discovering CA's is a largely unexplored territory with great remaining potential (Salmon and House, 2015). An example of this was the discovery of SSpCA (α-class). It was shown to have approximately 50% residual activity after 2 hr incubation at 100 °C. However, it should be noted that the overall activity of the enzyme was lower than that of hCAII (Di Fiore *et al.*, 2013). One concern with enzymes from thermophiles is that although they are more tolerant to higher temperatures, they might not be stable under the high pH's and solvent concentrations encountered in EECCS. Therefore, enzyme engineering might be a better approach to obtain enzymes that have all the desired properties needed for EECCS (Savile and Lalonde, 2011). However, lessons learned from studying extreme thermophiles like SSpCA can be used to direct enzyme evolution of engineered CA's. SSpCA for example was found to have a compact hydrophilic core, a high amount of ions, hydrogen bonds and charged surface residues. Which has been proposed to contribute to the increased enzyme stability (Di Fiore *et al.*, 2013).

Enzyme engineering has also rendered thermostable CA's. In one example, Alvizo and colleagues from the company Codexis successfully engineered a CA to withstand temperatures up to 107 °C in 4.2M MDEA with pH's over 10. Stability tests for up to 14 weeks were run with the new enzyme. Compared to the wild type the engineered enzyme after 9 rounds of engineering has obtained a $4*10^6$ fold stability improvement (Alvizo *et al.*, 2014). Other engineering efforts include strategies such as targeted insertion of disulfide bonds to increase thermal stability. A recent example of this was carried out by Jo and colleagues. Where an engineered enzyme with one introduced disulfide bond showed 87% residual activity at 80 °C after 30 min incubation. A significant improvement from the wild type, which had only 19% residual activity under the same conditions (Jo *et al.*, 2016) CA stability has been found to be more dependent on temperature than other factors like loading and pH (Gundersen, von Solms and Woodley, 2014; Ye and Lu, 2014)

6.3.4 Solvents

CA's reaction mechanism is water dependent (Scheme 6.1), save one CA isozyme, solvents must therefore be water based. However, pure water has a very low CO_2 loading capacity, so at least one other component must be added to the solvent to increase loading capacity. Most often this is done by adding inorganic carbonate salts or amines. An ideal solvent used should have the following key properties; high absorption rates, high loading capacity, low energy regeneration requirement, high selectivity for CO_2, high stability, low volatility, low viscosity, low foaming, environmentally friendly and inexpensive (Salmon and House, 2015). Some of the most frequently used solvents are outlined here with the most important results. In general CA's has been found to be surprisingly stable to various solvents tested (Gundersen, von Solms and Woodley, 2014; Salmon and House, 2015). In addition at least one CA displays broad pH stability (Gundersen, von Solms and Woodley, 2014).

6.3.4.1 Bicarbonate:

Bicarbonate-based solvents are perhaps the most studied solvent in EECCS, and often regarded as one of the solvents with the highest potential for use with CA's. It is favorable because it requires low regeneration energy, is less corrosive than amine solvents and the solvent does not degrade. Furthermore, it has the advantage that CA has been found to have high stability in bicarbonate, with activity remaining after several months (Gundersen, von Solms and Woodley, 2014; Ye and Lu, 2014), of course depending on the CA in question. However, the solvent is impeded by slow kinetics, compared to many amines (Hu *et al.*, 2016, 2017), therefore the use of a kinetic promoter is needed. A number of promoters can be used, from metal ions to amines, however, none of them has been commercially applied due to slow rates, high toxicity and other drawbacks (Ye and Lu, 2014; Zhang and Lu, 2015). CA's has higher reaction rates but suffers from instability under process relevant conditions (Savile and Lalonde, 2011; Salmon and House, 2015; Hu *et al.*, 2017). One drawback of this solvent which is rarely mentioned in the literature is the low CO_2 loading capacity of this solvent (Figure 6.7). The solvent has a high molecular weight, and due to precipitation, it cannot be used at very high concentration compared to amine solvents, it is usually used at 20 wt/wt % or less. This means that the loading capacity of

CO_2/solvent w/w % is relatively low compared to some of the other solvent such as MEA. Therefore, if a temperature shift is be used with to regenerate CO_2 from this solvent, large amounts of water must be heated up, which in turn is energetically unfavorable. In the case of electrochemical or pH swing to absorb/desorb that challenge is not as relevant. However, the capacity per cycle remains low and must be counteracted by a high flow rate. Increasing the cyclic capacity of the solvent has a significant positive effect on energy consumption (Qi *et al.*, 2016).

In one study the energy requirement of potassium carbonate systems was calculated to be 273-322 kJ/mol CO_2, MEA in the same system was calculated to be 262 kJ/mol CO_2. Increasing the reboiler duty lead to a higher overall energy consumption. The reboiler duty was increased from 0.85 kW (75.5 °C) to 1.1 kW (80 °C) capture efficiency increased from 84% to 89%, however, energy requirements were increased from approximately 1300 kJ/mol CO_2 to 1600 kJ/mol CO_2. It was also found that the reboiler duty accounted for the majority of the total energy consumed (Qi *et al.*, 2016).

Experiments using 30% K_2CO_3 solution and CA investigated enzyme kinetics with stopped flow showed that K_{cat} and K_M increased with increased temperature in the range 25 to 55 °C. Furthermore, catalytic efficiency (K_{cat}/K_M) increased with increasing pH in the range from pH 6.8 to 8.3. Finally, the results suggested that CA's are beneficial for desorption if the enzyme can tolerate the conditions (Hu *et al.*, 2017).

6.3.4.2 Other solvents:

Other than bicarbonate, CA's are also used with other bicarbonate forming solvents such as hindered or tertiary amines, also. Solvents like ammonia, amino acids, amines and carbonate salts have all been used with CA. (Salmon and House, 2015). For amine solvents, it has been found that the uncatalyzed rate increases linearly with increasing pKa (Penders-Van Elk, Oversteegen and Versteeg, 2016). On the contrary, it was found, with one CA studied, enzyme stability was lower with increasing solvent pKa (Gundersen, von Solms and Woodley, 2014). Furthermore, it was found that the concentration of the solvent (tested with several amines), in the case of bicarbonate forming solvents, did not affect catalyzed absorption rate, which indicates that the

amine acts as a base to regenerate the enzyme. The enzymes used here was an engineered hCAII (α-class) (Penders-van Elk, Fradette and Versteeg, 2015). This is likely true for most CA's, which is rate limited by enzyme regeneration.

Haritos *et al*. studied several solvents with and without enzyme. They used a parr reactor with a large headspace, with pressurized CO_2, initial pressure at 8 bar, and measured reaction rates from 4 bar, the set-up is shown in Figure 6.8. They found that with all solvents tested, save one, CA increased reaction rates significantly (Haritos, Dojchinov and Puxty, 2012). However, this set-up favors the use of the enzyme, due to high soluble CO_2 concentrations (arising from a high partial pressure), which is beneficial for the enzyme, which generally has high K_M values, in the range 3-70 mM (Salmon and House, 2015). In an industrial setting with atmospheric pressure and 10% CO_2 stream the CO_2 concentration in the solvent is significantly lower, also noted by the authors (Haritos, Dojchinov and Puxty, 2012). Furthermore, the same authors carried out another study in the same report, with 13% CO_2 in a bubble reactor. Here reported enhancement factors were lower, ranging from 1.09 to 1.75, MEA had the lowest enhancement factor. Although the other solvents had higher enhancement factors, their catalyzed reaction rates did not compare to MEA rates. However, these experiments had problems with foaming, occasionally more when the enzyme was present. It is therefore possible that the rate enhancement is in part caused by the increased surface area of the foam (Haritos, Dojchinov and Puxty, 2012).

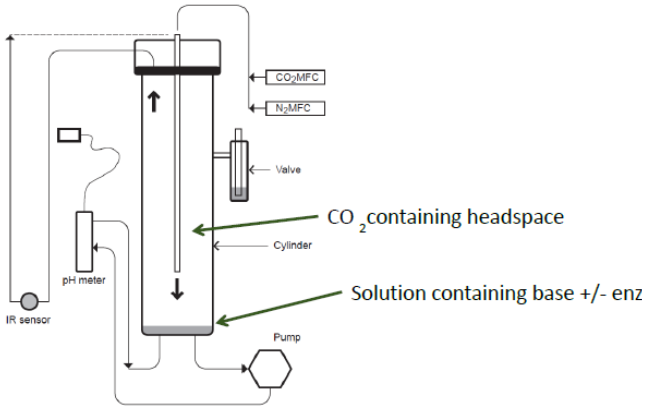


Figure 6.8: Diagram of a Parr reactor (Haritos, Dojchinov and Puxty, 2012).

6.3.5 Enzyme retention

Enzyme retention with CA's in CCS is most often carried out to localize enzymes in a specific region of the process (Savile and Lalonde, 2011; Salmon and House, 2015). Thereby preventing enzyme deactivation from high temperatures in other areas. Some of the most commonly used enzyme retention techniques are immobilizing enzymes on particles (Dean *et al.*, 1977; Haritos, Dojchinov and Puxty, 2012; Penders-van Elk *et al.*, 2013; Reardon *et al.*, 2014; Hooks and Rehm, 2015) or surfaces (Akermin, 2013; Reardon *et al.*, 2014), entrapment in gels (Zhao *et al.*, 2016) or foam (Migliardini *et al.*, 2014), on and in membranes (Bao and Trachtenberg, 2006; Figueroa *et al.*, 2008; Yong *et al.*, 2015, 2016). In addition, the use of ultrafiltration devices has been suggested to restrain free enzymes in solution (Gundersen *et al.*, 2017).

6.3.5.1 Enzymes on particles

Perhaps the most common enzyme immobilization is carried out by immobilizing enzymes on the surface of small particles. Several approaches have been taken on the subject, from simple nylon particles to magnetic beads (Lv *et al.*, 2015). Penders and colleagues suggested that due to the shear forces in a conventional reactive absorption column, covalently bound enzymes on particles would provide a better long-term result (Penders-van Elk *et al.*, 2013). Furthermore, it has been shown that particle size has a significant impact on the turnover number. In these

experiments, a particle size of over 20 μM had drastically lower turnover numbers (Penders-van Elk *et al.*, 2013). The results will depend on the kinetic rates of the enzyme in question, here a modified hCAII was used. Faster enzyme kinetics increases the effect of the particle size. Furthermore, Haritos and colleagues tested 4 different materials to immobilize bovine CAII, Eupergit C250L, Sepabeads EC-EP, Sepabeads ECHFA, and Nylon (6, and 6,6). Interestingly, the highest loading was found on the Sepabeads EC-EP but the Eupergit C250L has the highest activity. Testing of enzymes immobilized on the Eupergit C250L and comparing to the free enzyme at 70 °C for 15 min, where the free enzyme had no residual activity and the immobilized enzyme had 50% remaining activity. pH tolerance of the same beads was also tested by incubating the beads in 3M dimethylaminoethanolamine (DMAE), at pH 12.4 and 20 °C for 24hrs. 75% of the activity remained (Haritos, Dojchinov and Puxty, 2012). The company Akermin has immobilized CA's on the surface of floating particles. This biocatalyst immobilization was shown to increase kinetic rates compared to coated packing, and modeling showed a reduced energy requirement of 30% compared to NETL- case 12 (Akermin, 2013; Reardon *et al.*, 2014).

Other notable examples include CA's covalently bound to magnetic particles, which showed some increased stability, but most importantly easier separation (Lv *et al.*, 2015). CA's entrapped in microcapsules where it was found that internal enzymes were only responsible for 0.3% of the activity, all most all activity was found to come from the enzymes directly on the surface (Dean *et al.*, 1977). Finally, a polyester forming enzyme was fused with CA. The polyester forming enzyme forms beads of 100-500 nM and remains bound to the bead in the cell. The cells were then disrupted and contained CA's on surface display. In addition to an interesting way of immobilizing the enzymes, the beads showed increased stability (Hooks and Rehm, 2015)

6.3.5.2 Enzymes on surfaces

Enzymes immobilized on surfaces by immobilizing enzymes directly on structured packing has been investigated both theoretically and experimentally. This set-up has an inherent mass-transfer limitation, arising from the fact that CO_2 must not only diffuse into the solvent but furthermore, it must diffuse through the entire liquid layer before it reaches the enzyme. As

discussed in Chapter 1, enzyme immobilization has an inherent mass transfer penalty, the two major factors in for the extent of the limitation in a particular system is the K_M of the enzymatic reaction and the substrate concentration, if the substrate concentration is much higher than the K_M, mass transfer limitations are minimal (Tischer and Kasche, 1999). However, in the case of EECCS, both of these factors are problematic. CO_2 does not have a high solubility in water, which is furthermore lowered by the low partial pressure of CO_2 in flue-gas, for example, 10% CO_2 at 40 °C, would yield a maximum soluble CO_2 of approximately 2 mM, where the reported range of CA's K_M's are ranging from 3-70 mM (Salmon and House, 2015). Furthermore, enzyme immobilization carries a secondary penalty, it will change the microenvironment immediately surrounding the carrier, meaning that the product concentration and pH around the enzymes will be different than the bulk of the reaction (Tischer and Kasche, 1999). This is particularly important because CA's can suffer from product inhibition, the extent of product inhibition will depend highly on the CA in question (Pierre, 2012; Gladis, 2017). Furthermore, the reaction rate is highly pH dependent (Salmon and House, 2015). Penders and colleagues concluded that for solvents with a reasonably fast reaction, the mass transfer rate is too slow for enzymes to have an impact on the reaction rate with immobilized enzymes. However, the same authors postulated that slower solvents such as carbonate salts with pH's under 10, could have a small enhancement effect from CA (Penders-van Elk *et al.*, 2013). Indeed, this was shown by Akermin in a demonstration project, in a 25 feet column with a gas flow of 30 Nm^3/hr and liquid flow of 275 LPH. Where CA's were immobilized on the surface of Sulzer M500X structured packing. High enzyme retention was demonstrated, after 400 hours over 80% enzyme was retained. Enhancement from the enzyme was 6-fold compared with un-catalyzed K_2CO_3 and 90% captured. This set-up was compared to the MEA case; NETL case 12. Their case showed decreased energy requirements, lower capital costs and lower LCOE (Levelized cost of energy). The largest energy saving was in the reduction of energy in the reboiler. 97 °C in reboiler for K_2CO_3 vs 157 °C in MEA case. (Akermin, 2013; Reardon *et al.*, 2014). Although Akermin showed good long-term stability in their experiments, nonetheless eventually the coating needs to be reloaded. Which

will pose a logistic challenge, on a commercial scale (Qi *et al.*, 2016). In another test Akermin showed 2800hrs of 90% capture without loss of enzyme performance (Reardon *et al.*, 2014).

6.3.5.3 Membranes

The use of membranes is a complex and diverse aspect of CCS, with many possible options. Some of these options also directly affect the setup of absorption and desorption. An advantage to using CA's in membrane applications is that the enzyme, due to its surfactant properties, increased the solubility of some solvents, such as carbonate salts. These solvents otherwise have problems fully wetting membrane pores (Zhao *et al.*, 2016). One of the major drawbacks of using membranes is solvent evaporation, this is especially difficult to overcome when using small wetted pores. One solution to this has been to immobilize CA's in hydrogels, however, the mass transfer limitation to such gels was too high to give any added benefit of the enzyme (Zhao *et al.*, 2016). Furthermore, the inherent fouling of some membranes causes a problem with operating membranes on scale (Savile and Lalonde, 2011). Finally, there are technical difficulties with operating some of the more complex membrane set-ups. In general, a more complex set-up has lower operational cost and are more energy efficient, but higher capital costs. Here a brief description of some of the key studies carried out with CA's and membranes are discussed.

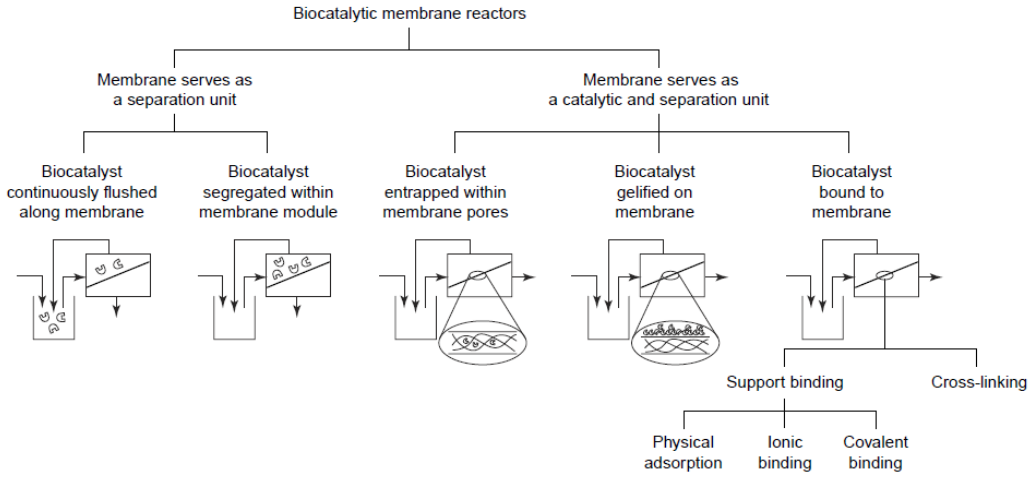


Figure 6.9: Overview of biocatalytic membrane reactors (Giorno and Drioli, 2000).

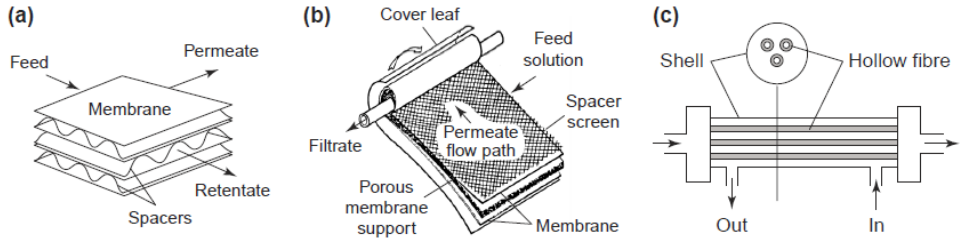


Figure 6.10: Illustration of common membranes modules flat-sheet membranes assembled in (**a**) plate and frame, and (**b**) spiral wound modules; (**c**) a hollow fibre membrane assembled in a tube-and-shell module (Giorno and Drioli, 2000).

6.3.5.4 Ultrafiltration (UF)

The simplest application of membranes in EECCS is the use of ultrafiltration membranes. Where a membrane is used to separate the majority of the enzymes from the rich solvent before the solvent is sent to the stripper. This does not impose any modification on the reaction set-up outlined in Figure 6.5. The use of ultrafiltration was theoretically investigated and showed good results for significantly extending enzyme viability (Gundersen *et al.*, 2017). With UF, separation of smaller particles requires more work. Therefore, combining this technology with immobilization on particles could be energetically beneficial and yield higher enzyme retention rates. The use of UF in EECCS is covered in detail in Chapter 8.

6.3.5.5 Contained liquid membranes

Contained liquid membranes (CLM), are a gas to gas application. Which is operated in the same way as simple selective membranes, shown in Figure 6.11 below. Here, absorption and desorption are carried out in the same unit. Where CO_2 selectively dissolves into the liquid in the membrane and is desorbed on the other side, producing a 100% CO_2 stream. They can be applied as simple membranes or as hollow fiber membranes, which increases surface area but adds complexity in production. Experimentally, desorption is often done by using a sweep-gas such as argon or nitrogen. However, in an industrial setting, it would be done with vacuum. It has the advantage

that no heat is applied, which reduces energy use (Figueroa *et al.*, 2008). Furthermore, lower heat is beneficial for the enzyme stability. However, energy is needed to pressurize the inlet gas and to produce vacuum on the outlet side. Solvent loss through evaporation in the pores may also pose a problem (Russo *et al.*, 2013). Furthermore, higher capture ratios require exponentially higher requirements. Such technology can be imagined to best applied for applications where the CO_2 concentration on the inlet side is fairly high and was very high capture rates are not necessary. Experimentally, this technology has yielded good results on laboratory scale. Bao and Trachtenberg showed that CA in bicarbonate had higher rates than both uncatalyzed bicarbonate and the secondary amine diethanolamine (DEA) (Bao and Trachtenberg, 2006).

A similar strategy is layer by layer wetted hollow fiber membranes (LbL). Which also showed increased capture rates compared to uncatalyzed bicarbonate solutions, with approximately a 3 fold increase in absorption rates (Yong *et al.*, 2016). Carbozyme has developed a hollow fiber membrane system for EECCS, as showed in Figure 6.11.

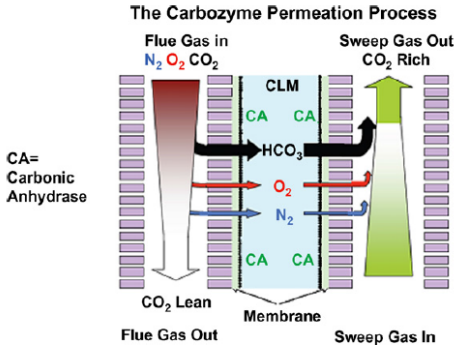


Figure 6.11: Carbozyme CO_2 capture with CA and membrane process (Figueroa *et al.*, 2008)

6.3.5.6 Membrane contactors

Finally, CA's the use of in membrane contactors has been investigated, however, the research in this area is currently still limited (Zhao *et al.*, 2016). One biomimetic set-up aims at combating some of the drawbacks of using membranes and enhancing the advantages of CA's. A two-layered Janus membrane that combines a hydrophilic and a hydrophobic layer. The gas interface has a

hydrophobic perfluodecyltrichorosilane layer, which reduces solvent evaporation, and allows for easy CO_2 penetration, Figure 6.12. CA is immobilized on the surface of hydrophilic carbon nanotubes, located at the solvent interface, thereby reducing mass transfer limitations. Furthermore, the enzyme immobilization retains the enzyme in the absorber, while the solvent can be regenerated in a desorber. The set-up was shown to have increased absorption properties, compared to both the uncatalyzed set-up and compared to free enzymes in solution.

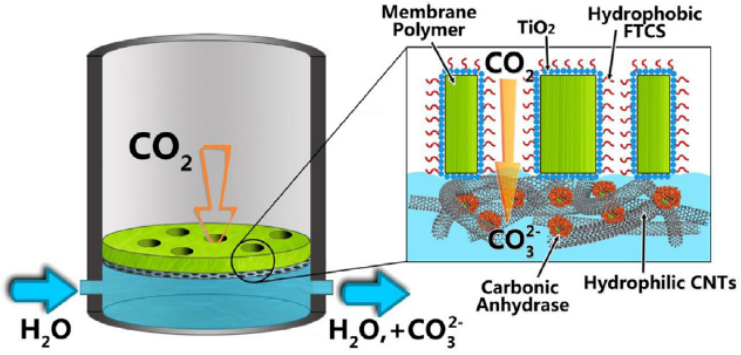


Figure 6.12: CA in membrane contactor: Janus membrane with an hydrophobic/hydrophilic interface (Zhao *et al.*, 2016).

6.4 Conclusion and future outlook

This chapter has discussed the status quo of EECCS, a rapidly advancing field. The technology has high potential due to highly efficient enzymes and the possibility of using energy efficient solvents. Enzymes are produced cost efficiently on a large scale for other applications, it is therefore likely that CA's would have competitive pricing if demand increases. However, before enzymes are ready to be commercially applied more research is needed, and it is important to find solutions that will work well on a large-scale. Furthermore, it could be envisioned that CA's are used for specialized CO_2 applications, where high-quality CO_2 can be sold for a higher price, for example in the soda industry as mentioned above, or applications such as food or fine chemicals. The following chapters will investigate enzyme stability under process relevant conditions, and methods to overcome stability challenges in detail.

6.5 References:

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Section II

Chapter 7:
Enzyme Stability under process relevant conditions

This chapter is an extended version of the published article 'Enzymatically Assisted CO_2 Removal from Flue-Gas'. It was published in the journal Energy Procedia (2014), pages 624-632, by the authors Maria T. Gundersen, Nicolas von Solms and John M. Woodley. Modifications have been made to update the information with current knowledge, ease readability and to make the publication coherent with the rest of the thesis. A copy of the article as published can be found in Annex A.

7.1 Introduction

As discussed in detail in Chapter 6, the enzyme carbonic anhydrase (CA) catalyzes CO_2 fixation in Nature, by reversibly hydrating CO_2 to bicarbonate, Scheme 7.1.

$$CO_2 + H_2O \rightleftharpoons HCO_3^- H^+$$

Scheme 7.1. Carbonic anhydrase hydrolysis of carbon dioxide.

For viable industrial use of CA in CCS, it is important that the enzyme is both stable and kinetically active under operating conditions. Several excellent reviews highlight the advances made in this field (Savile and Lalonde, 2011; Salmon and House, 2015). This chapter takes a holistic view of enzyme stability under process relevant conditions. A developmental enzyme supplied by Novozymes was applied under process relevant conditions to evaluate enzyme stability. Due to the volume of CO_2 emerging from an average power plant, large capture equipment is required, thus any change in the capture set-up, such as changing the solvent or column packing will involve large capital and labor costs, and should therefore be avoided for as long as possible. Thus, enzymes used in such systems must remain active for extended periods of time under operating conditions. In addition, enzymes can only account for a small fraction of

the overall cost of the capture process, since the cost of the technology must compete with current carbon taxes. The total cost of the process will also encompass solvents, stripping, compression and storage costs. To that extent, a supplier which can deliver large quantities of enzymes at low costs, found in applications such as detergents and textile industry is needed for an economically viable process. It has been suggested that the cost of the enzyme will likely be drastically reduced if it were produced in larger quantities, and would therefore not pose an economic problem, as long as the enzyme does not need to be replenished too often (Penders-van Elk, Fradette and Versteeg, 2015; Salmon and House, 2015).

7.2 Experimental

Here a brief experimental description follows, to ensure that the reader may follow the presented results in the next section. Full experimental procedures can be found in the full paper as published in Annex A.

7.2.1 Methods

7.2.1.1 Activity assay

Activity was measured by a modified assay by Chirică and colleagues (Chirică *et al.*, 1997), Figure 7.1. Each sample was tested by adding 1 mL of 0.1 M Tris-HCl buffer, pH 7.6, in a plastic cuvette, 10 µL sample or control was added, the sample was then left to equilibrate for at least 1 hour, and then 10 µL para-nitro-phenyl (PNP) solution (54.3 mg PNP in 3 mL acetonitrile) was added. The samples were mixed by inverting samples covered with parafilm, twice. Each sample set ran in parallel contained two blank samples and three reaction samples. Each duplicate reaction sample was run separately, thereby obtaining 6 data points per condition tested per data point. Absorption was recorded at 348 nM for 320 s, and the activity was determined from the slope of the absorption between 60 s and 300 s, subtracting the slope of the blank samples. The samples containing MEA and MAPA/MDEA had a high background absorption, thus the sample preparation was modified: 0.05 mL sample or blank was added to 0.45 mL 0.1 M Tris-HCl buffer, pH 7.6, in centricon vial 10 kDa cut off, spun down at 14,000 rpm for 10 min, 0.5 mL new buffer

was added, this process was repeated 3 times. 0.1 mL of this sample was added to 0.9 mL buffer and the procedure was followed as indicated above.

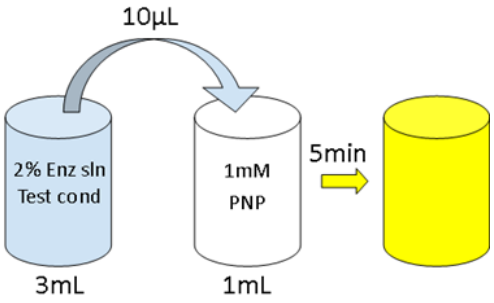

Figure 7.1: Experimental setup for stability tests. CA (2%) exposed to test condition: solvent, temperature or pH. Activity was tested with the *p*-nitrophenyl assay at regular time intervals, and compared to initial activity under identical conditions.

7.3 Results and Discussion

The experiments in this chapter were carried out to evaluate the stability of enzymes under EECCS relevant conditions. Enzyme stability was evaluated using the activated ester assay; PNP. Which measures ester hydrolysis through a color change. As mentioned in Chapter 6, many but not all CA's has this activity (James *et al.*, 2014; Salmon and House, 2015), although it is not the defining activity of the enzyme class (Supuran, 2016). Furthermore, it should be noted that the activity on this substrate may not accurately correspond to CO_2 hydrolysis rates. Several studies have shown that ester hydrolysis rates and CO_2 hydration rates are not comparable (Bond *et al.*, 2001; Salmon and House, 2015). Therefore, activity is not reported in units, but rather percent activity compared to the initial activity. Furthermore, reaction rates between different samples are not compared in this study, with different pHs or different solvents. Thus, here only the residual activity of the enzyme, under identical conditions, where background activity is thoroughly controlled for, is compared. Therefore, in this particular setting, the PNP assay is

deemed valid. However, caution should be offered in the general use of this assay for comparatively measuring CA activity.

In a post-combustion carbon capture (PCCC) conditions over the course of the process will vary with respect to temperature and pH, CO_2 absorption lowers the pH of the solvent, and the solvent is heated in desorption to release CO_2, in addition, the solvent can both be varied in type and concentration. Therefore, these three variables are first examined singularly, and subsequently compared synergistically, where the additive effects of the parameters are examined. Figure 7.2 shows a typical PCCC set-up where temperature and pH gradients over the process are indicated by color.

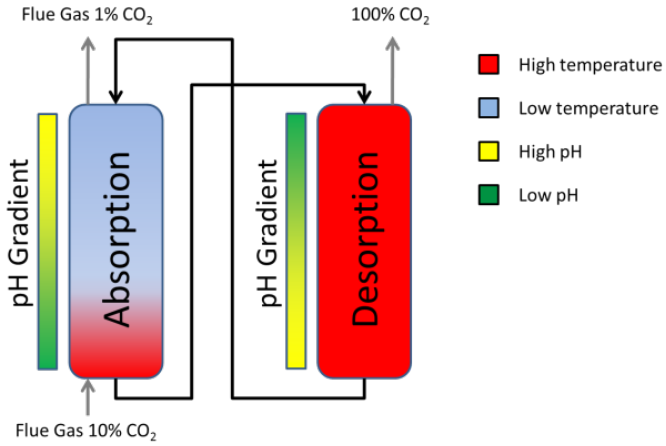

Figure 7.2: Schematic example of varying pH and temperatures of the solvent in a post-combustion solvent-based capture process.

7.3.1 pH stability

pH stability was evaluated by measuring residual activity after 100 hours of incubation at the respective pH at room temperature in the pH range from 4 to 12, in increments of 1 pH unit. Only pH values above pH 7 are used in EECCS applications, since below this pH the hydration rate of the reaction is strongly reduced, due to thermodynamic limitations (Russo *et al.*, 2013). The results are shown in Figure 7.3. No activity was detected after 100 hours at pH 4 and moderate

activity (45-70 %) with pH's 5, 6 and 12. It is therefore unlikely that the enzyme will be stable under these conditions over extended periods of time (months). The enzyme was stable in the pH range from 7 to 11 after 100 hours incubation. Thereby defining the operating space for this enzyme, and furthermore, the maximal and minimal loading of any given solvent, as the pH of a solvent is defined by the CO_2 loading. However, this limitation is restricted to the enzyme in question. If a process is deemed more efficient at a higher maximum pH, several options are possible to overcome this obstacle. Another enzyme with a higher pH tolerance can be chosen, or the enzyme can be optimized by enzyme engineering to withstand higher pHs.

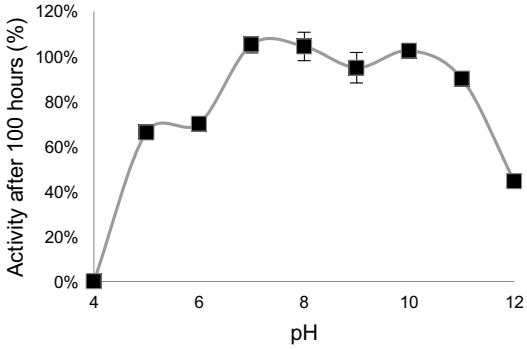

Figure 7.3: Residual activity after 100hours of incubation at pH 4 to 12.

7.3.2 Temperature stability

Temperature stability was evaluated in the range of 50-80 °C, and the enzyme was found to have residual activity at all temperatures for up to 48 hours (Table 7.1), but the activity was drastically reduced at higher temperatures. In addition, some experiments were made to recover activity from the high temperature experiments (70 °C and 80 °C) by incubating the samples at lower temperatures for some time before measuring the sample again (data not shown). This regained up to 10 % activity. This is particularly interesting in a cyclical EECCS setup (Figure 7.2) where the enzymes are not immobilized, because the enzymes would cycle between high and low temperatures, albeit in such a set up the incubation time at both high and lower temperatures would be significantly shorter than in these experiments. After 48 and 72 hours no activity was

detected at the temperatures 80 °C and 70 °C, respectively. It should be noted that the accuracy of the reported values may be questionable as the activity is increasing over time with the 50 °C sample; this is likely due to the increased internal energy of the enzyme at the higher temperature, which in turn results in higher reaction kinetics. This may be an artifact stemming from the fact that the activity assay was taken too soon after the enzyme was taken from the incubation temperature. Therefore, the residual activity reported here should be evaluated in terms of activity/no activity, rather than the reported activity measures. This was corrected in later experiments below. Nonetheless, the results give an indication that this enzyme is stable under operating conditions under 60 °C. The results correlate well with results found with other CA's, which have been found to be stable at high temperatures (Savile and Lalonde, 2011). For example Codexis engineered a thermostable enzyme, which retained 40 % activity after 40 hours incubation at 75 °C, comparable to what is found here (Savile and Lalonde, 2011). In addition another engineered CA was found to retain up to 54 % residual activity after 2 hours incubation at 80 °C (Borchert and Saunders, 2010). Thus, these results are comparable to the former results, indicating that the enzyme is stable at temperatures up to 60 °C for extended periods of time, although these results demonstrate that if the enzyme is to be exposed to temperatures above 70 °C, it must be limited to short periods. In practical terms this means that this enzyme is for example unlikely to survive a treatment in a reboiler. The results here are again particular to the enzyme in question, and a general conclusion for all CA's cannot be drawn. As mentioned in the previous chapter, other more thermostable CA's has been found, such as the SSpCA, which can withstand temperatures of 100 °C (Di Fiore *et al.*, 2013). However, it can be argued that increasing the temperature of the process is counterproductive, as increased temperatures would add an energetic penalty to the process. Especially for temperatures over 100 °C, as many of the EECCS processes has high water content. However, an enzyme which tolerates higher temperatures is likely to be stable for extended periods at a lower temperature. In addition, higher temperature stability would prevent deactivation at localized pockets with higher temperatures. For example, close to heating coils, and other heat inducing devices.

Table 7.1: Residual activity after incubation at temperatures from 50 °C to 80 °C for up to 100 hours. All results are given in % residual activity

	Time (hours)				
T (°C)	1.5	25	48	72	100
50	105	121	132	196	280
60	84	87	64	66	74
70	47	41	49	36	0
80	7	9	2	0	0

7.3.3 Long-term solvents stability

A long-term stability study was carried for 150 days. This is one of the longest stability studies carried out. Other notable studies include a 6-month stability study from Ye and Li, also using a microbial enzyme provided by Novozymes (Ye and Lu, 2014). Industrially the companies Akermin and CO2 Solutions have both independently run filed tests in pilot plants for over 100 days (CO2 Solutions inc, 2017; Akermin, 2013; Reardon *et al.*, 2014). The study here was undertaken at two pH values and six different solvents, with 1 M and 3 M concentrations, to evaluate if the enzyme could be used long-term under operating conditions. The results show that the enzyme was highly stable for extended periods of time. The solvents tested were chosen as they were previously proven to be useful in CO_2 capture. Specifically, the primary amine solvent monoethanolamine (MEA) as it has been reported as a candidate for industrial applications, and is single most commonly used solvent (Wang *et al.*, 2010), and thus serves as a good benchmarking solvent. It has excellent absorption rates, it is however haunted by problems such as corrosion, low stability, and high energy needed for desorption. 2-Amino-2-methyl-1-propanol (AMP), N-methyldiethanolamine (MDEA) and 3-(Methylamino)propylamine (MAPA)/MDEA have all shown great promise in CO_2 absorption, but with slightly lower absorption rates, thus they are good candidates for EECCS. 2-Aminoisobutyric acid (AIB) was previously shown to have higher desorption rates than MEA at 80 °C, but slower absorption rates (Hook, 1997), thus it serves as a good target for enzyme enhanced technologies, in addition, AIB had higher solubility than other comparable amino acids like alanine, therefore a higher

concentration can be used, resulting in a higher loading and higher cyclic capacity. The use of potassium carbonate (K_2CO_3) is discussed in detail in the previous Chapter, as it is the most commonly used solvent with CA for enzyme enhanced carbon and capture (EECCS). It has the advantages of favorable thermodynamics and lowered desorption temperatures, however, a drawback with this solvent is low solubility and overall low cyclic capacity. Enzyme stability results are shown in Table 7.2. All data points illustrate that activity is lost over time and a higher deactivation is found with a higher pH. Finally, the activity was compared after 100 hours and 150 days, to evaluate if a short-term study could efficiently reveal which solvents were stable long the long term. The results showed poor correlation and it is concluded that within this data set a prediction of long-term stability cannot be made from short-term studies.

Table 7.2: Remaining activity after 5 and 150 days

Solvent (concentration)	pH	Residual Activity 5 days (%)	Residual Activity 150 days (%)
MEA (3M)	8.3	95 ± 0.4	73 ±1 0.8
	10	76 ± 1.8	33 ± 4.8
AMP (3M)	9	99 ± 0.3	42 ± 1.6
	10	104 ± 7.7	12 ± 0.6
MDEA (3M)	9	92 ± 2.8	62 ± 4.0
	10	91 ± 3.0	54 ± 2.5
AIB (3M)	8	106 ± 4.9	91 ± 3.0
	10	95 ± 0.1	35 ± 0.9
K_2CO_3 (1M)	8	116 ± 6.8	83 ± 3.6
	10	85 ± 1.2	29 ± 2.4
MAPA (1M)/ MDEA (2M)	8.6	86 ± 10.3	85 ± 0.5
	10	99 ± 4.8	69 ± 4.4

However, as seen from Figure 7.4, a correlation was found between deactivation and solvent pKa values. In comparison higher pKa values been found to have a positive effect on solvent kinetics (da Silva and Svendsen, 2007; Penders-Van Elk, Oversteegen and Versteeg, 2016). This suggests that a compromise might need to be made in implementation between enzyme activity and

stability. However, in the case of CA, it has been known since the 1930's that buffers and solvents can have more severe impacts on the catalytic activity of CA (Roughton and Booth, 1938), albeit of mammalian source. Thus, the actual enhanced activity with the solvents should be investigated in detail. Such work has been carried out on several occasions, in particular, Haritos and colleagues have investigated enzyme enhanced CO_2 capture in a pressurized stirred cell reactor, where a rate enhancement was found with all eleven solvents tested. However, as discussed in the previous Chapter, this set-up was particularly favorable to enzyme enhancement (Haritos, Dojchinov and Puxty, 2012). Other studies, with an experimental set-up that is closer to that of a closer to an absorption column, a wetted wall apparatus, have found conflicting results to these studies (Gladis *et al.*, 2017). It should be noted that higher enzyme stability may be associated with lower reaction rates (Kuchner and Arnold, 1997).

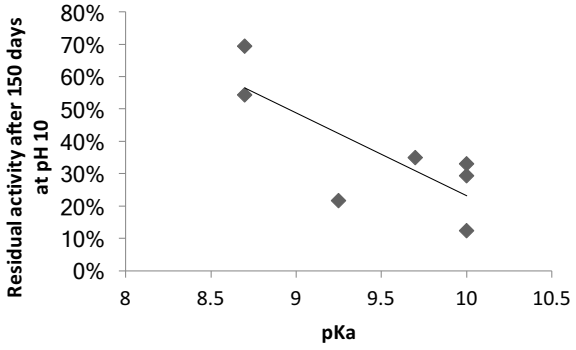

Figure 7.4: Correlation between pKa and long-term (150 days) stability. Pka values were predicted with the software ChemDraw.

7.3.4 Additive effects on stability

Finally, the additive effects of solvent strength and temperature with the solvents NaCl (pH 7), K_2CO_3 (pH 9), AMP (pH 8) and MDEA (pH 8.5) was investigated for 100hrs (Table 7.3). Increasing the concentration from 1 M to 3 M lead to a slight decrease in stability. The salts NaCl and K_2CO_3 led to an overall a higher enzyme stability than the amines AMP and MDEA, with the

exception of K2CO3 at 25 °C, 3, it showed in fact a lower stability than the same concentration at 50 °C This is likely due to the lower solubility of this solvent at the lower temperature, rather than the increased temperature increased enzyme stability. These experiments did not show a large impact on stability by increasing the temperature from 25 °C to 50 °C. However, since both of these temperatures are within the stable range of this CA, determined by previous experiments in this chapter, it does not exclude an additive effect at higher temperatures.

Table 7.3: Residual activity of CA after 100 hours with varying temperature and solvent concentration with the solvents, NaCl, K_2CO_3, AMP and MDEA

Solvent	1 M, 25 °C	1 M, 50 °C	3 M, 25 °C	3 M, 50 °C
NaCl	76%	91%	90%	78%
K_2CO_3	125%	100%	63%	80%
AMP	91%	87%	70%	79%
MDEA	88%	89%	83%	75%

7.4 Conclusion

This chapter has evaluated enzyme stability in terms of pH, temperature and solvents, the latter at different concentrations and types of solvents, and the effect of these three factors added together. The carbonic anhydrase used here showed long-term stability for some, but not all process-relevant conditions. The results found in this chapter which has the highest relevance for EECCS is the decreased stability at higher temperatures. Which poses a practical challenge for the desorption step due to enzyme activation. However, this may be overcome by several strategies, such as using thermostable enzymes (Savile and Lalonde, 2011). Localizing enzymes in areas of the process with lower temperatures, technologies such as enzyme immobilization or ultrafiltration membranes, which has shown great promise to extend enzyme viability for intermediate temperatures (discussed in detail in the next Chapter). Furthermore, this study has drawn two important conclusions. Firstly, it was found that long term and short-term stability studies, did not show a good correlation. Therefore, longer studies are needed to evaluate enzyme stability under particular conditions, before choosing and implementing a new process. Finally,

it was discovered that enzyme stability is decreased with increasing pKa, the opposite trend that is found with un-catalyzed solvent kinetics. These findings are important to evaluate the potential of EECCS on an industrial scale. The results in this paper indicate that EECCS is possible, but will require careful process and reaction engineering for a viable process.

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Section II

Chapter 8:
Ultrafiltration as a means to extend enzyme longevity

This chapter is an extended version of the published article 'Operating considerations of ultrafiltration in enzyme enhanced carbon capture'. It was published in the journal Energy Procedia (2017), pages 735-743, by the authors Maria T. Gundersen, Arne Gladis, Philip Loldrup Fosbøl, Nicolas von Solms and John M. Woodley. Modifications have been made to update the information with current knowledge, ease readability and to make the publication coherent with the rest of the thesis. A copy of the full article as published can be found in Annex A.

8.1 Abstract

Enzyme enhanced carbon capture and storage (EECCS) has the advantage of using energy efficient solvents, and can therefore potentially reduce the carbon footprint of carbon capture and storage (CCS). A challenge in the implementation of this technology is the high temperatures encountered in the desorber, which deactivate enzymes. One solution to this challenge is the use of ultrafiltration to keep the enzymes away from the stripper. In this chapter, a base case of a CCS facility is defined and used to model the impact of such membranes for the use in a full-scale CCS commercial plant. The base case has an approximate capture capacity of 1 MTonn CO_2/year. The timeframe used in this model is one-year continuous operation. This chapter compares soluble enzymes dissolved in a liquid solvent with and without the use of ultrafiltration membranes. Three membranes with 90%, 99% and 99.9% enzyme retention were modeled. Enzyme retention is defined as the percentage of enzyme that does not enter the stripper column in each cycle. These membranes were then further modeled with five stripper temperatures of 60 °C, 70 °C, 80 °C, 90 °C and above 100 °C. Enzyme deactivation was extrapolated from data in Chapter 7 and followed a 1st order rate (Gundersen, von Solms and Woodley, 2014). Furthermore, deactivation

rates increase with increasing temperatures. The key finding from this work is that for all stripper temperatures used in this model, deactivation rates were too high for continuous operation over 1 year, maintaining at least 50 % residual activity, without adding additional enzyme. Increased stripper temperatures increased the membrane retention requirement. To retain over 50% activity over a whole year at 60 °C and 70 °C stripper temperature required a membrane of 90% or higher enzyme retention, at stripper temperatures of 90 °C a membrane of 99.9% retention was required for the same result. Finally, it was investigated if stripper temperatures over 100 °C, where instant deactivation was modeled could be used. It was found that with enzyme retention of 99.9%, with instant deactivation, after 1 month 50% of the enzyme was deactivated. Thus, the use of membranes in enzyme enhanced CCS, with the enzyme studied here, is restricted to temperatures below 100 °C, or if another enzyme is used, temperatures where the enzyme in question can withstand without instant deactivation.

8.2 Introduction

As discussed in detail in Chapter 6, carbonic anhydrase (CA), is an attractive catalyst in CCS to enable the use of energy efficient solvents, by enhancing reaction rates (Savile and Lalonde, 2011). A drawback of using enzymes is that they are often not stable under operating conditions encountered in a CCS capture facility. This was explored in detail in the previous chapter, by investigating the stability of one CA, especially suitable for CCS in terms of pH, temperature and solvent type at CCS relevant conditions. The enzyme studied was found to be considerably more stable than most enzymes under similar extreme conditions. However, in long-term studies carried (months), it was found that the enzyme was particularly sensitive to high temperatures (Gundersen, von Solms and Woodley, 2014; Ye and Lu, 2014). On a commercial scale, it is neither practical nor economically viable, to frequently add enzymes to the solvent, to make up for the activity loss. Therefore, for commercial implementation, a strategy must be devised to elongate enzyme stability. This chapter explores such a strategy, namely ultrafiltration (UF).

This chapter explores the feasibility and impact of using UF membranes for restricting enzymes from entering the stripper column. Other strategies, such as immobilization

on a fixed surface in the absorber would yield similar results in terms of enzyme longevity. However, there are several significant drawbacks with immobilization technology. Most importantly immobilization on a fixed surface locates enzymes further from the gas-liquid interphase, therefore mass transfer resistance is increased, which in turn yields slower absorption kinetics (Penders-van Elk *et al.*, 2013). Furthermore, immobilizing enzymes adds a cost penalty to the process. In a general example of enzyme immobilization, calculated that the added cost of immobilization would render the cost of the enzyme to be four-fold higher (Tufvesson *et al.*, 2011). Finally, although immobilization will elongate enzyme viability, the enzyme must occasionally be exchanged. This poses technical complexity, especially on the large scale seen with CCS (Qi *et al.*, 2016).

UF, on the other hand, allows for solubilized enzymes to be dispersed in the solution, therefore it does not add additional mass transfer resistance. Furthermore, exchanging the enzymes periodically would only require exchanging parts of the solvent. A simpler operation than exchanging packing inside a column. UF membranes are already in use in on a large scale with applications such as water purification. In such applications, the membranes are often applied as several units in series or parallel, to give a high surface area. A model for investigating the stability of such enzymes in a theoretical commercial plant with and without UF membranes was constructed. Enzyme stability was calculated within the model framework for stripper temperatures ranging from 60 °C to over 100 °C. The membranes are furthermore explored with enzyme retention from 90% up to 99.9%. The results were modeled for 1 year of continuous operation.

8.3 Experimental

In order to develop a model, a base case was first defined, to serve as a fundament for the calculations. The parameters for the base case is defined below in Table 8.1 and illustrated in Figure 8.1. The parameters were based on publically available data from the Boundary Dam CCS facility and were supplemented with information from experts in the field.

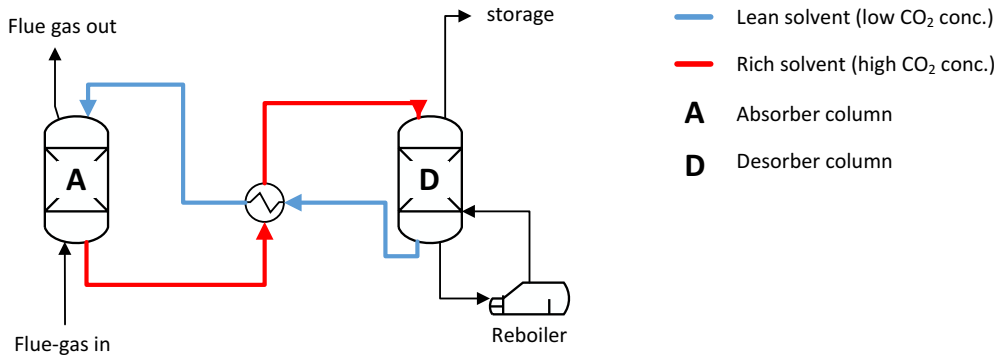

Figure 8.1: Flowchart of a typical reaction based carbon capture process. The flue gas enters the bottom of the absorber, and flows towards the gas outlet, a lean (unloaded) solvent (**blue**) counter currently flows down the column and reacts with the gas to absorb CO_2. The rich (loaded) (**red**) solvents exit the absorber at the base of the column. Where it is passed through a heat exchanger before it enters the desorber column. CO_2 is stripped from the gas in the desorber by heat or vacuum, thereby regenerating a lean solvent. Passing through the heat exchanger before it re-enters the absorber column.

Table 8.1: Base case data, solvent mass: $2.06 * 10^6$ kg, flow rate: $2 * 10^6$ kg/hr

	Residence time (min)
Absorber	11.4
Stripper	5.3
Hold-up	45.1
Total	61.8

8.3.1 Deactivation rates

Enzyme deactivation rates were extrapolated from data in Chapter 7 (Gundersen, von Solms and Woodley, 2014), it follows a first-order reaction rate, according to Equation 8.1:

Equation 8.1:

$$A_t = A_i e^{-kt}$$

Where A_t is the activity remaining at a certain time point, A_i is the initial activity (100%), k is the deactivation rate constant and t is the time at that time point. In addition, a stripping

temperature above 100 °C was used in the calculations, where instant enzyme deactivation is assumed.

Table 8.2: Rate constants for temperature dependent enzyme deactivation

Temperature (°C)	50	60	70	80	90
Deactivation rate (h^{-1})	0	0.003	0.0054	0.0536	0.3860

8.3.2 Ultrafiltration membranes

Three UF membranes were used in this study, they had an enzyme retention of 90%, 99% and 99.9%. Furthermore, the membrane flux of water (L/(m^2*h*bar)) was used for evaluating membrane size and cost. Data for water permeability for two specific membranes were obtained from the commercial membrane producer Alfa Laval, Table 8.3.

Table 8.3: Physical properties of commercial membranes used in this model

Type	Material	Selectivity (%)	Water permeability (L/(m2*h*bar))	Source
GR80PP	Polyethersulphone	90	50	Alfa Laval
UFX10pHt	Polysulphone	99.9	400	Alfa Laval

8.4 Results and Discussion

A model was established to investigate residual enzyme activity, with and without UF membranes, under several operating conditions. In its simplest form enzyme enhanced carbon capture and storage (EECCS), can be carried out by adding soluble enzyme to a liquid solvent, and run the capture facility as before, as described in Figure 8.1. Using soluble enzymes in solution gives the least mass transfer resistance, as discussed in the introduction of this chapter, and in detail in Chapter 6 (Penders-van Elk *et al.*, 2013). Finally, it has the lowest capital and operational costs since no additional cost for membranes and compression are added. However, as discussed in detail in Chapter 6 and 7, the enzyme deactivates at a significant rate at higher temperatures (Savile and Lalonde, 2011; Gundersen, von Solms and Woodley, 2014; Ye and Lu, 2014; Salmon and House, 2015). Therefore, the stability of carbonic anhydrase was investigated

at different operating temperatures with the base case CCS facility outlined above. Enzyme viability was modeled, in terms of residual activity in a continuous operating power plant for one year. Here five different stripper temperatures were used, 60 °C, 70 °C, 80 °C, 90 °C and above 100 °C. In the model, it was assumed that absorption was carried out at low temperature, with minimal enzyme deactivation. Therefore, deactivation in the model only occurred in the stripper. Reduced deactivation at lower temperatures has previously been reported (Gundersen, von Solms and Woodley, 2014; Ye and Lu, 2014). Figure 8.2 displays residual enzyme activity for the duration of one year, from initial activity (100%), for free enzymes, under the five stripper temperatures used in this model, 60 °C, 70 °C, 80°C, 90°C and 100°C. The model shows that significant activity loss was found after a few minutes with the highest temperature (100 °C). However, some residual enzyme activity still remains after 1 year with the two lowest temperatures. As can be noted from Figure 6.7 in Chapter 6, a lower stripper temperature will reduce the cyclic capacity of the solvent. However, this could be overcome by decreased partial pressure in the stripper. In this model, a uniform temperature in the stripper unit was assumed. It was also tested if a non-uniform temperature model, where 10% of the stripper column was modeled to be 10 °C higher and 10% to be 10°C lower than the bulk of the solvent, would influence the outcome of the model. A slight decrease in stability was observed, but the results follow the same general trend as the data in Figure 8.2 (data not shown).

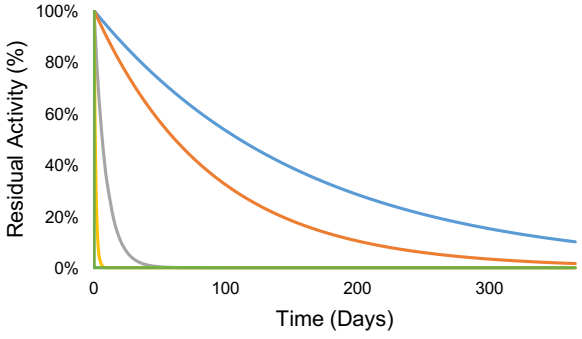

Figure 8.2: Residual enzyme activity after one year with five operating temperatures in the stripper: 60 °C (**blue**), 70 °C (**Orange**), 80 °C (**Grey**), 90 °C (**Yellow**) and over 100 °C (**Green**).

These results indicated that the soluble enzymes modeled here would not be suitable for the type of set-up tested here without modification, or frequent enzyme replenishment. Therefore, the use of a UF unit was implemented in the model. Where the rich solvent was passed through a UF membrane, where the majority enzyme was separated from the bulk of the solvent, and diverted directly back to the absorber, thereby circumventing the high temperatures in the stripper, Figure 8.3.

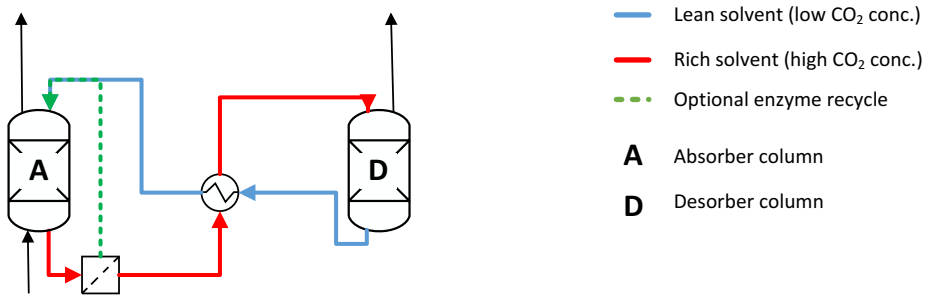

Figure 8.3: Process diagram of an ultrafiltration unit in a CCS facility. The process is similar to that which is described above (Figure 8.1). However, the rich solvent will be passed through an ultrafiltration device where most of the solvent will pass through, and some of the rich solvent will be diverted back to the lean solvent with the enzymes, not passing through the desorber column.

Here residual activity is calculated for three UF membranes with enzyme retention of 99.9%, 99% and 90% and compared with the same conditions without the use of a UF membrane. The rate of enzyme deactivation was calculated using five different stripper temperatures 60 °C, 70 °C, 80 °C, 90 °C and <100 °C. The comparison of the models can be found in Figure 8.4: A), B), C), D) and E), respectively.

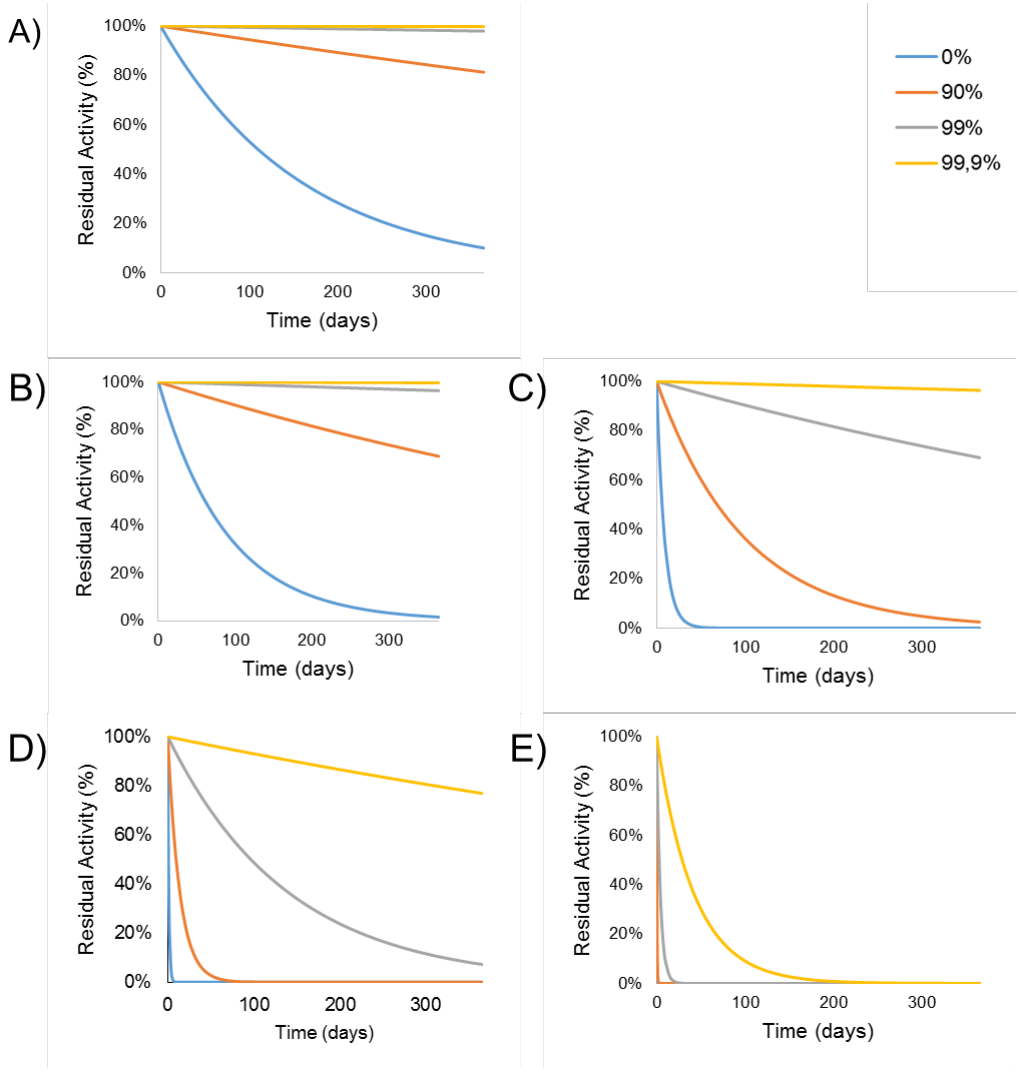

Figure 8.4: Effect of enzyme stability, measured by residual activity over time, of ultrafiltration enzyme separation with various stripper temperatures: A) 60 °C, B) 70 °C, C) 80 °C, D) 90 °C, E) over °100 C (instant deactivation). Membrane retention for all figures: **Blue**: No membrane, **Orange**: 90% retention, **Grey**: 99% retention and **Yellow** 99.9% retention.

The efficiency of the membrane has a significant impact on the enzyme viability. The least selective membrane with an enzyme retention of 90%, only has a small impact on enzyme

viability for all temperatures above 70 °C. For stripping temperatures over 70 °C membranes with higher selectivity perform significantly better. For example, at stripping temperatures of 80 °C less than 50% of the residual activity remains after 70 days, whereas the membrane with 99% selectivity leaves 94% of the enzymes active after a full year of operation. The most selective membrane with 99.9% selectivity, has more than 75% residual activity after 1 year for temperatures up to and including 90 °C. Finally, it was investigated if UF is a viable option with temperatures above 100 °C, where instant deactivation was assumed. Without the use of UF membranes, all activity is lost within 1 hour of operation. Although the use of membranes, especially the membrane with the highest retention at 99.9% significantly increases the lifespan of the enzymes, high activity loss is still observed, due to the high number of cycles. In this model 1 cycle lasts approximately 1 hour, therefore almost 9000 cycles are carried out per year, and the additive effect of even a small activity loss has a big impact. With instant deactivation at 100 °C, after 1 month less than 50% of the activity remains after 1 month and after 6 months only 1% of the initial activity remains. These results highlight both the effect of the membranes and the need to adjust the selectivity to the deactivation rate of the enzymes used.

8.5 Discussion

CA's are useful catalysts in CCS because they can enhance the absorption rate of CO_2 into kinetically limited solvents, such as tertiary amines and carbonate salts, enabling the uses of such low-energy solvents (Penders-van Elk *et al.*, 2012; Ye and Lu, 2014; Monteiro *et al.*, 2015; Salmon and House, 2015). However, a drawback of using enzymes as catalysts is limited to thermal degradation, which is particularly problematic due to the high temperatures encountered in the stripper unit. Therefore, the use of the use of UF to extend enzyme viability was explored. There are two key aspects that set a CCS process apart from other applications where enzymes are applied, which should be considered. Firstly, the scale of a CCS facility must be kept in mind. In the base case used in this chapter a large amount of solvent is used. Thus, the cost of the enzyme may be a significant contribution, especially is enzymes are to be frequently replenished. Furthermore, such a large scale poses technical operational difficulties. For example, the absorber

column used in this model is based on the absorber column in the boundary dam CCS facility, which is over 7 meters wide and almost 50 meters tall, with a volume of over 2000 cubic meters. Any modification to a set-up of this scale will be both costly and complex. The solutions implemented, should therefore be as simple as possible, rendering the required results. Secondly, as mentioned above, the number of cycles should be kept in mind. This base case has almost 9000 cycles per year. In addition to the cumulative effect on deactivation, as mentioned above, with such a high amount of cycles, replenishing enzymes on regular frequent intervals would significantly dilute the solvent, and would likely over time change the physical properties of the solvent, such as the viscosity. Experimentally, it has been observed that the precipitation of deactivated enzyme, deposited on heat coils of the reboiler, and thereby significantly reducing the effect of the coils (Salmon *et al.*, 2015). This can be problematic on a large scale. To avoid such complications in a commercial scale plant, it here argued that the use UF can be a possible solution. Figure 8.3 depicts how such a process may be carried out with solubilized enzyme in solution with UF. In comparison, this study showed very high deactivation rates without the use of UF. Even at the lowest stripper temperature of 60 °C, enzymes must be replenished 3 times annually to retain 50% or more of the initial activity. Deactivation rates, and thereby the need for replenishment intensifies significantly when the temperature increases. Increasing the temperature by 20 °C, increased the need for replenishment to maintain the same activity by 20-fold. As discussed above this does not only add cost in terms of increased enzyme requirements but also, perhaps more importantly on this scale, it also poses a practical problem with solvent dilution and precipitation.

UF, on the other hand, restricts CA in the cooler area of the process, thereby limiting deactivation. The model had 3 different enzyme retentions of 90%, 99% and 99.9%, and five stripper temperatures of 60 °C, 70 °C, 80 °C, 90 °C and above 100 °C. In this model, the enzymes are returned to the absorber with 10% of the rich solvent stream, without any desorption. The non-retained enzyme, will pass through the stripper column and deactivate at the same rate as the soluble enzyme. Although this set-up has a clear advantage, especially at higher temperatures, with increased enzyme viability, it also comes with several drawbacks. Firstly, 10%

of the solvent is not regenerated, thus the capacity of each cycle and the overall capacity of the facility is reduced by 10%. Furthermore, the kinetic penalty of such a set-up is likely to be higher than 10%, since the reaction rates in the absorber decrease with loading. The model indicates that a UF membrane with 90% retention is a viable solution for stripper temperatures up to 70 °C, for the enzyme tested here. For temperatures between 80 °C and 90 °C, a more selective membrane should be used. Furthermore, it was found that due to the high amount of cycles that UF is only a suitable option in EECCS when a deactivation is not instant. In the calculations above 100 °C, where instant deactivation was modeled, even the most selective membrane at 99.9% selectivity was not suitable for long-term use. In practical terms, this means that primary amines, such as monoethanolamine (MEA), frequently used as solvents in CCS, may not be suitable solvents with EECCS since the desorption with MEA is operated in the 120 - 140 °C range.

It is clear from the results in Figure 8.4, that the use of a membrane with a higher enzyme retention has a better performance with respect to retention of enzyme activity. However, increased enzyme retention often comes at a cost. The capital costs of such membranes are likely to be higher, and it would be expected that they are more difficult to produce and maintain at a high level of perfection, since any tear/leak would be detrimental to enzyme activity at higher stripping temperatures. In addition, the flux of the membranes should be considered, since it will influence the membrane size needed for such a setup. Table 8.4 indicates the membrane size needed to maintain the target flux of two commercial membranes. Estimated membrane size for the two commercial membranes used here is between 1,000 and 10,000 m^2. It should be mentioned, that in general the membrane flux and selectivity is correlated, where a higher flux is correlated with a lower selectivity (Mehta and Zydney, 2005). The inverse trend, shown in the two membranes here, arises because the 99.9% selectivity membrane (UFX10pHT), a technical membrane developed especially for enzyme retention in aqueous solutions, in collaboration between Alfa Laval and Novozymes. Large-scale UF membranes are implemented in other industries such as the water purification industry. Where numerous membrane units are connected in series. As such the membrane sizes estimated here are evaluated to be of a feasible size.

Furthermore, the cost of the two membranes was been calculated using an estimated cost of commercial membranes of 50$/m^2, which assumes a membrane lifetime of 4 years (Peters *et al.*, 2011). This cost estimate was confirmed by personal communication with Alfa Laval to be in the correct range. In addition, for an efficient UF, it is needed to operate with a higher pressure, here a pressure of 4 bar has been used. In general, the cost of cross-flow UF is dominated by membrane replacement and pumping (Rossignol *et al.*, 1999). However, in the case of CCS, the solvent is routinely compressed after exiting the absorber, so the cost will be dominated by capital costs, rather than operational costs.

Table 8.4: Required membrane sizes of ultrafiltration membranes used in this study, operated at 4 Bar, with a flux of 2.1*10^6 L/h

Type	Selectivity	Water permeability (L/(m^2*h*bar))	Membrane size (m^2)	Membrane cost (USD)	Source
GR80PP	90	50	10,600	530,000	Alfa Laval
UFX10pHt	99.9	400	1,330	66,500	Alfa Laval

8.6 Conclusion

This chapter evaluates the use of UF to elongate enzyme viability in EECCS. A model using three different enzyme retention membranes of 90%, 99% and 99.9% enzyme retention was modeled, with 5 stripper temperatures of 60 °C, 70 °C, 80 °C, 90 °C and over 100 °C. The target used in this chapter was to maintain over 50% activity after 1-year operation. UF showed increased viability with all temperatures tested here. To meet the set criteria requirements for membrane selectivity increased with increasing stripper temperature. At 60 °C and 70 °C, the 90% retention membrane was sufficient to meet the target. For temperatures of 80 °C and 90 °C, the enzyme retention required increased to 99% and 99.9%, respectively. For the highest temperature of over 100 °C, where instant deactivation was assumed, none of the membranes used in this model were sufficient to meet the target. The key conclusion drawn from this study is that UF is a good method for extending enzyme viability for intermediate stripper temperatures, but may not be suitable at very high stripper temperatures. Thus, the use of enzyme enhanced CCS might be

restricted to temperatures below 100 °C, or temperatures the enzyme can withstand for shorter time periods if the use of UF units is in use.

8.7 References

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Section II

Chapter 9:
Conclusion and future work, Section II

9.1 Introduction

Enzyme enhanced carbon capture (EECCS), is a developing technology. At the present time, no commercial EECCS plants are operating, however, the first plant is scheduled to open late 2018 (CO2 solutions inc., 2017). This chapter therefore draws conclusions from previous chapters in Section II and looks ahead to the future work still needed in the field.

9.2 Conclusion

Chapter 7 and Chapter 8, looked in detail at enzyme stability and ultra-filtration as a means to extend enzyme viability. Here the main conclusions from those two chapters are summarized.

9.2.1 Stability

This research confirms previous reports, that a key issue to implementation of EECCS is enzyme stability under process relevant conditions (Savile and Lalonde, 2011; Gundersen, von Solms and Woodley, 2014; Ye and Lu, 2014).

9.2.1.1 pH tolerance

The pH of the process will change over the course of the process, with higher pH in the lean solvent (Figure 7.2). The pKa and the loading/unloading of the solvent will determine the pH variation in the solvent. It is therefore important to know the pH stability of the enzyme in question when a solvent is chosen, or *vice versa* to know the solvents expected pH range when choosing an enzyme. Chapter 7 tested enzymes stability with respect to pH, by exposing it to a particular pH for 100hrs.

The results indicated:

- pH 4: No activity remained - This CA does not tolerate this pH
- pH 5, 6 and 12: ~50% activity remained - This CA tolerates these pHs at short intervals
- pH 7: 11: 100% activity remained - This CA is tolerant to these pHs

An average CCS process will fluctuate in the pH range of 7.5-11, it is therefore assumed that for most CCS processes, the enzyme tested here would be suitable in terms of pH. Furthermore, the enzyme was tested with 7 solvents (see 9.2.1.2 below for solvent list), where a high (pH 10) and a low (pH 8-9) pH was tested. It was found that for all the solvents tested, a higher pH had a negative impact on long-term stability (150 days).

9.2.1.2 Solvent tolerance

A plethora of possible solvents can be used in EECCS, the most common solvents are amines and inorganic salts. Here CA was tested with 7 different solvents, of those solvents 6 were tested both short-term, 100 hrs and long-term 150 days.

The solvents tested were (see Chapter 7 or abbreviations for full names), remaining activity after 150 days is presented in the brackets after each solvent:

- Amines:
    - Primary amines: MEA (pH 8.3: 73%, pH 10: 33%)
    - Tertiary or hindered amines AMP (pH9: 42%, pH 10: 12%), MDEA (pH 9: 62%, pH 10: 54%), MAPA/MDEA (pH 8.6: 85%, pH 10: 69%)
    - Amino acids: AIB (pH 8: 91%, pH 10: 35%)
- Salts: K_2CO_3 (pH 8: 83%, pH 10: 29%), NaCl (not tested for 150 days)

The following trends were observed:

- Increasing solvent pKas decreased enzyme stability

- Changing the pH from 8 to 10 had the greatest impact on solvents with a lower solubility (K_2CO_3 and AIB).
- The enzyme exhibited the lowest stability in AMP at both the high and low pH tested.

9.2.1.3 Temperature stability

Finally, thermal stability was tested over 100 hrs, with temperatures ranging from 50 °C to 80 ° C. After 100 hrs it was found that all initial activity remained at 50 °C, some activity remained at 60 °C and no activity remained at 70 °C and 80 °C. Which showed lower stability than what was modeled in Chapter 8, where the enzyme was only exposed to high temperatures for 5 minutes, each time, rather than prolonged exposure over several days. These results are concerning because most conventional CCS process operates the stripper at high temperatures, often in the range of 120-140 °C. These results indicate that the enzyme tested here is not likely to be stable in this range. Although a thermostable CA has been engineered to tolerate higher temperatures (Alvizo *et al.*, 2014), and a thermostable CA has been found in a thermophile (Del Prete *et al.*, 2016), it is deemed unlikely that CA's will tolerate such high temperatures for extended periods. Therefore, the process must either be altered to lower stripper temperatures or restrict the enzymes from entering the stripper.

9.2.2 Process solutions in EECCS

The results above indicate that process strategies must be implemented in order to extend enzyme viability in EECCS, especially with respect to temperature. Chapter 8 discusses one such option, namely ultrafiltration (UF), the results of that chapter is summarized here.

9.2.2.1 Ultrafiltration

Although one enzyme has been shown to retain some activity at temperatures of 100°C (Del Prete *et al.*, 2016). A likely scenario for a viable long-term solution in EECCS will likely restrict enzymes from entering the stripper. Chapter 8, modeled enzyme viability with 5 temperatures 60 °C, 70 °C, 80 °C, 90 °C and above 100 °C, with no membrane and 3 UF membranes with selectivity of 90 %, 99% and 99.9% over one year, the results are summarized in Table 9.1.

Table 9.1: Remaining activity (%) after 1year exposure to stripper temperatures from 60 °C to >100 °C, with UF membranes.

Temperature (°C)	Membrane selectivity (%)			
	0	90	99	99.9
60	10%	81%	98%	100%
70	2%	69%	96%	100%
80	0%	2%	69%	96%
90	0%	0%	7%	77%
>100	0%	0%	0%	0%

These results indicate that UF can be a powerful process strategy to increase enzyme viability, but that the membrane selectivity must be adjusted to the deactivation rates observed. Higher deactivation rates require more selective membranes. Furthermore, the results in this model indicate that UF is not suitable in situations where instant enzyme deactivation occur.

9.2.3 Conclusion

The results presented here indicate that EECCS is indeed technically feasible. However, a viable process requires careful enzyme selection, and optimized reaction and process engineering. Furthermore, it should be noted that several other competing technologies in CCS is also available, and a careful economic evaluation should be undertaken to evaluate if EECCS is the best option in each case. In conclusion, the results presented in this thesis indicate that EECCS is currently a viable solution for specialized applications where an economic benefit beyond that of avoiding carbon taxes can be obtained. Examples include the production of CO_2 for the soda industry or natural gas upgrade. It is not deemed a suitable solution for climate mitigation on a large scale at the present time.

9.3 Future work

A mentioned above, although EECCS is a technically feasible technology, it does not, in my opinion, take full advantage of the enzyme. As described in Chapter 1 of this thesis, enzymes are often applied because they are highly selective or have other qualities which cannot easily be

obtained by conventional chemical synthesis. In the case of EECCS, some of the advantages of the enzyme are not fully exploited.

9.3.1 Reactive at room temperature

CA's can catalyze the both the absorption and desorption reaction at a low temperature, unlike some of the chemical reactions that are exothermic, which produce heat during absorption and needs heat to desorb. The capture solvents consist of 70-85% water, heating up the water, especially above the boiling point, requires a large amount of energy. Currently, the strategy is to increase the heat stability of the enzymes. However, it might, in my opinion, be more appropriate to look at methods for operating the process at a lower overall temperature. With some of the solvents used in EECCS, it is not strictly necessary to operate at a higher temperature. Other methods for desorption could be employed to remove the CO_2 from the stripper.

The reversible conversion between CO_2 and bicarbonate in cells is one of the main methods for retaining pH homeostasis in the human body. It is highly pH dependent and will react towards an equilibrium. One could envision that a similar technique could be used in EECCS, where the absorber operates at higher pH of 8-10 and the desorber operates at lower pHs of 4-6. This pH swing could be obtained for example by electrochemical methods, or by addition of certain chemicals during some stages of the process. As has been proposed by Hamborg and colleagues (Hamborg *et al.*, 2011).

Another strategy that takes advantage of the low vapor pressure of the inorganic salt based solvents, such as K_2CO_3, is the use of a compressible sweep gas. Which could be used in combination with the pH swing or alone. The suggestion is that a gas that liquefies at a lower temperature than CO_2 can be used to strip CO_2 out of the solvent. For example, an ether or another organic solvent, which has a low boiling post and requires little energy to vaporize. After stripping the gas will be cooled to separate out the water, then compressed to liquefy the CO_2 for transport. Here the stripping solvent would liquefy first and could be separated out and reused for another cycle. This strategy can replace the use of partial vacuum, which is often used in EECCS.

9.3.2 High reaction speed

Another important aspect of using enzymes is, of course, their catalytic abilities to enhance reaction speed. The enzyme CA is one of the fastest known enzymes, with reaction speeds of up to 10^6 reactions per second, however in EECCS reaction rates are several orders of magnitude lower. It is therefore suggested to use other reactor set-ups or other methods to enhance reaction rates, both in absorption and desorption.

A common configuration in biotechnology is a bubble column, of the type that is used in fermentation. The use of stirring and small bubble sizes would increase surface area and decrease mass transfer rates. Testing this type of reactor in EECCS would be worth further investigation. However, it should be noted that some of the enzyme formulations used in EECCS cause foaming, so foaming must be controlled or the biocatalyst formulation must be altered to cause less foaming.

Furthermore, the configuration can be optimized to minimize mass transfer limitations. One interesting configuration that has been suggested is rotating packed beds. Which was recently shown to out-perform column absorption under identical conditions (Leimbrink *et al.*, 2017).

Finally, as discussed on several occasions in this section, one reason for the slow reaction rate is the discrepancy between low CO_2 solubility and CA's K_M's. It is proposed to enhance this by increasing the CO_2 solubility in the solution. Simply by decreasing the temperature of the absorber, CO_2 solubility increases. Further enhancement can be obtained by adding a chemical adsorption solvent, like zeolites, commonly used in CCS to capture CO_2.

9.3.3 Selectivity

It should be noted that enzymes are often applied for their excellent selectivity and ability to be used in concert. It is therefore a natural line of thought that the CO_2 captured with enzymes, could be further reacted in the same reactor to produce higher value compounds. This has been tested on several occasions and is part of carbon capture and utilization (CCU), an area not covered here. Although it is possible, it should be considered whether CO_2 is the best starting

material, both energetically and economically. Furthermore, it should be considered the scale of operation. The volume of CO_2 needed for climate mitigation is several orders of magnitude above the volume that could be used in CCU. Although CCU might be useful for generating value for CCS, it might not be an ideal solution for reducing atmospheric CO_2.

Finally, a thorough techno-economic assessment of EECCS compared to other technologies, and comparing the options within EECCS is needed to drive the field forward.

9.4 Conclusion

In conclusion, EECCS might be technically feasible when strategies like enzyme engineering and ultrafiltration are added. However, the current state of EECCS does not optimize the use of the enzymes, but rather add enzymes to an already existing method. Innovation is needed to bring this technology to the next level. Although EECCS is technically mature enough for implementation, other competing strategies are more developed and are easier to implement. In my opinion, EECCS is therefore not currently ready for general use as a CCS technology for climate mitigation. However, for specialized applications, such as natural gas upgrade, or producing food grade CO_2 (such as for the beverage industry), EECCS might be a good option right now. The key to success for these applications is that CA can provide good selectivity and avoid the use of potentially harmful amine solvents. Furthermore, these applications provide an economic incentive beyond that of avoiding carbon tax.

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Chapter 10:
Lessons learned

10.1 Introduction

With the increasing number of commercial processes (Woodley, Breuer and Mink, 2013; Salmon and House, 2015), and the use of biocatalysis in non-natural reactions (Siegel *et al.*, 2010; Coelho *et al.*, 2013), it is clear that the use of biocatalysis will continue to expand. This chapter summarizes the common lessons learned from the previous chapters, and offer a tool to evaluate the impact of an implementation strategy on common challenges found in biocatalysis.

10.2 Integrated process development

The results in both sections of this thesis point towards the importance of integrated process development early in the implementation of a new biocatalytic process, for a successful outcome. Here 6 challenges in biocatalytic applications are outlined, with specific examples from each section. Furthermore, 4 common process strategies to overcome these challenges are outlined. Finally, in an easy to follow table the effect on each strategy has on the challenges outlined is presented.

10.2.1 Challenging areas of biocatalysis

In this section, the 6 key challenges in implementation of biocatalysis are briefly summarized, this is by no means a complete list, but includes the 6 areas believed to be the most relevant hindrances and the most relevant to comparing the 4 key strategies listed in section 10.2.2 below.

10.2.1.1 Enzyme stability

Enzyme stability is often a challenge in biocatalysis. Enzymes are taken from their natural protective environment inside a cell and applied under very different conditions. ω-TAs, for example, are exposed to organic solvents and two-phase reaction conditions which can lower enzyme stability. CA's in CCS are routinely exposed to high temperatures and high solvents conditions. In both these cases, enzyme stability is greatly reduced.

10.2.1.2 Thermodynamics

Thermodynamics is an independent property of the reaction, which is not affected by the enzyme. Often unfavorable thermodynamics is unavoidable, due to the nature of the reactants and products. However, identifying unfavorable thermodynamics early in development can enable strategies to limit the effects of unfavorable thermodynamics. For a successful reaction process, favorable thermodynamics or strategies to overcome unfavorable thermodynamics is needed. In the case of ω-TAs, thermodynamics is a frequent hurdle impeding implementation. In Section II, the low solubility of CO_2 in the solvent, which impedes kinetics and mass transfer rates, a result of unfavorable thermodynamics.

10.2.1.3 Kinetics

Although CA's are some of the fastest enzymes known, with measured reaction rates at 10^6 reactions per second (Savile and Lalonde, 2011), CA's applied in CCS are often several orders of magnitude slower than that (Salmon and House, 2015). Furthermore, in the case of ω-TAs enzyme inhibition and low reaction rates with un-natural substrates, prevents high yields at high substrate concentrations. Kinetic limitations under process relevant conditions limits the efficient use of biocatalysis in industrial applications.

10.2.1.4 Development time

Any new technology must be developed and adjusted to the application. Some development times here are deemed to be longer such, as enzyme engineering, which can take a significant time as several rounds of optimization is needed to reach the desired target (Savile *et al.*, 2010;

Alvizo *et al.*, 2014). Other applications such as reaction engineering are less complex.

10.2.1.5 Capital costs

Capital costs are the costs of the physical set-up. In this comparison, for simplicity, it is assumed that the costs of the facility and external costs are the same for all set-ups, and only the cost of the enzyme technology is evaluated.

10.2.1.6 Operational costs

Operational costs here include the costs of operating the facility and any reoccurring cost. Such as the enzyme replacements or additional pumping costs with membranes. As in section 3.1.4, only the cost of the additional enzyme technology is evaluated, auxiliary costs are assumed to be the same for all scenarios.

10.2.2 Biocatalysis implementation strategies

The implementation strategies discussed in this section is highlighted and discussed in terms of the 5 challenges outlined in the section above.

10.2.2.1 Enzyme engineering

Enzyme engineering has played an important role in both case studies presented here. The engineered enzyme for the manufacture of a Sitagliptin intermediate opened up the possibility of using a non-natural substrate yielded an enzyme compatible with optimal process conditions (Savile *et al.*, 2010). Furthermore, in the case of CA, enzyme engineering provided with a significant increase in thermal stability (Alvizo *et al.*, 2014). It is clear from these two examples that enzyme engineering broadened the potential application of the respective enzymes. However, enzyme engineering has two key issues. Firstly it has a long development time, even for the most advanced commercial specialists took 1 year to develop the ω-TA used in Sitagliptin manufacture (Truppo, 2017). Furthermore, the outcome is uncertain, it is for example not possible to predetermine how high temperatures an enzyme will be able to withstand. Especially, concerning EECCS applications, where temperatures are much higher than in most other

biocatalytic applications. It was therefore evaluated that stability and operating costs are moderate, and development time is long for this application.

10.2.2.2 Enzyme Immobilization

Enzyme immobilization is the attachment of enzymes on surfaces, on small particles or as self-aggregates, discussed in Chapter 1. Immobilization can be beneficial in retaining the enzymes in one area of the process to increase stability (Section II), or to ease with enzyme recycle or product separation (conventional biocatalysis). However, it carries high mass transfer limitations and can impede reaction kinetics. Numerous options are readily available, and the technology is ready for implementation. Finally, the use of immobilization drastically increases the capital costs of the enzyme. One paper reports 4 fold increase in enzyme costs from enzyme immobilization (Tufvesson *et al.*, 2011), therefore capital cost and complexity of the process are increased.

10.2.2.3 Process engineering

In this thesis, numerous options for process engineering has been shown, from simple ISPR strategies in section I (Savile *et al.*, 2010; Tufvesson, Bach and Woodley, 2014) to complex contained liquid membrane systems in Section II (Bao and Trachtenberg, 2006; Russo *et al.*, 2013). Although it is difficult to draw a general conclusion for all process engineering strategies, they are often applied to enhance thermodynamics and kinetics of the reaction. However, they often add development time and capital costs.

10.2.2.4 Reaction engineering

In both the case studies here reaction engineering has been important. In the case of ω-TAs several reaction engineering strategies has been possible. Chapter 3 showed both the importance and impact of careful donor selection (Gundersen *et al.*, 2015; Meier *et al.*, 2015). Furthermore, Chapter 4 investigated the use of organic solvents to increase yield. In Section II, the solvent used in CCS can greatly impact both thermodynamics (loading) and reaction kinetics. Arguably, for successful implementation, early and careful reaction engineering is paramount and can alleviate both thermodynamic and kinetic constraints, without adding any additional costs.

Table 10.1: Evaluation of biocatalytic implementation strategies

Strategy	Stability	Thermodynamics	Kinetics	Development time	Capital Cost	Operating costs
Enzyme Engineering						
Enzyme Immobilization						
Process Engineering						
Reaction Engineering						

10.3 Conclusion

In conclusion, the results of this thesis indicate that early an integrated enzyme, process and reaction engineering is vital to success. Arguably, that this is not only true for the two case studies presented here, but to commercial biocatalytic process in general.

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Appendix A

Included Publications:

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Amine donor and acceptor influence on the thermodynamics of ω-transaminase reactions

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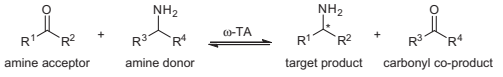
ABSTRACT

In recent years biocatalytic transamination using ω-transaminase has become established as one of the most interesting routes to synthesize chiral amines with a high enantiomeric purity, especially in the pharmaceutical sector where the demand for such compounds is high. Nevertheless, one limitation for successful implementation and scale-up is that the thermodynamics of such conversions are frequently found unfavourable. Herein we report experimental measurements of apparent equilibrium constants for several industrially relevant transamination reactions in a systematic manner to better understand the effect of amine acceptor and donor choice. For example, we have found that *ortho*-substitution of acetophenone like molecules, had a significant impact on the thermodynamic equilibrium. Likewise, the effect of cyclic amine acceptors was evaluated and compared to similar non-cyclic structures. It was found that an aliphatic six membered ring was favourable and a conjugated bicyclic five membered ring structure, unfavourable. Finally, we evaluated and compared the use of five different donor molecules, and calculated their ΔG^{app} values. This is particularly important in the further implementation of such reactions because it may be used to help select suitable donor/acceptor combinations. The results presented here give guidance, with respect to thermodynamics, in order to further extend the application of biocatalytic transamination.

1. Introduction

Biocatalysis, where one or more synthetic chemical reactions are catalysed by enzymes, has over recent decades proved to be a particularly powerful complement to conventional approaches for the synthesis of chiral molecules, as illustrated by numerous commercial processes.[1] In particular the synthetic application of biocatalysis capitalizes upon the excellent stereo- and regioselectivity and mild reaction conditions of enzymes,[2,3] implying a potentially attractive synthesis of valuable chiral products.

One particularly interesting group of chiral products is optically active amines, which are amongst the most important groups of chiral molecules from a commercial perspective, with many interesting pharmaceutical applications.[3,4] There are several biocatalytic methods to produce optically active amines and one of the most established methods uses the enzyme ω-transaminase (ω-TA) (EC.2.6.1.18).[5] The enzyme catalyses the transfer of an amine group from an amine donor to an amine acceptor, yielding an optically active amine target product and a non-chiral carbonyl co-product (see Scheme 1).



Scheme 1. Generalized scheme of biocatalytic transamination.

Despite its applicability, the utilization of this enzyme is frequently hampered by low reaction yields, often stemming from the unfavourable thermodynamics of the respective couples of substrates (amine acceptor and amine donor) and products (target product and carbonyl co-product). Additionally, low yields can be the result of biocatalyst related issues, such as enzyme inhibition or instability of the enzyme. Such limitations can be overcome by enzyme modification through protein engineering.[6] However, the thermodynamics of the reaction, which are very often found to be the primary limitation, is independent of the enzyme used to catalyse the reaction and therefore alternative solutions need to be sought.[7]

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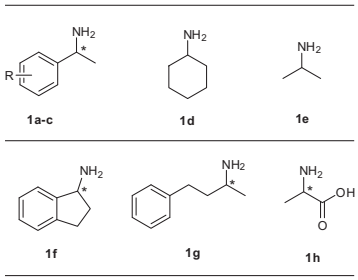
0957-4166/

The thermodynamic yield of a reaction is influenced by the structural and energetic differences between the couples of amine donor/amine acceptor and the target product/carbonyl co-product, respectively. Since the structure of the target product is determined by the structure of the acceptor molecule, chemical flexibility in the reaction is provided by the choice of amine donor alone. Although in principle this means that a plethora of possible compounds could be used as the donor, in practice, to date only a few selected compounds have been used, or even tested, in large part due to limited availability and poor enzyme specificity. With this in mind, we reasoned that it would be useful to evaluate a wider range of donors and in particular establish their effect on the reaction thermodynamic yield. At the same time it is clear that the influence of donor selection on the thermodynamic equilibrium is dependent upon the given acceptor molecule to be converted. Hence we have also sought to understand the effect of using different acceptors on the thermodynamics, with a given donor molecule.

Although it is clear that overcoming thermodynamic constraints is paramount for the successful implementation of ω-transaminase-based processes,[8,9] remarkably little information about reaction thermodynamics has been reported in the scientific literature, with a few exceptions.[10,11] Herein, we report experimentally determined concentration-based equilibrium constants (K_{eq}^{app}) for a variety of ω-transaminase catalysed reactions. In each reaction, we have chosen amine acceptor molecules in such a way as to explore the influence of both substitution and the effect of ring strain. Likewise various donors have been selected, for transamination with a given acceptor, to also understand the effect of different donors on thermodynamic equilibrium. In Table 1 the various donors and target products of all the reactions used herein are listed. The corresponding amine acceptors can be derived from these.

In order to simplify the interpretation of the results throughout, we have kept either the donor, or the acceptor, molecule fixed and changed the other reactant. Hence we have compared variable half reactions. The alteration to a given molecule occurs on both the reactant and product side. Hence, we are comparing a certain structural change and the effect that has on both the reactant and product side. For example, when comparing the reaction of **1b**/**2b** with **1c**/**2c**, substituting the fluorine for an alcohol has an overall negative effect on the thermodynamic equilibrium of that of half reaction, but does not indicate that **1b** is more stable than **1c** in isolation.

Table 1
Structures of amine donors



(**1a**) R = H, (**1b**) *o*-F, (**1c**) *o*-OH, (**1d**) cyclohexylamine, (**1e**) isopropylamine, (**1f**) aminoindane, (**1g**) 3-amino-1-phenylbutane, (**1h**) alanine. The corresponding amine acceptors are named with the designation 2 followed by the same letter.

2. Results and discussion

2.1. Influence of amine acceptor

In initial experiments we sought to determine the effects of two types of acceptor modification on the thermodynamic equilibrium. In the first type we examined the effect of substituted aryl compounds similar to those of acetophenone, by *ortho*-substitution to investigate the effect of the substituent group. In the second type, we explored the effect of ring strain between cyclohexane and indane, compared to similar compounds in the absence of the ring or just one ring, respectively.

2.1.1. Substitution in the acceptor molecule

Several acceptors **2a**, **2b**, **2c** (see Table 1) were chosen to test in ω-transaminase catalysed reactions in order to understand the effects of substitution at the *ortho*-position of the amine acceptor on the measured apparent equilibrium constant.

The reaction equilibrium at completion was measured using both **1a** and **1e**, as the amine donor, and variously substituted acetophenone molecules as the acceptor.

We found the K_{eq}^{app} of **2a** with **1a** and **1e** to be 1 and 0.006. In comparison the *ortho*-substituted compounds **2b** and **2c** gave K_{eq}^{app} of **2b**: 1.8 and 0.1 and **2c**: 0.3 and 0.01 with the same donors **1a** and **1e**, respectively. The results indicate that fluorine substitution gives a higher apparent equilibrium constant than hydroxyl substitution in the *ortho*-position, and therefore is more favourable for product formation. We postulate there is a higher fraction of one of the stable un-reactive tautomers of the molecule, resulting in a lower conversion. Previous work supports that a significant fraction of such tautomers may exist in solution.[5]

Talwar et al.[12] have used a cyclometallated iridium catalyst to carry out the transfer reaction reporting 14 reactions with substituted acetophenones, where all of the substituted reactants have either the same or higher isolated yields than acetophenone itself. No strong electron donating substituents were used. Likewise, Paul et al.[13] report similar trends. Finally Tufvesson et al. have shown that substitution may significantly impact thermodynamics of acetophenone-like compounds.[14] These published studies are qualitative, support the results presented here.

2.1.2. Ring strain in the amine acceptor

A further aspect of interest in the acceptor molecules is the effect of ring strain. Here the effect on the thermodynamic equilibrium might in some cases be easier to predict, since there is an observed difference in the bond angles of the ketone of the amine acceptor and the amine in the amine donor; approximately 120° to 109°, respectively. In order to test this, we measured the apparent equilibrium constant using **2d** and **2f** where we predict conversion to **1d** to be favourable and **1f** to be unfavourable in comparison to the respective non-cyclic structure, **2e**, and monocyclic structure, **2a**. In all cases, **1a** was used as the amino donor.

In the reaction between **2d**/**1d**, compared to **1e**/**2e**, the ring has a positive effect on the measured apparent equilibrium constant: in this case the ring of the target product is less strained. We suggest that this is caused by the formation of the cyclohexane with six sp^3 carbons, the most stable saturated hydrocarbon conformation, compared to the starting material with five sp^3 carbons and one sp^2 carbon.

Likewise, **2f** can be compared with **2a**, with an additional five-membered ring. Since the angle strain of the sp^2 is stipulated to be more favourable than the sp^3 carbon of the product, this would result in increased strain. However the amino donor in the reaction pair **2f**/**1f** would be less flexible and therefore more susceptible to steric hindrance. We therefore considered that these two acceptors

would give similar apparent equilibrium values. Interestingly, the results (Table 2) indicate that **2f** gives an apparent equilibrium constant of merely one hundredth of the value for **2a**. These results are also partially supported by the low observed conversion yields in another study by Fesko et al.[15]

Table 2
Effect on ring strain, using five- and six- membered rings, all using **1a** as the donor

Acceptor	K_{eq}^{app}
2d	213
2e[11]	30
2f	0.01
2a	1

2.2. Influence of amine donor

Finally, we have compared the data from the experiments here with some other data previously published to investigate the effect of donor choice. As mentioned, the ω-transaminase reaction is used to produce a chiral amine, thus the structure of the amino acceptor is determined solely by the structure of the desired product. However, the amine donor may be freely selected and this will clearly influence the apparent thermodynamic constant. Here we have compared the measured apparent equilibrium constant of five different donors (using acetophenone as the acceptor molecule). Using these values, the ΔG^{app} of each reaction couple was calculated. This is particularly interesting, because by comparing the half reaction between each of these donor choices, it is possible to use ΔG^{app} to estimate the impact of changing the donor in any given reaction, where the ΔG^{app} or K_{eq}^{app} is known for either one of the donors listed in the table. The results are presented in Table 3.

Table 3
K_{eq}^{app} and ΔG^{app} for the acceptor **2a** coupled to the donors; **1a**, **1d**, **1e**, **1f**, **1g** or **1h**

Donor	K_{eq}^{app}	ΔG^{app} (kJ/mol)
1a	1	0
1d	$6.0 * 10^{-3}$	13
1e[11]	$3.3 * 10^{-2}$	8.6
1g	0.18	4.3
1h[11]	$4.0 * 10^{-5}$	26

Out of the five compounds selected for testing, we found that the K_{eq}^{app} of the selected reaction varies by five orders of magnitude from the most beneficial donor, **1a** to the worst case alanine **1h**. Another reaction with alanine using **2f** as the acceptor likewise gave a very poor equilibrium constant of $<10^{-4}$. These data clearly indicate the strong influence of donor selection. Interestingly, two of the donors are achiral **2d** and **2e**, offering an advantage economically (higher concentrations and less expensive starting material) by avoiding an enantiomerically pure starting material.

3. Conclusion

The data reported here show interesting patterns for selection of the amine acceptor and donor couples. The data presented in Table 3 are potentially the basis for at least initial calculations of reaction feasibility. Nevertheless the effects of substitution and ring strain on the acceptor molecules reported here are not supported by all published data. For example, for ring strain, the opposite trend was found by Talwar et al.[12] This indicates not only the difficulty of making effective measurements but also the need to report a wider dataset.

The use of data such as those measured in the experiments reported here are to enable an estimate of process feasibility, and to establish if supplementary methods are required to achieve sufficiently high reaction yields. For example, an excess of the amine donor can be used (provided it is not inhibitory to the enzyme or difficult to separate downstream). Other possibilities include in situ product removal or in situ co-product removal. Nevertheless, all such schemes have operational limitations. For example, using an in situ co-product removal approach to reach high reaction yields by 'pulling' reactions towards the target product through removal of the carbonyl co-product, by evaporation under reduced pressure[2] or extraction into a second, organic solvent phase,[9] is limited to those cases in which the carbonyl co-product is significantly more volatile or hydrophobic, respectively, than the amine acceptor. Indeed, high selectivity in in situ product removal or in situ co-product removal approaches is a prerequisite for effectively shifting equilibrium. For this reason, careful choice of the amine donor and acceptor pair, considering not just kinetics, but also thermodynamics are important for further applications of the ω-transaminase reaction.

4. Experimental

4.1. Materials

All chemicals were of reagent grade or higher and purchased from a chemical vendor and used without further alteration. Enzymes ATA-42/ATA-81, were provided from c-LEcta GmbH (Leipzig, Germany), ATA-42 was used for reactions 4 and 8 all other reactions were carried out with ATA-81.

4.2. Reactions

All reactions were carried out in duplicate with a minimum of 3 different donor/acceptor concentrations between 2 mM and 10 mM, thus a minimum of 6 reactions for each reaction. All were carried out at a 0.4 mL scale in 1.5 mL Eppendorf tube with: 1 mg/mL lyophilized ω-transaminase, 1 mM pyridoxal-5′-phosphate, 2 to 10 mM substrate, 5% (v/v) DMSO in 0.1 M Tris–HCl buffer, pH 7. Equilibrium was reached by 48 h incubation in a thermoshaker (HCL, Bovenden, Germany) at 30 °C with constant agitation (400 rpm). Each reaction was carried out in duplicate, with duplicate blank samples where enzyme activity was quenched by NaOH before substrate addition, see analytical analysis below.

4.3. Analytical

After 48 h, the reaction was quenched by adding the aqueous NaOH (5 M, 100 μL). 0.02 mL eternal standard solution (150 mM 4-bromo-acetophenone in DMSO) was added. An extraction was carried out with 400 μL ethyl acetate. After separation the sample was dried in magnesium sulfate. Furthermore 0.15 mL of the ethyl acetate was transferred to a GC vial and derivatized by the addition of 15 μL triethylamine and 10 μL acetic anhydride. Conversion was measured by Gas chromatography on a Clarus 500 (Perkin–Elmer) with a 25 m * 0.25 mm CP-Chirasil-Dex CB column (Agilent J&W GC scientific). 2 μL sample was injected, with a thermal gradient from 120 to 200 °C for 13 min, 1.4 mL/min He was used as a carrier gas. FID detection was carried out at 250 °C.

4.4. Experimental set up for reactions: 1f/2f and 1 h/2 h

4.4.1. Materials

All chemicals were of reagent grade or higher and purchased from a chemical vendor and used without alteration. The ω-

transaminases from *Vibrio fluvialis* and *Aspergillus terreus* as described and produced by Meadows et al.[16] and Fesko et al.[17] were used as cell-free *Escherichia coli* extracts.

4.4.2. Reactions

All reactions were carried out on a 5.0 mL scale in 10 mL screw-capped glass vials containing 5 mM of the respective acceptor substrate and amine donor in 50 mM potassium phosphate buffer pH 7.5 containing 0.1 mM pyridoxal-5′-phosphate. As biocatalyst 1.25 mL cell-free extract containing the respective transaminase was added resulting in final total protein concentrations of approximately 10 mg/mL. The reactions were incubated on an IKA KS130 basic shaker at 28 °C with constant agitation (480 rpm). The reactions were followed in time until the equilibrium was reached by taking 250 μL samples, diluting and stopping by addition into 750 μL of a 1:1 mixture of acetonitrile and 0.5% (v/v) formic acid.

4.4.3. Analytical

All samples were analysed by HPLC on a Hypersil BDS C18 column (250 × 4.0 mm, I.D. 5 μm) and eluted with a gradient of Eluent A (0.1% formic acid in water) and Eluent B (0.1% formic acid in acetonitrile) from 97.5% A/2.5% B to 30% A/70% B in 10 min with a flow of 1.0 mL/min at 40 °C and an injection volume of 5 μL. Substrates and products were detected by UV at 256 nm and 210 nm and quantified based external standards of the respective substrate and product compounds.

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CHEMCATCHEM Communications

A Practical and Fast Method To Predict the Thermodynamic Preference of ω-Transaminase-Based Transformations

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A simple, easy-to-use, and fast approach method is proposed and validated that can predict whether a transaminase reaction is thermodynamically unfavourable. This allowed us to deselect, in the present case, at least 50% of the reactions because they were thermodynamically unfavourable as confirmed by experiment. Once a larger data base is established, in silico screening of several new reactions (new target molecules) can easily be performed each day.

ω-Transaminases (EC 2.6.1.18) have over the last decade gained significant interest as biocatalysts to produce enantiopure chiral amines owing to their high selectivity and broad substrate repertoire. If a prochiral amino acceptor undergoes an aminotransferase reaction with an amino donor, the reaction then yields an amine product and a keto co-product. ω-Transaminases can either be applied in the synthetic direction, the main focus of this communication, or in kinetic resolution.[1] This provides a useful way to produce chiral amines for pharmaceuticals or fine chemicals, as demonstrated by several commercial applications.[2,3] However, in practice several challenges are often encountered during process development such as enzyme inhibition, poor substrate binding, and/or unfavorable thermodynamics. To shift the equilibrium towards product formation, one has to, in practice, often add an excess amount of the amino donor and/or remove the co-product.

A common solution is to add an excess amount of the amine donor;[4] however, the isolation of an amine product from a reaction mixture still containing a large excess amount of a chemically very similar amino donor represents a major downstream processing challenge. Another possible solution is to remove the co-product, either physically or chemically. A simple strategy used if the co-product is volatile is to evaporate it,[5] which thus physically removes it from the reaction. Using isopropylamine as the amino donor in an excess amount under reduced pressure, for instance, usually results in high conversions towards the target product,[3,5] but it can also lead to the formation of a Schiff base of the amino donor with the acceptor ketone and low atom efficiency on the amino donor side or the requirement of large amino donor recycling streams. Chemical strategies such as enzymatic cascades[6] and the use of amine donors, which produce a co-product that undergoes a spontaneous reaction,[7,8] both efficiently convert the co-product into another compound. The successfully applied strategy to remove pyruvic acid as a co-product originating from the amino-donor alanine (using enzymatic cascades by employing lactate dehydrogenase coupled to glucose dehydrogenase) generally works well[6] but comes with the disadvantage of having to produce or purchase at least three enzymes instead of only one transaminase.

Whereas such protocols may be successful, they are not always applicable on large scale and they always incur additional time in process development and additional costs for the process itself. An alternative strategy could be the judicious selection of the amino donor. Whereas the ketone substrate is fixed to yield a particular product, as the corresponding desired amine is given as the product, the amino donor can be chosen freely. Consequently, the optimal scenario is to identify an amino donor that gives a high equilibrium value (towards the products). The opposite strategy is also valid, although not as desirable, by using an amino donor that gives a low equilibrium value (towards the substrates) such that a kinetic resolution can be effectively employed.[9] Of course the multitude of amines available gives a plethora of possible reactions. Therefore, to truly find the optimal reaction one must perform numerous time- and cost-intensive experiments. Thus, it would be preferential to have an in silico approach to predict the thermodynamic equilibrium with sufficient accuracy to decide whether a particular substrate combination is suitable for subsequent experimentation. In this way, the method proposed herein identifies reactions by using amino donors to give either high or low equilibrium values for potential use in synthetic or resolution reactions, respectively. Intermediate equilibrium values are eliminated. The rationale is therefore to use such an in silico method to focus subsequent experiments, for which more accurate thermodynamic and kinetic data will be obtained to assist in the process design. Evidently, a favorable thermodynamic equilibrium does not imply that the enzyme will be kinetically active towards the substrate. Steric hindrance in the active site of the enzyme can prevent productive substrate binding and can also prevent a thermodynamic equilibrium from actually being reached. Therefore, further experiments are required to determine both enzyme kinetics and inhibition for every transaminase/substrate couple.

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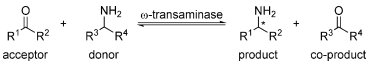
As the first stage in this procedure, in this paper, we present a simple and straightforward method to predict the thermodynamic equilibria for transaminase reactions. Ab initio calculations are widely used to calculate reaction (free) energies and kinetic parameters of chemical reactions, but for enzymatic reactions this is less well developed. Our approach is based on ab initio calculations of total energies of the molecules involved. It allows a good prediction of the equilibria of transaminase reactions. However, it should be noted that the key strength of this approach lies with predicting whether or not a reaction is thermodynamically favorable: hence, predicting which combinations of acceptor and donor are thermodynamically favorable. In this way, the approach can be used to eliminate those reactions that will not give high conversion or alternatively identify those same reactions as good reactions for kinetic resolution. Furthermore, it must be considered that whereas favorable thermodynamics are necessary for an effective reaction, they are not necessarily sufficient. Other factors such as enzyme reactivity or inhibition may still prevent high conversion from being reached. Thus, the proposed modeling approach allows the elimination of thermodynamically highly unfavorable reactions so that subsequent focus can be placed on those for which the chances of success are highest. Furthermore, in practice often only a few amino donors are applied, namely, 1-phenylethylamine (often referred to as α-methylbenzylamine), isopropylamine, and alanine, and given that transformations involving one of these are not always successful, it would be highly interesting whilst developing process routes to have a method that can effectively and efficiently look for alternative donors that do allow for high conversion.

Computational procedures

The B3LYP hybrid density functional was used in combination with a 6-311+(d,p) basis set while using the Spartan10 program.[10] With the transaminase transformation involving the reaction between a ketone and an amine leading to another ketone and another amine (Scheme 1), the ab initio calculations are applied to all four structures (structure optimization or, equivalently, energy minimization). The energy difference between the left-hand side and the right-hand side of the reaction, that is, the reaction energy (ΔE), is straightforwardly calculated from these four individual energies. If the energy is negative, the reaction is favored in the synthetic direction, if positive the reaction is not favored. This is a simplification, as experimentally the equilibrium concentration can be converted into a Gibbs free-energy (ΔG) value. In addition, in the presented method we neglected the entropy contribution, solvent effects, and the influence of the dynamic nature of the structures. However, as is often in quantum calculations, if the solvent and other experimental conditions (such as temperature and pH) are kept the same in all experiments, a cancelation of the contributions might be expected, which would lead to a simple model involving molecular energies alone. The results indicate that this applies in the current work.



Scheme 1. General reaction scheme of ω-transaminases. If $R^1 \neq R^2$ and $R^1, R^2 \neq H$, the product is a chiral amine.

The predicted conversion (K) was calculated by applying the known expression [Eq. (1)]:

$$\exp\left(-\frac{\Delta G}{RT}\right) = K = \frac{[A][B]}{[C][D]} \quad (1)$$

for the reaction A+B→C+D, in which R is the gas constant and T is the absolute temperature. Assuming we start with equal amounts of A and B, we can rephrase this as [Eq. (2)]:

$$\exp\left(-\frac{\Delta G}{RT}\right) = K = \frac{x^2}{(1-x)^2} \quad (2)$$

This can be solved with the solution conversion [Eq. (3)]:

$$x = \frac{\left[\exp\left(-\frac{\Delta G}{RT}\right) + \left(\frac{\Delta G}{RT}\right)\right]}{\left[1 - \exp\left(-\frac{\Delta G}{RT}\right)\right]} \times 100\% \quad (3)$$

Validation of the method

The potential of ab initio calculations was evaluated for transaminase reactions by comparing simulated values with experimental data from the relevant scientific literature.[11–14] The reaction yield was estimated by using a 1:1 ratio of donor/acceptor starting material. Calculated and experimentally obtained values were compared, as shown in Figure 1 (see the Supporting Information for the raw data). The data chosen for the comparison were all run at low concentrations (under 100 mM), and the reported values were considered to be at equilibrium, either stated by the authors or confirmed by numerical comparisons, for which briefly ΔG of the reactions were compared to uncover any inconsistencies in the reported values.

The method predicts with high accuracy (within $\approx 5\ kJ\,mol^{-1}$) low conversion yield reactions. Indeed, it can be seen that experiments with less than 25% conversion yield are predicted to have conversions below 40%. This highlights the key aim of this method, that is, to eliminate thermodynamically unfavorable reactions. The same trend is observed for very favorable reactions. It is interesting to note that many of the reactions that lie far from the line have a compound containing an aryl group, either acetophenone, a well-known inhibitor for this enzyme,[9] or a similar structure. However, this trend is not observed for all reactions with these structures. It is, therefore, probable that the error lies with the experimental results rather than with the predictive method.

It is difficult, actually not realistic, to achieve ab initio molecular energies, for example, heats of formation, that are accurate within $4\ kJ\,mol^{-1}$.[16] Given that we look here at simple transformations for which a ketone on the one molecule is exchanged for an amine on another molecule, one may expect

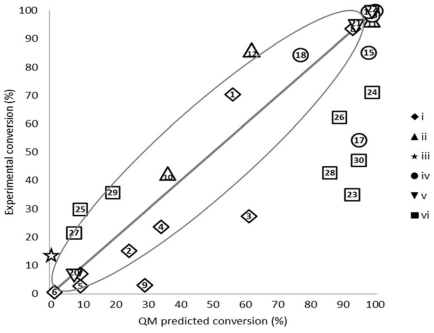


Figure 1. Comparison of predicted and measured conversions with a 1:1 donor/acceptor reaction. Data were extracted from i) Gundersen et al.,[13] ii) Gundersen et al.,[11] iii) Tufvesson et al.,[12] iv) Fesko et al.,[14] and v) Meadows et al.[15] The line represents a match between experimental and predicted quantum mechanics (QM) values. The ellipsoid area gives an indication of the error in the predicted conversion, assuming an error of 4 kJ mol^{-1} in the quantum-calculated reaction energy. The number in each point refers to the reaction number, as listed in the Supporting Information.

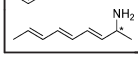
that the accuracy of such an energy difference, namely, the reaction energy for the reaction displayed in Scheme 1, is also of the order of 4 kJ mol^{-1}. The impact of the accuracy on the conversion is highly dependent on the absolute value of the conversion indicated by the ellipsoid in Figure 1. This is understandable, as a very significant energy difference will, despite an error of 4 kJ mol^{-1}, still remain large and conversion will remain close to 0 or close to 100%, respectively. For intermediate cases, for example, conversion of approximately 50%, the effect of 4 kJ mol^{-1} is roughly 20% in conversion. This is confirmed by comparison with the experimental data, save the data discussed above.

Application of method

As mentioned above, the key feature of the approach we propose is to predict whether a particular reaction is thermodynamically unfavorable and, subsequently, to remove that reaction from the options. This can be further extended into screening a large set of donors for any given reaction. This may be particularly valuable, as all that is required is to calculate the energy for each compound once. The ΔE of the reaction consists of the sum of the four individual energies from each compound in the reaction. In this way, a set of donors can be applied to any desired acceptor. For example, if we already have a set of 50 pairs calculated, by just calculating the energies of one new amine–ketone pair we immediately have the reaction energies of this pair with 50 other pairs. Upon going through these 50 energies we can immediately eliminate those reactions that are thermodynamically unfavorable and focus on those that have a potential high conversion. Consequently, we can, in a practical case, run 4–8 new pairs overnight on a standard contemporary laptop with 4 processors, and the next day we have 200–400 new predicted conversion results.

In another practical example, we illustrate this on a small scale. Most experimental work is very much dominated by the use of relatively few amino donors, although the enzyme has a relatively broad substrate specificity.[17] The donors alanine (ALA), isopropylamine (IPA), and 1-phenylethylamine (PEA) dominate the field. For example, PEA is one of the most generally accepted amino donors by ω-transaminases; it is generally thermodynamically favorable but is haunted by substrate and product inhibition, which hampers scale up to high substrate and product concentrations, as indicated above. In addition, the substrate is chiral and thus either must be used as an optically pure compound or only half of the substrate can be used. ALA, on the other hand, is easily obtained in an optically pure form but inherently suffers from poor thermodynamics. From this, we may see that using other donors can be favorable under many settings. Therefore, by using our approach to find other, thermodynamically favored amino donors would be highly beneficial. Table 1 illustrates a hypothetical case study. Four acceptors are evaluated with four suggested donors. As an example, we show that two of these donors are not good choices for the four ketones shown in the upper row of the table, whereas PEA is effective in two out of the four cases.

Table 1. Predicted (quantum mechanics) conversion values from a 1:1 acceptor/donor reaction.

Donors/acceptors				
	0.11	6.29	4.79	0.002
	4.36	74.10	68.20	0.08
	79.52	99.59	99.46	6.49
	95.85	99.93	99.91	29.22

The fourth donor, however, is predicted to give high yield for three out of the four ketones. From this we can easily eliminate reactions that will give a low conversion, and we can also evaluate for which acceptor molecules a simple and economic donor such as IPA may be sufficient. Likewise, we can find for which acceptors a high reaction yield is difficult to obtain. In such cases, we could either expand the donor selection or select the reaction with the lowest yield and run the reaction as a racemic resolution.

We proposed and validated, by application to 30 experimentally known cases, a simple, easy-to-use, and fast approach to predict whether a transaminase reaction is thermodynamically unfavorable. This allowed us to deselect, in the present case,

at least 50% of the reactions because they turned out to be thermodynamically unfavorable, as confirmed by experiment. We showed that once a larger data base is established, one can easily perform in silico screening of a dozen new reactions (new target molecule) each day and directly assess which combinations with one of all the previously calculated amines would be a suitable donor molecule for that new reaction.

Keywords: ab initio calculations · amines · biocatalysis · molecular modeling · transaminases

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Supporting Information

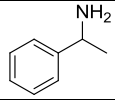
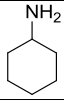
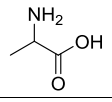
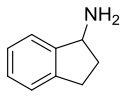
A Practical and Fast Method To Predict the Thermodynamic Preference of ω-Transaminase-Based Transformations

Robert J. Meier,*[a] Maria T. Gundersen,[b] John M. Woodley,[b] and Martin Schürmann[a]

cctc_201500347_sm_miscellaneous_information.pdf

Reaction number	Donor	Acceptor	Conversion (%)		Reference
			QM	Exp	
1	D1	A1	69.8	56.9	Gundersen[13]
2	D2	A1	14.7	24.9	Gundersen[13]
3	D1	A2	26.9	61.0	Gundersen[13]
4	D1	A3	23.2	34.2	Gundersen[13]
5	D2	A3	2.2	9.9	Gundersen[13]
6	D3	A5	0.0	1.0	Gundersen[13]
7	D1	A5	6.5	9.1	Gundersen[13]
8	D1	A6	93.0	93.6	Gundersen[13]
9	D6	A7	20.5	29.8	Gundersen[13]
10	D1	A8	36.0	41.4	Gundersen[11]
11	D1	A9	99.4	95.5	Gundersen[11]
12	D5	A9	62.8	85.0	Gundersen[11]
13	D5	A8	0.7	13.7	Tufvesson[5]
14	D1	A10	100.0	99.4	Tufvesson[12]
15	D1	A11	98.8	84.6	Tufvesson[12]
16	D6	A10	99.9	97.6	Tufvesson[12]
17	D6	A11	95.6	53.7	Tufvesson[12]
18	D6	A6	77.5	83.8	Tufvesson[12]
19	D6	A12	98.4	98.9	Tufvesson[12]
20	D3	A13	7.7	7	Meadows[15]
21	D7	A13	94.9	95	Meadows[15]
22	D1	A13	99.7	100	Meadows[15]
23	D5	A14	93.1	34.5	Fesko[14]
24	D1	A14	99.9	70.5	Fesko[14]
25	D5	A15	9.3	29.2	Fesko[14]
26	D1	A15	89.7	61.7	Fesko[14]
27	D5	A16	7.2	21.0	Fesko[14]
28	D1	A16	86.9	42.1	Fesko[14]
29	D5	A17	19.4	35.2	Fesko[14]
30	D1	A17	95.3	46.6	Fesko[14]

Structures: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	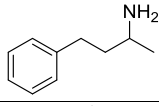	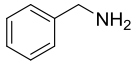	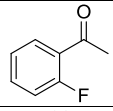
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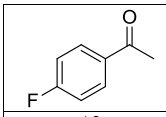	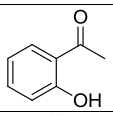	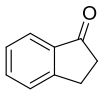	
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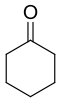	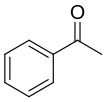	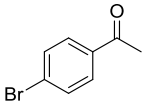	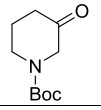
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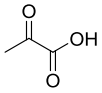		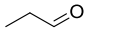	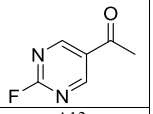
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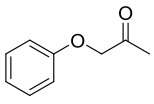	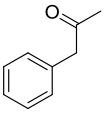	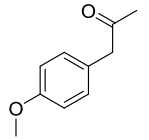	
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A Rapid Selection Procedure for Simple Commercial Implementation of ω-Transaminase Reactions

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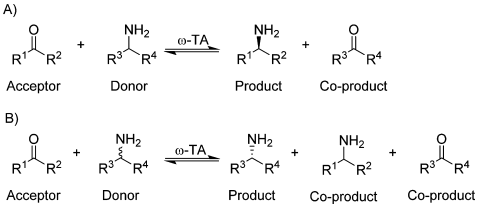
ABSTRACT: A stepwise selection procedure is presented to quickly evaluate whether a given ω-transaminase reaction is suitable for a so-called "simple" scale-up for fast industrial implementation. Here "simple" is defined as a system without the need for extensive process development or specialized equipment. The procedure may be used when investment in intensive process development cannot be justified or when rapid execution is paramount, for applications such as small singular batches. The three-step evaluation procedure consists of: (1) thermodynamic assessment, (2) biocatalyst activity screening, and (3) determination of product inhibition. The method is exemplified with experimental work focused on two products: 1-(4-bromophenyl)ethylamine and (*S*)-(+)-3-amino-1-Boc-piperidine, synthesized from their corresponding pro-chiral ketones each with two alternative amine donors, propan-2-amine, and 1-phenylethylamine. Each step of the method has a threshold value, which must be surpassed to allow "simple" implementation, helping select suitable combinations of substrates, enzymes, and donors. One reaction pair, 1-Boc-3-piperidone with propan-2-amine, met the criteria of the three-step selection procedure and was subsequently run at 25 mL scale synthesizing (*S*)-(+)-3-amino-1-Boc-piperidine at concentrations up to 75 g/L. However, the highest product yield (70%) was obtained at a lower substrate concentration of 50 g/L.

1. INTRODUCTION

Over the past decade, biocatalysis has become an established and useful complement to conventional chemical catalysis for the synthesis of fine chemicals. Most often, biocatalytic methods have been selected due to exceptional selectivity (regio- and/or enantioselectivity).[1] In fact, the majority of industrially applied biocatalytic reactions today yield optically pure chiral products that are used in the fine chemical industry as building blocks for agrochemicals and pharmaceuticals.[2] In particular, biocatalytic transamination chemistry has been identified as one of the key emerging areas for the pharmaceutical industry[1,3] as a means of producing optically pure chiral amines. This paper focuses on the biocatalytic synthesis (and resolution) of chiral amines of high optical purity using ω-transaminase (ω-TA) (E.C. 2.6.1.18), which is a type of amino transferase. ω-TA was chosen as a catalyst for this work due to its outstanding stereoselectivity and broad ketone substrate repertoire. Two ω-TA-catalyzed paths are available toward optically pure chiral amines, using either asymmetric synthesis or kinetic resolution. Although the latter is challenged by a maximum 50% yield,[4−6] both are considered as potential options for the "simple" scale-up.

ω-TA catalyzes an amino transfer reaction, illustrated in Scheme 1. Briefly, in the synthetic direction (Scheme 1A) the amino donor (an amine), and the amino acceptor (a prochiral ketone), here referred to simply as the "donor" and "acceptor", respectively, react with the enzyme in a sequential fashion producing the desired target chiral amine product and a coproduct. Detailed descriptions of the sequential ping-pong bi-bi enzymatic reaction mechanism can be found elsewhere.[7,8] In the resolution reaction (Scheme 1B) the same reaction takes place, but now the amino donor is added as a racemic mixture.

Scheme 1. Examples of Potential ω-TA Reactions Using (A) a Synthetic Route and (B) a Resolution Route



Through reaction therefore, one isomer is left unreacted, which becomes the desired optically pure product.

The amino moiety alone is transferred between the two starting substrates, and therefore in the synthetic direction, the molecular structure of the chiral product will be determined by the structure of the acceptor molecule. This means that the donor molecule can be freely chosen, since it neither affects the target product structure nor the stereoselectivity. In principle therefore, a plethora of possible donors could be chosen, although in the scientific and patent literature only a handful of amine donors have been reported. The authors have recently proposed a novel quantum mechanical method to determine the free energy of compounds and hence the thermodynamic feasibility of using novel amino donors for this reaction type, irrespective of kinetic considerations.[9] This along with a wider implementation of this technology in the future is likely to lead to a broader range of different amino donors.

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Table 1. Compounds Used in This Study[a]

Reactants	Acceptor ketones		Amino donors	
				
	1	2	3	4
Products	**Target chiral amine products**		**Co-products**	
				
	5	6	7	8

[a]Acceptor ketones: 4-bromoacetophenone (1), 1-Boc-3-piperidone (2); amino donors: propan-2-amine (3), 1-phenylethylamine (chiral) (4); target chiral amine products: 1-(4-bromophenyl)ethylamine (chiral) (5), 3-amino-1-boc-piperidine (chiral) (6); coproducts: acetone (7) and acetophenone (8).

Despite the interest in such reactions, they are often demanding to implement on an industrial scale due to frequent thermodynamic and kinetic challenges.[10] While many technical solutions are available to overcome these challenges, the proposed solutions are frequently complex and often require significant process development time. Indeed, for some applications, a fast and simple process development is not only desirable but may be essential for commercial success. In these cases, it will be more important to rapidly develop a simple process, than to obtain an economically optimal process. Such situations include pharmaceutical synthesis in the early phases of clinical testing and other cases such as small singular batches, where investment in extensive process development cannot be justified. Using this logic, and from a knowledge of the properties of a given ω-transaminase-catalyzed reaction and the available enzymes to catalyze the reaction, we reasoned that it should be possible to categorize a particular reaction as "complex" (requiring extensive development) or "simple" (with easy implementation and scale-up). We therefore suggest that an evaluation method allowing the identification and selection of "simple" reactions (and eliminating the "complex" ones) would prove a valuable tool for process chemists.

The scope of this manuscript is therefore to present a stepwise decision-making procedure to quickly identify if a "simple" scale-up is feasible for a given reaction. Hence, solutions such as biocatalyst modification by protein engineering,[10] amino donor recycling,[11,12] and equilibrium shifting methods[13,14] have not been considered here. The three-step decision-making procedure involves an evaluation of: (1) thermodynamics, (2) biocatalyst activity, and (3) product inhibition. Each step is evaluated against a threshold value, which must be met in order to identify a given case as suitable for "simple" implementation.

2. RESULTS

In order to exemplify the method, experimental data on two chiral target products were evaluated, 1-(4-bromophenyl)-ethylamine (**5**) and (*S*)-(+)-3-amino-1-Boc-piperidine (**6**). These compounds were selected because both products are commercially attractive and additionally biocatalytic transaminations to synthesize both **5**[15] and **6**[11,16] have been reported previously. In these reactions optical purity was necessary and evaluated, but a specific stereoisomer was not required. The prochiral ketone substrates, the amino acceptors, 4-bromoacetophenone (**1**) and 1-Boc-3-piperidone (**2**), corresponding to the products above, were reacted with two possible donor molecules propan-2-amine (**3**) and 1-phenylethylamine (**4**) (Table 1). Both amino donors have frequently been used in a wide variety of biocatalytic transamination. Between them, they represent different classes of donor. For instance, donor **3** serves as an inexpensive achiral donor. In contrast amino donor **4** is a more costly chiral compound which has also been reported to be inhibitory[17] with downstream processing complications due to separation issues when the product shares structural similarity. However, donor **4** also offers a significant thermodynamic advantage, since the carbonyl coproduct, acetophenone (**8**) formation is highly favorable (Table 1). Academically, a more common amino donor that has often been reported is the use of alanine (or pyruvate for the resolution reaction).[4,18] We have previously shown that the thermodynamics using this donor very strongly favors the reverse resolution reaction,[19] and therefore we have not considered this further in this work.

2.1. Method Development. In order to enable rapid evaluation, the three selection criteria are each assigned a threshold value, which must be met to enable implementation of a simple scale-up. The proposed procedure is outlined in Figure 1. In the figure, full lines indicate that the reaction has met (green lines) or failed (red lines) the individual criteria. Likewise, dashed lines indicate an alternative strategy by

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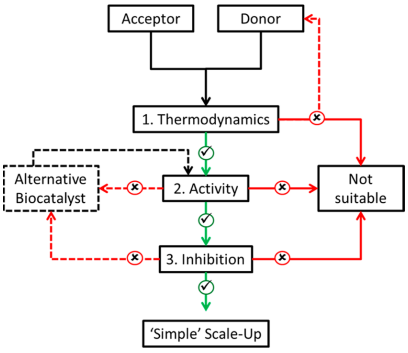

Figure 1. Decision making procedure for a simple scale-up. Green lines marked with a check mark or red lines marked with an X indicate if a given criterion is met or not met, respectively. Dashed lines and boxes indicate options for reassessment if a criterion is not met. Each criteria has cut off values for simple implementation. 1. The thermodynamic criteria is meet when K_{eq} is less than 0.02 (resolution reactions) or greater than 1 (synthetic direction). 2. The activity criterion requires a specific activity greater than 0.05 g/g/h. 3. The inhibition criteria is met at less than 50% activity loss, with 5% of target concentration product present. Possible remediation options, if a given criterion for a simple scale-up is not met, can be to consider an alternate amino donor or test an alternative biocatalyst (dashed lines).

adjusting one of the variable reaction components (the amino donor or the biocatalyst). The threshold values for each criterion are also indicated in the legend of Figure 1, the justification for which is given in the following section.

2.1.1. Thermodynamic Assessment. Unfavorable thermodynamics presents one of the main barriers to the implementation of the transaminase−catalyzed reactions on an industrial scale.[4] The thermodynamic equilibrium constant (K_{eq}) of the reaction is important since it determines the maximum reaction yield for a given concentration of substrates. Thus, we reasoned it is one of the most important parameters for determining the optimal process configuration.[6,13,20] For this reason, we suggest the first step in the procedure should be to determine if a candidate reaction has a suitable thermodynamic equilibrium constant to make a "simple" scale-up feasible.

In the synthetic mode, thermodynamic feasibility is here defined as a K_{eq} above 1.0, since lower values of K_{eq} would require a high excess (more than 20-fold) of the amino donor to obtain sufficient reaction yields (95% or higher), for eventual industrial implementation. Use of such an excess makes the reaction costly and practically difficult to carry out at high substrate concentrations. In a similar way, we reasoned that for reactions with a low K_{eq}, a kinetic resolution would be a better choice for the reaction. On the other hand, the resolution reaction requires more stringent conversion requirements since the separation of the amine product from the unreacted half of the racemic donor starting material is of course quite challenging. Hence we have chosen a K_{eq} threshold of 0.02 in the resolution direction, meaning only values lower than this are suitable for a simple scale-up.

In this work, the concentration-based equilibrium constant was experimentally determined using a previously described method.[20] Since the value is obtained for comparative purposes, practical (rather than standard) conditions were used, meaning it is more accurate to describe the constant as "apparent", K_{eq}^{app}. In principle to save time as an alternative to experimental measurement, in silico methods could be used to estimate such values, although the accuracy is perhaps questionable. Here the K_{eq}^{app} for the two chiral amine products **5** and **6** were measured experimentally using the two donors **3** and **4**, as described above. The K_{eq}^{app} for the four reactions (Table 2) varied by a factor of 10^4 from the most challenging

Table 2. Experimental Values for K_{eq}^{ap}

	donors	
acceptors	3	4
1	0.025[a]	0.5
2	32	450

[a]Data previously reported in ref 13.

pair, **1** and **3**, at 0.025 to the most favorable pair, **2** and **4**, which had a K_{eq}^{app} of 450, in the synthetic direction. Thus, thermodynamics is indeed highly variable between the four selected reaction pairs. After applying the threshold criteria one of the two products, **5**, was eliminated from further investigation. This may indicate that highly conjugated aryl compounds are not suitable for simple scale up and should be assisted by other process technologies and strategies. For example, it has been reported that one of the compounds we have used as a donor here **4**, could also synthesized and successfully scaled in combination with in situ product removal, alleviating both the thermodynamic and inhibitory strains.[14] None of the reaction pairs evaluated here was found suitable for the resolution reaction, although alanine, the amine donor often found most suited for resolutions reactions was not tested as discussed previously.[19]

Clearly it is possible to carry forward more than one amine donor to the subsequent evaluation steps, although this is not helpful for the procedure, which aims to focus effort on those cases with the biggest chance of simple scale-up success. In this case, due to the low cost and high water solubility, amine donor **3** was selected for further evaluation.

2.1.2. Biocatalyst Activity Screening. No matter how favorable the thermodynamics, without sufficient activity the reaction will not be completed in a reasonable time, and issues like enzyme inactivation may arise. Hence, the next step of the procedure is to find a suitable biocatalyst with sufficient activity. Candidates for biocatalyst screening can be obtained from commercial screening kits or in-house enzymes. For the "simple" scale-up, strategies such as protein engineering are not considered. Low activity of an enzyme preparation will negatively impact downstream processing, by adding extra proteinaceous material which impedes product recovery. Therefore, the maximum biocatalyst loading was set to 10% v/v irrespective of the biocatalyst formulation. Additionally, product concentration should be in the range of ≥50 g/L[6,10] to assist downstream product recovery. Finally, due to biocatalyst stability concerns, we reasoned it necessary to complete the reaction within 96 h. On this basis, we calculated a minimal biocatalyst specific activity (sometimes termed "biocatalyst productivity"), as a threshold value for the "simple" of 0.05 g/g/h (g product/g biocatalyst/hour).

For this case study a small screen with four enzymes was conducted, using the reactant pair **2** and **3** selected from the previous section. In this screen four selected enzymes were tested, two of which were known to be (*R*)-selective and two

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(*S*)-selective. We reasoned that for this case study the particular stereoselectivity of the enzyme did not influence the overall procedure. Additionally, since this screen was conducted with an achiral amine donor and the pro-chiral ketone, the selectivity of the enzyme would not affect the reactivity with these substrates. The screen showed a large variation between the least and most reactive candidates (Table 3). Details of the

Table 3. Specific Activities Obtained with Reactant Pair 2 and 3, with Selected Enzymes

enzyme	selectivity	specific activity (g/g/h)
Ars-ωTA	S	0.048
Tar0	R	0.003
Tar1	R	0.012
ATA 47	S	0.054

individual enzymes (ATA-47, Tar0, Tar1, and Ars-ωTA) are given in the experimental section of the paper. Tar0 was found to give a specific activity of 0.003 g/g/h, whereas the best candidate (ATA-47) gave a 20-fold higher value of 0.054 g/g/h. ATA-47 was therefore carried to the next step. Likewise the enzyme Ars-ωTA had a high specific activity of 0.048 g/g/h, close to the threshold value.

2.1.3. Determination of Product Inhibition. The final step of the procedure considers product inhibition of the enzyme, which due to the requirement for high product concentrations (50 g/L) in industrial processes,[2] is a frequent hurdle for process intensification of enzyme reactions in general, and ω-TAs in particular.[14] Hence, we set the threshold value here at a 50% reduction in reaction rate in the presence of 2.5 g/L product, under the assay conditions used here (see Experimental Section). Here only product inhibition is assessed, since substrate inhibition can relatively easily be overcome by substrate feeding.

In order to experimentally test for product inhibition, the initial reaction rate of ATA-47 was measured using 100 mM **3** and 10 mM **2**, in the presence of various concentrations of the product **6**, up to 10 mM. Importantly, the substrate concentrations were chosen to avoid limiting the reaction by thermodynamic constraints. Inhibition was observed with 10 mM product and amounted to a 10% initial rate reduction, compared to initial conversion rates in the absence of product. Initial conversion rates were assumed when less than 10% of limiting starting material was converted.

2.1.4. Discussion. First, with respect to thermodynamics, the procedure enables the elimination of unfavorable cases. Clearly each donor or acceptor molecule has an associated free energy which contributes to the net thermodynamics of a given reaction. In this way for instance a comparison of the equilibrium constants of two reactions (with different acceptors, but using the same donor) can be used to interpret the effect of changing acceptors. In an analogous way, one could determine the K_{eq} for a given acceptor with one donor and extrapolate the K_{eq} to other donors with the same acceptor, given one knows the difference in ΔG between the reactions, as discussed elsewhere.[19]

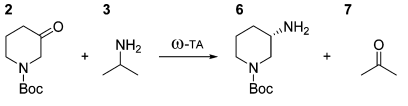
Second, the biocatalyst activity is assessed, since low activity will have drawbacks in the form of low space-time yields and may prevent the reaction from going to completion due to enzyme deactivation. One solution would be to apply high biocatalyst concentration, but these may negatively impact downstream processing by hindering product recovery. Thus, the threshold for the enzyme is defined as minimum specific activity, which for the "simple scale-up" was set at 0.05 g/g/h. Biocatalyst recycle was not considered for the simple scale-up.

Finally a determination of product inhibition is carried out. This is a frequent hurdle for process intensification of ω-TA's,[14] due to the high product concentrations (50 g/L) required to simplify the product recovery.[2] In contrast to the high concentration intensity of commercial processes, enzymes are designed to work under physiological (dilute) conditions. This frequently leads to process intensification challenges with biocatalytic reactions. For example transaminases display a ping-pong bi-bi reaction mechanism, with two sequential half reactions,[8] and this type of reaction mechanism is often plagued by inhibition from competitive dead-end complexes of products bound to the *apo*-enzyme or the incorrect form of the *holo*-enzyme. Hence, understanding the inhibition profile of a potential product is vital in evaluating the possibility of a simple scale-up. As such, we advocate that, if severe inhibitory effects are observed with low product concentrations, it implies a high risk of inhibition under process scale concentrations.

The three-step evaluation method has been successfully applied to a case study, and one reaction pair with one biocatalyst was deemed suitable for "simple" scale-up.

2.2. Intensification and Scale-Up. In the previous sections, the selection procedure for a simple scale-up toward the synthesis of **6** identified acceptor **2** with donor **3** (Scheme 2) using ATA-47 as suitable. In the event ATA-47 was

Scheme 2. Synthetic Transaminase Reaction Carried Out[a]



[a]Compounds: 1-Boc-3-piperidone (**2**), propan-2-amine (**3**), (*S*)-(+)-3-amino-1-Boc-piperidine (*S*)-(+)-**6**), acetone (**7**).

substituted by ArS-ωTA since the difference in activity was negligable and the latter enzyme has been reported to have excellent stereoselectivity.[21,22]

2.2.1. Reaction Optimization: pH and Donor Loading. Prior to scale-up, a small optimization study was undertaken to evaluate if reaction rates could be enhanced by simple optimization within the biocatalyst stability range. A range of pH and donor loadings was explored in an attempt to improve kinetics, with both short (0.5−2 h) and long (18 h) reaction times; the latter time point was chosen to investigate enzyme stability under the given conditions.

The rate dependency on pH was tested between pH 7 and 9, with 40 mM acceptor and 500 mM donor (Figure 2). Other studies have found up to 40% variation in yield in this pH range for similar reactions.[23] Here the fastest reaction rates were identified at pH 9 for all time points. The greatest difference was found in the 18 h reaction times, where average reaction rates are 45% faster at pH 9 compared with pH 7, indicating that this is the best pH, within the pH range tested, with respect to kinetics, and that the enzyme is more stable under these conditions. Since the pK_a of the amine donor **3** is 10.6,[24] meaning a higher pH would render a higher fraction of the substrate uncharged and thus reactive, in principle operating at a higher pH would therefore be beneficial from the perspective

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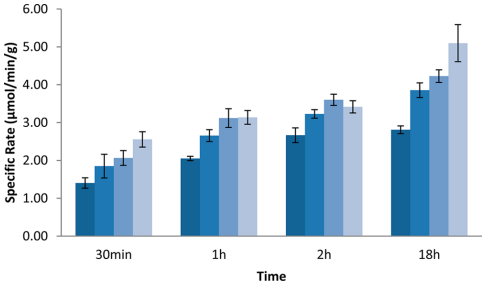

Figure 2. Specific rates measured at four reaction time points at four different reactions, each carried out at different pH values (pH 7.0, pH 7.5, pH 8, and pH 9 from left to right at each time point, respectively).

of the reaction rate. Nevertheless, in this study we limited the pH range to keep the study simple and manageable, consistent with the philosophy of this work, and therefore did not test the reaction at higher pH values than 9.

Furthermore, the same method was used to determine optimal donor loading. Donor concentrations could potentially be limiting, dependent upon K_M[12]. Clearly an excess concentration of the donor (over acceptor) could be used which might also drive the equilibrium.[23,25] This was tested experimentally but at all concentrations tested, the rate was unaffected by donor concentration (Figure 3), suggesting a K_M beneath 100 mM. For subsequent experiments 1 M **3** was used.

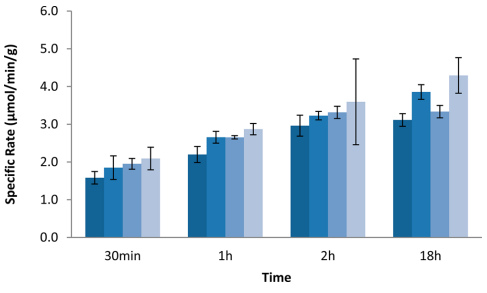

Figure 3. Specific rates found at four time intervals at four donor concentrations (0.1, 0.5, 1, and 2 M from left to right at each time point, respectively) used to investigate the optimal donor loading for the reaction.

2.2.2. Reaction Intensification. As indicated above a viable scale-up depends on reaction intensification (i.e., the synthesis of high product concentrations).[10] This is important in the simple scale-up because too low a concentration will add volume to the reaction and thus complicate the process. The reaction of **2** and **3** using Ars-ωTA was therefore intensified by increasing the substrate concentration up to 75 g/L. Three reactions were done in scintillation vials at concentrations of 25, 50, and 75 g/L. The reactions proceeded smoothly (Figure 4) at both 25 g/L and 50 g/L but not at 75 g/L, the latter most likely due to mass transfer limitations from low solubility and decomposition of the starting material in aqueous conditions. The latter was further investigated and confirmed (data not shown). To the best of our knowledge no other study has

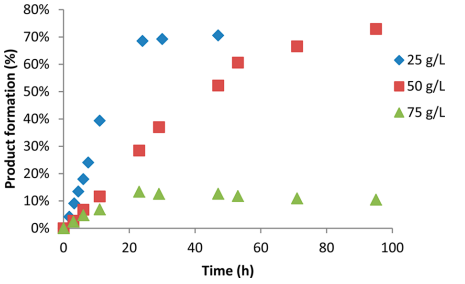

Figure 4. Reaction profile over 96 h with initial substrate concentrations of 25, 50, and 75 g/L.

investigated the stability of this compound in water, either for biocatalysis[11] or chemical catalysis. In the 25 and 50 g/L reactions final conversions of acceptor **2** to chiral amine target **6** of 70% were observed. Figure 5 shows that the initial reaction rates are similar at all substrate concentrations tested, indicating that the reaction is not kinetically controlled (above K_M).

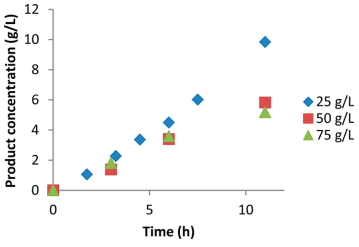

Figure 5. Initial product formation for the first 12 h of the reaction with initial substrate concentrations of 25, 50, and 75 g/L.

2.2.3. Product Identification. Finally, the reaction was run at 25 mL scale for 96 h to isolate product. At 50 g/L substrate concentration the final reaction composition was analyzed to contain 89% **6** and 11% **2** (with an isolated product yield of around 70%). This composition is in excellent agreement with that found in the 50 g/L 1 mL scintillation vial experiment, which gave 91% target chiral amine **6** and 9% ketone **3**, after 96 h.

3. CONCLUSION

A simple stepwise procedure has been described, to facilitate the selection of suitable substrate-donor-enzyme combinations to allow so-called "simple" scale-up. Each step in the procedure has a threshold value which must be met to allow simple implementation. We believe that this method will prove useful both to select good candidates for this technology and to eliminate those that may require further development. A simple case study was used to illustrate the power of the procedure, sequentially eliminating unsuitable substrates, donors, and enzymes. Beyond this case study, we furthermore suggest that analogous procedures could be used for the evaluation of other "simple" biocatalytic processes.

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4. EXPERIMENTAL SECTION

4.1. Materials. Three plasmids encoding the enzymes, Tar0, Tar1, and Ars-ωTA, were kindly provided by Professor NJ Turner (University of Manchester, Manchester, UK). Tar0 encoded the ω-transaminase from *Arthrobacter* sp. KNK168 (Sequence 2 from US 7169592) inserted between the *Nde* I and *Xho* I (with C-terminal His tag) site of pET21a (Accession number ABN35871). Tar1 encoded ω-transaminase from *Arthrobacter* sp. KNK168 (Sequence 110 from US 8293507, Tar1) inserted between the *Nde* 1 and *Xho* 1 (with N-terminal His tag) site of the pET16b (Accession number AFX11601). Ars-ωTA encoded mutated ω-transaminase from *Arthrobacter citreus* (Sequence 16 from US 7172885,) inserted between the *Nde* 1 and *Xho* 1 (with C-terminal His tag) sites of the pET21a (Accession number ABN37907). The commercial enzyme ATA-47 (30902-2; activity 0.41 U/mg; batch LH1-01-02) was purchased from c-LEcta GmbH (Leipzig, Germany).

Deionized water (18 Ω) was used for all experiments. All chemicals where purchased from chemical vendors at reagent grade or higher and used without modification. GC and NMR solvents were of analytical grade, and products used for the enzyme expression were of biological grade.

4.2. Methods. *4.2.1. Enzyme Expression. Section 2.2.1.* The plasmid that encodes Ars-ωTA with its *C*-terminal hexahistidine tag, was transformed into *E. coli* BL21 (DE3) (Novagen from Merck KGaA, Darmstadt, Germany), using standard procedures,[26] and maintained with 100 μg/mL ampicillin. Briefly, Ars-ωTA was expressed in autoinducing medium as follows: a 1% glycerol stock inoculum was used to inoculate 400 mL of ZYP-5052 medium.[27] The culture was incubated at 37 °C, shaking at 250 rpm for 24 h, in a Sartorius Stedim CERTOMAT BS-1. The culture was centrifuged at 8000 rpm (Beckman Coulter Avanti J-26S XP centrifuge, JLA 8.1 rotor) at 4 °C for 30 min, the supernatant were decanted and the cell pellet stored at −20 °C. The average yield was 9 g pellet mass per liter of culture.

Sections 2.1.2, 2.2.2, and 2.2.3. A sample of 1 mL of *E. coli* BL21 (DE3) expressing Ars-ωTA enzyme was inoculated in 50 mL vegetable peptone broth with 20 g/L glucose and 15 μg/mL kanamycin and cultivated for 6−7 h at 37 °C, 250 rpm in a rotary shaker incubator. This preculture was used to seed two 1 L fermentation vessels. Both fermenters were run with the same fermentation procedure, which consisted of culturing the cells in a sugar free semidefined base medium, controlled at pH 7.2, 30 °C, 20% dissolved oxygen, and feeding at a predefined linear rate with base medium containing 400 g/L glucose from the point of inoculation. The culture was induced when 100 OD_{600} was reached by the addition of 0.5 mM IPTG final concentration and reduction in culture temperature to 25 °C. Following 24 h elapsed fermentation time the feed rate was reduced to a predefined constant rate until cell harvest at 41 h. The final cell population reached 212 and 192 OD_{600}, 80 and 81 g/L dry cell weight, respectively. A portion of 1 L of fermentation broth from each reactor was harvested by centrifugation at 6000 g for 35 min; the supernatant was discarded, and the pellet was frozen at −80 °C.

Enzyme Purification. *E. coli* cells (25 g) expressing Ars-ωTA were added to 250 mL 0.1 M phosphate buffer (pH 7) and sonication at 2 °C. The lysate was concentrated by ammonium sulfate to 50−60% ammonium sulfate fraction. The precipitate was resuspended in 25 mL 0.1 M phosphate buffer with 30 mM imidazole, and purified using a His-Trap (Ni-NTA), Ars-ωTA was eluted with 500 mM imidazole. The purified enzyme solution was exchanged into phosphate buffer (0.1 M, pH 7) using an Amicon Ultra 15 Centrifugal filter (10 k) unit and SDS-PAGE analysis performed to confirm that the Ars-ωTA had been purified (>85%) and concentrated (35 mg/mL).

4.2.2. Reaction Conditions. Sections 2.1.1 and 2.1.3. Each reaction, performed in duplicate, contained: 1 g L^{-1} ATA-47, 2 mM PLP, 5% DMSO, 0.1 M tris-HCl buffer pH 7.5, and up to 10 mM pro-chiral ketone acceptor **2** or 1.5 mM pro-chiral ketone acceptor **1** together with 10 mM amino donor **4** or 100 mM **3**, and was run for up to 48 h in 4 mL reaction vessels, at 30 °C in a thermos-shaker. K_{eq} values were determined by measuring conversion at varying concentrations of substrates and products according to a previously described protocol.[20] Inhibition studies were made by measuring initial rates (less than 10% of the limiting substrate consumed), in the presence of increasing product.

Section 2.2.1. All samples were carried out in 0.5 mL reactions in a 96 well plate format. Short reactions of 30 min, 1 h, and 2 h were run with 20 g/L lyophilized cells of Ars-ωTA; the 18 h reactions were run with 2 g/L lyophilized cells. All reactions were run with 10 g/L acceptor **2** in 0.1 M Tris-HCl buffer. The pH optimum was tested at pH's 7, 7.5, 8, and 9, with 0.5 M donor **3**. Donor optimization was tested with concentrations of donor **3** of 0.1, 0.5, 1, and 2 M, carried out at pH 7.5. All experiments were carried out in triplicate.

Section 2.2.2. Reactions were done at 1 mL scale in a 96 well plate, with 11 identical reactions per substrate concentration. The reaction contained 0.1 M Tris-HCl buffer pH 9.0 with 0.4 g/L purified Ars-ωTA, 0.5 M donor **3**, 0.1 g/L PLP, 5−10% DMSO, and 25, 50, or 75 g/L pro-chiral acceptor **2**. The reaction was agitated 250 rpm at 25 °C. Samples were taken at regular time points throughout the experiment.

Section 2.2.3. Reactions were performed at 25 mL scale in an Easymax vessel, which was stirred at 400 rpm, maintained at 25 °C, with substrate concentrations of 25 and 50 g/L. Otherwise the composition in the reactor was identical to that described in section 2.2.2.

Section: 2.1.2. Experiments were carried out by resuspending 500 mg wet cells in 4.75 mL of 500 mM **3** hydrochloride, 50 mM potassium phosphate buffer pH 7.0, and shaking in an orbital shaker at 250 rpm, maintained at 30 °C, for 30 min. 0.25 mL of a 200 g/L solution of pro-chiral ketone acceptor **2** in DMSO was added and the reaction returned to the shaker for 18 h. Reactions were analyzed by GC. The activity for ATA-47 was extrapolated from rates measured in the experiments carried out as described in sections 2.1.1 and 2.1.3.

4.2.3. Product Isolation. Section 2.2.3. The pH of the reaction was adjusted to 13 with 5 M NaOH and extracted with MTBE (3 × 20 mL). The combined organic extracts were filtered through Celite to remove emulsion and dried ($MgSO_4$), filtered, and concentrated in vacuo. Being volatile, the excess amine donor **3** was removed with the organic solvents during concentration.

4.2.4. Work up of Samples for Analysis. Sections 2.1.1 and 2.1.3. The analytical samples were prepared as follows; 0.1 mL of sample was added to 0.4 mL of 1 M NaOH with 10 mM dibenzyl ether as external standard. The compounds were extracted with 0.3 mL of MTBE, and the organic layer was dried with anhydrous $MgSO_4$, which was removed by centrifugation.

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Section 2.2.1. The sample was mixed for indicated time at 700 rpm, 30 °C. Samples were sacrificed by addition of 0.5 mL MeCN and spun down. 0.2 mL of the supernatant was transferred to a new plate with 0.8 mL of MeCN and $MgSO_4$. Finally 0.5 mL was transferred to an analysis plate and derivatized with 15 μL of Et_3N and 10 μL of Ac_2O, preceding analysis.

Sections 2.1.2 and 2.2.2. A 1 mL reaction was mixed thoroughly with 9.0 mL MeCN containing 4.5 mg/mL dibenzyl ether. 1.0 mL of this mixture was put in a GC vial and derivatized with 30 μL of Et_3N/20 μL of Ac_2O prior to GC analysis (Chiraldex Dex-CB column 25 m × 0.25 mm × 0.25 μm, oven temp 170 °C for 15 min, Carrier He @ 20 psi, injector/detector 200 °C). Quenched 50 g/L and 75 g/L reactions were further diluted 1:1 with MeCN prior to derivatization and analysis.

Section 2.2.3. 100 μL of the reaction was removed and diluted with MeCN (900 μL). This mixture was derivatized with 30 μL of Et_3N/20 μL of Ac_2O and analyzed by GC.

4.2.5. Analytical. Sections 2.1.1 and 2.1.3. All analytical work was carried out with gas chromatography, with a PerkinElmer (Santa Clara, CA, USA) Clarus 500 apparatus, with PerkinElmer Elite-5 column. 1 μL was injected with a 30:1 split ratio and ran with a constant flow rate of 1.6 mL min^{-1} helium with a temperature gradient from at 120 to 230 °C.

Sections 2.1.2, 2.2.1, 2.2.2, and 2.2.3. Chiraldex Dex-CB column 25 m × 0.25 mm × 0.25 μm, oven temp. 170 °C for 15 min, carrier He @ 20 psi, injector/detector 200 °C.

4.3. ^{1}H NMR of Isolated Product. *N*-Boc-3-amino-piperidine ^{1}H NMR (400 MHz, CD_3OD) 3.96 (1H, m), 3.82 (1H, m), 2.78 (1H, br), 2.67 (1H, m), 2.60 (1H, br s), 1.91 (1H, m), 1.67 (1H, m), 1.44 (9H, s), 1.40 (1H, m), 1.26 (1H, m).

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Notes

The authors declare no competing financial interest.

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Enzymatically Assisted CO_2 Removal from Flue-Gas

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Abstract

The enzyme carbonic anhydrase is an enzyme known to enhance CO_2 absorption rates. However, for economic viability in enzyme based absorption technology long term stability under process relevant conditions is needed. Thus, here enzyme stability for extended times are investigated with respect to pH, temperature and solvent. Temperatures and pH stability were tested for up to 100 hours incubation and the enzyme was temperature stable up to 60 °C and in the pH range from 7 to 11, with some residual activity between pH 5 and 12. Furthermore, enzyme stability was tested for 7 different capture solvents for 150 days, at 1 M or 3 M solvent concentrations, 40 °C and pH between 8-9 and 10. Residual activity was found with all samples ranging from 12 to 91 % of the initial activity. This study show that this enzyme can indeed be used for extended periods in process relevant conditions, and thus shows promise for industrial implementation as a catalyst in carbon capture.

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Keywords: Carbonic anhydrase; post combustion carbon capture, biocatalysis

1. Introduction

The enzyme carbonic anhydrase (CA) (EC 4.2.1.1) catalyzes CO_2 fixation in nature, by hydrating CO_2 to bicarbonate (Figure 1). The reaction is catalyzed by a divalent zinc ion in the active site of the enzyme. CA is one of the fastest enzymatic reactions known, with reaction rates up to 10^6 s^{-1}[1]. The enzyme originates from a number of different sources and it has developed several times through convergent evolution. In fact five different enzyme

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classes which display little resemblance to each other, save the dependence on the zinc ion, are currently known[1]. Early research on the enzyme was focused on mammalian sources of the enzyme [2,3]. However, for industrial applications, such as carbon capture, today much focus has shifted towards microbial sources especially enzymes from thermophiles which often yield higher stability[1].

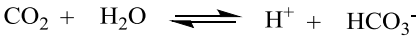

Figure 1. Carbonic anhydrase hydrolysis of carbon dioxide

To enable industrial implementation of CA in PCCC, it is vital that the enzyme is both stable and kinetically active under operating conditions. A recent review of thermostable enzymes[1] highlights the advances made in this field. However, in this study we take a holistic view of the process where a developmental enzyme supplied by Novozymes, is evaluated both in terms of temperature, pH and solvent loading stability for extended periods of time. All these factors will have a cumulative impact on enzyme deactivation during an actual process, making it important to understand the combined effects. It is important to note that the formulation of the enzyme may vary, to be used either as free enzymes, immobilized enzymes or particles, which all add benefits and drawbacks, not discussed here. Nonetheless, it is clear that the volume of CO_2 from an average power plant demands large capture equipment, thus any change in the capture set up, such as changing the solvent or enzyme packing will involve large capital and labor costs, and should therefore be avoided for as long as possible. Thus the enzymes used in such systems must be stable for long periods of time under operating conditions. In addition enzymes can only account for a small fraction of the overall cost of the capture process, since the cost of the technology must compete with current carbon taxes. The total cost of the process will also encompass solvents, stripping, compression and storage costs. To that extent a supplier which can deliver large quantities of enzymes at low costs, like we find in applications such as detergents and textile industry is needed for an economically viable process.

Nomenclature

AIB	2-Aminoisobutyric acid
AMP	2-Amino-2-methyl-1-propanol
CA	Carbonic anhydrase (Enzyme)
MAPA	3-(Methylamino)propylamine
MDEA	N-Methyldiethanolamine
MEA	Monoethanolamine
PCCC	post-combustion carbon capture
PNP	4-nitrophenyl acetate

2. Experimental

2.1. Materials

A developmental carbonic anhydrase of microbial origin was supplied by Novozymes A/S, Denmark in the form of a cell-free brown liquid. All other materials used were of reagent quality and purchased from chemical vendors.

2.2. Methods

2.2.1. Sample preparation

pH samples was prepared with a 0.2 M boric acid, 0.05 M citric acid and 0.1 M tertiary sodium phosphate complex buffer[4] suitable for the entire range of pH's tested.

Temperature tests were conducted with 0.1 M Tris-HCl buffer, pH 7.6, a Good buffer, which has a small pH change with temperature change.

Solvent solution was prepared by adding 1 M or 3 M in MilliQ water, and the pH adjusted by bubbling in CO_2 gas or by 6 M HCl solution.

Three separate vials of 3 mL solution were prepared for each sample described above, 2 % enzyme solution was added to two of the vials and the third was kept as a blank. The samples were then incubated as descried in the results section in a thermoshaker, at 450 rpm.

2.2.2 Activity assay

Activity was measured by a modified assay by Chirică and colleagues[5], Figure 2. Each sample was tested by adding 0.1 M Tris-HCl buffer, pH 7.6, in a plastic cuvette, 10 µL sample or blank was added, the sample was then left to equilibrate for at least 1 hour, and then 10 µL para-nitro-phenyl (PNP) solution (54.3 mg PNP in 3 mL acetonitrile) was added. The samples were mixed by inverting samples covered with parafilm twice. Each sample set ran in parallel contained two blank samples and three reaction samples. Absorption was recorded at 348 nM for 320 s, and the activity was determined from the slope of the absorption between 60 s and 300 s, subtracting the slope of the blank samples. The samples containing MEA and MAPA/MDEA had a high background absorption, thus the sample preparation was modified: 0.05 mL sample or blank was added to 0.45 mL 0.1 M Tris-HCl buffer, pH 7.6, in centricon vial 10 kDa cut off, spun down at 14,000 rpm for 10 min, 0.5 mL new buffer was added, the process was repeated 3 times. 0.1 mL of this sample was added to 0.9 mL buffer and the procedure was followed as indicated above.

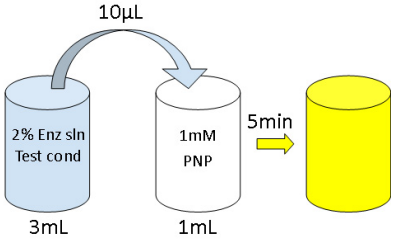

Figure 2. Experimental set up. 2 % CA solution in 3 mL test condition. 10 µL sample transferred to a 1 mM *p*-nitrophenyl (PNP) solution, color change (348 nm) detected over 5 min. All activities are % residual activity compared to initial activity under identical conditions

3. Results and Discussion

The study here evaluates the applicability of enzymes in PCCC. Enzyme stability was evaluated using an activated ester assay; PNP. Which measures the hydrolysis on the ester; this induces a color change. It should however be noted that the activity on this substrate may not accurately correspond to CO_2 hydrolysis rates. For this reason we do not report the activity values nor do we compare reaction rates between different samples in this study, with different pH's or solvents. Thus, here only the residual activity of the enzyme, under identical conditions, where background activity is thoroughly controlled for, is measured. In a PCCC application the conditions over the course of the process will vary with respect to temperature and pH (the pH drops as CO_2 is absorped in the solvent), in addition the solvent can both be varied in type and concentration. We therefore examine these three variables singularly and finally we compare the additive effects of the parameters, illustrated in Figure 3.

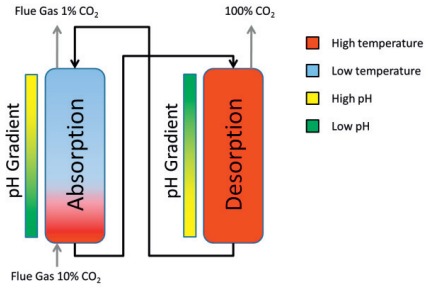

Figure 3. Example of varying pH and temperatures of the solvent in a typical post combustion solvent based capture process.

3.1 pH stability

pH stability was evaluated by measuring residual activity after 100 hours incubation at the respective pH at room temperature in the pH range from 4 to 12, with increments of 1 pH unit. Where only the pH values above pH 7 are initially interesting for PCCC applications, since below this pH the hydration rate of the reaction is strongly reduced[6]. Results are shown in Figure 4. No activity was detected after 100 hours at pH 4 and moderate activity (45-70 %) with pH's 5, 6 and 12. The enzyme proved stable in the pH range from 7 to 11 after 100 hours incubation. This defines the operating space for enzymes, and furthermore the maximal and minimal loading of any given solvent. Dependent on the solvent chosen this will also determine the maximum and minimum CO_2 loading used, since the pH of the solvent will depend on the loading.

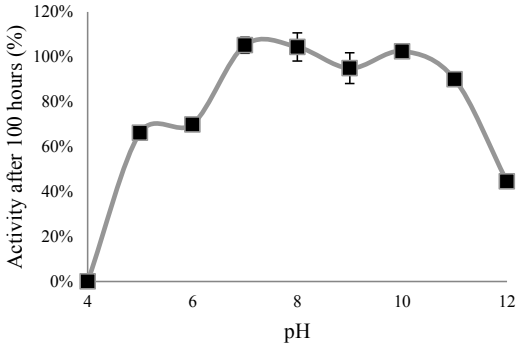

Figure 4. Residual activity after 100hours of incubation at pH 4 to 12

3.2 Temperature stability

Temperature stability was evaluated in a range from 50-80 °C, and the enzyme was found to have residual activity for at temperatures for up to 48 hours (Table 1), but the activity was drastically reduced at higher temperatures. In addition some experiments were made to recover activity from the high temperature experiments (70 °C and 80 °C) by incubating the samples at lower temperatures for some time before measuring the sample again (data not shown). From this up to 10 % activity was regained. After 48 and 72 hours no activity remained from the temperatures 80 °C and 70 °C, respectively. It should be noted that the accuracy of these values may be questionable as the activity is increasing over time with the 50 °C sample; this is likely due to the increased internal energy of the enzyme at the higher temperature, which in turn results in higher reaction kinetics. This may be an artifact stemming from that the activity assay was taken too soon after the enzyme was taken from the incubation temperature. This was corrected in later experiments below. Furthermore, this may suggest that some of the other residual activity values may be lower than the reported values. Nonetheless, the results give an indication that the enzyme is stable under operating conditions. The results correlate well with results found with other engineered CA's [1], which have been found to be stable at high temperatures. For example Codexis has previously engineered a thermostable enzyme, which retained 40 % activity after 40 hours incubation at 75 °C, comparable to what is found here[1]. In addition another engineered CA was found to retain up to 54 % residual activity after 2 hours incubation at 80 °C[7]. Thus, our results are comparable to the former results, indicating that the enzyme is stable at temperatures up to 60 °C for extended periods of time, although these results demonstrate that if the enzyme is to be exposed to temperatures above 70 °C, this must be limited to short periods of time. In practical terms this means that this enzyme is unlikely to survive a treatment in a reboiler for example.

Table 1. Residual activity after incubation at temperatures from 50 to 80 °C for up to 100 hours. All results are given in % residual activity

	Time (hours)				
T (°C)	1.5	25	48	72	100
50	105	121	132	196	280
60	84	87	64	66	74
70	47	41	49	36	0
80	7	9	2	0	0

3.3 Long term solvents stability

Here we also present a long term stability study, tested for 150 days, which is to the best of our knowledge the longest stability test under process relevant conditions for PCCC to date. The study was undertaken at two pH values and 7 different solvents, either using 1 M or 3 M concentrations. This was done in order to evaluate if the enzyme could be used long-term under operating conditions. The results show that the enzyme was highly stable for extended periods of time. The solvents tested were chosen as they were previously proven to be useful in CO_2 capture. Specifically the primary amine solvent MEA as it has been reported as a candidate for industrial applications, and is single most commonly used solvent[8], and thus serves as a good benchmarking solvent. It has excellent absorption rates, it is however haunted by problems such as corrosion, low stability, and high energy needed for desorption. AMP, MDEA and MAPA/MDEA have all shown great promise in CO_2 absorption, but with slightly lower absorption rates, thus they are good candidates for enzyme activation. AIB was previously shown to have higher desorption rates than MEA at 80 °C, but slower absorption rates[9], thus it serves as a good target for enzyme enhanced technologies, in addition AIB had higher solubility than other comparable amino acids like alanine. Potassium carbonate (K_2CO_3) has been used in enzyme based[10] and chemically enhanced[11] carbon capture on several occasions, and has the advantages of favorable thermodynamics and lowered desorption temperature, however a drawback with this solvent is lowered solubility. Finally the solvent AC was used as a cheap and available source of ammonium. Results for activity are showed in Table 2. All data points illustrate that activity is lost over time and a higher deactivation is found with a higher pH. Furthermore there is a negative correlation between pKa (calculated) values and stability. Finally the activity was compared after 100 hours and 150 days, to evaluate if a short term study could efficiently reveal which solvents were stable long the long term. The results correlated poorly and we conclude that within this data set a prediction of long term stability cannot be made from short term studies.

Table 2. Remaining activity after 5 and 150 days

Solvent (concentration)	pH	Residual Activity 5 days (%)	Residual Activity 150 days (%)
MEA (3M)	8.3	95 ± 0.4	73 ±1 0.8
	10	76 ± 1.8	33 ± 4.8
AMP (3M)	9	99 ± 0.3	42 ± 1.6
	10	104 ± 7.7	12 ± 0.6
MDEA (3M)	9	92 ± 2.8	62 ± 4.0
	10	91 ± 3.0	54 ± 2.5
AIB (3M)	8	106 ± 4.9	91 ± 3.0
	10	95 ± 0.1	35 ± 0.9
K_2CO_3 (1M)	8	116 ± 6.8	83 ± 3.6
	10	85 ± 1.2	29 ± 2.4
MAPA (1M)/ MDEA (2M)	8.6	86 ± 10.3	85 ± 0.5
	10	99 ± 4.8	69 ± 4.4
AC (3M)	8	99 ± 4.8	71 ± 5.1
	10	100 ± 4.7	22 ± 3.4

However as seen from Figure 5, a correlation was found between deactivation and pKa values. In comparison higher pKa values has previously been found to have a positive effect on solvent kinetics[12]. This suggests that a compromise might be made on implementation between enzyme activity and stability. However in the case of CA it it has been known since the 1930's that buffers and solvents can have more severe impacts on the catalytic activity of CA[2], albeit of mammalian source, thus the actual enchanced activity with the solvents should be investigated in

detail. Such work has been carried out on several occasions, in particular Haritos and colleagues has investigated enzyme enhanced CO_2 capture in a stirred cell reactor, where a rate enhancement was found with all eleven solvents tested[10]. This gives further evidence that the enzyme may indeed enhance reaction rates for CC applications. It should be noted however that at times higher enzyme stability can be associated with lower reaction rates[13].

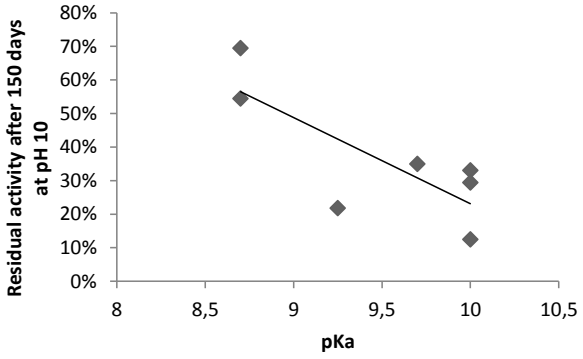

Figure 5. Correlation between pKa and long term (150 days) stability. Pka values were predicted with the software ChemDraw

3.4 Additive effects on stability

Finally, we have investigated the addititve effects of solvent strength and temperature with the solvents NACl, K_2CO_3, AMP and MDEA, Table 3. From these experiments we did not find a significant impact either by altering the concentation of the solvent or the temperature. When analyzing these results with the results we see that the two factors pH and solvent type seems to have a significant impact on enzyme stability but temperatures upto 50 °C and molar concentrations up to 3 M had no significant impact.

Table 3. Residual activity of CA after 100 hours with varying temperature and solvent concentration with the solvents, NaCl, K_2CO_3 , AMP and MDEA.

Solvent	1 M, 25 °C	1 M, 50 °C	3 M, 25 °C	3 M, 50 °C
NaCl	76%	91%	90%	78%
K_2CO_3	125%	100%	63%	80%
AMP	91%	87%	70%	79%
MDEA	88%	89%	83%	75%

4. Conclusion

We have evaluated enzyme stability in terms of pH, temperature and solvents, the latter at different concentrations and types of solvents, and the effect of these three factors added together. The carbonic anhydrase used here, showed long term stability for some, but not all process relevant conditions. In conclusion the findings in this paper hint potential future application of CA for use in PCCC applications.

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Operating considerations of ultrafiltration in enzyme enhanced carbon capture

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Abstract

Today, enzyme enhanced carbon capture and storage (CCS) is gaining interest, since it can enable the use of energy efficient solvents, and thus potentially reduce the carbon footprint of CCS. However, a limitation of this technology is the high temperatures encountered in the stripper column, which can deactivate the enzymes. One solution to this challenge is the use of ultrafiltration to retain the enzyme in the absorber unit. In this report, a base case of a CCS facility is used to model the impact of such membranes for use in a full scale CCS commercial plant. The base case has an approximate capture capacity of 1 MTonn CO_2/year, and is here operated for one year continuously. This publication compares soluble enzymes dissolved in a capture solvent with and without the use of ultrafiltration membranes. The membranes used here have an enzyme retention of 90%, 99% and 99.9%. Enzyme retention is the amount of enzyme that is retained in the absorption column in each cycle. These membranes were modeled with five stripper temperatures 60 °C, 70 °C, 80 °C, 90 °C and above 100 °C. Enzyme deactivation follows a 1st order rate and increases with increasing temperatures. It was found that for all stripper temperatures used in this model, deactivation rates were too high for continuous operation over 1 year, without adding additional enzyme, if an activity of at least 50% should be maintained. With increasing stripper temperatures the membrane retention requirement increased. To retain over 50% activity over a whole year at 70 °C stripper temperature required a membrane of 90% or higher enzyme retention, at stripper temperatures of 90 °C a membrane of 99.9% retention was required for the same result. Finally, it was investigated if stripper temperatures over 100 °C, where instant deactivation was modeled could be used. It was found that with enzyme retention of 99.9%, with instant deactivation, after 1 month 50% of the activity is lost. Thus the use of membranes in enzyme enhanced CCS might be restricted to temperatures below 100 °C, or temperatures the enzyme can withstand for shorter time periods.

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Keywords: biocatalysis; carbon capture and storage; carbonic anhydrase; ultrafiltration

1. introduction

To limit further climate change, atmospheric CO_2 among other greenhouse gases must be reduced. One option for doing so is carbon capture and storage (CCS). This paper will focus on enzyme enhanced CCS, using carbonic anhydrase (CA) EC 4.2.1.1. Enzymes are beneficial for such processes since they enhance reaction rates, especially for bicarbonate forming solvents[1]. However, enzymes are not designed to operate under process conditions encountered in a CCS capture facility. Therefore one of the challenges encountered when using enzymes in such processes is the stability under these conditions, where enzymes may lose activity over time. Previous work has explored this by investigating the stability of one CA, especially suitable for CCS in terms of pH, temperature and solvent type at CCS relevant conditions. Although, the enzyme in question was significantly more stable than most enzymes under such conditions, long term studies (over several months) found that the enzyme was sensitive to higher temperatures[2,3]. Here the impact of these results, if these enzymes were to be used on an industrial scale, are investigated by modeling the stability of such enzymes in a theoretical commercial plant. Enzyme stability within a model framework for stripper temperatures ranging from 60 °C to over 100 °C compared for soluble enzymes with and without the implementation of ultra-filtration membranes. The membranes are explored with enzyme retentions up to 99.9%. The results are modeled for 1 year continuous operation of the facility.

The enzyme CA catalyzes hydration of CO_2 into bicarbonate (Reaction 1). It is therefore particularly useful in solvents which form bicarbonate, such as tertiary and hindered amines, and carbonate salts. These types of solvents have the advantage in that they have relatively low energy for desorption requirements, compared to solvents like primary amines, because they do not form covalent bonds with the absorbed CO_2. However, they are often impeded by slow absorption kinetics, which can either result in poor capture capacity or increased operating and capital costs due to a bigger absorber column. The addition of CA or another catalyst can alleviate this effect by enhancing reaction kinetics. Just like a conventional chemical catalyst, the enzyme does not change the thermodynamics of the reaction, it simply speeds up the reaction rate. This publication does not investigate reaction kinetics, since excellent examples of this can be found in literature[4–6].

Reaction 1:

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$$

Nomenclature

CCS	Carbon capture and storage
CA	Carbonic Anhydrase

2. Model framework

The base case is defined in Table 1 and illustrated in Figure 1, this is based on some publically available data from the Boundary Dam CCS facility, and is supplemented with information from experts in the field.

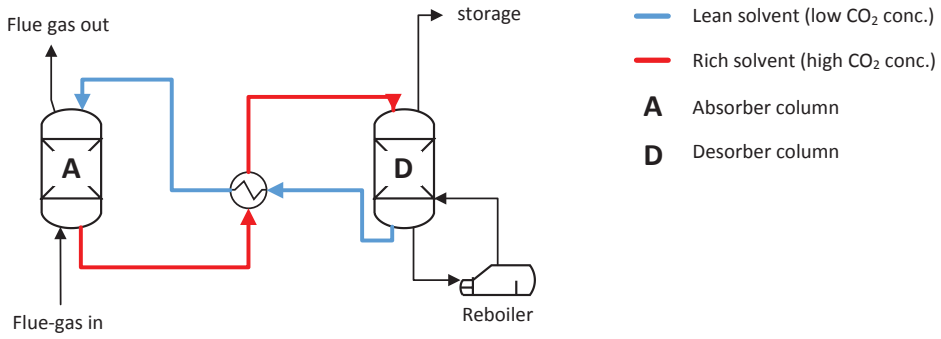

Figure 1: A typical solvent based carbon capture process. The flue gas enters the bottom of the absorber, a lean solvent (blue) counter currently reacts with the gas. At the base the rich solvent (red) is passed through a heat exchanger before it enters the desorber column. The CO_2 is the stripped from the gas and the lean solvent is regenerated. It will again pass through the heat exchanger before it re-enters the absorber column.

Table 1: Base case data, with a solvent volume of 2060 tonne, and a flow rate of 2000 tonne/hr.

	Residence time (min)
Absorber	11,4
Stripper	5,3
Hold-up	45,1
Total	61,8

Deactivation rates

The enzyme deactivation rates were obtained from previously published data[2], and follow first order reaction rates according to the following formula

$$A_t = A_i e^{-kt},$$

Where A_t is the activity remaining at a certain time point, A_i is initial activity (100%) ,k is the deactivation rate constant and t is the time at that time point.

Table 2: Deactivation with temperature

Temperature (°C)	50	60	70	80	90
Deactivation rate (h^{-1})	0	0.003	0.0054	0.0536	0.3860

In addition a stripping temperature above 100 °C was used in the calculations, instant enzyme deactivation is assumed.

Ultra-filtration membranes

Ultra-filtration membranes used in this study were calculated to have an enzyme retention of 90%, 99% and 99.9%. Furthermore the membrane flux was calculated for two specific membranes from the commercial membrane producer Alfa Laval, Table 3.

Table 3: Properties of commercial membranes used in this model.

Type	Selectivity (%)	Water permeability (L/m2*h*bar)	Source
Commercial	90	50	Alfa Laval
Commercial	99,9	400	Alfa Laval

3. Results

Enzymes can be used in a CCS facility by simply adding soluble enzyme to the solvent, and run the facility as before, as described in figure 1. This method of adding enzymes provides the maximal effect of the enzyme in terms of catalytic rates due to the lowest mass transfer limitations. For example, when enzymes are immobilized mass transfer limitations increase because the enzymes are not dispersed in the liquid. In fact it has been found that enzymes immobilized on packing is not a viable option for enzyme enhances CCS, due to mass transfer limitations[4]. In addition, this set-up enables the enzymes to catalyse both absorption and desorption. Finally, it has the lowest capital and operational costs since no additional cost for membranes and compression are added. However, as investigated in previous work, the enzyme deactivates at a significant rate at higher temperatures[2]. Therefore, the stability of such enzymes are investigated at different operating temperatures with the base case CCS facility outlined above. Enzyme viability is calculated, in terms of residual activity in a continuous operating power plant for one year. Here five different stripper temperatures were used, 60 °C, 70 °C, 80 °C, 90 °C and above 100 °C. It was assumed that absorption is done at a lower temperature and enzyme activity loss is limited, therefore deactivation is only occurring in the stripper. The reduced deactivation at lower temperatures has been reported in scientific literature[2,3]. Figure 2 outlines trends over a time period of one year, from initial activity (100%). From this figure it can be observed that significant activity loss is found after a few minutes with the highest temperature (100 °C). However, some enzyme activity still remains after 1 year with the two lowest temperatures. In this model we have assumed a uniform temperature in the stripper unit. It was also tested if a non-uniform temperature model, where parts of the stripper were warmer and colder than the bulk solvent, would influence the outcome of the model. From this we see a slight decrease in stability, but the results follow the same general trends as the data in Figure 2 (data not shown).

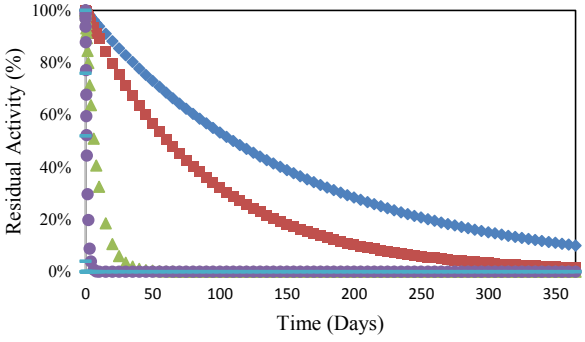

Figure 2: Residual enzyme activity after one year with five operating temperatures in the stripper: 60 °C (blue diamond), 70 °C (red squares), 80 °C (green triangles), 90 °C (purple circles) and over 100 °C (light blue dashes).

Furthermore, the use of an ultrafiltration unit was considered. Here the rich solvent will be passed over an ultrafiltration membrane where a limited amount of enzyme will pass through. The enzyme which does not pass through the membrane is then shuttled back to the absorption column with 10 % of the rich solvent. Figure 3). The stability of the enzymes of this process depends on the amount of enzyme which passes through the membrane and the temperature the enzyme is exposed to in the stripper.

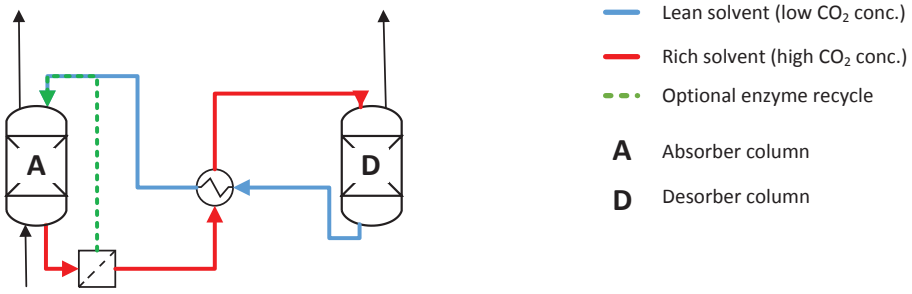

Figure 3: One possible set-up of an ultrafiltration unit in a CCS facility. The process is similar to that which is described above (Figure 1). However, the rich solvent will be passed through an ultrafiltration device where most of the solvent will pass through, and some of the rich solvent will be diverted back to the lean solvent with the enzymes, not passing through the desorber column.

Here three membranes with enzyme retentions of 99.9%, 99% and 90% are used for the calculations, and compared with soluble enzyme. The rate of deactivation of the enzymes which pass through the membrane and experience the conditions in the stripper unit are calculated using five different stripper temperatures 60 °C, 70 °C, 80 °C, 90 °C and above 100 °C. The comparison of the models can be found in Figure 4: a, b, c, d and e, respectively. It is observed that the efficiency of the membrane has a significant impact on the enzyme viability. The membrane with the poorest enzyme retention (90%) has only a small impact on enzyme viability for all temperatures above 70 °C. For stripping temperatures over 70 °C membranes with higher selectivity preform significantly better. The membrane with the highest selectivity (99.9% selectivity) preforms well at temperatures up to and including 90 °C. Finally, it was investigated if temperatures above 100 °C, where instant deactivation is assumed, is a viable option with the use of ultrafiltration. Without the use of ultrafiltration membranes all activity is lost within 1 hour of operation. Although the use of membranes, especially the membrane with the highest enzyme retention at 99.9% significantly increases the life span of the enzymes, high activity loss is still observed. 50% of the activity is lost after 1 month and after 6 months only 1% activity remains.

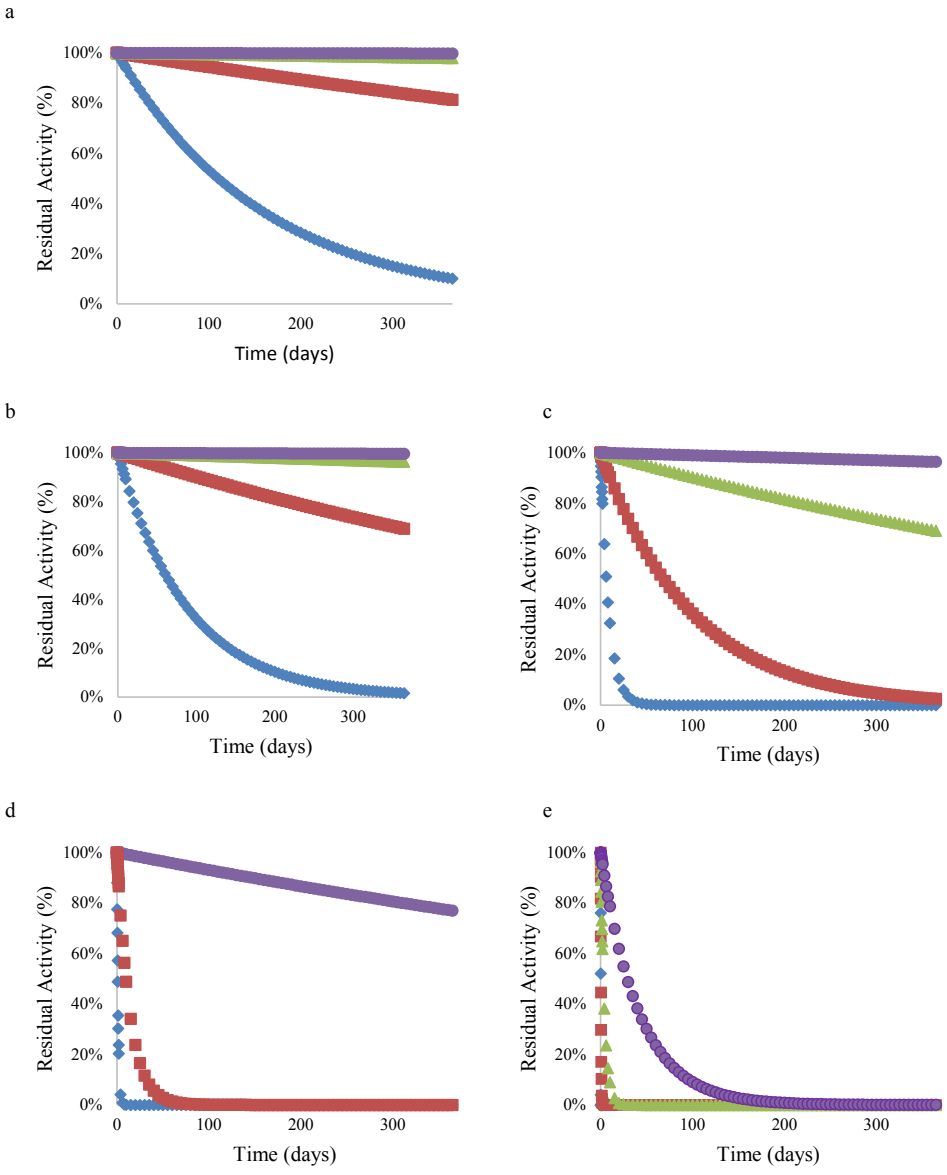

Figure 4: Effect of enzyme stability, measured by residual activity over time, of ultrafiltration enzyme separation with various stripper temperatures: (a) 60 °C, (b) 70 °C, (c) 80 °C, (d) 90 °C, (e) over °100 C (instant deactivation). Membrane retention for all figures: Blue diamond: No membrane, Red squares: 90% retention, Green triangles: 99% retention and Purple circles 99.9% retention.

Discussion

Enzymes can enhance the absorption rate of CO_2 into kinetically limited solvents, such as tertiary amines and carbonate salts[3,5,7]. However, enzymes are often limited in CCS applications due to thermal stability, which is problematic due to the high temperatures encountered in the stripper unit. We have therefore explored the use of ultrafiltration units in comparison to free enzymes in solution. There are two key issues that makes a CCS process challenging to operate compared to other applications where enzymes are used. Firstly, the scale of a CCS facility must be kept in mind. In the base case used in this paper the addition of only 1% enzyme would be 20 tonnes enzymes. Thus, the cost of the enzyme would be a significant contribution. Secondly, the number of cycles should be kept in mind. This base case has a 1 hour cycle time, which equates to almost 9000 cycles per year. Thus the addition of enzymes on regular intervals would significantly dilute the solvent, and would likely over time change physical properties of the solvent, such as the viscosity. Figure 2 indicates how such a process would look like with a solubilized enzyme without the use of any ultrafiltration units. With the deactivation rates indicated here, it was found that even with the lowest stripper temperature 60 °C, enzymes must be added 3 times a year to maintain an activity over 50% of initial activity. When the temperature increases this trend intensifies, such that at stripper temperatures of 80 °C, enzyme must be added 60 times in a year to maintain the same activity of 50% or higher. As discussed above this does not only add costs to the process, but it also poses a practical problem with solvent dilution, and increased enzyme concentrations.

One solution could be the use of an ultrafiltration membrane unit, which restricts the enzymes in one area of the process, the absorber, so the enzymes does not enter the high temperature areas of the stripper. This means enzyme deactivation is minimized. Here, calculations have been carried out with 3 enzyme retentions, 90%, 99% and 99.9%. Operated at the same five stripper temperatures as outlined above from 60 °C to above 100 °C. In the scenario described here, 10% of the rich solvent stream is diverted while the majority of the enzymes are diverted back to the lean solvent, thus not being regenerated. The non-retained enzyme, will pass through the stripper column and deactivate at the same rate as the soluble enzyme. It should be noted that such a set-up poses several disadvantages. Firstly, 10% of the solvent is not regenerated, thus the capacity of each cycle and the overall capacity of the facility is reduced by 10%. Furthermore, the kinetic penalty of such a set-up is likely to be higher than 10%, since the reaction rates in the absorber decreases with loading.

Our calculations show that the 90% enzyme retention membrane works well up to 70 °C, with temperatures above that more stringent requirements set for the enzyme retention capacity. Furthermore, it was found that ultrafiltration is only suitable when a deactivation process is taking place. In the calculations above 100 °C, where instant deactivation was, even the 99.9% membrane is not suitable for long term use, without replenishing enzymes. This is due to the high number of cycles in such a process as discussed above. Practically, this means that primary amines, such as monoethanolamine, frequently used as solvents in CCS, may not be suitable solvents with enzyme enhanced CCS, since stripper temperatures above 120 °C are used for this type of enzyme retention. Nonetheless, using other methods such as enzyme immobilization in the absorber column, might still be attractive. Furthermore, it indicates that the use of a conventional reboiler in a set-up as described here might be unsuitable, shown in Figure 1. One could rather envision using a stripper set-up with vacuum, steam or a combination of the two. Indeed such setups has been applied in practice with success in enzyme enhanced CCS[8].

It is clear from the results in Figure 4, that the use of a membrane with a higher enzyme retention has a better performance with respect to retention of enzyme activity. However, increased enzyme retention often comes at a cost. The capital costs of such membranes are likely to be higher, and it would be expected that they are more difficult to produce and maintain at a high level of perfection, since any tear/leak would be detrimental to enzyme activity at higher stripping temperatures. In addition the flux of the membranes should be considered, since it will influence the membrane size needed for such a setup. Table 4 indicates the membrane size needed to maintain the target flux of two commercial membranes. In addition, for an efficient ultrafiltration, it is needed to operate with a higher pressure, here a pressure of 4 bar has been used. In fact, it has been stated the cost of cross-flow ultrafiltration is dominated by membrane replacement and pumping[9].

Table 4: Required membrane sizes of ultrafiltration membranes used in this study, operated at 4 Bar, with a flux of $2.1*10^6$ L/h.

Type	Selectivity	Water permeability ($L/m^2*h*bar$)	Membrane size (m^2)	Source
Commercial	90	50	10600	Alfa Laval
Commercial	99,9	400	1330	Alfa Laval

The calculated membrane sizes are relatively high. However such setups are uses commercially in other industries such as the water purification industry. Where numerous membrane units are connected in series. As such the membrane sizes estimated here would be feasible for such a set-up.

Conclusion:

The use of ultrafiltration in enzyme enhanced CCS was evaluated. A model using three different enzyme retention membranes was used in combination with five different stripper temperatures. It was found that to retain over 50% activity for one year an ultrafiltration unit was required in all cases tested here. With higher the stripper temperatures the requirement for the membrane selectivity increased. For the highest temperature, where instant deactivation was assumed, the most selective membrane with 99.9% enzyme retention, did not meet the requirement. Thus, the use of enzyme enhanced CCS might be restricted to temperatures below 100 °C, or temperatures the enzyme can with stand for shorter time periods, if the use of ultrafiltration units are in use.

Acknowledgements

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