Universidad de Oviedo

Departamento de Química Orgánica e Inorgánica

Programa de Doctorado: Síntesis y Reactividad Química

SELECTIVE ENZYMATIC TRANSFORMATIONS FOR THE VALORISATION OF ORGANIC COMPOUNDS.

ENZYME IMMOBILISATION STRATEGIES TO DEVELOP BIOTRANSFORMATIONS IN ORGANIC SOLVENTS.

Tesis Doctoral

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Memoria presentada para optar al grado de Doctora en Química por Antía Pintor Labandeira

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Idioma: Transformaciones enzimáticas selectivas para la valorización de compuestos orgánicos. Estrategias de inmovilización de enzimas para desarrollar biotransformaciones en disolventes orgánicos.	Inglés: Selective enzymatic transformations for the valorisation of organic compounds. Enzyme immobilisation strategies to develop biotransformations in organic solvents.

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RESUMEN (en español)

La Biocatálisis se define como el uso de sustancias naturales para acelerar reacciones químicas. En este contexto la naturaleza proteica de los enzimas, incluyendo su quiralidad intrínseca, hacen que este tipo de moléculas orgánicas sean catalizadores altamente selectivos y capaces de trabajar bajo diversas condiciones de reacción. Esta Tesis Doctoral opta tanto a mención internacional como industrial, y se ha desarrollado dentro de un proyecto de financiación europeo involucrando globalmente a 9 universidades y 13 industrias. Para una mejor comprensión, su contenido se ha dividido en una introducción general y cuatro capítulos, donde se pone de manifiesto las ventajas y posibilidades del uso de enzimas inmovilizadas.

La Introducción desglosa el concepto de Química Sostenible, destacando el rol de la Biocatálisis en esta disciplina. Para ello, se describe la forma de clasificar las enzimas en función de su actividad catalítica, y se definen distintos indicadores utilizados para evaluar la sostenibilidad de un proceso. Además, se discuten las principales ventajas de la inmovilización enzimática, haciendo hincapié en la descripción de los soportes EziG usados en esta Tesis Doctoral.

El Capítulo 1 describe la obtención quimioselectiva de derivados acilados a partir de furfurilamina y 5-hidroximetilfurfurilamina y usando la lipasa de *Candida antarctica* de tipo B (CALB) inmovilizada en diversos soportes comerciales. Además, se ha evaluado la escalabilidad y sostenibilidad del proceso. Los resultados de esta investigación han sido publicados en el artículo “Chemoselective lipase-catalyzed synthesis of amido derivatives from 5-hydroxymethylfurfurylamine” (*ACS Sustainable Chem. Eng.* **2023**, *11*, 10284–10292).

En el Capítulo 2 se ha estudiado la transaminación del 5-hidroximetilfurfural usando diferentes transaminasas. Los enzimas con mayor actividad fueron seleccionados para una optimización de la reacción, incluyendo cantidad de donor de amino y biocatalizador. Además, se inmovilizaron tres de las transaminasas que condujeron a los mejores resultados en fase homogénea, y se estudió la posibilidad de realizar las reacciones en disolvente orgánico. Los principales hallazgos de esta investigación han sido recogidos en el trabajo titulado “Biotransamination of furan-based aldehydes with isopropylamine: Enzyme screening and pH influence” que ha sido recientemente aceptado para su publicación con menores modificaciones en la revista ChemBioChem.

En el Capítulo 3 se han estudiado las condiciones adecuadas para el desarrollo de una cascada lipasa-transaminasa para la formación de una amida quiral en disolvente orgánico. Así, se ha desarrollado una cascada bienzimática estereoselectiva consistente en la transformación de una cetona en una amina quiral que posteriormente es acilada en un sistema de flujo continuo. Con este propósito y seleccionando la 1-fenoxipropil-2-ona como sustrato modelo, empleando la transaminasa *Arthrobacter* sp. variante 11, que es selectiva hacia la formación de la amina de configuración *R*, y la CALB. Los resultados de esta investigación han sido recogidos en la publicación “Development of an amine transaminase-lipase cascade for chiral amide synthesis under flow conditions” (*Green Chem.* **2023**, *25*, 6041–6050)

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Finalmente, en el Capítulo 4 se ha investigado la inmovilización de dos enzimas redox, como son una una alqueno reductasa y una alcohol deshidrogenasa para la reducción selectiva de cetonas insaturadas. Los enzimas se inmovilizaron en soportes EziG con el fin de ser empleados de manera conjunta en disolvente orgánico, optimizando las condiciones de reacción. Además, se ha explorado la posibilidad de co-inmovilizar ambas enzimas en el mismo soporte, así como el uso de diferentes tratamientos de post-inmovilización destinados a optimizar la retención de actividad enzimática en el disolvente orgánico elegido. Actualmente, se está trabajando en la redacción del correspondiente artículo de investigación

RESUMEN (en Inglés)

Biocatalysis refers to the utilisation of natural compounds to accelerate chemical reactions. In this context, the proteinaceous composition of enzymes, along with their inherent chirality, makes these organic molecules exceptional selective catalysts, and capable of operating under a broad spectrum of reaction conditions. This Doctoral Thesis applies for both international and industrial recognition. It was developed within an European-funded project, involving 9 universities and 13 companies. To enhance comprehension, its content is organised into a general introduction and four chapters, which explains the advantages and potentials of using immobilised enzymes.

The Introduction elucidates the concept of Sustainable Chemistry, emphasising the current role of Biocatalysis. Additionally, it described the enzyme classification based on their catalytic activity, and defines diverse indicators used to evaluate process sustainability. Moreover, the section discusses the primary advantages of enzyme immobilisation, highlighting the description of the EziG supports used in this Doctoral Thesis.

Chapter 1 describes the chemoselective synthesis of acylated derivatives from furfurylamine and 5-hydroxymethylfurfurylamine using *Candida antarctica* lipase type B (CALB), which is immobilised on a series of commercial supports. The scalability and sustainability of the process was also evaluated. The outcomes of this investigation are documented in the research article entitled “Chemoselective lipase-catalyzed synthesis of amido derivatives from 5-hydroxymethylfurfurylamine” (*ACS Sustainable Chem. Eng.* **2023**, *11*, 10284–10292).

Chapter 2 explores the transamination of 5-hydroxymethylfurfural using different transaminases. The enzymes displaying high activity were selected for subsequent optimisation of the reaction, involving factors such as amino donor quantity and biocatalyst concentration. Furthermore, three of the transaminases that yielded the best results under homogeneous conditions in aqueous buffer were immobilised. Additionally, the possibility of conducting reactions in organic solvents was investigated. The findings of this study are summarised in the article “Biotransamination of furan-based aldehydes with isopropylamine: Enzyme screening and pH influence” that was recently accepted with minor modifications in the journal ChembioChem.

Chapter 3 delves into identifying the optimal conditions to develop a lipase-transaminase cascade to form chiral amides in organic solvents. As a result, a stereoselective bienzymatic cascade was developed, involving the conversion of a ketone into a chiral amine, which was subsequently acylated within a continuous flow system. For this purpose, the transaminase *Arthrobacter* sp. variant 11, selective towards the formation of the amine *R*-enantiomer, and CALB were chosen. The model substrate used was 1-phenoxypropan-2-one. The findings of this investigation are documented in the publication titled " Development of an amine transaminase-lipase cascade for chiral amide synthesis under flow conditions " (*Green Chem.* **2023**, *25*, 6041–6050).

Lastly, Chapter 4 focuses on the development of two redox enzymes immobilisation, namely alkene reductase and alcohol dehydrogenase, to selectively reduce unsaturated ketones. These enzymes were immobilised on EziG supports to enable their utilisation in organic solvents, after optimising the reaction conditions for their synergistic function. Furthermore, the feasibility of co-immobilising both enzymes on the same support, along with various post-immobilisation treatments aimed at enhancing enzymatic activity retention within the chosen organic solvent, was explored. The corresponding research article is currently under preparation.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN SINTESIS Y REACTIVIDAD QUÍMICA

ABBREVIATIONS AND ACRONYMS

2-MeTHF	2-methyltetrahydrofuran
2-PrOH	2-propanol
Å	Angstrom(s)
AADH	Amino acid dehydrogenase
Ac	Acetyl
ADH	Alcohol dehydrogenase
ADH	Alcohol dehydrogenase
AE	Atom economy
Af	Transaminase from *Aspergillus fumigatus*
AKL	*Pseudomonas fluorescens*
ANL	*Aspergillus niger*
API	Active pharmaceutical ingredient
Arg	Arginine
ArR	Transaminase from *Arthrobacter* sp.
ArRmut11	Transaminase from *Arthrobacter* sp. round 11 variant
ArS	Transaminase from *Arthrobacter citreus*
Asp	Aspartic acid
At	Transaminase from *Aspergillus terreus*
ATA	Amine transaminase
AtR	Transaminase from *Aspergillus terreus*
BAMF	2,5-bis(aminomethyl)furan
BHMF	2,5-bis(hydroxymethyl)furan
Bm	Transaminase from *Bacillus megaterium*
Bn	Benzyl
BPR	Back pressure regulator
br	Broad (spectral)
CALA	Lipase type A from *Candida antarctica*

Abbreviations and Acronyms

CALB	Lipase type B from *Candida antarctica*
Cb	Ene-reductase from *Clostridium beijerinckii*
CFE	Cell free extract
CLEA	Cross-linked enzyme aggregate
CLEC	Cross-linked enzyme crystal
cm	Centimetre(s)
cm^{-1}	Wavenumber(s)
CPG	Controlled pore glass
CRL	Lipase from *Candida rugosa*
Cv	Transaminase from *Chromobacterium violaceum*
d	Doublet (spectral)
DEPT	Distortionless enhancement by polarisation transfer
DES	Deep eutectic solvents
DFF	2,5-diformylfuran
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DMSO	Dimethyl sulfoxide
E. coli	Escherichia coli
EC	Enzyme Commission
Eh	Ene-reductase from *Entamoeba histolytica*
Enz	Enzyme
EQ	Environmental quotient
equiv	Equivalent
ERED	Ene-reductase
ESI	Electrospray ionisation (mass spectrometry)
Et	Ethyl
Et_2O	Diethyl ether
EtOAc	Ethyl acetate

EU	Europe Union
EWG	Electro-withdrawing group
EziG	Support from EnginZyme
FA	Furfurylamine
FDA	U.S. Food and drug administration
FDCA	2,5-furandicarboxylic acid
FDH	Formate dehydrogenase
FFCA	5-formyl-2-furancarboxylic acid
FID	Flame ionisation detector (gas chromatography)
FMN	Flavin mononucleotide
$FMNH_2$	Flavin mononucleotide, reduced form
g	Gram(s)
GA	Glyoxyl-agarose
GC	Gas chromatography
GDH	Glucose dehydrogenase
h	Hour(s)
His	Histidine
HMF	5-hydroxymethylfurfural
HMFA	5-hydroxymethyl-2-furfurylamine
HMFCA	5-hydroxymethyl-2-furancarboxylic acid
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
I	Intermediate
i.d.	Internal diameter
ID	Identification
IMAC	Immobilised metal affinity chromatography
IPA	Isopropylamine
^{i}Pr	*iso*-Propyl

Abbreviations and Acronyms

IPTG	Isopropyl β-D-1-thiogalactopyranoside
IR	Infrared spectroscopy
IRED	Imine reductase
IY	Immobilisation yield
J	Coupling constant (spectral)
kPa	Kilopascal
KPi	Potassium phosphate
KYE1	Ene-reductase from *Kluyveromyces lactis*
L	Litre(s)
LCA	Life cycle assessment
LDH	Lactate dehydrogenase
m	Multiplet (spectral); metre; milli
M	Molar (moles per litre)
m/*z*	Mass-to-charge ratio
M^+	Parent molecular ion (mass spectrometry)
MBA	Methylbenzylamine
Me	Methyl
MeOH	Methanol
MHz	Megahertz
min	Minute(s)
MNP	Magnetic nanoparticle
MOPS	(3-(*N*-morpholino)propanesulfonic acid)
Mp	Melting point
MS	Mass spectroscopy
MTBE	Methyl *tert*-butyl ether
NAD^+	β–Nicotinamide adenine dinucleotide
NADH	β–Nicotinamide adenine dinucleotide, reduced form
$NADP^+$	β–Nicotinamide adenine dinucleotide 2'-phosphate

NADPH	β– Nicotinamide adenine dinucleotide 2'-phosphate, reduced form
^{n}Bu	*n*-Butyl
NCR	Ene-reductase from *Zymomonas mobilis*
nm	Nanometre(s)
NMR	Nuclear magnetic resonance
^{n}Pr	*n*-Propyl
ºC	Degrees Celsius
OCH	6-oxocamphor hydrolase from *Rhodococus ruber* NCIMB 9784
OD	Optic density
OYE	Old Yellow Enzyme
P	Product
PDB	Protein data bank
PIT	Post-immobilisation treatment
PL	Protein loading
PLP	Pyridoxal 5'-phosphate
PMP	Pyridoxamine phosphate intermediate
ppm	Part(s) per million (spectral)
PSL	Lipase from *Pseudomonas cepacia*
PTDH	Phosphite dehydrogenase
Q	Unfriendliness quotient
q	Quartet (spectral)
qui	Quintet (spectral)
RA	Recovered activity
rac	Racemic
R_f	Retention factor
rpm	Revolutions per minute
S	Substrate

Abbreviations and Acronyms

s	Singlet (spectral); second
Sc	Ene-reductase from *Saccharomyces cerevisiae*
Ser	Serine
SP	Silica particle
sxt	Sextet (spectral)
t	Triplet (spectral)
TA	Transaminase
TB AIM	Terrific broth autoinduction media
TBU	Tributyrin units
TeS	Ene-reductase from *Thermoanaerobacter ethanolicus*
TeS-WIC	Ene-reductase WIC variant from *Thermoanaerobacter ethanolicus*
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
TLL	Lipase from *Thermomyces lanuginosus*
TOS	Time on stream
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
TsER	Enoate reductase from *Thermus scotoductus*
TsOYE	Ene-reductase from *Thermus scotoductus*
U	Enzymatic units
U.S.	United States
UV	Ultraviolet
v/v	Volume per unit volume (volume to volume ratio)
Vf	Transaminase from *Vibrio fluvialis*
Vf	Transaminase from *Vibrio fluvialis*
Vf-mut	Transaminase from *Vibrio fluvialis* variant
vs	Versus

w/w	Weight per unit weight (weight to weight ratio)
Yers	Ene-reductase from *Yersinia bercovieri*
μ	Micro
δ	Chemical shift in parts per million downfield from tetramethylsilane
ε	Molar absorptivity

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GENERAL INTRODUCTION

0.1. Sustainable Chemistry

The increasing global awareness of climate change and its environmental impact, led to a growing concern in the mid 1980s regarding the significant amount of waste generated by the chemical industry.[1] This generated a need for cleaner and more resource-efficient alternatives in chemistry minimising waste production. In 1990, the U.S. Pollution Prevention Act within the U.S Environmental Protection Agency,[2] marked a fundamental shift from traditional "end-of-pipe" waste treatment methods to prioritising waste prevention strategies, giving rise to the "green chemistry" concept. In this context, the main goal for green chemistry is the exploitation of raw materials (preferably renewable), while minimising or eliminating waste generation throughout all stages of chemical product manufacturing and application processes.[3] Additionally, green chemistry, also known as sustainable chemistry, strives to avoid any potential use of toxic and/or hazardous reagents and solvents. This fundamentally transforms environmental protection efforts into proactive pollution prevention measures, rather than relying on reactive solutions primarily focused on remediation after pollution has already occurred.

With the release of Anastas and Warner's book entitled “Green Chemistry. Theory and Practice”,[4] , the concept of green chemistry gained official recognition. The publication introduced the 12 principles of green chemistry, which emphasised the importance of designing chemical

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[1] R. A. Sheldon, *Chem. Soc. Rev.* **2012**, *41*, 1437–1451.
[2] Summary of the Pollution Prevention Act. http://www.epa.gov/ laws-regulations/summary-pollution-prevention-act (accessed Aug 10, 2023).
[3] R. A. Sheldon, *Comptes Rendus l’Académie des Sci. - Ser. IIC - Chem.* **2000**, *3*, 541–551.
[4] P. T. Anastas, J. C. Warner, *Green Chemistry: Theory and Practice*, Oxford University Press, Nueva York (EE. UU.), **1998**.

processes with minimal harm to human health and the environment.[5] Referred to as "benign by design," these principles serve as a comprehensive guide for implementing environmentally friendly practices in chemical production, and they can be summarised using the acronym PRODUCTIVELY;[6]

1. **P**revent waste
2. **R**enewable materials
3. **O**mit derivatisation steps
4. **D**egradable chemical products
5. **U**se safe synthetic methods
6. **C**atalytic reagents
7. **T**emperature, pressure ambient…
8. **I**n-process monitoring
9. **V**ery few auxiliary substances
10. **E**-factor, maximise feed in product
11. **L**ow toxicity of chemical products
12. **Y**es, it is safe

In addition to green chemistry, there is another important concept known as sustainable development. This term was first introduced in 1987 with the publication of the Brundtland report called "Our Common Future" by the World Commission on Environment and Development.[7] The report

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[5] H. C. Erythropel, J. B. Zimmerman, T. M. De Winter, L. Petitjean, F. Melnikov, C. H. Lam, A. W. Lounsbury, K. E. Mellor, N. Z. Janković, Q. Tu, L. N. Pincus, M. M. Falinski, W. Shi, P. Coish, D. L. Plata, P. T. Anastas, *Green Chem*. **2018**, *20*, 1929–1961.

[6] S. L. Y. Tang, R. L. Smith, M. Poliakoff, *Green Chem*. **2005**, *7*, 761–762.

[7] W. Commission on Environment, *Report of the World Commission on Environment and Development: Our Common Future*, Oxford University Press, Oxford (U.K.), **1987**.

acknowledged that industrial and societal growth must ensure a satisfactory quality of life for an increasing global population while also being able to be maintained over time. Sustainable development was defined as "development that meets the needs of the present generation without compromising the ability of future ones to meet their own needs".[7] In contrast to green chemistry, sustainable development also takes into account an economic dimension. Therefore, the long-term viability of a specific technology depends on its economic competitiveness.

In today's chemical industry, a significant factor contributing to excessive waste production arises from the use of stoichiometric amounts of reagents, predominantly inorganic substances.[8] This issue is of particular concern in the manufacturing processes of fine chemicals and pharmaceuticals, due to the high complexity of these molecules, which need lengthy synthetic procedures involving numerous steps. One potential solution to address this challenge involves adopting catalytic methodologies that result in higher atom economy, and thus, reducing waste generation.

0.2. Biocatalysis

Biocatalysis can be defined as the use of natural catalysts, such as enzymes and other biological materials, to facilitate chemical reactions. [9,10] The scope of biocatalysis includes fermentation and biotransformations using whole cells or isolated enzymes. This approach represents a genuinely green and sustainable technology, aligning harmoniously with 10 out of the 12 principles of green chemistry.[8] The remaining two principles (4 and 11)

[8] R. A. Sheldon, J. M. Woodley, *Chem. Rev.* **2018**, *118*, 801–838.
[9] M. T. Reetz, *J. Am. Chem. Soc.* **2013**, *135*, 12480–12496.
[10] V. Gotor-Fernández, M. J. Hernáiz Gómez-Dégano, *An. Quim*. **2017**, *113*, 27–35.

are somewhat less applicable as they relate more to the final product rather than the design of the process itself.

Historically, enzymes have been employed for thousands of years to facilitate the process of alcoholic fermentation, enabling the production of wine or beer.[11] Nevertheless, it was not until 1848 that processes mediated by enzymes received enough attention. In this year, Louis Pasteur successfully performed the kinetic resolution of tartaric acid using the fungus *Penicillium glaucum*.[12] However, the establishment of biocatalysis as a mature technology did not occur until 1985, when Zaks and Klibanov discovered that certain enzymes could catalyse transformations in organic media.[13] In recent years, the use of enzymes as catalysts has been recognised as a feasible solution to many challenges encountered in chemical industry,[14] involving the design and development of organic transformations in a more efficient and sustainable manner.[15,16,17]

Enzymes have been considered privileged catalysts due to the high levels of selectivity they can induce in the processes they accelerate, primarily attributed to its three-dimensional nature.[18] Like any other catalyst, enzymes can exhibit three different types of selectivity:[19]

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[11] C. M. Heckmann, F. Paradisi, *ChemCatChem* **2020**, *12*, 6082–6102.
[12] L. Pasteur, *Ann. Chim. Phys.* **1848**, *24*, 442–459.
[13] A. Zaks, A. M. Klibanov, *Prod. Natl. Acad. Sci. USA* **1985**, *82*, 3192–3196.
[14] J. Chapman, A. E. Ismail, C. Z. Dinu, *Catalysts* **2018**, 8, 20–29.
[15] E. L. Bell, W. Finnigan, S. P. France, A. P. Green, M. A. Hayes, L. J. Hepworth, S. L. Lovelock, H. Niikura, S. Osuna, E. Romero, K. S. Ryan, N. J. Turner, S. L. Flitsch, *Nat. Rev. Methods Prim.* **2021**, *1*, 46.
[16] R. A. Sheldon, D. Brady, *ACS Sustain. Chem. Eng*. **2021**, *9*, 8032–8052.
[17] R. A. Sheldon, D. Brady, *ChemSusChem* **2019**, *12*, 2859–2881.
[18] D. Ringe, G. A. Petsko, *Science* **2008**, *320*, 1428–1429.
[19] R. Mu, Z. Wang, M. C. Wamsley, C. N. Duke, P. H. Lii, S. E. Epley, L. C. Todd, P. J. Roberts, *Catalysts* **2020**, *10*, 832.

- Chemoselectivity is the ability to modify a specific functional group while leaving others, with similar reactivity, unchanged within the same molecule.
- Regioselectivity is the capacity to distinguish between identical functional groups located in different parts of a determined molecule.
- Stereoselectivity is the capability to differentiate between the two enantiomers in a racemic mixture, or to obtain a single diastereoisomer when multiple options are possible.

While the first two methods offer numerous advantages for applications in organic synthesis by avoiding laborious processes of functional group protection and deprotection, the stereoselective action of enzymes allows to produce enantiomerically pure products from non-chiral molecules or racemic mixtures. This has led to an increasing interest in biotransformations within the pharmaceutical industry.[20,21,22]

Taking all of this into consideration, enzymes effectively meet both economic and environmental requirements for a sustainable chemical process. Thus, some key advantages can be here summarised such as:

- The ability to produce catalysts from easily accessible renewable resources. Moreover, these catalysts are biodegradable, non-hazardous, and non-toxic.
- Enzymatic catalysis avoids the use of scarce precious metals and the subsequent expenses related to trace removal of these metals from final products.

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[20] J. I. Ramsden, S. C. Cosgrove, N. J. Turner, *Chem. Sci.* **2020**, *11*, 11104–11112.
[21] S. Wu, R. Snajdrova, J. C. Moore, K. Baldenius, U. T. Bornscheuer, *Angew. Chem. Int. Ed.* **2021**, *60*, 88–119.
[22] J. P. Adams, M. J. B. Brown, A. Diaz-Rodriguez, R. C. Lloyd, G. D. Roiban, *Adv. Synth. Catal.* **2019**, *361*, 2421–2432.

- Enzymesoperate under mild reaction conditions, using water, ambient temperature, and atmospheric pressure.
- Finally, they circumvent the need for activating, protecting, and deprotecting functional groups, resulting in more streamlined, cost-effective routes and reduced waste generation when compared to traditional processes.

In terms of classification, enzymes are categorised according to the reactions they catalyse, allowing them to be grouped into six major families. In 2018, a seventh class of enzymes was discovered, known as translocases, however, they do not exhibit catalytic activity, rather, their role involves the transportation of molecules across cell membranes. Each enzyme is designated by a four-digit numerical code, prefixed by the letters EC, which stands for Enzyme Commission (Table 0.1):[23]

[23] K. Faber, *Biotransformations in Organic Chemistry. A textbook*, 7ª Ed. Springer, Berlín (Germany), **2017**.

Table 0.1. Enzyme classification based on the reaction they catalyse.

Code	Family	Catalysed reaction
EC 1	Oxidorreductases	Oxidation-reduction reactions, involving the transfer of electrons between molecules.
EC 2	Transferases	Transfer of functional groups (such as methyl, phosphate, or amino groups) from one molecule (donor) to another (acceptor).
EC 3	Hydrolases	Hydrolysis reactions, where bonds are cleavaged by the addition of a nucleophile, generally water.
EC 4	Lyases	Removal of atoms or functional groups to form double bonds or rings.
EC 5	Isomerases	Rearrangement of atoms within a molecule, resulting in the formation of isomers.
EC 6	Ligases	Bond formation, often using energy from ATP.
EC 7	Translocases	Transport molecules across cell membranes

Despite what this classification establishes, in recent years, it has been reported that certain biocatalysts can catalyse reactions different from their natural role. This ability exhibited by some enzymes is referred to as catalytic promiscuity,[24,25,26] and its discovery dates back to 2003 when it

[24] M. López-Iglesias, V. Gotor-Fernández, *Chem. Rec.* **2015**, *15*, 743–759.

was first described that lipase from *Candida antarctica* type B (CALB), whose natural activity is ester hydrolysis, was able to catalyse the aldol reaction between to hexanal molecules to form C–C bonds.[27]

This thesis focuses on the use of enzymes belonging to the EC 1 group, namely oxidoreductases such as ene-reductases and alcohol dehydrogenases (ADHs), as well as enzymes from the EC 2 group, specifically amine transaminases (ATAs) as transferases. Additionally, enzymes from the EC 3 group, particularly lipases as hydrolases, have been utilised. Detailed descriptions of each enzyme type used will be provided in the introductions of their respective chapters.

0.3. Metrics of green chemistry

To assess the sustainability of all manufacturing processes, including those involving biocatalysis, it is necessary to incorporate quantitative measurements.[28] This enables industries to gain insights into their existing performance levels, while also establishing attainable targets for future improvements. Furthermore, such quantitative measures play an important role in justifying investment decisions when implementing industrial practices.[8] Two well-known indicators for assessing environmental impact are atom economy[29] and the E-factor, [30] which have been used since 1991 and 1992, respectively.

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[25] M. Svedendahl-Humble, P. Berglund, *Eur. J. Org. Chem*. **2011**, 3391–3401.
[26] E. Busto, V. Gotor-Fernández, V. Gotor, *Chem. Soc. Rev*. **2010**, *39*, 4504–4523.
[27] C. Branneby, P. Carlqvist, A. Magnusson, K. Hult, T. Brinck, P. Berglund, *J. Am. Chem. Soc*. **2003**, *125*, 874–875.
[28] J. Martínez, J. F. Cortés, R. Miranda, *Processes* **2022**, *10*, 1274.
[29] B. M. Trost, *Science* **1991**, *254*, 1471–1477.

Atom economy (AE) was defined by Trost as the proportion of reagents that are incorporated into the final product(s).[29] This is a theoretical concept that assumes ideal conditions, including the use of exact stoichiometric amounts of starting materials and the achievement of a 100% chemical yield. It does not consider substances like solvents and auxiliary chemicals used in product recovery, which do not appear in the stoichiometric equation. As a result, it does not fully account for the sustainability of a process. However, it is a useful metric for initial evaluation before conducting actual experiments.

The E-factor, in contrast, quantifies the complete amount of waste produced in the process. It not only takes the product yield into account, but also considers the waste generated from all auxiliary operations, such as solvent losses and chemicals used in the downstream process.[30] The E-factor is defined as the amount of waste generated (in kg) per kg of target product produced. Despite not quantifying the energy invested in the process, and representing a simple mathematical quantification of the produced waste without considering its nature, toxicity, and hazardous potential, the E-factor continues nowadays to be extensively employed by scientists as a simplified measure for assessing the environmental impact of a selected chemical process.[31,32,33]

The metrics described above consider only the mass of waste generated. However, to determine the environmental impact of waste it is important to consider not only the quantity but also their nature. To address this issue, other metrics have been introduced, such as the environmental

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[30] R. A. Sheldon, *Organic Synthesis - Past, Present and Future,* Chem. Ind., London (UK), **1992**.
[31] R. A. Sheldon, *Green Chem.* **2017**, *19*, 18–43.
[32] Y. Ni, D. Holtmann, F. Hollmann, *ChemCatChem* **2014**, *6*, 930–943.
[33] R. A. Sheldon, *Green Chem*. **2023**, *25*, 1704–1728.

quotient (EQ)[34] and life cycle assessment (LCA).[35] The former is obtained by multiplying the E-factor with an arbitrarily assigned unfriendliness quotient, denoted as Q. The latter, LCA, integrates waste quantities with quantifiable effects caused by the waste, such as global warming and smog formation.[36]

The quantification of the process sustainability through the application of these tools does not often favour biocatalysed processes over traditional chemical process. Specifically, carrying out reactions in water led to a higher value of the E-factor compared to those reactions performed in an organic solvent. This is due to the high polarity of water compared to the hydrophobic nature of most organic compounds, which means that enzymatic reactions need to be carried out under much more diluted conditions than conventional chemical transformations. Furthermore, in most cases, carrying out liquid-liquid extraction processes is necessary to isolate the product, which results in an additional use of organic solvent. Taking this into consideration, the development of biocatalytic processes in non-aqueous media is highly appealing from both an environmental and economic standpoint.

0.4. Biocatalysis in organic solvents

Enzymes have naturally evolved to function in aqueous environments, and for this reason, aqueous buffers have traditionally been the first reaction medium choice for biocatalytic reactions. While the use of aqueous

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[34] R. A. Sheldon, *CHEMTECH* **1994**, *24*, 38–47.
[35] C. Jimenez-Gonzalez, A. D. Curzons, D. J. C. Constable, V. L. Cunningham, *Int. J. Life Cycle Assess.* **2004**, *9*, 114–121.
[36] S. Arfelis Espinosa, A. Bala, P. Fullana-i-Palmer, *Green Chem.* **2022**, *24*, 7751–7762.

solutions might seem like the perfect choice due to their non-hazardous and non-toxic nature, in reality, it conceals some potential challenges such as substrate solubility and product recovery.

Moreover, the application of biocatalytic processes in purely aqueous solutions is feasible only if reagents are soluble enough to meet industrially acceptable conditions. General guidelines indicating product value have been reported, ranging from 50 g/L for high-priced products to 300 g/L for low-priced products.[37] This might be viable for most ions, sugars, or even for some high-value products that do not require high substrate concentrations. However, there is a large number of hydrophobic substrates for which solubility might be a challenge.

On the other hand, the downstream process might be challenging due to the inconvenience of product isolation by simple water evaporation, attributed to its high boiling point and associated costs. Alternatively, the product could be precipitated,[38,39] or extracted into a solvent with lower boiling point.[40],[41],[42] Nevertheless, these routes require additional optimisation to find the suitable conditions or the best extracting solvent for product isolation.

The article published in 1985 by Zaks and Klibanov,[13] demonstrating the applicability of enzymes in organic solvents, marked the beginning of non-aqueous biocatalysis. At first glance, the use of organic solvents might

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[37] J. Lima-Ramos, P. Tufvesson, J. M. Woodley, *Green Process. Synth.* **2014**, *3*, 195–213.
[38] S. Schmidt, T. P. de Almeida, D. Rother, F. Hollmann, *Green Chem*., **2017**, *19*, 1226–1229.
[39] D. Hülsewede, M. Tänzler, P. Süss, A. Mildner, U. Menyes, J. von Langermann, *Eur. J. Org. Chem*. **2018**, 2130–2133.
[40] S. Elgue, L. Prat, M. Cabassud, J. Cézerac, *Chem. Eng. J.* **2006**, *117*, 169–177.
[41] E. Papadakis, A. K. Tula, R. Gani, *Chem. Eng. Res. Des.* **2016**, *115*, 443–461.
[42] O. Fellechner, M. Blatkiewicz, I. Smirnova, *Chemie Ing. Tech.* **2019**, *91*, 1522–1543.

seem to contradict the sustainability of this approach. However, in practice, it often reduces solvent waste due to higher concentrations of starting materials. It also simplifies downstream processes, requiring fewer purification steps and potentially producing less wastewater. Moreover, waste generation can be reduced even more if the solvents are reused. Therefore, when considering the entire process, integrating organic solvents can actually lead to better environmental and economic results.[43] Moreover, using organic solvents allows the development of reactions that are sensitive to water, such as (trans)esterifications and amidations.[44] These reactions would otherwise be affected by competing hydrolysis reactions. Furthermore, it is worth noting that the choice of reaction medium might affect enzyme specifity, including potentially enhancing or inverting enantioselectivity.[45,46,47]

In some cases, small amounts of water are added to the solvent until saturation levels, resulting in a single phase system, helping to maintain enzyme activity. This added water creates a protective layer around the biocatalyst, ensuring its functionality.[48] The optimal amount of water (water activity) depends on factors such as the specific enzyme, catalyst formulation, and solvent characteristics. For instance, lipases generally require much less water compared to oxidoreductases.[49]

However, even when adding small amounts of water to prevent the removal of essential water, enzymatic rates are usually lower in organic

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[43] M. M. C. H. van Schie, J. D. Spöring, M. Bocola, P. Domínguez de María, D. Rother, *Green Chem.* **2021**, *23*, 3191–3206.
[44] A. M. Klibanov, *Acc. Chem. Res*. **1990**, *23*, 114−120.
[45] G. Carrea, G. Ottolina, S. Riva, *Trends Biotechnol*. **1995**, *13*, 63−70.
[46] A. M. Klibanov, *Nature* **2001**, *409*, 241–246.
[47] F. G. Mutti, W. Kroutil, *Adv. Synth. Catal*. **2012**, *354*, 3409–3413.
[48] P. J. *Halling, Philos. Trans. R. Soc., B* **2004**, *359*, 1287–1297.
[49] R. H. Valivety, P. J. Halling, A. R. Macrae, *FEBS Lett*. **1992**, *301*, 258–260

solvents compared to aqueous conditions for most enzyme families.[50] Among these, NAD(P)-dependent redox reactions are particularly challenging to control due to the insolubility of the polar cofactor in organic solvents. This makes it difficult to transfer hydride equivalents between the reductase and the recycling enzyme in a coupled-enzyme system.[51]

Furthermore, enzyme stability is limited under these harsh conditions, although this can be improved by modifying the biocatalyst structure and formulation, such as immobilising the enzyme.

0.5. Enzyme immobilisation

As previously mentioned, enzymes naturally function in aqueous environments. However, operational conditions in many industrial processes often involve the use of organic solvents. Thus, one of the primary challenges for biocatalysis applications in industry is the limited enzyme stability under these process conditions.[52] Twenty-five years ago, it was necessary to modify the processes to accommodate the available enzymes. Today, there are several strategies to tackle these challenges, such as directed enzyme evolution or enzyme immobilisation, aiming to optimize the enzymes for a predefined process. This Doctoral Thesis centers around the strategic implementation of enzyme immobilisation to enable the design of bioprocesses in organic solvents. Consequently, some key concepts will be explained in the following sections.

0.5.1. Advantages and disadvantages of immobilised enzymes

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[50] A. M. Klibanov, *Trends Biotechnol.* **1997**, *15*, 97−101.
[51] J. Grunwald, B. Wirz, M. P. Scollar, A. M. Klibanov, *J. Am. Chem. Soc*, **1986**, *108*, 6732–6734.
[52] A. Basso, S. Serban, *Mol. Catal.* **2019**, *479*, 110607.

Introduction

One of the widely mentioned advantages of using an immobilised enzyme is the possibility to be recovered and reused, leading to a more cost-effective process due to the potential multiple catalyst uses.[53] While this might be true for some cases, nowadays, the costs associated with large-scale enzyme production have been reduced significantly, thanks to current advances in microbial strain improvement through genetic engineering and the design of better protein overexpression systems. Additionally, the optimisation of fermentation conditions and improved enzyme recovery processes have further contributed to this cost reduction.[54,55] Moreover, alternative methods are also being developed for enzyme reuse, including the utilisation of two-liquid (aqueous-organic) systems, where the product remains in the organic phase while the enzyme is in the aqueous phase.[56,57] The soluble enzyme can be separated and recycled, as long as it remains active, by liquid-liquid separation after each reaction cycle. Consequently, in addition to enzyme recovery and reuse, additional advantages might also be needed to justify enzyme immobilisation.[58]

An important benefit of employing immobilised enzymes, which applies to all heterogeneous catalysts, is the simplification of downstream processing through simple catalyst separation methods like filtration or centrifugation.[52] Furthermore, it is easier to prevent product contamination caused by the enzyme, especially in food technology where such contamination may be highly undesirable.[59] Similarly, for pharmaceutical

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[53] R. A. Sheldon, S. van Pelt, *Chem. Soc. Rev.* **2013**, *42*, 6223–6235.
[54] A. Fasim, V. S. More, S. S. More, *Curr. Opin. Biotechnol*. **2021**, *69*, 68–76.
[55] M. Becker, S. Lütz, K. Rosenthal, *Molecules* **2021**, *26*, 573.
[56] J. M. Woodley, *Comput. Chem. Eng*. **2017**, *105*, 297–307.
[57] J. M. Bolivar, B. Nidetzky, *Green Process. Synth*. **2013**, *2*, 0091.
[58] J. M. Bolivar, J. M. Woodley, R. Fernández-Lafuente, *Chem. Soc. Rev.* **2022**, *51*, 6251–6290.
[59] O. L. Tavano, A. Berenguer-Murcia, F. Secundo, R. Fernández-Lafuente, *Compr. Rev. Food Sci. Food Saf.* **2018**, *17*, 412–436.

products, it is mandatory, according to FDA regulations, to eliminate all proteins before formulating small molecule APIs.[60]

When dealing with multi-enzyme processes (co-factor regeneration systems, and/or cascade reactions) the co-immobilisation of enzymes and cofactors offers a dual advantage. On one hand, it facilitates enzyme reusability while also enables the continuous regeneration and reuse of valuable cofactors, which can be costly.[61,62,63,64,65] Moreover, from a kinetic standpoint, co-immobilisation is beneficial because the second enzyme operates on product 1 (P1) within the pore at significantly high concentrations, eliminating the need for P1 to diffuse out of the first biocatalyst and then penetrate the second one (Figure 0.1).

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[60] M. E. Hassan, Q. Yang, Z. Xiao, L. Liu, N. Wang, X. Cui, L. Yang, *3 Biotech* **2019**, *9*, 440.
[61] A. I. Benítez-Mateos, M. L. Contente, S. Velasco-Lozano, F. Paradisi, F. López-Gallego, *ACS Sustainable Chem. Eng*. **2018**, *6*, 13151–13159.
[62] A. I. Benítez-Mateos, E. San Sebastián, N. Ríos-Lombardía, F. Morís, J. González-Sabín, F. López-Gallego, *Chem. Eur. J.* **2017**, *23*, 16843–16852.
[63] S. Velasco-Lozano, A. I. Benítez-Mateos, F. López- Gallego, *Angew. Chem., Int. Ed*. **2017**, *56*, 771–775.
[64] A. H. Orrego, D. Andrés-Sanz, S. Velasco-Lozano, M. Sánchez-Costa, J. Berenguer, J. M. Guisán, J. Rocha- Martín, F. López-Gallego, *Catal. Sci. Technol*. **2021**, *11*, 3217–3230.
[65] A. I. Benítez-Mateos, C. Huber, B. Nidetzky, J. M. Bolivar, F. López-Gallego, *ACS Appl. Mater. Interfaces* **2020**, *12*, 56027–56038.

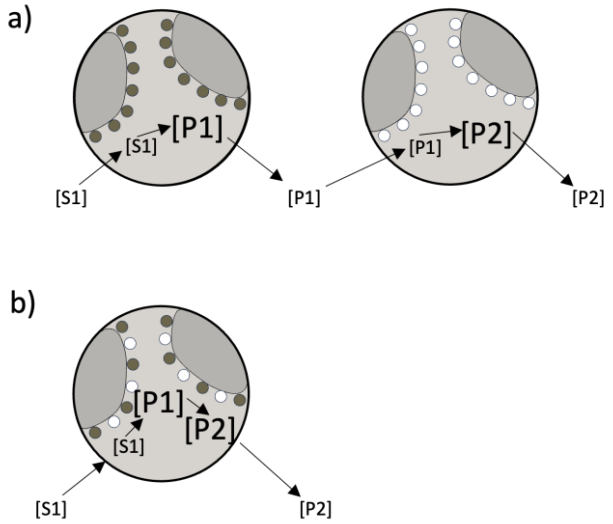

Figure 0.1. a) Enzymes immobilised on different beads. The substrate is modified by the first enzyme, the product 1 leaves the support pores and is diluted before going inside the other biocatalyst with the second enzyme immobilised within the pores; b) Enzymes co-immobilised on the same bead. The substrate is modified by the first enzyme and the second enzyme acts in the presence of high concentrations of product 1.

Furthermore, it has been demonstrated that enzyme immobilisation can enhance enzyme stability under non-optimal conditions, such as high temperatures, or the presence of organic cosolvents in the reaction media.[66,67,68,69] This not only allows the enzyme to be utilised for multiple

[66] C. García-Galán, Á. Berenguer-Murcia, R. Fernández-Lafuente, R. C. Rodrigues, *Adv. Synth. Catal.* **2011**, *353*, 2885–2904.
[67] R. C. Rodrigues, Á. Berenguer-Murcia, D. Carballares, R. Morellon-Sterling, R. Fernández-Lafuente, *Biotechnol. Adv*. **2021**, *52*, 107821.
[68] R. C. Rodrigues, C. Ortiz, Á. Berenguer-Murcia, R. Torres, R. Fernández-Lafuente, *Chem. Soc. Rev*. **2013**, *42*, 6290–6307.
[69] C. Mateo, J. M. Palomo, G. Fernández-Lorente, J. M. Guisan, R. Fernández-Lafuente, *Enzyme Microb. Technol*. **2007**, *40*, 1451–1463.

reaction cycles, but also expands the range of conditions under which the enzyme can effectively function, ultimately leading to improved process performance. In certain cases, operating reactions under non-optimal enzymatic conditions becomes essential for successful scale-up and achieving commercially relevant catalyst performance.[70,71,72] Factors such as product yields, solubility, stability of the substrate/product, and other reaction parameters, must align with the requirements of scale-up and also meet the demands of a commercially viable performance.

Immobilisation can also be combined with enzyme purification, as seen in cases such as lipase immobilisation on hydrophobic supports and tagged enzymes on supports with the corresponding affinity tag.[73,74,75] Furthermore, the suitability of a heterogeneous catalyst for flow setups, like packed bed or plug flow reactors, makes that immobilisation plays a crucial role in the ongoing shift from batch to continuous processes.[76]

In conclusion, nowadays enzyme immobilisation is receiving renewed interest as a pivotal technology for process intensification, illustrated with

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[70] G. Volpato, R. C. Rodrigues, R. Fernández-Lafuente, *Curr. Med. Chem*. **2010**, *17*, 3855–3873.
[71] R. Fernández-Lafuente, C. M. Rosell, J. M. Guisán, *Enzyme Microb. Technol*. **1991**, *13*, 898–905.
[72] R. Fernández-Lafuente, O. Hernández-Jústiz, C. Mateo, M. Terreni, G. Fernández-Lorente, M. A. Moreno, J. Alonso, J. L. Garcáa-López, J. M. Guisán, *Biomacromolecules* **2001**, *2*, 95–104.
[73] R. C. Rodrigues, J. J. Virgen-Ortiz, J. C. S. dos Santos, Á. Berenguer-Murcia, A. R. Alcántara, O. Barbosa, C. Ortiz, R. Fernández-Lafuente, *Biotechnol. Adv*. **2019**, *37*, 746–770.
[74] O. Barbosa, C. Ortiz, Á. Berenguer-Murcia, R. Torres, R. C. Rodrigues, R. Fernández-Lafuente, *Biotechnol. Adv*. **2015**, *33*, 435–456.
[75] O. Barbosa, R. Torres, C. Ortiz, Á. Berenguer-Murcia, R. C. Rodrigues, R. Fernández-Lafuente, *Biomacromolecules* **2013**, *14*, 2433–2462.
[76] M. P. Thompson, I. Peñafiel, S. C. Cosgrove, N. J. Turner, *Org. Process Res. Dev.* **2019**, *23*, 9–18.

examples related to combining enzyme purification with catalyst preparation, making in-line product isolation smoother through easier downstream processing, and enhancing reactions by utilising non-conventional reaction media or implementing continuous processes.

0.5.2. Immobilisaton technologies

There are different strategies that can be followed to immobilise enzymes. Typically, they are categorised into three main groups (Figure 0.2), which includes their binding to a support (carrier), entrapment (encapsulation) and cross-linking.

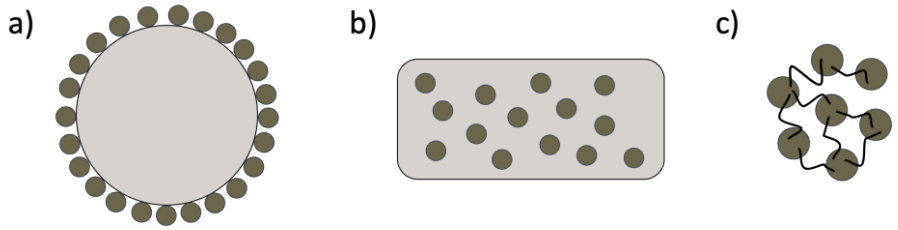

Figure 0.2. Different methodologies for enzyme immobilisation: a) Binding to a carrier; b) Entrapment or encapsulation; c) Cross-linking.

0.5.2.1. Binding to a support

The first approach, which involves binding to a support or carrier, can happen through covalent or weak non-covalent interactions.[53] On the one hand, non-covalent interactions include various physicochemical linkages, such as hydrophobic forces, van der Waals interactions, charge-charge attractions, and binding through affinity tags. On the other hand, covalent attachment entails the formation of more resistant covalent bonds between reactive groups (like epoxides and aldehydes) on the support's surface and lysine groups situated on the protein's surface.

Perhaps the most straightforward approach for protein immobilisation involves physical adsorption on pre-existing supports, through non-covalent bonds like hydrophobic forces and/or van der Waals interactions.[66] However, while adsorption offers simplicity, it often lacks the strength required to firmly anchor the enzyme to the carrier, particularly under industrial conditions characterised by elevated reactant and product concentrations, along with high ionic strength.

Ionic binding is typically stronger. For example, affinity immobilisation relies on interactions between metal chelates on the support's surface and affinity tags incorporated into the proteins. The most common tag is the poly-His tag, containing six histidine residues. This methodology enables the immobilisation of the enzyme of interest directly from the lysate, avoiding the binding of any exogenous enzymes that may be present, and therefore eliminating the need for prior purification.[77] This coupling of immobilisation and enzyme purification is particularly advantageous for industrial applications given the high cost associated with enzyme purification.

The covalent binding of enzymes to supports often utilizes surface-functionalised carriers bearing groups such as epoxides or amines. This results in the formation of robust covalent bonds with surface lysines on the enzyme, contributing to lower enzyme leaching compared to other strategies. However, this approach might have a higher degree of complexity. In most cases, preliminary support activation is necessary (with exceptions like epoxyacrylic supports such as Sepabeads or Eupergit).[66] Furthermore, reaction conditions play a pivotal role as they can lead to the

[77] K. Engelmark Cassimjee, M. Kadow, Y. Wikmark, M. Svedendahl Humble, M. L. Rothstein, D. M. Rothstein, J. E. Bäckvall, *Chem. Commun.* **2014**, *50*, 9134–9137.

inactivation of reactive groups on the support's surface. Consequently, it is advisable to implement a method for blocking any remaining free reactive groups after immobilisation.[69] Additionally, the strong bonding between the enzyme and the support has a main drawback consisting of the enzyme immobilisation in a more rigid form, potentially resulting in reduced activity of the heterogeneous catalyst. Furthermore, the irreversible deactivation of the enzyme renders both the enzyme itself and the support unusable.

Common solid supports for enzyme immobilisation include inorganic solids like silicas or zeolites, as well as synthetic resins and biopolymers such as polysaccharides. The choice of support allows for tailoring mechanical properties to suit specific reactor configurations. For example, reactor setups with mechanical stirring may need highly flexible supports, such as natural polymers like agarose or cellulose. On the other hand, fixed bed reactors benefit from rigid supports, like inorganic options such as porous glass and silicates.[78]

0.5.2.2. Entrapment or encapsulation

Enzymes that are entrapped or encapsulated are either soluble or aggregated enzymes confined within a bulk matrix. This matrix can take the form of a polymer network, often composed of organic or inorganic polymers like polyacrylamide and silica sol-gel, respectively. Alternatively, it can be a membrane-based device, such as a hollow fiber or a microcapsule.[53] Entrapment generally involves synthesising the polymeric matrix in the presence of the enzyme, theoretically allowing the enzyme to be encapsulated in its active state. However, the primary drawback of this

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[78] J. M. Guisan, *Immobilization of Enzymes and Cells*., Humana Press Inc., Totowa (USA), **2006**.

approach is that the physical restraints are generally too weak to entirely prevent enzyme leakage.[76] Hence, additional covalent attachment is often required.[79]

0.5.2.3. Cross-linking

Cross-linking involves the preparation of enzyme macroparticles using a bifunctional reagent, typically glutaraldehyde.[80] This is a carrier-free methodology and can lead to the development of cross-linked enzyme crystals (CLECs)[81] and cross-linked enzyme aggregates (CLEAs).[82] While this approach may seem simple at first glance, optimising the procedures might be tedious and often results in a poor recovery of enzyme activity.[53] For example, the preparation of CLECs involves the challenging task of protein crystallisation. In contrast, CLEAs preparation offers a simpler technological approach where aggregation can be achieved using various agents, constituting a relatively straightforward process. However, achieving consistent laboratory-made CLEAs with reproducible particle sizes is challenging, as it strongly depends on factors such as stirring rate, protein concentration, and the rate of precipitant addition.[66] Furthermore, during scaling up, recovering CLEAs can become complex due to the viscosity of certain precipitants.

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[79] L. Betancor, F. López-Gallego, A. Hidalgo, M. Fuentes, O. Podrasky, G. Kuncova, J. M. Guisan, R. Fernández- Lafuente, *Biomacromolecules* **2005**, *6*, 1027–1030.
[80] O. Barbosa, C. Ortiz, Á. Berenguer-Murcia, R. Torres, R. C. Rodrigues, R. Fernández-Lafuente, *RSC Adv.* **2013**, *4*, 1583–1600.
[81] J. J. Roy, T. E. Abraham, *Chem. Rev*. **2004**, *104*, 3705–3721.
[82] R. A. Sheldon, *Org. Process Res. Dev*. **2011**, *15*, 213–223.

Additionally, the crystalline structure of CLECs makes them both rigid and susceptible to abrasion.[81,83] Even slight agitation can result in the formation of fine powders that could pose challenges for long-term reactor management. On the other hand, CLEAs are typically considered too soft for industrial applications across various types of reactor configurations (although basket reactors may be an exception).[84]

Furthermore, the diverse immobilisation strategies can be synergistically combined to enhance catalyst performance. For instance, CLEAs or CLECs can be immobilised on a siliceous support,[85] or entrapped within a membrane.[86]

0.5.3. EziG supports

In this Doctoral Thesis, the chosen supports were those developed by EnginZyme AB (Sweden). The EziG carriers are based on controlled pore glass (CPG) coated with a functional organic polymer bearing chelating groups, thereby enabling the selective binding of metal ions (Figure 0.3).[77]

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[83] J. D. Vaghjiani, T. S. Lee, G. J. Lye, M. K. Turner, *Biocatal. Biotransform*. **2000**, *18*, 151–175.
[84] G. Sheelu, G. Kavitha, N. W. Fadnavis, *J. Am. Oil Chem. Soc*. **2008**, *85*, 739–748.
[85] J. Lee, J. Kim, H. Jia, M. I. Kim, J. H. Kwak, S. Jin, A. Dohnalkova, H. C. Park, H. N. Chang, P. Wang, J. W. Grate, T. Hyeon, *Small* **2005**, *1*, 744–753.
[86] N. Hilal, R. Nigmatullin, A. Alpatova, *J. Membr. Sci.* **2004**, *238*, 131–141.

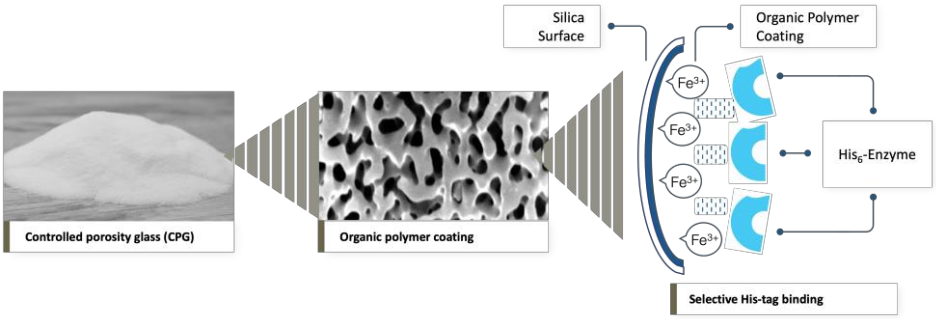

Figure 0.3. Schematic representation of EziG technology.

Proteins are attached to the support through affinity immobilisation by incorporating a His-tag at either the C- or N-terminus. Among the potential binding metal ions, Fe^{3+} was deliberately chosen for its high binding capacity, minimal environmental impact, and almost absence of toxicity.[87] Significantly, this selectivity enables direct immobilisation from the cell lysate, eliminating the need for pre-purification steps, while reaching protein loadings of up to 20% w/w.[77] CPG provides a favourable environment for enzymes, and its porous structure notably enhances the efficient mass transfer of reactants and products across the material.[77,88] Furthermore, CPG materials are generally stable in most organic solvents and aqueous media with a pH below 10.

Currently, three different types of EziG supports are available, each of them characterised by varying surface properties that differ in the degree of carrier hydrophobicity (Table 0.2): EziG-Opal has a hydrophilic surface with a long-chain aminoalkyl coating, EziG-Coral presents a hydrophobic surface attributed to its polyvinyl benzyl chloride coating, and EziG-Amber

[87] P. Domínguez de María, G. de Gonzalo Calvo, *Biocatalysis : An Industrial Perspective*, Royal Society of Chemistry., London (UK), **2022**.
[88] M. P. Thompson, S. R. Derrington, R. S. Heath, J. L. Porter, J. Mangas-Sánchez, P. N. Devine, M. D. Truppo, N. J. Turner, *Tetrahedron* **2019**, *75*, 327–334.

exhibits a semi-hydrophobic surface resulting from its copolymer of styrene and acrylonitrile coating. For a given enzyme and reaction, all three supports should be evaluated to identify the best choice for the application in question.

Table 0.2. Specifications for the three different types of EziG carriers found in the EziG product data sheet.

Carrier	Surface	Coating	Particle size [µm]	Pore diameter [nm]	pH range
Opal	Hydrophilic	Long amino alkyl chain	75-125	50±5	5-10
Coral	Hydrophobic	Polyvinyl benzyl chloride	75-125	30±5	5-10
Amber	Semi-hydrophilic	Copolymer of styrene and acrylonitrile	75-125	30±5	5-10

These carriers have been assessed for the immobilisation of various enzyme classes,[88] such as arylmalonate decarboxylases, [89] lipases, [90] norcoclaurine synthases, [91] co-immobilised dehydrogenases, [92] and

[89] M. Aßmann, C. Mügge, S. K. Gaßmeyer, J. Enoki, L. Hilterhaus, R. Kourist, A. Liese, S. Kara, *Front. Microbiol*. **2017**, *8*, 448.
[90] K. Engelmark. Cassimjee, P. Hendil-Forssell, A. Volkov, A. Krog, J. Malmo, T. E. V. Aune, W. Knecht, I. R. Miskelly, T. S. Moody, M. S. Humble, *ACS Omega* **2017**, *2*, 8674–8677.
[91] H. Lechner, P. Soriano, R. Poschner, H. C. Hailes, J. M. Ward, W. Kroutil, *Biotechnol. J.* **2018**, *13*, 1700542.

hydroxynitrile lyases.[93] Furthermore, they have demonstrated their suitability for diverse applications, including reactions in organic solvents and continuous processes.[94,95]

0.5.3. Immobilisation metrics

The terminology used to evaluate enzyme immobilisation can frequently be inconsistent and/or confusing.[53] In this context, this section aims to provide clear and comprehensive definitions for the metrics employed in this Doctoral Thesis: immobilisation yield (IY), protein loading (PL) and recovered activity (RA).

The immobilisation yield (IY) describes the percentage of enzyme that bounds to the support from the provided enzyme solution. Typically, this yield is calculated by assessing the remaining enzyme activity in the solution after immobilisation and then deducting this activity from the initial activity (Equation 0.1). It is advisable to conduct a blank experiment (using only the enzyme solution without the support material) in parallel with the immobilisation process to account for potential free enzyme deactivation under the immobilisation conditions.

$$\text{IY}\,[\%] = \frac{\text{Activity of free enzyme} - \text{Activity of supernatant}}{\text{Activity of free enzyme}} \times 100 \qquad \text{(Equation 0.1)}$$

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[92] W. Böhmer, T. Knaus, F. G. Mutti, *ChemCatChem* **2018**, *10*, 731–735.
[93] J. Coloma, T. Lugtenburg, M. Afendi, M. Lazzarotto, P. Bracco, P. L. Hagedoorn, L. Gardossi, U. Hanefeld, *Catalysts* **2020**, *10*, 899.
[94] W. Böhmer, T. Knaus, A. Volkov, T. K. Slot, N. R. Shiju, K. Engelmark Cassimjee, F. G. Mutti, *J. Biotechnol.,* **2019**, *291*, 52–60.
[95] W. Böhmer, A. Volkov, K. Engelmark Cassimjee, F. G. Mutti, *Adv. Synth. Catal.,* **2020**, 1858–1867.

Introduction

Protein loading (PL) refers to the quantity of the target protein immobilised on the support material (Equation 0.2). In this Doctoral Thesis, enzymes are immobilised from CFEs, rather than pure protein solutions. Consequently, the content of the target protein within the CFE must be determined. To achieve this, a portion of the CFE is purified to obtain a pure protein solution containing only the target enzyme. By quantifying the amount of the target protein within this purified solution, the concentration of the target protein in the CFE can be obtained. Using this data, along with the immobilisation yield, allows for the calculation of target protein loading on the support material.

$$\text{PL [\% w/w]} = \frac{\text{Amount of target protein offered (mg)} \times \text{IY (\%)}}{\text{Amount of support (mg)}} \qquad \text{(Equation 0.2)}$$

Neither the immobilisation yield nor protein loading takes into consideration the activity of the immobilised enzyme; in other words, both metrics only account for the quantity of target protein that becomes immobilised, whether in its active or inactive state. Therefore, the concept of recovered activity (RA) is introduced to address the actual enzymatic activity of the immobilised enzyme. It is a comparison of the enzymatic activity between immobilised and free states. The calculations include the immobilisation yield to exclusively assess the quantity of immobilised enzyme, rather than considering the total amount offered to the support material. Recovered activity is obtained by comparing the specific activity of the immobilised enzyme to that of the CFE in an aqueous buffer (Equation 0.3). Specific activity is defined as the activity per mg of target protein and it is determined here from initial reaction rates, taking into account the quantity of enzyme used in the reaction.

$$\text{RA [\%]} = \frac{\text{Specific activity of immobilised enzyme}}{\text{Specific activity of the free enzyme}} \times 100 \qquad \text{(Equation 0.3)}$$

GENERAL OBJECTIVES

The Introduction of this Doctoral Thesis highlighted the potential of enzymes as catalysts for sustainable and greener processes, describing various indicators used to evaluate process sustainability. Moreover, the section emphasised the primary advantages of enzyme immobilisation, with a particular focus on detailing the EziG supports used in this Doctoral Thesis. Enzyme immobilisation is described as a key technology that enables the implementation of enzymatic reactions in organic solvents, addressing a recurring problem encountered when implementing biocatalytic processes at an industrial scale - the low solubility of substrates in aqueous solutions. Considering this context, this Doctoral Thesis focuses on the application of enzyme immobilisation to facilitate enzymatic reactions in organic solvents.

N-Substituted furfuryl amines hold significant importance as precursors for biologically active compounds. Therefore, there is substantial interest in developing sustainable and highly selective synthetic pathways for their synthesis and for the obtention of N-protected furfuryl amines. Chapter 1 and 2 are dedicated to the biocatalytic modification of bio-based furan derivatives. Specifically, Chapter 1 describes the chemoselective synthesis of acylated derivatives from furfurylamine (FA) and 5-hydroxymethylfurfurylamine (HMFA), using *Candida antarctica* lipase type B (CALB) immobilised on various commercial supports. The presence of both amine and hydroxyl functional groups in HMFA opens up a wide array of synthetic opportunities, facilitating the formation of a diverse range of N- or O-protected compounds. On the other hand, Chapter 2 delves into the transamination of 5-hydroxymethylfurfural (HMF) using various transaminases, with a primary emphasis on optimising the reaction regarding the amount of amino donor and biocatalyst concentration. Moreover, three of the transaminases that exhibited the most promising outcomes under homogeneous conditions in an aqueous buffer were immobilised to evaluate the feasibility of the reaction in organic solvent.

General Objectives

The combination of transaminases and lipases offers a very attractive approach for the formation of chiral nitrogenated compounds. However, there are only a limited number of examples reported in literature. Therefore, this chapter presents the design of a bienzymatic cascade combining the transaminase *Arthrobacter* sp. variant 11 (ArRmut11), and CALB, chosen for the stereoselective synthesis of (*R*)-*N*-(1-phenoxypropan-2-yl)acetamide from 1-phenoxypropan-2-one. The bienzymatic process is executed under both batch and continuous conditions for comprehensive evaluation.

The incorporation of redox enzymatic reactions in organic solvents presents an additional challenge, the cofactor recycling in this environment. Consequently, Chapter 4 is centred on the development of two redox enzymes, namely alkene reductase (ERED) and alcohol dehydrogenase (ADH), for the selective reduction of unsaturated ketones within an organic solvent. The immobilisation process and post-immobilisation methods need to be optimised for both enzymes to enhance their suitability for use in organic solvents. Since the spatial proximity of the two enzymes is crucial when cofactor recycling is involved, the co-immobilisation of both enzymes is also explored.

CHAPTER 1.

Chemoselective Lipase-Catalysed Synthesis of Amido Derivatives from 5-Hydroxymethylfurfurylamine

Introduction

1.1.1. Lipases

Lipases belong to the hydrolase family of enzymes (EC 3.1.1.3) and are well-known for their ability to catalyse ester bond hydrolysis using water as nucleophile.[96] In living organisms, lipases play a crucial role in the digestion and absorption of dietary fats by breaking down triglycerides into glycerol and fatty acids.

From a structural perspective, lipases have a compact globular fold that exhibits a characteristic α/β hydrolase fold. This fold contains a catalytic triad composed of serine, histidine, and acidic (aspartic or glutamic) residues, which are responsible for the catalytic activity of the enzyme. The general mechanism of lipases involves several stages (Scheme 1.1): substrate binding, activation of a serine residue, nucleophilic attack, formation of an acyl-enzyme intermediate and subsequent hydrolysis, and finally, product release while the enzyme starts a new catalytic cycle.

The catalytic cycle begins when the substrate binds to the active site of the enzyme, triggering the serine deprotonation by the adjacent histidine residue. This generates a reactive serine residue that acts as a nucleophile and attacks the carbonyl carbon of the ester bond in the substrate, leading to the formation of a covalent acyl-enzyme intermediate. In hydrolytic reactions, a water molecule enters the active site and interacts with the histidine residue within the catalytic triad. This interaction prompts the deprotonation of the water molecule. Subsequently, the deprotonated water acts as a nucleophile and attacks the acyl-enzyme intermediate, leading to the hydrolysis of the ester bond. This hydrolysis step results in the release of the fatty acid moiety from the enzyme and regenerates the active form of the lipase.

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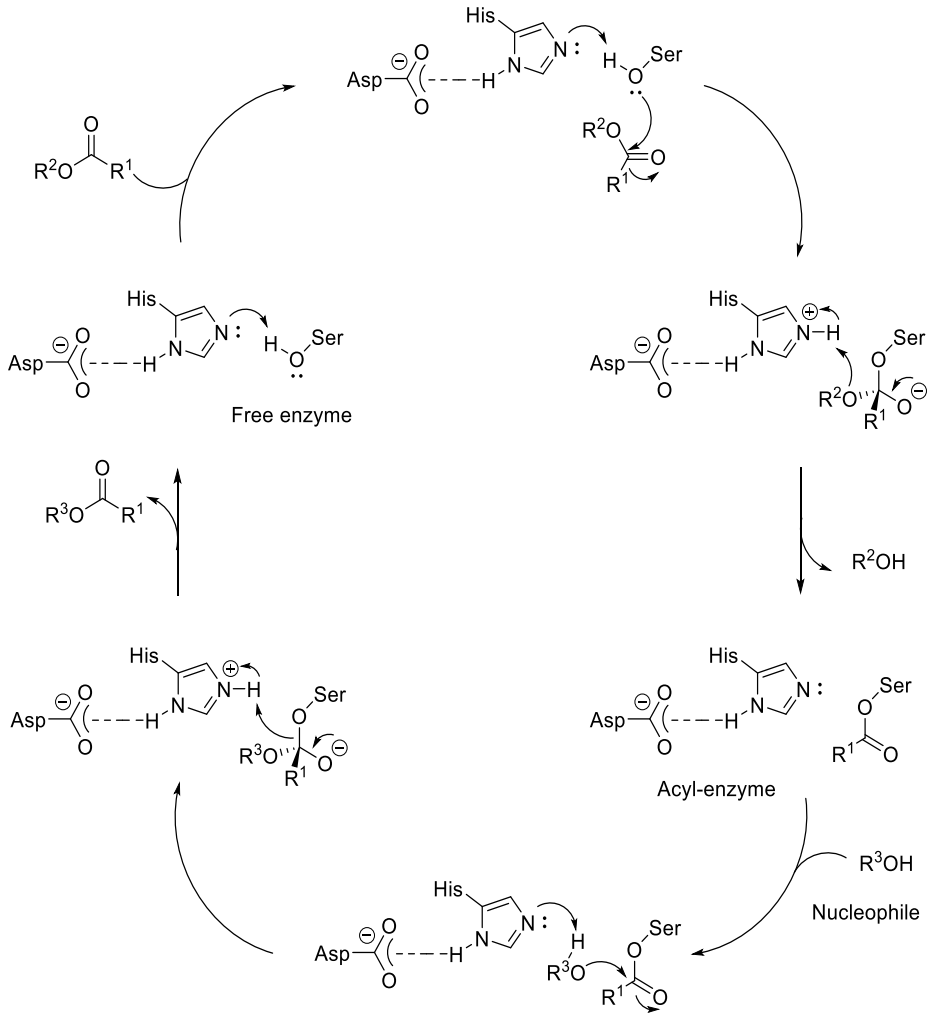
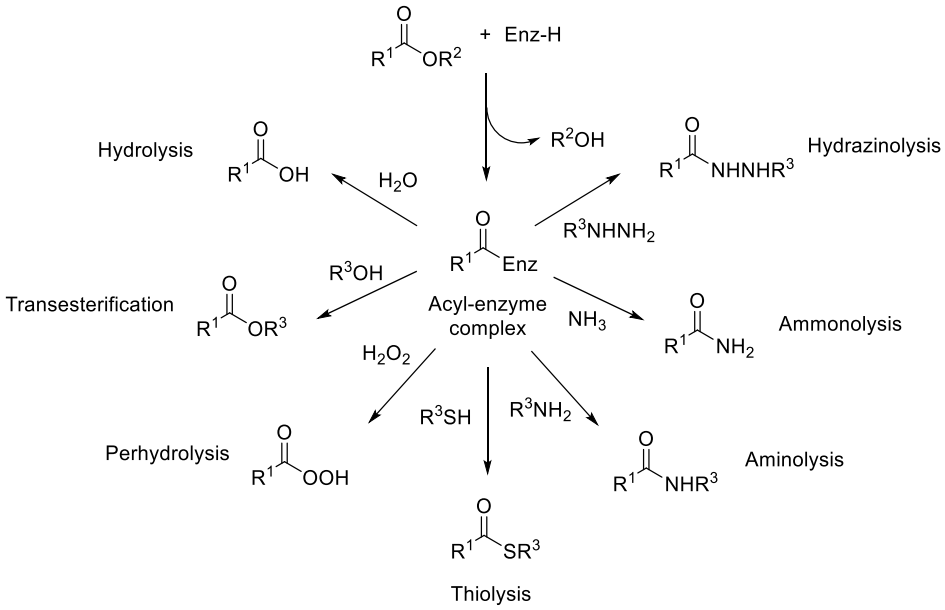
[96] R. D. Schmid, R. Verger, *Angew. Chem. Int. Ed.* **1998**, *37*, 1608–1633.



Scheme 1.1. Mechanism of lipase-catalysed reaction.

Beyond their natural role, lipases also have a significant synthetic potential since they can, depending on the nucleophile of choice, effectively catalyse (trans)esterification, aminolysis, ammonolysis, hydrazinolysis,

thiolysis and also perhydrolysis reactions (Scheme 1.2).[97] In these cases, the mechanism is the same as the one explained for the hydrolysis but the natural nucleophile water is replaced by an alcohol, amine, ammonia, hydrazine, thiol or hydroperoxide.



Scheme 1.2. Lipase-catalysed biotransformations of an ester in organic solvents.

1.1.1.1. Candida antarctica lipase B (CALB)

[97] V. Gotor-Fernández, R. Brieva, V. Gotor, *J. Mol. Catal. B Enzym.* **2006**, *40*, 111–120.

CALB is a monomeric enzyme with a size of 33 kDa and a polypeptide chain composed of 317 amino acid residues.[98] Structurally, CALB is a member of the α/β protein family, characterised by a central β-sheet composed of 9 β-strands, surrounded by 10 α-helices (Figure 1.1a).[99,100] The active site, where catalysis occurs, is formed by the catalytic triad of Ser^{105}, His^{224}, and Asp^{187}, and it is located at a sharp turn, known as the nucleophile elbow, situated between the start of an α-helix and the end of an adjacent β-strand.

A typical feature of lipases is the presence of an amphipathic surface loop/α-helix, often referred to as the "lid", "cap", or "flap".[96] This lid covers the catalytic site in its closed (inactive) conformation and upon contact with a hydrophobic surface swings to expose the site in its open (active) conformation, facilitating the accessibility of substrates (Figure 1.1b). This mechanism of action is known as interfacial activation, and it allows the adsorption of lipases to hydrophobic surfaces such as glycerides, their natural substrates.[101]

The existence of a lid in CALB structure has been a subject of debate until 2015, when its crystal structure was elucidated at a high resolution of 0.91 Å.[102] This study revealed that the CALB contains both open and closed conformations within the same asymmetric unit. Unlike typical lipases, CALB does not have a conventional lid, instead, its α-helices 5 and 10 function similarly to a lipase lid, responsible for the closed conformation of

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[98] Z. Tan, G. Chen, S. Chen, J. Zhang, J. Liu, X. Ma, H. Liao, Z. Hu, F. Ge, F. Ju, H. Shi, M. Bilal, *Mol. Catal.* **2023**, *546*, 113271.
[99] J. Uppenberg, M. T. Hansen, S. Patkar, T. A. Jones, *Structure* **1994**, *2*, 293–308.
[100] J. Uppenberg, N. Ohmer, M. Norin, K. Hult, G. J. Kleywegt, S. Patkar, V. Waagen, T. Anthonsen, T. A. Jones, *Biochemistry* **1995**, *34*, 16838–16851.
[101] R. Verger, *Trends Biotechnol.* **1997**, *15*, 32–38.
[102] B. Stauch, S. J. Fisher, M. Cianci, *J. Lipid Res.* **2015**, *56*, 2348–2358.

CALB. Even with that small lid, CALB retains its capacity to be adsorbed on hydrophobic surfaces, so it is considered an interfacial enzyme.[103]

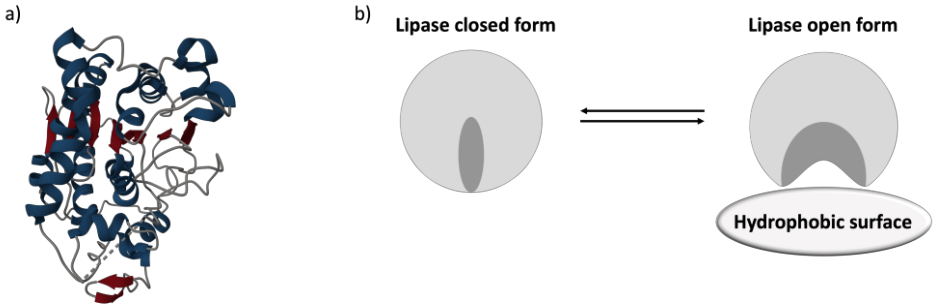

Figure 1.1. a) Crystal structure of CALB (PDB ID: 4K6G) displayed in cartoon representation and showing α-helices (blue), β-sheets (red) and loop regions (grey). b) Representation of closed and open conformation of lipases showing the interfacial activation.

1.1.2. EziG-CALB

Lipases, in general, are enzymes with broad applicability in the industry, including the production of cosmetic ingredients,[104] active pharmaceutical ingredients (APIs) and their intermediates,[105,106] as well as

[103] C. Ortiz, M. L. Ferreira, O. Barbosa, J. C. S. Dos Santos, R. C. Rodrigues, Á. Berenguer-Murcia, L. E. Briand, R. Fernandez-Lafuente, *Catal. Sci. Technol.* **2019**, *9*, 2380–2420.
[104] M. B. Ansorge-Schumacher, O. Thum, *Chem. Soc. Rev.* **2013**, *42*, 6475–6490.
[105] A. C. L. D. M. Carvalho, T. D. S. Fonseca, M. C. De Mattos, M. D. C. F. De Oliveira, T. M. L. G. De Lemos, F. Molinari, D. Romano, I. Serra, *Int. J. Mol. Sci.* **2015**, *16*, 29682–29716.
[106] F. J. Contesini, M. G. Davanço, G. P. Borin, K. G. Vanegas, J. P. G. Cirino, R. R. de Melo, U. H. Mortensen, K. Hildén, D. R. Campos, P. de O. Carvalho, *Catalysts* **2020**, *10*, 1–33.

the production of modified compounds for food and nutraceuticals.[107]In this sense, CALB is one of the most widely employed ones due to its excellent stereoselectivity control and stability.[108,109,110] Moreover, extensive research has been conducted on the immobilisation of CALB, which is a crucial requirement for its effective use in organic solvents for synthetic applications.[111,112] Notably, the immobilisation of CALB on hydrophobic resins is a very straightforward process, owing to its specific surface properties, retaining remarkable activity and stability even at elevated temperatures in organic solvents.

Novozyme 435 is the most widely used commercially available preparation of CALB.[103] It is immobilised on a macroporous acrylic polymer resin called Lewatit VP OC 1600 by adsorption through interfacial activation. However, it should be noted that this method can also lead to the immobilisation of certain aggregates.[113,114,115] Novozyme 435 serves as a highly effective biocatalyst for various industrial reactions such as biodiesel production,[116] the generation of enantiomerically enriched ketoprofen,[117] and the synthesis of glycerol carbonate.[118]

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[107] A. L. Reyes-Reyes, F. V. Barranco, G. Sandoval, *Catalysts* **2022**, *12*, 1–24.
[108] E. M. Anderson, K. M. Larsson, O. Kirk, *Biocatal. Biotransform*. **1998**, *16*, 181–204.
[109] V. Gotor-Fernández, E. Busto, V. Gotor, *Adv. Synth. Catal.* **2006**, *348*, 797–812.
[110] O. Kirk, M. W. Christensen, *Org. Process Res. Dev.* **2002**, *6*, 446–451.
[111] K. F. Schilke, C. Kelly, *Biotechnol. Bioeng.* **2008**, *101*, 9–18.
[112] P. Adlercreutz, *Chem. Soc. Rev.* **2013**, *42*, 6406–6436.
[113] B. Chen, J. Hu, E. M. Miller, W. Xie, M. Cai, R. A. Gross, *Biomacromolecules* **2008**, *9*, 463–471.
[114] B. Chen, M. E. Miller, R. A. Gross, *Langmuir* **2007**, *23*, 6467–6474.
[115] Y. Mei, L. Miller, W. Gao, R. A. Gross, *Biomacromolecules* **2003**, *4*, 70–74.
[116] Y. Shimada, Y. Watanabe, T. Samukawa, A. Sugihara, H. Noda, H. Fukuda, Y. Tominaga, *J. Am. Oil Chem. Soc.* **1999**, *76*, 789−793.
[117] A. L. Ong, A. H. Kamaruddin, S. Bhatia, *Process Biochem*. **2005**, *40*, 3526−3535.
[118] S. C. Kim, Y. H. Kim, H. Lee, D. Y. Yoon, B. K. Song, *J. Mol. Catal. B Enzym*. **2007**, *49*, 75−78.

Despite its broad application, several concerns have arisen regarding Novozyme 435 application.[103] One common issue in biocatalysts prepared using interfacial activation is the potential leaching of the enzyme at high temperatures or in the presence of organic cosolvents and detergents.[119] This problem is particularly relevant when lipases are used, because in many cases, the substrates/products are detergent-like molecules (e.g., fatty acids, partial glycerides, and phospholipids).[120] Another challenge arises from the hydrophilic nature of the support, which can lead to the retention of hydrophilic by-products, such as water or glycerin, within the biocatalyst, causing apparent enzyme inactivation.[121] Furthermore, the support material may dissolve in certain media, leading to both enzyme leaching and product contamination with polymeric components from the support.[122,123]

An alternative option is offered by EnginZyme AB (Sweden), where CALB is immobilised on EziG, a material based on controlled pore glass (section 0.5.3).[77] As previously described, this immobilisation approach allows for simultaneous purification directly from crude extracts or culture media. Thus, the immobilisation procedure was streamlined by doing the immobilisation from the culture medium, where CALB is secreted after expression in *E. coli* BL21.[90] The results, measured in terms of activity (TBU/g), were compared to the ones obtained with CALB immobilised on EziG from cell free extract (CFE) and Novozyme 435. Among the available

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[119] N. Rueda, J. C. S. dos Santos, R. Torres, C. Ortiz, O. Barbosa, R. Fernández-Lafuente, *RSC Adv*. **2015**, *5*, 11212–11222.
[120] J. J. Virgen-Ortíz, V. G. Tacias-Pascacio, D. B. Hirata, B. Torrestiana-Sanchez, A. Rosales-Quintero, R. Fernandez-Lafuente, *Enzyme Microb. Technol.* **2017**, *96*, 30–35.
[121] E. Séverac, O. Galy, F. Turon, C. A. Pantel, J. S. Condoret, P. Monsan, A. Marty, *Enzyme Microb. Technol.*, **2011**, *48*, 61–70.
[122] C. José, R. D. Bonetto, L. A. Gambaro, M. P. Guauque Torres, M. L. Foresti, M. L. Ferreira, L. E. Briand, *J. Mol. Catal. B: Enzym.* **2017**, *71*, 95–107.
[123] C. José, L. E. Briand, *React. Kinet., Mech. Catal*. **2010**, *99*, 17–22.

supports from EnginZyme, EziG Amber was chosen as it demonstrated the highest efficiency.[90]

The EziG preparation made from the culture medium displayed a similar activity compared to Novozyme 435, whereas the EziG preparation from the CFE showed more than eight-fold higher activity (Table 1.1).[90] This difference in activity between samples prepared from culture medium and CFE could be caused by the binding of contaminants in the former case, leading to higher occupied space on carrier surface and resulting in lower enzyme loading. The difference between Novozyme 435 and EziG-CALB could be due to more efficient mass transfer through EziG, surpassing the commonly used porous acrylic beads.

Table 1.1. Enzyme activities of CALB immobilised on EziG Amber (from CFE and from culture medium) compared to Novozyme 435.[90]

Immobilised CALB preparation	Standard assay (TBU/g)
EziG-CALB (from CFE)	21000
EziG-CALB (from culture medium)	2300
Novozyme 435	2500

1.1.3. HMF and derivatives

1.1.3.1. Production and applications

The conversion of lignocellulosic biomass into furan derivatives offers a highly promising approach for the production of biofuels and

valuable chemicals, such as adhesives and polymers.[124,125] Among these derivatives, 5-hydroxymethylfurfural (HMF, Scheme 1.3) is considered a key molecule for biomass valorisation and serves as a versatile synthetic building block.[126,127] The selective modification of its primary hydroxyl and formyl groups enables the production of a wide range of valuable compounds.[128] Chemical oxidations of HMF provide access to 2,5-diformylfuran (DFF), 5-hydroxymethyl-2-furancarboxylic acid (HMFCA), 5-formyl-2-furancarboxylic acid (FFCA), and 2,5-furandicarboxylic acid (FDCA).[129,130,131] Furthermore, reduction of HMF leads to the formation of 2,5-bis(hydroxymethyl)furan (BHMF), while amination of HMF results in the synthesis of 5-hydroxymethyl-2-furfurylamine (HMFA).[132] These transformations demonstrate the versatile nature of HMF as a precursor for various valuable compounds derived from lignocellulosic biomass.

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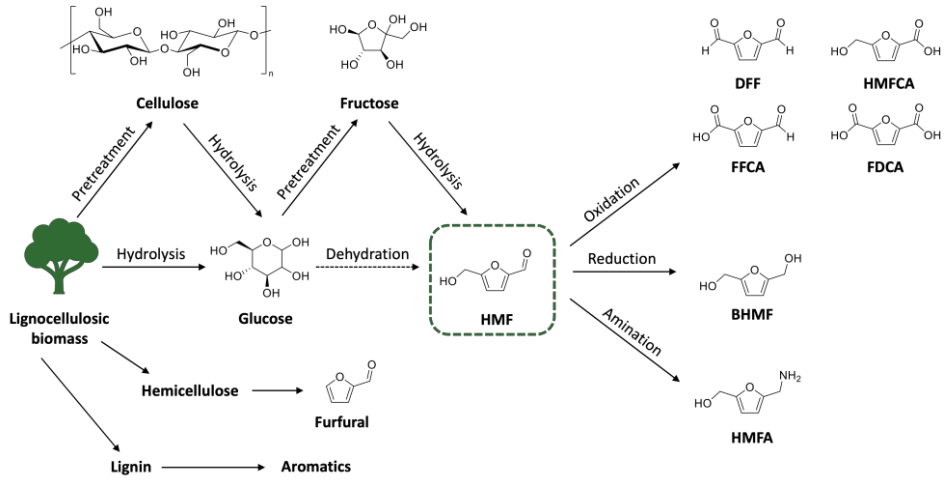
[124] Q. Hou, X. Qi, M. Zhen, H. Qian, Y. Nie, C. Bai, S. Zhang, X. Bai, M. Ju, *Green Chem.* **2021**, 119–231.
[125] C. Rosenfeld, J. Konnerth, W. Sailer-Kronlachner, T. Rosenau, A. Potthast, P. Solt, H. W. G. van Herwijnen, *ChemSusChem* **2020**, *13*, 5408–5422.
[126] A. A. Rosatella, S. P. Simeonov, R. F. M. Frade, C. A. M. Afonso, *Green Chem.* **2011**, *13*, 754–793.
[127] C. Xu, E. Paone, D. Rodríguez-Padrón, R. Luque, F. Mauriello, *Chem. Soc. Rev.* **2020**, *49*, 4273–4306.
[128] X. Kong, Y. Zhu, Z. Fang, J. A. Kozinski, I. S. Butler, L. Xu, H. Song, X. Wei, *Green Chem.* **2018**, *20*, 3657–3682.
[129] Z. Zhang, K. Deng, *ACS Catal.* **2015**, *5*, 6529–6544.
[130] G. Totaro, L. Sisti, P. Marchese, M. Colonna, A. Romano, C. Gioia, M. Vannini, A. Celli, *ChemSusChem* **2022**, *15*, e202200501.
[131] P. H. Tran, *ChemSusChem* **2022**, *15*, e202200220.
[132] C. C. Truong, D. K. Mishra, Y. W. Suh, *ChemSusChem* **2023**, *16*, e202201846.



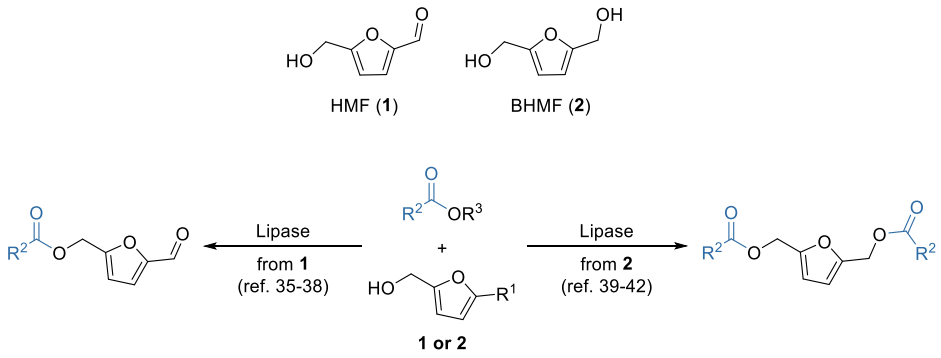
Scheme 1.3. Basic framework of the biorefinery process based on HMF as key building block for the production of valuable bio-based products.

1.1.3.2. HMF and derivatives valorisation with lipases

Among possible HMF valorisation products, a broad range of HMF esters can be synthesised using both enzymatic and chemical methods. In fact, these ester derivatives have demonstrated promising applications across diverse industries, including their use as monomers, fuels additives, surfactants, and fungicides.[133,134,135,136,137] Given the challenging reactivity of HMF, there is a strong interest in developing mild reaction conditions that minimise the formation of undesirable by-products. Consequently, the

[133] P. Mäki Arvela, E. Salminen, T. Riitonen, P. Virtanen, N. Kumar, J. P. Mikkola, *Int. J. Chem. Eng*. **2012**, ID674761.
[134] T. Thananatthanachon, T. B. Rauchfuss, *Angew. Chem.* **2010**, *122*, 6766–6768.
[135] G. J. M. G. Gruter, US2010/0212218, **2010**.
[136] A. J. Sanborn, US2009/0156841, **2009**.
[137] M. Mascal, US2009/ 0234142, **2009**.

use of lipases has been widely investigated for synthesising HMF derivatives through (trans)esterification reactions in recent years (Scheme 1.4).



Scheme 1.4. Lipase-catalysed transformations using HMF (**1**) and BHMF (**2**) as starting materials.

The reaction between HMF and various esters or carboxylic acids provides a selective method for functionalising the hydroxyl group while preserving the aldehyde functionality unaffected.[138,139,140,141] For instance, commercial immobilised CALB from c-LEcta has been employed to catalyse the (trans)esterification reaction between HMF and different acyl donors including carboxylic acids, natural oils, carbonates, methyl esters, and ethyl esters.[138] Notably, high yields were achieved in most cases, even at high HMF concentrations and in solvent-free conditions. Another notable example is the synthesis of HMF levulinate, which involves upgrading both

[138] M. Krystof, M. Pérez-Sánchez, P. Domínguez de María, *ChemSusChem* **2013**, *6*, 630–634.
[139] M. Krystof, M. Pérez-Sánchez, P. Domínguez de María, *ChemSusChem* **2013**, *6*, 826–830.
[140] Y. Z. Qin, M. H. Zong, W. Y. Lou, N. Li, *ACS Sustain. Chem. Eng.* **2016**, *4*, 4050–4054.
[141] J. Uribe, M. E. Lienqueo, N. Guajardo, *Catalysts* **2023**, *13*, 19.

HMF and levulinic acid, another essential platform chemical derived from biomass.[140] The enzymatic esterification process using Novozyme 435 has shown promising outcomes in various solvents such as *tert*-butanol, 2-methyl-2-butanol, cyclopentyl methyl ether, as well as the environmentally friendly biomass-derived 2-methyltetrahydrofuran (2-MeTHF). The resulting product, HMF levulinate exhibits high potential for use as a fuel additive. Additionally, the levulinyl group serves as a valuable protecting group for hydroxyl groups, enabling the utilisation of HMF levulinate as a versatile precursor for further upgrades of HMF.

Moreover, lipases have been utilised in the non-selective (trans)esterification of BHMF, which contains two alcohol moieties. Typically, these reactions lead to the formation of the corresponding diesters that offer potential applications as reliable additives for lower grade biodiesels.[142,143,144,145]

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[142] M. A. Lăcătuş, L. C. Bencze, M. I. Toşa, C. Paizs, F. D. Irimie, *ACS Sustain. Chem. Eng.* **2018**, *6*, 11353–11359.
[143] S. Baraldi, G. Fantin, G. Di Carmine, D. Ragno, A. Brandolese, A. Massi, O. Bortolini, N. Marchetti, P. P. Giovannini, *RSC Adv.* **2019**, *9*, 29044–29050.
[144] K. S. Arias, J. M. Carceller, M. J. Climent, A. Corma, S. Iborra, *ChemSusChem* **2020**, *13*, 1864–1875.
[145] M. A. Lăcătuş, A. I. Dudu, L. C. Bencze, G. Katona, F. D. Irimie, C. Paizs, M. I. Toşa, *ACS Sustain. Chem. Eng.* **2020**, *8*, 1611–1617.

Objectives

Despite extensive investigations involving HMF and BHMF, little research has been done towards the synthesis of the corresponding amino alcohol derivative, HMFA (**3**). The presence of both amine and hydroxyl functional groups in HMFA offers a plethora of synthetic possibilities, enabling the production of a wide range of N- or O-protected compounds. Based on the excellent activity and selectivity displayed by lipases in amide formation under mild conditions, in this chapter a thorough investigation has been carried out towards the selective lipase-catalysed acylation of the HMFA primary amine group. The study had the following specific objectives:

- Find a suitable lipase and solvent using furfurylamine (FA, **4**) as model substrate, given its broad commercial availability and cost-effectiveness. To use the best conditions found using the model substrate for the reaction with the target substrate, HMFA.

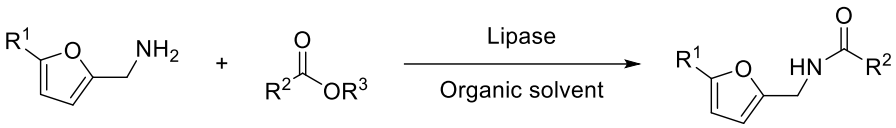
- Study the versatility of the lipase-catalysed acylation of HMFA using different acyl donors, bearing a variety of structural motifs, and to obtain a series of HMFA amide derivatives.

- Demonstrate the potential of the developed methodology by scaling-up the selective N-acetylation of HMFA to gram scale, and to assess the sustainability of the process with an environmental impact assessment.

- Explore the possibility of accessing orthogonally protected amido esters using other acyl donors for the O-acylation.

Results

In this study, the synthesis of a wide range of furfuryl amide derivatives is disclosed as depicted in Scheme 1.5. Initially, FA (**4**) will be considered as a benchmark substrate to test the activity of different lipases. The best candidates found in these initial tests will be used later for the production of HMFA (**3**) amide derivatives under optimized reaction conditions.



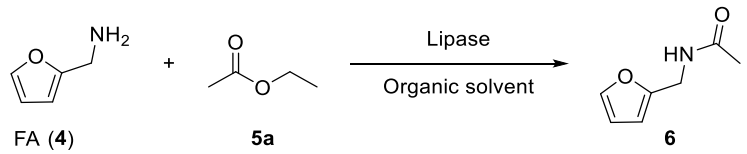
R^1=CH_2OH (**3**) or H (**4**)

Scheme 1.5. Lipase catalysed tranformations using HMFA (**3**) or FA (**4**).

1.2.1. Furfurylamine

Initially, a series of tests were conducted using FA (**4**) as the model substrate (Scheme 1.6). This selection was influenced by its broad commercial availability and cost-effectiveness. Additionally, the presence of only one reactive group in FA simplified the process of identifying lipases capable of acylating the primary amino group. Considering the high reactivity of amines, non-activated acylating agents were used to prevent any unwanted background reactions.[146] Consequently, ethyl acetate (EtOAc) was selected as the acyl donor for preliminary screenings performed in methyl *tert*-butyl ether (MTBE) as solvent.

[146] V. Gotor-Fernandez, V. Gotor, *Curr. Org. Chem.* **2006**, *10*, 1125–1143.



Scheme 1.6. Lipase-catalysed acetylation of FA (**4**) using ethyl acetate (EtOAc, **5a**) as acyl donor (**5a**).

1.2.1.1. Enzyme and solvent screening

Initially, a lipase screening was conducted under standard reaction conditions (3 equiv of EtOAc, MTBE, 30 ºC, 24 h and 250 rpm) to synthesize *N*-(furan-2-ylmethyl)acetamide (**6**). Among all the tested lipases under these specific conditions, CALB proved to be the most effective biocatalyst (Figure 1.2a). Remarkably, two different CALB formulations (Novozyme 435 and EziG-CALB) resulted in complete conversion into the acetamide **6**, even without any specific optimisation of the reaction parameters. These CALB immobilised catalysts demonstrated superior performance compared to the other screened lipases: *Thermomyces lanuginosus* (TLL), *Pseudomonas cepacia* (PSL), *Pseudomonas fluorescens* (AKL), *Candida rugosa* (CRL), *Candida antarctica* lipase type A (CALA) and *Aspergillus niger* (ANL). All of them were used as immobilised formulations obtained from the corresponding commercial supplier, except for AKL, that was used as lyophilised powder (see section 1.3.1.1).

To further explore the advantages of both CALB preparations, a time study was conducted. It was found that a short reaction time of 30 min led to conversions above 90% for both catalysts, while only 2 h were needed to achieve complete conversion. As part of a collaborative project between the University of Oviedo and the EnginZyme company inside the INTERfaces EU project, the synthetic potential of EziG-CALB was investigated more in detail. Thus, the use of this biocatalyst was prioritised, and a solvent screening was carried out (Figure 1.2b). From the eight tested solvents, complete conversions were achieved within 2 h using MTBE, Et_2O, EtOAc and 2-MeTHF. Notably, the latter solvent has been previously recognised as

an excellent biorenewable medium for hydrolase catalysed reactions,[147] , so from this point 2-MeTHF was selected for further studies.

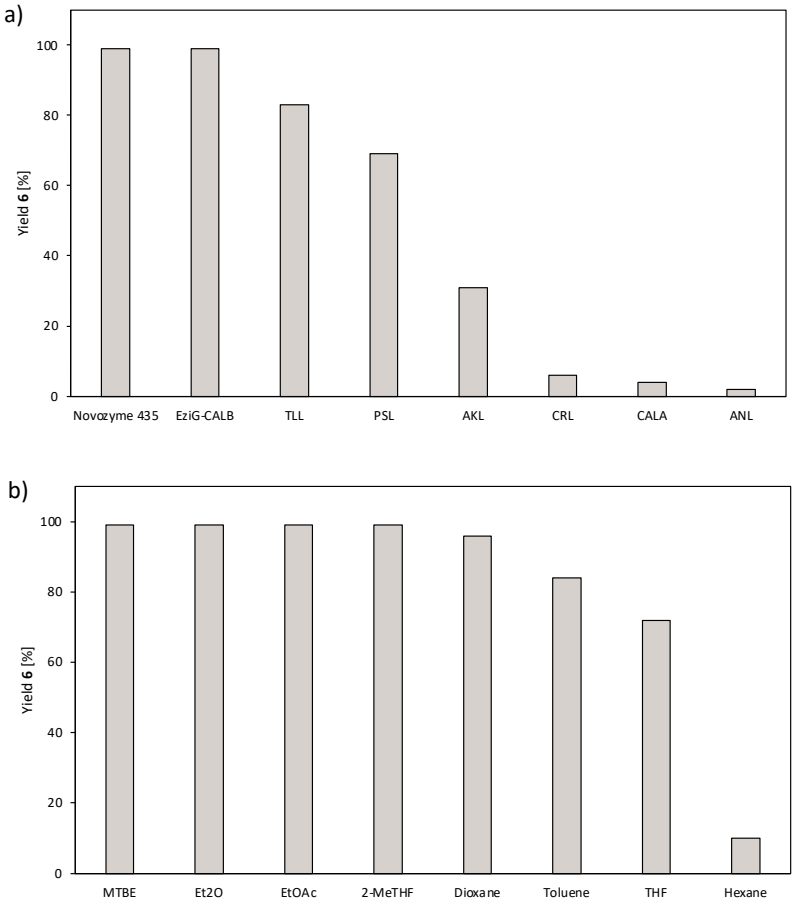


Figure 1.2. Lipase-catalysed acetylation of FA (**4**). A) Under standard conditions [100 mM of **4** in MTBE, 3 equiv of **5a** and lipase:**4** (1:1 w/w) at 30 ºC and 250 rpm for 24 h]; b) Solvent screening using 100 mM of **4**, 3 equiv of **5a** and EziG-CALB (1:1 w/w enzyme:**4**) at 30 ºC and 250 rpm for 2 h.

[147] A. R. Alcantara, P. Domínguez de María, *Curr. Green Chem.* **2018**, *5*, 86–103.

1.2.1.2. EziG-CALB recycling studies

To further investigate the capabilities of the EziG-CALB preparation, a recyclability study was performed by repeating the acetylation of furfurylamine over ten cycles. The objective was to assess the stability and reusability of the EziG-CALB catalyst over multiple reaction cycles, which are crucial factors in determining the potential implementation of a catalyst for industrial applications.

To ensure the detection of any catalyst deactivation, aliquots were taken from the reactions after 15, 30, and 60 min. This was done to assess the recyclability at lower conversions, rather than at complete conversion. The results depicted in Figure 1.3 were quite promising. Even after ten cycles, the EziG-CALB consistently achieved over 93% conversion after just 1 h of reaction. This high level of performance across multiple cycles suggests that the EziG-CALB catalyst is not only very active but also highly stable and reusable.

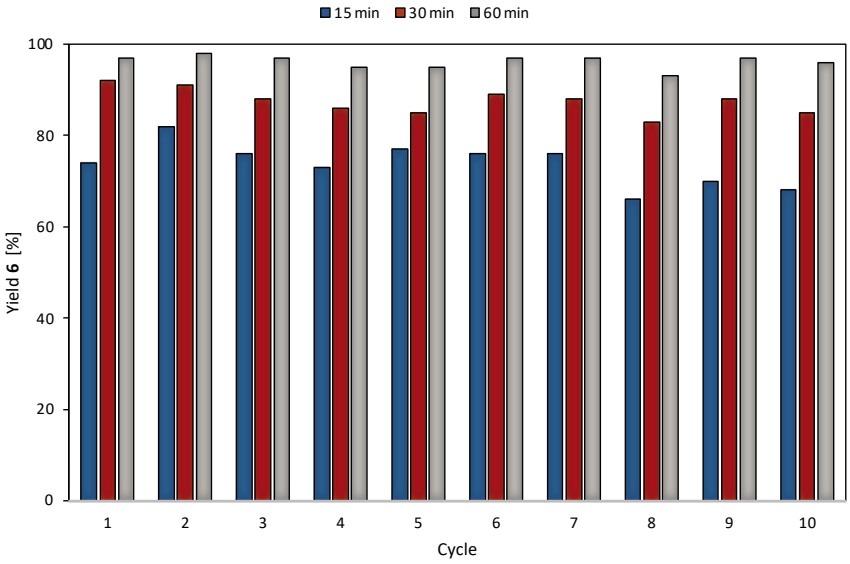


Figure 1.3. Recycling studies for the acetylation of 2-furfurylamine (**4**, 20 mg, 100 mM) using immobilised EziG-CALB (20 mg), EtOAc (3 equiv) in 2-MeTHF at 30 °C and 250 rpm. Conversion values for the ten reactions were determined by GC analyses of the reaction crudes, recovering the enzyme after each use by filtration and wash with 2-MeTHF.

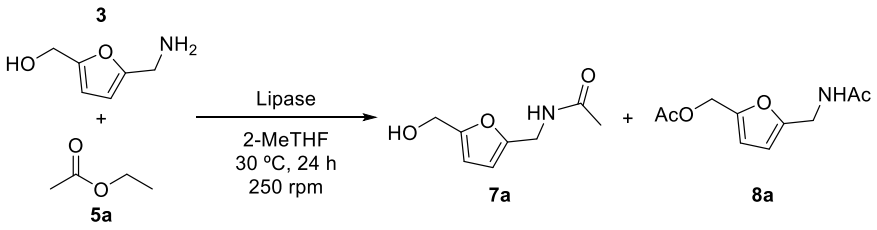
1.2.2. HMFA

1.2.2.1. Enzyme screening

Similarly to the FA acylation study, a lipase screening was initially conducted for HMFA. However, the screening was limited to the enzymes that demonstrated the best performance in the acetylation of FA (CALB, TLL, PSL and AKL, Table 1.2). Moreover, to develop a selective process, the amount of acyl donor was reduced from 3 to 1,3 equiv, given that HMFA contains two competing functional groups (alcohol *vs* amine). This adjustment was made with the aim of achieving only N-acetylation and preventing the formation of the N,O-diacetylated derivative. Indeed, there are examples in the literature where the CALB-catalysed acylation of amino

alcohols resulted in different mixtures of N- and O-acylated compounds, depending on the reaction medium and substrate used.[148,149]

Table 1.2. Screening of lipases for the enzymatic acetylation of HMFA (**3**)[a]



Entry	Enzyme	**7a** (%)[b]
1	-----	<3
2	Novozyme 435®	>99
3	EziG-CALB	>99
4	TLL	83
5	PSL	69
6	AKL	31

[a] Reaction conditions: HMFA (**3**, 100 mM in 2-MeTHF), 1.3 equiv of **5a** and 1:1 w/w enzyme:**3** ratio for 24 h at 30 ºC and 250 rpm. [b] Conversion calculated by GC analyses of the reaction crudes (see experimental section for details).

Satisfyingly, the use of 1.3 equiv of EtOAc allowed the chemo- and monoselective N-protection of HMFA, enabling the formation of the hydroxyamide **7a** with all the tested lipases. Remarkably, the formation of the diacetylated amido ester **8a** was not observed in any case. Specifically, both immobilised CALB preparations led to complete conversions (entries 2

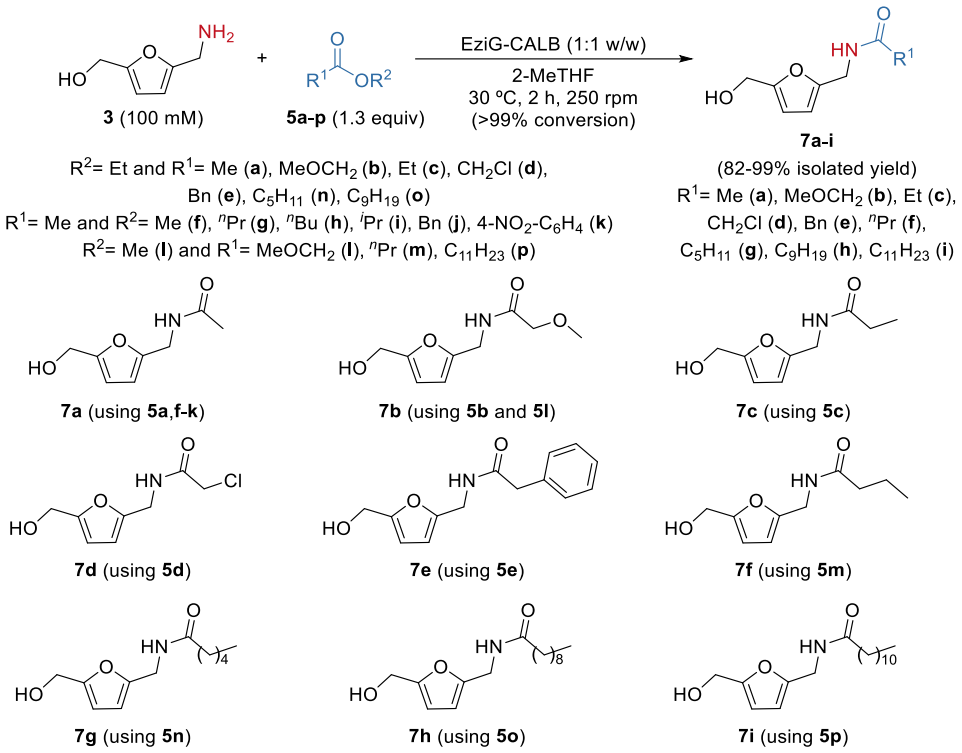
[148] F. Le Joubioux, Y. Ben Henda, N. Bridiau, O. Achour, M. Graber, T. Maugard, *J. Mol. Catal. B Enzym.* **2013**, *85–86*, 193–199.
[149] F. Le Joubioux, N. Bridiau, Y. Ben Henda, O. Achour, M. Graber, T. Maugard, *J. Mol. Catal. B Enzym.* **2013**, *95*, 99–110.

and 3) towards the N-acetylated product **7a**. Relatively high conversions were achieved with PSL and TLL, with 69% and 83% values, respectively. However, the AK lipase yielded a notably lower conversion of just 31%.

Alternatively, the use of EtOAc as solvent was investigated. This presents a distinct advantage as it could be used as both acyl donor and solvent. This dual role of EtOAc could significantly streamline the reaction protocol by reducing the number of components involved, potentially leading to a more efficient and cost-effective process. Unfortunately, when the reactions were carried out in EtOAc, the formation of the amido ester **8a** was observed as an unique product, which highlights the importance of the selection of adequate and mild reaction conditions for selective biotransformations.

1.2.2.2. Acyl donor study

The versatility of the lipase-catalysed acylation of HMFA was examined with a range of acyl donors. The aim was to synthesize a series of HMFA-derived amides **7a-I** and to analyze the influence of the acyl donor when different structural motifs were present in their structures (Scheme 1.7). All reactions were conducted at a substrate concentration of 100 mM, under mild conditions (30 ºC), and over short reaction times (2 h). The catalyst, EziG-CALB was used (at a 1:1 weight ratio of enzyme to substrate) along with 1.3 equiv of the corresponding acyl donor **5a**-**p** using 2-MeTHF as biorenewable solvent.



Scheme 1.7. Scope of the EziG-CALB catalysed chemoselective acylation of HMFA.

The use of acyl donors **5a-e**,**m-p**, bearing different acyl groups (Scheme 1.7; R^1= Me, $MeOCH_2$, Et, $ClCH_2$, Ph, ^{n}Pr, pentyl, nonyl and undecyl), led to outstanding results in terms of chemical yield (82-99%, see experimental section). This provided a straightforward approach to a series of HMFA-derived amides **7a-i**, which were recovered with excellent purity following a column chromatography-free protocol. Of particular interest is the synthesis of halogenated derivative **7d** which shows potential for further selective transformations.

Additionally, the impact of the alkoxy group from the non-activated esters was investigated. As such, various acyl donors were used to produce **7a** with **5f-k** (R^2= Me, ^{n}Pr, ^{n}Bu, ^{i}Pr, Bn, and 4-$NO_2C_6H_4$), **7b** with **5l** (R^2=

CH_2OMe), **7f** with **5m** (R^2= Me), and **7i** with **5p** (R^2= Me). Satisfyingly, the desired hydroxy amides were obtained in all cases, the only exception was a very sterically hindered ester, such as *tert*-butyl acetate, which did not result in any conversion towards **7a**.

In most cases, product isolation was obtained after simple enzyme filtration and subsequent evaporation of the filtrate, with only a few exceptions. For ethyl phenylacetate (**5e**) the reaction crude was dried on the freeze-dryer overnight. For benzyl acetate (**5j**) and 4-nitrophenyl acetate (**5k**), the formation of product **7a** was observed in complete conversion, but no purification was performed to remove the remaining acyl donor. For ethyl caprate (**5o**) methyl laurate (**5p**), to remove the excess of the acyl donor, the reaction crude was washed with cold Et_2O.

1.2.2.3. HMFA N-acetylated scale-up

Given the promising results obtained for the selective N-acetylation of HMFA, a reaction scale-up using 1 g of substrate was considered. However, before proceeding with scaling-up the process, the lipase-catalysed acetylation of HMFA was tested at increasing substrate concentrations (200, 300, 400, 500, and 1000 mM), while keeping the other reaction parameters constant (30 ºC, 1.3 equiv EtOAc and 15 mg EziG-CALB in 1.2 mL of 2-MeTHF).

The reactions were monitored using GC, observing complete conversions in all cases. However, the biotransformations with higher substrate concentrations required longer reaction times to reach full conversion (up to 22 h). Unfortunately, the use of HMFA concentrations higher than 200 mM resulted in the formation of moderate amounts of the diacetylated product **8a** (up to 7%, as seen in Figure 1.4). Therefore, the lipase-catalysed reaction with 200 mM of HMFA was selected to be conducted at a 1 g-scale (7.87 mmol). The desired hydroxy amide **7a** was obtained after 5 h and a simple column-free work-up, which consisted of enzyme filtration and solvent evaporation (85% isolated yield).

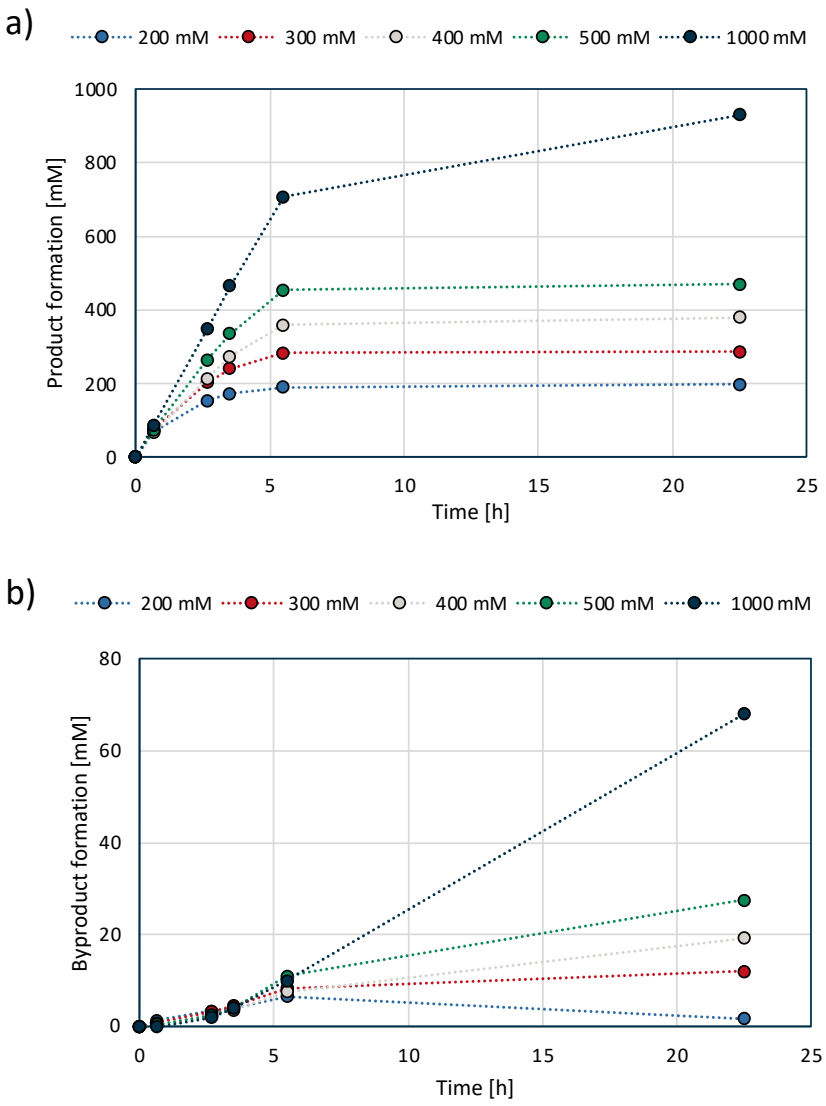


Figure 1.4. Time course study, using GC, for the acetylation of HMFA at different substrate concentrations (200-1000 mM) using immobilised EziG-CALB (15 mg), EtOAc (1.3 equiv) in 2-MeTHF at 30 °C and 250 rpm: a) Target product HMFA-NAc formation (mM); b) Byproduct diacetylated **8a** formation (mM).

1.2.2.4. Environmental impact assessment (E-factor)

Following these results, an environmental impact assessment was carried out, utilising the E-factor concept (Figure 1.5).[31] This analysis was focused on evaluating the contribution of the reagents, catalyst, and solvents used in the experimental procedure (Figure 1.7 in experimental section). The results revealed that the methodology had an E-factor value of 53.6, with the most significant contribution (97.9%) being the organic solvents used in the reaction and downstream process for enzyme washing. Interestingly, when excluding the solvent contribution, the enzymatic method demonstrated an excellent E-factor value of 1.1, highlighting its high potential, particularly if the organic solvents could be reused, further enhancing the environmental sustainability of the process.

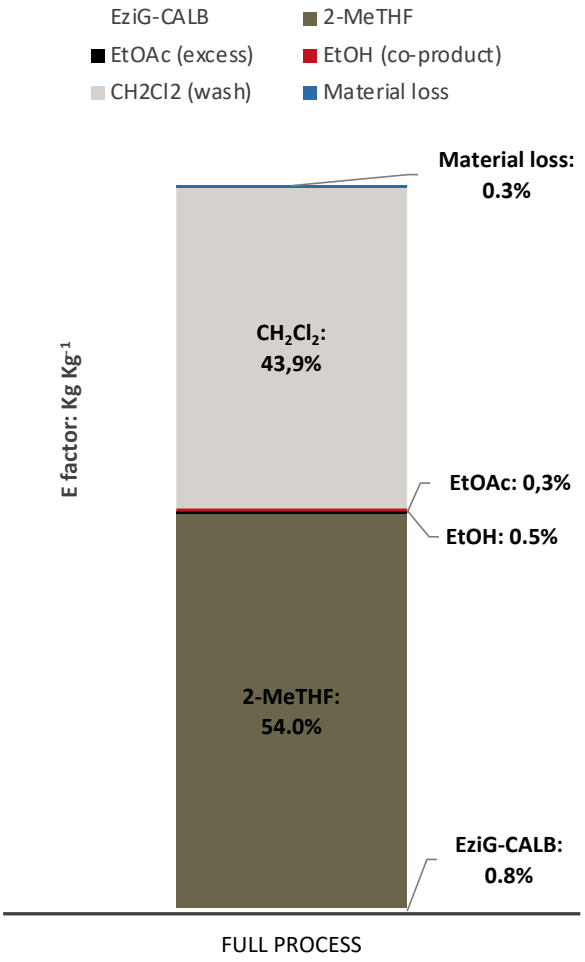
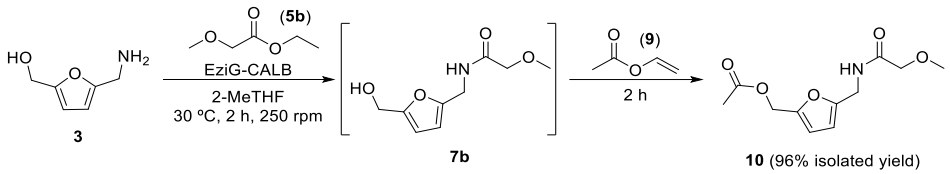


Figure 1.5. Contribution of the different components to the *E*-factor for the acetylation with EtOAc of **3** using EziG-CALB at 1 g scale.

1.2.3. Double acylation of HMFA

To further explore the potential of EziG-CALB, the possibility of accessing orthogonally protected amido esters, using a fully enzymatic approach, was explored (Scheme 1.8). This was facilitated by the excellent selectivity displayed by EziG-CALB in the N-acylation of HMFA.



Scheme 1.8. One-pot two-step enzymatic process to obtain orthogonally protected HMFA amido ester 10.

Initially, hydroxy amide **7b** was obtained using ethyl methoxy acetate (**5b**) as acyl donor and 2-MeTHF as solvent at 30 ºC. Then, after complete conversion, which took 2 h, an activated acyl donor such as vinyl acetate (**9**, 3 equiv) was added to the Erlenmeyer-flask. After additional 2 h, amide **10**, bearing two different acyl moieties as O- and N-substitutions, was isolated with a high yield (96%). Thus, this straightforward reaction sequence could provide easy access to a variety of amido esters by selecting the appropriate (non) activated esters.

Conclusions

As conclusions of this chapter, the following points can be highlighted:

- Two commercial immobilised CALB preparations on acrylic resin (Novozyme 435) and glass porous material (EziG-CALB) were found as excellent enzymes for the N-acylation of FA and HMFA.

- Particularly, EziG-CALB stood out as an exceptional biocatalyst, maintaining high activity across multiple cycles for the acylation of FA in the biobased organic solvent 2-MeTHF.

- The chemoselective N-acylation of HMFA enabled the synthesis of 9 novel hydroxy amides with good to excellent yields, using various acyl substituents under optimised reaction conditions.

- The approach demonstrated scalability up to 1 g of substrate and suitability for high substrate concentration (up to 1 M), showing a low environmental impact through E-factor calculations.

- An orthogonally N,O-diprotected amido ester derived from HMFA was successfully obtained via a one-pot two-step transformation using ethyl methoxyacetate and vinyl acetate as acyl donors for N- and O-protection, respectively.

Experimental part

1.3.1. General information

1.3.1.1. Materials and equipment

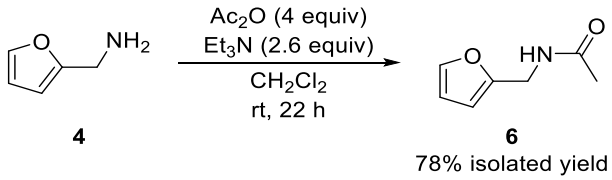
Chemical reagents were purchased from Sigma-Aldrich, VWR International, and Thermo Fisher Scientific, and used as received. Particularly, FA and HMFA that were used as substrates for lipase-catalysed reactions were acquired from Sigma-Aldrich. Regarding the enzyme availability, *Candida antarctica* type B lipase (CALB) was used as two different immobilised forms: Novozyme 435 was kindly donated by Novozymes, and it is supported on the resin Lewatit VP OC 1600, while EziG-CALB is produced by EnginZyme and is supported on a polymer-coated controlled porosity glass carrier EziG Amber. Regarding other enzymes employed in this contribution, immobilised *Candida rugosa* lipase (CRL), immobilised *Pseudomonas cepacia* (PSL), and lyophilised lipase AK from *Pseudomonas fluorescens* (AK) were purchased from Sigma-Aldrich; *Candida antarctica* type A lipase (CALA) and *Thermomyces lanuginosus* lipase (TLL) were obtained from Immozymes and Meito Sangyo, respectively, both used as immobilised preparations; finally, immobilised *Aspergillus niger* lipase (ANL) was obtained from Biocatalysts LTD. Thin-layer chromatography (TLC) analyses were conducted using Merck Silica Gel 60 F254 precoated plates and visualised with a UV lamp, and potassium permanganate or vanillin stains. Column chromatography purifications, when required, were performed using silica gel 60 (230-240 mesh).

^{1}H-, ^{13}C- and DEPT NMR experiments were recorded on a Bruker AV300 MHz spectrometer, using $CDCl_3$ or MeOD as solvent. All chemical shifts (δ) are given in parts per million (ppm) and referenced to the residual solvent signal as internal standard. IR spectra were recorded on a Jasco FT/IR-4700 spectrophotometer, and ν_{max} values are given in cm^{-1} for the main absorption bands of the synthesised compounds. High resolution mass spectra (HRMS) experiments were carried out by electrospray ionisation in positive mode (ESI^+) using a Micro Tof Q spectrometer.

Gas chromatography (GC) analyses were performed on an Agilent HP6890 GC chromatograph equipped with an FID detector.

1.3.2. Chemical synthesis of N-(furan-2-ylmethyl)acetamide (6)

Before studying the lipase-catalysed acylation of HMF (**3**), 2-furfurylamine (FA, **4**) was selected as a model substrate due to its commercial availability at low prize. The chemical synthesis of the corresponding acetamide, namely *N*-(furan-2-ylmethyl)acetamide (**6**), was initially performed to develop adequate GC methods to monitorise later the enzymatic reactions (Scheme 1.9).



Scheme 1.9. Chemical synthesis of *N*-(furan-2-ylmethyl)acetamide.

The experimental protocol was as follows: Acetic anhydride (Ac_2O, 389 µL, 4.12 mmol, 4 equiv) and triethylamine (Et_3N, 377 µL, 2.68 mmol, 2.6 equiv) were successively added to a solution of commercial FA (**4**, 100 mg, 1.03 mmol, 200 mM) in dry dichloromethane (5.15 mL). The reaction was stirred at room temperature (rt) after complete consumption of the starting amine (22 h). After this time the reaction mixture was quenched with water (10 mL), extracted with CH_2Cl_2 (3 x 10 mL), dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. The reaction crude was purified by column chromatography on silica gel (eluent gradient from 40 to 50% EtOAc/Hexane), yielding *N*-(furan-2-ylmethyl)acetamide (**6**) in 78% isolated yield (139.1 mg).

***N*-(furan-2-ylmethyl)acetamide (6)** Yellow oil (78% yield). R_f (EtOAc): 0.48. IR (neat): 3274, 3077, 1646, 1544, 734, and 599 cm^{-1}. ^{1}H-NMR (300

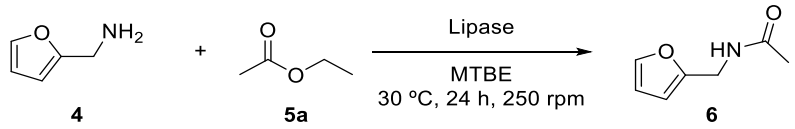
MHz, $CDzCl_3$) δ 7.35 (dd, J = 2.0, 0.9 Hz, 1H), 6.31 (dd, J = 3.2, 1.9 Hz, 1H), 6.22 (dd, J = 3.2, 0.9 Hz, 1H), 5.88 (br s, 1H), 4.42 (d, J = 5.5 Hz, 2H), 2.00 (s, 3H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) δ 169.9 (C), 151.4 (C), 142.3 (CH), 110.6 (CH), 107.6 (CH), 36.7 (CH_2), and 23.3 (CH_3) ppm. ESI-TOF-HRMS: $[M+Na]^+$ calculated for $C_7H_9NNaO_2$: 162.0532; found 162.0525.

1.3.3. Lipase-catalysed acetylation of amine 4

*1.3.3.1. General protocol for the lipase screening in the acetylation of amine **4***

The lipase-catalysed acetylation of FA (**4**) was developed with different hydrolases prior developing the optimisation of the reaction conditions. A general protocol is as follows: Amine **4** (20 mg, 0.2 mmol, 100 mM) was dissolved in MTBE (2 mL) in an Erlenmeyer-flask, then the corresponding hydrolase (20 mg, 1:1 w/w enzyme:**4** ratio) and EtOAc (59 μL, 0.6 mmol, 3 equiv) were successively added. The reaction was shaken at 250 rpm for 24 h at 30 °C, and after this time an aliquot was taken and analysed by GC (see Section 1.3.6). The conversion values are depicted in Table 1.3.

Table 1.3. Screening of lipases for the acetylation of **4** with EtOAc as acyl donor.



4 **5a** **6**

Entry	Lipase	Conversion (%)[a]
1	CALB (Novozyme 435®)	>99
2	CALB (EziG-CALB)	>99
3	*Thermomyces lanuginosus* lipase (TLL)	83
4	*Pseudomonas cepacia* lipase (PSL)	69
5	Lipase AK from *Pseudomonas fluorescens* (AKL)	31
6	*Candida rugosa* lipase (CRL)	6
7	*Candida antarctica* lipase type A (CALA)	4
8	*Aspergillus niger* lipase (ANL)	2

[a] Conversion values were determined by GC analyses of the reaction crude.

*1.3.3.2. Monitorisation of the CALB-catalysed acetylation of amine **4***

Both immobilised CALB forms, the one from Novozymes and the one from EnginZyme were used for the acetylation of amine **4**, and a reaction time course study was performed, taking regular aliquots after 0.5, 1 and 2 h (Figure 1.6).

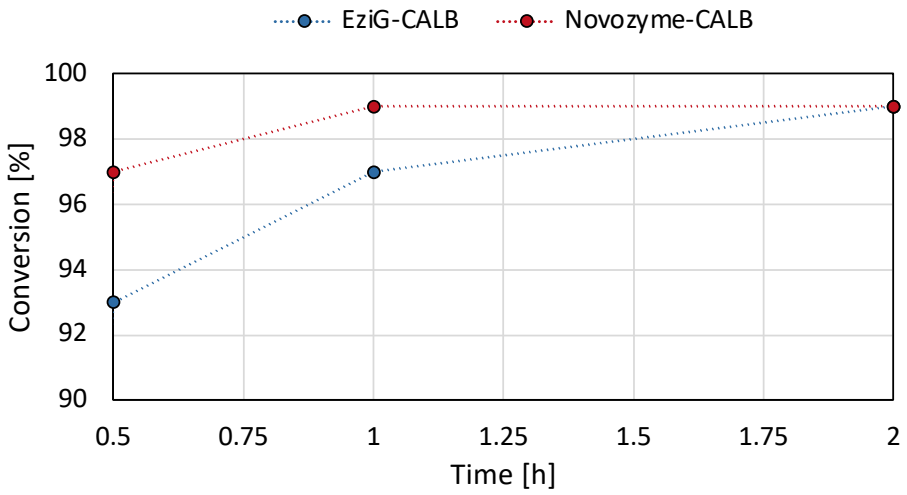


Figure 1.6. Time course study for the acetylation of FA (**4**, 20 mg, 100 mM) using immobilised CALB (Novozyme 435 or EziG-CALB, 20 mg), EtOAc (3 equivalents) in MTBE at 30 °C and 250 rpm. Conversion values were determined by GC analyses of the reaction crudes.

1.3.3.3. Solvent screening for the EziG-CALB-catalysed acetylation of FA (4)

The lipase-catalysed acetylation of FA (**4**) was developed following the general protocol described in Section 1.3.3.1, but during only 2 h and using different solvents as disclosed in Table 1.4.

Table 1.4. Screening of solvents for the acetylation of **4** using EziG-CALB.[a]

Entry	Solvent	6 (%)[b]
1	MTBE	>99
2	Et_2O	>99
3[c]	EtOAc	>99
4	2-MeTHF	99
5	1,4-Dioxane	96
6	Toluene	84
7	Tetrahydrofuran	72
8	Hexane	10

[a] Reaction conditions: **4** (20 mg, 100 mM), 3 equiv of **5a** and EziG-CALB (20 mg, 1:1 w/w enzyme:**4** ratio) for 2 h at 30 ºC and 250 rpm.
[b] Product percentage calculated by GC analyses.
[c] Used as solvent and acyl donor.

*1.3.3.4. Lipase-catalysed acetylation of furfurylamine (**4**) using EtOAc (**5a**) in an organic solvent.*

Amine **4** (20 mg, 0.2 mmol, 100 mM) was dissolved in a hydrophobic organic solvent (2 mL) such as tert-butyl methyl ether (MTBE), diethyl ether (Et_2O), ethyl acetate (EtOAc) or 2-methyltetrahydrofuran (2-MeTHF) inside an Erlenmeyer-flask. Then, Novozyme 435 or EziG-CALB (20 mg, 1:1 w/w enzyme:4 ratio) and EtOAc (59 µL, 0.6 mmol, 3 equiv) were successively added (the acyl donor was added only for the reactions with MTBE, Et_2O and 2-MeTHF). The reaction was shaken at 250 rpm for 2 h at 30 °C, and after this time an aliquot was taken and analysed by GC, observing the quantitative conversion. The reaction was filtered, and the enzyme was washed with CH_2Cl_2 (2 x 1 mL). The filtrate was evaporated under reduced pressure affording *N*-(furan-2-ylmethyl)acetamide (**6**) as a yellow oil. The spectroscopic data match with the ones obtained via

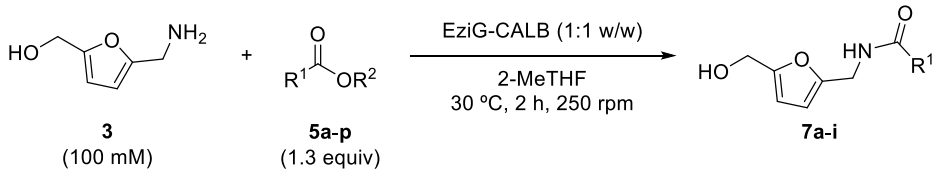
chemical acetylation of **4** using acetic anhydride and triethylamine (see section 1.3.2).

1.3.4. Acetylation of HMFA (3) using different lipases and acyl donors

1.3.4.1. Lipase screening for the acetylation of HMFA with EtOAc

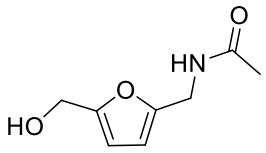
HMFA (**3**, 15 mg, 0.12 mmol, 100 mM) was dissolved in 2-MeTHF (1.2 mL) inside an Erlenmeyer-flask, then the corresponding hydrolase (15 mg, 1:1 w/w enzyme:**3** ratio) and EtOAc (**5a**, 15 μL, 0.15 mmol, 1.3 equiv) were successively added. The reaction was shaken at 250 rpm for 24 h at 30 °C, and after that time an aliquot was taken and analysed by GC (see section 1.3.6.). The conversion values are depicted in Table 1.2 (results and discussion section 1.2.2.1).

*1.3.4.2. General procedure for the EziG-CALB-catalysed selective N-acylation of HMFA (**3**).*



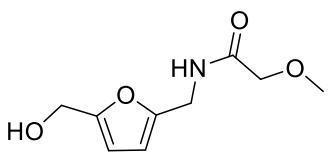
The corresponding acyl donor **5a-p** (0.16 mmol, 1.3 equiv) was added to a mixture of HMFA (**3**, 15 mg, 0.12 mmol, 100 mM), EziG-CALB (1:1 w/w enzyme:3), and 2-MeTHF (1.2 mL). The mixture was shaken for 2 h at 250 rpm at 30 °C, and after this time, the reaction crude was analysed by TLC and/or GC analyses. The reaction was filtered, and the enzyme was washed with CH_2Cl_2 (2 x 1 mL). The filtrate was concentrated under reduced pressure, affording the corresponding hydroxy amides **7a-i** with excellent purities that were then fully characterised (results and discussion section 1.2.2.2). The only exceptions are the reactions with: ethyl

phenylacetate (**5e**), where the reaction crude was dried on a freeze-dryer overnight; and those using benzyl acetate (**5j**), 4-nitrophenyl acetate (**5k**), ethyl caprate (**5o**) and methyl laurate (**5p**) because the product formation was observed in complete conversion, but the unreacted acyl donor was not separated. For instance, the wash of the reaction crudes containing the hydroxy amides **7h** and **7i** with cold Et_2O (3 x 2 mL) allowed the isolation of the product with excellent purity.



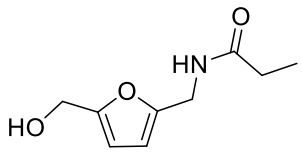
7a

***N*-[(5-(Hydroxymethyl)furan-2-yl)methyl]acetamide (7a).** Yellow oil (19.8 mg, 99% isolated yield). R_f (EtOAc): 0.25. IR (neat): 3390, 3280, 1623, 1545, 998, and 796 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 6.44 (br s, 1H), 6.16 (d, *J* = 3.1 Hz, 1H), 6.12 (d, *J* = 3.2 Hz, 1H), 4.51 (s, 2H), 4.33 (d, *J* = 5.5 Hz, 2H), 3.15 (br s, 1H), and 1.94 (s, 3H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) δ 170.4 (C), 154.0 (C), 151.3 (C), 108.7 (CH), 108.4 (CH), 57.3 (CH_2), 36.8 (CH_2), and 23.2 (CH_3) ppm. ESI-TOF-HRMS: $[M+Na]^+$ calculated for $C_8H_{11}NNaO_3$: 192.0631; found 192.0634.



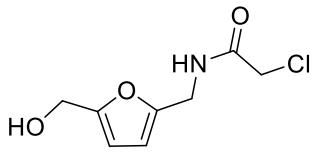
7b

***N*-[(5-(Hydroxymethyl)furan-2-yl)methyl]-2-methoxyacetamide (7b).** Yellow oil (23.3 mg, 99% isolated yield). R_f (EtOAc): 0.25. IR (neat): 3298, 2918, 2849, 1649, 1195, and 1116 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 6.93 (br s, 1H), 6.18 (d, *J* = 3.1 Hz, 1H), 6.15 (d, *J* = 3.2 Hz, 1H), 4.52 (s, 2H), 4.41 (d, *J* = 5.9 Hz, 2H), 3.88 (s, 2H), 3.37 (s, 3H), and 2.97 (br s, 1H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) δ 169.8 (C), 154.2 (C), 150.9 (C), 108.6 (CH), 108.4 (CH), 71.9 (CH_2), 59.3 (CH_3), 57.3 (CH_2), and 35.9 (CH_2) ppm. ESI-TOF-HRMS: $[M+Na]^+$ calculated for $C_9H_{13}NNaO_4$: 222.0737; found 222.0739.



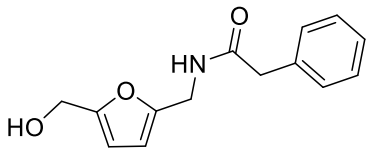
7c

***N*-[(5-(Hydroxymethyl)furan-2-yl)methyl]propionamide (7c).** Yellow oil (21.4 mg, 99% isolated yield). R_f (EtOAc): 0.40. IR (neat): 3350, 3173, 1645, 1539, 997, and 790 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 6.17 (d, *J* = 3.2 Hz, 1H), 6.13 (d, *J* = 3.2 Hz, 1H), 4.52 (s, 2H), 4.35 (d, *J* = 5.5 Hz, 2H), 2.95 (br s, 2H), 2.20 (q, *J* = 7.6 Hz, 2H), and 1.12 (t, *J* = 7.6 Hz, 3H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) δ 174.1 (C), 154.0 (C), 151.5 (C), 108.7 (CH), 108.2 (CH), 57.3 (CH_2), 36.7 (CH_2), 29.6 (CH_2), and 9.8 (CH_3) ppm. ESI-TOF-HRMS: $[M+Na]^+$ calculated for $C_9H_{13}NNaO_3$: 206.0788; found 206.0790.



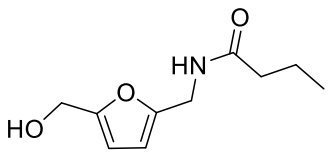
7d

2-Chloro-*N*-[(5-(hydroxymethyl)furan-2-yl)methyl]acetamide (7d). Yellow oil (23.7 mg, 99% isolated yield). R_f (EtOAc): 0.56. IR (neat): 3278, 3079, 1652, 1537, 1010, and 792 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.05 (br s, 1H), 6.19 (apparent q, *J* = 3.4 Hz, 2H), 4.54 (s, 2H), 4.43 (d, *J* = 5.7 Hz, 2H), 4.04 (s, 2H), and 3.59 (s, 1H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) δ 166.2 (C), 154.2 (C), 150.4 (C), 108.9 (CH), 108.8 (CH), 57.5 (CH_2), 42.6 (CH_2), and 37.0 (CH_2) ppm. ESI-TOF-HRMS: $[M+Na]^+$ calculated for $C_8H_{10}ClNNaO_3$: 226.0241; found 226.0245.



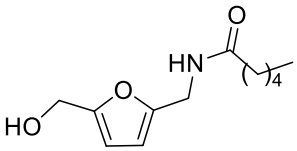
7e

***N*-[(5-(Hydroxymethyl)furan-2-yl)methyl]-2-phenylacetamide (7e).** White powder (28.6 mg, 99% isolated yield). R_f (EtOAc): 0.65. Mp: decomposition observed between 141-161 ºC. IR (neat): 3355, 3280, 1631, 1539, 1003, 999, and 691 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.40–7.19 (m, 5H), 6.16 (d, *J* = 3.2 Hz, 1H), 6.07 (d, *J* = 3.1 Hz, 1H), 5.98 (br s, 1H), 4.51 (s, 2H), 4.35 (d, *J* = 5.7 Hz, 2H), and 3.57 (s, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ 171.2 (C), 153.9 (C), 151.3 (C), 134.7 (C), 129.6 (2CH), 129.1 (2CH), 127.5 (CH), 108.7 (CH), 108.2 (CH), 57.4 (CH_2), 43.7 (CH_2), and 36.9 (CH_2) ppm. ESI-TOF-HRMS: $[M+Na]^+$ calculated for $C_{14}H_{15}NNaO_3$: 268.0944; found 268.0947.



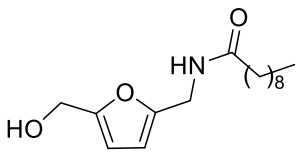
7f

***N*-[(5-(Hydroxymethyl)furan-2-yl)methyl]butyramide (7f).** Brown powder (20.8 mg, 90% isolated yield). R_f (EtOAc): 0.50. Mp: 79-81 ºC. IR (neat): 3277, 2962, 2931, 2872, 1625, 1543, 1275, 996, and 758 cm^{-1}. ^{1}H-NMR (300 MHz, MeOD-d_4) δ 6.22 (d, *J* = 3.2 Hz, 1H), 6.17 (d, *J* = 3.2 Hz, 1H), 4.46 (s, 2H), 4.33 (s, 2H), 2.18 (t, *J* = 7.4 Hz, 2H), 1.57-1.70 (sept, *J* = 7.4 Hz, 2H), 0.93 (t, *J* = 7.4 Hz, 3H). ^{13}C-NMR (75 MHz, MeOD-d_4) δ 174.5 (C), 154.1 (C), 151.5 (C), 107.8 (CH), 107.4 (CH), 56.0 (CH_2), 37.4 (CH_2), 35.8 (CH_2), 18.9 (CH_2), 12.6 (CH_3). ESI-TOF-HRMS: $[M+Na]^+$ calculated for $C_{10}H_{15}NNaO_3$: 220.0944; found 220.0939.



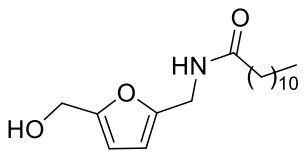
7g

***N*-[(5-(Hydroxymethyl)furan-2-yl)methyl]hexanamide (7g).** Orange powder (21.7 mg, 82% isolated yield). R_f (EtOAc): 0.63. Mp: 94-97 ºC. IR (neat): 3283, 2954, 2930, 2871, 2449, 1623, 1540, and 1022 cm^{-1}. ^{1}H-NMR (300 MHz, MeOD-*d*4) δ 6.22 (d, *J* = 3.2 Hz, 1H), 6.17 (d, *J* = 3.1 Hz, 1H), 4.46 (s, 2H), 4.33 (s, 2H), 2.20 (t, *J* = 7.5 Hz, 2H), 1.61 (quint, *J* = 7.5 Hz, 2H), 1.39–1.21 (m, 4H), 0.91 (t, *J* = 6.8 Hz, 3H). ^{13}C-NMR (75 MHz, MeOD-*d*4) δ 174.7 (C), 154.1 (C), 151.5 (C), 107.8 (CH), 107.4 (CH), 56.0 (CH_2), 35.8 (CH_2), 35.5 (CH_2), 31.1 (CH_2), 25.3 (CH_2), 22.0 (CH_2), 12.9 (CH_3). ESI-TOF-HRMS: $[M+Na]^+$ calculated for $C_{12}H_{19}NNaO_3$: 248.1257; found 248.1258.



7h

***N*-[(5-(Hydroxymethyl)furan-2-yl)methyl]decanamide (7h).** Yellow powder (29.8 mg, 90% isolated yield). R_f (50% EtOAc/Hexane): 0.21. Mp: 115-118 ºC. IR (neat): 3283, 2918, 2849, 1626, 1542, 1002, and 808 cm^{-1}. ^{1}H-NMR (300 MHz, MeOD-*d*4) δ 6.22 (d, *J* = 3.2 Hz, 1H), 6.17 (d, *J* = 3.1 Hz, 1H), 4.46 (s, 2H), 4.33 (s, 2H), 2.20 (t, *J* = 7.5 Hz, 2H), 1.61 (quint, *J* = 6.8 Hz, 2H), 1.30 (d, *J* = 3.5 Hz, 12H), 0.90 (t, *J* = 6.8 Hz, 3H). ^{13}C-NMR (75 MHz, MeOD-*d*4) δ 175.2 (C), 154.6 (C), 152.1 (C), 108.4 (CH), 107.9 (CH), 56.5 (CH_2), 36.3 (CH_2), 36.1 (CH_2), 32.2 (CH_2), 29.8 (CH_2), 29.6 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 26.1 (CH_2), 22.9 (CH_2), 13.6 (CH_3). ESI-TOF-HRMS: $[M+Na]^+$ calculated for $C_{16}H_{27}NNaO_3$: 304.1883; found 304.1883.



7i

***N*-[(5-(Hydroxymethyl)furan-2-yl)methyl]dodecanamide (7i).** Pale yellow powder (33.1 mg, 91% isolated yield). R_f (50% EtOAc/Hexane): 0.26. Mp: 119-121 ºC. IR (neat): 3282, 2918, 2849, 1624, 1464, 1199, and 1002 cm^{-1}. ^{1}H-NMR (300 MHz, MeOD-*d*$_4$) δ 6.22 (d, *J* = 3.2 Hz, 1H), 6.17 (d, *J* = 3.2 Hz, 1H), 4.46 (s, 2H), 4.33 (s, 2H), 2.20 (t, *J* = 7.5 Hz, 2H), 1.60 (apparent t, *J* = 7.3 Hz, 2H), 1.30 (d, *J* = 4.4 Hz, 16H), 0.90 (t, *J* = 6.8 Hz, 3H). ^{13}C-NMR (75 MHz, MeOD-*d*$_4$) δ 175.2 (C), 154.6 (C), 152.1 (C), 108.4 (CH), 107.9 (CH), 56.5 (CH_2), 36.3 (CH_2), 36.1 (CH_2), 32.2 (CH_2), 29.9 (2 CH_2), 29.8 (CH_2), 29.6 (CH_2), 29.6 (CH_2), 29.4 (CH_2), 26.1 (CH_2), 22.9 (CH_2), 13.6 (CH_3). ESI-TOF-HRMS: $[M+Na]^+$ calculated for $C_{18}H_{31}NNaO_3$: 332.2196; found 332.2196.

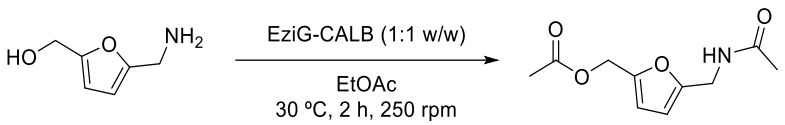
1.3.4.3. Study of the influence of the substrate concentration in the EziG-CALB-catalysed N-acylation of HMFA (3)

Ethyl acetate (1.3 equiv) was added to a mixture of HMFA (**3**, 30-150 mg, 0.24-1.2 mmol, 200-1000 mM), EziG-CALB (15 mg), and 2-MeTHF (1.2 mL). The corresponding mixture was shaken between 2 and 22 h at 30 °C and 250 rpm, taking aliquots regularly that were analysed by GC. The reactions were stopped once the complete disappearance of the starting material was achieved. In all cases, it was obtained the hydroxy amide **7a** as major component (93-99% yield). Higher concentrations of the substrate favoured the formation of the diacetylated product **8a** to some extent, particularly under prolonged reaction times (1-7%).

*1.3.4.4. Scale-up of the EziG-CALB-catalyzed N-acylation of HMFA (**3**).*

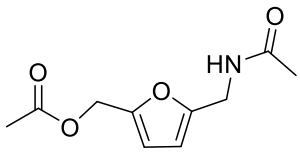
Ethyl acetate (1.0 mL, 10.22 mmol, 1.3 equiv) was added to a mixture of HMFA (**3**, 1.00 g, 7.87 mmol, 200 mM), EziG-CALB (500 mg, 1:2 w/w enzyme:3), and 2-MeTHF (38.3 mL). The mixture was shaken for 5 h at 250 rpm at 30 °C, and after this time, the reaction crude was analysed by GC analyses. The reaction was filtered, and the enzyme was washed with CH_2Cl_2 (2 x 10 mL). The filtrate was concentrated under reduced pressure, affording the hydroxy amide **7a** (1.13 g, 85% yield) and 99% purity through GC analysis.

*1.3.4.5. General procedure for the EziG-CALB-catalysed diacetylation of HMFA (**3**).*



3 (100 mM) **8a**

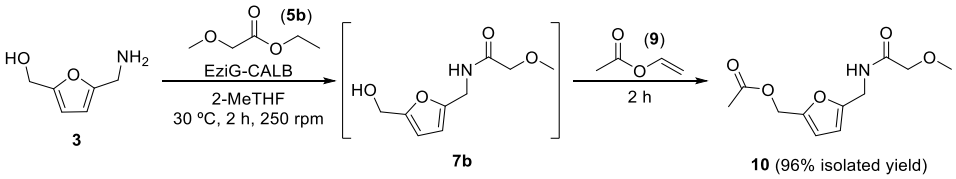
A suspension of HMFA (**3**, 15 mg, 0.12 mmol, 100 mM) and EziG-CALB (1:1 w/w enzyme:**3**) in EtOAc (1.2 mL) was shaken for 2 h at 250 rpm at 30 °C, and after this time, the enzyme was filtered and washed with CH_2Cl_2 (2 x 1 mL). The filtrate was concentrated under reduced pressure, affording the amido ester **8a** with excellent purity that was fully characterised.



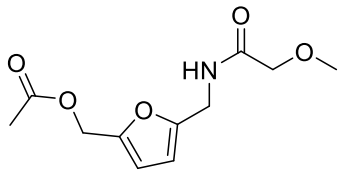
8a

5-(Acetamidomethyl)furan-2-yl)methyl acetate (8a). Yellow oil (78 mg, 99% isolated yield). R_f (EtOAc): 0.65. IR (neat): 2988, 2940, 1733, 1370, 1230, and 1048 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 6.33 (d, J = 3.2 Hz, 1H), 6.19 (d, J = 3.1 Hz, 1H), 5.93 (br s, 1H), 4.99 (s, 2H), 4.40 (d, J = 5.5 Hz, 2H), 2.07 (s, 3H), and 2.00 (s, 3H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) δ 170.7 (C), 169.9 (C), 152.3 (C), 149.3 (C), 111.8 (CH), 108.6 (CH), 58.2 (CH_2), 36.7 (CH_2), 23.3 (CH_3), and 21.0 (CH_3) ppm. ESI-TOF-HRMS: $[M+Na]^+$ calculated for $C_{10}H_{13}NNaO_4$: 234.0739; found 234.0737.

1.3.4.6. General procedure for the one-pot sequential double acylation of HMFA using EziG-CALB.



EziG-CALB (20 mg) and ethyl methoxyacetate (**5b**, 24 µL, 0.21 mmol, 1.3 equiv) were added to a solution of HMFA (**3**, 20 mg, 0.16 mmol, 100 mM) in 2-MeTHF (1.5 mL). The mixture was shaken for 2 h at 30 °C and 250 rpm, until the complete consumption of the starting amine was observed by GC analysis towards the formation of the methoxyacetamide **7b**. Thereafter, vinyl acetate (**9**, 44 µL, 0.47 mmol, 3 equiv) was added to the reaction mixture, and the reaction was shaken for additional 2 h at 30 ºC and 250 rpm observing the disappearance of **7b** and the formation of the product **10** (GC analysis). The reaction was filtered, and the enzyme washed with CH_2Cl_2 (2 x 1 mL). The filtrate was concentrated under reduced pressure, recovering **10** with excellent purity (36.4 mg, 96% isolated yield).



10

{5-[(2-Methoxyacetamido)methyl]furan-2-yl}methyl acetate (10). Yellow oil. R_f (EtOAc): 0.60. IR (neat): 3343, 3135, 3112, 1739, 1658, 1262, and 751 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 6.85 (br s, 1H), 6.33 (d, J = 3.2 Hz, 1H), 6.21 (d, J = 3.2 Hz, 1H), 5.00 (s, 2H), 4.46 (d, J = 5.8 Hz, 2H), 3.92 (s, 2H), 3.41 (s, 3H), and 2.07 (s, 3H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) δ 170.7 (C), 169.6 (C), 152.0 (C), 149.3 (C), 111.7 (CH), 108.7 (CH), 72.0 (CH_2), 59.3 (CH_3), 58.2 (CH_2), 35.9 (CH_2), and 21.0 (CH_3) ppm. ESI-TOF-HRMS: $[M+Na]^+$ calculated for $C_{11}H_{15}NNaO_5$: 264.0844; found 264.0842.

1.3.5. Environmental assessment of the enzymatic acetylation

Environmental *E*-factor calculations (Figures 1.5 and 1.7) were performed based on the scale-up of the transformation at 1 g of substrate **3** (200 mM) with EtOAc as acylating agent using EziG-CALB. The transformation was treated as proceeding to the corresponding isolated yield, hence all losses in yield are accounted for as "material loss".

	to obtain 1,13 g product (g)	to obtain 1 kg Amine (g)	to obtain 1 kg Amine (kg)	Waste (kg per Kg of P)	Waste (kg/Kg of P)-excl solv
Substrate (g)	1,00	885,0	0,88	0,00	0,00
EtOAc	0,69	613,3	0,61	0,00	0,00
EziG-CALB	0,50	442,5	0,44	0,44	0,44
2-MeTHF	32,71	28946,9	28,95	28,95	0,00
EtOAc (excess)	0,21	185,0	0,18	0,18	0,18
EtOH (co-product)	0,31	272,6	0,27	0,27	0,27
CH_2Cl_2 (wash)	26,60	23539,8	23,54	23,54	0,00
Material loss	0,20	177,0	0,18	0,18	0,18
			E-factor	53,6	1,1

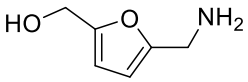
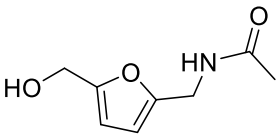
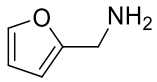
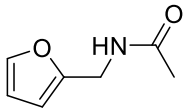
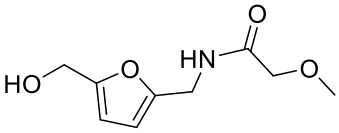
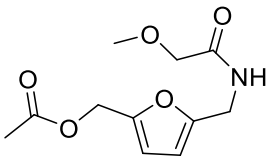
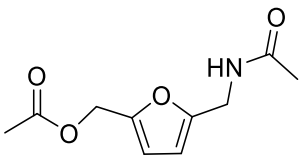
	Upstream	Downstream	Upstream (Kg Kg^{-1})	Downstream (Kg Kg^{-1})
EziG-CALB	0,44		0,8%	
2-MeTHF	28,95		54,0%	
EtOAc (excess)	0,18		0,3%	
EtOH (co-product)	0,27		0,5%	
CH_2Cl_2 (wash)		23,54		43,9%
Material loss		0,18		0,3%
E- factor contribution	55,7%	44,3%		
E- factor total	53,6			

Figure 1.7. Calculated *E*-factor for the acetylation of **3** with EtOAc using EziG-CALB at 1-g scale.

1.3.6. Analytical methods (Gas chromatography analyses)

GC analyses were performed using a HP-1 column (30 m x 0.32 mm x 0.25 μm) was for the determination of conversion values and product percentages (Table 1.6). Method specifications: 5 mL/min Hydrogen, 79 kPa. Injection 1 μL with 20:1 split ratio. Injection temperature: 250 °C, Detector temperature: 250 °C, Detector type: FID. Oven temperature: 60 °C, hold 2 min, 15 °C/min ramp to 225 °C, hold 5 min. Treatment of results: Relative peak area.

Table 1.6. GC retention times.

Compound	Retention time (min)	Compound	Retention time (min)
 **3**	6.2	 **7a**	9.9
 **4**	2.4	 **6**	6.7
 **7b**	10.7	 **10**	11.3
 **8a**	12.7		

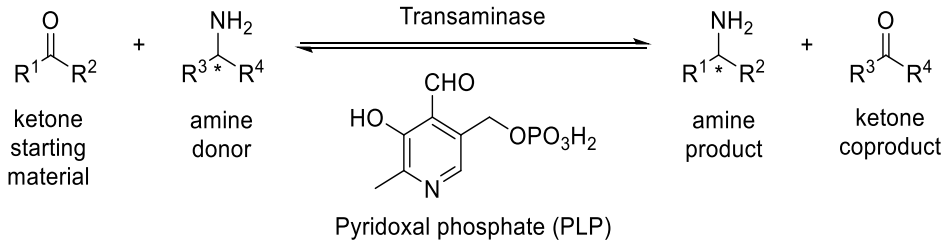
CHAPTER 2.

Biotransamination of 5-Hydroxymethylfurfural in aqueous and organic media

Introduction

2.1.1. Transaminases

Transaminases (TAs), or aminotransferases, belong to the transferase family of enzymes and mediate the reversible transfer of an amino group from an amine donor (amine or amino acid) to an amine acceptor (carbonylic compound) using pyridoxal phosphate as cofactor (Scheme 2.1).[150,151,152]

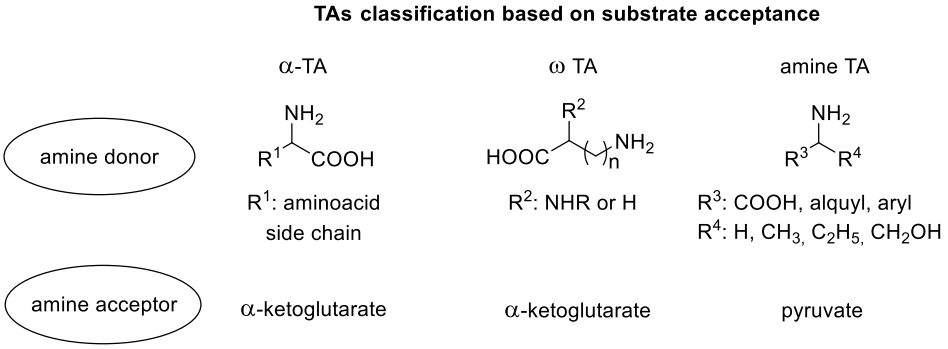


Scheme 2.1. General transformation catalysed by transaminases.

They are typically used by organisms to synthesize α-amino acids from a α-keto acid and an amine source (typically another amino acid). As this process is reversible, TAs are also used to metabolise amino acids from proteins. They are classified as transferases (EC 2.6.1.x) and the subclassification number depends on the specific substrate specificity and the amine donor.[153,154] For example, EC 2.6.1.18 corresponds to a β-alanine-pyruvate transaminase. In a broader classification TAs can also be classified in two sub-classes (Scheme 2.2): α-transaminases (α-TAs) and ω-

[150] N.J. Turner and L. Humphreys, *Biocatalysis in organic synthesis: The retrosynthesis approach*, Royal Society of Chemistry, Cambridge, UK, **2018**.
[151] F. Guo, P. Berglund, *Green Chem.* **2017**, *19*, 333–360.
[152] I. Slabu, J. L. Galman, R. C. Lloyd, N. J. Turner, *ACS Catal.* **2017**, *7*, 8263–8284.
[153] J. Rudat, B. R. Brucher, C. Syldatk, *AMB Express* **2012**, *2*, 1–10.
[154] S. Mathew, H. Yun, *ACS Catal.* **2012**, *2*, 993–1001.

transaminases (ω-TAs).[155] The α-TAs are highly specific and exclusively accept substrates containing a carboxylic acid in the α position to the amino group. In contrast, the ω-TAs transfer amino groups located far from the carboxylic acid. Among ω-TAs, amine transaminases (ATAs) are enzymes that do not require substrates bearing carboxylic groups, they can accept ketones or aldehydes. This unique feature makes them exceptionally valuable in organic synthesis, as they offer the possibility to produce a broad range of chiral amines in a straightforward manner.[156,157]



Scheme 2.2. Transaminase classification based on the type of amino group transferred.

ATAs are homo-dimeric enzymes with two half-active sites located on each monomer. For the enzyme to be catalytically active, these two

[155] M. Höhne, U. T. Bornscheuer, *Enzyme Catalysis in Organic Synthesis*, Wiley-VCH, Weinheim, Alemania, **2012**.
[156] J. Ø. Madsen, J. M. Woodley, *ChemCatChem* **2023**, *15* e202300560.
[157] S. A. Kelly, S. Mix, T. S. Moody, B. F. Gilmore, *Appl. Microbiol. Biotechnol.* **2020**, *104*, 4781–4794.

monomers must be assembled together (Figure 2.1).[158] The active site comprises three different pockets, one for PLP binding and two additional ones (small and large) for binding (amine substrates (amino donor and amine acceptor).

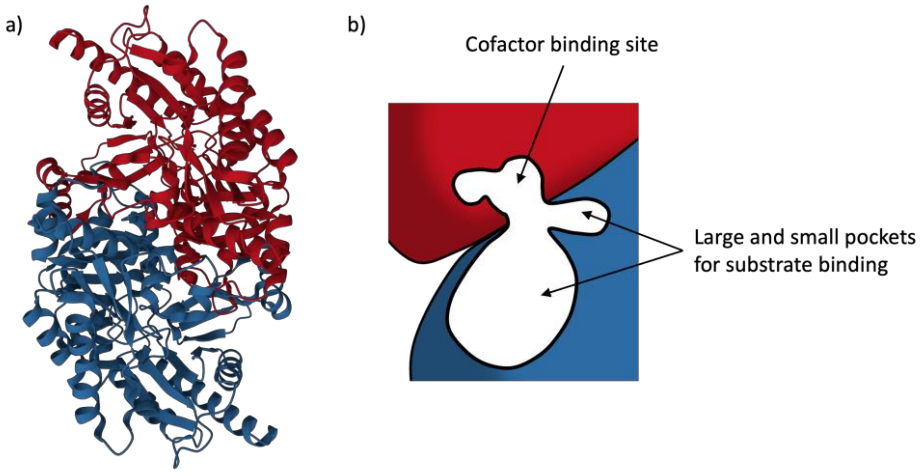


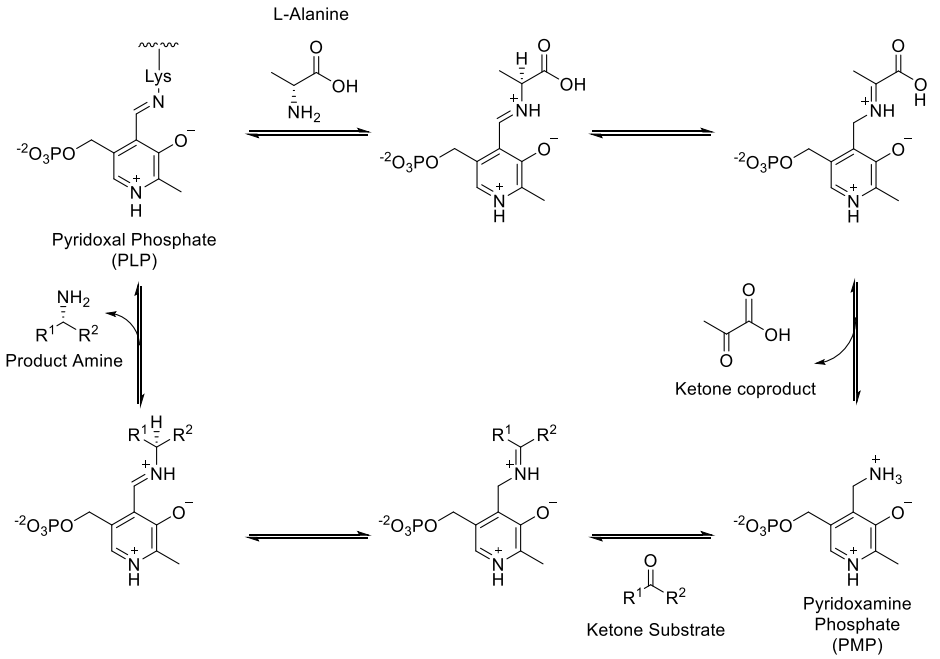
Figure 2.1. Amine transaminase structure: a) Crystal structure of *Chromobacterium violaceum* ATA (PDB ID: 6S4G) displayed in cartoon representation and showing the two homo-dimers; b) Representation of one active site located at the interface between subunits.

The PLP-binding plays a crucial role in the reversible covalent fixation of the cofactor. The small substrate pocket selectively accommodates small organic groups, such as methyl groups, whereas, the large pocket exhibits greater versatility, allowing the binding of larger

[158] H. Meersseman Arango, L. van den Biggelaar, P. Soumillion, P. Luis, T. Leyssens, F. Paradisi, D. P. Debecker, *React. Chem. Eng.* **2023**, *8*, 1505–1544.

organic groups such as alkyl chains, carboxylic acids, ether functions, aromatic groups , and others.[159]

The mechanism of TAs has been investigated in great detail and is nowadays well understood (Scheme 2.3).[150] The amino group transfer is mediated by the cofactor PLP, which is reversibly bound to a lysine group from the enzyme through an imine bond. The reaction follows a ping-pong bi-bi mechanism, which can be divided into two half-reactions, each comprising four steps: imine formation, tautomerisation, hydrolysis, and amino group transfer.



Scheme 2.3. General mechanism of transaminase-catalysed reactions.

[159] R. C. Simon, N. Richter, E. Busto, W. Kroutil, *ACS Catal.* **2014**, *4*, 129–143.

In the first half reaction (top and right part of Scheme 2.3), the PLP cofactor reacts with the amine donor (*e.g.* alanine) to produce the pyridoxamine phosphate intermediate (PMP). This step involves a series of imine formation reactions. Initially, the imine bond established between the PLP and the lysine residue is cleaved, forming a new imine, in this case between the amine donor and the PLP, to generate an aldimine. Tautomerisation of the aldimine then forms a ketimine, which is hydrolysed to give the PMP intermediate, transferring the nitrogen to the cofactor.

In the second half reaction (bottom and left part of Scheme 2.3), the process is reversed, leading to the transfer of the amino group from the PMP to an acceptor carbonylic compound. Similarly, a ketimine is formed between the PLP and the carbonylic compound. This ketimine is then tautomerised to produce an aldimine, which is hydrolysed to generate the final product and regenerate the PLP, thus closing the catalytic cycle.

2.1.2. Amine donor and equilibrium shift

The equilibrium of TA reactions often lies in favour of the starting materials rather than the products. As a result, thermodynamically, amine formation is not favoured. To enhance TA performance in organic synthesis, various physical and chemical strategies have been developed and described to shift the reaction equilibrium towards the desired product formation.[151,152,160,161]

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[160] P. Tufvesson, J. Lima-Ramos, J. S. Jensen, N. Al-Haque, W. Neto, J. M. Woodley, *Biotechnol. Bioeng.* **2011**, *108*, 1479–1493.
[161] R. Abu, J. M. Woodley, *ChemCatChem* **2015**, *7*, 3094–3105.

One approach to drive the equilibrium is by removing the product or coproduct using physical vaporisation/extraction methods or chemical reactions. For instance, when alanine is used as amine donor, pyruvate is formed as the coproduct. It is possible to remove it from the reaction with the use of lactate dehydrogenase (LDH), which transforms pyruvate into lactate. This process requires the addition of NAD(P)H with its corresponding regeneration system (usually glucose/glucose dehydrogenase or formate/formate dehydrogenase).[162,163,164]

Although this method is effective, it requires a stoichiometric (or sometimes larger) amount of the amine donor which can be costly. Alternatively, an amino acid dehydrogenase (AADH), such as alanine dehydrogenase, can be used. As in the previous method, this enzyme uses a nicotinamide cofactor, and therefore, a third enzyme is required to recycle the cofactor.[165,166]

A more recent approach involves the use of so-called smart cosubstrates. When used in quasi-stoichiometric amounts, these cosubstrates can shift the equilibrium in the direction of amination after undergoing spontaneous cyclisation and/or polymerisation reactions.[167,168,169]

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[162] N. Richter, J. E. Farnberger, D. Pressnitz, H. Lechner, F. Zepeck, W. Kroutil, *Green Chem.* **2015**, *17*, 2952–2958.
[163] Mathew, G. Shin, M. Shon, H. Yun, *Biotechnol. Bioprocess Eng*. **2013**, *18*, 1–7.
[164] D. Pressnitz, C. S. Fuchs, J. H. Sattler, T. Knaus, P. Macheroux, F. G. Mutti, W. Kroutil, *ACS Catal.* **2013**, *3*, 555–559.
[165] D. Koszelewski, M. Göritzer, D. Clay, B. Seisser, W. Kroutil, *ChemCatChem* **2010**, *2*, 73–77.
[166] M. D. Truppo, J. D. Rozzell, J. C. Moore, N. J. Turner, *Org. Biomol. Chem.* **2009**, *7*, 395–398.
[167] S. Grigoriu, P. Kugler, E. Kulcinskaja, F. Walker, J. King, P. Hill, V. F. Wendisch, E. O'Reilly, *Green Chem.* **2020**, *22*, 4128-4132.
[168] A. Gomm, S. Grigoriu, C. Peel, J. Ryan, N. Mujtaba, T. Clarke, E. Kulcinskaja, E. O'Reilly, *Eur. J. Org. Chem.* **2018**, 5282–5284.

Another option would be to use a large excess of the amine donor (≥50 equivalents) to shift the equilibrium towards the desired product. One of the most widely used, owing to its accessibility and low cost, is isopropylamine (IPA, $^{i}PrNH_2$).[170,171,172,173] Besides being recognised by several natural transaminases, the by-product formed is acetone, which can be easily removed, offering a significant operational advantage, especially when the involved substrates and products are non-volatile.[174] However, this significant excess of IPA can be problematic in some cases, as the transaminase may be inhibited due to the high concentration of the amine donor present in the reaction medium.

2.1.3. ATAs immobilisation

Immobilisation has emerged as a widely used approach to enhance the operational and storage stability of TAs. Different strategies, such as covalent grafting, entrapment, affinity tags, adsorption, and aggregation, have been extensively explored to develop efficient heterogeneous biocatalysts for transamination reactions.[158,175]

Covalent immobilisation of TAs has been extensively studied on various support materials, such as chitosan, cellulose, or polymeric resins. For example, *Vibrio fluvialis* TA (Vf-TA) was immobilised on epoxy-

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[169] S. E. Payer, J. H. Schrittwieser, W. Kroutil, *Eur. J. Org. Chem.* **2017**, 2553–2559.
[170] A. W. H. Dawood, M. S. Weiβ, C. Schulz, I. V. Pavlidis, H. Iding, R. O. M. A. de Souza, U. T. Bornscheuer, *ChemCatChem* **2018**, *10*, 3943–3949.
[171] C. Iglesias, P. Panizza, S. R. Giordano, *Appl. Microbiol. Biotechnol.* **2017**, *101*, 5677–5687.
[172] P. Tufvesson, C. Bach, J. M. Woodley, *Biotechnol. Bioeng.* **2014**, *111*, 309–319.
[173] K. Engelmark Cassimjee, C. Branneby, V. Abedi, A. Wells, P. Berglund, *Chem. Commun.* **2010**, *46*, 5569–5571.
[174] P. Kelefiotis-Stratidakis, T. Tyrikos-Ergas, I. V. Pavlidis, *Org. Biomol. Chem.* **2019**, *17*, 1634–1642.
[175] M. D. Patil, G. Grogan, A. Bommarius, H. Yun, *Catalysts* **2018**, *8*, 254.

functionalised cellulose and used in the asymmetric synthesis of (*S*)-phenylethylamine. The heterogeneous catalyst exhibited excellent temperature stability and reusability, remaining consistently active over a broad temperature range from 30 to 60 °C for four repetitive cycles.[176] More recently, Poppe and co-workers covalently immobilised the Try60Cys mutant of *Chromobacterium violaceum* TA (Cv-TA) on bisepoxide-activated polymeric resins.[177] the immobilised ATA was applied in the kinetic resolution of *rac*-4-phenylbutan-2-amine to its corresponding (*R*)-enantiomer, which is a precursor of the antihypertensive dilevalol. Remarkably, an excellent activity (50% conversion) and selectivity (99% *ee*) were achieved. Notably, this enzyme preparation could preserve the activity even at a high concentration of DMSO (50% v/v), demonstrating an excellent recyclability after being used for 19 consecutive cycles in batch mode.

There are also some examples where covalent immobilisation was combined with crosslinking techniques. For example, Lee and co-workers successfully immobilised the ATA from *Vibrio fluvialis* JS17[178] on chitosan beads using glutaraldehyde as a cross-linker agent, recovering 18% activity compared with the free enzyme. Similarly, Bornscheuer and co-workers followed the same approach to immobilise a series of ATAs (from *Giberella zeae*, *Neosartorya fischeri*, *Aspergillus fumigatus*, *Ruegeria pomeroyi*, and

[176] S. P. De Souza, I. I. Junior, G. M. A. Silva, L. S. M. Miranda, M. F. Santiago, F. Leung-Yuk Lam, A. Dawood, U. T. Bornscheuer, R. O. M. A. De Souza, *RSC Adv.* **2016**, *6*, 6665–6671.
[177] E. Abaházi, P. Sátorhelyi, B. Erdélyi, B. G. Vértessy, H. Land, C. Paizs, P. Berglund, L. Poppe, *Biochem. Eng. J.* **2018**, *132*, 270–278.
[178] S. S. Yi, C.-w. Lee, J. Kim, D. Kyung, B. G. Kim, Y. S. Lee, *Process Biochem.* **2007**, *42*, 895–898.

Rhodobacter sphaeroides), leading to improved thermal stability for *Giberella zeae* and *Neosartorya fischeri*.[179]

Kroutil and co-workers explored the encapsulation as immobilisation technique for several commercially available ATAs (ATA-113, ATA-117 and Vf-ATA)[180] using a sol-gel matrix with celite inclusion as a porous additive. Notably, the encapsulated enzymes demonstrated remarkable activity even under extreme conditions at pH 11, where the free enzymes were severely deactivated. Specifically, the immobilised ATA-117 exhibited excellent operational stability, retaining approximately 78% of its initial activity after eight repetitive uses without compromising its enantioselectivity. Similarly, Päiviö and Kanerva,[181] the kinetic resolution of racemic amines was performed using the transaminase from *Arthrobacter* sp. (ArR-TA) encapsulated in a sol-gel matrix with controlled hydrophobicity, achieved using methyltriethoxysilane during the sol-gel synthesis. The resulting catalysts were shown to be reusable for up to 5 cycles, further demonstrating the potential of encapsulation in enhancing the stability and efficiency of enzymatic reactions.

Affinity immobilisation offers distinct advantages as it facilitates both purification (of tagged proteins) and immobilisation processes simultaneously. This approach was followed by Bäckvall and co-workers, to immobilise *Arthrobacter* sp. (ArR-TA) and *Chromobacterium violaceum* (Cv-TA), using a His-tag, on three EziG supports (glass carrier with chelated Fe^{3+} ions).[77,95] The immobilised Cv-TA was applied to the continuous flow production of (*S*)-methylbenzylamine by kinetic resolution

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[179] H. Mallin, U. Menyes, T. Vorhaben, M. Höhne, U. T. Bornscheuer, *ChemCatChem* **2013**, *5*, 588–593.
[180] D. Koszelewski, N. Müller, J. H. Schrittwieser, K. Faber, W. Kroutil, *J. Mol. Catal. B Enzym.* **2010**, *63*, 39–44.
[181] M. Päiviö, L. T. Kanerva, *Process Biochem.* **2013**, *48*, 1488–1494.

of the racemic amine in MTBE as the solvent and at a temperature of 50 °C.[77] The cofactor, PLP, was co-immobilised with the enzyme, so no external addition was needed. The reactor was run for 96 consecutive hours without any detectable loss of enzymatic activity and a space-time yield of 335 g L^{-1} h^{-1}. In a more recent work, the immobilised ArR-TA was used for the asymmetric synthesis of 1-phenoxypropan-2-amine in neat toluene in a packed-bed flow reactor.[95] This innovative approach holds promise for enhancing the efficiency and stability of the enzyme.

Hughes and co-workers followed the adsorption approach to investigate the immobilisation on polymeric resins through hydrophobic interactions.[182] They successfully immobilised and engineered ATA-117 transaminase on Sepabeads® resin EXE 120, which is a polystyrene resin grafted with octadecyl moieties. This solid biocatalyst exhibited remarkable activity for the asymmetric synthesis of Sitagliptin in an organic medium, using water-saturated isopropyl acetate as the solvent, and was capable of operating at high temperatures, reaching up to 60 °C. Similarly, Neto et al. recently reported the immobilisation of ATA-47 and Ate-TA using commercially available polymeric resins as supports.[183] These carriers demonstrated exceptional reusability, allowing the preparation to be used for 250 h of operation while retaining over 50% of the initial activity. Furthermore, the immobilised enzyme preparations demonstrated significantly improved thermal stability, allowing the enzyme to be stored at room temperature for more than 60 days.

2.1.4. Furan-based amine derivatives

[182] M. D. Truppo, H. Strotman, G. Hughes, *ChemCatChem* **2012**, *4*, 1071–1074.
[183] W. Neto, M. Schürmann, L. Panella, A. Vogel, J. M. Woodley, *J. Mol. Catal. B Enzym.* **2015**, *117*, 54–61.

In the previous chapter (section 1.1.3), the potential application of lignocellulosic biomass has been described, with emphasis on the potential of HMF as a key molecule for biomass valorisation (Scheme 1.3). Specifically, HMFA was used to synthesize diverse amides in a highly selective manner.[184] Within this context, other furan-based amines such as furfuryl amine (FA) or 2,5-bis(aminomethyl)furan (BAMF) are crucial intermediates for various industrial applications. FA, for instance, serves as a precursor of furmethide,[185] a substance used in the treatment of glaucoma, as well as pyridostigmine drugs[186] employed against myasthenia gravis. On the other hand, BAMF stands out as an interesting monomer for a wide variety of functionalised polymers.[187]

Traditionally, these amine derivatives are obtained through metal-catalysed reductive amination[188] of the carbonyl precursors furfural or HMF,[124,125,127,189,190] which involves combining ammonia with hydrogen or a reducing agent. Various metals have been explored for this application, with the most used ones being cobalt,[191,192] nickel,[193,194,195,196,197] and

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[184] A. Pintor, I. Lavandera, A. Volkov, V. Gotor-Fernández, *ACS Sustain. Chem. Eng.* **2023**, *11*, 10284–10292.

[185] J. Jakubík, L. Bačáková, E. E. El-Fakahany, S. Tuček, *Mol. Pharmacol.* **1997**, *52*, 172–179.

[186] C. Müller, V. Diehl, F. W. Lichtenthaler, *Tetrahedron* **1998**, *54*, 10703–10712.

[187] P. S. Choong, N. X. Chong, E. K. W. Tam, A. M. Seayad, J. Seayad, S. Jana, *ACS Macro Lett.* **2021**, *10*, 635–641.

[188] K. Saini, S. Kumar, H. Li, S. A. Babu, S. Saravanamurugan, *ChemSusChem* **2022**, *15*, e202200107.

[189] F. Chacón-Huete, C. Messina, B. Cigana, P. Forgione, *ChemSusChem* **2022**, *15*, e202200328.

[190] Z. Jiang, Y. Zeng, D. Hu, R. Guo, K. Yan, R. Luque, *Green Chem.* **2023**, 25, 871–892.

[191] V. G. Chandrashekhar, K. Natte, A. M. Alenad, A. S. Alshammari, C. Kreyenschulte, R. V. Jagadeesh, *ChemCatChem* **2022**, *14*, e202101234.

[192] X. Zhuang, J. Liu, S. Zhong, L. Ma, *Green Chem.* **2022**, *24*, 271–284.

[193] Z. Wei, Y. Cheng, K. Zhou, Y. Zeng, E. Yao, Q. Li, Y. Liu, Y. Sun, *ChemSusChem* **2021**, *14*, 2308–2312.

ruthenium[198,199,200]. However, these methods often require harsh conditions, including elevated hydrogen pressures and high temperatures. As a result, they favour the formation of product mixtures due to the reactivity of the imine intermediate formed from the carbonyl moieties and ammonia, as well as the unselective reduction of the furan ring (Scheme 2.4).[188] These limitations present significant challenges in achieving high selectivity and efficiency in the synthesis of the desired amine derivatives.

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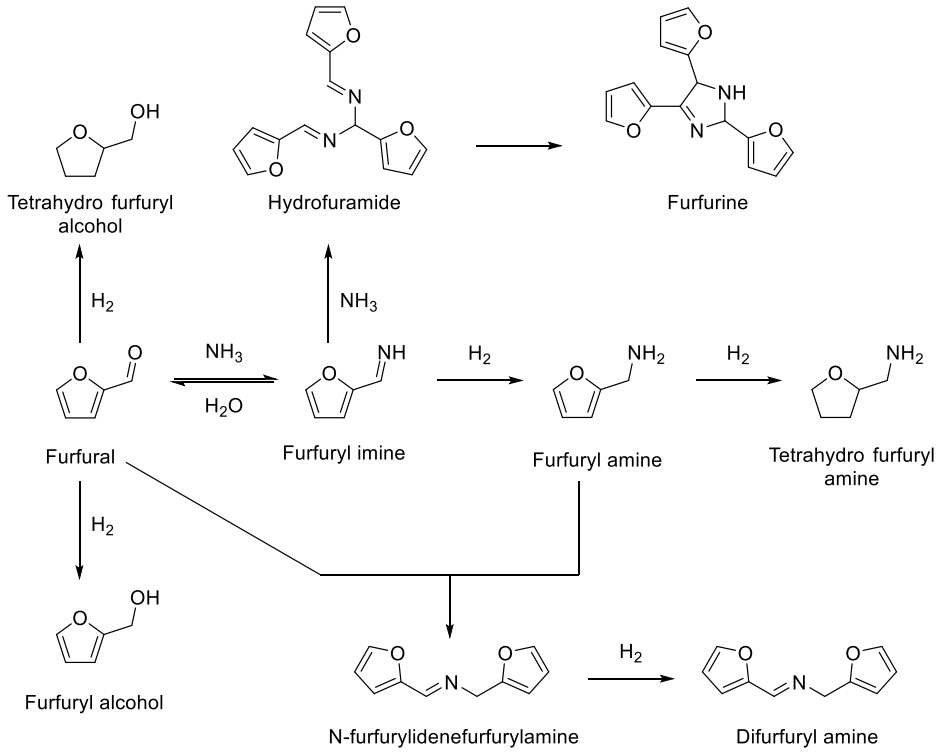
[194] J. Zhang, J. Yang, X. Li, H. Liu, A. Wang, C. Xia, J. Chen, Z. Huang, *ACS Sustain. Chem. Eng.* **2022**, *10*, 5526–5537.
[195] Z. Pan, Q. Zhang, W. Wang, L. Wang, G.-H. Wang, *ACS Sustain. Chem. Eng.* **2022**, *10*, 3777–3786.
[196] Q. Hu, S. Jiang, Y. Wu, H. Xu, G. Li, Y. Zhou, J. Wang, *ChemSusChem* **2022**, *15*, e202200192.
[197] Z. Wei, Y. Cheng, H. Huang, Z. Ma, K. Zhou, Y. Liu, *ChemSusChem* **2022**, *15*, e202200233.
[198] H. Zou, J. Chen, *Appl. Catal. B: Environ.* **2022**, *309*, 121262.
[199] L. Gou, L. Xie, Y. Wang, L. Dai, *Appl. Catal. A: Gen.* **2022**, *647*, 118902.
[200] X. Li, S. D. Le, S. Nishimura, *Catal. Lett.* **2022**, *152*, 2860–2868.



Scheme 2.4. Reductive amination of furfural to furfuryl amine including the structure of potential byproducts.

With these considerations in mind, there is a strong interest in exploring alternative solutions that enable the selective synthesis of amines from corresponding carbonyl compounds under mild conditions. Among the different alternatives, the use of ATAs is particularly appealing due to demonstrated efficacy as a synthetic method for furan-based amines.[151,152,175,201,202] Notably, ATAs from *Chromobacterium violaceum*

[201] M. Fuchs, J. E. Farnberger, W. Kroutil, *Eur. J. Org. Chem.* **2015**, 6965–6982.
[202] S. A. Kelly, S. Pohle, S. Wharry, S. Mix, C. C. R. Allen, T. S. Moody, B. F. Gilmore, *Chem. Rev.* **2018**, *118*, 349–367.

(Cv-TA)[203] and *Aspergillus terreus* (At-TA)[204] have been identified as highly efficient catalysts for synthesizing FA[205,206,207,208] and HMFA[205,209] in buffered systems. Additionally, the use of bio-based organic solvents[210,211] and specially designed deep eutectic solvents (DES)[212,213] has been explored to improve substrate solubility in these reactions.

Recent studies have shown that thermostable mutants derived from At-TA are exceptionally effective in the transamination of furfural[214,215] and HMF[216,217] under high substrate concentrations. However, it is worth noting that many of these transformations have been conducted using an excess of alanine as the amine donor, and in the case of At-TA-catalysed reactions, the costly D-alanine need to be employed.

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[203] U. Kaulmann, K. Smithies, M. E. B. Smith, H. C. Hailes, J. M. Ward, *Enzyme Microb. Technol.* **2007**, *41*, 628–637.
[204] M. Höhne, S. Schätzle, H. Jochens, K. Robins, U. T. Bornscheuer, *Nat. Chem. Biol.* **2010**, *6*, 807–813.
[205] A. Dunbabin, F. Subrizi, J. M. Ward, T. D. Sheppard, H. C. Hailes, *Green Chem.* **2017**, *19*, 397–404.
[206] P. Zhang, X. Liao, C. Ma, Q. Li, A. Li, Y. He, *ACS Sustain. Chem. Eng.* **2019**, *7*, 17636–17642.
[207] X.-L. Liao, Q. Li, D. Yang, C.-L. Ma, Z.-B. Jiang, Y.-C. He, *Appl. Biochem. Biotechnol.* **2020**, *192*, 794–811.
[208] E. M. Carter, F. Subrizi, J. M. Ward, T. D. Sheppard, H. C. Hailes, *ChemCatChem* **2021**, *13*, 4520–4523.
[209] Z. Wang, H. Chai, J. Ren, Y. Tao, Q. Li, C. Ma, Y. Ai, Y. He, *ACS Sustain. Chem. Eng.* **2022**, *10*, 8452–8463.
[210] X. Feng, L. Zhang, X. Zhu, Y. Xia, C. Ma, J. Liang, Y.-C. He, *Catal. Lett.* **2021**, *151*, 1834–1841.
[211] J.-H. Di, L. Gong, D. Yang, Y.-C. He, Z.-Y. Tang, C.-L. Ma, *J. Biotechnol.* **2021**, *334*, 26–34.
[212] Q. Li, J. Di, X. Liao, J. Ni, Q. Li, Y.-C. He, C. Ma, *Green Chem.* **2021**, *23*, 8154–8168.
[213] J. Di, N. Zhao, B. Fan, Y.-C. He, C. Ma, *Appl. Biochem. Biotechnol.* **2022**, *194*, 2204.–2218.
[214] Z. Tang, Q. Li, J. Di, C. Ma, Y.-C. He, *Bioresour. Technol.* **2023**, *369*, 128424.
[215] L. Li, Q. Li, J. Di, Y. He, C. Ma, *ACS Sustain. Chem. Eng.* **2023**, *11*, 7515–7525.
[216] J. Di, Q. Li, C. Ma, Y.-C. He, *Bioresour. Technol.* **2023**, *369*, 128425.
[217] R. Gao, Q. Li, J. Di, Q. Li, Y.-C. He, C. Ma, *Ind. Crops Prod.* **2023**, *193*, 116199.

Objectives

Following the interest in the biocatalytic modification of bio-based furan derivatives, in this chapter are presented the results obtained from the biotransamination of 5-hydroxymethylfurfural having the objectives described below:

- Find suitable ATA candidates for the HMF transamination in buffer to produce HMFA.

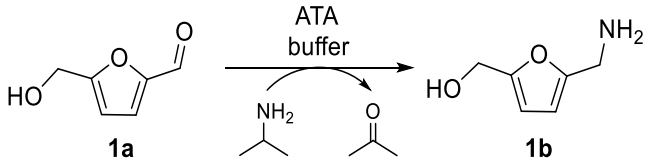
- Optimise the ATA-catalysed reaction in buffer systems in terms of substrate concentration, enzyme loading and amine donor (IPA) equivalents.

- Immobilisation of the best enzyme candidates on three different EziG supports, namely, Amber, Coral and Opal, to explore the biotransamination in organic solvent using heterogenous catalysts.

Results

2.2.1 5-Hydroxymethylfurfural (HMF) transamination

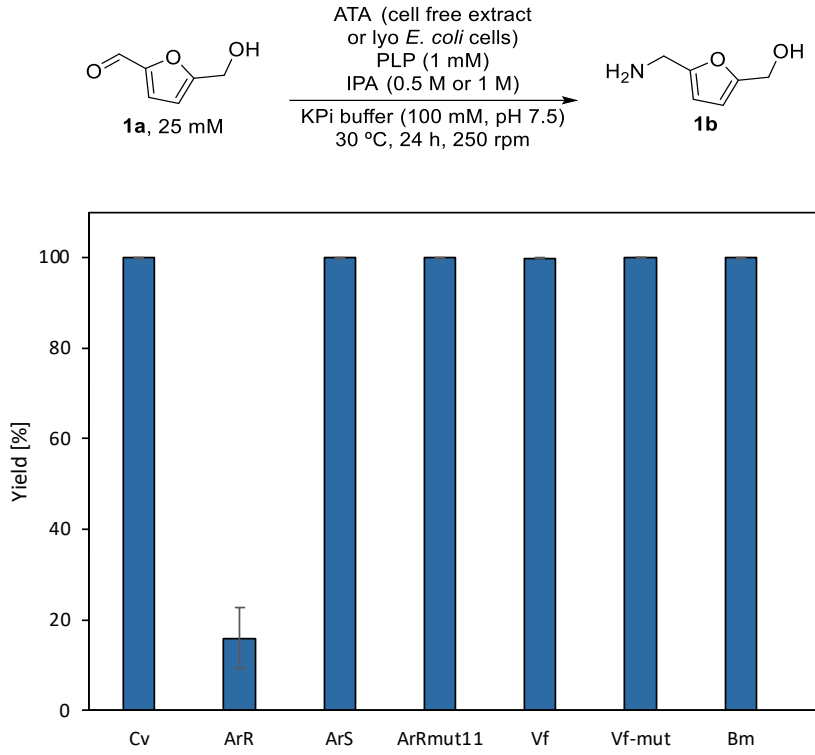
5-Hydroxymethylfurfural (HMF, **1a**, 25 mM) was used as a substrate for transamination reactions using a selection of ATAs under the standard conditions widely employed for this type of reaction such as 100 mM KPi buffer, and pH 7.5 (Scheme 2.5).[205]



Scheme 2.5. General scheme for the biotransamination reaction of HMF.

The ATAs were employed either as crude cell-free extracts (CFE) or as *E. coli* lyophilised cells, as detailed in the experimental section. The decision not to use all of them as CFE stemmed from the fact that certain ATAs (Vf-TA, Vf-mut-TA, Bm-TA and AtR-TA) were exclusively available in the form of *E. coli* lyophilised cells, without access to their respective plasmids or cell stocks. Moreover, limited stock of this ATAs hindered the process of obtaining the necessary lysate after cell lysis.

The concentration of IPA was set at 0.5 M for enzymes used as cell free lysate due to possible inhibition, while 1 M was selected for those used as *E. coli* lyophilised powder. After incubation at 30 °C and 250 rpm for 24 h, the reactions were stopped by the addition of trifluoroacetic acid (TFA) in water (10% v/v) and the samples were analysed by reverse phase HPLC. The results showed that quantitative yields (>99%) were achieved with several enzymes under the tested conditions (Scheme 2.6). Accordingly, Cv-TA, ArS-TA, ArRmut11-TA, Vf-TA, Vf-mut-TA, and Bm-TA were identified as the most promising candidates for studying the transformations at higher substrate concentrations.



Scheme 2.6. Enzyme screening results for the transamination of HMF (25 mM) substrate using IPA (0.5 M or 1 M) as amine donor for 24 h at 30 °C. Yield values were calculated by reverse phase HPLC analysis of the crude reaction mixtures using calibration curves (see section 2.3.7.1 in the experimental section).

Notably, full conversions for most candidates were obtained when substrate concentration was increased to 50 mM while maintaining the same enzyme loading and using 0.5 M of IPA (Figure 2.2).

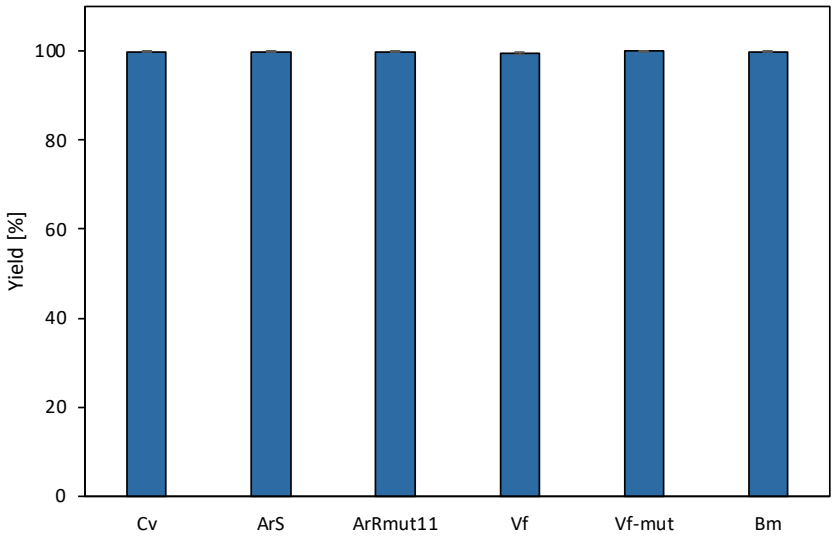
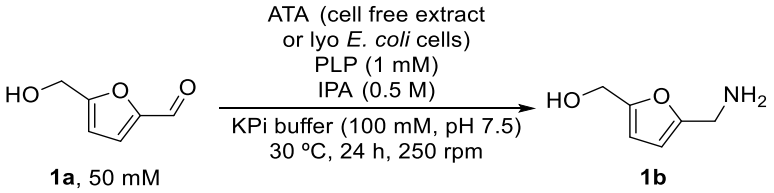


Figure 2.2. Biotransamination of HMF (50 mM) using IPA (0.5 M) of as amine donor for 24 h at 30 °C. Yield values were calculated by reverse phase HPLC analysis of the crude reaction mixtures using calibration curves (see section 2.3.7.1 in the experimental section).

To minimise enzyme consumption in the reactions, the enzyme loading was optimised, determining the minimum ATA amount per mmol of substrate required to obtain full conversion (Table 2.1). This optimisation was particularly crucial for those enzymes used as *E. coli* lyophilised cells due to limited stock availability, but also for the enzymes used as CFE to save reagents and time. The conditions used for this study were the same as reported above, these are 50 mM of **1a** and 0.5 M of IPA. This optimisation enabled significant reduction in employed enzyme amount, with a 24-fold for Cv-TA, 12-fold for ArS-TA and ArRmut11, 10-fold for Bm-TA and 1.7-fold for Vf-TA and Vf-mut-TA.

Table 2.1. Minimum enzyme/substrate relation needed to obtain full conversion into HMFA.[a]



Entry	Enzyme	Minimum enzyme / substrate ratio
1	Cv-TA	0.4[b]
2	ArS-TA	0.8[b]
3	ArRmut11-TA	0.8[b]
4	Vf-TA	120[c]
5	Vf-mut-TA	120[c]
6	Bm-TA	20[c]

[a] Biotransamination of HMF (**1a**, 50 mM) using IPA as amine donor (0.5 M) during 24 h at 30 °C and 250 rpm. Conversions were determined using reverse phase HPLC against substrate standards (see section 2.3.7.1 in the experimental section).
[b] mL $mmol^{-1}$.
[c] mg $mmol^{-1}$.

Subsequently, with the lowest enzyme/substrate ratio identified for these ATAs and keeping a constant IPA excess (10 equiv), HMF concentration was increased to 100 and 200 mM (Figure 2.3.a). Under these conditions, at 100 mM of HMF similar results to those obtained with 50 mM were obtained, observing only an important activity drop for Vf-TA and Bm-TA. When increasing the HMF concentration to 200 mM, the conversion notably decreased to less than 50% for Cv-TA, Vf-TA, and Bm-TA. However, ArRmut11-TA, ArS-TA, and Vf-mut-TA still provided

values higher than 80%, with the latter two ATAs showing nearly quantitative conversions (>96%).

Additionally, the influence of the enzyme loading on the reaction conversion was investigated, by doubling the enzyme amount while keeping IPA concentration constant (500 mM, Figure 2.3.b). Although complete conversions were achieved when increasing **1a** concentration from 50 to 100 mM, the transformation reactions yielded only 50-70% of amine **1b** at 200 mM of substrate. These results are likely related to the reaction equilibrium, emphasising the need for a higher IPA excess, since only 2.5 equiv were used, which might not be enough to completely shift the reaction towards the product formation.

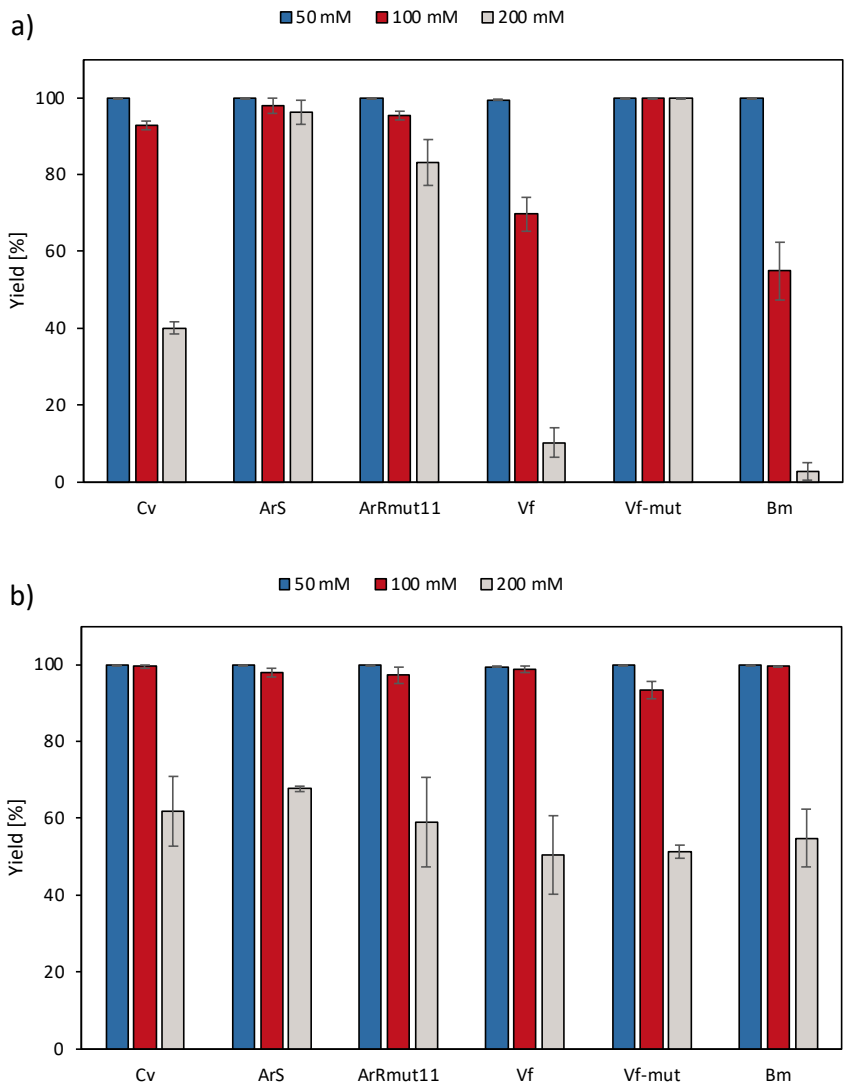


Figure 2.3. Transamination of HMF (**1a**, 50, 100, and 200 mM) for 24 h at 30 °C and 250 rpm using: a) The enzyme quantity in the ratio specified at Table 2.4 in the experimental section and IPA (10 equiv) as amine donor; and b) Doubling the amount of ATA while keeping constant the IPA concentration (500 mM),. Yield values were calculated by reverse phase HPLC analysis of the crude reaction mixtures using calibration curves (see section 2.3.7.1 in the experimental part).

2.2.3. Transition to organic solvent - enzyme immobilisation

To achieve the highest performance when running the reactions in organic solvent, selected ATAs were immobilised to reduce the activity loss after exposure to the organic medium. Among the best candidates found in previous studies, Cv-TA, ArS-TA and ArRmut11-TA, were selected based on their broad availability and possibility of obtaining the CFE (needed for immobilisation).

The ATA immobilisation process was carried out by incubating the enzyme solution containing 15 mg/mL of CFE and 0.3 mM of PLP with three different EziG carriers: Opal, Coral and Amber. The progress of the immobilisation was monitored by withdrawing samples after 2 h to test the remaining enzymatic activity of the supernatant using a spectrophotometric assay for the detection of produced acetophenone from racemic 1-phenylethylamine. The assay mixture contained 1-phenylethylamine (5 mM) and pyruvate (5 mM) in 20 mM sodium phosphate buffer pH 8.0, and the formation of ketone was monitored at 245 nm where acetophenone has an extinction coefficient of 12 mM^{-1} cm^{-1}. By comparing the decrease in the rate of acetophenone formation between the starting enzyme solution and the supernatant during immobilisation, the immobilisation yield was determined (Table 2.2).

Table 2.2. Immobilisation yield [%] of the three target transaminases on EziG Amber, Coral and Opal. 0.5 mL of 15 mg/mL of CFE resuspended in 20 mM sodium phosphate buffer with 0.3 mM PLP, pH 8.0, was added to 10 mg of carrier material. All the measurements were performed in duplicates and the result is presented as the means of the individual samples.

ATA	Amber	Coral	Opal
Cv	99 ± 0.3	98 ± 0.3	53 ± 5
ArS	98 ± 0.2	99 ± 0.5	99 ± 4
ArRmut11	86 ± 2	89 ± 10	42 ± 6

The results revealed that both Amber and Coral were effective supports for the three target enzymes, with immobilisation yields higher than 85% in all cases. For ArS-TA immobilised on Opal, a high immobilisation yield was also obtained, whereas with the other two ATAs, Cv-TA and ArRmut11-TA, this support provided moderate immobilisation yields (approximately 50%). In the view of these results, Amber and Coral were selected for further studies, now in organic solvent.

2.2.4. Biotransformations using ATA immobilised on EziG in organic solvent

Next, the biotransformations were run with two different commercial carbonylic substrates, which are 1-phenoxypropan-2-one (**2a**) and HMF (**1a**), the first one bearing a ketone functionality while the second one contains a reactive aldehyde group.

2.2.4.1. Reaction with model substrate: 1-phenoxypropan-2-one

After conducting the initial immobilisation tests, it was important to confirm the activity of selected ATAs in organic solvent using a model substrate, 1-phenoxypropan-2-one. Water is essential in the local environment surrounding the protein, ensuring its three-dimensional structure remains intact while preventing self-aggregation.[218] Each ATA-catalysed reaction requires its own level of water activity in the reaction mixture for optimum functionality. Typically, less than a monolayer of water is sufficient for an enzyme to exhibit activity in an organic solvent. To control and set this water activity, the most straightforward approach involves adding water to the reaction mixture.

In this Doctoral Thesis, the immobilised ATAs were utilised as wet formulations. Since the immobilisation was carried out in an aqueous buffer, the immobilised enzyme could not be directly used in the reaction due to the excessive water content in the formulation. Although this water excess could protect the enzyme from the bulk organic solvent, it also hinders the uniform distribution of the catalyst within the reaction mixture and impedes efficient mass transfer of the target compound to the enzyme. To reduce the amount of water in a controlled manner and to prevent a decrease in enzymatic activity, an additional step was introduced to the immobilisation protocol consisting of a series of washing steps, first with 2-PrOH containing a predefined volume of deionised water (%v/v), and second with the organic solvent used in the reaction to remove the remaining 2-PrOH.

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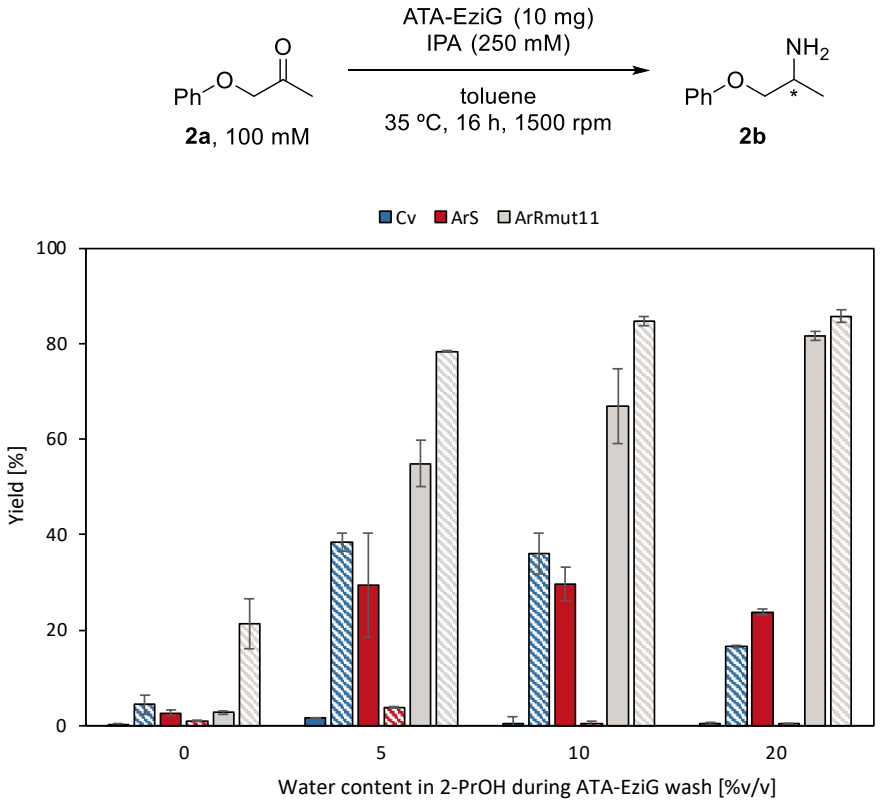
[218] A. Zaks and A. M. Klibanov, *J. Biol. Chem.*, 1988, **263**, 8017–8021.

Ideally, the quantification of water content in the reaction media could have been determined through Karl Fischer titration. However, due to equipment limitations, such characterisation could not be performed. Consequently, the precise quantification of water amount within the immobilised preparation was not accomplished. As a result, each water activity level was characterised as "water content in 2-PrOH during ATA-EziG wash" referring to %v/v of water in 2-PrOH used during the washing steps after immobilisation.

To assess the activity of the three ATAs immobilised on Coral and Amber (Opal was excluded at this stage due to very low immobilisation yields), a series of reactions in toluene with 1-phenoxypropan-2-one (100 mM) and IPA (250 mM) as an amine donor were conducted (Scheme 2.7). Four different water activity levels were explored using various 2-PrOH washing solutions (0, 5, 10, and 20 %v/v water content).



Scheme 2.7. Biotransamination of 1-phenoxypropan-2-one using ATA-EziG enzyme preparations containing various levels of water. Reaction conditions: 100 mM 1-phenoxypropan-2-one, 250 mM IPA, water saturated toluene, ATA-EziG (10 mg of EziG with 0.5 mL of 15 mg/mL CFE) in 1 mL total reaction volume, 16 h reaction time, 35 °C, and 1500 rpm. Yield values were were determined using GC, after derivatisation of the samples with acetic anhydride (see section 2.3.7.2 in the experimental section). Legend – Full: enzymes immobilised on Amber. Dashed: enzymes immobilised on Coral.

The obtained results showed that ArRmut11-TA immobilised on both Amber and Coral carriers exhibit good activity under the initial conditions. For Cv-TA immobilised on Coral and ArS-TA immobilised on Amber, moderate activities were observed. Interestingly, the water content in the preparation of the immobilised ATA, and consequently the water activity in

the reaction, played a significant role in the observed activity. In the case of ArRmut11-TA, higher water contents, 20% v/v, were found to be desirable, whereas for Cv-TA and ArS-TA, lower water contents, 5% v/v, resulted in higher conversions. This emphasizes the importance of carefully controlling the water activity to optimize the enzymatic performance of the immobilised ATAs.

2.2.4.2. Reaction with target substrate: 5-hydroxymethylfurfural (HMF)

The initial conditions tested in the target reaction were the same as those previously used for the model reaction with 1-phenoxypropan-2-one, but ethyl acetate (EtOAc) was used instead of toluene due to the higher solubility of HMF in EtOAc.

the best immobilised ATAs found in the 1-phenoxypropan-2-one transamination were selected for the reaction with HMF, namely Coral for Cv-TA and ArRmut11-TA, and Amber for ArS-TA. Prior to mixing with the organic solvent, ATA washes with 10% v/v water in 2-PrOH were performed. In addition, a negative control without catalyst was included to determine whether the reaction was indeed catalysed by the ATAs. The reactions were analysed after derivatisation with acetic anhydride, and the amounts of substrate (HMF) and product (HMFA) were determined using calibration curves prepared with commercial standards (see experimental section for further details).

For all the three enzymes and the positive control, it was observed that the amount of HMF substrate was decreasing. However, it did not correspond with the amount of HMFA formed, indicating low carbon balance between the substrate and the product in the reaction (Figure 2.4). To confirm that the support was not responsible for these observations, the reactions were repeated with the three ATAs immobilised on Opal, showing consistent results with those previously obtained. Furthermore, in the

negative control, a decrease of more than 20% in the substrate concentration was observed, but no HMFA formation was detected.

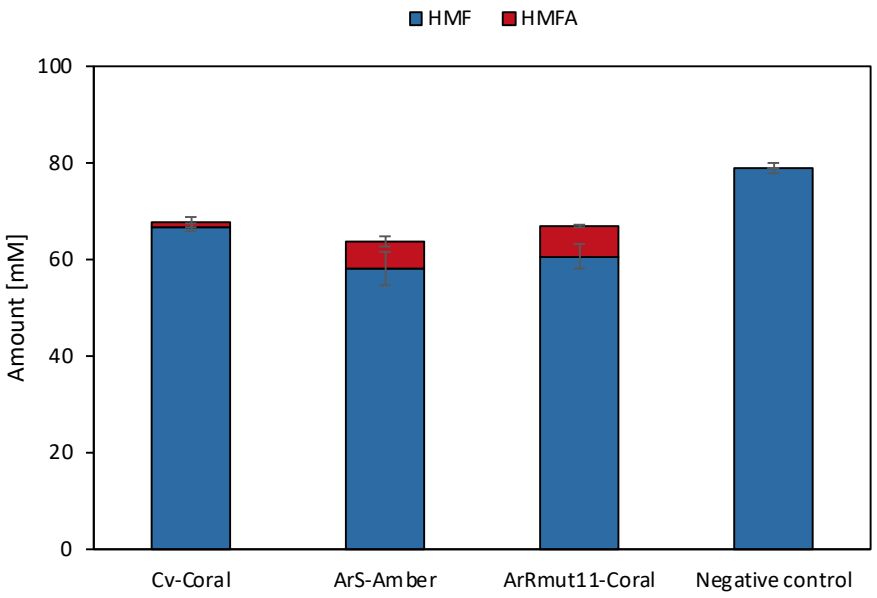


Figure 2.4. Biotransamination of HMF using Cv-Coral, ArS-Amber and ArRmut11-Coral. Reaction conditions: 100 mM HMF, 250 mM IPA, water saturated ethyl acetate, ATA-EziG (10 mg of EziG with 0.5 mL of 15 mg/mL CFE) in 1 mL total reaction volume after 16 h and 35 °C at 1500 rpm. Amount values [mM] were determined using GC analyses, after derivatisation of the samples with acetic anhydride (see section 2.3.7.2. in experimental part).

This suggests that there might be some compound(s) in the reaction mixture that are not being detected by the GC analysis. Thus, the samples were reinjected using a longer method with higher temperatures (ranging from 225 °C to 325 °C). However, no additional peaks were observed in the resulting chromatograms.

Subsequently, the samples were injected without prior derivatisation (Figure 2.5) to explore the possibility of additional products in the reaction mixture. Interestingly, the HMF peak was not detected in any of the cases, but two new signals were observed in the GC chromatogram. The peak with

the largest area was identified as the imine formed between HMF and IPA. This can be attributed to the high reactivity of IPA, present in excess to drive the equilibrium toward amine synthesis, with the aldehyde group of HMF, resulting in the formation of the HMF-IPA-Imine (**1c**). The presence of this particular peak has been interpreted as residual substrate in all enzymatic reaction samples. The other initially unknown peak observed was associated with the imine formed between the carbonyl group of HMF and the amine from HMFA, referred here as HMF-HMFA-Imine (**1d**). The results from reinjected samples without derivatisation are presented in area % due to the unavailability of standards for both imines (Figure 2.5). It was observed that the predominant compound in the reaction mixture is HMF-IPA-Imine (**1c**), followed by HMF-HMFA-Imine (**1d**), and, if detected, HMFA (**1b**). Remarkably, the relative area of HMF-IPA-Imine is higher than 85% in all cases, indicating that there is minimum conversion of HMF to the target HMFA.

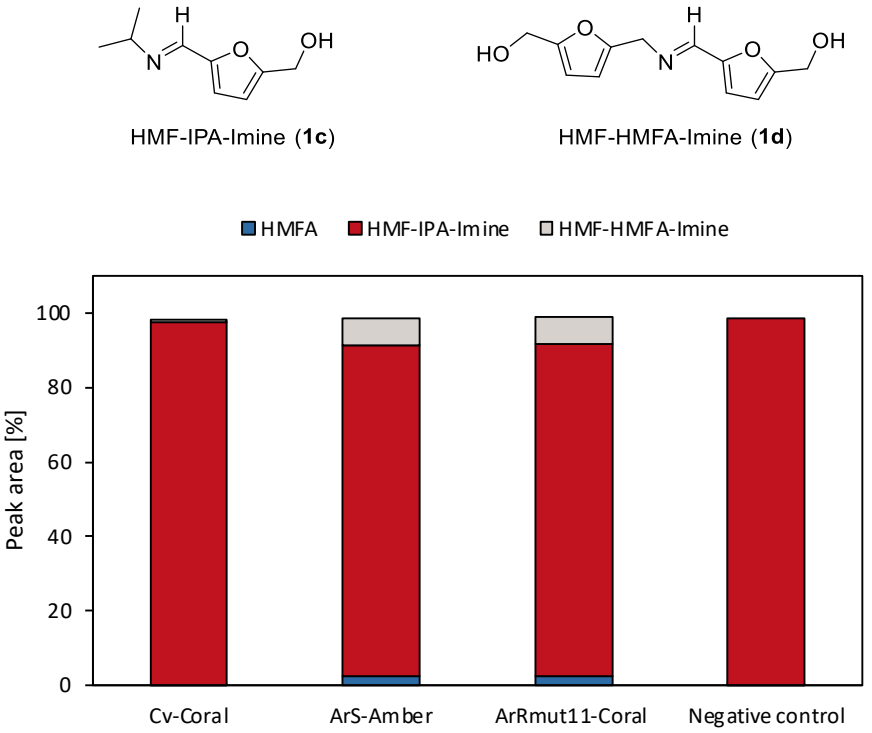


Figure 2.5. Product distribution data from the biotransformations of HMF in ethyl acetate using ATA-EziG. Biotransformation conditions: 100 mM HMF, 250 mM IPA, water saturated ethyl acetate, ATA-EziG (10 mg of EziG with 0.5 mL of 15 mg/mL CFE) in 1 mL total reaction volume after 16 h reaction time at 35 °C and 1500 rpm. Negative control was included, containing the same reaction mixture but without catalyst. Peak areas [%] were determined using GC analysis (see section 2.3.7.3 in the experimental section).

In contrast to the previous observations in buffer reactions, where substantial conversions to HMFA were achieved at up to 100 mM substrate concentration, in the organic solvent, chemical imine formation appears to be more favoured than the biotransamination of HMF.

2.2.5. Shifting imine equilibrium

The reaction of primary amines with carbonyl compounds to form imines, also known as Schiff bases or azomethines, is a well-known chemical process. In theory, imine formation is in equilibrium with the carbonyl compound, aldehyde, or ketone. Here, to shift the equilibrium towards HMF aldehyde formation, two approaches have been explored: 1) Increasing the water content in the immobilised ATA preparations; and 2) Introducing a small amount of protic acid into the reaction media.

2.2.5.1. Water profile for ArRmut11

When an imine is formed from an aldehyde and a primary amine, one molecule of water is released per imine molecule. Therefore, the formation of imines is favoured when water is removed from the reaction mixture. Consequently, the addition of water and an increase in the protic strength of the reaction medium could shift the imine equilibrium towards the carbonyl compound.

In this study, the amount of water in the reaction mixture was determined by the water content in the 2-PrOH solutions used to wash the immobilised formulations. The biotransformation of HMF with immobilised ArRmut11 was studied (Figure 2.6) in ethyl acetate using six different water activity levels, set by various 2-PrOH washing solutions (0, 5, 10, 20, 40, and 80% v/v water). The results indicated that the optimal washing conditions were between 5 and 10% v/v of water in 2-PrOH, as this resulted in a lower relative area of HMF-IPA-Imine, which is considered as unreacted HMF. This suggests that increasing the water content does not significantly affect the aldehyde-imine equilibrium to the extent that it could impact the biotransformation.

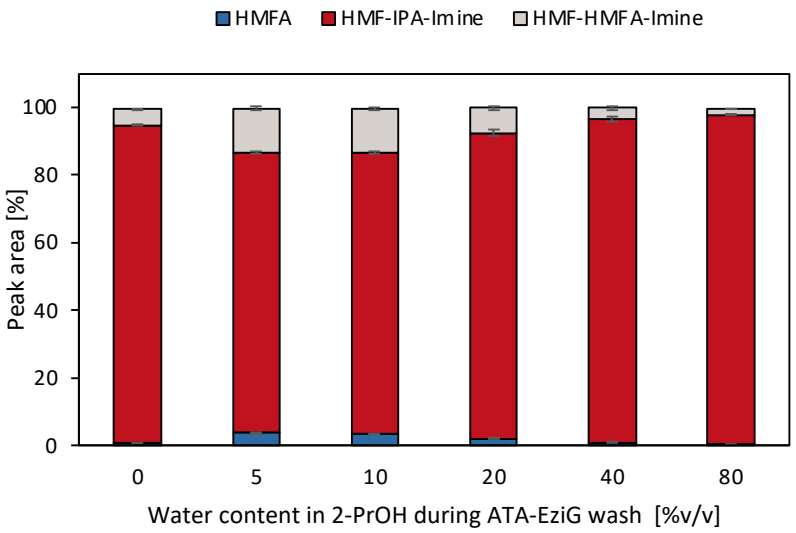


Figure 2.6. Product distribution data from the biotransformations of HMF in ethyl acetate using ArRmut11-EziG washed with 2-PrOH containing varying levels of water. Biotransformation conditions: 100 mM HMF, 250 mM IPA, water saturated ethyl acetate, ATA-EziG (10 mg of EziG with 0.5 mL of 15 mg/mL CFE) in 1 mL total reaction volume, 16 h reaction time, 35 °C, 1500 rpm. Peak areas [%] were determined using GC (see section 2.3.7.3. in experimental part).

Additionally, two different biocatalyst amounts were tested, 10 mg and 30 mg, but the observed trends were the same. The only difference was that the relative area of HMF-IPA-Imine was lower when 30 mg of catalyst was used compared to 10 mg.

2.2.5.2. Protic solvent in the reaction mixture

After determining the best washing conditions for the enzyme preparation, the addition of protic cosolvents in small amounts to the reaction media was investigated. Increasing the protic strength could help to shift the imine equilibrium towards the carbonyl compound, making it available for the transaminase to convert in this case HMF into HMFA. To

explore this, four protic cosolvents with different characteristics were tested: 2-PrOH, 2-pentanol, methanol (MeOH), and 1-phenylethanol (Figure 2.7.). However, the results showed that the presence of protic solvents in small amounts did not yield the desired outcome and, instead, negatively impacted the enzymatic reaction. In fact, the obtained results were worse than those observed previously when no cosolvent was added. Further investigation may be needed to understand the underlying reasons for this unexpected outcome and to identify alternative strategies for enhancing the desired imine-to-carbonyl conversion in the biotransformation process.

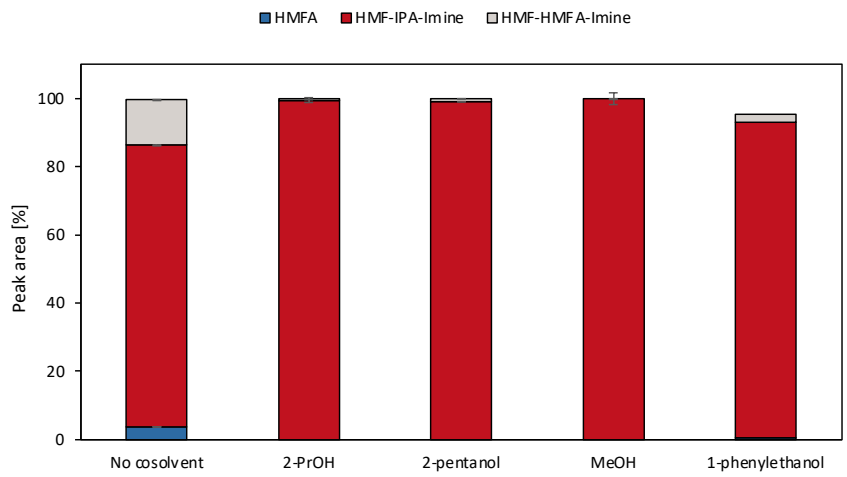


Figure 2.7. Product distribution data for the biotransformations of HMF in ethyl acetate using ArRMut11-EziG-Amber washed with isopropyl alcohol containing 10%v/v of water. Reaction conditions: 100 mM HMF, 250 mM IPA, water saturated ethyl acetate, 10%v/v protic cosolvent, ATA-EziG-Amber (10 mg of EziG with 0.5 mL of 15 mg/mL CFE) in 1 mL total reaction volume after 16 h at 35 °C and 1500 rpm. Peak areas [%]were determined using GC analysis (see section 2.3.7.3. in the experimental section).

Conclusions

Overall, the following conclusions can be highlighted after studying the use of different ATAs for transamination reactions in both aqueous and organic media:

- Six suitable ATA candidates were identified for the HMF transamination studies in aqueous buffer, and reaction conditions were optimised. From these, three, Cv, ArS and ArRmut11, were selected to investigate enzyme immobilisation.

- All these three ATAs were immobilised on three different EziG supports, Amber, Coral and Opal. The best results in terms of immobilisation yields were obtained with Amber and Coral supports.

- The immobilised enzymes were tested for the transamination of 1-phenoxypropan-2-one using isopropylamine as amine donor in organic solvent.

- The catalysts that showed highest activity (Cv-Coral, ArS-Amber and ArRmut11-Coral) with the 1-phenoxypropan-2-one were tested for HMF biotransamination in organic solvent. It was determined that imine formation between HMF and IPA amine donor was a limiting factor for implementation of ATA-catalysed reaction in organic solvent.

Experimental part

2.3.1. General information

All chemicals were purchased from Sigma Aldrich unless stated otherwise. EziG supports (Amber, Coral and Opal) were provided by EnginZyme AB. Gas chromatography (GC) analyses were performed on an Agilent HP6890 GC chromatograph equipped with a FID detector and analytical reverse phase analysis was performed using an Agilent 1100 Series HPLC equipped with a photodiode array detector.

2.3.2. ATA expression and cell lysis

Table 2.3. Name of ATAs used in this contribution.

Abbreviation	Original organism
Cv	*Chromobacterium violaceum*
ArR	*Arthrobacter* sp. (*R*)-selective
ArS	*Arthrobacter citreus*
ArRmut11	*Arthrobacter* sp. (*R*) round 11 variant
Vf	*Vibrio fluvialis*
Vf-mut	*Vibrio fluvialis* variant
Bm	*Bacillus megaterium*
AtR	*Aspergillus terreus* (*R*)-selective

2.3.2.1. General protocol for the recombinant expression of ATAs

The cells were grown at 37 °C in terrific broth medium supplemented with ampicillin (100 μg mL^{-1}) or kanamycin (50 μg mL^{-1}). Expression was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at an OD_{600} between 0.6 and 0.8 and carried out at 30 °C overnight. The cells were harvested at 4000 rpm for 20 min and resuspended in buffer (50 mM potassium phosphate buffer (KPi) buffer, pH 7.5, 0.1 mM PLP).

2.3.2.2. General protocol for cell lysis

Cell lysis of all the samples was performed using a sonicator (1 s pulse on; 4 s pause off; 2.5 min; 40% amplitude). The lysed cells were centrifuged at 4500 rpm for 1 h at 4 °C. Then, the supernatant was recovered and frozen with liquid nitrogen and lyophilised. The cell free extracts containing ATAs were stored at -20 °C, remaining stable for several months.

2.3.3. Measurement of ATA activities

The activity of the ATAs used as cell free extract (CFE) was determined with an adjusted standard photometric assay. Here, the substrate 1-phenylethylamine is converted to acetophenone, which can be detected spectrophotometrically at 245 nm. The absorbance increases with rising product concentration. To measure the activities, reactions were set up in a 200 µL volume with 20 mM sodium phosphate buffer pH 8.0 containing 5 mM 1-phenylethylamine, and 5 mM sodium pyruvate. For the reaction, 100 µL of cell free lysates were used. The production of acetophenone was measured for 30 minutes at 245 nm. The volumetric activity as U mL^{-1} of the enzyme samples was calculated using the Beer−Lambert law with the molar extinction coefficient of acetophenone ε_{245} = 12 mM^{-1} cm^{-1}.

Table 2.4. Units measured for ATAs used in CFE preparations. 1 unit is defined as 1 µmol of formed product per min.

Abbreviation	U (µmol min^{-1}) /mL of CFE
Cv	1.30
ArR	0.98
ArS	0.28

2.3.4. Biotransamination experiments

Reactions in buffer. Reactions were performed on a total reaction volume of 500 µL in an Eppendorf vial, containing the substrate (25-200 mM), PLP (1 mM), potassium phosphate buffer (100 mM, pH 7.5), and the crude cell lysate (240 µL) or *E. coli* lyophilized cells heterologously expressing the corresponding ATA (5 mg). The concentration of IPA was 0.5 M, for enzymes used as cell free lysates, and 1 M for the enzymes used as *E. coli* lyophilized cells. After incubation at 30 °C and 250 rpm for 16 h, the reactions were stopped by the addition of 10% (v/v) trifluoroacetic acid (TFA) in water (25 µL). Denatured protein was removed by centrifugation (3000 rpm, 4 min) and the supernatant diluted, filtered with a 45 µm micro-filter, and analysed by analytical HPLC. The substrate HMF was detected at 280 nm and the target amine HMFA at 210 nm. Conversions were obtained using calibration curves prepared with substrate and product standards (see section 2.3.7.1.).

Reactions in organic solvent. Reactions were performed on a total reaction volume of 1 mL in an Eppendorf vial, containing the substrate (100 mM), IPA (250 mM), water saturated organic solvent and ATA-EziG (10 mg of EziG with 0.5 mL of 15 mg/mL CFE) incubation at 35 °C and 1200 rpm for 16 h. Reactions were analysed by GC (see sections 2.3.7.2 and 2.3.7.3.).

2.3.5. ATAs immobilisation

2.3.5.1. General immobilisation procedure

The freeze-dried powder of ATA-His-tag was rehydrated in 20 mM sodium phosphate buffer, pH 8 containing 0.3 mM PLP to obtain a CFE concentration of 15 mg/mL (freeze-dried powder/buffer solution). The prepared CFE was resuspended on an end-over-end rotator for 1 h (20 rpm, rt) and then centrifuged for 5 min (7000 rpm, rt). After centrifugation, the CFE (500 µL) was transferred to a new tube containing the 10 mg of the

support. Tubes were covered with foil and immobilisation was performed on an end-over-end rotator, 20 rpm, at room temperature for 2 h. Then, the supernatants were removed, and the immobilised supports were washed first with immobilisation buffer (1 x 1 mL, 30 sec each), second with 2-PrOH washing solutions (2 x 1 mL, 30 sec each) and third with the solvent used for the reaction (1 x 1 mL, 30 sec), then the catalyst was used directly after removal of remaining solvent.

2.3.5.2. Immobilisation yield

To measure the immobilisation yield, the CFE and supernatant from the immobilisations were diluted with 20 mM sodium phosphate buffer, pH 8. An aliquot of the diluted enzyme solution (100 μL) was mixed with a reaction mixture (100 μL) containing 10 mM 1-phenylethylamine, 20 mM sodium pyruvate in 20 mM sodium phosphate buffer, pH 8. The formation of acetophenone was measured by the absorbance (A_{245}) at 245 nm every 49 s for 20 min using a plate reader. Reaction rates with each supernatant from immobilisations and fresh enzyme solution were extracted from linear regression of the data points (A_{245}/min). The immobilised yield was calculated by determining the percentage of enzymatic activity left in the supernatant after immobilisation, relative to the enzymatic activity in the CFE.

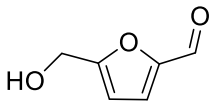
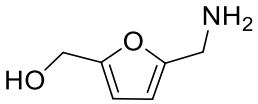
2.3.6. Analytical methods

2.3.6.1. Reverse phase HPLC analyses – reactions in buffer

Analytical reverse phase analysis was used for quantitative analysis of transaminase reactions of HMF (**1a**), using a Mediterranea Sea C18 column (18.5 μm x 250 mm x 4.6 mm) from Teknokroma at 30 °C. Elution was carried out at 1 mL min^{-1} with a linear gradient 15–75% of acetonitrile/H2O

(v/v) containing 0.01% TFA over 30 min, with detection at 280 (for HMF, **1a**) or 210 nm (for HMFA, **1b**). The injection volume was 10 µL. Product concentrations were obtained from calibration curves prepared with commercial standards.

Table 2.5. HPLC retention times for **1a** and **1b**.

Compound	Retention time (min)	Compound	Retention time (min)
 **1a**	5.5	 **1b**	2.6

2.3.6.2. Gas chromatography analyses – reactions in organic solvent – derivatized samples

GC analyses were performed using the HP-5 column (30 m x 0.32 mm x 0.25 µm) for HMF (**1a**) reactions and CP-Chirasil Dex DB (25 m x 0.25 mm x 0.25 µm) for 1-phenoxypropan-2-one (**2a**) reactions.

Reactions were analysed after derivatisation with acetic anhydride. For this, after stopping the mixing and allowing the catalyst to settle, an aliquot (40 µL) of the supernatant was transferred to a new tube containing acetic anhydride (130 µL) and 1-methylimidazole (40 µL) to promote the derivatisation. After gently mixing, the solution was left standing for 30 min at room temperature. Deionised water (460 µL) was added to the mixture, gently mixed and ethyl acetate containing 40 mM dodecane as internal standard (460 µL) was added. The reaction was extracted for 5 min (1500 rpm, 25 °C) and the tubes were centrifuged for 5 min (15000 rpm) to promote phase separation. The organic layer (200 µL) was transferred to another tube and dried over Na_2SO_4. An aliquot of the dried organic layer

(120 µL) was transferred to a GC vial and analysed by gas chromatography coupled to flame ionisation detector (GC-FID) to determine the conversion.

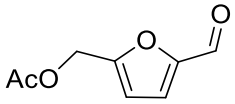
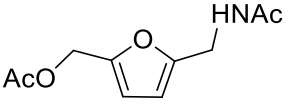
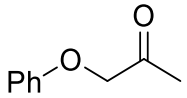
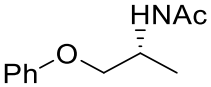
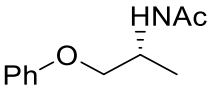
Method specifications used for HMF (1a)

HP-5 (30 m x 0.32 mm x 0.25 µm). 5 mL/min Hydrogen, 79 kPa. Injection 1 µL with 20:1 split ratio. Injection temperature: 250 °C, Detector temperature: 250 °C, Detector type: FID. Oven temperature: 60 °C, hold 2 min, 15 °C/min ramp to 225 °C, hold 2 min. Treatment of results: Calibration curve with 1,4-dioxane as an internal standard

Method specifications used for 1-phenoxypropan-2-one (2a)

CP-Chirasil Dex DB (25 m x 0.25 mm x 0.25 µm). 2 mL/min Hydrogen, 47 kPa. Injection 1 µL with 20:1 split ratio. Injection temperature: 200 °C, Detector temperature: 250 °C, Detector type: FID. Oven temperature: 100 °C, hold 2 min, 15 °C/min ramp to 195 °C, hold 2 min. Treatment of results: Calibration curve with dodecane as an internal standard

Table 2.6. GC retention times for methods with sample derivatisation.

Compound	Retention time (min)	Compound	Retention time (min)
 Ac-**1a**	7.0	 **Ac-1b**	10.5
 **2a**	5.5	 Ac-(*R*)-**2b**	8.5
		 Ac-(*R*)-**2b**	8.6

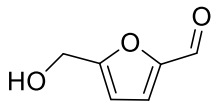
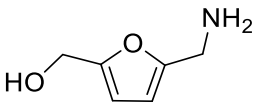
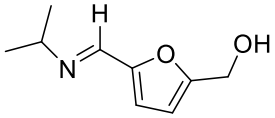
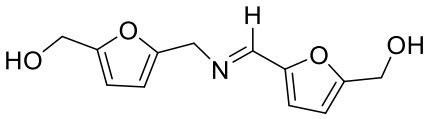
2.3.7.2. Gas chromatography analyses – reactions in organic solvent – direct analyses (without derivatisation)

GC analyses were performed on an Agilent HP6890 GC chromatograph equipped with an FID detector and HP-5 column (30 m x 0.32 mm x 0.25 μm).

Method specifications

HP-5 (30 m x 0.32 mm x 0.25 μm). 5 mL/min Hydrogen, 79 kPa. Injection 1 μL with 20:1 split ratio. Injection temperature: 250 °C, Detector temperature: 250 °C, Detector type: FID. Oven temperature: 60 °C, hold 2 min, 15 °C/min ramp to 225 °C, hold 2 min. Treatment of results: Area % with 1,4-dioxane as an internal standard.

Table 2.7. GC retention times for methods without sample derivatisation.

Compound	Retention time (min)
 **1a**	5.9
 **1b**	5.6
 **1c**	7.5
 **1d**	13.4

CHAPTER 3.

Development of an amine transaminase-lipase cascade for chiral amide synthesis under batch and flow conditions

Introduction

3.1.1. Enzymatic cascades

Nature employs various biocatalysts to facilitate the preparation of complex organic compounds. These systems typically involve multiple enzymes, each with a well-defined role, adapted to specific steps in the process without participating in other reactions along the synthetic route. A classic example is the Krebs cycle, a metabolic pathway associated with cellular respiration, which orchestrates the action of eight different enzymes.[219] Biocatalytic cascades mimic the natural metabolic pathways found in living organisms, but they are engineered for specific purposes in industrial and synthetic applications.[21, 220] The advantages of biocatalytic cascades include high specificity, mild reaction conditions, and the opportunity to obtain high-value products in a few operational steps while generating minimal waste.[221,222] However, optimising biocatalytic cascades can be challenging due to the interdependence of different enzymatic steps and potential compatibility issues.

Depending on how the reaction steps are organised, it is possible to distinguish between two main different cascade modes (Figure 3.1):[223]

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[219] I. Jochmanova, K. Pacak, *Clin. Cancer Res*. **2016**, *22*, 5001-5011.
[220] Biocatalysis for Practitioners. Techniques, Reactions and Applications (Eds.: G. de Gonzalo, I. Lavandera), Wiley-VCH, Weinheim (Germany), **2021**.
[221] J. H. Schrittwieser, S. Velikogne, M. Hall, W. Kroutil, *Chem. Rev.* **2018**, *118*, 270–348.
[222] E. T. Hwang, S. Lee, *ACS Catal.* **2019**, *9*, 4402–4425.
[223] R. Siedentop, C. Claaßen, D. Rother, S. Lütz, K. Rosenthal, *Catalysts* **2021**, *11*, 1183.

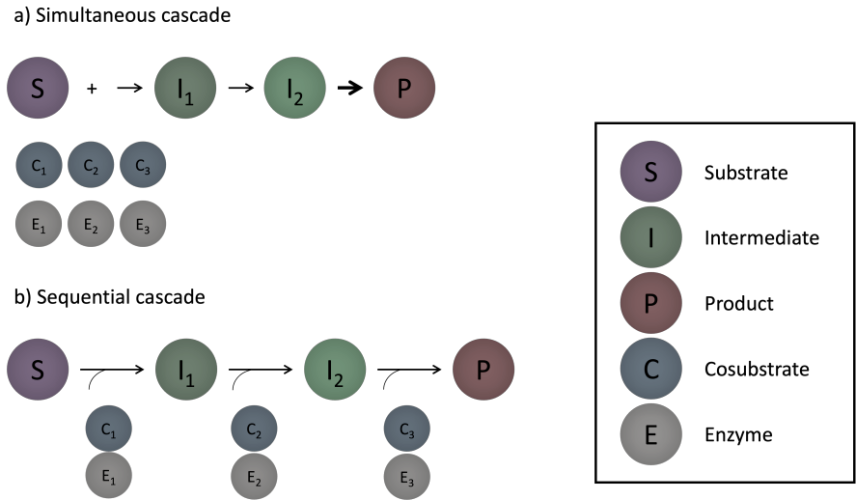

Figure 3.1. Cascade mode depending on how reaction steps are organised.

- Simultaneous or concurrent (Figure 3.1.a): In this approach, all reaction components are introduced into the same reaction vessel (a one-pot system) simultaneously, and upon completion, the final product is isolated. The reaction conditions, such as temperature, pressure, agitation, and solvent, remain constant for all catalysts involved. The simultaneous cascade stands as the most straightforward strategy for reactions in one pot and it is a very useful approach for processes involving intermediates that are highly unstable, avoiding their isolation or manipulation. Nevertheless, in certain cases, adding all reaction components simultaneously might not be feasible due to potential incompatibilities between enzyme(s) and substrate(s) or product(s).
- Sequential (Figure 3.1.b): In this methodology, the reaction steps are separated either in terms of time or space, with a single isolation step at the end of the process. After a specific synthesis step concludes, the reaction conditions are altered in some way, such as adding new reactants or catalysts, modifying temperature, pH, reactant concentrations, reaction solvent, or other variables. For example, for separation in time, the enzymes responsible for the next reaction step can be added in a timely fashion.

Another classification can be done based on the possible interconnections between the catalytic steps (Figure 3.2).[224]

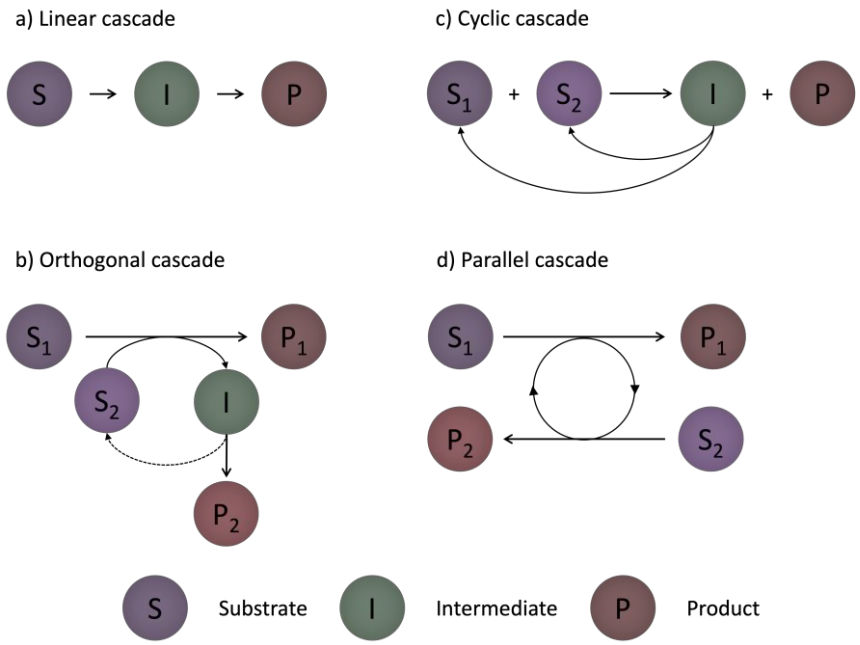

Figure 3.2. Cascade modes depending on possible interconnections between catalytic steps.

- Linear (Figure 3.2.a): The product of one reaction serves as the starting substrate for the next. This cascade configuration is the most commonly applied.[221]
- Orthogonal (Figure 3.2.b): The equilibrium of a reaction is shifted towards the formation of the desired product, or alternatively, some by-product that might deactivate the catalyst of the main reaction is removed.[159]

[224] E. Ricca, B. Brucher, J. H. Schrittwieser, *Adv. Synth. Catal.* **2011**, *353*, 2239–2262.

- Cyclic (Figure 3.2.c): Particularly used in deracemisation or stereoinversion processes, primarily outlined for the synthesis of optically active alcohols, amines, and amino acids.[225,226]
- Parallel (Figure 3.2.d): Two reactions happen simultaneously, interconnected by the action of a compound, for example, a cofactor.[227,228]

There are also hybrid forms that mix these reaction variations, particularly when an extensive number of reaction stages are combined. It is worth noting that in the literature, the terms "enzyme cascade," "multi-enzymatic cascade/reaction," and "multi-step reaction" are often used interchangeably, with "cascade" encompassing any sequence of reactions comprising more than one step.

The advantages of adopting multienzymatic systems over traditional stepwise chemical methods and consecutive batch-reactions are evident in the increasing number of processes adopting such type of systems. This transition avoids the need for isolating (unstable) intermediates, streamlines the work-up process by reducing the number of steps, and generally results in high-yielding overall processes. However, there are still some limitations to consider when integrating one-pot systems during cascade assembly such as: (i) Potential enzyme inhibition by any of the reagents, products or catalysts; (ii) Determining optimal substrate concentrations for different enzyme classes; (iii) Identification of suitable pH ranges and temperatures for the individual enzymes; or (iv) Selection of an appropriate reaction medium that enables desired activity for all catalysts involved.[224] A

[225] M. M. Musa, F. Hollmann, F. G. Mutti, *Catal. Sci. Technol*. **2019**, *9*, 5487–5503.
[226] C. Aranda, G. Oksdatch-Mansilla, F. R. Bisogno, G. de Gonzalo, *Adv. Synth. Catal.* **2020**, *362*, 1233–1257.
[227] C. Rodríguez, I. Lavandera, V. Gotor, *Curr. Org. Chem.* **2012**, *16*, 2525-2541.
[228] S. Mordhorst, J. N. Andexer, *Nat. Prod. Rep.* **2020**, *37*, 1316–1333.

potential solution to these challenges is the adoption of a flow setup, which allows for the compartmentalisation and segregation of different enzymes in separate reactors, enabling the implementation of transformations that would not be feasible under normal batch conditions.[229,230]

3.1.2. Flow biocatalysis

Flow chemistry, also known as continuous flow chemistry, refers to processes conducted in a continuous stream of fluids rather than in a flask (batch mode). In flow chemistry, reactants are continuously pumped into a flow reactor, where they mix and react as they flow through a series of channels or tubes. This approach offers several advantages over traditional batch processess:[231,232,233]

1. Easier control: Improved control over reaction conditions, such as temperature, pressure, and mixing.[234] In a continuous flow system, reactants are mixed more uniformly and consistently than in batch, leading to better control over reaction kinetics and more predictable outcomes. Moreover, flow reactors typically have a higher surface area-to-volume ratio compared to traditional batch vessels, enabling faster and more efficient heat transfer.

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[229] A. P. Mattey, G. J. Ford, J. Citoler, C. Baldwin, J. R. Marshall, R. B. Palmer, M. Thompson, N. J. Turner, S. C. Cosgrove, S. L. Flitsch, *Angew. Chem. Int. Ed.* **2021**, *60*, 18660–18665.
[230] S. C. Cosgrove, A. P. Mattey, *Chem. Eur. J.*, **2022**, *28*, e202103607.
[231] S. G. Newman, K. F. Jensen, *Green Chem.* **2013**, *15*, 1456–1472.
[232] M. B. Plutschack, B. Pieber, K. Gilmore, P. H. Seeberger, *Chem. Rev.* **2017**, *117*, 11796 11893.
[233] G. Gambacorta, J. S. Sharley, I. R. Baxendale, *Beilstein J. Org. Chem.* **2021**, *17*, 1181–1312.
[234] L. Capaldo, Z. Wen, T. Noël, *Chem. Sci.* **2023**, 4230–4247.

2. Safety: In general, because reactions occur within a closed system and at a smaller scale, so potential hazards and risks can be minimised. Moreover, reactants are continuously flowing through the system, which reduces the risk of sudden and unexpected reactions. Moreover, uniform and consistent mixing of reactants reduces the possibility of localised high reactant and product concentrations that can lead to unsafe reactions.
3. Productivity: Continuous flow processes can lead to more efficient reactions (due to enhanced mass transfer, improved mixing and heat transfer, etc.) and reduced reaction times. This can result in higher productivity and less energy consumption.
4. Scalability: The principles of flow chemistry are scalable, making it easier to translate laboratory-scale reactions to larger production scales.
5. Good versatility: Multiple reaction steps or processes can be integrated into a single flow system, leading to streamlined synthesis routes and reduced purification steps.
6. Waste reduction: Flow systems can help minimize the formation of byproducts and waste, resulting in greener and more sustainable processes.

While flow chemistry offers a modular approach that provides synthetic chemists a wide range of options for experimental work, there are certain components that typically constitute a flow setup (Figure 3.3): A sampling unit (such as a reservoir, syringe, or sampling loop), a pumping unit, a mixing unit, a reactor, and ultimately, a collection unit for capturing the reaction product.[235] Additionally, to extend functionality and introduce advanced process control features, some extra modules can be incorporated. For

[235] M. Santi, L. Sancineto, V. Nascimento, J. B. Azeredo, E. V. M. Orozco, L. H. Andrade, H. Gröger, C. Santi, *Int. J. Mol. Sci.* **2021**, *22*, 990.

example, back pressure regulators (BPR) and inline phase separators, can be employed to either pressurize the system or carry out downstream extraction within the flow setup. Moreover, the introduction of inline switching valves and analytical cells enables the real-time monitoring of reactions.[236]

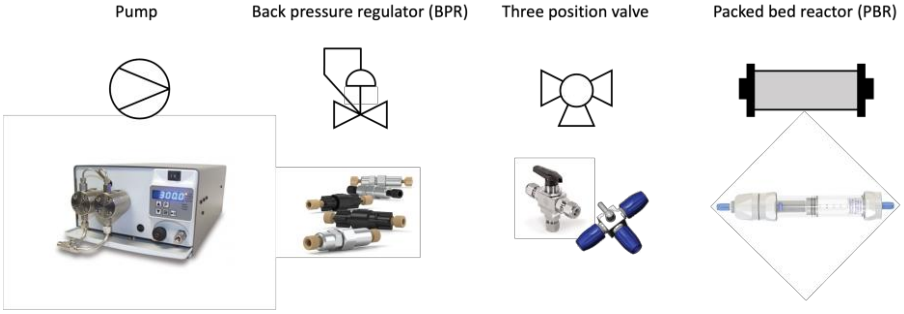

Figure 3.3. Schematic representation of some basic components of a flow setup.

In this context, only the components used to construct the flow setup detailed in the chapter are described:

- Reagent reservoirs store the reactants and to ensure a continuous supply of reactants for uninterrupted reactions.
- Tubing and connections join the various components of the setup. It must be chemically compatible with the reagents and provide a smooth flow path.
- Pumps introduce the feed (liquid or gas) into the system. Different types of pumps, such as HPLC pump, syringe pump, peristaltic pump, or piston pump can be used.

[236] R. A. Sheldon, D. Brady, M. L. Bode, *Chem. Sci.* **2020**, *11*, 2587–2605.

- Valves control the flow of reagents, directing them into the appropriate channels and reactors. They allow for precise control over reaction sequences and the introduction of different reagents.
- Reactors are where chemical reactions take place. They can vary in design, such as reactor coils, microreactors or packed bed reactors (PBR) containing immobilised reagents.
- Heating or cooling elements are used to regulate the temperature, which is crucial to control reaction rates and selectivity. These elements, such as electric heaters or heat exchangers, are integrated into the setup to maintain the desired temperature conditions.
- Back pressure regulator (BPR) can be used to control the system pressure. It operates by a valve opening when a specific pressure is reached. Implementing a BPR enables operations above the boiling point of a solvent and helps to increase the amount of gas dissolved in the liquid phase when performing gas-liquid transformations. The integration of a BPR within a system can also enhance pump performance, particularly in the case of HPLC pumps.

Flow chemistry has been applied across a wide range of fields, including pharmaceuticals, fine chemicals, materials science, and more recently, biocatalysis as well.[235,237,238,239,] The integration of advanced flow technologies, combined with the benefits of enzymatic catalysis,[238,240] has led to diverse sustainable synthetic tools that include an extensive variety of chemical reactions.

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[237] L. Tamborini, P. Fernandes, F. Paradisi and F. Molinari, *Trends Biotechnol.* 2018, **36**, 73–88.
[238] J. Britton, S. Majumdar, G. A. Weiss, *Chem. Soc. Rev.* **2018**, *47*, 5891–5918.
[239] S. Ötvös and C. O. Kappe, *Green Chem*. **2021**, *23*, 6117–6138.
[240] M. Crotti, M. S. Robescu, J. M. Bolivar, D. Ubiali, L. Wilson, M. L. Contente, *Front. Catal.* **2023**, *3*, 1–10.

In the following section some applications of transaminases in flow are described.

3.1.2.1. ATA examples in flow

A wide array of examples demonstrates the diverse implementation of ATAs in continuous flow mode, highlighting the numerous opportunities they present.[158] These applications include the utilisation of ATAs for both kinetic resolutions and asymmetric synthesis, using different enzyme forms such as whole cells, immobilised enzymes with or without PLP cofactor, and in combination with other enzymes for the development of multienzymatic cascades in continuous mode.

Some examples describe the application of immobilised whole cells on different supports, such as Ca-Alginate beads,[241] chitosan,[242] hollow silica microspheres[243] and, methacrylate beads,[244] to be used for kinetic resolutions and chiral syntheses. For example, (*R*)-mexiletine, an active pharmaceutical ingredient (API) was synthesised using *Escherichia coli* whole cells, containing an overexpressed (*R*)-selective transaminase and entrapped PLP cofactor (Scheme 3.1).[244] The cells were strategically immobilised on methacrylate beads through the grafting of the peptidoglycan layer from the cell wall. The asymmetric synthesis was performed in organic solvent, MTBE, using IPA as the amine donor. Notably, this process rendered the target amine with 94% yield. Moreover, the use of MTBE prevented PLP leaching, as it facilitated that the cofactor

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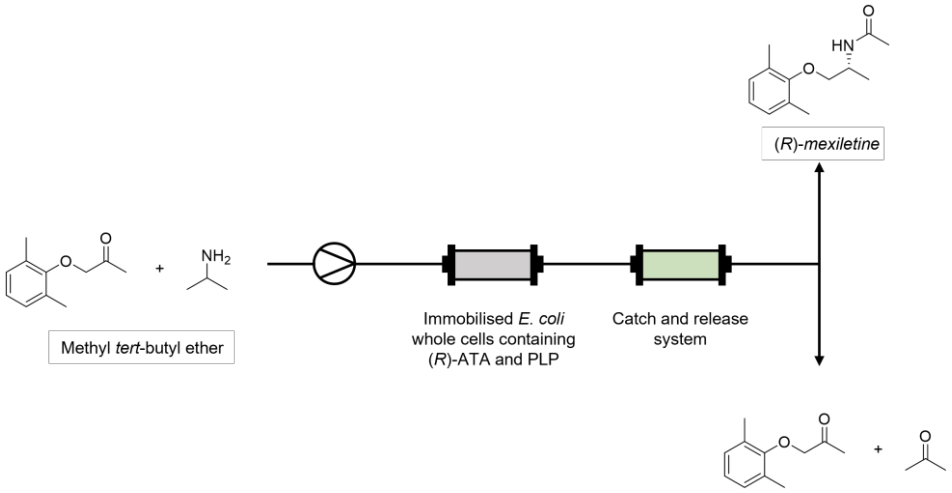
[241] J. S. Shin, B. G. Kim, D. H. Shin, *Enzyme Microb. Technol.* **2001**, *29*, 232–239.
[242] G. Rehn, P. Adlercreutz, C. Grey, *J. Biotechnol*. **2014**, *179*, 50–55.
[243] Z. Molnár, E. Farkas, Á. Lakó, B. Erdélyi, W. Kroutil, B. G. Vértessy, C. Paizs, L. Poppe, *Catalysts* **2019**, *9*, 438.
[244] L. H. Andrade, W. Kroutil, T. F. Jamison, *Org. Lett.*, **2014**, *16*, 6092–6095.

remained in the aqueous phase within the beads. The biocatalysts were stable for up to 10 days and the continuous reaction was integrated with downstream purification step (i.e. a catch-and-release system), enabling the recovery of highly pure chiral amine product in a straightforward way.



Scheme 3.1. Schematic representation of the continuous process leading to the asymmetric synthesis of (*R*)-mexiletine in organic solvent.

There are quite a few more examples of stand-alone ATAs in flow compared to whole cells, using either cell-free extracts (CFE) or pure formulations for immobilisation. Among the different supports evaluated for immobilising ATAs within flow applications, porous glass-based materials94[95,229, 245] and functionalised polymeric resins[177, 246 , 247 , 248 , 249]

[245] A. W. H. Dawood, J. Bassut, R. O. M. A. de Souza, U. T. Bornscheuer, *Chem. – Eur. J.* **2018**, *24*, 16009–16013.
[246] C. M. Heckmann, B. Dominguez, F. Paradisi, *ACS Sustain. Chem. Eng*. **2021**, *9*, 4122–4129.
[247] M. L. Contente, F. Dall'Oglio, L. Tamborini, F. Molinari, F. Paradisi, *ChemCatChem* **2017**, *9*, 3843–3848.
[248] M. L. Contente, F. Paradisi*, Nat. Catal*., **2018**, *1*, 452–459.

emerge as the prevailing choices. In this section, the discussion will be focused only on examples that involve the use of EziG materials, which are the support employed in this Doctoral Thesis.

Bornscheuer and co-workers presented the development of a chemobiocatalytic cascade combining an immobilised ATA variant from *Aspergillus fumigatus* (Af-TA) on EziG support and a Pd catalyst.[245] Specifically, this cascade involved two key steps: the palladium-catalysed Suzuki-Miyaura coupling reaction for producing biphenyl ketones, followed by transamination to obtain chiral biaryl amines. While the soluble Pd specie ($PdCl_2$, as a homogeneous coupling catalyst) was continuously fed into the system, Af-TA was fixed in a single PBR. This means that two reaction solutions (one containing crude Suzuki-Miyaura mixture producing ketone and another with IPA/PLP) were pumped through the PBR containing the immobilised ATA. The cascade resulted in 43% overall conversion using a flow rate of 0.1 mL h^{-1} (210 minutes residence time) when using 30% v/v DMF as cosolvent for the biotransamination.

More recently, Flitsch and co-workers reported the combined use of three enzymes such as alcohol oxidase, transaminase, and imine reductase (IRED) to obtain a variety of primary and secondary amines (Scheme 3.2).[229] The implementation of the multienzymatic cascade under batch conditions was not successful due to issues arising from incompatible substrates and enzyme combinations (cross-reactivity and inhibition issues). To overcome such limitations, a sequential flow mode strategy was attempted, where different amine reagents were introduced at each step to avoid undesired competitive reactions. In this system, the first reaction

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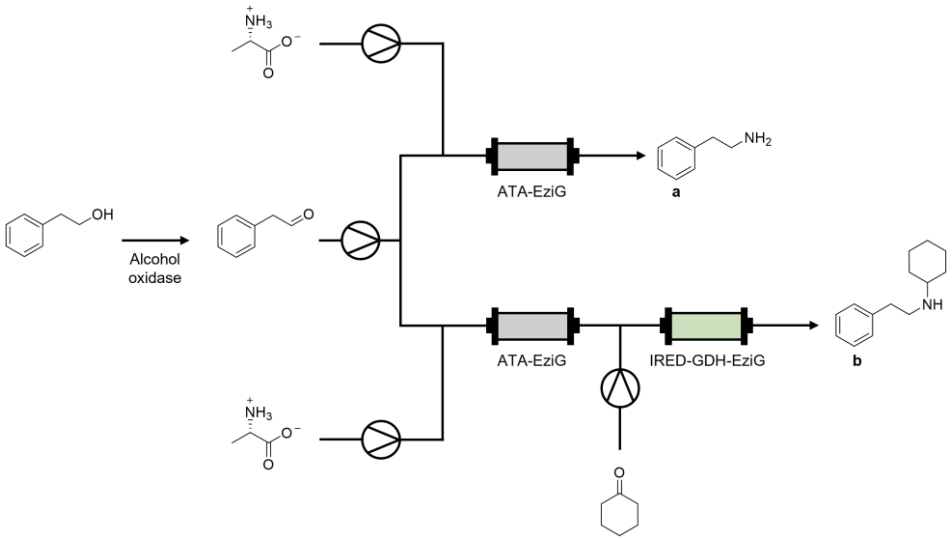
[249] R. Semproli, G. Vaccaro, E. E. Ferrandi, M. Vanoni, T. Bavaro, G. Marrubini, F. Annunziata, P. Conti, G. Speranza, D. Monti, L. Tamborini, D. Ubiali, *ChemCatChem*, **2020**, *12*, 1359–1367.

involved an alcohol oxidase to form aldehyde intermediates from alcohols, using catalase-generated oxygen (from the decomposition of hydrogen peroxide). Then, the resulting aldehyde was pumped through a series of PBRs containing the immobilised ATA or IRED. The second reaction was the transamination, used to generate target primary amines or intermediate amines that were subsequently non-catalytically carbonylated (into imines) and then reduced by IRED forming targeted secondary amines. This approach showcases the potential of flow methods to overcome the limitations inherent to some batch processes.



Scheme 3.2. Schematic representation of a multienzymatic cascade in continuous process leading to primary (a) and secondary amines (b).

Mutti and co-workers described the utilisation of (*R*)-selective transaminase from *Arthrobacter* sp. immobilised on EziG-Amber support, to continuously produce the (*S*)-MBA by kinetic resolution of the racemic amine.[94] The cofactor, PLP, was co-immobilised with the enzyme, avoiding external supplementation. The reactor was run for 96 consecutive h without any detectable loss of enzymatic activity and a space-time yield of 335 g L^{-1} h^{-1}. The same research group has recently employed the same biocatalyst immobilised in a PBR for the asymmetric synthesis of 1-phenoxypropan-2-

amine in neat organic solvent.[95] Herein, the water content in the organic solvent plays a key role in the enzyme activity, requiring specific optimisation tailored for each biocatalyst and solvent combination. Under the best conditions found, the flow reactor showed excellent performance obtaining a space-time yield of 1.99 g L^{-1} h^{-1}.

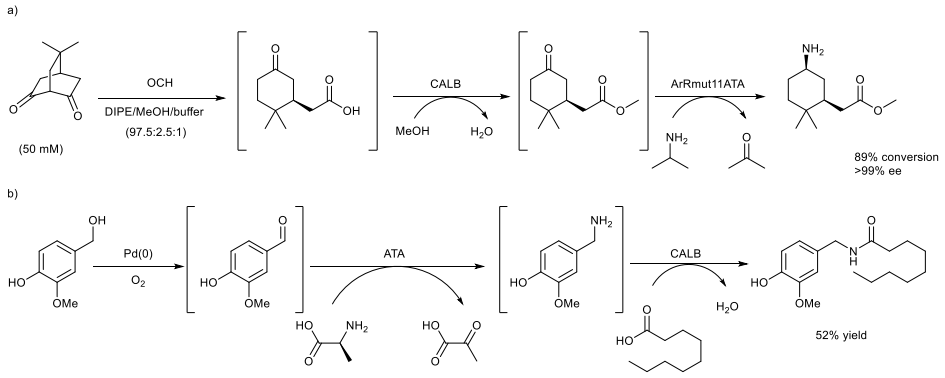
3.1.3. ATA + lipase cascade for amide synthesis

The application of immobilised lipases in flow is broad[250] and extends beyond the specific discussion provided here. Therefore, the next section will be focused on the examples reported in the literature regarding the combination of lipases and ATAs for the synthesis of chiral nitrogenated compounds.

Kroutil and co-workers described a three-step method for the synthesis of enantiopure 3-substituted cyclohexylamine derivatives bearing two chiral centres.[251] Prochiral bicyclic diketones were used as starting materials, in which the first chiral centre was introduced using a C-C hydrolase (6-oxocamphor hydrolase from *Rhodococus ruber* NCIMB 9784, OCH). This enzyme catalysed the conversion of bicyclic diketones into the corresponding keto acids, which were subsequently esterified with methanol using CALB. In the final step, stereocomplementary transaminases were employed to introduce the second chiral centre via amination reaction, resulting in the formation of the desired amino ester diastereoisomers (Scheme 3.3.a). Depending on the specific choice of the transaminase and

[250] I. Itabaiana, L. S. De Mariz E Miranda, R. O. M. A. De Souza, *J. Mol. Catal. B Enzym.* **2013**, *85–86*, 1–9.
[251] E. Siirola, F. G. Mutti, B. Grischek, S. F. Hoefler, W. M. F. Fabian, G. Grogan and W. Kroutil, *Adv. Synth. Catal.*, **2013**, *355*, 1703–1708.

substrate used during the process, it was possible to obtain either cis- or trans-diastereomers in optically pure forms. Notably, when using ArRmut11-TA (*E. coli* lyophilised cells), all three steps could be performed in one-pot mode in organic media, requiring the filtration of the hydrolases before performing the transamination reaction.



Scheme 3.3. Enzymatic cascades combining ATAs and lipase.

A year later, Berglund, Córdova and co-workers detailed a new methodology for the synthesis of capsaicinoids from vanillyl alcohol. The approach combined a palladium catalyst, an ATA, and a lipase (Scheme 3.3.b).[252] The two first steps of the cascade, alcohol oxidation catalysed by Pd and amination catalysed by ATA, were combined in a one-pot sequential approach, where the reaction conditions were changed after completion of the initial reaction. To run the lipase-catalysed reaction, it was necessary to lyophilise the mixture obtained from ATA reaction before subjecting it to the lipase-catalysed aminolysis in organic solvent. Although there was no intermediate purification step performed between transamination and

[252] M. Anderson, S. Afewerki, P. Berglund, A. Córdova, *Adv. Synth. Catal.* **2014**, *356*, 2113–2118.

acylation processes, it is noteworthy that the reaction environment required adjustment for each reaction step.

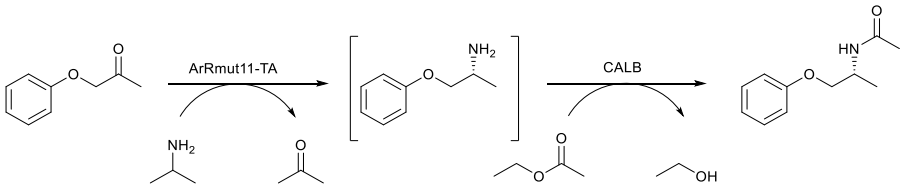
Objectives

The combination of transaminases and lipases is a very attractive approach for the formation of chiral nitrogenated compounds, however only a few examples developed in batch mode have been reported in literature. Hence, this chapter outlines the design of a bienzymatic cascade combining ArRmut11-TA and CALB, for the stereoselective synthesis of (*R*)-*N*-(1-phenoxypropan-2-yl)acetamide from 1-phenoxypropan-2-one. Thus, the following specific objectives are ambitioned.

- Immobilisation of ArRmut11-TA on three available EziG supports to find the best combination to perform transamination experiments in an organic solvent.
- Explore possible conditions under which both enzymes, ArRmut-11 and CALB, can retain their highest activity to work together in a biotransamination-acylation cascade.
- To assemble the bienzymatic cascade in batch mode, ideally following a one-pot simultaneous mode, and to optimise the reaction conditions.
- Cascade implementation in a packed bed reactor and to compare the batch and flow experiments in terms of productivity.
- To evaluate the environmental impact of the system using the E-factor metrics.

Results

This chapter describes the development of a bienzymatic cascade for the stereoselective preparation of optically active (*R*)-*N*-(1-phenoxypropan-2-yl)acetamide from the corresponding prochiral 1-phenoxypropan-2-one (Scheme 3.4). For this proof of concept, (*R*)-selective *Arthrobacter* sp. round 11 variant transaminase (ArRmut11) and the *Candida antarctica* lipase type B (CALB) were selected as biocatalysts.



Scheme 3.4. Bienzymatic cascade combining ArRmut11-TA and CALB for the synthesis of optically active (*R*)-*N*-(1-phenoxypropan-2-yl)acetamide from the prochiral 1-phenoxypropan-2-one.

3.2.1. Transition to organic solvent - enzyme immobilisation

As previously explained in chapter 2, immobilisation strategies enhance the enzymatic activity when running the reactions in organic solvent. Consequently, the immobilisation of ArRmut11-TA was performed on the three types of EziG materials, while CALB was immobilised on EziG-Amber, based on previous studies showing its superior efficiency as support material.[77]

Enzyme immobilisation was performed by incubating the desired amount of cell free extract (CFE) in buffer with the corresponding EziG carrier material. The progress of immobilisation was monitored by measuring the remaining enzymatic activity of the supernatant using a spectrophotometric assay for ArRmut11-TA and active-site titration assay for CALB (see the experimental section for further details).

To evaluate the activity of the heterogeneous catalysts, immobilised ArRmut11-TA was used in aqueous media for the amination of 1-phenoxypropan-2-one with isopropylamine (IPA). This model reaction was used to analyse and to compare the performance of the immobilised enzyme across the three different support materials, utilising recovered activity as the primary metric. Regarding lipase activity, the model reaction for immobilised CALB was the kinetic resolution of 1-phenylethan-1-ol through acylation with vinyl acetate in organic solvent.

The results summarised in Table 3.1 show that ArRmut11-TA was successfully immobilised on the three different EziG supports and CALB on EziG-Amber. For ArRmut11, the highest immobilisation yield was achieved with Amber, 69%, which corresponded to a protein loading of 5.7% w/w. Similar results were obtained with Coral, 66% immobilisation yield and 5.4% w/w protein loading, followed by Opal with 57% immobilisation yield and 4.7% w/w. Remarkably, the protein purification prior immobilisation led to a further improvement in protein loading up to 8.1 w/w% as shown in Figure 3.4.

Surprisingly, the highest recovered activity with 66% value was obtained with Opal, followed by Amber and Coral, with values of 43 and 33%, respectively. This indicates that the immobilisation efficiency is higher with Opal compared to Amber. While more enzyme is being immobilised on Amber, the amount of enzyme that remains active after immobilisation is higher with Opal.

For CALB, 67% immobilisation yield was obtained, which corresponds to 7.5 w/w% protein loading, and the conversion towards the O-acetylated 1-phenylethan-1-ol was 15% after 0.5 h. Under the same conditions, Novozyme 435 (the most well known commercial preparation of CALB) gave 13% conversion.

Table 3.1. Immobilisation of ArRmut11-TA and CALB on EziG supports[a]

Enzyme	Support	Immobilisation yield[b] [%]	Target protein loading[c] [w/w%]	Recovered activity[d] [%]
ArRmut11-TA	Amber	69±6	5.7±0.5	43±1.3
ArRmut11-TA	Coral	66±6	5.4±0.5	35±1.4
ArRmut11-TA	Opal	57±3	4.7±0.2	66±1.6
CALB	Amber	67±2	7.5±3.7	-

[a] For ArRmut11-TA: 7.5 mg of CFE containing 0.825 mg of target protein were used with 10 mg of support in all cases. For CALB: 1 L of liquid CFE was used with 50 g of support. [b] Immobilisation yield= [(activity of the free enzyme - activity of the supernatant after immobilisation)/activity of the free enzyme]*100. [c] Protein loading= (Amount of target protein offered to the support* Immobilisation yield/10). [d] Recovered activity= (specific activity of immobilised enzyme/specific activity of the free enzyme)*100. Activities for free and immobilised enzyme were obtained from the initial rates (see Figure 3.14 in the experimental section).

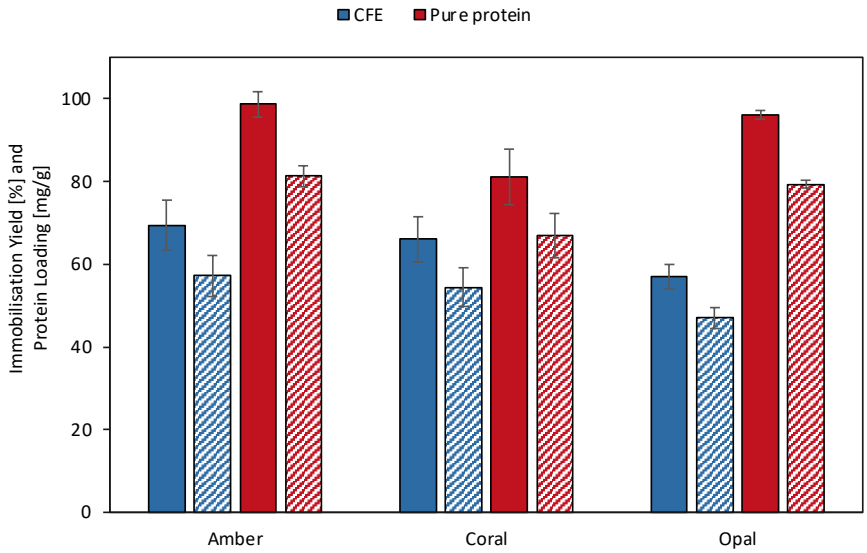


Figure 3.4. Immobilisation yield (filled) and protein loading (dashed) of ArRmut11-TA on EziG Amber, Coral and Opal. Immobilisation from CFE: 0.5 mL of 15 mg/mL of CFE resuspended in 20 mM sodium phosphate buffer with 0.3 mM PLP, pH 8.0, was added to 10 mg of carrier material. Immobilisation from pure protein: 434 µL of the pure protein solution in 50 mM MOPS buffer with 0.3 mM PLP, pH 7.6, was added to 10 mg of carrier material. In both cases: Tubes were covered with foil and immobilisation was performed on an end-over-end rotator, 20 rpm, at room temperature. 50 µL of the enzyme supernatant were withdrawn after 2 h for kinetic analysis and diluted for the spectrophotometric assay containing 10 mM 1-phenylethylamine and 10 mM pyruvate in 20 mM sodium phosphate buffer pH 8.0. Total volume for the spectrophotometric assay was 200 µL. All the measurements were performed in duplicates and the results are presented as the mean of the individual samples.

3.2.2. ArRmut11-TA in organic solvent

To remain active in organic solvent, enzymes require a certain amount of water in the local environment to preserve the 3-dimensional structure,

while preventing undesired self-aggregation. In theory, less than a monolayer of water is required, yet in practice, the precise amount of water within the reaction mixture needs to be optimised for each individual case.[95]

3.2.2.1. Water content in ArRmut11 immobilised on EziG

Like in the previous chapter, in this project, the immobilised ArRmut11-TA enzyme was used as wet formulation. To maintain a controlled and minimised amount of water content within the immobilised enzyme, the same procedure as previously explained was applied (see section 2.2.4.1). Thus, it was possible to set the water content in the immobilisation preparation through a series of washing steps: first with 2-propanol (2-PrOH) containing set volume (%v/v) of deionised water and second with an organic solvent to remove the 2-PrOH.

To evaluate the enzymatic activity of ArRmut11-TA immobilised on the three different supports, the target reaction was the amination of 1-phenoxypropan-2-one (100 mM) using IPA (250 mM) as amine donor, and ethyl acetate (EtOAc) as solvent. The water amount was set through a two-step process: initially, by washing with a 10% v/v water in 2-PrOH solution, followed by conducting the reaction in EtOAc with an additional 3% v/v water. This final step was added following empirical observations, which showed that additional amount of water within the reaction mixture was leading to higher conversions (results obtained with ArRmut11-Amber are shown in Figure 3.5).

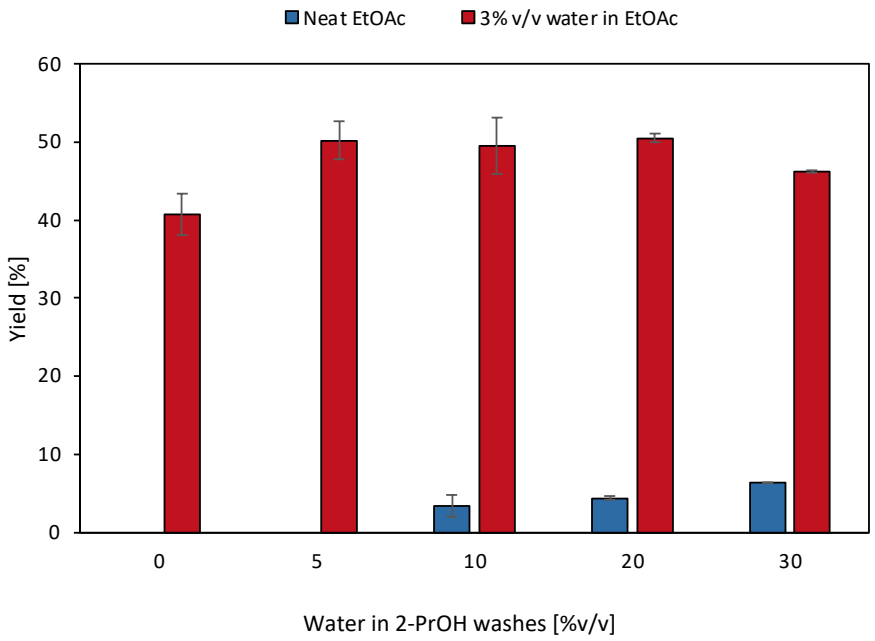


Figure 3.5. Yield data from the transamination of 1-phenoxypropan-2-one using ArRmut11-TA immobilised on Amber containing varying levels of water (0-30% v/v). Reaction conditions: 100 mM 1-phenoxypropan-2-one, 250 mM IPA, neat or 3% v/v water in ethyl acetate, ArRmut11-EziG-Amber (10 mg of EziG with 0.5 mL of 15 mg/mL CFE) in 1 mL total reaction volume after 18 h, 37 °C and 1200 rpm. Reactions were performed in duplicate, and yields were determined using GC analysis, after derivatisation of the samples with acetic anhydride (see section 3.3.7 in the experimental section).

Under the designated operational conditions (3% v/v water in EtOAc), the yields achieved using the immobilised enzyme on Amber, Coral and Opal carriers were 58%, 50% and 58%, respectively, as illustrated in Figure 3.6. In addition to immobilised preparations, freeze-dried CFE was also evaluated in organic solvent reactions, resulting in 26% conversion to the target amine. The amount of target protein used in the reactions depended on the catalyst formulation (CFE or immobilised) and on the immobilisation yield obtained for each support. Notably, for CFE, it was 0.825 mg, while

for Amber, Coral and Opal, the values were 0.621 mg, 0.588 mg and 0.495 mg respectively.

These results showed that although less amount of target protein was used in the reactions when using immobilised enzyme, higher activities were obtained. This phenomenon could be attributed to the potential stabilisation effect by immobilisation, particularly under non optimal conditions such as organic solvents as reaction media.

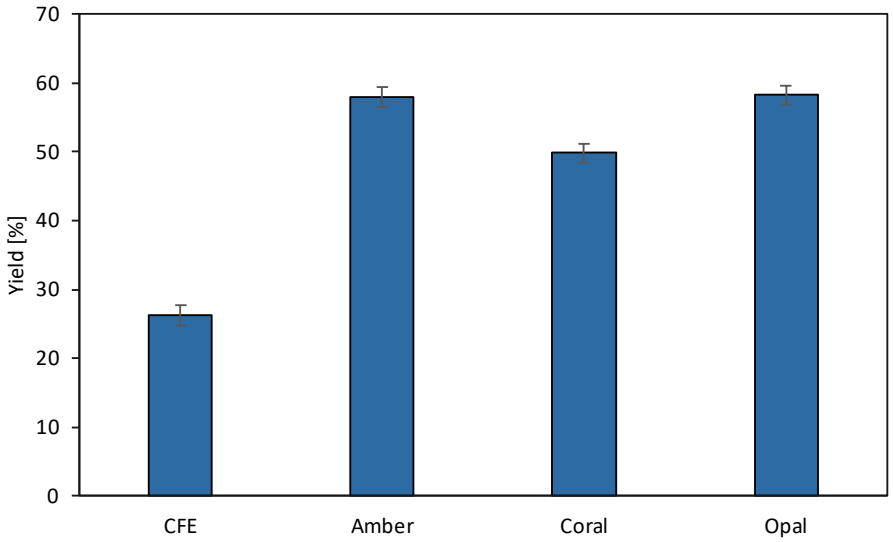


Figure 3.6. Activity of the CFE and immobilised ArRmut11-TA in organic solvent based on the conversion of 1-phenoxypropan-2-one to (*R*)-1-phenoxypropan-2-amine. Reaction conditions: 100 mM 1-phenoxypropan-2-one, 250 mM IPA, 3% v/v water in ethyl acetate, 1 mL total reaction volume after 18 h at 37 °C and 1200 rpm. The amount of CFE used in the reactions was 7.5 mg, corresponding to 0.825 mg of target protein. The amount of immobilised catalysts was 10 mg, which corresponds to an amount of immobilised target protein of 0.621, 0.588 and 0.495 mg for Amber, Coral and Opal, respectively. Reactions were performed in duplicate, and yields were determined using GC analysis, after derivatisation of the samples with acetic anhydride (see section 3.3.7 in the experimental section).

For all enzyme formulations when moving to organic solvent, a change in the stereoselectivity was observed, with an increase from 30% in

buffer to 94% in organic solvent. The cause has not been investigated, but a similar behaviour was observed for the *Halomonas elongata* TA, where subtle changes in the reaction conditions affected the enzyme enantiopreference.[253]

3.2.2.2. Catalyst recyclability

Beyond evaluating activity and selectivity, another important consideration that should be assessed is the operational stability under the process conditions. To analyse the stability of the immobilised enzymes, recycling studies were carried out for batch reactions using both, heterogeneous catalysts, and the freeze dried CFE. The experimental conditions were the same as the previous ones, and all the immobilised preparations and CFE were washed twice in between cycles with 3% v/v water in EtOAc to remove any residual substrate or product that could be adsorbed on the catalyst surface.

The findings revealed a consistent decline in activity across all immobilised formulations and CFE. After a single cycle, all the tested catalysts lost 20-25% of their initial activity (Figure 3.7), and after four recycles, approximately 80% of the initial activity was lost. The similarity in observed trends among the three tested supports and the CFE indicates that the stability is an enzyme dependent characteristic and changing the support would not lead to any significant improvement.

To streamline the experimental procedure, a support choice had to be made, leading to the selection of Amber. Although there were no significant variations in terms of activity towards the target reaction and recyclability

[253] C. M. Heckmann, L. Robustini, F. Paradisi, *ChemBioChem* **2022**, *23*, e202200335.

among the various supports tested, this decision was based on slightly higher protein loading. Furthermore, this was aligned with the use of the same support for CALB immobilisation, maintaining consistency and simplifying the overall experimental setup.

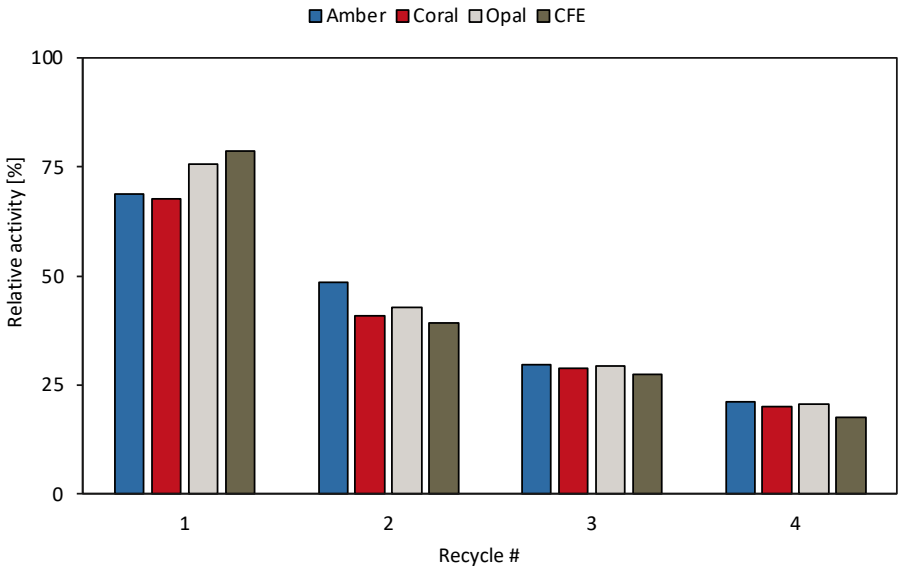


Figure 3.7. Recyclability studies of the CFE and immobilised ArRmut11-TA on the three different supports. Reaction conditions: 100 mM 1-phenoxypropan-2-one, 250 mM IPA, 3% v/v water in EtOAc, 10 mg of immobilised catalyst or 7.5 mg CFE in 1 mL total reaction volume after 18 h at 37 °C and 1200 rpm. Between runs the catalysts were washed twice with 1 mL of 3% v/v water in EtOAc. Relative activity is the activity obtained in each recycle relative to that calculated in the first reaction cycle (set as 100%, Figure 3.6). Reactions were performed in duplicate, and yields were determined using GC analysis, after derivatisation of the samples with acetic anhydride (see section 3.3.7 in the experimental section).

3.2.3. Sequential ATA-lipase cascade in batch

To find the operational window between transaminase and lipase reactions, the role of reaction media was explored, particularly focusing on the water content in EtOAc. While the transaminase activity benefits from higher water levels, such water excess might hinder the lipase-catalysed acylation step. To address this dynamic, both reactions were carried out in EtOAc containing varying water concentrations, ranging from 0 to 3% v/v (Figure 3.8). Moreover, the lipase reaction was run in presence of IPA (250 mM), to ensure the compatibility with the first step of the reaction cascade.

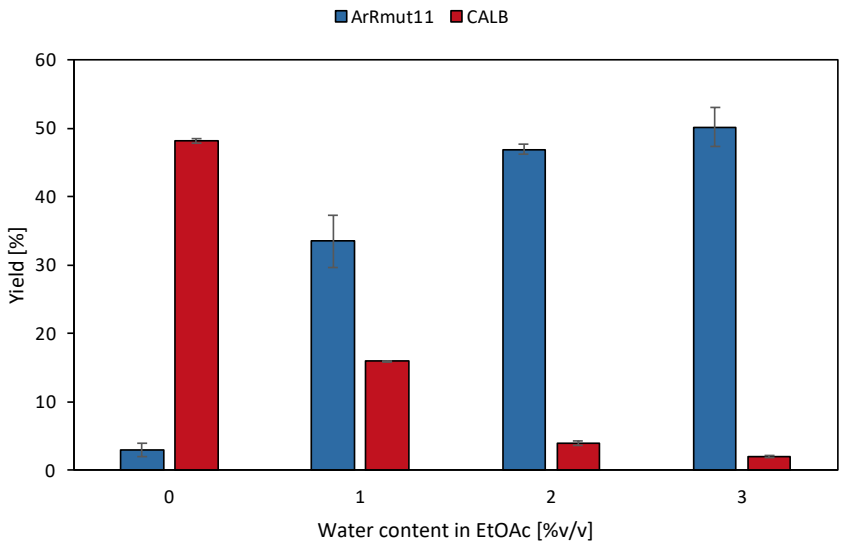


Figure 3.8. Yield data from the transamination of 1-phenoxypropan-2-one using ArRmut11-TA immobilised on Amber, and for the kinetic resolution of the racemic 1-phenoxypropan-2-amine using CALB immobilised on the same support. ATA-catalysed reaction: 100 mM 1-phenoxypropan-2-one, 250 mM IPA, different water contents in EtOAc, ArRmut11-TA-EziG (10 mg of EziG with 0.5 mL of 15 mg/mL CFE) in 1 mL total reaction volume after 18 h at 37 °C and 1200 rpm. Lipase-catalysed reaction: 100 mM racemic 1-phenoxypropan-2-amine, 250 mM IPA, different water contents in EtOAc 1 mL total reaction volume after 24 h at 37 °C and 1200 rpm. Reactions were performed in duplicate, and yields were determined using GC analysis (see section 3.3.7 in the experimental section).

As previously described, the immobilisation of ArRmut11-TA was carried out on Amber, and the resulting preparation was washed with 10% v/v water in 2-PrOH solution. The obtained results were in alignment with previous observations. Consequently, when the reactions were run in neat EtOAc, the target amine was not detected. However, upon addition of 1% v/v water, conversions higher than 30% into the enantiopure amine were achieved.

In parallel, the lipase-catalysed reactions were performed in the presence of water to simultaneously validate its impact on the amidation step. It was observed that even with 1% v/v water content in the reaction mixture, the activity of the lipase with racemic 1-phenoxypropan-2-amine declined, exhibiting a reduction of over 50% (as depicted in Figure 3.8). Furthermore, higher water concentrations were incompatible with this reaction step. The obtained results validated the hypothesis regarding the importance of water content in the reaction, enabling an operational window for both enzymes under the same reaction conditions. Specifically, it was determined that adding a 1% v/v water in EtOAc was necessary for both enzymes to be sufficiently active.

Having adjusted the optimal water content in the organic solvent, the next objective entailed reassessing the water amount in the wash steps for the immobilised ArRmut11-TA. This study aimed to ensure the compatibility of the water content within the immobilised ATA formulation and the lipase-catalysed reaction. For this purpose, different heterogeneous ArRmut11-TA samples were prepared with variation in washing conditions (0-10% v/v water in 2-PrOH). Subsequently, these catalysts were subjected to conditions previously identified as compatible with the lipase-catalysed step (1% v/v water in EtOAc). After completion of the ATA reaction after 18 h, the supernatants were transferred to new tubes and CALB was added, following a sequential approach to run the cascade reaction in batch (Figure 3.9).

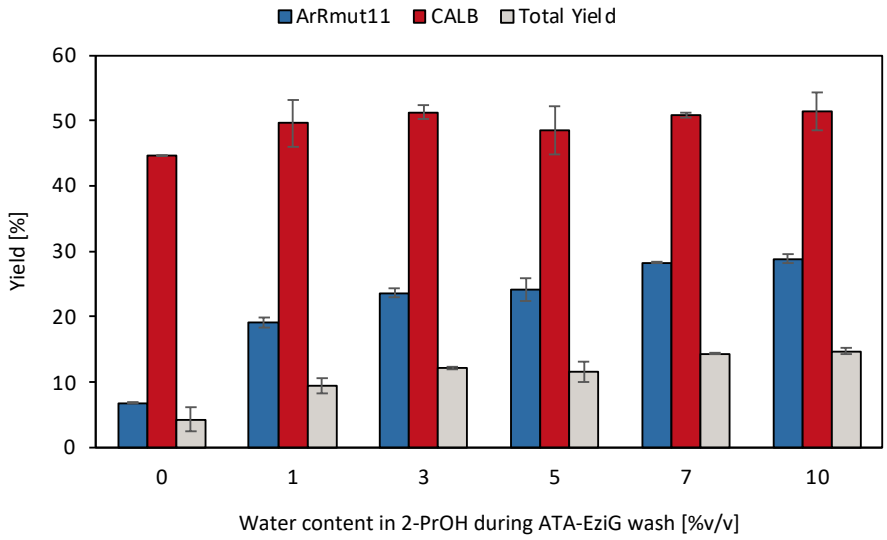


Figure 3.9. Yield data for the biocascade starting from 1-phenoxypropan-2-one using ArRmut11-TA immobilised on Amber containing varying levels of water and CALB immobilised on the same support. Reaction conditions: 100 mM 1-phenoxypropan-2-one, 250 mM IPA, 1% v/v water in EtOAc, ArRmut11-TA-EziG (10 mg) in 1 mL total reaction volume after 18 h, at 37 ºC and 1200 rpm, and then CALB-Amber (10 mg) was added and the reaction was run for additional 20 h under the same conditions. Reactions were performed in duplicate, and yields were determined using GC analyses (see section 3.3.7 in the experimental section).

The results obtained from the ATA-catalysed reactions were in agreement with previous findings, yielding approximately 30% target product when a minimum of 10% v/v water in 2-PrOH was used as washing solution. The study did not include washing conditions with higher water content because it was previously demonstrated that from 10 to 20% v/v water in 2-PrOH, the resultant enzymatic preparations showed similar activity levels (Figure 3.8). When ATA reaction mixture was used directly, the lipase-catalysed acylation proceeded as expected exhibiting similar activity observed previously at 1 v/v% water content in EtOAc with commercial racemic amine (Figure 3.9). Despite incomplete conversions in ATA-catalysed reactions that limited the substrate availability in the second

step of the cascade, it seemed that the lipase activity was not influenced by the water content in the washing solutions used for immobilised ArRmut11-TA (Figure 3.9).

The total yield in the cascade, calculated from amide formation, was increasing from 4% to 15% with higher water percentage in the washing solution (0-10% v/v, Figure 3.9). These results showed that ATA-catalysed reaction is the limiting step in the cascade, and that the water content during the washing step had a limited impact on the lipase activity. Consequently, for the following experiments, 10% v/v water in 2-PrOH was selected, ensuring highest activity of immobilised catalyst in the initial step of the cascade. Under the most favourable batch conditions, the maximum yield for the amide reached 15%, corresponding to a productivity value of 2.8 mg g^{-1} h^{-1}. Furthermore, the entire cascade was also run in a one-pot approach, with both enzymes in the reaction vessel from the beginning. However, in this case, no conversion was observed to either the intermediate or the final products (amine or amide).

To understand the reasons behind these observations, the reaction mixture was analysed more in detail. Initially, the formation of *N*-isopropylacetamide as by-product was detected and it was speculated that the consumption of IPA by the acetylation reaction might deplete the offered amine donor for the transaminase reaction. Consequently, reactions were run with increasing concentrations of IPA to ensure sufficient amine equivalents for the transaminase step. However, the results remained unchanged with no detected conversion to the intermediate amine. Another possible explanation is that either the acetylated IPA (*N*-isopropylacetamide) or the co-product ethanol, generated during the acetylation reaction, are inhibitors of ArRmut11. To investigate this hypothesis, the ATA reaction was carried out in the presence of different concentrations of ethanol (1-10 mM, corresponding to equimolar amounts of formed *N*-isopropylacetamide). However, in all cases, conversion to the desired amine was observed.

Simultaneously, the potential inhibition of the transaminase by any component present in the lipase formulation was evaluated. The ATA-catalysed reaction was performed under the previously established conditions, using the solvent (ethyl acetate with 1 v/v% water) that had been preincubated with EziG-CALB. Interestingly, no conversion was observed under these reaction conditions. Furthermore, the addition of IPA to this preincubated solvent led to a liquid-liquid phase separation. Such behaviour could be attributed to salt-induced liquid-liquid phase separation triggered by the buffer salts from the immobilised CALB. It is noteworthy that subjecting immobilised CALB to a washing process aimed to remove impurities and buffer salts before the solvent incubation procedure did not avoid the phase separation. Although the precise cause behind this phenomenon remains unknown, it is clear that the addition of immobilised CALB alters the reaction mixture, inducing phase separation and affecting ArRmut11 activity. These results highlight the need for compartmentalisation of both catalysts to make the cascade system feasible.

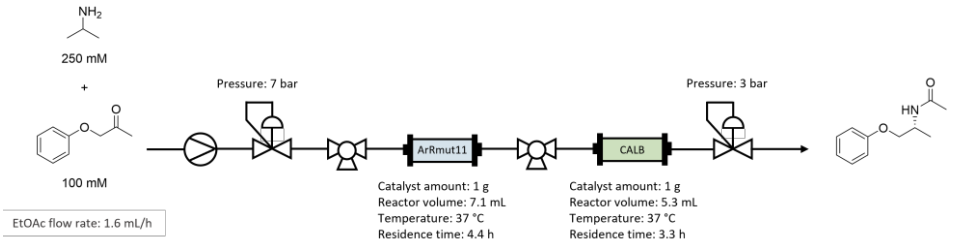
3.2.4. ATA-lipase cascade in continuous mode

The bienzymatic system was implemented in a continuous flow setup, having both catalysts in separated reactors. This decision was driven by the findings explained in the previous section. Therefore, immobilised ArRmut11-TA and CALB were used as separate PBRs, thus effectively compartmentalising them. The composition of the reaction mixture used for the flow mode was identical to the best batch experiments: 100 mM 1-phenoxypropan-2-one, 250 mM IPA and 1% v/v water in EtOAc as the solvent.

Two glass column reactors (15 mm i.d., 10 cm length) were filled individually with 1 gram of the corresponding immobilised catalyst. A slurry of immobilised ArRmut11-TA in 1% v/v water in EtOAc was poured into one column, the bed was allowed to settle, and the solvent excess was

drained off. In another column, EziG-CALB catalyst was packed from dry formulation, without additional solvent.

The flow setup was assembled as shown in Scheme 3.5 and Figure 3.15 (experimental section). To equilibrate the columns containing the catalysts, an initial washing step with 1% v/v water in EtOAc was performed. Then, a solution containing 1-phenoxypropan-2-one (100 mM) and IPA (250 mM) in EtOAc with 1% v/v water was pumped through the reactors at 1.6 mL h^{-1} flow rate. The ArRmut11-TA PBR volume was calculated to 7.1 mL, corresponding to a residence time of 4.4 h. For the CALB PBR the volume was 5.3 mL, and the residence time was 3.3 h. The system operated continuously for 11 days, which corresponds to 60.4 and 80.6 reactor volumes, for ArRmut11-TA and CALB, respectively.



Scheme 3.5. Continuous flow setup for the ArRmut11-TA and CALB cascade reaction for the production of (*R*)-*N*-(1-phenoxypropan-2-yl)acetamide.

As illustrated in Scheme 3.5, two additional 3-position valves were incorporated in the setup. The first one was positioned after the pump and before the reactors, enabling the analysis of the feed composition. The second valve was placed in between the ArRmut11-TA and CALB reactors, and it was used to monitor the progress of the ATA-catalysed reaction to evaluate the stability of this enzyme. Finally, the performance of the full cascade was measured at the outlet of the second reactor containing CALB. The integration of consecutive sampling valves highlights the potential to adopt such an approach in an automated fashion and may allow for the design of closed loop self-optimising continuous biocatalytic systems.

To obtain stable pressure and temperature levels throughout the experiment, the system was run for 25.5 h before the first samples which was considered as stabilisation time, corresponding to approximately three reactor volumes. After this time, aliquots were taken for analyses of the feed composition, ArRmut11-TA outlet and CALB outlet. Remarkably, (*R*)-*N*-(1-phenoxypropan-2-yl)acetamide was obtained with 99% *ee* and an initial cascade productivity of 9.6 mg g^{-1} h^{-1}. However, these initial values decreased to half after 11 days, due to ATA activity loss (Figure 3.10). While the activity of the lipase catalyst appeared to be stable, the gradual decrease in ATA activity resulted in a concurrent decline in the substrate concentration available for the lipase step. Thus, for the acylation the ratio CALB/amine ratio is continuously increasing, and for this reason activity losses for the lipase could not be detected.

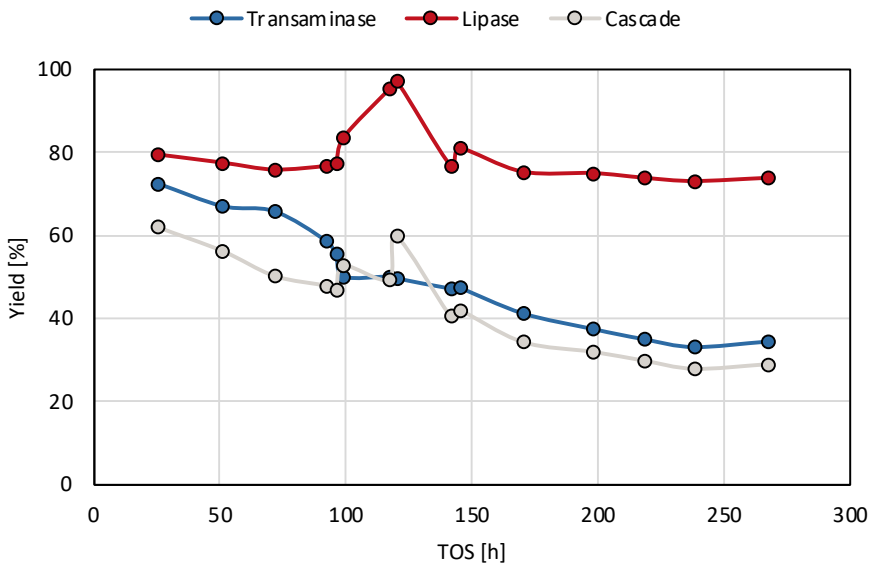


Figure 3.10. In flow stability test of the ArRmut11-TA and CALB cascade (TOS: time on stream). For the ATA-catalysed amination the yield is calculated based on the initial 1-phenoxypropan-2-one concentration (100 mM) and (*R*)-phenoxypropan-2-amine measured at the outlet of the ATA reactor. For the lipase-catalysed acylation the yield is calculated based on the (*R*)-phenoxypropan-2-amine concentration measured at outlet of the ATA reactor and (*R*)-*N*-(1-

phenoxypropan-2-yl)acetamide at the outlet of the lipase reactor. The cascade yield corresponds to the total conversion into (*R*)-*N*-(1-phenoxypropan-2-yl)acetamide and is based on the product formed and the initial ketone concentration (100 mM). The yields were determined using GC analyses (see section 3.3.7 in the experimental section).

It is worth mentioning that one unknown peak was observed across all the outlets, i.e., feed, ATA, and lipase. This peak displayed a notably higher intensity in the feed, and its relative area remained consistent throughout the flow run (40%). However, it was not possible to isolate this compound, since after solvent evaporation (leaving the solvent to be evaporated at room temperature and atmospheric pressure) the area of the peak was decreasing to residual amount, which was not enough for characterisation. Alternatively, the reaction mixture was analysed by ^{1}H-NMR (preparing the mixture in toluene-d_8) and GC-MS (preparing the mixture in EtOAc). These analyses conclusively identified the peak as an imine resulting from the reaction between 1-phenoxypropan-2-one and IPA, specifically *N*-isopropyl-1-phenoxypropan-2-imine (as depicted in Figures 3.11 and 3.12).

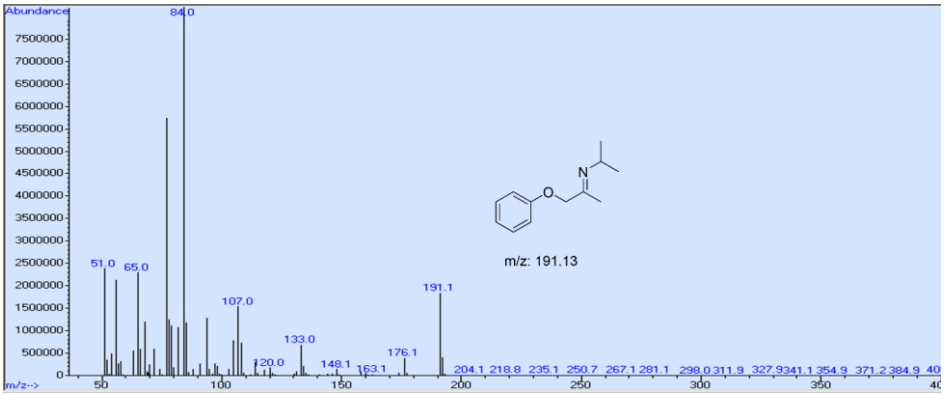


Figure 3.11. MS analysis of *N*-isopropyl-1-phenoxypropan-2-imine (peak at 6.0 min). Calculated m/z: 191.1, obtained: 191.1.

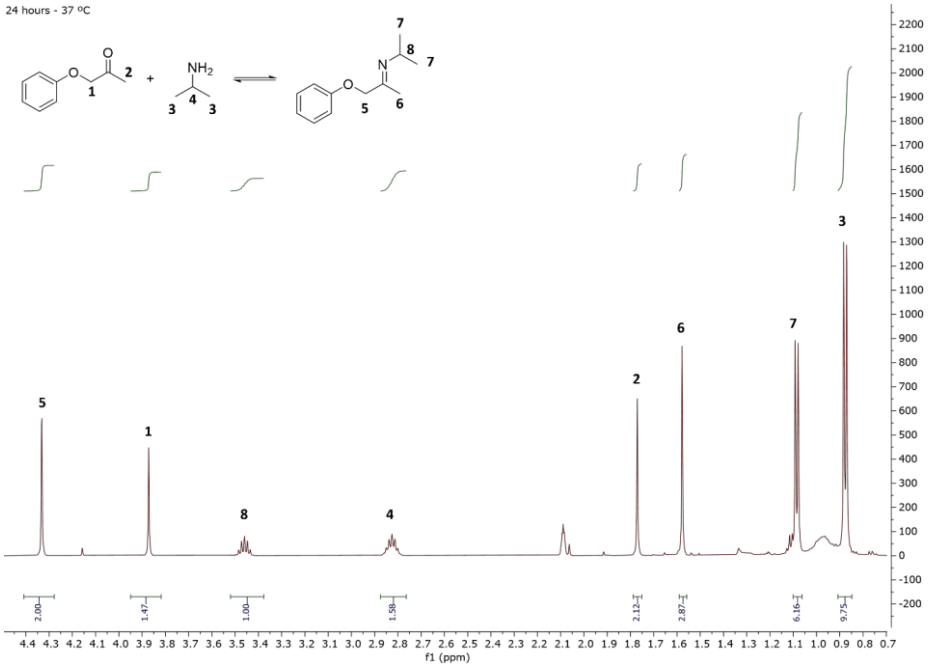


Figure 3.12. ^{1}H-NMR of the reaction mixture in deuterated toluene.

3.2.4.1. Transaminase stability in flow

One essential factor to contemplate when implementing a catalyst in an industrial setting is the long-term stability. For some cases, this stability can be enhanced by reducing operational temperatures. Consequently, in the next experiments the reactors were run under the same conditions but at room temperature (not controlled), to see whether mild conditions would lead to an improved stability of the immobilised enzymes.

The flow rate was reduced by three-fold, to 0.54 mL h^{-1}, in order to achieve same conversion values as in the previous runs. This adjustment facilitated a comparison between the two continuous mode experiments at the same conversion level. This readjustment was done according to the data obtained from batch reactions, at room temperature the activity was three times lower compared to 37 °C conditions.

The results showed a decrease in the initial productivity to 2.6 mg g^{-1} h^{-1}. Moreover, the activity retention observed after 56 h on stream of the room temperature flow run appeared to be identical to the one at 37 °C, showing that in this case catalyst stability may not be temperature dependent (Figure 3.13).

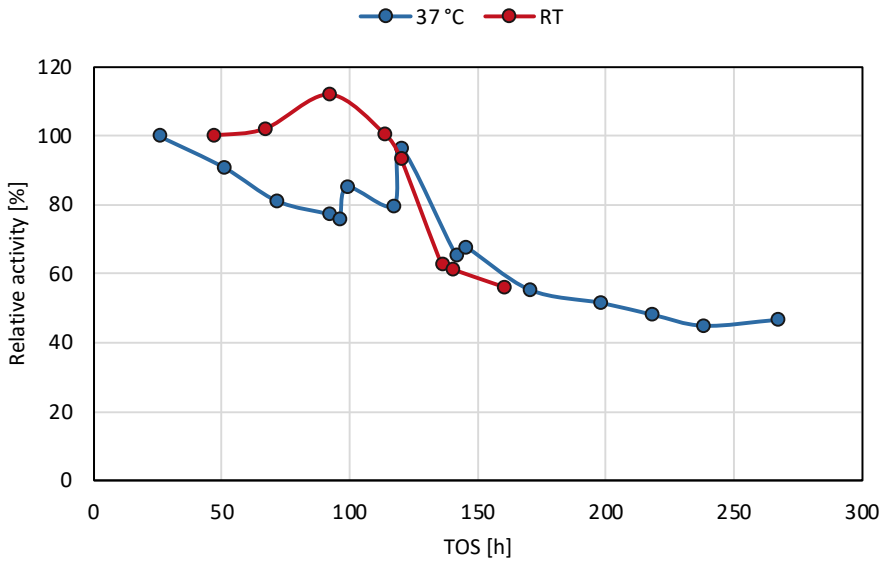


Figure 3.13. Stability comparison of the cascade (in flow) at two different temperatures, 37 °C and room temperature. For the ATA-catalysed amination the yield is calculated based on the initial 1-phenoxypropan-2-one concentration (100 mM) and (*R*)-phenoxypropan-2-amine measured at the outlet of the ATA reactor. For the lipase-catalysed acylation the yield is calculated based on the (*R*)-phenoxypropan-2-amine concentration measured at outlet of the ATA reactor and (*R*)-*N*-(1-phenoxypropan-2-yl)acetamide at the outlet of the lipase reactor. The cascade yield corresponds to the total conversion into (*R*)-*N*-(1-phenoxypropan-2-yl)acetamide and is based on the product formed and the initial ketone concentration (100 mM). The yields were determined using GC (see section 3.3.7 in the experimental section).

3.2.4.2. Environmental impact assessment (E-factor)

Introduced by Sheldon in 1992, the E-factor serves as a valuable metric for assessing process sustainability. This metric takes into account not only product yield but also waste components such as solvent and material losses from (multi)step processes.[31] To consider the environmental implications of the continuous biocatalytic process described here, the E-factor was calculated considering the waste generated from the different components involved. The utilised yield was averaged across the entire flow run (39.6%), while the total volume was calculated from the flow rate (Table 3.2). Undoubtedly, the main contribution to the E-factor was EtOAc, employed as both solvent and acylating agent, followed by IPA and water. The unreacted substrate was considered as material loss. The high E-factor (117.5) in this case highlights the need to run continuous processes at high conversions with stable catalyst formulations.[32] However, this number could largely diminish if a solvent recycling system would be implemented.

Table 3.2. Calculation of E-factor for the transaminase-lipase continuous biocatalytic process.

Component	Mass wasted for 3.23 g of amide (g)[a]	Contribution to E factor (kg/kg)
Ethyl acetate	362.40	112.20
Water	4.22	1.31
Isopropylamine	5.29	1.64
EziG-CALB	1.00	0.31
EziG-ArRmut11-TA	1.00	0.31
Acetone (co-product)	0.97	0.30
Ethanol (co-product)	0.77	0.24
Material loss	3.83	1.19
Total		117.50

[a] Taking an average yield value of 39.6%.

Conclusions

The following conclusions have been drawn after exploring the combination of an amine transaminase and a lipase for chiral amide synthesis:

- A bienzymatic cascade combining ArRmut11-TA and CALB lipase for stereoselective preparation of (*R*)-*N*-(1-phenoxypropan-2-yl)acetamide was successfully developed.
- The immobilisation of ArRmut11-TA on various EziG supports was evaluated. Although similar results were obtained across all tested supports, EziG-Amber was chosen since this one previously provided the best results for CALB-catalysed reactions.
- The target cascade was investigated using EtOAc as the solvent, identifying the water content in the reaction mixture as a critical parameter for cascade assembly. A trade-off between enzyme activities was unavoidable for the cascade to produce the target compound, with the finding that 1% v/v water in the reaction mixture was viable, resulting in a 30% activity retention for both enzymes.
- In batch one-pot mode, very low to no conversion was observed, whereas the continuous flow setting demonstrated to be successful. Thus, ArRmut11-TA and CALB were compartmentalised within distinct packed bed reactors (PBR) in the flow setup.
- Significantly enhanced metrics were achieved in the flow setup, up to 3.4-fold increase in productivity towards the enantiopure (*R*)-amide compared to the traditional batch cascade approach.

Experimental part

3.3.1. General information

All chemicals were purchased from Sigma Aldrich unless stated otherwise. EziG supports were provided by EnginZyme AB. Gas chromatography (GC) analyses were performed on an Agilent HP6890 GC chromatograph equipped with a FID detector.

3.3.2. Enzyme expression and cell lysis

The enzymes were overexpressed in *E. coli* BL21(DE3), 50 µL of glycerol stock were inoculated to terrific broth autoinduction media (TB AIM 500 mL) with 100 µg/mL of kanamycin. The cultures were incubated at 200 rpm and 37 °C for 5 h and then at 200 rpm and 30 °C for 19 h. After 24 h the OD was measured, and the cells were harvested by centrifugation at 8000 rpm for 10 min at 4 °C. The collected cell pellets were resuspended in MOPS buffer and cell lysis of all the samples was performed using a Microfluidizer at 1200 bar over five passes. After lysis the OD was checked again to ensure the cells had been properly disrupted. The lysed cells were centrifuged at 15000 rpm for 1 h at 4 °C and the CALB was stored as liquid CFE in the fridge. For ArRmut11-TA the supernatant was recovered and PLP was added to a final concentration of 0.01 mM before freeze drying. Freeze drying of the CFE was performed using a freeze dryer and stored in the fridge.

3.3.3. ArRmut11-TA protein purification

For the samples that were purified, the supernatant was collected and filtered (0.45 µm). The purification was performed on a Ni-IDA agarose column using standardised immobilised metal affinity chromatography (IMAC) protocol with ÄKTA explorer. The binding buffer was 50 mM MOPS, 150 mM NaCl, pH 7.6. The elution buffer was the same as the

binding buffer but with 1 M imidazole. Fractions with containing target enzyme were merged and buffer exchanged to MOPS buffer (50 mM, pH 7.6) with 0.3 mM PLP using a PD10 desalting column. The concentration of pure proteins in solution was determined by measuring the absorbance of the solution at 280 nm.

3.3.4. Determination of target enzyme content in CFE

To quantify the amount of target enzyme present in the CFE, a known quantity of the extract was subjected to purification, and the purified enzyme was quantified based on its absorbance at 280 nm. Specifically, 300 mg of CFE were purified, yielding 18 mL of purified enzyme solution with a concentration of 1.9 mg/mL. Based on this measurement, the target enzyme constituted 11% of the CFE.

3.3.5. Enzyme immobilisation

The freeze-dried powder of ArRmut11-TA-His was rehydrated in 20 mM sodium phosphate buffer, pH 8 containing 0.3 mM PLP to obtain a CFE concentration of 15 mg/mL (freeze-dried powder/buffer solution). The prepared CFE was resuspended on an end-over-end rotator for 1 h (20 rpm, rt) and then centrifuged for 5 min (7000 rpm, rt). After centrifugation, the CFE (500 μL) was transferred to a new tube containing the 10 mg of the support. Tubes were covered with foil and immobilisation was performed on an end-over-end rotator, 20 rpm, at room temperature for 2 h. Then, the supernatants were removed, and the immobilised supports were washed first with immobilisation buffer (1 x 1 mL, 30 sec each), second with 2-PrOH washing solutions (2 x 1 mL, 30 sec each) and third with the solvent used for the reaction (1 x 1 mL, 30 sec), then the catalyst was used directly after removal of remaining solvent.

When immobilised from the pure protein solution, the same procedure was done but 434 µL of pure enzyme solution were incubated with the support and PLP to 0.3 mM final concentration. In both cases the amount of target protein offered to the support (10 mg) was 0.825 mg.

For the immobilisation of CALB, the CFE was buffered with 20 mM MOPS pH 7.5 and EziG-Amber was then added. The ratio 1:20 EziG:CFE was chosen based on previous optimisations done at EnginZyme (data not shown). The immobilisation was performed with end-over-end mixing for 3 h before rinsing with the same buffer and vacuum drying for 16 h.

3.3.5.1. Immobilisation yield

For ArRmut11-TA, the CFE and supernatant from the immobilisations were diluted with 20 mM sodium phosphate buffer, pH 8. An aliquot of the diluted enzyme solution (100 µL) was mixed with a reaction mixture (100 µL) containing 10 mM 1-phenylethylamine, 20 mM sodium pyruvate in 20 mM sodium phosphate buffer, pH 8. The formation of acetophenone was measured by the absorbance (A_{245}) at 245 nm every 49 sec for 20 minutes using a plate reader. Reaction rates with each supernatant from immobilisations and fresh enzyme solution were extracted from linear regression of the data points (A_{245}/min). The immobilised yield was calculated by determining the percentage of enzymatic activity left in the supernatant after immobilisation, relative to the enzymatic activity in the CFE.

For the CALB, the immobilised enzyme content was calculated by the tributyrin (TBU) hydrolysis activity assay, by comparing the activity of the starting CFE and the supernatant during immobilisation.

3.3.5.2. Recovered activity of immobilised catalysts

The recovered activity was obtained from the specific activity of immobilised enzyme compared to the specific activity of CFE in aqueous buffer as follows: Recovered activity=(specific activity of immobilised enzyme/specific activity of the free enzyme)*100). The specific activity was determined using initial reaction rates, considering the amount of enzyme used in the reaction. For the immobilised enzyme, the immobilisation yield was included in the calculations to consider only the amount of immobilised enzyme. The activity of ArRmut11-TA was determined by its ability to convert 1-phenoxypropan-2-one to 1-phenoxypropan-2-amine using IPA as amine donor. A reaction mixture (1 mL) containing 50 mM 1-phenoxypropan-2-one, 250 mM isopropylamine and 5% v/v DMSO in 20 mM sodium phosphate buffer, pH 8 was added to the immobilised catalyst, and the mixture was incubated for 5, 15, 30 and 45 min (1200 rpm, 37 °C). After that time, the reaction was quenched by adding 5 M NaOH. Then the reaction was extracted, and the yield of 1-phenoxypropan-2-amine was obtained by GC-FID analyses. Each reaction was performed in duplicate, and a single reaction was conducted for each time point. The activity of the immobilised CALB was tested in the kinetic resolution of racemic 1-phenylethan-1-ol with vinyl acetate as the acyl donor. The reaction mixture contained 1 M 1-phenylethan-1-ol, 600 mM vinyl acetate and 1% v/v dodecane in MTBE.

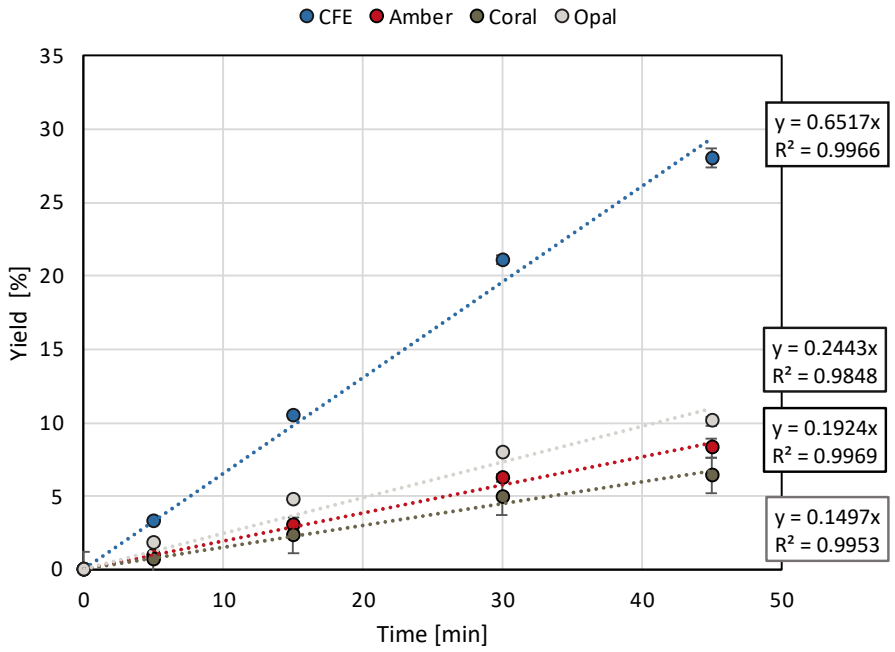


Figure 3.14. The recovered activity gives the percentage of activity that is retained after immobilisation. The recovered activity was obtained from the specific activity of immobilised enzyme compared to the specific activity of CFE in aqueous based assay. The specific activity was determined using initial reaction rates, considering the amount of enzyme used in the reaction. For the immobilised enzyme, the immobilisation yield was included in the calculations to consider only the amount of immobilised enzyme. Biotransformation conditions: 50 mM 1-phenoxypropan-2-one, 250 mM IPA, 5% v/v DMSO in 20 mM sodium phosphate buffer, pH 8. CFE (7.5 mg) and ArRmut11-TA-EziG (10 mg of EziG with 0.5 mL of 15 mg/mL CFE) in 1 mL total reaction volume, 37 °C, 1200 rpm. The reactions were run for 0, 5, 15, 30 and 45 min. Each reaction was performed in duplicate, and a single reaction was conducted for each time point. Yields were determined using GC after quenching with 5 M NaOH and extraction (yield considering total amine formation, including both (*R*)- and (*S*)-enantiomers).

3.3.6. Continuous flow setup

Continuous flow reactions were performed using the following equipment: DIONEX dual piston HPLC pump (flow rate 1.6 mL h^{-1}), PTFE/Steel tubing (1/16" ID), IDEX stainless steel BPR (back pressure regulator) holder fitted with a 7 bar cartridge, restek adjustable BPR with 5 µL dead volume, glass columns (15 mm i.d.), UNIQSIS heater block unit and OMNIFIT 3 way switching valves (Figure 3.15).

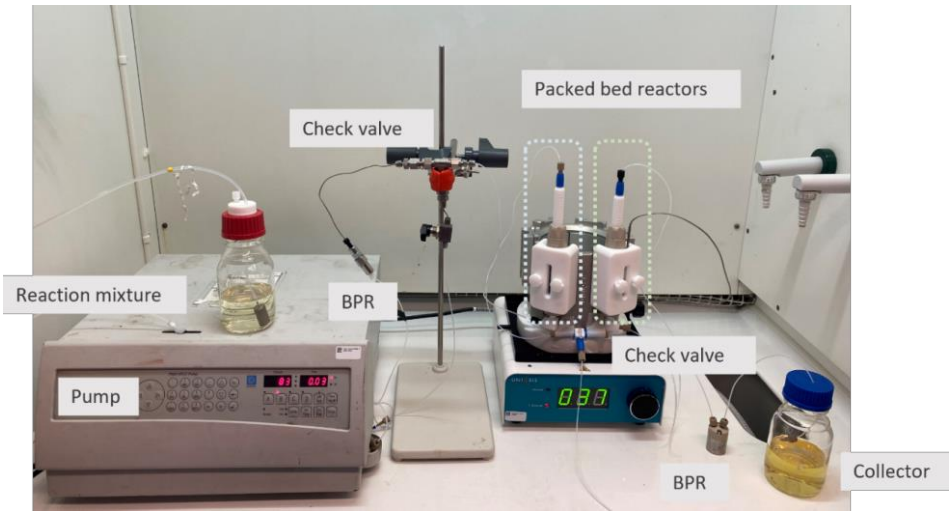


Figure 3.15. Labelled image of the flow setup.

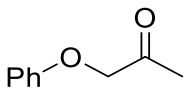
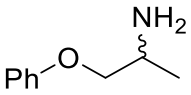
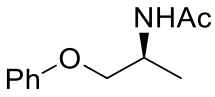
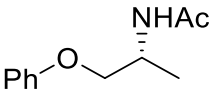
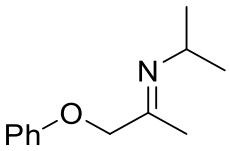
3.3.7. Analytical methods

GC analyses were performed using a CP-Chirasil Dex DB column (25 m x 0.25 mm x 0.25 µm) to analyse the reactions and an HP-5MS column (30 m x 0.25 mm x 0.25 µm) to identify the unknown peaks. The ATA-catalysed reactions were analysed after derivatisation with acetic anhydride to facilitate the separation of both amine enantiomers. The lipase and cascade reactions in flow were analysed directly by sample dilution.

For reactions analysed after derivatisation with acetic anhydride the following protocol was applied. After stopping the mixing and allowing the catalyst to settle, an aliquot (40 µL) of the supernatant was transferred to a new tube containing acetic anhydride (130 µL) and 1-methylimidazole (40 µL) to promote the derivatisation. After gently mixing, the solution was left standing for 30 min at room temperature. Deionised water (460 µL) was added to the mixture, gently mixed and ethyl acetate containing 40 mM dodecane as internal standard (460 µL) was added. The reaction was extracted for 5 min (1500 rpm, 25 °C) and the tubes were centrifuged for 5 min (15000 rpm) to promote phase separation. The organic layer (200 µL) was transferred to another tube and dried over Na_2SO_4. An aliquot of the dried organic layer (120 µL) was transferred to a GC vial and analysed by gas chromatography coupled to flame ionisation detector (GC-FID) to determine the conversion.

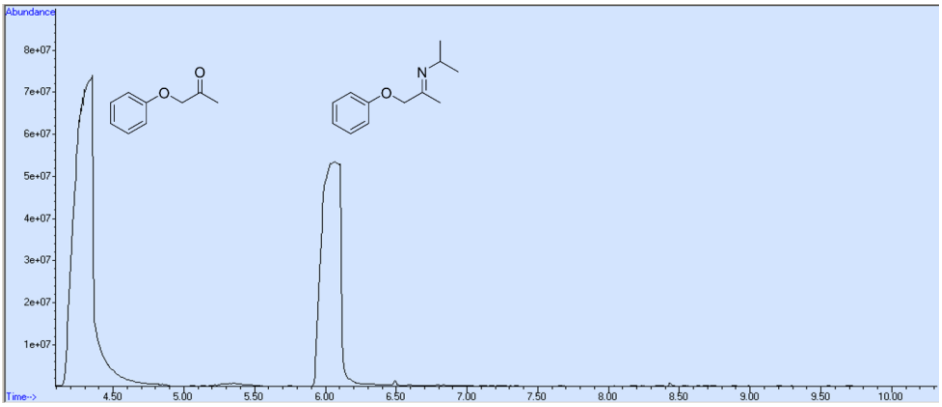
Method specifications. 2 mL/min Hydrogen, 47 kPa. Injection 1 µL with 20:1 split ratio. Injection temperature: 200 °C, Detector temperature: 250 °C, Detector type: FID. Oven temperature: 100 °C, hold 2 min, 15 °C/min ramp to 195 °C, hold 2 min. Treatment of results: Calibration curve with dodecane (10 mM) as an internal standard.

Table 3.3. GC retention times for methods with CP-Chirasil Dex DB / HP-5MS.

Compound	Retention time (min)	Compound	Retention time (min)
	7.7 / 4.4		5.9
	8.7		8.8
	6.5 / 6.1		

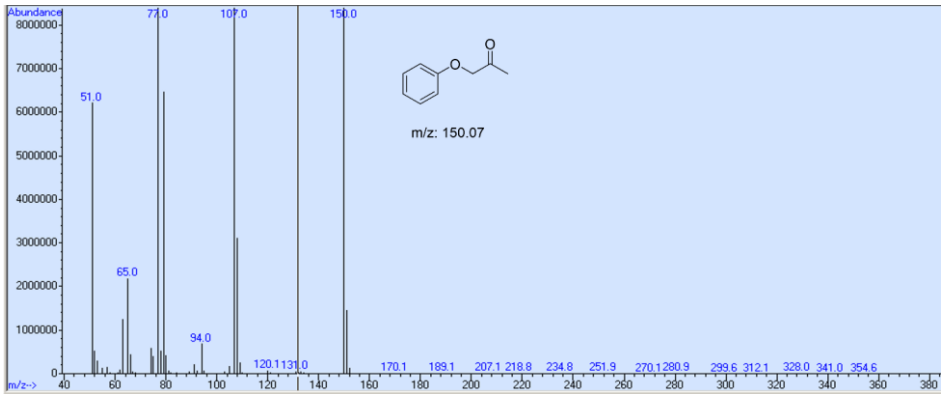
3.3.7.1. GC chromatogram of the 1-phenoxypropan-2-one incubated with isopropylamine

Figure 3.16. GC-FID chromatogram of 1-phenoxypropan-2-one (peak at 4.3 min) and *N*-isopropyl-1-phenoxypropan-2-imine (peak at 6.0 min).



3.3.7.2. MS analysis of the 1-phenoxypropan-2-one

Figure 3.17. MS analysis of 1-phenoxypropan-2-one (peak at 4.3 min). Calculated m/z: 150.1, obtained: 150.0.



CHAPTER 4.

Bioreduction of alkene bonds using an immobilised ene-reductase in organic solvent

Introduction

4.1.1. Ene-reductases (EREDs)

Ene-reductases (EREDs) catalyse the asymmetric reduction of carbon-carbon doble bounds (C=C) enabling the formation of up to two stereogenic centers. The most predominant family of EREDs are the flavin mononucleotide (FMN) containing Old Yellow Enzyme (OYE) family of oxidoreductases (EC 1.6.99.1).[254] These enzymes exhibit high chemo-, regio-, and stereoselectivity towards alkenes bearing electro-withdrawing groups (EWGs) such as carbonyl (ketone/aldehyde/acid/ester/lactone/cyclic imide), nitro or nitrile.[255]

During the asymmetric reduction, a stereoselective [256]transfer of a hydride from the $FMNH_2$ cofactor occurs onto the C-β carbon atom, while a proton (ultimately derived from the solvent) is added by a Tyr residue onto the C-α atom from the opposite side (Figure 4.1). This reduction proceeds via the transfer of [2H] in a Michael-type addition reaction operating in a *trans*-fashion.[257,258] The catalytic cycle is completed by the reduction of the oxidised flavin cofactor at the expense of a nicotinamide cofactor [NAD(P)H]. Presumably, the prosthetic flavin is sufficiently exposed, which enables EREDs to exhibit flexible specificity towards either NADH or NADPH as cofactor, allowing for greater adaptability and customization in selecting the appropriate cofactor recycling system.

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[254] A. Scholtissek, D. Tischler, A. H. Westphal, W. J. H. van Berkel, C. E. Paul, *Catalysts* **2017**, *7*, 130.
[255] H. S. Toogood, N. S. Scrutton, *Curr. Opin. Chem. Biol.* **2014**, *19*, 107–115.
[256] F. Parmeggiani, E. Brenna, D. Colombo, F. G. Gatti, F. Tentori, D. Tessaro, *ChemBioChem* **2022**, *23*, e202100445.
[257] C. K. Winkler, G. Tasnádi, D. Clay, M. Hall, K. Faber, *J. Biotechnol.* **2012**, *162*, 381–389.
[258] Durchschein, M. Hall, K. Faber, *Green Chem.* **2013**, *15*, 1764–1772.

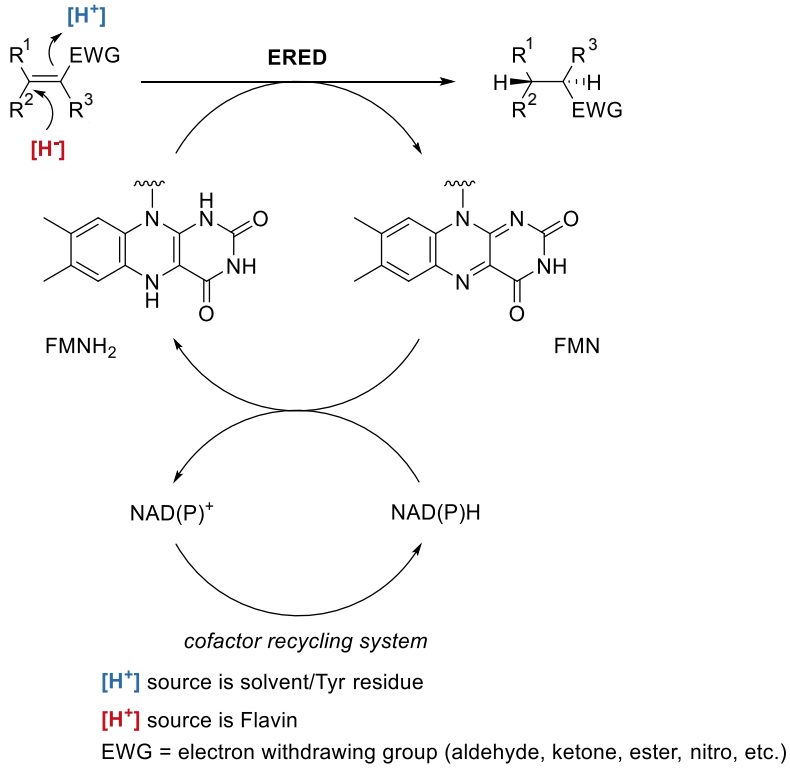

Figure 4.1. Mechanism of asymmetric reduction of activated alkenes by EREDs.

The stereogenic centre formed at C-β is configurationally stable, but the one at C-α may undergo epimerisation due to the presence of the EWG.[259] Predicting the selectivity of EREDs might be challenging. For instance, in the reduction of both (*E*) and (*Z*) isomers from tetra-substituted alkenes, the obtained enantiomer is the result of a *trans*-hydrogenation in both cases.[255] Therefore, the stereochemistry of the final product is influenced not only by the configuration of the alkene but also by the substrate's positioning inside the active site.

[259] G. Oberdorfer, K. Gruber, K. Faber, M. Hall, *Synlett* **2012**, *23*, 1857–1864.

4.1.1.1. Ene-reductase from Zymomonas mobilis (NCR-ERED)

The ene-reductase from *Zymomonas mobilis* (NCR-ERED) is a member of the Old Yellow Enzyme (OYE) class I, which includes traditional EREDs from plants and bacteria.[254] Like a typical class I OYE, NCR-ERED adopts an $(\alpha,\beta)_8$-barrel structure, also known as a TIM barrel, with the flavin mononucleotide (FMN) non-covalently bound on top of the barrel (Figure 4.2).[260] This enzyme has three additional barrel elements: 1) an N-terminal β-hairpin that seals the barrel's bottom, 2) a capping subdomain formed by secondary structure elements between β3 and α3, and 3) an α-helix between β8 and α8 that helps with FMN binding.

The enzyme is as a homodimer, composed of two virtually identical monomers, but described as a monomeric protein in solution.[260] Furthermore, the dimer interface's organisation in the asymmetric unit suggests no functional dimerisation: the contact surface is minimal, and the monomers' interactions are entirely hydrophilic, with numerous water molecules bridging the gap.

[260] S. Reich, H. W. Hoeffken, B. Rosche, B. M. Nestl, B. Hauer, *ChemBioChem* **2012**, *13*, 2400–2407.

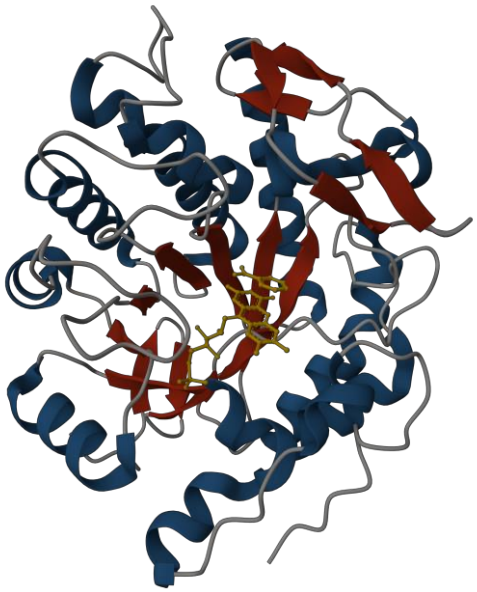

Figure 4.2. Crystal structure of the NCR ene-reductase (PDB ID: 4A3U) displayed in cartoon representation and showing prosthetic FMN and cofactor NAD^+ (yellow sticks), α -helices (blue), β -sheets (red) and loop regions (grey).

NCR-ERED is particularly interesting for synthetic applications due to its high tolerance to both organic solvents and high substrate concentrations, [261] a feature that is highly beneficial for industrial applications where reactions often take place in non-aqueous environments.

4.1.2. Cofactor regeneration

[261] T. Reß, W. Hummel, S. P. Hanlon, H. Iding, H. Gröger, *ChemCatChem* **2015**, *7*, 1302–1311.

A crucial factor to consider when using cofactor dependent enzymes in for large-scale production is the selection of a hydride source that is cost-effective, since supplying abundant quantities of NAD(P)H is not economically feasible. To address this challenge several strategies have been extensively described in literature and compiled in comprehensive reviews.[255,262,263]

The most used approach is the use of a cofactor recycling system by introducing an additional enzyme/substrate pair that also relies on NAD(P)H. Commonly employed cofactor recycling systems include formate dehydrogenase/formate, glucose dehydrogenase/glucose, phosphite dehydrogenase/phosphite, and alcohol dehydrogenase/2-propanol (Figure 4.3).[264]

[262] H. S. Toogood, T. Knaus, N. S. Scrutton, *ChemCatChem* **2014**, *6*, 951–954.
[263] H. S. Toogood, N. S. Scrutton, *ACS Catal.* **2018**, *8*, 3532–3549.
[264] W. Hummel, H. Gröger, *J. Biotechnol.* **2014**, *191*, 22–31.

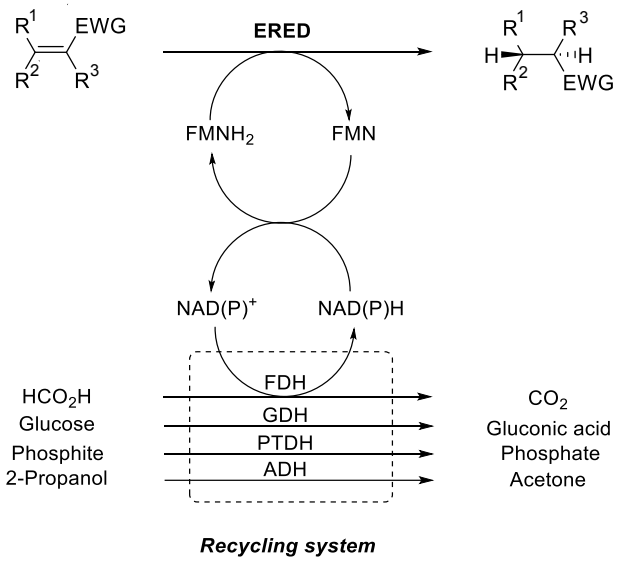

Figure 4.3. Commonly used recycling systems for NAD(P)H in ERED catalysed reactions: formate dehydrogenase (FDH), glucose dehydrogenase (GDH), phosphite dehydrogenase (PTDH) and alcohol dehydrogenase (ADH).

Furthermore, the combination of multiple recycling enzymes has also been described. For instance, a cascade involving an ADH, formaldehyde dismutase, and FDH has shown to be highly efficient for the reduction of ketoisophorone to (*R*)-levodione, reaching full conversion and high enantioselectivity (92% *ee*).[265] In this case, three equivalents of NADH are generated, while methanol is oxidized to carbon dioxide. Nevertheless, the current limitation of this system lies in the relatively low catalytic efficiency of the initial ADH oxidation step, requiring high methanol concentrations which might be detrimental for enzyme activity. This approach is especially interesting for cascade reactions, when the biocatalysts can be deliberately

[265] S. Kara, J. H. Schrittwieser, S. Gargiulo, Y. Ni, H. Yanase, D. J. Opperman, W. J. H. van Berkel, F. Hollmann, *Adv. Synth. Catal.* **2015**, *357*, 1687–1691.

selected to use the same nicotinamide cofactor. This arrangement generates a closed-loop system, where the catalytic activity of one enzyme generates the cofactor form required for the second biocatalyst and vice versa. The potential of this methodology was demonstrated with the isomerisation of allylic alcohols to the corresponding saturated ketones by an ADH from *Thermus* sp. and enoate reductase from *Thermus scotoductus* (TsER).[266]

The fact that the nicotinamide cofactor is used to regenerate FMN and does not directly interact with the substrate enables its replacement with synthetic biomimetics (mNADHs). These synthetic cofactors have demonstrated successful applications in stoichiometric amounts, resulting in high conversions and enantioselectivities with diverse substrates.[267,268] The cost efficiency can be further enhanced by adding a recycling system like those employed with natural cofactors. For example, the NADH-oxidase from *Lactobacillus pentosus*[269] or the GDH from *Sulfolobus solfataricus*[270] were successfully used for this purpose, offering promising options for recycling the synthetic cofactors.

Another cofactor managing strategy is to eliminate the requirement for NAD(P)H by supplying an alternative hydride donor (co-substrate) for flavin recycling. A variety of 2-enones or 1,4-diones have been identified as potential hydride donors.[271] However, this led to the formation of phenolic compounds that can decrease the ERED activity. To tackle this challenge, a

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[266] S. Gargiulo, D. J. Opperman, U. Hanefeld, I. W. C. E. Arends, F. Hollmann, *Chem. Commun.* **2012**, *48*, 6630−6632.
[267] T. Knaus, C. E. Paul, C. W. Levy, S. de Vries, F. G. Mutti, F. Hollmann, N. S. Scrutton, *J. Am. Chem. Soc.*, **2016**, *138*, 1033−1039.
[268] C. E. Paul, S. Gargiulo, D. J. Opperman, I. Lavandera, V. Gotor-Fernández, V. Gotor, A. Taglieber, I. W. C. E. Arends, F. Hollmann, *Org. Lett.* **2013**, *15*, 180−183.
[269] C. Nowak, B. Beer, A. Pick, T. Roth, P. Lommes, V. Sieber, *Front. Microbiol.* **2015**, *6*, 957.
[270] C. Nowak, A. Pick, P. Lommes, V. Sieber, *ACS Catal.* **2017**, *7*, 5202–5208.
[271] C. Stueckler, T. C. Reiter, N. Baudendistel, K. Faber, *Tetrahedron* **2010**, *66*, 663–667.

more recent study explored the use of other co-substrates that would form (quasi)aromatic, non-inhibitory dehydrogenation byproducts.[272] From this investigation, six promising cost-effective co-substrates were identified, including menthone. All of them were grouped as substituted 2-cyclohexenones, cyclohexanediones, ketoheterocycles, and other hydrogen donors.

A less explored approach involves regeneration of cofactors through electrochemical reactions using chemical mediators.[273] This strategy allows for the direct delivery of electrons to the active site of enzyme. For instance, methyl viologen was employed successfully in a biphasic bioreactor to facilitate the reduction of 2-cyclohexen-1-one with OYE pentaerythritol tetranitrate reductase.[274] The stability and reusability of both enzyme and mediator were effectively maintained over a period of 12 h.

Finally, novel strategies were explored to utilise photosynthetic energy for ERED catalysed reductions. These included the use of natural photosynthesis pathways to drive the production of reduced nicotinamide cofactors[275], and the use of a photoelectrochemical (PEC) cell to facilitate enzyme-bound FMN reduction.[276] Another method involved the direct photoactivation of FMN in the presence of several photosensitisers such as

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[272] C. K. Winkler, D. Clay, M. Entner, M. Plank, K. Faber, *Chem. Eur. J.* **2014**, *20*, 1403–1409.
[273] A. Tosstorff, C. Kroner, D. J. Opperman, F. Hollmann, D. Holtmann, *Eng. Life Sci.* **2017**, *17*, 71–76.
[274] K. Fisher, S. Mohr, D. Mansell, N. J. Goddard, P. R. Fielden, N. S. Scrutton, *Catal. Sci. Technol.* **2013**, *3*, 1505–1511.
[275] K. Köninger, Á. Gómez Baraibar, C. Mügge, C. E. Paul, F. Hollmann, M. M. Nowaczyk, R. Kourist, *Angew. Chem. Int. Ed.* **2016**, *55*, 5582–5585.
[276] E. J. Son, S. H. Lee, S. K. Kuk, M. Pesic, D. S. Choi, J. W. Ko, K. Kim, F. Hollmann, C. B. Park, *Adv. Funct. Mater.* **2018**, *28*, 1705232.

light-activated Ru(II) or Ir(III) complexes.[277] In this setup, the electron donor triethanolamine and the mediator methyl viologen were present to aid the process. Remarkably, these innovative strategies yielded products with comparable yields and levels of enantiopurity when compared to reactions that relied on NADPH as the primary hydride donor, demonstrating the potential and practicability of utilising photosynthetic energy in ERED catalysed bioreductions.

4.1.3. Immobilised ERED

As previously mentioned in this Doctoral Thesis, the use of immobilised enzymes offers numerous advantages, such as enhanced stability under non-optimal conditions and simplified downstream processing. However, the use of supported ERED is still relatively unexplored, with limited information available on the most effective methods and approaches for achieving stable and efficient immobilised EREDs

Ubali and co-workers have studied the immobilisation of OYE3 from *Saccharomyces cerevisiae* using two different immobilisation techniques,[278] such as covalent binding onto glyoxyl-agarose (GA) and affinity-based adsorption on controlled pore glass EziG. The protein loadings achieved with these methods was relatively low, with 2 mg/g and 4.2 mg/g when utilizing GA and EziG, respectively. To evaluate the recyclability of the heterogeneous catalysts, a series of reactions were conducted in which α-

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[277] M. K. Peers, H. S. Toogood, D. J. Heyes, D. Mansell, B. J. Coe, N. S. Scrutton, *Catal. Sci. Technol.* **2015**, *6*, 169–177.
[278] F. Tentori, T. Bavaro, E. Brenna, D. Colombo, D. Monti, R. Semproli, D. Ubiali, *Catalysts* **2020**, *10*, 260.

methyl-*trans*-cinnamaldehyde (5 mM) was reduced to (*S*)-α-methyl-β-phenylpropanal. For $NADP^+$ cofactor recycling they both used the GDH system, however their set-up slightly differ. For GA immobilisation, GDH was added as a soluble enzyme in each reaction cycle, while when using EziG, a co-immobilised OYE3/GDH catalyst was used, taking advantage of both enzymes having a His-tag.

OYE3-GA exhibited excellent recyclability and could be used for multiple reaction cycles, with a maximum conversion of 40% achieved after 12 runs. Remarkably, OYE3-GA maintained its complete activity up to the fifth reaction cycle. In contrast, OYE3/GDH-EziG showed an initial 30% activity loss after two reaction cycles. In this case, when the conversion percentage became negligible in the eleventh run, fresh soluble GDH was introduced into the biotransformation process resulting in a significant increase in reaction conversion to 30%. This observation indicates that the gradual decrease in conversions observed for OYE3/GDH-EziG may have been caused by either immobilised GDH losing its activity or leakage from the carrier.

Hall and co-workers employed a similar approach involving the co-immobilisation of OYE3 and NCR EREDs with GDH from *Bacillus megaterium*.[279] In this case, a glutaraldehyde-activated Relizyme HA403/M carrier was employed for enzyme immobilisation, directly from the crude cell-free extract. The resulting co-immobilised catalysts demonstrated compatibility with organic cosolvents up to 20% volume in reactions. The bi-functional redox biocatalyst displayed remarkable reusability, with only 17% activity loss after 5 cycles for OYE3. Preparative-scale synthesis at a substrate concentration of 50 mM yielded three chiral compounds with high

[279] F. Nagy, I. Gyujto, G. Tasnádi, B. Barna, D. Balogh-Weiser, K. Faber, L. Poppe, M. Hall, *J. Biotechnol.* **2020**, *323*, 246–253.

enantiopurity (up to 97% *ee*). However, poor product recovery (up to 42% isolated yield) was observed due to product absorption to the support. To avoid this drawback, the washing of the catalyst with a solvent was attempted, finding a significant enzyme deactivation after subsequent cycles.

In another example, YqjM from *Bacillus subtilis* was co-immobilised with GDH using cross-linked enzyme aggregates (CLEAs) and biomimetic immobilisation techniques.[280] The latter method involved the formation of ERED-GDH-silica particles (SPs) through a one-step approach using a silicic acid precursor. Both ERED-GDH-CLEAs and ERED-GDH-SPs exhibited high immobilisation yields (93.5% and 92.4%, respectively) and moderate immobilised activity (44.9% and 44.5%, respectively).

Recently, the Vincent group reported the co-immobilisation of a commercial ERED namely ENE101 from Johnson Matthey and a nickel-iron hydrogenase 1 from *Escherichia coli* onto a carbon support.[281] In this system, the hydrogenase reduces the flavin cofactor using H_2 as the reductant. However, the study does not provide specific information regarding the immobilisation approach and detailed results. Only the loading of the hydrogenase and ERED were mentioned, with values of 39 pmoles per 100 μg of carbon for the hydrogenase and 65 pmoles per 100 μg of carbon for the ERED.

Berensmeier and co-workers developed a novel selective $(His\text{-}Arg)_4$ peptide-tag for the directed immobilisation of proteins on magnetic

[280] H. Li, W. Xiao, P. Xie, L. Zheng, *Enzyme Microb. Technol.* **2018**, *109*, 66–73.
[281] J. S. Rowbotham, M. A. Ramirez, O. Lenz, H. A. Reeve, K. A. Vincent, *Nat. Commun.* **2020**, *11*, 1454.

nanoparticles (MNPs).[282] The study focused on using the OYE enzyme from the cyanobacterium *Nostoc* sp. PCC7120, which was engineered to effectively utilise NADH as a cofactor instead of NADPH. The immobilisation process resulted in enhanced thermostability, and a longer half-life compared to the free enzyme. Moreover, the authors reported very high enzyme loading of approximately 380 mg per gram of MNPs, with 67% residual activity. Recyclability studies showed around 50% activity loss after the first reuse, although the subsequent nine cycles maintained stable activity without further decline.

Very recently, the use of immobilised ERED from *Thermus scotoductus* (TsOYE) in micro-aqueous organic solvent was demonstrated. Immobilisation of TsOYE was performed by adsorption on Celite 545, R-632, R-633 and R-648.[283] The obtained immobilisation yields were ranging from 54 to 59%, corresponding to 6.7-7.6 mg/g protein loading. Optimisation of reaction conditions (10% buffer in organic solvent), facilitated the scale-up to 50 mmol of 2-methyl-*N*-phenylmaleimide, obtaining high conversion with TsOYE-Celite R-633 and R-648. The product was easily isolated by separation and evaporation of the organic phase, affording 91% of (*R*)-2-methyl-*N*-phenylsuccinimide, avoiding extraction difficulties and product hydrolysis typically observed in aqueous media. TsOYE-Celite R-633 was further selected to evaluate reusability over multiple operational cycles, while adding salt pairs in each cycle to ensure the maximum enzyme activity retention. Full conversion was obtained after the second cycle, and ca. 70% conversion after five consecutive cycles. The slight systematic decrease in conversion after the

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[282] A. A. Zanker, N. Ahmad, T. H. Son, S. P. Schwaminger, S. Berensmeier, *Biotechnol. J.* **2021**, *16*, e2000366.
[283] R. Villa, C. Ferrer-Carbonell, C. E. Paul, *Catal. Sci. Technol.* **2023**, DOI 10.1039/d3cy00541k.

third cycle was attributed to loss of immobilised enzyme material when removing the supernatant after each reaction.

4.1.4. Application of ERED reactions in organic solvent

The industrial application of ERED-catalysed reactions,[263] commonly carried out in buffered media, are limited by substrate-media incompatibilities derived from low solubilities. This represents a major problem for the implementation of biocatalytic processes at/on industrial scale.[284] Therefore, the use of organic solvents is highly attractive, as it not only improves the substrate solubility but also facilitates downstream processing by eliminating the need for extraction steps required otherwise when reactions take place in buffer. Moreover, organic solvents have been reported to enhance stereoselectivity, making them particularly advantageous for asymmetric reactions.[285,286]

Despite the many advantages of organic solvent systems, their implementation in EREDs reactions remains largely unexplored, although a noteworthy example was already reported in the literature in 2011.[287] Bommarius and co-workers investigated the effects of organic solvents, both water miscible and immiscible, on the catalytic efficiency and stereoselectivity of KYE1 from *Kluyveromyces lactis* and YersER from *Yersinia bercovieri*. Initially, they tested biphasic systems, with only 20%

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[284] T. K. Roy, R. Sreedharan, P. Ghosh, T. Gandhi, D. Maiti, *Chem. Eur. J.* **2022**, *28*, e202103949.
[285] B. V. Adalbjörnsson, H. S. Toogood, A. Fryszkowska, C. R. Pudney, T. A. Jowitt, D. Leys, N. S. Scrutton, *ChemBioChem* **2010**, *11*, 197–207.
[286] C. Stueckler, N. J. Mueller, C. K. Winkler, S. M. Glueck, K. Gruber, G. Steinkellner, K. Faber, *Dalt. Trans.* **2010**, *39*, 8472–8476.
[287] Y. Yanto, C. K. Winkler, S. Lohr, M. Hall, K. Faber, A. S. Bommarius, *Org. Lett.* **2011**, *13*, 2540–2543.

organic solvent in the reduction of 2-cyclohexen-1-one. Interestingly, no correlation was observed between logP and activity, as ethylene glycol, dimethyl sulfoxide, toluene and hexane rendered high conversions despite having logP values ranging from –1.43 to 3.5. were able to establish such correlation. Water miscible solvents employed in 20-35% v/v in buffer led to a 50% loss in activity, while water immiscible solvents maintained unaltered activity even at 70% v/v. These results were attributed to the reduced exposure of the enzyme to the organic solvent in biphasic systems, resulting in lower enzyme deactivation.

Subsequently, Faber and co-workers expanded on this knowledge using the same model reaction (2-cyclohexen-1-one).[288] A similar trend was observed, where OYE1 achieved near-quantitative conversion in the presence of all selected solvents up to 20% v/v. However, beyond this threshold, activities began to decline for reactions with 30 and 50% v/v for water soluble solvents. In contrast, enzyme exhibited complete tolerance for water immiscible solvents at concentrations up to 70% v/v and modest deactivation was observed at 90% v/v. Particularly, with 90% v/v, the authors have tested NADH-free disproportionation of 2-cyclohexen-1-one and observed that the use of organic solvents with a logP value ≥1.8 resulted in full conversion, indicating that the limiting factor might be NADH solubility rather than enzyme deactivation. Unfortunately, further increases in organic solvent content to 97% v/v led to significant decrease in reaction rates for both NADH dependent reduction and disproportionation, attributed to enzyme deactivation.

Several approaches can be followed to enhance ERED performance in nonaqueous media. Strong preference for thermostable enzymes is one of the examples, as a correlation between thermostability and organic solvent

[288] D. Clay, C. K. Winkler, G. Tasnádi, K. Faber, *Biotechnol. Lett.* **2014**, *36*, 1329–1333.

tolerance has been demonstrated.[285] Building on this premise, enzyme engineering can be employed to obtain more stable enzymes. Additionally, enzyme immobilisation is a well-explored technique that improves enzyme stability under non-optimal conditions. In line with this, Paul and co-workers pursued the immobilisation of TsOYE on Celite, as described in the previous section 4.1.3.[283] The system showed high conversions to the target product with buffer saturated MTBE (1% v/v). However, as substrate concentration increased, the buffer content needed to be increased to 4 or 10% v/v, depending on the substrate, to achieve high conversions.

One major potential limitation of the previously described systems is the use of GDH/glucose as recycling system for NADPH. As substrate concentration increases, the amount of glucose required for cofactor recycling also needs to increase. However, the system performance is highly limited by the low solubility of glucose in organic solvents. As an alternative, this chapter describes the use of an ADH enzyme for cofactor recycling, where 2-propanol is employed as a highly soluble co-substrate in organic solvents.

Objectives

A major challenge in biocatalytic processes is the low solubility of organic compounds in aqueous media. The implementation of strategies that enable enzymes to function in organic solvents, such as enzyme immobilisation, could potentially address this issue by improving both substrate solubility and product isolation. This chapter outlines a strategy to obtain an active ERED coupled to an ADH system in organic solvent media, and has the following specific objectives:

- To find a model substrate for the application of EREDs in organic solvent, and develop robust analytical methods for bioreduction monitorisation.

- Select a suitable ADH for cofactor recycling, that is also able to retain high activity in organic solvent.

- Immobilisation of the target enzymes, ERED and ADH, evaluating different strategies regarding cofactor co-immobilisation and to apply the obtained catalyst in alkene bioreductions in organic solvents.

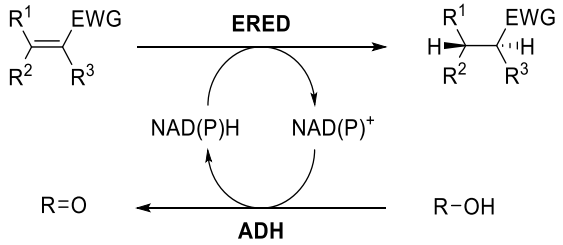
- To identify the best post-immobilisation treatments for both redox enzymes to retain high activity in organic solvent after water removal from the heterogeneous catalyst.

- Evaluate the potential implementation of the bienzymatic system in a packed bed reactor.

Results

4.2.1. Substrate scope for NCR-ERED

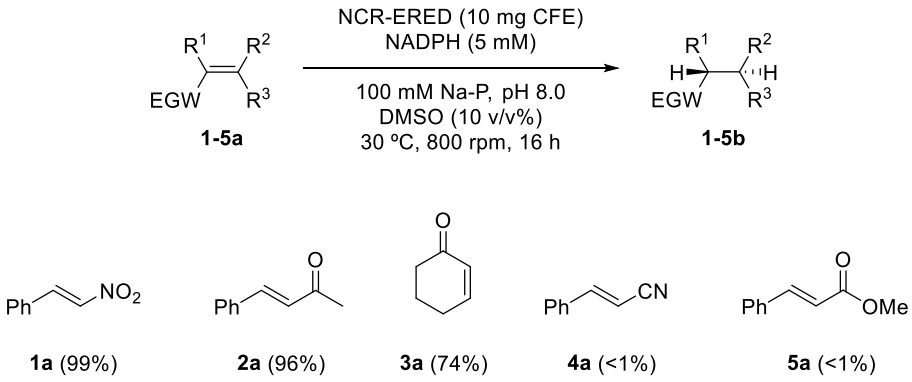
A substrate scope study was conducted with readily accessible commercial compounds, with the objective of identifying a suitable model substrate for the application of the NCR-ERED in an organic solvent system (Scheme 4.1).



Scheme 4.1. General scheme for the NCR-ERED bioreduction of alkenes using an ADH for cofactor recycling.

The selected alkene containing compounds carried diverse EWGs to explore the dependence of the enzyme activity from the electronegativity of the EWG (Scheme 4.2). The experiments were conducted using standard conditions with the reactions taking place in Na-P buffer at pH 8.0 containing 10% DMSO to enhance substrate solubility. NCR-ERED was used as cell free extract (CFE) and equimolar amounts of cofactor (NADPH) were added to test the reaction with the different substrates. All reactions were run overnight, and conversions towards the saturated compound were determined using GC-FID relative areas of substrate and product.

Scheme 4.2 summarises the results obtained from the substrate scope study, showing that compounds with EWG with low electronegativity (**4-5a**) were not reduced by NCR-ERED. In contrast, good to complete conversions were observed with substrates containing nitro- and keto- EWG (**1-3a**): 99% for *trans*-β-nitrostyrene, 96% with *trans*-4-phenyl-3-buten-2-one, and 74% for 2-cyclohexen-1-one.



Scheme 4.2. Substrate screening for NCR-ERED chemoselective reduction of C=C double bonds bearing different EWGs. For all the reactions: substrate (**1-5a**, 5 mM), NCR-ERED (10 mg, CFE), NADPH (5 mM) and DMSO (10% v/v) in 100 mM sodium phosphate, pH 8.0, 30°C, 16 h, 800 rpm. Conversions were determined by GC analysis from the relative area of substrate and product. Reactions were performed in duplicate and standard deviations were below 10%.

Based on the reactivity observed, the most promising compounds **1-3a** were chosen for further investigation, studying the action of different ADHs for cofactor recycling purposes.

4.2.2. NCR-ERED reaction with selected substrates using different ADHs

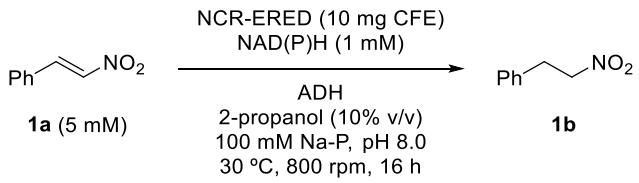
To facilitate efficient cofactor recycling, the study proceeded to investigate different ADHs, including *Clostridium beijerinckii* (CbADH), *Entamoeba histolytica* (EhADH), *Thermoanaerobacter ethanolicus* (TeSADH), WIC variant from *Thermoanaerobacter ethanolicus* (TeSADH-WIC) and *Saccharomyces cerevisiae* (ScADH).

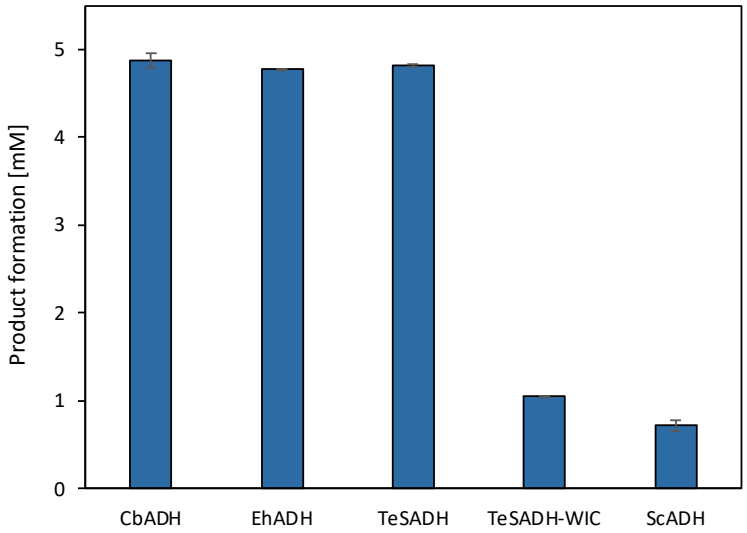
The enzymes CbADH, EhADH, and TeSADH-WIC were overexpressed in-house and used as cell free extracts (CFE), while TeSADH was purchased from Prozomix and used as CFE, and ScADH was obtained

from Sigma-Aldrich and used as a pure enzyme. For all of them, 2-propanol (10% v/v) was used as co-substrate and NADPH as the cofactor ,with the exception of ScADH where NADH was employed.

*4.2.2.1. trans-β-Nitrostyrene (**1a**)*

The initial tests with substrate **1a** showed that it had high reactivity for the NCR-ERED-catalysed reaction (Scheme 4.3). To investigate its potential, the reduction was tested using previously selected ADHs for cofactor recycling. The results showed that CbADH, EhADH and TeSADH led to high product formation (>90%), while application of TeSADH-WIC and ScADH resulted in a low conversion (less than 20%). In parallel, negative control reactions were run under the same conditions. In this case no remaining substrate **1a** was detected, suggesting potential instability. Further studies were conducted to assess substrate stability, such as incubation in different buffers and pHs, but the results were consistent across all conditions tested with observed compound decomposition. Therefore, it was decided that no further studies would be conducted using substrate **1a**.



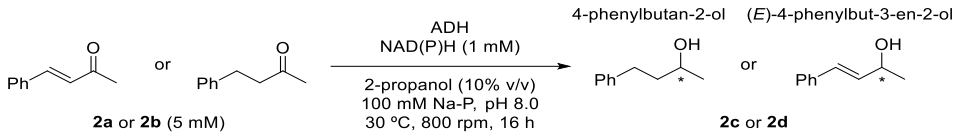


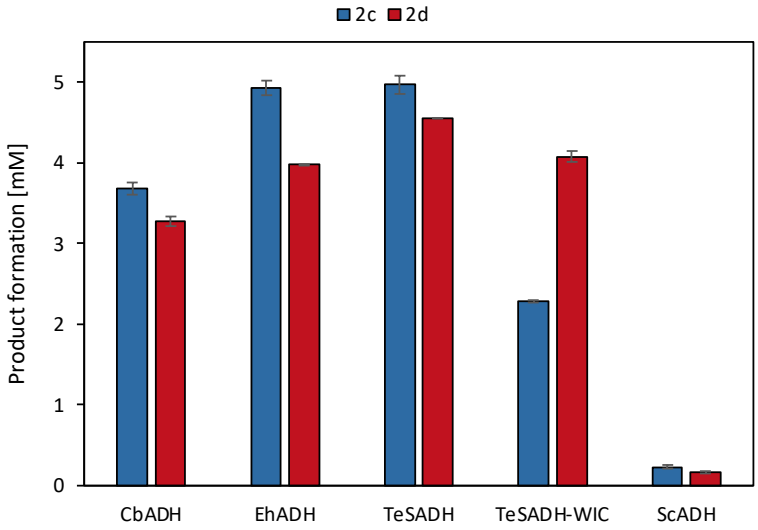
Scheme 4.3. Bioreduction of *trans*-β-nitrostyrene (**1a**) using NCR-ERED and different ADHs for cofactor recycling. For all the reactions: substrate **1a** (5 mM), NCR-ERED (10 mg, CFE), NAD(P)H (1 mM) and 2-propanol (10% v/v) in 100 mM sodium phosphate, pH 8.0, 30 °C, 16 h, 800 rpm. ADHs amount - CbADH, EhADH, TeSADH and TeSADH-WIC: 10 mg, CFE; ScADH: 0.1 mg = 30 U, pure enzyme. Conversions into **1b** were determined by GC analysis. Reactions were performed in duplicate and standard deviations were below 10%.

*4.2.2.2. trans-4-Phenyl-3-buten-2-one (**2a**)*

Next, a substrate bearing a ketone as EWG was evaluated, this is the unsaturated ketone **2a** (Scheme 4.4). Additionally, the possible reduction of the carbonyl group of the resulting saturated ketone **2b** need to be considered, but also the possible formation of alcohols **2c** and **2d** which requires the development of robust analytical methods. All available ADHs were screened for their reactivity towards both saturated and unsaturated ketones **2a** and **2b**, respectively. Only ScADH showed low reactivity with either substrate (less than 10% product formation), whereas all other ADHs

showed moderate activity with both compounds (conversions higher than 40%). Such promiscuity could lead to the formation of mixed products upon assembly of the cofactor regeneration system.



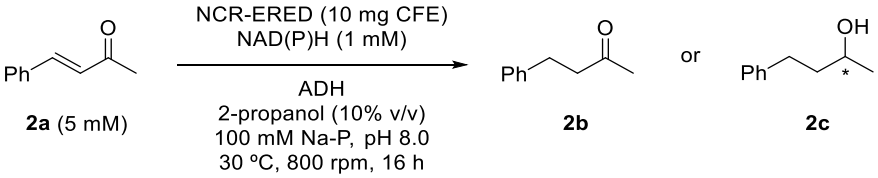


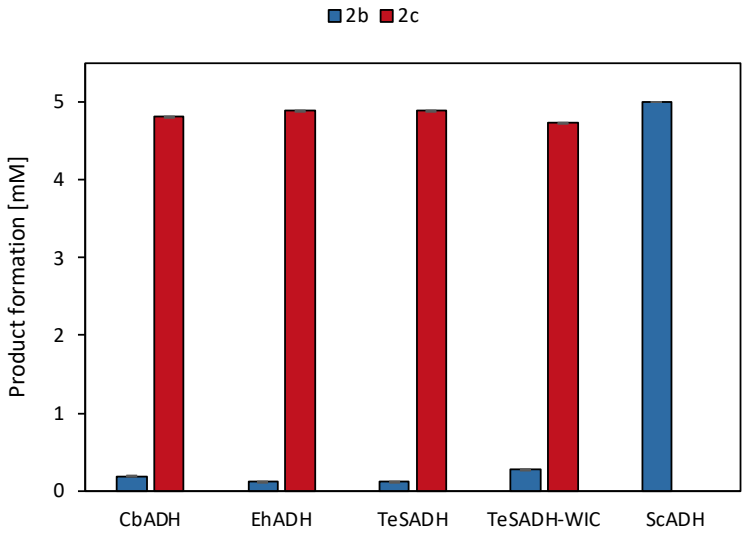
Scheme 4.4. Bioreduction of *trans*-4-phenyl-3-buten-2-one (**2a**) and 4-phenylbutan-2-one (**2b**) to obtain 4-phenylbutan-2-ol (**2c**) and *trans*-4-phenyl-3-buten-2-ol (**2d**), respectively, using different ADHs. For all the reactions: substrate **2a** or **2b** (5 mM), NAD(P)H (1 mM) and 2-propanol (10% v/v) in 100 mM sodium phosphate, pH 8.0, 30 °C, 16 h, 800 rpm. ADHs amount - CbADH, EhADH, TeSADH and TeSADH-WIC: 10 mg, CFE; ScADH: 0.1 mg = 30 U, pure enzyme. Conversions were determined by GC analysis. Reactions were performed in duplicate and standard deviations were below 10%.

Despite the initial results, it was decided to proceed with all the ADHs and perform the reaction with NCR-ERED as described in the previous

section for substrate **2a**. The obtained results (Scheme 4.5) showed that only ScADH allowed the chemoselective reduction of the unsaturated ketone **2a** to the saturated ketone **2b**. Interestingly, with the other ADHs no mixture of alcohols was observed despite concerns derived from previous experiments. Only a minimal amount of saturated ketone (less than 5%) was detected. Additionally, the resulting alcohol contained a chiral centre. Chiral analysis revealed an enantiomeric excess (*ee*) towards the (*S*)-enantiomer of 99% with CbADH, EhADH and TeSADH and 88% with TeSADH-WIC. With these results it was possible to show that the choice of the ADH enables the selective production of saturated ketones or alcohols as desired.

To confirm that lack of reactivity of ScADH with **2a** or **2b** was not due to its inability to accept 2-propanol as a co-substrate, the reactions with different alcohols such as ethanol, 1-propanol and 1-butanol were also tested (Figure 4.4). The results were comparable to those previously observed with 2-propanol, except for 1-butanol, which led to moderate product formation (3.1 mM), corresponding to 62% conversion. These findings provide evidence that ScADH does not accept **2a** or **2b** as substrates and highlight the importance of using appropriate alcohols for this reaction.





Scheme 4.5. Bioreduction of *trans*-4-phenyl-3-buten-2-one (**2a**) using NCR-ERED and different ADHs for cofactor recycling. For all the reactions: substrate **2a** (5 mM), NCR-ERED (10 mg, CFE), NAD(P)H (1 mM) and 2-propanol (10% v/v) in 100 mM sodium phosphate, pH 8.0, 30 °C, 16 h, 800 rpm. ADHs amount - CbADH, EhADH, TeSADH and TeSADH-WIC: 10 mg, CFE; ScADH: 0.1 mg = 30 U, pure enzyme. Conversions were determined by GC analysis. Reactions were performed in duplicate and standard deviations were below 10%.

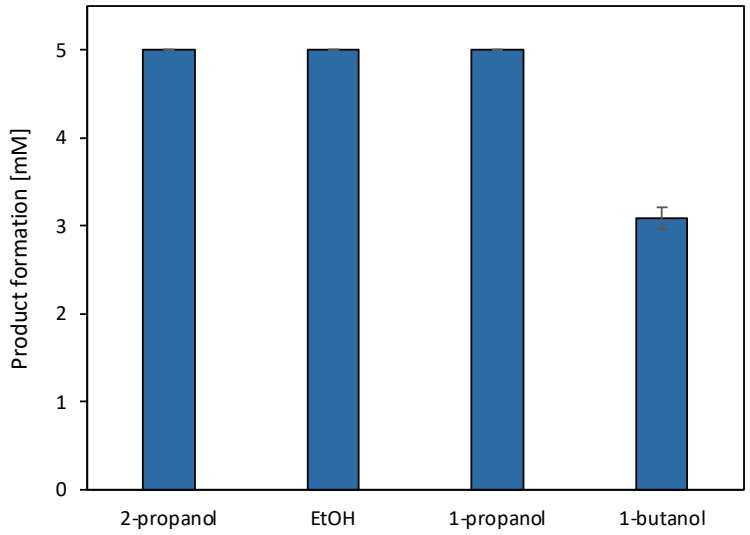
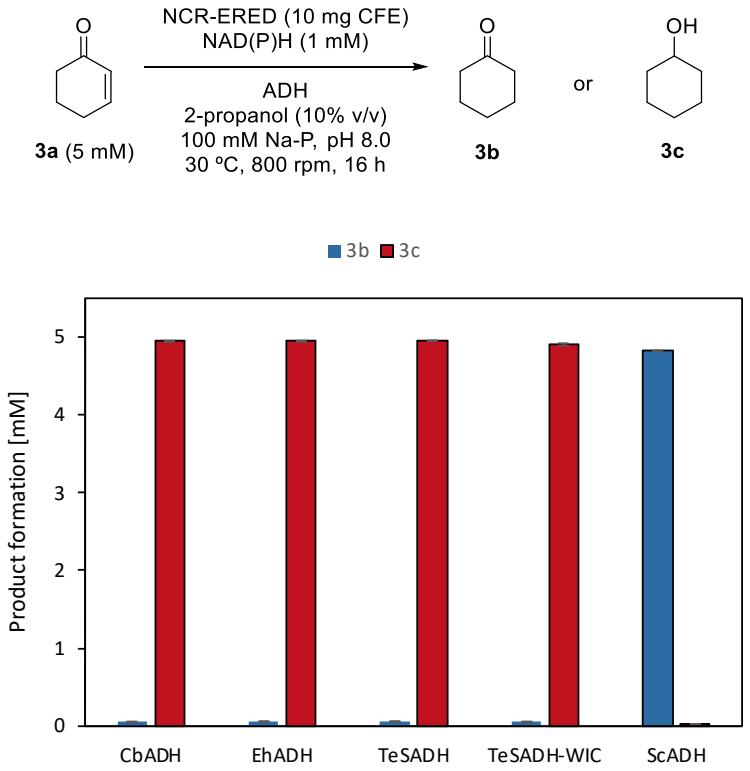


Figure 4.4. Bioreduction of *trans*-4-phenyl-3-buten-2-one (**2a**) using NCR-ERED and ScADH using different alcohols for cofactor recycling. For all the reactions: substrate **2a** (5 mM), NCR-ERED (10 mg, CFE), ScADH (0.1 mg, pure enzyme), NADH (1 mM) and alcohol (10% v/v) in 100 mM sodium phosphate, pH 8.0, 30 °C, 16 h, 800 rpm. Conversions were determined by GC analysis. Reactions were performed in duplicate and standard deviations were below 10%.

*4.2.2.3. Cyclohex-2-en-1-one (**3a**)*

To further investigate the selectivity of the different ADHs for saturated ketones and alcohols, another unsaturated ketone, cyclohex-2-en-1-one (**3a**), was tested under the same conditions as previously used for substrates **1a** and **2a**. The findings were consistent with those obtained with **2a**, with ScADH demonstrating selectivity for the saturated ketone, and the other ADHs exhibiting good selectivity for the saturated alcohol (Scheme 4.6). As **3a** is a simpler substrate for analysis compared to **2a**, it was chosen as the model substrate for further experiments.



Scheme 4.6. Bioreduction of cyclohex-2-en-1-one (**3a**) using NCR-ERED and different ADHs for cofactor recycling. For all the reactions: substrate (5 mM), NCR-ERED (10 mg, CFE), NAD(P)H (1 mM) and 2-propanol (10% v/v) in 100 mM sodium phosphate, pH 8.0, 30 °C, 16 h, 800 rpm. ADHs amount - CbADH, EhADH, TeSADH and TeSADH-WIC: 10 mg, CFE; ScADH: 0.1 mg = 30 U, pure enzyme. Conversions were determined by GC analysis. Reactions were performed in duplicate and standard deviations were below 10%.

4.2.3. Enzyme immobilisation

To ensure highest activity retention in organic solvent, the enzymes were immobilised on porous glass supports from EnginZyme. A support screening for the NCR-ERED and the different ADHs was performed, with the selection of supports based on immobilisation yield rather than recovered activity.

The enzyme immobilisations were performed by incubating the corresponding enzyme solution containing the desired amount of enzyme (specified in the experimental part) with three different EziG carriers: Opal, Coral and Amber. The incubation was run for 2 h at room temperature, and after this time the immobilisation yield was calculated by comparing the decrease in the rate of NAD(P)H formation between the starting enzyme solution and the supernatant from immobilisation (see the experimental section for specifications). The results presented in Table 4.1 indicate that support Coral characterised with the highest immobilisation yields for most of the tested enzymes. Therefore, this support was selected for further experiments with all enzymes.

Table 4.1. Immobilisation yields [%] of NCR-ERED and the different ADHs on EziG Amber, Coral and Opal. The enzyme solutions were prepared as follows: for NCR-ERED, CbADH, EhADH, TeSADH and TeSADH-WIC immobilisation using CFE, 20 mg/mL; for ScADH immobilisation using pure enzyme 0.1 mg/mL. The enzyme solutions were prepared with 20 mM potassium phosphate buffer pH 7.0. For the immobilisation 0.5 mL from the enzyme solution were incubated with 10 mg of support on an end-over-end rotator, 20 rpm, at room temperature. 50 µL of the enzyme supernatant was withdrawn after 2 h for kinetic analysis and diluted for the spectrophotometric assay. All the measurements were performed in duplicates and the results are presented as the mean of the individual samples.

Enzyme	Amber	Coral	Opal
NCR-ERED	21 ± 1	23 ± 1	16 ± 1
CbADH	87 ± 4	97 ± 1	66 ± 7
EhADH	61 ± 2	79 ± 4	80 ± 1
TeSADH	41 ± 6	48 ± 4	41 ± 5
TeSADH-WIC	17 ± 3	23 ± 5	21 ± 4
ScADH	99 ± 1	99 ± 1	99 ± 1

4.2.4. Exploring the organic solvent tolerance of ADHs

To streamline the experimental process, the focus was placed on using one or two ADHs that exhibit high tolerance to organic solvents. To determine which ADHs are suitable for this task, it is essential to test their tolerance levels towards organic solvents. The study to select the best candidate was divided into two phases. In the first one, after immobilising

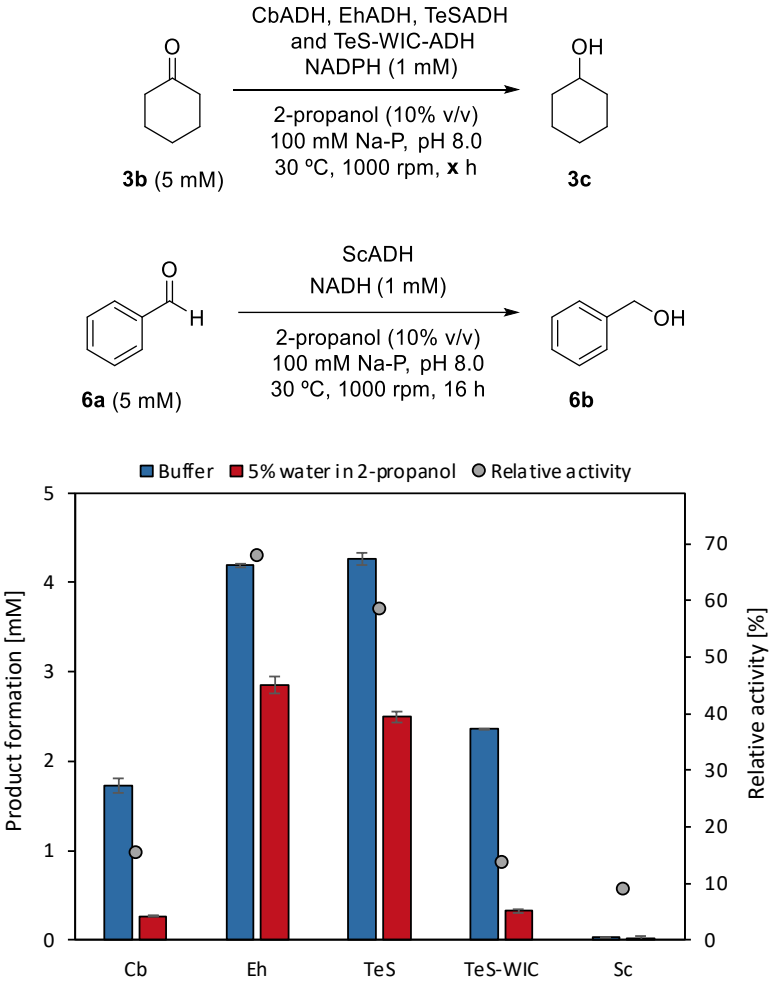
the enzymes, a wash with 2-propanol solution containing 5% v/v water was done before running the reaction in buffer. Then, in the second one the best-performing candidates from step 1 were tested by running the reductions in 2-propanol containing varying amounts of water. This will identify the most tolerant ADHs to organic solvents and determine the optimal conditions for the reaction with NCR-ERED.

4.2.4.1. Phase 1: washing with 2-propanol containing 5% v/v water and aqueous reaction in buffer

All enzymes were immobilised on Coral using previously established procedure. After removing supernatant, the supported enzymes were washed twice with immobilisation buffer (20 mM potassium phosphate, pH 7.0). Subsequently, all the resulting catalysts were subjected to a 5% v/v water wash in 2-propanol before running the reactions in buffer. The activity was compared to that of the immobilised enzymes that were washed only with buffer (not exposed to organic solvent).

The substrate used in the target reaction for enzymes CbADH, EhADH, TeSADH and TeSADH-WIC was 2-cyclohexanone (**3b**), while for ScADH was benzaldehyde (**6b**, Scheme 4.7). To enable a comparison of the activity between catalysts exposed to organic solvent and those that were not, the reactions were not carried out until completion. Therefore, the reaction times varied depending on the enzyme:

- 1 h for CbADH
- 15 min for EhADH and TeSADH
- 16 h for TeSADH-WIC and ScADH.



Scheme 4.7. Bioreduction of cyclohexanone or benzaldehyde using different immobilised ADHs on Coral after washing with buffer or 5% v/v water in 2-propanol. For all the reactions: substrate (5 mM), immobilised ADH (10 mg), NAD(P)H (1 mM) and 2-propanol (10% v/v) in 100 mM sodium phosphate, pH 8.0, 30 °C, 0.25-16 h, 1000 rpm. Conversions were determined by GC analysis. Reactions were performed in duplicate and standard deviations were below 10%.

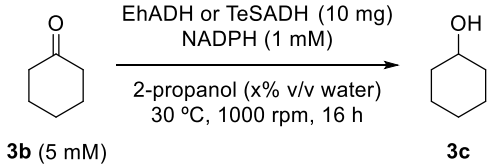
All enzymes, except ScADH, retained some activity even after exposure to organic solvents (Scheme 4.7). However, in this case no activity was observed with the immobilised enzyme that was washed with buffer. Such lack of activity could be attributed to the combination of the following reasons: The substrate used was unsuitable for measuring activity (with free enzyme under the same conditions only 10% conversion was obtained), and/or the enzyme became inactive upon immobilisation. Since no alternative substrate was identified for ScADH, the studies involving this enzyme were suspended. Among the other ADHs, enzymes EhADH and TeSADH exhibited the highest retention of activity after exposure to organic solvents, showing 68% and 59% activity compared to the non-exposed counterparts. Therefore, these two enzymes were selected as the best candidates for the next phase of the study.

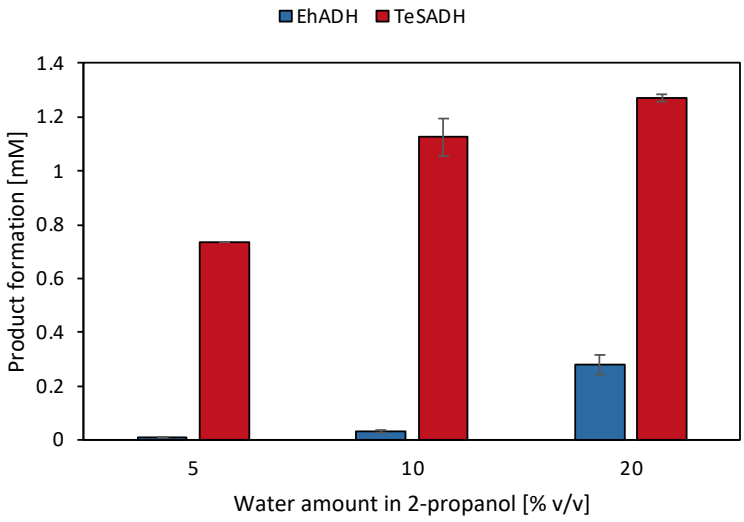
4.2.4.2. Phase 2: reaction with 2-propanol containing different amounts of water

The top-performing candidates from phase 1, EhADH and TeSADH, were subjected to further testing with 2-propanol. The reaction conditions were identical to those described previously, but the reaction time was increased to 16 h and the solvent was 2-propanol with varying water content (5-20% v/v). Additionally, solutions of 2-propanol containing buffer instead of water were tested, but the results obtained were identical and hence not presented here. The procedure was the same as explained in the previous section 4.2.4.1 when after immobilisation the catalysts were washed with the immobilisation buffer and after that with the 2-propanol solution that was used for the reactions.

In all the 2-propanol solutions tested, both enzymes showed low activity. However, the activity of both enzymes increased with higher amounts of water (Scheme 4.8.). In particular, TeSADH was able to convert around 25% of substrate to the target product when the reaction was run in

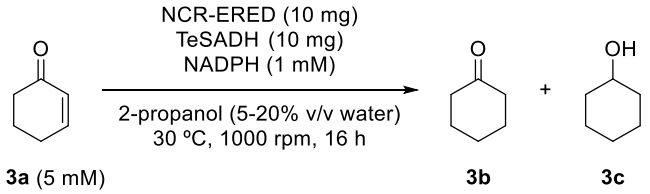
2-propanol with 20% v/v water. To optimize the reaction conditions, TeSADH was further tested under the best conditions found in the previous experiments (20% v/v water in 2-propanol). The reactions were run with double the amount of substrate (10 mM) while keeping the same amount of enzyme (10 mg support), and with the same amount of substrate (5 mM) and double the amount of enzyme (20 mg support). It was observed that when the amount of enzyme was increased two-fold, the conversion also increased two-fold. However, when double substrate concentration was used with the same amount of enzyme, the conversion remained the same (ca. 25%). This suggests that increasing the substrate concentration is more effective for improving the productivity of the reaction than increasing the enzyme concentration, since the amount of product formed increased by two-fold in the first instance. Based on these results, the substrate concentration was increased to 10 mM for further experiments.

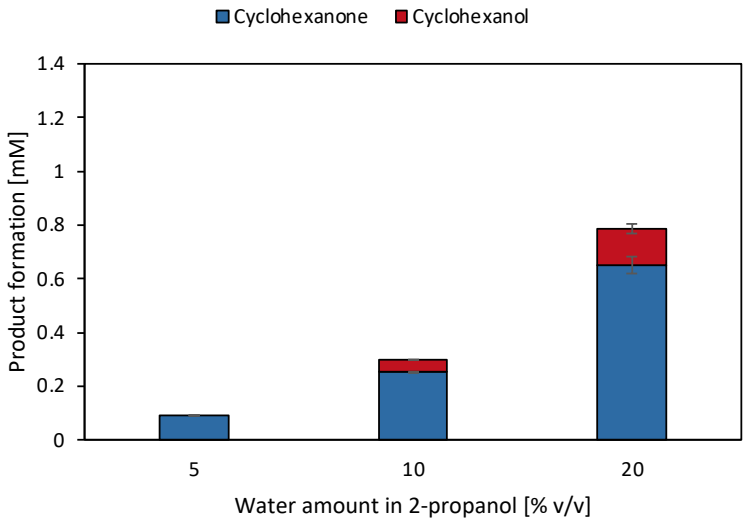




Scheme 4.8. Bioreduction of cyclohexanone (**3b**) using EhADH or TeSADH immobilised on Coral in 2-propanol with varying amounts of water. For all the reactions: substrate (5 mM), immobilised ADH (10 mg), NADPH (1 mM), 30 °C, 16 h, 1000 rpm. Conversions were determined by GC analysis. Reactions were performed in duplicate and standard deviations were below 10%.

In parallel, following reaction condition optimisation for TeSADH, NCR-ERED was subjected to screening under the exact same conditions to assess its activity retention (Scheme 4.9). The reaction was carried out using 2-cyclohexen-1-one (**3a**) as substrate, and otherwise identical conditions as previously described for ADHs were maintained. Similar to the trend observed with the ADHs, enzyme activity was found to increase with higher water content in the organic solvent. Specifically, with 20% v/v water in 2-propanol, the substrate conversion was measured to 16%. The reaction yielded a mixture of saturated ketone (**3b**, 13%) and alcohol (**3c**, 3%), with the starting ketone being the predominant compund of the resulting mixture.





Scheme 4.9. Bioreduction of 2-cyclohexen-1-one (**3a**) using NCR-ERED and TeSADH immobilised on Coral in 2-propanol with varying amounts of water. For all the reactions: substrate (5 mM), immobilised enzymes (10 mg), NADPH (1 mM), 30 °C, 16 h, 1000 rpm. Conversions were determined by GC analysis against substrate and product standards. Reactions were performed in duplicate and standard deviations were below 10%.

While the observed activity levels do not indicate high catalyst performance, the results are promising in the sense that both enzymes appear to retain some activity in the organic solvent. Further optimization of the reaction conditions could potentially lead to improved activity and yield.

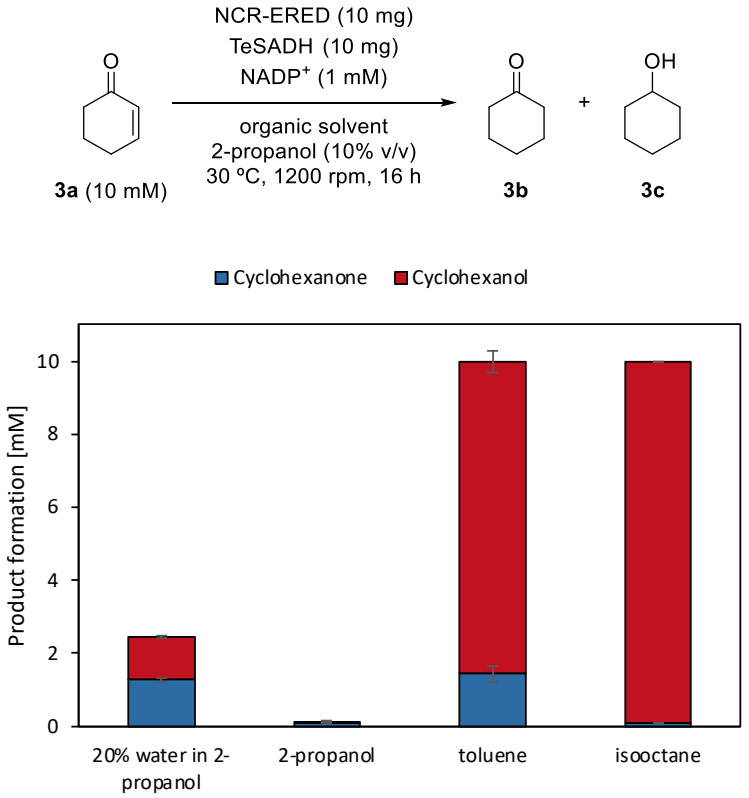
4.2.5. Study of the reaction in organic solvents with different hydrophobicities (logP)

The ERED-ADH cascade was studied in various organic solvents with increasing logP values: 2-propanol, toluene, and isooctane. For the reaction

in 2-propanol, water was not externally added to the system, while for reactions in toluene and isooctane, the solvent was water saturated. Positive control reactions in 2-propanol containing 20% v/v water were also conducted.

After immobilisation, all the prepared enzyme formulations were first rinsed with the immobilisation buffer (20 mM potassium phosphate, pH 7.0), followed by a wash with the solvent used for the reactions. Additionally, the cofactor $NADP^+$ was introduced into the reaction mixture externally, from a concentrated buffer stock solution, which resulted in 1% v/v buffer in organic solvent.

The results depicted in Scheme 4.10 indicate a clear trend where higher solvent hydrophobicity (higher logP) corresponds to higher activity. Negligible conversions were found with 2-propanol, while nearly full conversion to the target cyclohexanol (**3c**) were achieved in toluene and isooctane. The results were slightly better in isooctane compared to toluene because almost no saturated ketone intermediate **3b** was detected in this case. However, for further experiments toluene was selected as solvent due to higher compared to isooctane water solubility (0.033 and 0.006% v/v, respectively).



Scheme 4.10. Bioreduction of 2-cyclohexen-1-one (**3a**) using NCR-ERED and TeSADH immobilised on Coral in an organic solvent with varying logP. For all the reactions: substrate (10 mM), immobilised enzymes (10 mg), $NADP^+$ (1 mM) and 2-propanol (10% v/v), 30 °C, 16 h, 1200 rpm. Conversions were determined by GC analysis against substrate and product standards. Reactions were performed in duplicate and standard deviations were below 10%.

The application of the catalyst in its wet form led to the formation of a second liquid phasic in the system (Figure 4.5) when mixed the organic solvent (toluene and isooctane). This resulted in an uneven catalyst distribution in the reaction mixture which can lead to potential issues when scaling up the process or hinder its implementation in a flow setup. Therefore, to address this challenge, subsequent investigations focused on

exploring methods to reduce the excess water content in the heterogeneous catalyst.

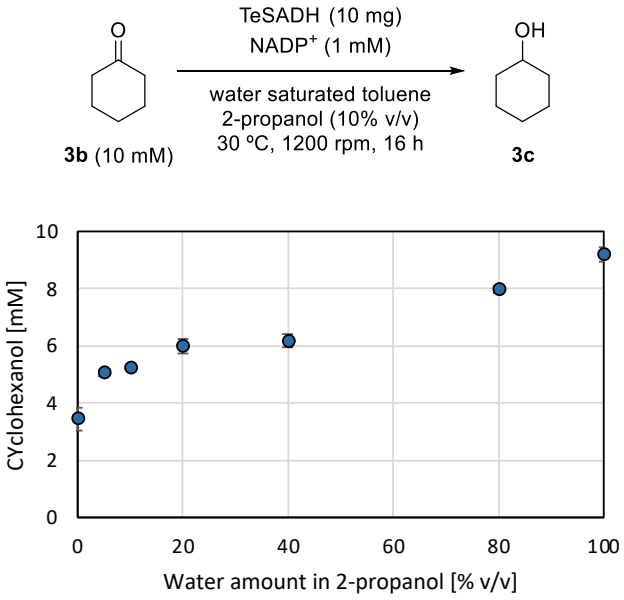
Figure 4.5. Second liquid phasic in the reaction mixture with catalyst.

4.2.6. TeSADH water profile – washing with 2-propanol solutions containing varying amounts of water

To partially remove water from the immobilised formulation, a previously established methodology, used for transaminases supported on EziG, was explored. Notably, this method has also been proven effective in organic solvents. This approach consisted of washing the immobilised enzymes with 2-propanol solutions containing a fixed amount of water to partially remove its excess from the immobilised biocatalyst formulation.

To find the optimal water amount that maximises activity retention and promotes uniform distribution of the catalyst in the reaction mixture, the immobilised TeSADH was subjected to several washes with 2-propanol containing varying water amounts (0-100% v/v). The obtained results, presented in Scheme 4.11., are in accordance with those previously obtained for the transaminases. As anticipated, there is a direct correlation between the water content in 2-propanol and the activity observed in organic solvent. An increase in conversion to cyclohexanol from 34 to 91% was observed

when the water amount in 2-propanol was increased from 0 to 100% (Scheme 4.11). The highest water content in 2-propanol was limited to 10% v/v to ensure homogeneous distribution of catalyst in the reaction mixture. Subsequently, for further optimization of the complete cascade involving NCR-ERED and TeSADH, only 5 and 10% v/v water in 2-propanol were tested. Unfortunately, the low water amount in 2-propanol led to a 50% reduction of the enzyme activity. For this reason, different post-immobilisation treatments were evaluated, aiming to retain highest activity during the water removal step.



Scheme 4.11. Water profile obtained for TeSADH immobilised on EziG-Coral and washed with 2-propanol solutions containing varying amounts of water (0-100% v/v). For all the reactions: cyclohexanone (10 mM) as substrate, immobilised TeSADH-Coral (10 mg), $NADP^+$ (1 mM), 2-propanol (10% v/v) for 16 h, at 30 °C and 1200 rpm. Product formation (cyclohexanol, **3c**) was determined by GC analysis. Reactions were performed in duplicate and standard deviations were below 10%.

4.2.7. Post-immobilisation treatments (PITs)

To maximise the activity retention after water removal through the 2-propanol wash, different PITs were examined to evaluate their effectiveness (Table 4.2.). Each PIT consisted of two washes using different compounds dissolved in the immobilisation buffer (20 mM KPi, pH 7.0). The selected compounds included the cofactor ($NADP^+$) as well as two different sugars (sucrose and maltodextrin). The concentration used for each compound was chosen based on considerations of solubility and cost-contribution.

The complete post-immobilisation workflow involved a sequence of four steps, including two washes with aqueous solutions (the PITs), followed by a wash with 2-propanol (containing either 5 or 10% v/v water), and finally, a wash with the solvent used in the reaction. This process is time-consuming and laborious, especially when different conditions are being evaluated and the number of samples is high. To address this challenge, the use of a liquid handling robot (Opentrons) was implemented to automate the washes. This automation not only streamlines the workflow, but also significantly reduces manual work, enabling greater efficiency and productivity.

To study the influence of the PITs on the enzymes independently, the impact of different PITs on TeSADH was initially evaluated. Following the identification of the most favourable conditions for this enzyme, the PITs were then examined for NCR-ERED.

Table 4.2. Description of the six different post immobilisation treatments (PITs) examined. Each PIT consisted of two washes, which could be of the same or different composition. All solutions were prepared in the immobilisation buffer (20 mM KPi, pH 7.0), and the compound concentrations are indicated in mM or % w/w.

	Wash 1	**Wash 2**		**Wash 1**	**Wash 2**
1	KPi buffer	KPi buffer	**4**	2% maltodextrin	2% maltodextrin
2	2 mM $NADP^+$	2 mM $NADP^+$	**5**	2 mM $NADP^+$	20% sucrose
3	20% sucrose	20% sucrose	**6**	2 mM $NADP^+$	2% maltodextrin

4.2.7.1. PITs study for TeSADH

To evaluate the influence of PITs on TeSADH behaviour, the enzyme was immobilised as previously on Coral and PITs were immediately applied after immobilisation. In the next step, the immobilised formulations were washed with 5 or 10% v/v water in 2-propanol to partially remove water from the catalyst. Subsequently, the preparations were further washed with water-saturated toluene to equilibrate the catalyst environment with the reaction mixture. The activity assessment consisted of running a reaction with cyclohexanone (**3b**, 10 mM) in water-saturated toluene.

The results, presented in Figure 4.6, clearly indicate that only 20% w/w sucrose wash significantly impacted the catalyst performance with more than a 2-fold improvement in activity. In contrast, the performance of the catalyst treated with other methods showed minimal deviation from the control samples, which were only washed with buffer. In view of these results, PIT number 3, which consisted of two consecutive washes with 20% w/w sucrose, was selected as the optimal treatment for TeSADH.

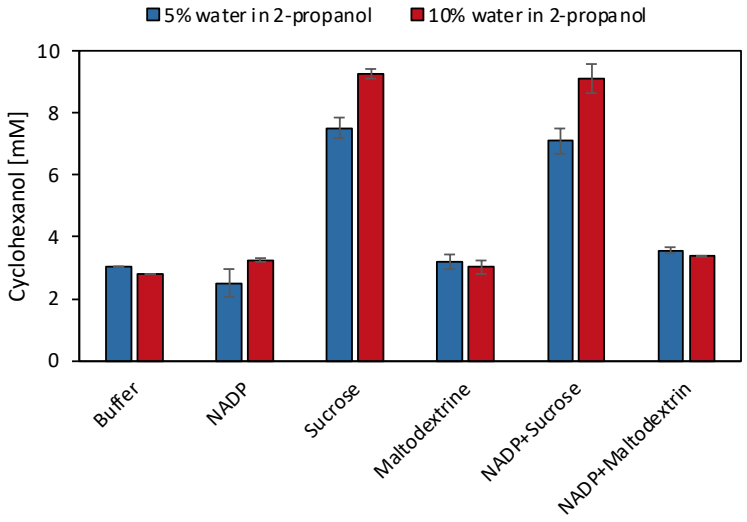


Figure 4.6. PITs influence on TeSADH performance in organic solvent. After PITs, the catalyst was washed with 5 or 10% v/v water in 2-propanol to partially remove the water from the immobilised preparation. Reaction conditions: Cyclohexanone (**3b**, 10 mM), immobilised TeSADH-Coral (10 mg), $NADP^+$ (1 mM), 2-propanol (10% v/v) for 16 h at 30 °C and 1200 rpm. Product formation (cyclohexanol, **3c**) was determined by GC analysis against substrate and product standards. Reactions were performed in duplicate and standard deviations were below 10%.

4.2.7.2. PITs study for NCR-ERED

Following the identification of the optimal treatment for TeSADH, a similar study was conducted for immobilised NCR-ERED. In this case, the reaction cascade was used as activity test to assess the catalyst performance. The evaluation of the best PIT for NCR-ERED involved the measurement of total product formation, comprising of cyclohexanone from the ERED reaction and cyclohexanol from the ADH reaction.

Both enzymes were immobilised on Coral. For TeSADH, the chosen PIT involved two washes with 20% w/w sucrose. In the case of NCR-ERED, the previously evaluated PITs with TeSADH were applied. After PITs, the immobilised catalysts (both ERED and ADH) were transferred to the same Eppendorf tube and subjected to two additional washes. First, the catalysts were washed with 2-propanol solutions containing either 5 or 10% v/v water, using the same concentration for both enzymes. Second, a wash with water-saturated toluene was performed.

The results shown in Figure 4.6 were in accordance with previous observations for TeSADH, highlighting that only the treatment with 20% w/w sucrose significantly impacts the catalyst performance. Furthermore, there were minimal differences between washing with 5 or 10% v/v water in 2-propanol, although slightly higher activities were observed after washing with the 5% solution. Taking these results into consideration, for further experiments, the chosen PIT for both enzymes involved two consecutive washes with 20% w/w sucrose, followed by one wash with 5% v/v water in 2-propanol, and one last wash using a water-saturated organic solvent (the one used in the reaction mixture).

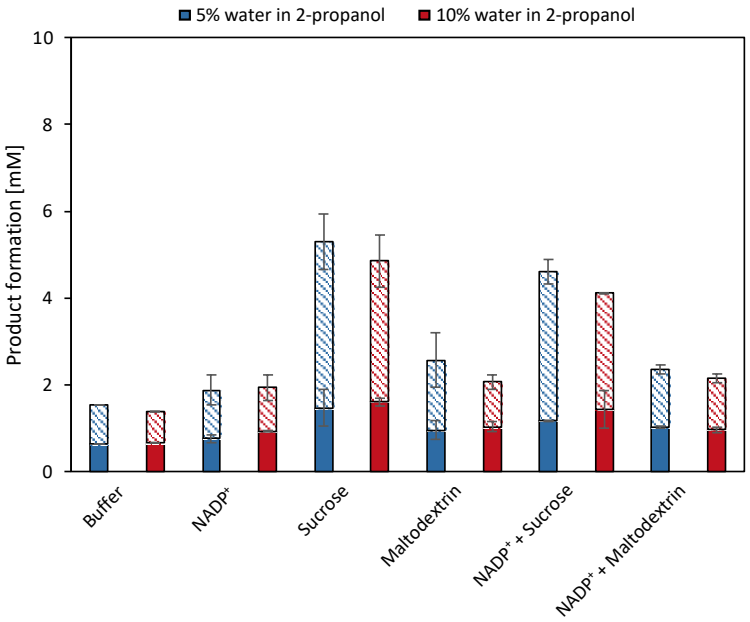


Figure 4.6. PITs influence on NCR-ERED performance in organic solvent. After PITs, the catalysts (both NCR-ERED and TeSADH) were washed with 5 or 10% v/v water in 2-propanol to partially remove the water from the immobilised preparation. Full bars represent the amount of cyclohexanone (**3b**) while dashed bars represent amount of cyclohexanol (**3c**). Reaction conditions: 2-cyclohexen-1-one (**3a**, 10 mM), immobilised TeSADH-and NCR-ERED-Coral (10 mg), $NADP^+$ (1 mM), 2-propanol (10% v/v) for 16 h at 30 °C and 1200 rpm. Product formations (cyclohexanone and cyclohexanol) were determined by GC analysis. Reactions were performed in duplicate and standard deviations were below 10%.

4.2.8. $NADP^+$ co-immobilisation: comparison of enzyme immobilisation on separate supports and co-immobilisation on the same support particle.

At this stage, a methodology was developed to improve the operation of ERED and ADH in organic solvents. However, the obtained enzyme activities were moderate (around 50% conversion), and the addition of $NADP^+$ externally from a concentrated buffer solution resulted in aggregate formation within the reaction mixture (prepared in toluene). This was caused by particle agglomeration induced by the addition of aqueous buffer (final concentration 1% v/v, Figure 4.7.).

Figure 4.7. Aggregate formation of the catalyst within the reaction mixture (in toluene).

To overcome this challenge, the immobilisation of $NADP^+$ on the support was explored as an alternative approach. This method aimed to eliminate the need for external $NADP^+$ supplementation while potentially enhancing enzyme activity by inducing close proximity between the enzymes and the cofactor. To test this hypothesis, two different approaches were evaluated, first the cofactor was immobilised individually with the two enzymes immobilised on separate beads. Second, the cofactor was immobilised together with both enzymes on the same bead.

For the immobilisation of the cofactor ($NADP^+$), three different approaches were evaluated and compared to the experiment where $NADP^+$ was added externally from a concentrated buffer solution:

- The first approach involved immobilisation of the enzymes as previously described, followed by incubation with 10 mM $NADP^+$ for 1 h.

- In the second approach, the enzymes were incubated with 10 mM $NADP^+$ for 1 h, and the resulting mixture was then immobilised.

- The third approach consisted of the incubation of 10 mM $NADP^+$ with the support for 1 h, followed by the immobilisation of the enzymes (for more details about immobilisation protocol and results see experimental part).

The amount of $NADP^+$ in the reaction for the latter three cases was determined by the difference between the added $NADP^+$ (10 mM) and the concentration measured in the supernatants after immobilisation. $NADP^+$ concentration was determined by measuring the absorbance at 340 nm. The amount of $NADP^+$ in the reaction mixture following the first, second and third approach was 1.5, 1.2 and 1.9 mM, respectively.

After immobilisation the catalysts were subjected to PIT with 20% w/w sucrose, followed by the washes with 5% v/v water in 2-propanol and water saturated toluene (0.033% v/v water).

The results, presented in Figure 4.8., illustrate the outcomes of these different approaches:

- "Immob. 1" represents samples where $NADP^+$ was supplemented externally
- "Immob. 2" corresponds to immobilised enzymes incubated with $NADP^+$
- "Immob. 3" represents samples where the enzymes were preincubated with $NADP^+$ before immobilisation
- "Immob. 4" denotes samples where $NADP^+$ was preincubated with the support prior to enzyme immobilisation

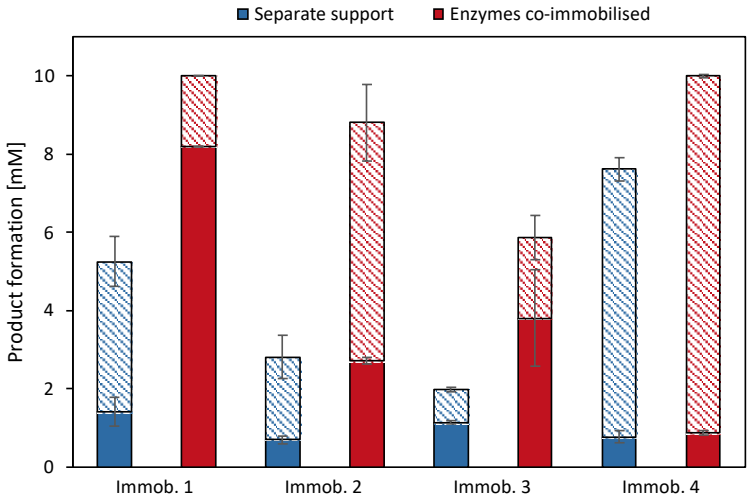


Figure 4.8. Influence of the immobilisation approach on the ERED-ADH cascade performance in organic solvent. For more details about each immobilisation procedure see experimental part. Full bars represent the amount of cyclohexanone (**3b**) while dashed bars represent amount of cyclohexanol (**3c**). For all the reactions: 2-cyclohexen-1-one (**3a**, 10 mM), immobilised enzymes (10 mg each, separately or co-immobilised), $NADP^+$ (1-2 mM), 2-propanol (10% v/v) for 16 h at 30 °C and 1200 rpm. Product formation (cyclohexanone and cyclohexanol) was determined by GC analysis against substrate and product standards. Reactions were performed in duplicate.

The findings revealed that for all the tested conditions higher activities were obtained when both enzymes were co-immobilised on the same support (Coral). A closer examination of the immobilisation approach highlighted a significant enhancement in the catalyst performance when the cofactor was pre-incubated with the support prior to enzyme immobilisation compared to the other methods. Remarkably, this optimised approach resulted in almost complete conversion to the target alcohol after overnight reaction. This development represents the achievement of a self-sufficient redox catalyst with concurrent ERED and ADH activities, along with cofactor regeneration, and high operation performance within a monophasic organic solvent system (water saturated toluene).

The support selection was performed in earlier stages of the research, primarily based on the immobilisation yields obtained for NCR-ERED and TeSADH. However, recognising the significant performance enhancement resulting from cofactor co-immobilisation, two additional support materials, Amber and Opal, were evaluated at this stage. The three different immobilisation approaches were applied for co-immobilisation of both enzymes.

The results showed that when Opal was used, the obtained conversion was notably low (Figure 4.9). Conversely, the results with Amber and Coral were comparable, with slightly higher conversion when Coral was used. For this reason, Coral was kept as support of choice for both enzymes.

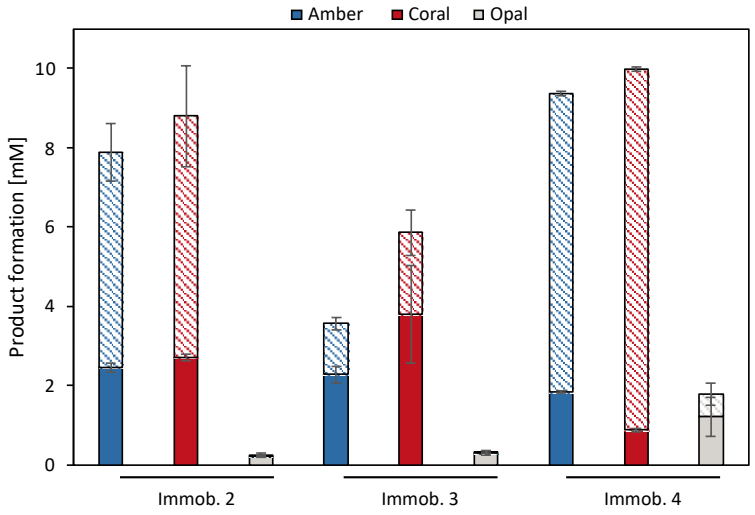


Figure 4.9. Support influence on the ERED-ADH cascade performance when $NADP^+$ was co-immobilised with both enzymes. For more details about each

immobilisation procedure, see experimental part. Full bars represent the amount of cyclohexanone (**3b**) while dashed bars represent the amount of cyclohexanol (**3c**).formed. Reaction conditions: 2-cyclohexen-1-one (**3a**, 10 mM), immobilised enzymes (10 mg each), $NADP^+$ (1-2 mM), 2-propanol (10% v/v) for 16 h at 30 °C and 1200 rpm. Product formation (cyclohexanone and cyclohexanol) was determined by GC analysis. Reactions were performed in duplicate.

4.2.9. Catalyst recyclability

To assess catalyst reusability in batch reactions and gain insights into its potential stability in a packed bed reactor, recyclability studies were conducted. The aim was to determine if the catalyst could be reused without the need for additional cofactor supplementation. However, due to the non-selective cofactor attachment to the support (based on ionic interactions), it was highly likely that the cofactor leached into the reaction mixture and became unavailable for subsequent reaction cycles.

Having this in mind, different recycling approaches were explored with respect to cofactor supplementation. The first approach involved the addition of the cofactor from a concentrated buffer solution, resulting in a $NADP^+$ concentration of 1 mM in the reaction mixture (and 1% v/v buffer in the reaction mixture). In the second approach, an attempt was made to maintain the same concentration of 1 mM using the cofactor from a concentrated solution prepared in 2-propanol. However, upon addition of the stock solution to the reaction mixture precipitation was observed. For the third approach, $NADP^+$ was not added externally, relying solely on the immobilised form within the catalyst.

The catalyst used for the recycling experiments consisted of both ERED and ADH co-immobilised with $NADP^+$ on the same bead of Coral support. In the first cycle, $NADP^+$ was exclusively added within the immobilised form, without any external supplementation.

The results of the recyclability study are presented in Figure 4.10, revealing important insights into the availability and utilisation of the cofactor throughout the recycling cycles. Notably, it was observed that catalyst reuse could be achieved only when the cofactor was added from a buffer solution after a single reaction cycle.

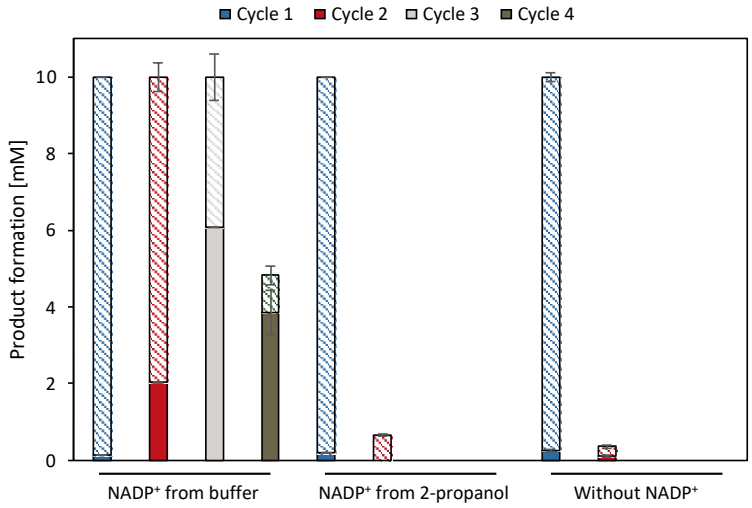


Figure 4.10. Recyclability studies of co-immobilised NCR-ERED and TeSADH on Coral. For cofactor recycling, different approaches were followed: addition from a concentrated buffer solution, addition in organic solvent and without external supplementation. Full bars represent the amount of cyclohexanone (**3b**) while dashed bars represent the amount of cyclohexanol (**3c**).formed. Reaction conditions: 2-cyclohexen-1-one (**3a**, 10 mM), co-immobilised enzymes (20 mg), $NADP^+$ (1-2 mM), 2-propanol (10% v/v) for 16 h at 30 °C and 1200 rpm. Product formation (cyclohexanone and cyclohexanol) was determined by GC analysis. Reactions were performed in duplicate.

Analysing each approach for cofactor supplementation independently provided different outcomes. Firstly, the results obtained when $NADP^+$ was dissolved in an organic solvent (2-propanol) and used for recycling, had a similar trend to the recycles done without cofactor supplementation. This suggests that the cofactor availability in these cases might not be enough to

sustain the desired reaction. Secondly, in the cycles where $NADP^+$ was added from a buffer solution, the cofactor availability was sufficient for catalytic activity, as evidenced by the formation of both cyclohexanone from the ERED reaction and cyclohexanol from the ADH reaction, up to the fourth cycle. Remarkably, full conversion of 2-cyclohexen-1-one was still achieved up to the last cycle, indicating that the ERED activity was maintained, while the ADH activity exhibited a gradual decline, as depicted in Figure 4.10.

To assess the availability of the cofactor for enzymatic activity when added directly to the organic solvent, freshly co-immobilised enzymes (without cofactor) were evaluated under the same experimental conditions as before. Two scenarios were examined: the addition of the cofactor directly dissolved in the organic solvent and the absence of cofactor supplementation (Figure 4.11). In both cases, the enzyme activity was lower compared to when $NADP^+$ was co-immobilised with the enzymes in the first place. However, product formation was still observed in all cases. Specifically, when $NADP^+$ was directly added to the organic solvent, no residual substrate was detected (indicating high ERED activity), and nearly 60% cyclohexanol formation was achieved. Conversely, without the addition of $NADP^+$ (relying solely on the cofactor present in the CFE), over 50% of the substrate remained unreacted, and the yield of cyclohexanol was less than 20%. These results emphasize that the availability of the cofactor was reduced when added directly to the organic solvent. Nonetheless, it is important to note that this factor alone does not fully account for the lack of recyclability observed under this condition.

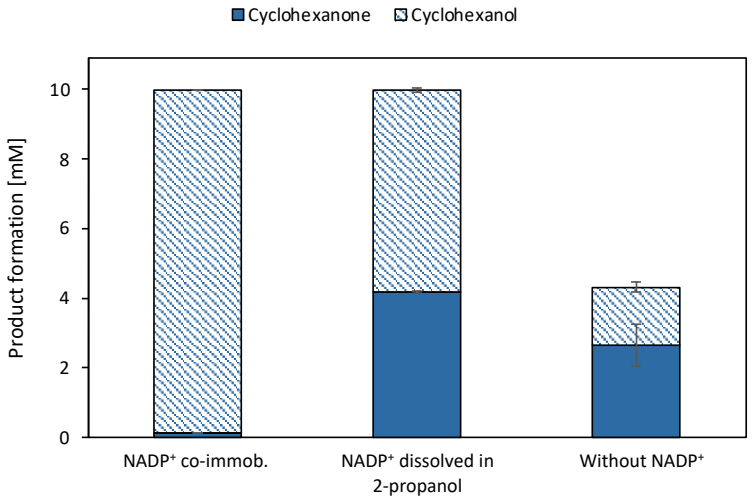


Figure 4.11. Activity of co-immobilised NCR-ERED and TeSADH on Coral with different $NADP^+$ supplementation approaches: co-immobilised on the support with both enzymes, added in the organic solvent and without external supplementation (only $NADP^+$ from CFE). Reaction conditions: 2-cyclohexen-1-one (**3a**, 10 mM) as substrate, co-immobilised enzymes (20 mg), $NADP^+$ (1-2 mM), 2-propanol (10% v/v) for 16 h at 30 °C and 1200 rpm. Product formation (cyclohexanone and cyclohexanol) was determined by GC analysis. Reactions were performed in duplicate.

Another contributing factor for the observed lack of recyclability might have been the deactivation of TeSADH, which was noticed during the recycling experiments done with $NADP^+$ added from a buffer solution. To address this issue, the ERED:ADH enzyme ratio was adjusted to 1:3 while keeping the total enzyme amount constant (see experimental section for detailed information). Subsequently, the recycling studies were repeated with $NADP^+$ supplementation from the buffer stock solution. Notably, an improvement in activity was observed following this adjustment. It became possible to achieve over 80% total conversion to the desired product, cyclohexanol, for at least three cycles (corresponding to two catalyst recycles). However, after four cycles (three recycles), both ERED and ADH exhibited more than 50% loss in activity as shown in Figure 4.12.

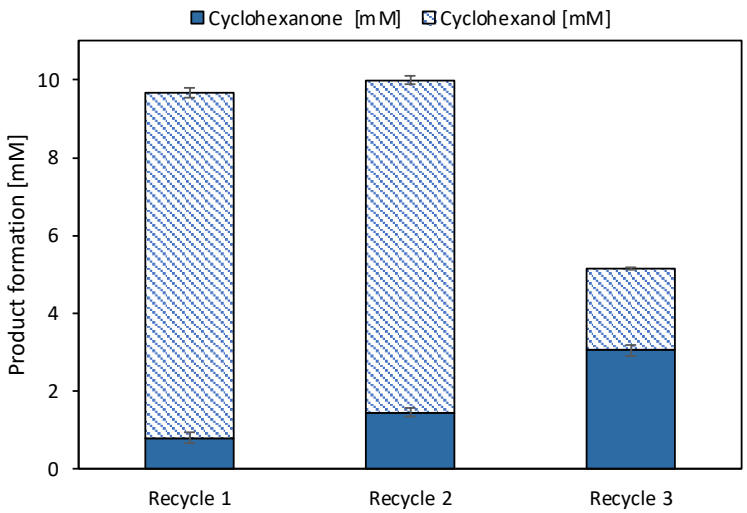


Figure 4.12. Recyclability studies of co-immobilised NCR-ERED and TeSADH on Coral using a 1:3 ratio. For cofactor recycling, $NADP^+$ was supplemented for each cycle (except the first one) from a 100 mM concentrated buffer solution. Reaction conditions: 2-cyclohexen-1-one (**3a**, 10 mM), immobilised enzymes (20 mg), $NADP^+$ (1-2 mM), 2-propanol (10% v/v) for 16 h at 30 °C and 1200 rpm. Product formations (cylohexanone and cyclohexanol) were determined by GC analysis. Reactions were performed in duplicate.

The methodology outlined here is well-suited for integration into a continuous flow system, with both enzymes co-immobilised and packed in the same reactor. To optimise this process, the cofactor can be conveniently supplied from a concentrated stock solution in buffer, while the substrate can be added from a solution in organic solvent. The incorporation of a liquid-liquid separation system facilitates the efficient separation of the organic phase, containing the product, from the aqueous phase, containing the cofactor, which could be recirculated and reused. In summary, the synergy between organic solvents and continuous flow reactors presents a promising solution to address the recurrent challenge of cofactor recycling.

Conclusions

Overall, the following acivements can be highlighted:

- After screening a series of enzymes and substrates under aqueous conditions, 2-cyclohexen-1-one was identified as a suitable alkene to develop a biocascade identifying NCR-ERED and TeSADH as suitable redox biocatalysts
- Both enzymes were successfully immobilised on three EziG supports for their application in organic solvent. Coral was found as the best support for both of them, and the heterogeneous catalysts were tested in organic solvents with different hydrophobicity (logP), showing higher activities with the more hydrophobic ones (toluene and isooctane).
- The water content in the immobilised formulations was studied, resulting in 5% v/v water in 2-propanol being the optimal solution to have an homogeneous distribution of the catalyst in the reaction mixture (toluene) while maintaining enzyme activities.
- The post-immobilisation treatment with 20% w/w sucrose in buffer enabled the highest activity enzyme retention after treatment with the 2-propanol solution.
- Both enzymes were co-immobilised on the same bead together with $NADP^+$ cofactor, leading to the production of a self-sufficient catalyst active in organic solvent.
- Unfortunately, recyclability studies with the self-sufficient catalyst showed that over 90% of the activity was lost after the first cycle.
- Adjusting the ERED:ADH ratio and supplementing $NADP^+$ from a buffer stock solution, it was possible to reuse the catalyst during two additional cycles. In this case, no ERED activity loss was detected, while ADH experienced less than 20% activity loss.

Experimental part

4.3.1. General information

4.3.1.1. Enzymes, solvents, and reagents

All chemicals were purchased from Sigma Aldrich unless stated otherwise. EziG supports were provided by EnginZyme AB. The plasmid containing NCR-ERED gene was generously provided by Prof. Helen Hailes (University College of London), while the plasmid containing TeSADH-WIC gene was kindly donated by Prof. Wolfgang Kroutil (Uni Graz). TeSADH was purchased from Prozomix and ScADH from Sigma-Aldrich. Both, CbADH and EhADH were internally produced at EnginZyme.

Table 4.3. Summary of the enzymes used, and their characteristics related to cofactor preference (NADPH or NADH) and source (indicated as CFE or pure form). Additionally, Prozomix and Sigma-Aldrich are specified as commercial sources for TeSADH and ScADH enzymes, respectively.

Abbreviation	Original organism	Enzyme form/source	Cofactor preference
NCR-ERED	*Zymomonas mobilis*	CFE / in-house	NAD(P)H
CbADH	*Clostridium beijerinckii*	CFE / in-house	NADPH
EhADH	*Entamoeba histolytica*	CFE / in-house	NADPH
TeSADH	*Thermoanaerobacter ethanolicus*	CFE / Prozomix	NADPH
TeSADH-WIC	variant from TeSADH	CFE / in-house	NADPH
ScADH	*Saccharomyces cerevisiae*	pure / Sigma-Aldrich	NADH

4.3.2. Enzyme expression and cell lysis

The NCR-ERED (*Zymomonas mobilis*), CbADH (*Clostridium beijerinckii*), EhADH (*Entamoeba histolytica*) and TeSADH-WIC (variant

from TeSADH) were overexpressed in *E. coli* BL21(DE3). For the expression, terrific broth autoinduction media (TB AIM 500 mL) with 100 µg/mL of kanamycin (NCR-ERED, CbADH and EhADH) or ampicillin (TeSADH-WIC) were inoculated with 50 µL of glycerol stock. The cultures were incubated at 200 rpm and 37 °C for 5 h and then at 200 rpm and 30 °C for 19 h.

After 24 h the OD was measured, and the cells were harvested by centrifugation at 8000 rpm for 10 min at 4 °C. The collected cell pellets were resuspended in MOPS (NCR-ERED, CbADH and EhADH) or Tris-HCl (TeSADH-WIC) buffer, and cell lysis of all the samples was performed using a Microfluidizer at 1200 bar over five cycles.

The lysed cells were centrifuged at 15000 rpm for 1 h at 4 °C. Then, freeze drying was performed using a freeze dryer and the freeze dried CFE was stored in the fridge (4 °C).

4.3.3. Determination of target enzyme content in CFE

To quantify the amount of target enzyme present in the CFE (for both NCR-ERED and TeSADH), a known quantity of the extract was subjected to purification, and the purified enzyme was quantified based on its absorbance at 280 nm. For NCR-ERED, 150 mg of CFE were purified, yielding 8.5 mL of purified enzyme solution with a concentration of 3.89 mg/mL. Based on this measurement, the target enzyme constituted 22% of the CFE. For TeSADH, 179.5 mg of CFE were purified, yielding 30 mL of purified enzyme solution with a concentration of 0.85 mg/mL. Based on this measurement, the target enzyme constituted 14% of the CFE.

4.3.4. Enzyme immobilisation

The general immobilisation protocol was as follows: the enzyme powder was rehydrated in 20 mM potassium phosphate buffer (pH 7.0 or pH 8.0) and resuspended on an end-over-end rotator for 15 min (20 rpm, rt)

and then centrifuged for 10 min (5000 rpm, rt). After centrifugation, the enzyme solutions were transferred to a new tube containing the support. Immobilisation was performed on an end-over-end rotator, 20 rpm, at rt for 2 h. For those enzymes handled as CFE, the concentration was 10 mg/mL or 20 mg/mL (freeze-dried powder/buffer solution) and for the ScADH the concentration was 30 U/mL which corresponds to 0.1 mg protein/mL. Then, the supernatants were removed, and the immobilised supports were washed with the corresponding solution (buffer, 20% (w/w) sucrose in buffer, 5% (v/v) water in 2-propanol) depending on the application.

Specifications about the amounts of each component are specified below for each immobilisation approach (Table 4.4).

Separate particle immobilisation: 0.5 mL from 20 mg/mL CFE solution incubated with 10 mg of support.

Enzyme co-immobilisation: 1 mL from CFE solutions incubated with 20 mg of support. For the 1:1 (ERED:ADH) ratio based on CFE amount, the CFE concentration was 10 mg/mL each. For the 1:3 (ERED:ADH) ratio based on CFE amount, the CFE concentration was 5 mg/mL ERED and 15 mg/mL ADH.

Table 4.4. Amount of CFE / target enzyme (mg) offered to the support (10 or 20 mg for separate and co-immobilisation, respectively).

	ERED	**ADH**
Separate	10 / 2.2	10 / 1.4
Co-immobilised 1:1 (ERED:ADH)	10 / 2.2	10 / 1.4
Co-immobilised 1:3 (ERED:ADH)	5 / 1.1	15 / 2.1

4.3.4.1. Immobilisation yield

The immobilised yield was calculated (**Equation 1**) by determining the percentage of enzymatic activity left in the supernatant after immobilisation relative to the enzymatic activity in the free enzyme solution. For all the enzymes a spectrophotometric assay was used, where the depletion or formation of NAD(P)H was measured by the absorbance (A_{340}) at 340 nm every 20 s for 5 min using a plate reader. Reaction rates with each supernatant from immobilisations and fresh enzyme solution were extracted from linear regression of the data points (A_{340}/min). Free enzyme solutions and supernatants from immobilisations were diluted with 20 mM potassium phosphate buffer, pH 7.0. To initiate the reactions, 100 µL of a specific reaction mixture was combined with 100 µL of the diluted enzyme solution, the composition of which varied depending on the enzyme, as indicated below.

NCR-ERED separate immobilisation: 5 mM cyclohex-2-en-1-one, 1 mM NADPH and 5% DMSO in 20 mM sodium phosphate buffer, pH 8.0.

NCR-ERED co-immobilisation with TeSADH: 0.5 mM *trans*-β-nitrostyrene, 0.1 mM NADPH and 5% (v/v) DMSO in 20 mM potassium phosphate buffer, pH 7.0.

CbADH, EhADH, TeSADH, TeSADH-WIC: 100 mM 2-propanol and 1 mM $NADP^+$ in 20 mM sodium phosphate buffer, pH 8.0.

ScADH: 100 mM ethanol and 2 mM NAD^+ in 20 mM sodium phosphate buffer, pH 8.0.

Table 4.5. Immobilisation results for the different immobilisation strategies indicating the corresponding amount of target proteins used in the reactions.

	Separate		**1:1 co-immobilised**		**1:3 co-immobilised**	
	ERED	ADH	ERED	ADH	ERED	ADH
Target protein offered [mg]	2.2	1.4	2.2	1.4	1.1	2.1
Immobilisation yield [%]	22.8	60.3	20.2	89.0	33.0	72.2
Support amount [mg]	10	10	20	20	20	20
Protein loading [w/w %]	5.0	8.4	2.2	6.2	1.8	7.6
Protein concentration in the reaction [mg/mL]	0.50	0.84	0.45	1.25	0.36	1.52

4.3.5. Analytical methods

The reactions performed in buffer were analysed after extraction with ethyl acetate, while those in organic solvent were injected without requiring any previous work-up.

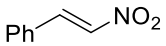
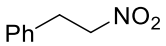
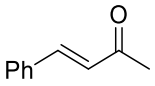
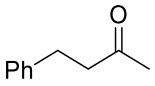
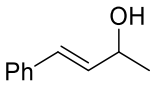
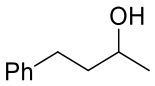
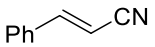
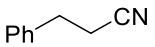
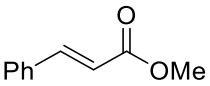
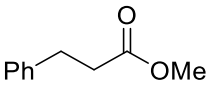
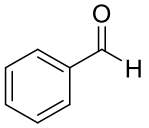
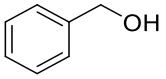
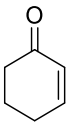
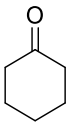
Method specifications used for achiral analyses:

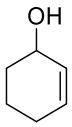
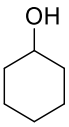
The column used was the HP-5 (30 m x 0.32 mm x 0.25 µm). 5 mL/min Hydrogen, 79 kPa. Injection 1 µL with 20:1 split ratio. Injection temperature: 250 °C, Detector temperature: 250 °C, Detector type: FID. For compounds **3a-d** the program was: 70 °C, hold 2 min, 15 °C/min ramp to 225 °C, hold 2 min. For the other compounds the program was: 100 °C,

hold 2 min, 15 °C/min ramp to 225 °C, hold 2 min. Treatment of results: Calibration curve with dodecane (10 mM) as an internal standard.

Table 4.6. GC retention times for achiral analyses for achiral analyses.

Compound	Retention time (min)	Compound	Retention time (min)

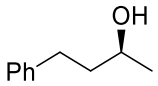
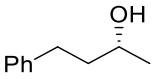
 **1a**	4.9	 **1b**	3.9
 **2a**	4.5	 **2b**	3.3
 **2d**	4.1	 **2c**	3.2
 **4a**	3.8	 **4a**	3.3
 **5a**	4.8	 **5b**	3.7
 **6a**		 **6b**	
 **3a**	1.8	 **3b**	1.5

 **3d**	1.5	 **3c**	1.5

Method specifications used for chiral analyses:

The column used was the CP-Chirasil Dex DB (25 m x 0.25 mm x 0.25 μm). 2 mL/min Hydrogen, 47 kPa. Injection 1 μL with 20:1 split ratio. Injection temperature: 220 °C, Detector temperature: 275 °C, Detector type: FID. Oven temperature: 100 °C, hold 2 min, 2.5 °C/min ramp to 120 °C, 10 °C/min ramp to 150 °C,hold 2 min. Treatment of results: Relative area of the peaks.

Table 4.7. GC retention times for achiral analyses for chiral analyses.

Compound	Retention time (min)	Compound	Retention time (min)
 (*S*)-**2c**	12.9	 (*R*)-**2c**	13.1

GENERAL CONCLUSIONS

This Doctoral Thesis explores the application of immobilised enzymes, including lipases, transaminases, ene-reductases, and alcohol dehydrogenases, all within the context of organic solvents as reaction media. The outcome obtained from these experiments demonstrated the practicality and viability of employing EziG supports for this specific purpose.

In Chapter 1, *N*-acylation reactions involving FA and HMFA was explored, particularly focusing on two commercial CALB preparations: Novozyme 435 on acrylic resin and EziG-CALB on glass porous material. EziG-CALB demonstrated exceptional performance, maintaining high activity over multiple cycles during FA acylation in the eco-friendly solvent 2-MeTHF. This approach enabled the synthesis of nine novel hydroxy amides with excellent yields, offering versatility for various acyl substituents under optimised conditions. The method proved scalable (up to 1 gram of substrate) and suitable for high substrate concentrations (up to 1 M), while environmental impact calculations showcased its sustainability. Additionally, an orthogonally *N*,*O*-diprotected amido ester from HMFA was successfully obtained through a one-pot, two-step transformation, highlighting the potential of immobilised enzymes for green and scalable synthesis processes in organic solvents.

In Chapter 2, several ATAs were evaluated for the transamination of HMF. Six promising ATA candidates were identified and their reaction conditions were carefully optimised in an aqueous buffer. From this selection, three key candidates—Cv, ArS, and ArRmut11—were chosen for further immobilisation on three distinct EziG supports, resulting in Amber and Coral supports being the most favourable ones. Subsequently, the immobilised enzymes were evaluated for their efficacy in catalysing the transamination of 1-phenoxypropan-2-one, using IPA as the amine donor in organic solvent. While catalysts such as Cv-Coral, ArS-Amber, and ArRmut11-Coral displayed the highest activity in this context, when using the target substrate HMF, a critical limitation emerged in the form of imine

formation between HMF and the IPA amine donor, hindering the implementation of ATA-catalysed reactions in organic solvents for this particular substrate. These findings contribute valuable insights into the challenges and opportunities of employing ATAs for biotransformations in organic solvents.

Chapter 3 described the development of a bienzymatic cascade, combining ArRmut11-TA and CALB lipase, for the precise stereoselective synthesis of (*R*)-*N*-(1-phenoxypropan-2-yl)acetamide. Further exploration delved into the immobilisation of ArRmut11-TA on various EziG supports, ultimately selecting EziG-Amber for its demonstrated suitability. The influence of water content in the reaction mixture became evident, with a delicate balance between enzyme activities necessary for success. An important finding was that 1% v/v water content allowed for a 30% activity retention for both enzymes. Moreover, the transition from batch one-pot mode to a continuous flow setup proved beneficial. Compartmentalising ArRmut11-TA and CALB within separate packed bed reactors remarkably enhanced productivity, achieving up to a remarkable 3.4-fold increase in enantiopure (*R*)-amide synthesis when compared to the conventional batch cascade approach. These collective findings underscore the immense potential and efficiency gains offered by continuous flow systems in the context of bienzymatic cascades.

Chapter 4 presents a series of significant findings in the context of redox enzymatic reactions within organic solvents. After careful screening, 2-cyclohexen-1-one emerged as a promising model substrate for the development of a biocascade, with NCR-ERED and TeSADH enzymes, identified as compatible redox biocatalysts. The successful immobilisation of these enzymes on various EziG supports, notably Coral, enabled their application in organic solvents, where higher hydrophobicity solvents like toluene and isooctane demonstrated highest enzyme activity. Moreover, the crucial role of water content in the immobilised formulations was revealed, with a 5% v/v water content in 2-propanol found optimal to ensure both a

homogeneous catalyst distribution and sustained enzyme activities. Additionally, post-immobilisation treatment with a 20% w/w sucrose solution in buffer proved effective in retaining enzyme activity when removing water from the biocatalyst. Despite initial challenges in catalyst recyclability, adjustments in the ERED:ADH ratio and the addition of $NADP^+$ from a buffer stock solution enabled the reuse of the catalyst for two additional cycles, with minimal activity loss. These findings collectively contribute valuable insights into optimising and leveraging redox biocatalysts in organic solvent environments.