

DEEP EUTECTIC SOLVENTS: PROPERTIES AND BIOCATALYTIC APPLICATIONS

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der RWTH Aachen
University zur Erlangung des akademischen Grades einer Doktorin der Naturwissenschaften
genehmigte Dissertation

vorgelegt von

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Tag der mündlichen Prüfung: 04.12.2014

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The present doctoral Thesis was carried out at the ITMC (Institut für Technische und Makromolekulare Chemie) at the RWTH Aachen University (Rheinisch-Westfälische Technische Hochschule Aachen) in Germany, under the supervision of Prof. Dr. Walter Leitner (RWTH Aachen), from September 2010 until March 2014. It was financially supported by the DFG graduate school 1166 BioNoCo – Biocatalysis in Non-Conventional Media.

Affidavit

I hereby declare that I wrote this work independently and only the specified sources and recourses were used. This work has not been presented to any examination office. Part of the work has been published (see list of publications and conference contributions).

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Hiermit erkläre ich, dass ich diese Arbeit eigenständig verfasst und nur die angegebenen Quellen und Hilfsmittel verwendet habe. Die Arbeit wurde bisher keiner Prüfungsbehörde vorgelegt. Teile der Arbeit wurden bereits veröffentlicht (siehe List of Publications und Conference Contributions).

ACKNOWLEDGEMENTS

Many people deserve my sincere acknowledgements, without them this thesis could not have been written.

My first thanks goes to Prof. Dr. Walter Leitner for giving me the opportunity to work on this interesting topic, with all the research facilities I needed.

I express my deepest gratitude to Dr. Pablo Domínguez de María. Since the beginning of my project I thought having been extremely lucky meeting such a wonderful person and I never changed my mind. The enthusiasm - that definitely characterizes him – kept me constantly motivated and gave me the strength to go ahead during these years to do all my best. I could always count on him, knowing he would listen to my problems and help me to find possible solutions. I learned a lot from him and I will be always grateful.

The financial support from DFG Graduate School 1166 BioNoCo (Biocatalysis in Non-Conventional Media) must be as well acknowledged and thus all the members belonging to it.

I would like to thank my research student Charlotte Knittel for her work, Philip Engel and Heiner Giese (AVT) for their help in the viscosity measurements, Hannelore Eschmann, Julia Wurlitzer and Elke Biener from the GC/HPLC-department and Ines Bachmann-Remy from the NMR-department. I thank Christian Lehmann from the group of Prof. Dr. Schwaneberg (Institute of Biotechnology) for our fruitful cooperation.

I am thankful for the nice working atmosphere at the ITMC and I would like to dedicate special thanks to the people who always helped me and supported me, colleagues and friends Philipp, Christoph, Henning, María, Lotte, Giancarlo and Tina. I spent a great time with them and will never forget it.

I thank and dedicate this thesis to my parents and my brother, for being always with me even when physically far. You gave me the strength to put myself out to arrive where I am now; I will never thank you enough. I thank all my closest friends, knowing I can count on you is essential to me. Last but not least, I thank my fiancé Claudio to be always so patient with me and to sustain me in all the moments. Thanks to him I understood that love is the only thing that really matters in life.

ABSTRACT

Deep eutectic solvents (DES) have emerged over the last decade as a novel class of ionic liquids (ILs). In its broadest sense, DESs are usually formed by mixing a quaternary ammonium salt (typically choline chloride and derivatives) with hydrogen bond donor molecules such as amines, amides, alcohols, carboxylic acids, sugars or polyols. The mixing of these two components upon gentle heating and in a specific molar ratio leads to a depression of the melting point, resulting in most of the cases in a liquid at room temperature. DESs share several properties with conventional ILs (e.g. similar conductivities, polarities, viscosities, densities, surface tensions, refractive indexes, chemical inertness, etc.) and they are usually cheap due to the relatively inexpensive components and their simple synthesis, where no waste is produced and no further purification steps are needed. Furthermore, DESs' components are often biodegradable and non-toxic.

The first part of this PhD Thesis focused on the synthesis of novel bio-based DESs using choline chloride and, as hydrogen bond donors (HBDs), several carboxylic acids and some saccharide-derived polyols. The novel DESs were characterized by measuring melting points, pH, water contents and viscosities. Likewise, kinetic studies on water absorption were conducted and solubilities in protic and aprotic organic solvents assessed. DESs were able to solve protic compounds (e.g. alcohols) whereas a second phase was formed with aprotic compounds (e.g. ketones, esters). Based on this property a novel procedure to separate mixtures of alcohol-ester, alcohol-ketone and amine-ketone was established, leading in most of the studied cases to 70-90% of efficiency and 90-99% of purity of the separated compounds.

The second part of this PhD Thesis focused on various biocatalytic applications in these solvents. Several reactions were assessed (such as transesterifications, enantioselective epoxidations, peptide syntheses, carboligations and reductions) by using isolated enzymes (*Candida antarctica* lipase B, α -chymotrypsin from bovine pancreas, benzaldehyde lyase from *Pseudomonas fluorescens*) and whole cells (baker's yeast). In most of the reported cases DESs were compatible with those biocatalysts, even despite the denaturing behavior of strong hydrogen bond donors towards proteins (e.g. urea). The amount of water present in the reaction mixture resulted to be critical, analogous to observations done in other non-conventional media. For each reaction and enzyme the most suitable DES was identified.

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LIST OF ABBREVIATIONS AND UNITS

[Bmim]	1-Butyl-3-methyl-imidazolium
AcChCl	Acetyl choline chloride
Acet	Acetamide
AcOEt	Ethyl acetate
AP	N-acetyl-phenylalanine
APEE	N-Acetyl-phenylalanine ethyl ester
APG	N-Acetyl-phenylalanine-glycinamide
ASTM	American Society for Testing and Materials
BAL	Benzaldehyde lyase from <i>Pseudomonas fluorescens</i>
ca.	Circa, about
CALA	<i>Candida antarctica</i> lipase A
CALB	<i>Candida antarctica</i> lipase B
ChCl	Choline chloride
cP	Centipoise
DCDMH	Dichloro-5,5-dimethylhydantoin
DES	Deep eutectic solvent
E	Enantioselectivity
EAC	Ethylammonium chloride
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EG	Ethylene glycol
<i>et al.</i>	<i>et alia</i> (and others)
G-DES	ChCl:glycerol DES (1:2 molar ratio)
GC	Gas chromatography
GH	Glycinamide hydrochloride
Gly	Glycerol
h	Hour
HBD	Hydrogen bond donor
HOBt	Hydroxybenzotriazole
HPLC	High pressure liquid chromatography
I-DES	ChCl:isosorbide DES (1:2)
iCALB	Immobilized <i>Candida antarctica</i> lipase B

IPA	Isopropenyl acetate
K	Kelvin
K_M	Michaelis-Menten constant
IL	Ionic liquid
log P	Logarithm of the partition coefficient
MA	Malonic acid
min	Minute
mL	Milliliter
mM	Millimolar
mol	Mole
mp	Melting point
MTHF	2-Methyltetrahydrofuran
mS	Millisiemens
n.d.	Not determined
PCL	<i>Pseudomonas cepacia</i> lipase
PE	<i>rac</i> -1-phenylethanol
R^2	Coefficient of correlation
rpm	Revolutions per minute
RT	Room temperature
RTILs	Room temperature ionic liquids
S-DES	ChCl:sorbitol DES (1:1 molar ratio)
SG-DES	ChCl:D-sorbitol:glycerol DES (1:1:0.5 molar ratio)
T	Temperature
TBAF	Tetrabutylammonium fluorid
TEA	Triethylamine
ThDP	Thiamindiphosphate cofactor
U	Urea
U-DES	ChCl:urea DES (1:2 molar ratio)
vol.	Volume
vol. %	Volume percentage
vol./vol.	Volume ratio
wt %	Weight percentage
X-DES	ChCl:xylitol DES (1:1 molar ratio)

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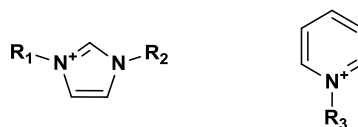
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1. INTRODUCTION

Ionic liquids (ILs) are defined as salts with melting points below 100°C. In the past 20 years, room temperature ionic liquids (RTILs) have attracted increasing interest in several fields such as catalysis, electrochemistry, material synthesis and more recently in the pre-treatment of biomass.^[1] Even though the most remarkable developments in RTIL are recent, their discovery dates back to the beginning of the 20th century when Walden *et al.* reported on the molten salt ethyl-ammonium nitrate [EtNH₃][NO₃].^[2] At that time, the potential of the discovery was not taken into consideration. Many years later, in 1960s, the first generation of ILs was synthesized and from then on developments in the area started. First ILs were mainly composed by imidazolium and pyridinium cations with chloroaluminate or other metal halide anions resulting in water and air sensible (Figure 1). To overcome this problem, a second generation was subsequently developed by Wilkes and Zaworotko^[3] where weakly coordinating anions such as [BF₄]⁻ or [PF₆]⁻ were introduced. The resulting ILs were water and air stable and for this reason became the most studied ones. Other key features were their moderate polarity and hydrophobicity resulting in water immiscible solvents. Most recently, a third generation of ILs has been developed, involving rather different components. Thus, choline derivatives may be used as a cation, combined with sugars, amino acids or organic acids, alkylsulfates, or alkylphosphates as anions. These ILs are typically more biodegradable and hydrophilic than previous ILs, and they tend to be soluble in water. Generally speaking, ILs are rather expensive, with prices about 20-fold higher than organic solvents (taking in consideration the current price targets from Solvent Innovation GmbH). In the last decade, a new class of solvents, having similar features of the ILs, has emerged, namely deep eutectic solvents (DESs). These solvents cannot be considered as conventional ILs, since some of their components are uncharged.

First generation ILs

Typical cations:



R= Alkyl

Typical anions:

AlCl_4^- , Al_2Cl_7^-

Second generation ILs

Typical cations:



R= Alkyl

Typical anions:

BF_4^- , PF_6^- , Me_2CO_2^- , $\text{N}(\text{SO}_2\text{CF}_3)_2^-$

Third generation ILs

Typical cations:



R= Oxygenated alkyl

Typical anions:

ROSO_3^- , saccharin, amino acids, R_2PO_4^-

Deep eutectic solvents (DESS)

Typical quaternary ammonium salt:



Typical hydrogen bond donor (HBD):



Figure 1. Examples of ILs and DESs (adapted from Gorke *et al.*^[4]).

1.1. Physicochemical properties of deep eutectic solvents (DESS)

DESS are usually formed by mixing a quaternary ammonium salt (in some cases replaced with a quaternary phosphonium salt) with a hydrogen bond donor (HBD).^[5-9] The first definition of deep eutectic solvent was given by Abbott *et al.* in 2003 to a mixture of choline chloride (ChCl) and urea.^[5] When these two components with high melting points (302°C for ChCl and 133°C for urea respectively) are mixed in a defined molar ratio, the eutectic point is reached, resulting in a liquid DES at room temperature with a melting point of 12°C. The great melting point depression derives from the interactions among the

components and more precisely between the hydrogen atoms from urea and the chloride ion (figure 2).^[5]

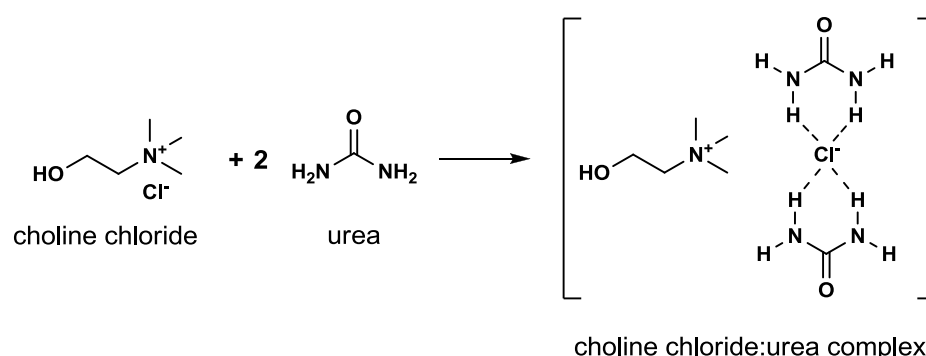
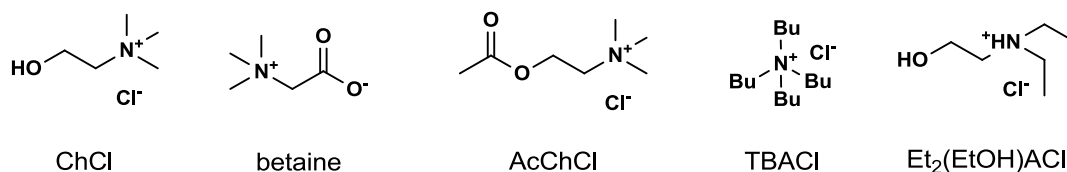


Figure 2. Proposed complexation occurring when the ChCl:urea DES (1:2 molar ratio) is formed (adapted from Abbott *et al.*^[5]).

One of the main features of these novel solvents is the possibility of having, simply by changing one or both components, a huge number of eutectic mixtures with different properties. Although ChCl is the most employed quaternary ammonium salt because of its low cost and biodegradability (it is indeed produced in large scale and added to the chicken feed), many other halides are suitable for producing DESs. Thus, considering the general formula $[R_1R_2R_3R_4N^+] X^-$, changing the alkyl groups and/or the anion, a broad variety of quaternary ammonium salts may become a potential DES's component. For the so called hydrogen bond donors (HBDs) several compounds may be used such as amines, amides, alcohols, carboxylic acids, sugars and polyols (Figure 3). DESs are generally cheap due to the relatively inexpensive components and their synthesis, which is simple and just require the stirring of the two components upon a gentle heating. The process leads to virtually no waste production and no further purification is needed. An important issue raised over the last decades is the impact of solvents on the environment. DESs have low vapor pressures and high boiling points and most of their components are biodegradable and non-toxic, as several recent papers have claimed.^[10,11] However, it must be mentioned that toxicological studies for DESs have just started to appear, and therefore more research along these lines is needed.

A solvent is defined by its properties (such as the conductivity, polarity, viscosity, density, surface tension, refractive index, chemical inertness). In the following subsections, several important properties of DESs will be briefly discussed.

Halide salts



HBDs

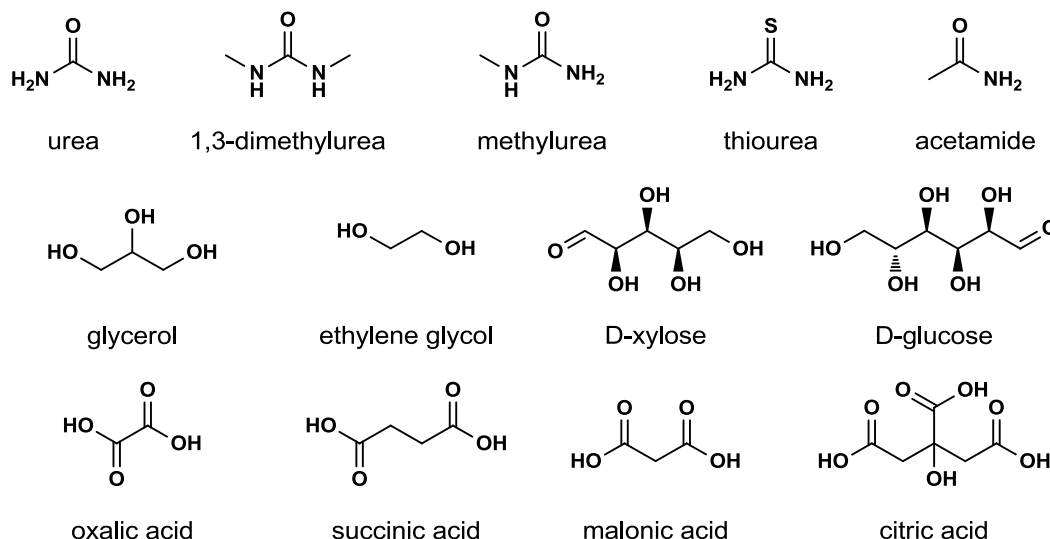


Figure 3. Structures of the most common halide salts and HBDs used in DESs (adapted from Zhang *et al.*^[9]).

1.1.1. Melting points

As already mentioned, when the halide salt is mixed in an adequate molar ratio with a HBD, a steep depression of the melting point (compared with the individual components) is observed. All reported DESs have melting points below 150°C and most of them are even liquid at 50°C or below, making them certainly attractive for many applications (e.g. in biocatalysis low temperatures are often used to avoid enzyme denaturation). Both components, the halide salt and the HBD, influence the final melting point of the mixture and so far no scientific systematic correlations have been elucidated. Moreover, the actual molar ratio of the halide salt to the HBD to reach the higher melting point depression is specific for each different DES. Aiming at shedding some light on these aspects, Abbott *et al.* proposed that the melting point of a DES might depend on the lattice energy that describes how the anion and the HBD interact and how the entropy changes when the liquid is formed.^[6]

1.1.2. Viscosities and conductivities

The viscosity describes the internal friction of a moving fluid or, in other words, the resistance of a substance to flow. Most of the reported DESs exhibit high viscosity values, often higher than 100 cP (Table 1) (for comparison, water viscosity is 0.89 cP at room temperature).

Table 1. Viscosity and conductivities of some DESs at specific temperatures (adapted from Abbott *et al.*, D'Agostino *et al.*^[12,13]).

Halide salt	HBD	Salt:HBD molar ratio	Viscosities (cP) (T)	Conductivity (mS·cm ⁻¹) (T)
ChCl	Urea	1:2	750 (25°C)	0.20 (40°C)
ChCl	Ethylene glycol	1:2	37 (25°C)	7.61 (20°C)
ChCl	Glycerol	1:2	359 (25°C)	1.05 (20°C)
EtNH ₃ Cl	CF ₃ CONH ₂	1:1.5	256 (40°C)	0.39 (40°C)
EtNH ₃ Cl	Acetamide	1:1.5	64 (40°C)	0.69 (40°C)
EtNH ₃ Cl	Urea	1:1.5	128 (40°C)	0.35 (40°C)
AcChCl	Urea	1:2	2214 (40°C)	0.02 (40°C)

The main reason for these high values might be related to the strong hydrogen bond network formed among the DES's components, that inevitably reduces the mobility of the molecular compounds.^[7,12–14] Other forces such as electrostatic or van der Waals interactions might contribute to their high viscosity as well. Considering the great potential of these solvents as sustainable media, the design and development of low-viscous DESs is more desirable as operational costs for several process operations (e.g. stirring, mixing, and pumping) could be significantly reduced. DES's viscosity is influenced by the chemical nature of the components, the water content and, of course, the temperature.^[9] An interesting effect observed in ChCl:glycerol mixtures was the decrease of the viscosity when higher amounts of the salt were added.^[15] Indeed, while the viscosity of glycerol at 20°C is ca. 1200 cP, the corresponding DES (ChCl:glycerol with a molar ratio of 1:2) exhibits a viscosity of ca. 400 cP. This might be the result of breaking the 3D intermolecular hydrogen bond interactions in the glycerol resulting in a less ordered system.^[15]

The conductivity is a measure of the ability of a material to conduct electric current. It depends on the available ions, their mobility and valence. Most of DESs exhibit poor ionic conductivities (lower than $2 \text{ mS}\cdot\text{cm}^{-1}$ at room temperature). The moderate conductivities might result from reduced ion mobility due to a large ion size and/or ion pairing or ion aggregation leading to a smaller amount of available charge carriers. In Table 1, ionic conductivities of various DESs at different temperatures are depicted.^[9]

1.1.3. Polarities and acid-base behavior of DESs

There are many scales to estimate the polarity of a solvent, whereby the $E_T(30)$ scale, introduced by Reichardt^[16], is most commonly used. For such scale standard solvatochromic compounds like betaine dye n°30 (Reichardt's dye) is used. The polarity is then calculated from the wavelength (nm) of maximum absorbance of the dye in solvents of different polarity at 25°C and normal pressure (1 bar). By using Reichardt's dye, a value for $E_T(30)$ can be calculated with the following Equation (1):

$$E_T(30) / \text{kcal}\cdot\text{mol}^{-1} = hcN_A / \lambda_{\text{max},30} = 28591 / \lambda_{\text{max},30} \quad (1)$$

where h = Planck constant, c = speed of light, N_A = Avogadro number, λ_{max} = the wavelength of the maximum absorption.

Moreover, a normalized scale (E_T^N) was introduced to obtain dimensionless values, using water ($E_T^N = 1.00$) and tetramethylsilane ($E_T^N = 0.00$) as reference solvents and applying the Equation (2).

$$E_T^N = \frac{[E_T(\text{solvent}) - E_T(\text{TMS})]}{[E_T(\text{water}) - E_T(\text{TMS})]} \quad (2)$$

In Table 2 the E_T^N values for some conventional organic solvents, ILs and DESs are summarized. As can be observed, the polarities of DESs are higher than those observed for short chain alcohols (e.g. ethanol, 2-propanol), some polar aprotic solvents (e.g. dimethylsulfoxide and dimethylformamide), and two imidazolium based ILs.

Table 2. E_T^N values for some conventional organic solvents, ILs and DESs (adapted from Ruß *et al.*^[14]).

Solvent	E_T^N	Solvent	E_T^N
Water	1.00	Dimethylformamide	0.40
Glycerol	0.81	[Bmim][acetate]	0.61
Ethylene glycol	0.78	[Bmim][propionate]	0.57
Ethanol	0.65	Glycerol-ChCl (2:1 molar ratio)	0.86
2-Propanol	0.55	Ethylene glycol-ChCl (2:1 molar ratio)	0.80
Dimethylsulfoxide	0.44	Urea-ChCl (2:1 molar ratio)	0.84

The Hammett function has been widely used to evaluate the acidity and basicity of no aqueous solvents. For a basic solution, the Hammett function measures the tendency of the solution to capture protons. When the indicators are weak acids, the Hammett function H_- is defined by Equation (3):

$$H_- = pK(HI) + \log\left(\frac{[I^-]}{[HI]}\right) \quad (3)$$

where $pK(HI)$ is the thermodynamic ionization constant of the indicator in water, $[I^-]$ and $[HI]$ are the concentrations of the anionic and neutral forms of the indicator, respectively.

As an example, the DES ChCl:urea (1:2 molar ratio), when 4-nitrobenzylcyanide was used as indicator, showed a value of 10.86 at 25°C, suggesting that this DES is weakly basic.^[17] The presence of water influences slightly the value (with 1–3 wt% of water the H_- value decrease from 10.77 to 10.65). Due to its weak alkalinity this DES is capable of absorbing a small amount of CO₂ leading to a H_- value of 6.25. It is possible to restore the initial value by just bubbling N₂ through the DES, suggesting that the acidity or basicity can be switched reversibly.

The chemical nature of the HBD has a strong influence on the corresponding DESs' acidity and the measured pH values for several phosphonium-based DESs clearly showed this strict correlation. Indeed, when glycerol was used as HBD the observed pH was neutral, whereas the presence of the trifluoroacetamide led to low pH values (2.5 at 20°C).^[8]

1.2. Overview of applications of DESs

Despite research and applications for DESs are still in their infancy, a number of promising concepts have been already proposed. In the following subsections the dissolution properties of DESs, their application as extracting agents and separation media, as well as the use of these solvents in biocatalysis will be mainly taken in consideration, since these topics are directly correlated to the work of this Thesis. Subsequently, applications for DESs in electrochemistry, material and organic synthesis will be briefly discussed as well.

1.2.1. Dissolution capabilities of DESs: metal oxides, highly-functionalized molecules, macromolecules and biomass

Being able to donate or accept electron pairs or protons to form hydrogen bonds, DESs have shown excellent dissolution properties for a broad range of molecules. For instance, DESs can dissolve various metal oxides^[6,18,19], thereby opening a novel strategy for the separation and metal recycling, a key aspect in electrochemistry technology. In Table 3 data on the solubility of different metal oxides in different ChCl-containing DESs are shown.

Table 3. Solubilities (ppm) at 50°C for some metal oxides in DESs (adapted from Abbott *et al.*^[18]).

Metal oxide	ChCl:malonic acid (1:1 molar ratio)	ChCl:urea (1:2 molar ratio)	ChCl:ethylene glycol (1:2 molar ratio)
TiO ₂	4	0.5	0.8
Co ₃ O ₄	5992	30	18.6
V ₂ O ₅	5809	4593	131
Cu ₂ O	18337	219	394
CrO ₃	6415	10840	7
MnO	6816	0	12
Mn ₂ O ₃	5380	0	7.5
MnO ₂	114	0.6	0.6
FeO	5010	0.3	2
ZnO	16217	1894	469
Fe ₃ O ₄	2314	6.7	15

As observed, solubilities differ significantly depending on the DES and the metal oxide used. The higher solubility of the oxides in the malonic acid-based DES compared to the other liquids is presumably due to the protons acting as an oxygen acceptor, thus changing the complex formed. The metals that exhibit higher solubilities tend, indeed, to have less oxygen attached to the metal center.

The dissolution of more complex and highly-functionalized molecules was later on investigated in DESs. In 2009, Morrison *et al.* looked into, for the first time, possible uses of DESs in dissolving poorly water-soluble compounds such as danazol, griseofulvin and itraconazole^[20] (Table 4). For these model compounds, solubilities in DESs were from 5 to 22000 fold higher than the ones observed in water. Few years later, Choi *et al.* synthesized novel DESs using components naturally produced by cell metabolism, the so-called NADESs (natural deep eutectic solvents).^[21] It was suggested that NADESs would provide liquid phases in organisms in which certain biosynthetic steps or storage of hydrophobic products may occur. To assess this, solubilities of some common natural products in NADESs were measured, and thus compounds like rutin, paclitaxel and ginkgolide B showed high solubilities in these solvents as well.^[22]

Table 4. Solubilities (mg·mL⁻¹) measured in several DESs. n.d. = not determined. (adapted from Morrison *et al.*, Spronsen *et al.*^[20,22]).

Compound	ChCl:urea	ChCl:malonic acid	ChCl:glucose	ChCl:sorbitol	Water
Benzoic acid	229	35	n.d.	n.d.	3
Danazol	0.048	0.160	n.d.	n.d.	< 0.0005
Griseofulvin	0.034	1.0	n.d.	n.d.	0.007
Itraconazole	< 0.001	22	n.d.	n.d.	< 0.001
Rutin	n.d.	n.d.	121.63	149.21	0.028
Paclitaxel	n.d.	n.d.	0.83	0.59	0.01
Ginkgolide B	n.d.	n.d.	5.85	1.70	0.15

On the basis of these dissolution properties, DESs were considered for their use in biomass valorization. Biomass processing pretreatment faces two main challenges: the difficulty in dissolving lignocellulosic biopolymers, and the set-up of efficient hydrolysis of

polysaccharides to sugars or high-value products. In this context, in 2011, Francisco *et al.* investigated a series of DESs prepared by combining some natural and renewable biomaterials and 26 new mixtures were screened as solvents for lignin, cellulose and starch.^[23] Most of the selected combinations showed high lignin solubility and very poor or negligible cellulose solubility, enabling the selective separation of lignin and cellulose. Investigations on microcrystalline cellulose (Avicel®) were afterwards done by Hertel *et al.* for some DESs showing a reduction of the crystallinity of 15-20%.^[24]

1.2.2. DESs as extracting agents and separation media

By making use of DESs' properties, several applications as extracting agents or as separation media have been recently developed. An important example relates to the biodiesel synthesis. Regardless of the feedstock used to make biodiesel (e.g. sunflower, rapeseed, rubber seeds and soy bean oil), the production procedure is analogous and requires the triglyceride oil extraction from the plant for its transesterification into alkyl esters (usually methyl or ethyl). Homogenous (e.g. KOH, NaOH) and heterogeneous catalysts (e.g. immobilized lipases, guanidinium-based immobilized organocatalysts, or chitosan for waste valorization)^[25-33] have been used. For each mole of triglyceride, three moles of ester and one mole of glycerol are produced. Importantly, the viscosity of the glycerol present in biodiesel may interfere with the high-pressure injection system of a modern diesel engine, causing damage to it. Conclusively glycerol must be removed through "end-of-pipe" treatments to reach the standards for biodiesel prior to its use in vehicles, as specified by the American Society for Testing and Materials (ASTM).^[34] Abbott *et al.* claimed that molecules unable to form hydrogen bonds with ChCl are immiscible with DESs.^[5] Taking advantage of this property the possibility of removing glycerol from biodiesel by using quaternary ammonium salts (Pr₄NBr, EtNH₃Cl, ClEtMe₃NCl, ChCl, and AcChCl) was tested. The idea behind is to form a DES that afterwards can be easily removed from the mixture, being immiscible with the produced biodiesel esters^[35] (schematic diagram shown in Figure 4).

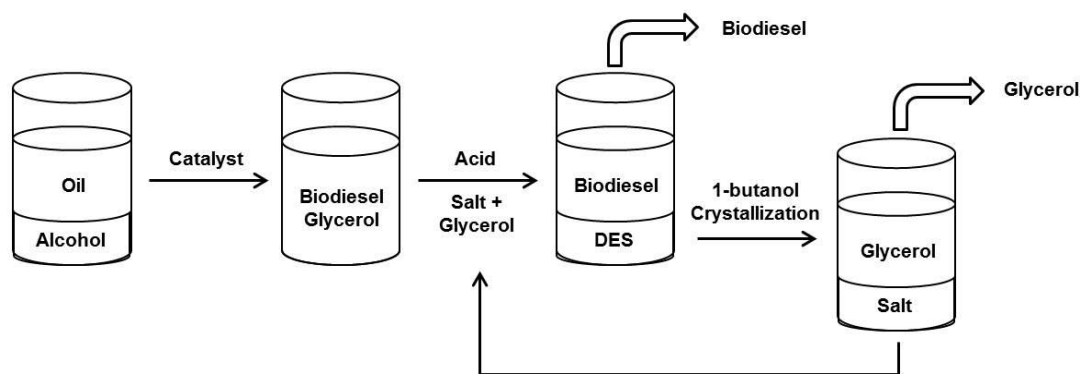


Figure 4. Schematic diagram for the removal of the glycerol after biodiesel production (adapted from Abbott *et al.*^[35]).

After this treatment a significant decrease of the content of glycerol in biodiesel was achieved and in some cases, depending on the salt used, values conforming the ASTM standards were obtained (0.00% molar fraction of glycerol observed, maximum value is 0.02%).^[34] Moreover, the used quaternary ammonium salt could be recovered by separating them from the DES by recrystallization with 1-butanol. Subsequently Hayyan *et al.* showed that ChCl:glycerol based DES could be used as a solvent in a continuous process for extracting glycerol from palm oil-derived biodiesel.^[34] More recent investigations showed that ChCl:ethylene glycol and ChCl:2,2,2-trifluoroacetamide DESs were also efficient for removing glycerol from palm oil-based biodiesel, as well as DESs composed by different salts like methyl triphenyl phosphonium bromide and HBDs such as glycerol, ethylene glycol, or triethylene glycol.^[36,37] Moreover, all tested DESs could also reduce the content of monoglycerides and diglycerides present in biodiesel.

Another application where DESs can be used as a separating media was discovered by Pang *et al.*^[38], demonstrating the possibility of using ammonium salts for the separation of phenols from oils. Toluene, paraxylene and hexane were chosen as model oils and different amounts of phenol or cresol were dissolved. Three different quaternary ammonium salts were tested (ChCl, Et₃NHCl and EtNH₃Cl) and all of them were successful in forming DESs with phenol (or cresol), separating it from the oils. Compared with other traditional methods the novel approach would avoid the use of corrosive alkalis and acids.

To overcome the problem of azeotropic mixtures separation processes like extractive/azeotropic distillation or liquid-liquid extraction, have been applied. Liquid-liquid extraction is an appealing alternative because it does not require high amounts of energy,

volatile organic compounds or high pressures. In this process, the separation occurs by the introduction of a third component into the azeotropic mixture (extraction solvent) which preferentially dissolves one of the components separating it from the other. Along these lines Oliveira *et al.* studied three different DESs (made by mixing CHCl_3 and HBDs such as glycerol, ethylene glycol and levulinic acid) for the separation of ethanol-heptane azeotropic mixtures.^[39] DESs containing HBD with hydroxyl groups showed to be more selective compared to the ones formed with the carboxyl group in the HBD. However in all cases the distribution coefficients and selectivity values were very promising for future investigations on scaling up the process.

1.2.3. DESs in biocatalysis

Biocatalysis can be defined as the use of enzymes, in isolated form or in whole cells, as catalysts for organic synthesis. Enzymes are proteins that operate under mild conditions in terms of pH, pressure and temperature, and can catalyze a broad spectrum of organic reactions such as oxidations/reductions, hydrolysis, C-C/C-N/C-O/C-S bonds formation, isomerizations etc., often with high chemo-, stereo- and regioselectivity.^[32,40,41] In Nature enzymes catalyze reactions in aqueous solutions. Even though this “conventional” medium can be identified as environmentally friendly and might require less investments in chemical plant safety, water-based downstream processing is typically cost and energy-intensive and, moreover, hydrophobic substrates and products are sparingly soluble in this media. In the last decades, biocatalysis in non-conventional media has attracted an increasing attention. Albeit in many cases enzymes display their highest catalytic activity in aqueous media, it has been shown that many enzymes and whole-cells can actually be active in non-aqueous media as well, such as organic solvents, supercritical fluids, gas phase or ILs.^[42] A further main advantage of working in such solvents is that thermodynamic equilibria can be shifted (e.g. to synthesis instead of hydrolysis). Following these thoughts, ILs have been applied in biocatalysis and enzymes showed comparable or higher activities in ILs than organic solvents and, in some cases, exhibited enhanced thermal and operational stabilities.^[43,44]

In 2008 Gorke *et al.* showed that some hydrolases were stable in DESs, keeping their activity in these solvents despite the expected denaturing properties of components like urea or other HBDs.^[45] Actually, *Candida antarctica* lipase B (CALB) – as lyophilized and immobilized form - catalyzed the transesterification of ethyl valerate with 1-butanol in eight different DESs and toluene (Table 5). The DESs were formed by using CHCl_3 or

ethylammonium chloride (EAC) as halides, and ethylene glycol (EG), glycerol (Gly), acetamide (Acet), urea (U) or malonic acid (MA) as HBDs. The enzymatic activity was observed in all tested DESs, and in two of them the conversions were comparable to those reported in toluene. Moreover, the aminolysis of ethyl valerate with 1-butylamine using the same enzyme led to similar high conversions (> 90%) in ChCl:Gly, ChCl:U or toluene. The reaction was slower in ChCl:Acet, presumably because the aminolysis with ethylamine from the DES predominated over aminolysis with butylamine.^[45]

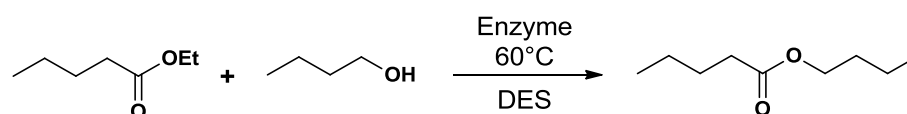


Table 5. Conversions (%) obtained in the lipase-catalyzed transesterification of ethyl valerate with butanol at 60°C in DES and toluene (adapted from Gorke *et al.*^[45]).

	ChCl: Acet	ChCl: EG	ChCl: Gly	ChCl: MA	ChCl: U	EAC: Acet	EAC: EG	EAC: Gly	Toluene
iCALB	23	11	96	30	93	63	23	93	92
CALB	96	32	96	58	99	92	33	91	92
CALA	1	3	70	1	2	3	20	2	76
PCL	0	0	22	0	1	0	0	1	5

Once these promising results appeared, other research groups focused their attention on biocatalysis in DESs.^[46–50] Zhao *et al.* reported the use of DESs – based on choline acetate (ChOAc) or ChCl and glycerol as HBD – for the lipase-catalyzed transesterification of ethyl sorbitate with 1-propanol catalyzed by Novozym[®] 435 (commercial iCALB).^[50] The enzyme showed high stabilities in ChOAc:glycerol DES keeping 92% and 50% of its activity after 48 h and 168 h of pre-incubation respectively. Based on such great stability the same group assessed the methanolysis of Miglyol[®] oil 812, a mixture of triglycerides of caprylic acid and capric acid. Nearly complete conversions were observed after few hours whereas days were needed with the traditionally used ILs.^[50] In an analogous area, Zhao *et al.* reported on the protease-catalyzed transesterification of N-acetyl-L-phenylalanine ethyl ester with 1-propanol^[51] (Table 6). The reaction was performed in some DESs and catalyzed by immobilized cross-linked proteases (subtilisin and α -chymotrypsin). Best results were obtained in ChCl:Gly with 3 vol.% of water using subtilisin immobilized on chitosan.

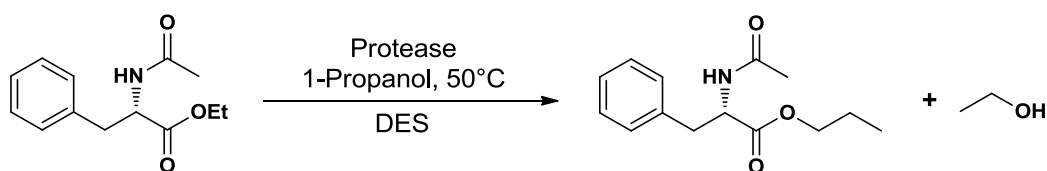


Table 6. Protease-catalyzed transesterification of N-acetyl-L-phenylalanine ethyl ester with 1-propanol at 50°C (S = subtilisin, Chit = chitosan, α -C = α -chymotrypsin). (adapted from Zhao *et al.*^[51]).

Solvent	Protease	Water content (vol.%)	Activity ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$)
ChOAC:Gly	S/Chit	2	0.42
ChOAC:Gly	S/Chit	2	0.40
ChOAC:Gly	S/Chit	4	0.90
ChCl:Gly	Free α -C	2	0.03
ChCl:Gly	α -C/Chit	2	0.03
ChCl:Gly	α -C/Chit	3	0.75
ChCl:Gly	S/Chit	3	2.90

Another point of discussion when using DESs and lipases was whether some HBDs (e.g. alcohols) could be actual substrates of the enzymes as well, and thus compete with other nucleophiles. Durand *et al.* evaluated DESs as media for CALB-catalyzed transesterifications of vinyl acetate with several alcohols.^[52] ChCl-based DESs with different HBDs were tested, showing that dicarboxylic acids or ethylene glycol HBDs led to side reactions that limited their application. Nevertheless, CALB exhibited high activity and selectivity in the other DESs showing their great potential as a media in biocatalysis. Subsequently, the same research group studied the alcoholysis of phenolic esters in DES-water binary mixtures (water content up to 20 wt%).^[53] The importance of water was addressed, as reducing the viscosity and thus improving the mass transfer, leading to a significant increase in the lipase's initial activity and final conversion rate. Moreover, it was hypothesized that the addition of a protic solvent in the DES reduced the interaction between the DES and the substrate, making the latter more available for reacting.

Apart from using DESs as solvents for biocatalysis, they were used as co-solvents in buffer media as well, and interesting results were reported. For instance, the styrene oxide hydrolysis catalyzed by epoxide hydrolase AD1 from *Agrobacterium radiobacter*, a 20-fold

increase in the conversion was observed when 25% of ChCl:glycerol DES was added to the buffer solution.^[45] The effect of the DESs as a co-solvent was also studied by Lindberg *et al.* on the hydrolysis of 1*S*,2*S*-2-methylstyrene epoxide by epoxide hydrolase StEH1.^[48] In presence of DES (up to 20%), the enzyme retained almost completely its activity, and major effects were observed on K_M (up to 20-fold increase) and on the regioselectivity of StEH1 (enhanced in the hydrolysis of 1*R*,2*R*-2-methylstyrene epoxide). Finally, Lehmann *et al.* observed enhanced stability and activity in mixtures of water with ChCl:glycerol DES for the cellulase variant 4D1, in comparison with the performance of the wild type.^[54]

Apart from isolated free enzymes, the use of whole-cells in biocatalytic processes is limited in non-aqueous solvents in general^[55–58], in ILs^[59–61] and DESs in particular. In the case of DESs, Gutiérrez *et al.* reported the incorporation of bacteria in ChCl:glycerol DES in its pure state by a freeze-drying process.^[47] Unexpectedly the bacteria preserved their integrity and viability, representing a proof-of-principle that could widen the scope of DESs and make them appealing also for other whole cell biotransformations. In the last part of this PhD Thesis, the first examples of whole-cell biocatalysis in DESs will be discussed.

1.2.4. Further applications: examples of DESs in electrochemistry, material and organic synthesis

Electrodeposition enables the formation of solid materials by electrochemical reactions in a liquid phase and it is performed to functionalize surfaces, especially for corrosion-resistance. This process can be performed in presence of various solvents but is limited by their redox potentials. For example, in aqueous solutions electrodeposition of metals is generally restricted to those having a redox potential higher than that of water. Over the past two decades electrodeposition of metals and alloys – which is carried out with difficulties in aqueous solvents – was successfully achieved in ILs and they have been used also in a broad range of applications in various electrochemical devices (e.g. lithium ion batteries, fuel cells, super capacitors, dye sensitized solar cells, etc.).^[62] Applications of DESs in electrochemistry, mainly as electrolytes for electrodeposition of metal and electropolishing have been reported.^[63–73] In this respect, ChCl:urea DES was used to perform the electrodeposition of several metals starting from some salts (e.g. CuGa, CdS, CdSe, ZnS).^[18,63,74–77] Being water-sensitive the electrodeposition of magnesium in aqueous solution is challenging. Hence, the efficiencies of several DESs made by mixing ChCl with different HBDs (ethylene glycol, malonic acid, glycerol, urea and ZnCl₂) were investigated for the electrodeposition of Zn onto

several Mg alloys. DESs containing ChCl and ethylene glycol were tested as electrolyte for the electrodeposition of Zn, Sn, ZnSn, composites of Cu and Al₂O₃ or SiC, Ag, CrCo alloys, as well as for polymers such as polypyrrole films.^[78–80] However, in spite of the promising results obtained, instability of DESs during the electrodeposition process remains still a major problem that needs to be well investigated prior to a possible industrial technology transfer. For example, studies on the stability of ChCl:urea DES showed the formation of gaseous chemicals at the cathode (trimethylamine) and anode (chlorine) during the nucleation of metal, suggesting a partial degradation of the DESs.^[81]

Conventional preparation of inorganic materials needs a thermal step for crystallization and structure formation. The solvothermal synthesis consists in growing single crystals from a non-aqueous solution by thermal treatment under pressure. Recently, a new type of solvothermal synthesis – so-called ionothermal synthesis – was developed. In this technique the ILs or DESs are used both as solvents and structure directing agents (SDAs).^[74,82–96] The method has become one of the most widely used synthetic strategies for open-framework structures (e.g. metal phosphates and phosphites, oxalatophosphates), metal organic frameworks (MOFs), covalent-organic frameworks (COFs), polymer–organic frameworks (POFs), mesoporous silicas and organosilicas, metal nanoparticles and nanostructured metal oxides. There are many advantages of using ionothermal rather than solvothermal syntheses. Thus, ionothermal synthesis can be readily conducted at ambient pressure, and due to the use of low volatile ILs or DESs the process is simpler and safer. Moreover, the possibility to vary the ionic nature of DESs provides several different reaction environments under which a large number of novel materials may be synthesized, changing material structure obtained by ionothermal synthesis.

In the field of catalysis, the choice of the solvent is critical because this not only influences the productivity of a reaction by enabling a better contact between reactants and catalysts, but also determines the downstream processing, recycling and disposal strategy. The quest of cheap and safe media for catalysis has become very important, and DESs have attracted increasing interest. Until now, many different types of reactions in organic synthesis have been performed with promising results in DES^[97–113] (Figure 5).

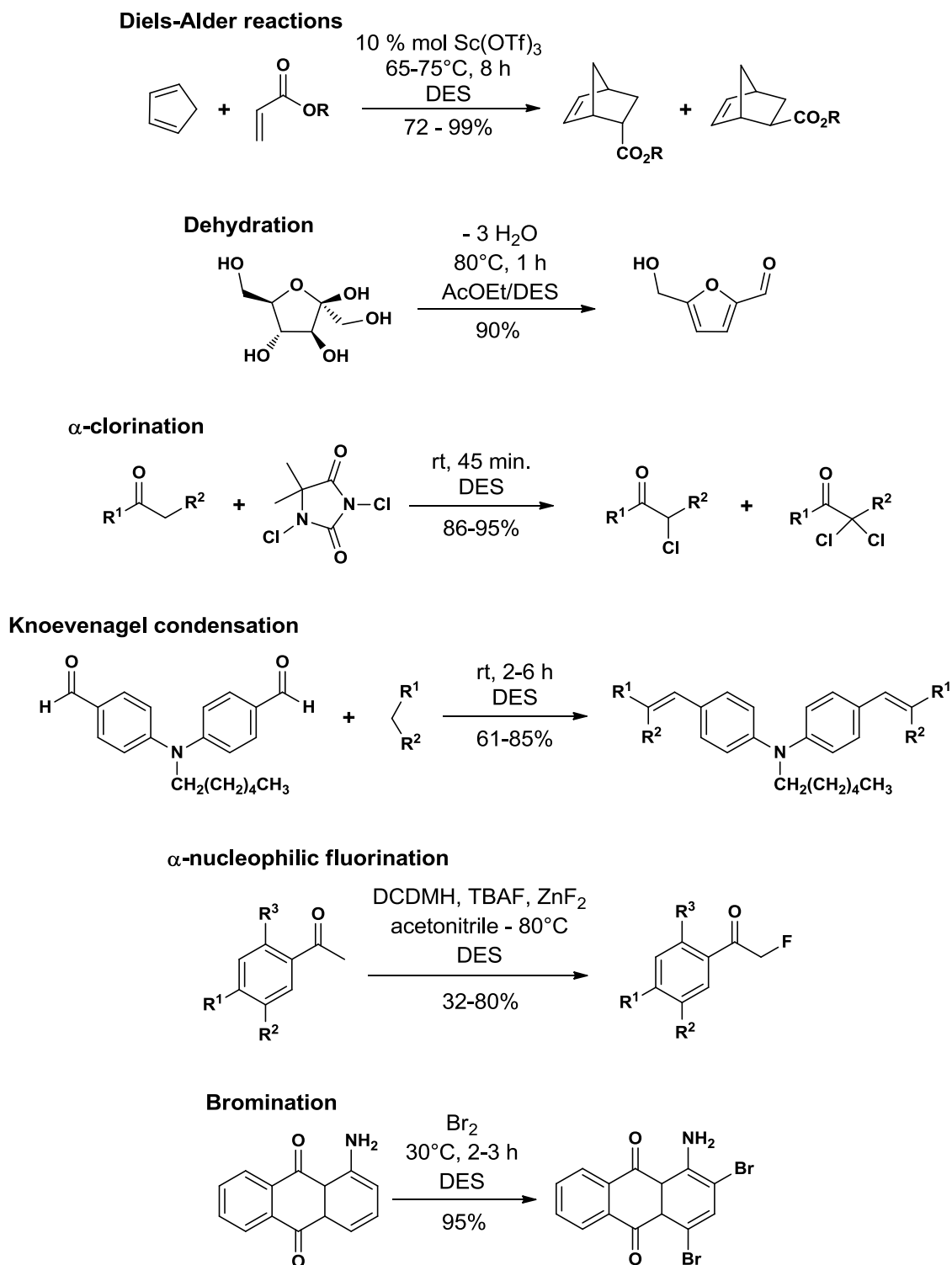


Figure 5. Some organic reactions in DESs (adapted from Imperato *et al.*, Hu *et al.*, Chen *et al.*, Phadtare *et al.*, Chen *et al.*, Sonawane *et al.* ^[97,99,100,103–105]).

As observed, Diels–Alder reactions are feasible in low-melting mixtures prepared from natural products, such as simple carbohydrates, sugar alcohols (e.g. fructose, sorbitol,

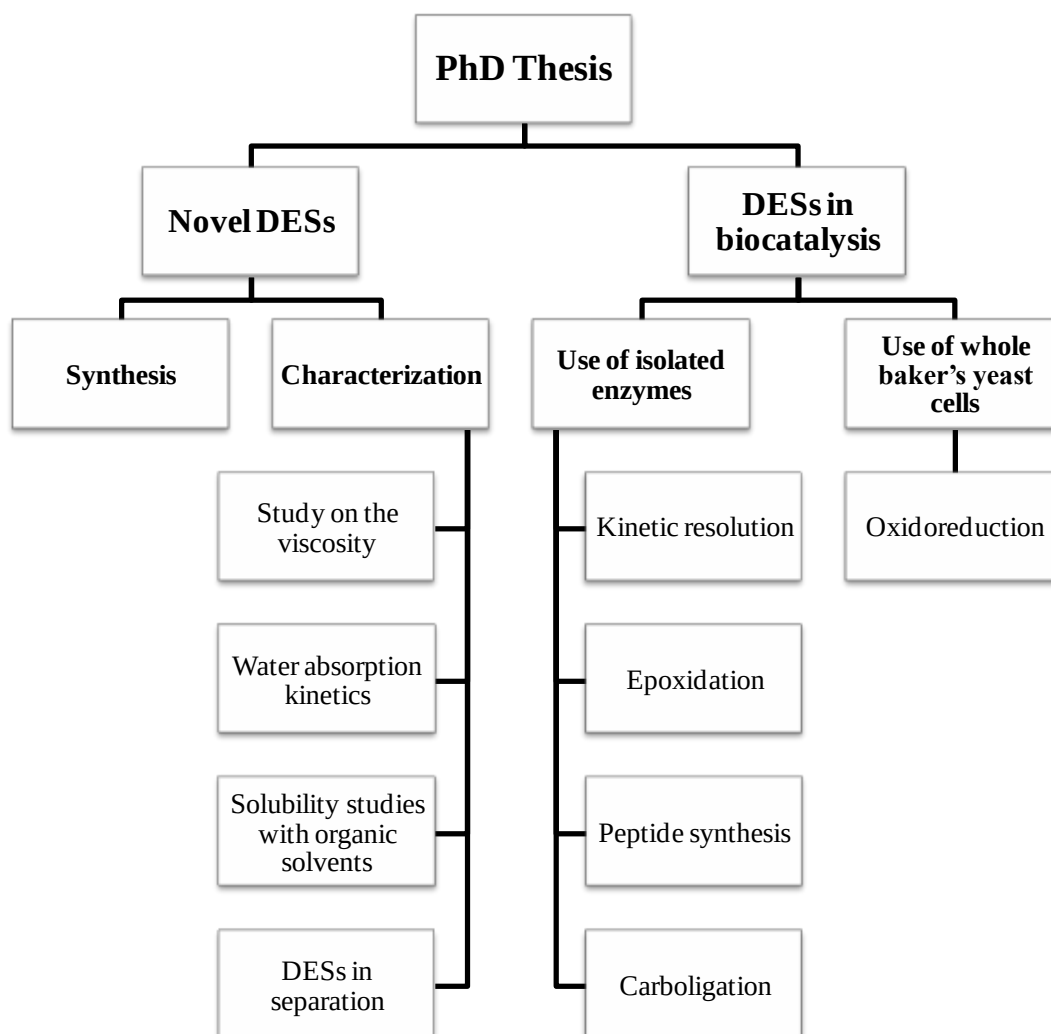
maltose, glucose) or citric acid, with urea or dimethylurea and inorganic salts.^[97] In addition, Hu *et al.* investigated the conversion of fructose to HMF in an ethyl acetate/DES biphasic system^[99], using ChCl-based DESs with different HBDs such as citric acid, oxalic acid, malonic acid, urea and some metal salts (e.g. ZnCl₂, CrCl₃). Yield and selectivity were, in some cases, higher than 90% and the DESs could be easily reused. Furthermore, chlorinated acetophenones and β -ketoesters were prepared straightforward at room temperatures and with high selectivity by using DCDMH in methanol or a ChCl:*p*-toluenesulfonic acid DES.^[100] The same research group developed a novel method for the direct one-pot fluorination of acetophenones to prepare α -fluoroacetophenones in the ChCl:*p*-toluenesulfonic acid DES.^[104] Sonawane *et al.* performed Knoevenagel condensations, showing that DESs were an efficient and convenient reaction media without using other toxic catalysts or hazardous organic solvents.^[105] Likewise, the catalyst-free bromination of 1-aminoanthra-9,10-quinones using molecular bromine in ChCl:urea DES was established by Phadtare *et al.*^[103], affording high isolated product yields.

2. AIM OF THE THESIS

As described in the introduction, DESs have numerous properties, such as their biodegradability and non-toxicity, easy and cost-effective synthesis, together with the great capability of dissolving a broad range of complex natural products and highly-functionalized molecules. Would it be possible to extend the number of the available DESs and which properties would the novel solvents have? For which applications might the (novel) DESs be used? Would they be suitable as (co)-solvents in biocatalysis and which advantages would they bring in comparison to the conventional media?

Trying to answer to all these questions, this PhD-Thesis will follow two research lines:

- 1) Synthesis of novel DESs with particular emphasis in the nature of HBDs, exploiting the possibility to create bio-based DESs and chiral solvents. Chemical and physical properties of the (novel) DESs, such as melting points, viscosities, acidities and water contents will be investigated and kinetic studies on water absorption will be undertaken. Moreover, the possibility of creating ternary DESs with two HBDs will be taken in consideration, in order to modulate the melting point and the corresponding viscosity of the resulting mixture. Likewise, the solubilities in protic and aprotic organic solvents will be assessed followed by a more comprehensive study on the interactions involved among the DESs' components. Finally the application of the (novel) DESs in separation of different mixtures will be explored.
- 2) Investigations on enzyme-catalyzed reactions, studying how different biocatalysts behave in these solvents. The activities of lipases will be validated and the effect of chiral DESs tested to see whether it is possible to modulate the enantioselectivity of a certain reaction. Special attention will be given to the reactions where challenging substrates are involved identifying the most suitable DESs able to solve these compounds. For the successful cases further investigations will be done to optimize the processes. Finally, for the first time the biocatalytic activity of microorganisms such as baker's yeast will be tested in DESs to understand whether whole cell biotransformation may be applied in DESs too.



3. RESULTS AND DISCUSSION

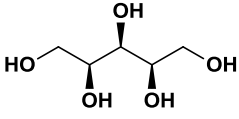
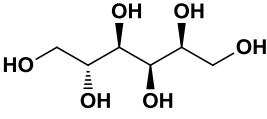
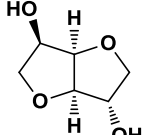
3.1. Novel DESs from biorenewable resources

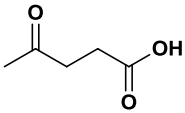
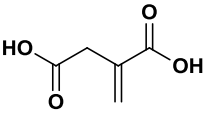
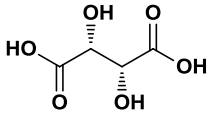
DESs are formed upon mixing of a quaternary ammonium salt with hydrogen-bond donors. One of the main features is their versatility by changing one or both components, leading to a broad number of DESs with different physicochemical properties and tailored for specific applications. Considering this fact – and given the largely unexplored area of DESs –, the first goal of this PhD Thesis was the synthesis and characterization of novel DESs with strong emphasis on the bio-based nature of HBDs, considering the increasing interest, in the last decades, for biomass-derived commodities and solvents.^[114–116]

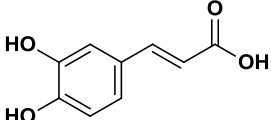
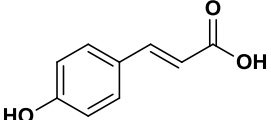
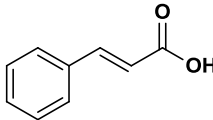
3.1.1. Preparation of binary bio-based DESs

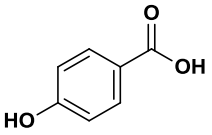
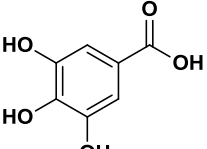
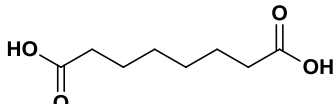
As halide component, the quaternary ammonium salt choline chloride (ChCl, B-complex vitamin) was chosen because of its relatively low price (ca. 50 €/Kg in Sigma Aldrich catalogue at low scale), non-toxicity and biodegradability.^[9,117] Different compounds were used as HBDs, such as bio-based carboxylic acids and sugar-related polyols. DESs' synthesis was straightforward through stirring the two compounds at 100°C for 15-120 minutes until a clear, homogenous liquid was formed, which was subsequently cooled down to room temperature. Several ChCl:HBD molar ratios (within a range of 1:0.5-1:2) were used to determine the eutectic point for each mixture (Table 7). All synthesized novel DESs exhibited melting points below 100°C, and liquid DESs at room temperature were obtained when xylitol, D-sorbitol, D-isosorbide and levulinic acid were used as HBDs. The depression in the melting point is strongly affected by the HBD due to the different nature of the interactions with ChCl. Interestingly when enantiomerically pure D-sorbitol, D-isosorbide or L-tartaric acid were used as HBDs chiral DESs were straightforwardly obtained. The different HBDs also influenced the viscosity of the DESs and the use of levulinic acid, xylitol and D-isosorbide led to low viscous liquids at room temperature.

Table 7. Novel ChCl-based DESs with renewable HBDs. mp measured only for solid DESs at RT, by heating them at a rate of 1°C/min and the mp was taken as the temperature at which the first liquid began to form. mp*= melting point of the HBD; mp = melting point of the synthesized DESs.

HBD			
	Xylitol	D-Sorbitol	D-Isosorbide
ChCl:HBD (molar ratio)	1:1	1:1	1:2
mp* (°C)	96	99	62
mp (°C)	Liquid at RT	Liquid at RT	Liquid at RT

HBD			
	Levulinic acid	Itaconic acid	L-Tartaric acid
ChCl:HBD (molar ratio)	1:2	1:0.5	1:0.5
mp* (°C)	32	166	171
mp (°C)	Liquid at RT	57 ± 3	47 ± 3

HBD			
	Caffeic acid	<i>p</i> -Coumaric acid	<i>trans</i> -Cinnamic acid
ChCl:HBD (molar ratio)	1:0.5	1:0.5	1:1
mp* (°C)	212	214	133
mp (°C)	67 ± 3	67 ± 3	93 ± 3

HBD			
	4-Hydroxybenzoic acid	Gallic acid	Suberic acid
ChCl:HBD (molar ratio)	1:0.5	1:0.5	1:1
mp* (°C)	215	251	142
mp (°C)	87 ± 3	77 ± 3	93 ± 3

3.1.2. Ternary systems with glycerol

Abbott *et al.* reported that the addition of ChCl to glycerol led to a mixture with lower density and viscosity^[15], revealing that this is not *just* a solution but that a DES is actually formed. Thus, while the viscosity of glycerol at 20°C is ca. 1200 cP, the corresponding ChCl:glycerol DES (1:2 molar ratio) exerts a much lower viscosity, of ca. 400 cP. The same research group observed a decrease of the viscosity when ethylene glycol was used with urea to form a ternary DES mixture. The DES with the composition ChCl:ethylene glycol:urea 1:1.5:0.5 molar ratio showed a viscosity of 56 cP, which is much lower in comparison with the one obtained with ChCl:urea (1:2 molar ratio), of ca. 800 cP.^[118] Based on these findings amounts of glycerol (up to 25 mol%) were added to the novel DESs, and ternary solvents with lower viscosity and melting points were obtained in all cases (Table 8). No DES was formed when suberic or gallic acid was used in ChCl:HBD molar ratios of 1:0.5 and 1:0.25 respectively without glycerol (both choline chloride and the HBD remained solid at 100°C after hours). Therefore, the possibility of envisaging DESs composed by ternary mixtures (ChCl, HBD and glycerol), not only leads to less viscous solvents, allowing mild reaction conditions for thermolabile substrates and/or catalysts, but also widen the number of possible combinations, and consequently achievable properties.

Table 8. Effect of glycerol addition on the DESs. mp[#] = melting point of the mixture without glycerol. mp = melting point of the three components mixture.

HBD	ChCl:HBD:Gly molar ratio	Mp [#] (°C)	Mp (°C)
Itaconic acid	1:0.5:0.5	57 ± 3	Liquid at RT
L-Tartaric acid	1:0.5:0.25	47 ± 3	Liquid at RT
4-Hydroxybenzoic acid	1:0.5:0.25	87 ± 3	63 ± 3
Caffeic acid	1:0.5:0.5	67 ± 3	Liquid at RT
<i>p</i> -Coumaric acid	1:0.5:0.25	67 ± 3	63 ± 3
<i>trans</i> -Cinnamic acid	1:1:0.5	93 ± 3	87 ± 3
Suberic acid	1:0.5:0.5	No DES formed	73 ± 3
Gallic acid	1:0.25:0.25	No DES formed	53 ± 3

Stimulated by these results, the focus was put on DESs composed by saccharides and polyols as HBDs. These DESs typically exhibit a neutral pH and such property could be

useful for some applications, especially when enzyme-friendly conditions are considered. Unfortunately as drawback they are highly viscous, especially at room temperature. To overcome this problem, the effect of the glycerol addition on the viscosity of some saccharide- and polyol-based DESs was studied (Figure 6).

In Figure 6 the viscosity η for each HBD as a function of the temperature is depicted. Over the addition of glycerol (25 mol%) a reduction of the viscosity, to less than 10% of the values of the binary mixtures, was observed, especially at lower temperatures. This might be the result of two main synergistic effects, the reduction of donor groups (hydroxyl groups) available to form hydrogen bonds with chlorine, and the disruption of the interactions within the sugar itself, with a consequent decrease of the DESs' density.

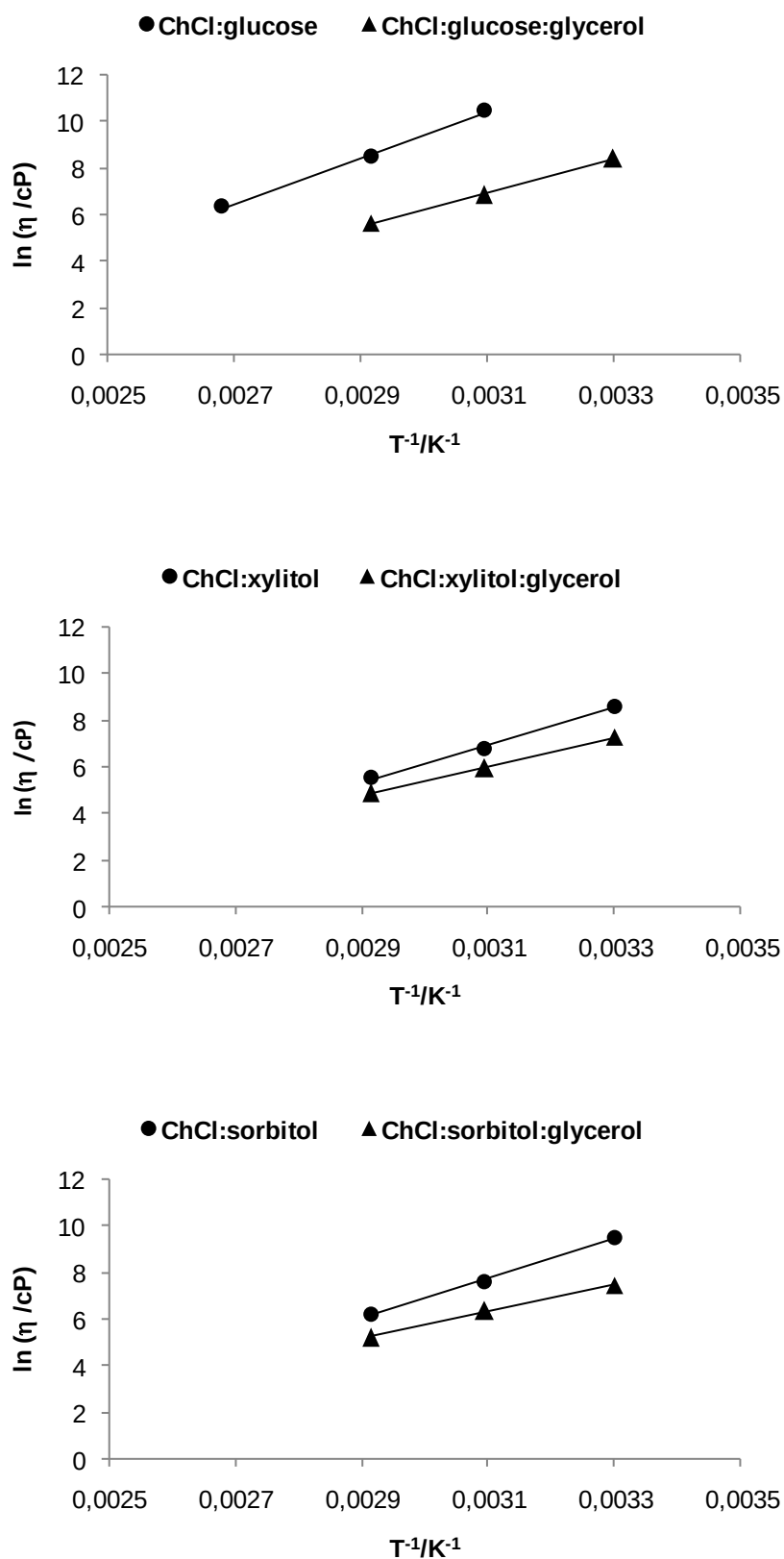


Figure 6. Viscosity as a function of the temperature of ChCl-based DESs, with and without glycerol addition (25 mol%).

The graphs depicted in Figure 6 clearly showed how the data followed an Arrhenius-like behaviour Equation:

$$\ln \eta = \ln \eta_0 + E_\eta / RT \quad (4)$$

where η_0 is a constant and E_η is the activation energy for viscous flow. In all studied cases DESs exhibited a Newtonian behavior. A fluid is said to be Newtonian if the viscous stresses that arise from its flow, at every point, are proportional to the local strain rate.^[119] In Table 9 the calculated E_η are given together with the coefficients of correlation R^2 . Data obtained on the new DESs were consistent with the ones calculated by Abbott *et al.* for some ILS.^[120]

Table 9. Activation energies for viscous flow (E_η) and coefficients of correlation (R^2) obtained by fitting the equation (4) to the data from the graph in Figure 6.

DES (molar ratio)	E_η (kJ·mol ⁻¹)	R^2
ChCl:glucose (1:1)	82.2	0.9973
ChCl:glucose:glycerol (1:0.5:0.5)	59.8	0.9984
ChCl:xylitol (1:1)	65.9	0.9948
ChCl:xylitol:glycerol (1:0.5:0.5)	51.7	0.9987
ChCl:sorbitol (1:1)	70.9	0.9985
ChCl:sorbitol:glycerol (1:0.5:0.5)	48.6	0.9984

3.1.3. Water absorption of levulinic acid-based DESs

When levulinic acid is used as HBD, the ChCl:HBD molar ratio that corresponds to the lowest eutectic point is 1:2 (Table 7). Interestingly, when levulinic acid was combined with ChCl in equimolar amounts, the mixture resulted in a white solid at room temperature. Owing to the hygroscopic nature of both components, the crystal tends to rapidly absorb water from the air humidity. It was observed that when a certain amount of water was incorporated the solid turned into a clear liquid at room temperature. To illustrate this, a layer of ca. 1 mm of ChCl:levulinic acid mixture (1:1 molar ratio) was spread over two glass Petri dishes, leaving one open while the other one closed (Figure 7). After few minutes the first drops appeared in the sample in contact with air, being completely liquefied within 1 hour. The water content

determined by Karl Fischer titration was 1.2 wt% in the solid mixture, increasing up to 9.5 wt% in the liquid form. The process was reversible by simply removing the water by evaporating it at 100°C in an open vessel. When the liquid was then cooled down it started to crystallize again. Because of its properties, such as biodegradability, possibility of reuse and low cost of the materials, this type of DES might be suitable for applications such as humidity sensors.

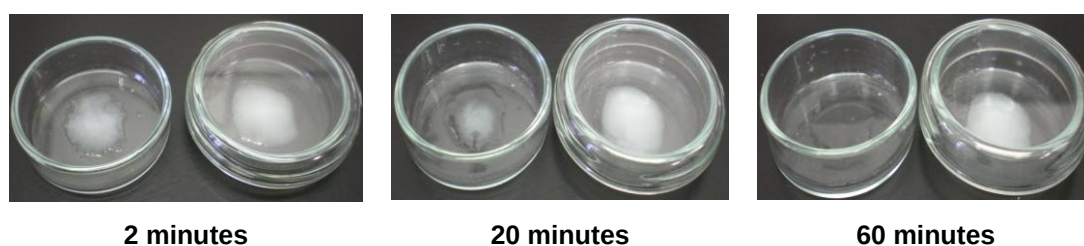
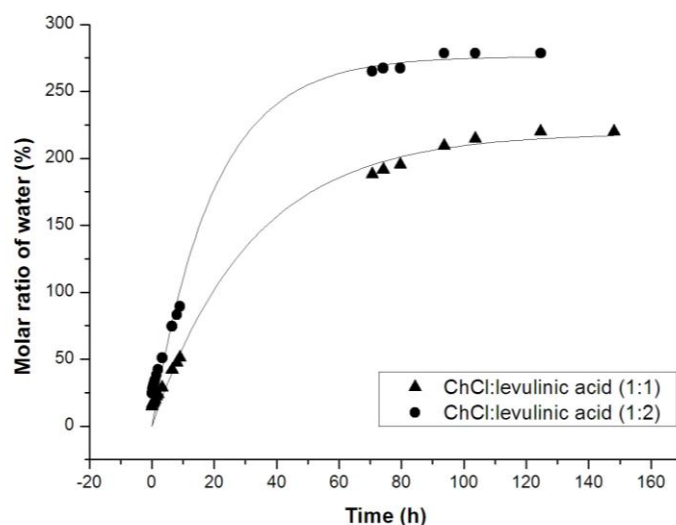


Figure 7. Glass Petri dishes with layers of ca. 1 mm thickness of ChCl:levulinic acid DES (1:1 molar ratio).

To study the effect more quantitatively gravimetric determinations of water absorption kinetics were performed on the ChCl:levulinic acid DES with two different compositions (1:1 and 1:2 molar ratios). The initial water contained in the DESs (measured by Karl Fischer titration, 1.2 wt%) was used as starting point. The water sorption was determined in a closed system at a specific humidity level of $50 \pm 3\%$ by measuring the weight variation over the time (Figure 8). The trends obtained were similar for both DESs and data could be accurately fitted into the Equation (5), recently proposed by Di Francesco *et al.*, to determine the water sorption kinetic for ILs^[121]:

$$M = M_{\infty}(1 - e^{-kt}) \quad (5)$$

where M is the water molar ratio, t is the time in hours, M_{∞} is the amount of water molecules absorbed by each DES molecule under equilibrium conditions and k is the sorption rate constant, providing information on how fast the equilibrium is reached. As a general observation, ChCl:levulinic acid mixtures with 1:2 molar ratio resulted to be more hydrophilic than equimolar combinations.



$$M = M_{\infty}(1 - e^{-kt})$$

DES	k (h^{-1})	M_{∞}	$k \cdot M_{\infty}$ (h^{-1})	R^2
ChCl:levulinic acid (1:1 molar ratio)	0.0314	219.0	6.88	0.9860
ChCl:levulinic acid (1:2 molar ratio)	0.0512	276.1	14.14	0.9770

Figure 8. Gravimetric determinations of water absorption on ChCl:levulinic acid DESs having two different compositions (1:1 and 1:2 molar ratio), in presence of a specific humidity level ($50 \pm 3\%$). Kinetic parameters obtained by fitting the equation (5) to the data of the graph.

3.1.4. Miscibility of the novel DESs with protic and aprotic solvents

According to Abbott *et al.*^[5], solvents forming hydrogen bonds with the halide salt tend to be miscible with DESs, whereas those not bearing hydrogen-bonding groups remain immiscible forming a second phase. Following these considerations, the miscibility of the novel DESs liquid at room temperature with several solvents was studied (Table 7). Most of the investigated DESs exhibited a behavior in agreement with the literature.^[5] Thus, DESs were completely soluble with protic solvents, such as water, methanol and ethanol, while with aprotic solvents, such as 2-methyltetrahydrofuran, ethyl acetate and acetone a biphasic system was formed.

Notably, in addition to these expected results, singular results were observed when levulinic acid and isosorbide were used as HBDs. The DESs formed with these molecules were miscible with protic solvents, however the addition of aprotic solvents led to the

precipitation of ChCl, presumably due to the disruption of the interactions among the DESs' components. To study this effect deeper, different volumes of acetone were added to the ChCl:levulinic acid DES. As shown in Figure 9, a surplus of ca. 10 equivalents of acetone led to the almost quantitative recovery of ChCl with no traces of impurities (analyzed by ^1H -NMR, data not shown).

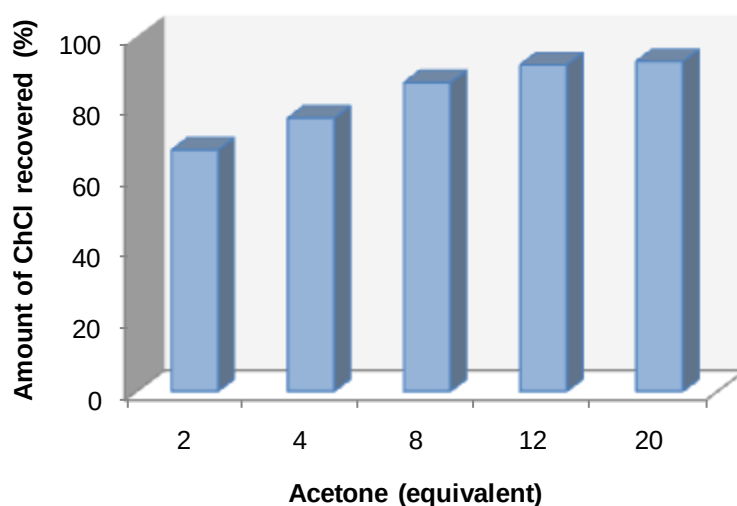


Figure 9. Efficiency in ChCl's recovery in correlation to the acetone equivalents added to ChCl:levulinic acid DES (1:2 molar ratio).

Based on these observed different properties, a new definition is proposed. Hence, when the addition of an aprotic solvent disrupted the interactions between the DES's components, leading to ChCl precipitation, then the DES was defined as "weak". Conversely, when the addition of an aprotic solvent resulted in a second phase, the DES was considered as "strong" (Figure 10). A broader study was performed on a number of ChCl-based DESs to understand what influences the strength of the interactions within DES's components (Table 10).

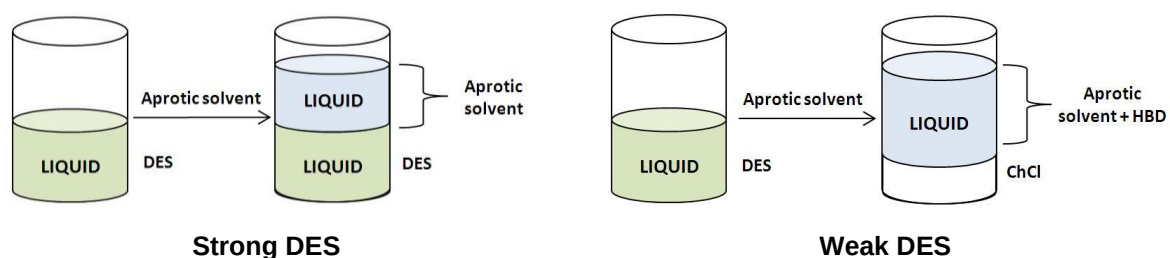
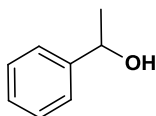
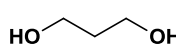
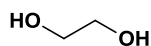
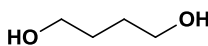
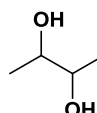
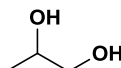
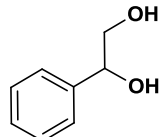
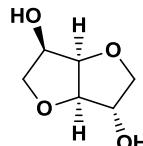
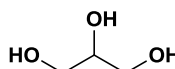
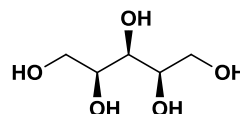
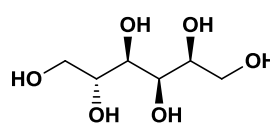
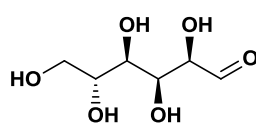
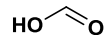
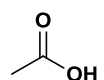
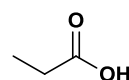
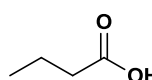
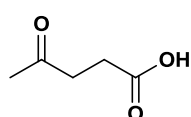
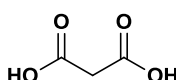
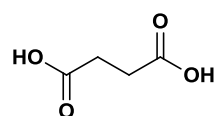


Figure 10. Visual representation of proposed "strong" and "weak" DESs.

Table 10. Classification of “strong” and “weak” DESs according to the proposed definition.

HBD (molar ratio ChCl:HBD)		Donor group (N° of donor groups)	Strength		
 Methanol (1:6)	 1-Phenylethanol (1:6)	OH (1)	Weak		
 1,3-Propanediol (1:3)	 Ethylene glycol (1:2)	 1,4-Butanediol (1:4)	OH (2)	Weak	
 2,3-Butanediol (1:5)	 1,2-Propanediol (1:3)	 1-Phenyl-1,2-ethanediol (1:2)			 D-Isosorbide (1:2)
 Glycerol (1:2)	 Xylitol (1:1)	OH (≥ 3)			Strong
 D-Sorbitol (1:1)	 Glucose (1:1)				
 Formic acid (1:2)	 Acetic acid (1:2)	COOH (1)	Weak		
 Propionic acid (1:4)	 Butanoic acid (1:4)			 Levulinic acid (1:2)	
 Malonic acid (1:1)	 Succinic acid (1:1)	COOH (2)	Strong		

DESs were prepared combining ChCl and HBDs bearing hydroxyl or carboxyl groups. Once the right ChCl:HBD molar ratio was found and a liquid at room temperature obtained, then acetone and AcOEt were added to mixtures in a DES:aprotic solvent ratio of 1:5 vol./vol.

Interestingly, it is possible to observe a correlation between the amount of hydrogen-donating groups within the HBD and the DES strength. When using HBDs bearing hydroxyl groups, the presence of only 1 or 2 functional groups in the HBD was not enough to form a strong DES and the addition of the aprotic solvent (ethyl acetate or acetone) broke the interactions leading to ChCl precipitation. On the other hand, for HBD containing three or more hydroxyl groups, the interactions were stronger leading to a biphasic system when the aprotic solvent was added. Conversely, when carboxylic acids were used as HBDs, the presence of only 2 groups (e.g. in of malonic and succinic acid) was already sufficient to form strong DESs probably due to the fact that the carboxyl group is highly polarized and more forceful interactions can be formed than the ones with the hydroxyl groups. This observation might trigger new applications in the field of DES.

3.1.5. DESs as separating agents for alcohol-ester mixtures

The above-studied property strong DESs possess, being miscible with protic solvents and immiscible with aprotic solvents, makes them certainly appealing for assessing separation applications. Several strategies in this area, published by different research groups, have been reported along the development of this PhD Thesis (see section 1.2.2.). Figure 11 shows a schematic representation of a novel separation method by using DESs.

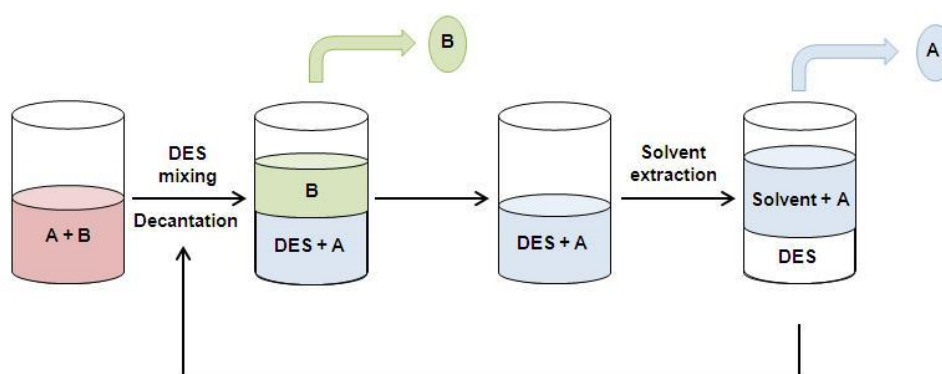


Figure 11. Schematic representation of the novel separation method using DESs. A = protic compound, B = aprotic compound.

To a mixture of an aprotic and a protic compound (e.g. alcohol-esters coming from enzymatic kinetic resolutions) a DES is added forming two phases with the reaction mixture.

The upper phase – mostly composed of aprotic compound –, can be easily removed and subjected to further extractions until a specific purity is reached. By using an organic solvent the protic compound is then extracted from the DES phase and the latter one can be reused for further cycles.

One example of practical relevance might be the kinetic resolution of racemic alcohols to afford optically active compounds. In this particular reaction the work-up is a key step because the separation of both enantiomers (the unreacted and the reacted one) must be carried out efficiently (high recovery and purity). Many possible ways have been used in literature and in some cases the classical chromatographic methods have been replaced by novel techniques such as the use of polymer-supported substrates^[122–126], fluorous solvents^[127,128], ILs as a media (alone or together with scCO₂) or acetylating agents^[127,129,130], membrane^[131–133], derivatization of products (e.g. by sulfation)^[134], anhydrides as acyl donors^[135] etc.

To validate the concept, the transesterification of *rac*-1-phenylethanol with two acyl donors (vinyl or isopropenyl acetate), catalyzed by immobilized lipase from *Candida antarctica* B was conducted under solvent-free conditions (Figure 12). Performing reactions in neat substrates avoids the use of hazardous solvents, and at the same time may provide conditions for high substrate loadings and high productivities. Interestingly, despite the ample use of lipases in organic media, the use of “solvent-free” conditions in lipase-catalyzed enantioselective reactions has been surprisingly not so extensively investigated, with only few examples reported, mostly using low substrate loadings.^[136–140]

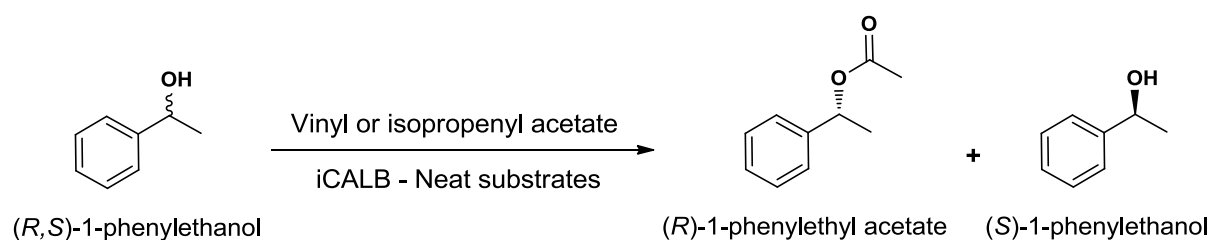


Figure 12. Kinetic resolution of racemic 1-phenylethanol catalyzed by iCALB in neat substrates.

In a first step, the alcohol:acyl donor ratio was assessed and after 22 h excellent conversions (maximum conversion for kinetic resolutions is 50%) were obtained (Figure 13).

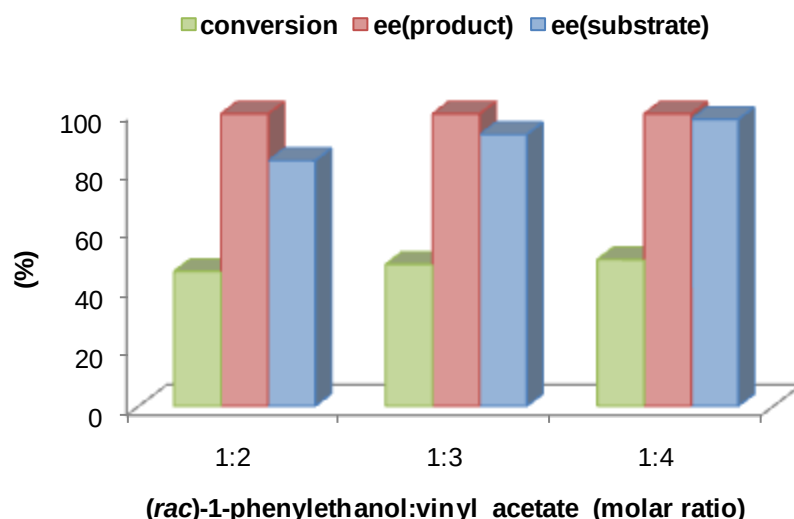


Figure 13. iCALB-catalyzed kinetic resolution of *rac*-1-phenylethanol in neat substrates using different amounts of vinyl acetate as acyl donor. Reaction conditions: enzyme loadings $10 \text{ mg} \cdot \text{mL}^{-1}$, 60°C , 22 h.

No differences were observed between the two acyl donors (data not shown for the isopropenyl acetate). The enantioselectivity (E), calculated using the Chen equation^[141], was high in all cases ($E > 200$), indicating the preference of the lipase to the *R* enantiomer. Subsequently, the process was optimized with regard to enzyme loadings and reaction time (Figure 14).

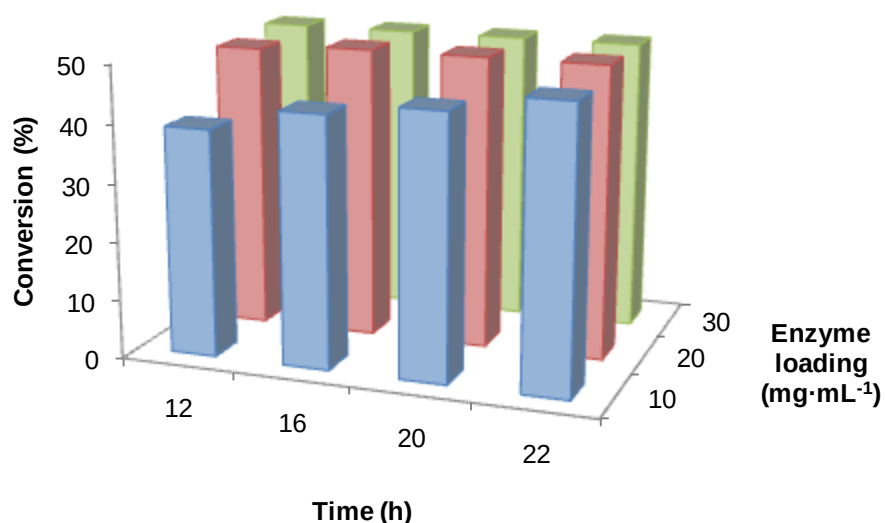


Figure 14. Influence of the enzyme loadings on the reaction rate using vinyl acetate. Reaction conditions: 2.6 mmol of *rac*-1-phenylethanol, 7.2 mmol of acyl donor, 60°C .

Maximum conversion was achieved after ca. 12 h with enzyme loadings of 30 mg·mL⁻¹ at 60°C with enantiomeric excesses of 99% for both the (*S*)-1-phenylethanol and (*R*)-1-phenylethyl acetate. The optimized process led to productivities of ca. 300 g·L⁻¹·d⁻¹ for the chiral acetate. Based on these optimized results, a preliminary economic assessment on the enzyme contribution cost was conducted (Table 11). Assuming reactions with only one enzyme cycle, the total contribution costs of starting materials would be in the range of ca. 0.3% of the final product price. The possibility of enzyme recycling was then studied. Once the reaction was performed, the recycle of the iCALB was done by filtering it off, washing it with 2-propanol and directly using it in the next catalytic cycle. As it can be observed in the Figure 15, half of the maximal activity was reached after 4 and 5 cycles when vinyl and isopropenyl acetate were used, respectively.

Table 11. Preliminary economic assessment on the enzyme contribution costs for the kinetic resolution of *rac*-1-phenylethanol. Reaction conditions: alcohol:acyl donor 1:3 molar ratio, enzyme loadings 30 g·L⁻¹. * Prices from Sigma-Aldrich, ** Price from c-LEcta GmbH.

	<i>rac</i> -1-Phenylethanol*	Vinyl acetate*	CALB**	Commercial available (<i>S</i>)-1-phenylethanol*
Price	96 €·Kg ⁻¹	22 €·L ⁻¹	700 €·Kg ⁻¹	
Amount	2 kg	5 L	0.2 Kg	
Cost	192 €	110 €	140 €	
Total cost	442 €·Kg⁻¹			145000 €·Kg⁻¹

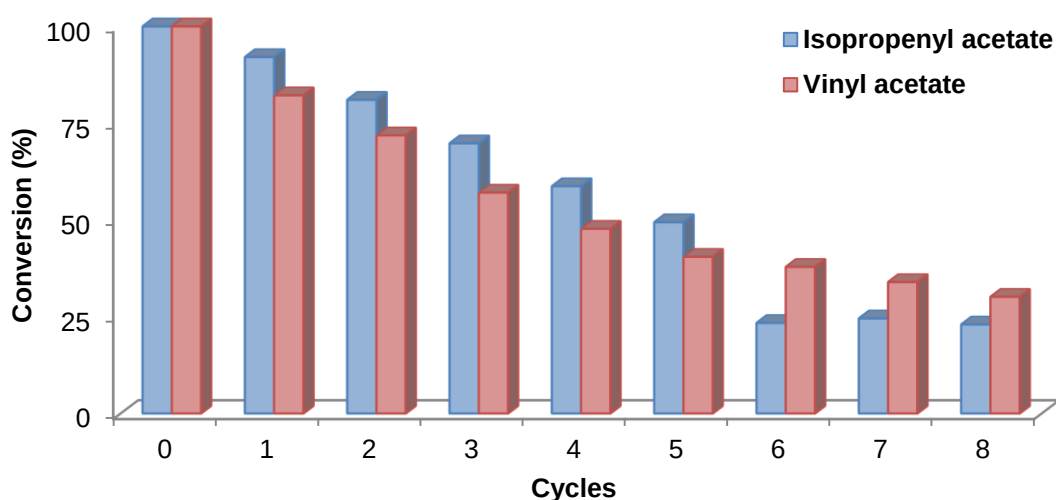


Figure 15. Enzyme recycling assessment (3 h per cycle). Reaction conditions: *rac*-1-phenyl ethanol:acyl donor 1:3 molar ratio; enzyme loading 30 mg·mL⁻¹, 60°C.

Once the kinetic resolution was performed, the enzyme was filtered off and the excess of the acyl donor straightforwardly stripped out. A ChCl:glycerol DES (1:2 molar ratio) was added to the (*S*)-1-phenylethanol and (*R*)-1-phenylethyl acetate mixture (mixture:DES 1:5 vol./vol.), mixed and centrifuged at 4000 rpm for 4 minutes. The upper phase – mostly containing the ester compound - was removed and analyzed by ^1H -NMR. This phase was subjected to further extractions with DES and after four cycles 67 wt% of the initial amount of the ester was recovered with 99% purity (Figure 16 a). Similarly, a mixture of *rac*-1-phenylethanol and its corresponding butyl ester was subjected to the same procedure leading to even higher efficiencies. After four extractive steps 82% of ester was recovered with purity higher than 99% (Figure 16 b).

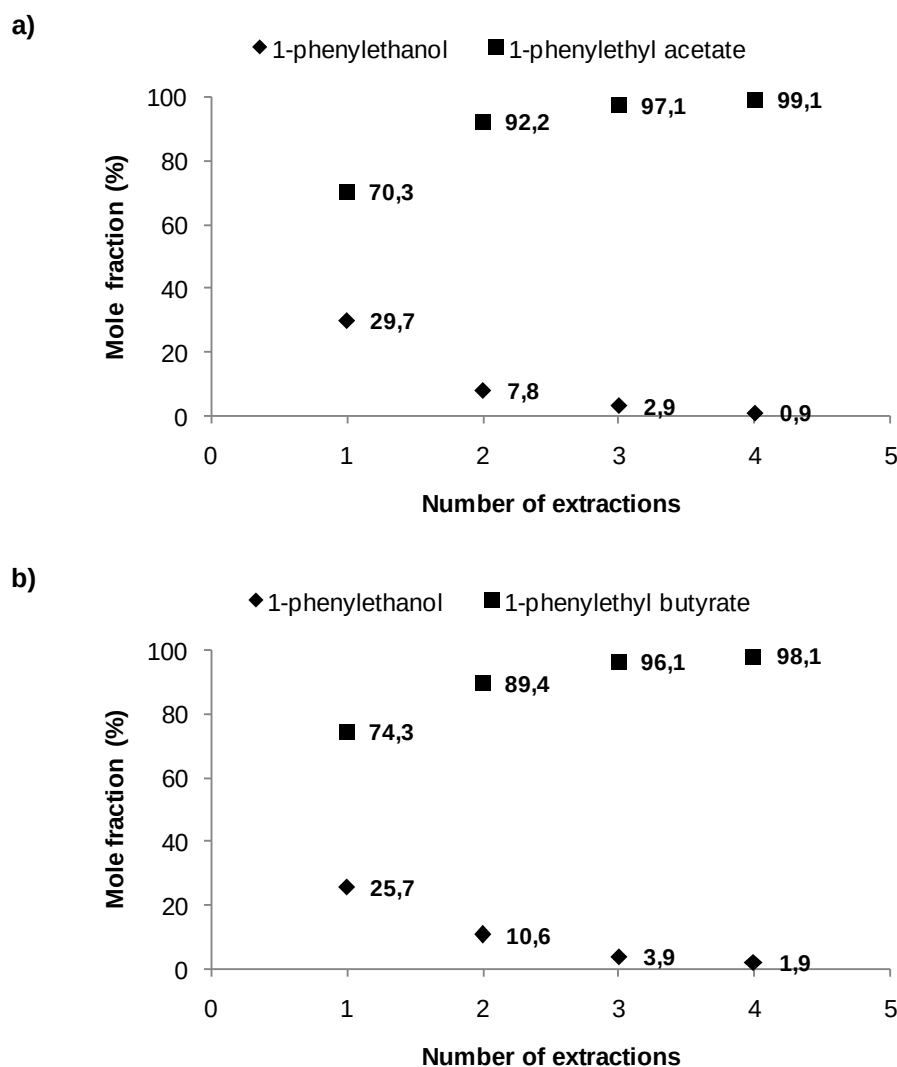
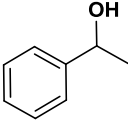
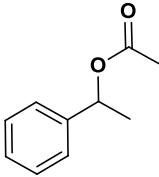
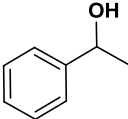
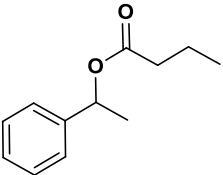
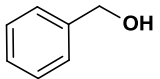
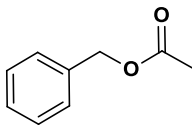
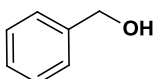
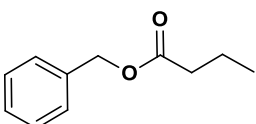
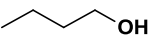
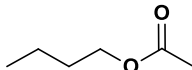
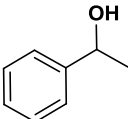
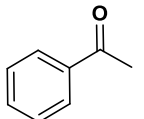
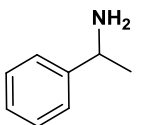
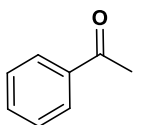


Figure 16. Composition of the upper phase after the extraction with ChCl:glycerol DES (1:2 molar ratio) of a mixture of 1-phenyl-ethanol and the corresponding ester: a) 1-phenylethyl acetate, b) 1-phenylethyl butyrate. Mixture:DES ratio of 1:5 vol./vol.

The same extractive conditions were applied to other alcohol-ester, alcohol-ketone and amine-ketone mixtures, to evaluate the versatility of this new separation method (Table 12).

Table 12. Extraction of equimolar alcohol-ester, alcohol-ketone, and amine-ketone mixtures with glycerol-based DES (ChCl:glycerol 1:2 molar ratio). Mixture:DES 1:5 vol./vol.

Mixture		N° of extractions	Purity of the recovered ester or ketone (%)	Recovered amount of the ester or ketone (%)
Protic compound	Aprotic compound			
		4	99	67
1-phenylethanol	1-phenylethyl acetate			
		4	98	82
1-phenylethanol	1-phenylethyl butyrate			
		3	99	83
benzyl alcohol	benzyl acetate			
		3	> 99	91
benzyl alcohol	benzyl butyrate			
		4	86	70
1-butanol	butyl acetate			
		3	94	40
1-phenylethanol	acetophenone			
		2	90	51
1-phenylethanamine	acetophenone			

As observed in Table 12, for all studied cases purities of 85–99% were achieved for the upper phase (the one containing the aprotic compound - the ester or the ketone), albeit with less efficiency when ketone was present in the mixture. This might be due to the fact that ketones are partially soluble in the DES phase and then do not get easily separated from the mixture as in the other cases. The broad adaptability of DESs may enable the design of other mixtures with tailored properties for the separation of specific mixtures. Likewise, the set-up of continuous extractive approaches may represent a promising research line for chemical engineering.

Once the upper phase, containing the ester or the ketone, was removed, the remnant mixture – containing alcohol and still some ester – was extracted from the DES phase. To an equimolar mixture of 1-phenylethanol and 1-phenylethyl-acetate ChCl:glycerol DES (1:2 molar ratio) was added (mixture:DES 1:5 vol./vol.), mixed and centrifuged at 4000 rpm for 4 minutes. The upper phase was removed and the DES phase (containing mostly the alcohol) was then subjected to extraction with several solutions of water:organic solvents. The amount of water was added to facilitate the extraction by breaking the interactions among the DES's components. Best results were obtained with both ethyl acetate or ethyl acetate:water mixture (Table 13), leading to efficiencies of ca. 90% in alcohol recovery.

Table 13. Extraction of the alcohol-ester mixture from the DES phase. Results obtained after 3 extractions by using ethyl acetate or ethyl acetate and water. The extracted glycerol is ca. 0.4% of the one present in the DES, and therefore it does not affect the DES properties for reusability. *2:1 vol./vol. **It is referred to the amount of alcohol-ester mixture recovered from the DES phase.

DES:solvent vol./vol.	Solvent	Mole fraction in the recovered mixture** (%)				Recovered Mixture** (%)
		Alcohol	Ester	Glycerol	ChCl	
5:1	AcOEt	85	9	6	0	86
5:0.75	AcOEt:water*	84	12	4	0	89

Furthermore, this novel method has been successfully applied by Krystof *et al.* to separate 5-hydroxymethylfurfural (HMF) from its esters, after the lipase-catalyzed transesterification of HMF with several acyl donors.^[142] Several DESs were synthesized and tested, and high ester purities (> 99%) and efficiencies (up to 90% in recovery of HMF-ester)

were achieved.

The results obtained with these diverse mixtures represent the first proofs-of-concept showing that this novel separation method can be successfully applied in different cases, avoiding the need of further chromatographic purification steps – or significantly simplifying them – in many synthetic applications (not only related to kinetic resolution of racemates). Due to their broad versatility, tailored DESs might provide better separation profiles (e.g. with higher efficiencies) for these or different mixtures as well.

3.2. Assessment of DESs in biocatalysis

The features of DESs such as biodegradability and non-toxicity, straightforward and inexpensive preparation, together with their excellent capability of dissolving a broad range of complex natural products and highly functionalized molecules, make them appealing novel solvents for biocatalysis. On this basis, the second part of this PhD Thesis focuses on the application of DESs in several enzyme-catalyzed reactions. By looking at the state-of-the-art, previous published results obtained with lipases showed that these enzymes could actually work with these solvents and the first experiments were done to validate these results, exploring as well the possibility of using new DESs. Later on several novel aspects were considered about DESs and biocatalysis and the developed work focused on:

- Use of chiral DESs with hydrolases to assess enantioselective peracid-mediated epoxidations based on biocatalytic diversity.
- Application of DESs in peptide synthesis as an example of dissolving challenging substrates with different polarities, investigating at the same time how proteases behave in these solvents.
- Application of DESs as novel (co)-solvents for lyases catalyzing different carboligation reactions (enantioselective C-C bond forming reactions).
- First evidence of using resting whole-cells (baker's yeast) as biocatalysts in DESs to perform enantioselective reductions of ketones affording optically active alcohols.

3.2.1. Kinetic resolution of racemic 1-phenylethanol

To validate the previous literature, the first reaction investigated in deep-eutectic-solvents was the kinetic resolution of *rac*-1-phenylethanol, catalyzed by the immobilized form of lipase from *Candida antarctica* B (Figure 17).

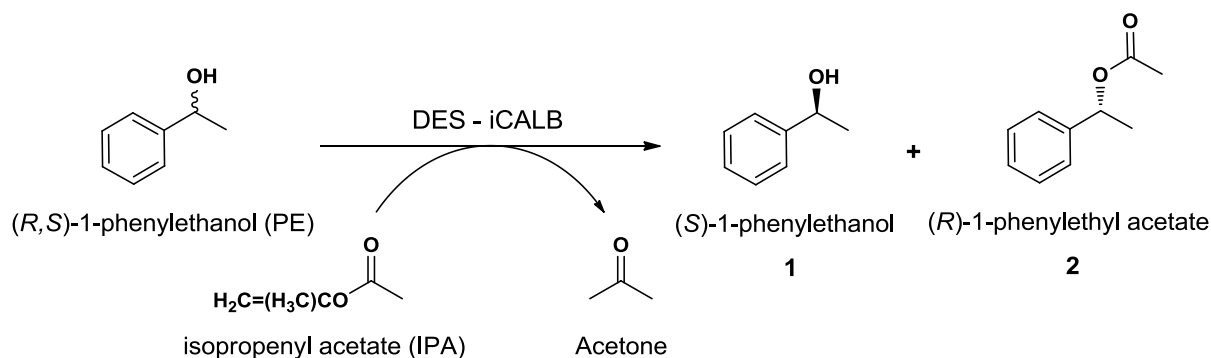
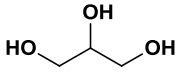
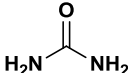
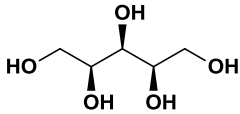
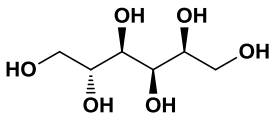
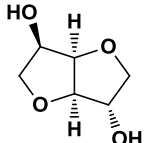
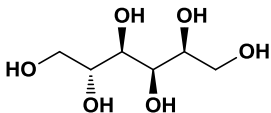
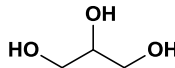


Figure 17. Kinetic resolution of *rac*-1-phenylethanol with isopropenyl acetate as acyl donor, biocatalyzed by iCALB, in DES.

The reaction is well known in literature in different non-conventional media such as organic solvents^[136,143–157], ILs^[60,129,130,158–163] or in solvent-free conditions (see section 3.1.5.). In these non-aqueous media the equilibrium can be shifted towards the (trans)-esterification of the alcohol rather than to the hydrolysis, which would proceed in water, the natural media of enzymes. Isopropenyl acetate was used as acyl donor, releasing isopropenyl alcohol that tautomerizes to acetone, thus making the reaction thermodynamically irreversible. Lipases, (triacylglycerol acyl hydrolases, EC 3.1.1.3) have been well established as valuable catalysts in organic synthesis due to their ability to perform enantioselective (trans)-esterifications in non-aqueous media – accepting a broad range of non-natural substrates –, and robustly working in absence of cofactors. Concerning the media, lipases have been used successfully both in apolar and polar organic solvents, as well in neat substrates.^[164] The application range of CALB does not exclusively involve carboxylic acids, but also esters such as methyl and ethyl esters, or other activated compounds such as trifluoroethyl esters, isopropenyl and vinyl, acrylic and methacrylic acid.^[164–166]

The screened ChCl-based DESs for this reaction were synthesized by using as HBDs molecules both already reported in literature such as glycerol and urea, and novel such as xylitol, D-sorbitol and D-isosorbide (structures and compositions in Table 14).

Table 14. Screened DESs in the reactions performed with iCALB.

HBD(1)	HBD(2)	ChCl:HBD(1) or ChCl:HBD(1):HBD(2) molar ratio	Name
 Glycerol	none	1:2	G-DES
 Urea	none	1:2	U-DES
 Xylitol	none	1:1	X-DES
 D-Sorbitol	none	1:1	S-DES
 D-Isosorbide	none	1:2	I-DES
 D-Sorbitol	 Glycerol	1:1:0.5	SG-DES

Transesterification reactions were first performed in organic solvents to benchmark the results in DESs. As shown in Table 15, the use of apolar solvents such as toluene and isooctane (log P 2.5 and 4.5 respectively) led to better results, with maximum conversions (50% for a kinetic resolution) and high enantiomeric excesses for both ester and the unreacted enantiomer of 1-phenylethanol ($E > 200$).

Table 15. Kinetic resolution of *rac*-1-phenylethanol in organic solvents and DESs. Reaction conditions: PE 40 mM, PE:IPA 1:3 molar ratio, enzyme loading 10 mg·mL⁻¹, 60°C, 24 h. Experiments performed in G8 vials, 6 mL volume. ee_1 : enantiomeric excess of the alcohol, ee_2 : enantiomeric excess of the produced ester. Conversions and ee calculated by gas chromatography (GC).

Organic solvent	Conversion (%)	ee_1 (%)	ee_2 (%)	E
MTHF	15	17	99	> 200
Toluene	50	99	99	> 200
Isooctane	50	99	99	> 200
U-DES	6	7	99	> 200
G-DES	1	1	99	> 200

The first two investigated DESs were G-DES and U-DES (Table 15), with lower conversions compared with the reactions in isooctane and toluene in both cases. However, the excellent enantioselectivity (E) observed in the organic solvents was maintained in DES. This low conversion values might be related to many causes. Even though the reaction temperature (60°C) allowed the solvents to be less viscous, the mixing was not optimal due to the volume and the reactor used for the reaction, causing the remaining of iCALB on the surface, rather than being completely dispersed in the solution. The miscibility of the reagents in the DESs might cause other mass-transfer limitation challenges. As already mentioned, strong DESs dissolve protic compounds whereas aprotic ones form a second phase. Thus, isopropenyl acetate in contact with strong U-DES or G-DES formed a second phase and released low substrate concentration to the active phase (DES) for the transesterification. To improve the mixing, a different set-up for the reactions was chosen. Keeping the size of the vial used in the experiments (G8 vial) unvaried, the volume of the solution was scaled down from 6 to 1 mL under the same reaction conditions (Table 16).

Table 16. Lipase-catalyzed kinetic resolution of *rac*-1-phenylethanol in DESs. Reaction conditions: PE 40 mM, PE:IPA 1:3 molar ratio, enzyme loading 10 mg·mL⁻¹, 60°C, 24 h. Experiments performed in G8 vials, 1 mL volume. Conversions and *ee* calculated by GC.

Organic solvent	Conversion (%)	<i>ee</i> ₁ (%)	<i>ee</i> ₂ (%)	E
Isooctane	50	99	99	> 200
U-DES	41	68	99	> 200
G-DES	2	1	99	> 200

As observed, a significant improvement of ca. 700-fold in conversion was reached when U-DES was used as a media under the new process set-up conditions. This demonstrates the high stability of iCALB in this solvent, despite the known denaturing behavior of urea towards proteins, quite consistent with previous observations for lipases in analogous DES.^[45,46,50,52,53] Conversely the low conversion observed in G-DES remained practically the same as before when the not optimized reaction conditions were set. A reason might be unwanted side reactions occurring between the DES's components and IPA (Figure 18). Bearing glycerol and ChCl hydroxyl groups, they may be subjected to enzymatically catalyzed transesterifications with the acyl donor as well. Consistent with these findings, Durand *et al.*, evaluating DESs in CALB-catalyzed transesterifications of vinyl acetate with several alcohols, observed side reactions as well when the HBD was a dicarboxylic acid or ethylene glycol (see section 1.2.3.).^[52]

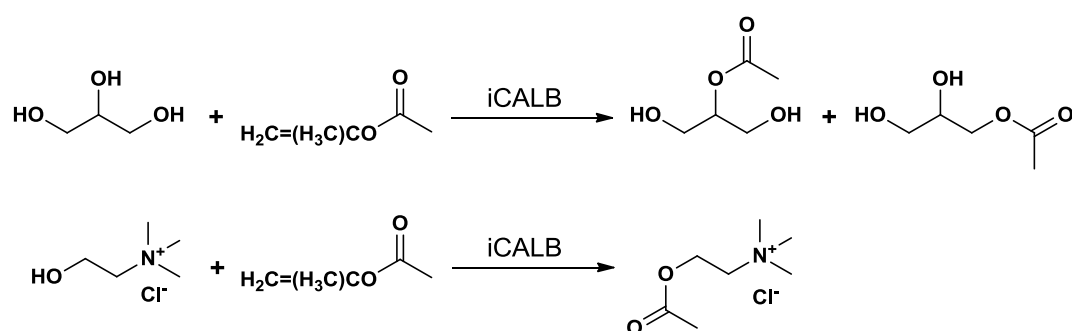


Figure 18. Possible transesterification reactions occurring between the DES's components and IPA. Similar secondary reactions have been reported in literature as well (adapted from Gorke *et al.*, Durand *et al.*^[46,52]).

Once proper reaction conditions were set for U-DES, other DESs were screened for that prototypical reaction (Figure 19). Other DESs led to lower conversions than those observed for U-DES, albeit higher than those obtained with G-DES. The HBDs xylitol, sorbitol and isosorbide, bearing hydroxyl groups too, were transesterified as well (as observed in GC analysis). Noteworthy, the enantioselectivity remained high in all cases ($E > 200$), indicating that the enzyme, not only keeps its activity in all investigated DESs but also its conformation and stereoselectivity.

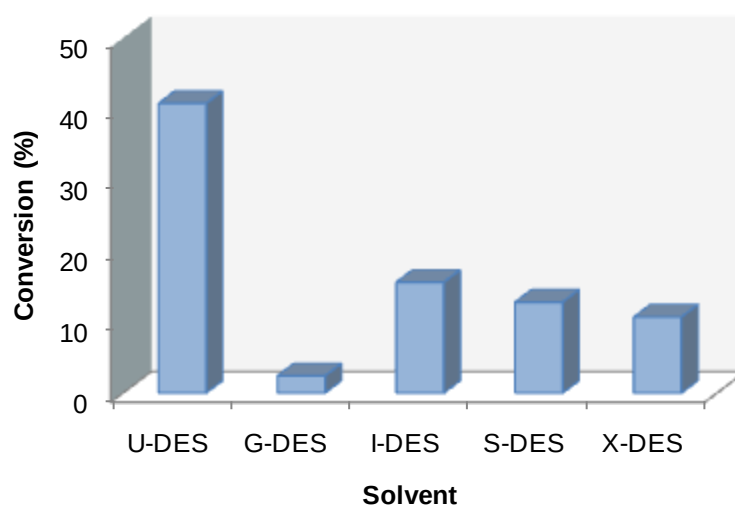


Figure 19. Lipase-catalyzed kinetic resolution of *rac*-1-phenylethanol in DESs. Reaction conditions: PE 40 mM, PE:IPA 1:3 molar ratio, enzyme loading $10 \text{ mg} \cdot \text{mL}^{-1}$, 60°C , 24 h. Experiments performed in G8 vials, volume of the solutions 1 mL. Conversions and *ee* calculated by GC.

3.2.2. Epoxidation of styrene via peracid formation

In section 3.1. the possibility to straightforwardly synthesize biomass-derived chiral DESs was shown when D-sorbitol, D-isosorbide or L-tartaric acid were utilized as HBDs. Renewable and inexpensive chiral DESs might represent a valuable tool to carry out enantioselective and asymmetric reactions. In this context, several biomass-derived DESs were screened in the lipase-mediated epoxidation of styrene *via* peracid formation (Figure 20). The lipase generates *in situ* the peroxycarboxylic acid, which subsequently epoxides alkenes. Peroxycarboxylic acids are commonly used as oxidant reagents in synthesis but their application is often hampered by their hazardous handling and storage. The possibility of generating peroxycarboxylic acids *in situ* directly from carboxylic acids (or esters) by using

lipases was firstly reported by Björkling *et al.* in the 90's^[167,168] and then later applied by several authors to different systems.^[46,169–173]

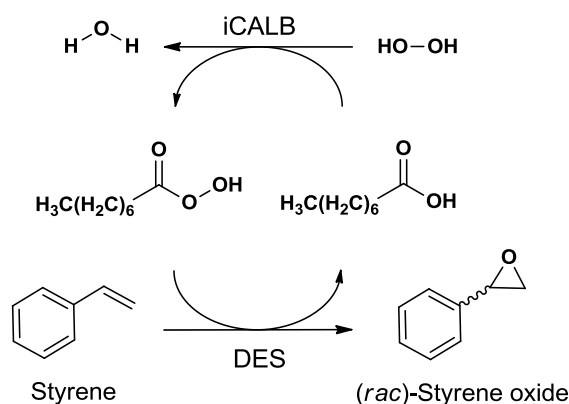


Figure 20. Epoxidation of styrene *via* peracid formation, catalyzed by *i*CALB in DESs.

Once the peracid is biocatalytically formed the alkene epoxidation proceeds non-enzymatically and thus leads to a racemic product. Chiral DESs were used as reaction media to investigate whether a chiral environment could influence the enantioselectivity of the reaction (Table 14). The use of a ternary DES, using glycerol as a second HBD, was necessary due to the high viscosity observed at room temperature when the HBD D-sorbitol was used. As previously described (section 3.1.2.), the addition of glycerol on a binary mixture displayed a relevant effect, especially at lower temperatures, with viscosity reductions higher than 90%. In Table 17 the obtained results are shown.

Table 17. Epoxidation of styrene *via* peracid formation, mediated by *i*CALB. Reaction conditions: styrene 0.1 mmol, octanoic acid 0.01 mmol, 0.12 mmol of H_2O_2 30% (added in 4 portions every hour), enzyme loading $10 \text{ mg} \cdot \text{mL}^{-1}$, RT, 24 h. Conversions and *ee* calculated by GC.

Solvent	Conversion (%)	<i>ee</i> of (<i>S</i>)-styrene oxide (%)
Ethyl acetate	11	1
Acetonitrile	1	1
Achiral DES	U-DES	3
	G-DES	3
Chiral DES	I-DES	2
	SG-DES	1

First experiments, performed in ethyl acetate and acetonitrile, led to low conversions and racemates as expected ($\pm 1\%$ deviation is within the instrumental error). At the same conditions, the reaction was carried out in chiral and achiral DESs afterwards. For all of the screened DESs low values of conversions were obtained and basis on these results it was difficult to say whether the enantioselectivities were really influenced by the chosen solvent or were just due to instrumental errors and traces of impurities. Further tuneability on the DESs might be considered to improve these results in future experiments and clearly more research would be needed to fully understand the results obtained.

3.2.3. Dipeptide synthesis catalyzed by α -chymotrypsin

As already discussed (section 1.2.1.), DESs enable the dissolution of a broad range of complex highly-functionalized natural products and macromolecules. In enzymatic peptide synthesis, where challenges arise from the low (and often different) solubility of substrates in the reaction media, the use of DESs may offer valuable options. Aqueous solutions (natural media for enzymes), are not recommended as water tends to shift the equilibrium towards the hydrolysis rather than the desired peptide synthesis. For such purposes, the production of peptides with the use of either chemo- or enzymatic catalysts have been performed in non-conventional media, such as organic solvents and ILs.^[174–193] Another applied approach is based on the formation of eutectic mixtures of substrates composed by amino acid derivates together with some hydrophilic solvents, called “adjuvants”.^[194–199] In this way it is possible to work with high substrate loadings. Zhao *et al.* reported an example of a protease-catalyzed transesterification in DESs^[51] but so far peptide synthesis reactions have not been explored in these solvents.

The α -chymotrypsin-catalyzed synthesis of protected N-acetyl-phenylalanine-glycinamide dipeptide (APG), starting from N-acetyl-phenylalanine ethyl ester (APEE) and glycinamide hydrochloride (GH), was investigated in DESs displayed in Figure 21 together with the reaction scheme. The chosen α -chymotrypsin (protease E.C. 3.4.21.1) is a well investigated catalyst in peptide synthesis^[41] and displays strong hydrolytic specificity for aromatic amino acids (e.g. tyrosine, tryptophan and phenylalanine) in aqueous solutions. Conversely, in media with low water-activity (trans)-esterification reactions with aromatic amino acid analogues are catalyzed by that enzyme, retaining activity in a wide range of organic solvents and other non-conventional media such as ILs.^[188,200–205]

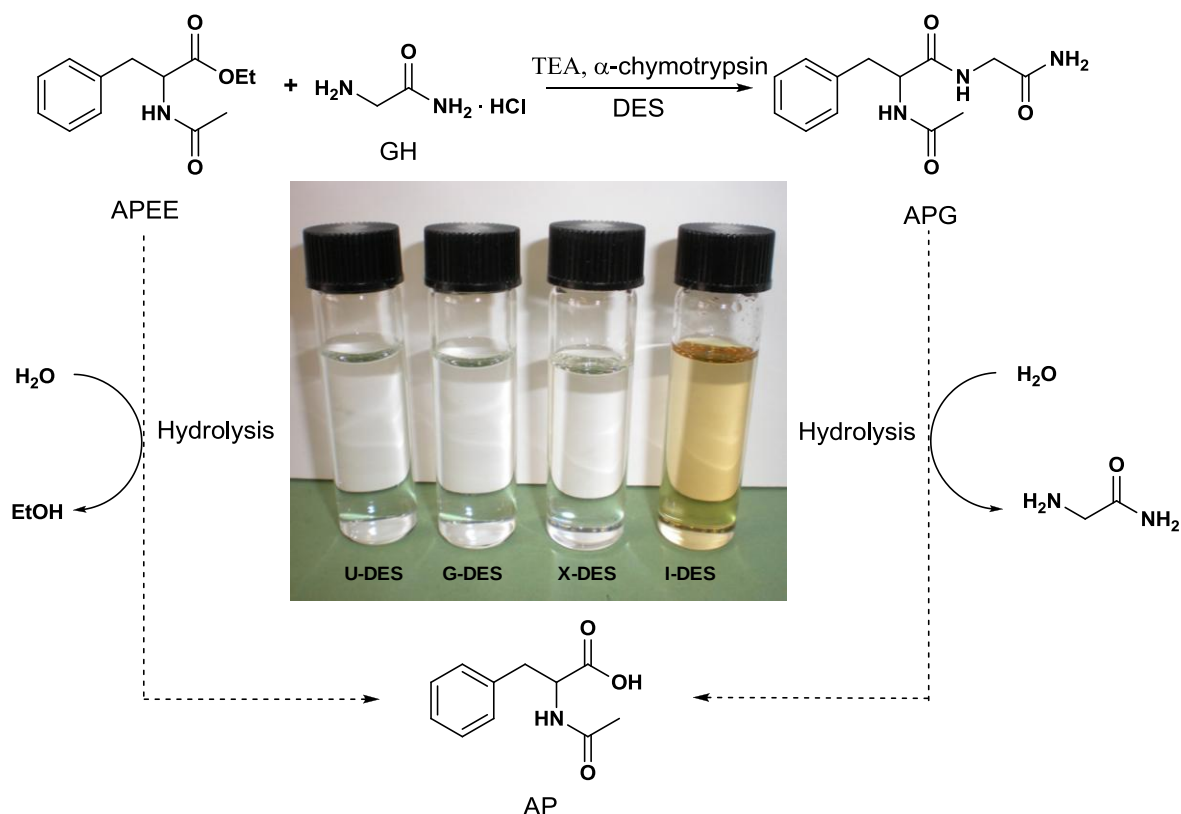


Figure 21. APG synthesis, α -chymotrypsin catalyzed, in DESs. Side reactions of hydrolysis occur when water is present leading to N-acetyl-phenylalanine (AP). TEA = triethylamine.

In peptide synthesis the reaction can be either thermodynamically or kinetically controlled. Usually the latter, requiring the use of amino acid esters instead of free acids, is preferred because of the lower energy barrier for the product formation (due to a faster acylation step of the enzyme).^[175,191] To have a kinetically controlled reaction an amino acid ester was used as a substrate. The investigated reaction was chosen as a model and the substrates, having different miscibility properties (APEE gets dissolved better in hydrophobic solvents while GH tends to be more soluble in hydrophilic ones) constitutes an excellent showcase of the issues generally observed with peptide synthesis. Moreover, the resulting product, the dipeptide APG can be also applied in therapeutics.^[203]

It is well known that lipases (especially CALB) retain and in some cases even show higher activity in anhydrous solvents^[206], while for other enzymes as α -chymotrypsin a certain amount of water is needed to keep their catalytic conformation.^[41,207–209] In peptide synthesis, this quantity must be carefully chosen due to hydrolysis reactions occurring when water is present (Figure 21). Indeed, once the acyl enzyme intermediate is formed, the water molecules compete with other nucleophiles e.g. amino acid derivatives, resulting in hydrolysis

or peptide synthesis reaction respectively. Thus, the synthesis of the desired product APG may be hampered and N-acetyl-phenylalanine (AP) formation occurs (Figure 21).^[179,210] To reduce the hydrolysis, and at the same time enhance the synthesis of the dipeptide, first experiments focused on the water effect on the reaction. Specifically, water amounts in the reaction mixture were varied within a range of 0-50 vol.% (Figure 22).

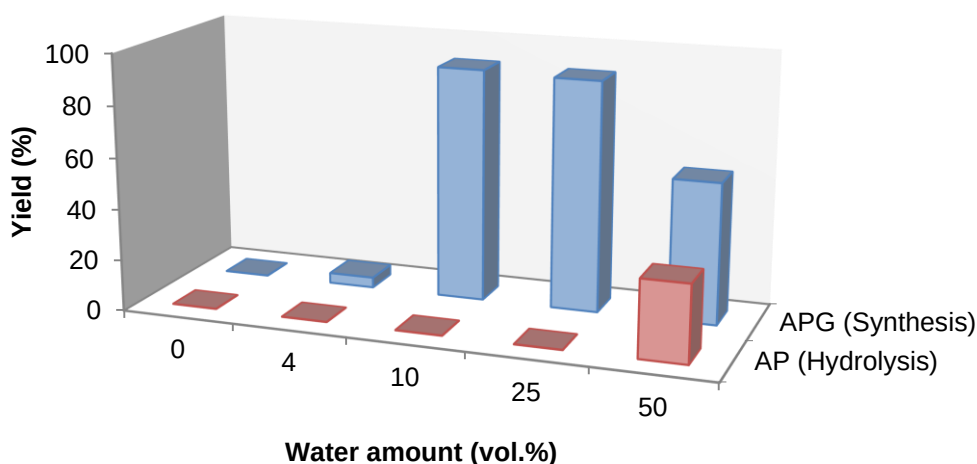


Figure 22. Effect of water amount on the APG synthesis. Reaction conditions: APEE 100 mM, GH 100 mM, TEA 200 mM, α -chymotrypsin 4 mg·mL⁻¹, G-DES (0-50 vol.% water), RT, 24 h. Conversions determined by ¹H-NMR spectroscopy.

The enzyme exhibited almost no activity in G-DES when low water amounts (up to 5 vol.%) were added to the reaction mixture. The reason might be the denaturation of the α -chymotrypsin under these severe dry conditions. Conversely, in the presence of 10 vol.% water the enzyme displayed good activity, leading to 90% of the desired dipeptide APG. Excellent results were also obtained for a water addition up to 25 vol.%. When higher amounts of water (50 vol.%) were added to the reaction mixture, the peptide synthesis still occurred, but significant hydrolytic activities were observed as well. Thus, in order to get high enzymatic activity and great selectivity for the synthetic reaction, water amounts within a range of 10-25 vol.% should be added to the reaction mixture. Water amounts of 10 vol.% were fixed for further experiments, taking the possibility to work with more hydrophobic substrates, e.g. oligopeptides, into consideration. Such reaction mixture of DES:water 90:10 vol./vol. should be suitable to dissolve a broad range of substrates and at the same time to assure an excellent enzymatic activity.

Once the amount water was fixed (10 vol.%), further investigations focused on the enzyme loadings in order to optimize the entire process, as well as to get more insights on the relationship between the synthesis and the hydrolysis reactions. Results for two different enzyme loadings (20 and 40 mg·mL⁻¹) are depicted in Figure 23.

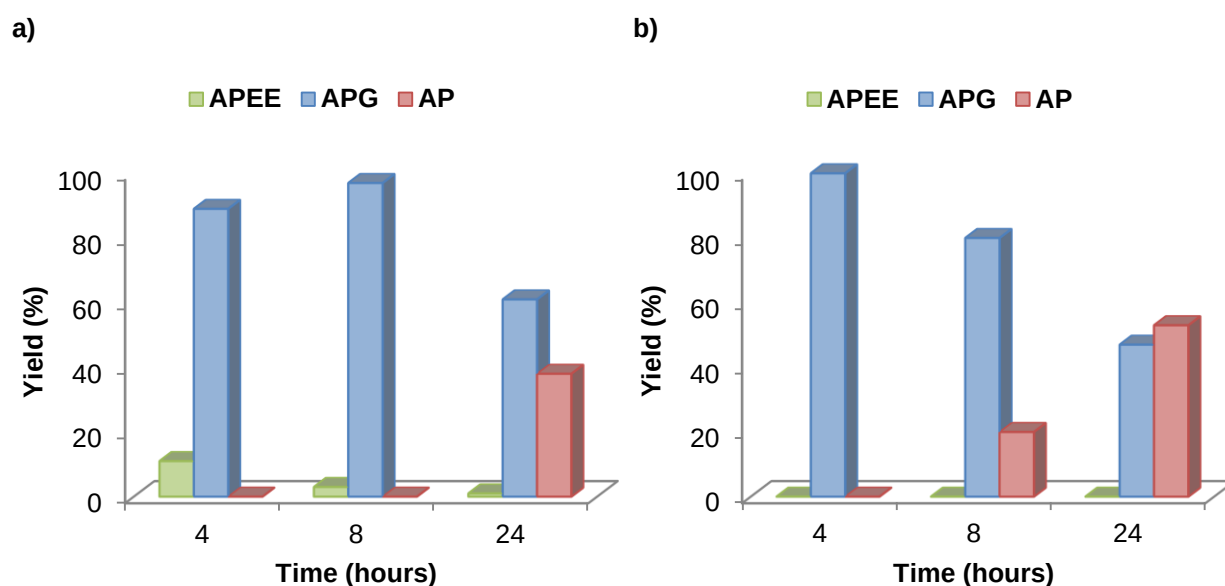


Figure 23. Enzyme loading influence on the APG synthesis. Reaction conditions: APEG 100 mM, GH 100 mM, TEA 200 mM, G-DES (10 vol.% water), RT, α -chymotrypsin in a) 20 mg·mL⁻¹ and in b) 40 mg·mL⁻¹. Conversions determined by ¹H-NMR spectroscopy.

As it can be observed, full conversions were achieved after 8 and 4 hours when the enzyme loading was 20 and 40 mg·mL⁻¹ respectively. In these cases no traces of AP were observed, leading to an excellent selectivity for the desired product. Longer reaction times led to (expected) significant amounts of the hydrolysis product.

A further set of experiments was done increasing the concentration of APEG. Remarkably, DESs displayed a great capability to dissolve high loadings of these *challenging* substrates, and thus the APEG concentration was increased from 20 g·L⁻¹ to 170 g·L⁻¹ (Figure 24). Gratifyingly, the process turned out to be quite robust, and full conversions with high selectivities (no hydrolysis observed) were achieved with substrate loadings of up to 80 g·L⁻¹ (within 4 h reaction time). Under industrially oriented conditions (full conversions and great selectivity together with high substrate loading and short reaction times), the results highlight the real applicability of DESs for this kind of biocatalytic reactions. The turnover number TON (also termed k_{cat}), in enzymology defined as the number of substrate molecules

converted by an enzyme into product per unit of time, was 14 s^{-1} . The value is in the same order of magnitude of other DESs-based processes found in literature.^[48]

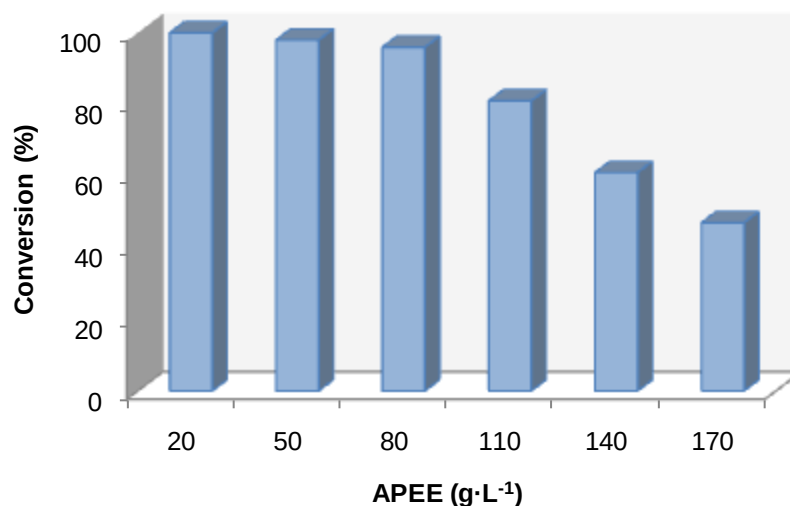


Figure 24. Substrate loading assessment in the APG synthesis. Reaction conditions: APEE:GH:TEA 1:1:2 molar ratio, G-DES (10 vol.% water), α -chymotrypsin $40\text{ mg}\cdot\text{mL}^{-1}$, RT, 4 h. Conversions determined by ^1H -NMR spectroscopy.

The α -chymotrypsin used was not immobilized but in a lyophilized form, that remained suspended as a powder in the reaction media. The stability of the enzyme in the solvents was then studied. Once the reaction was performed, the suspended enzyme was filtered, washed with 2-propanol and used again for another catalytic cycle. After the first cycle a decrease in the activity was observed, however the enzyme could be reused 3 times keeping the activity higher than 70% and retaining its selectivity towards the desired dipeptide formation (Figure 25). After 4 consecutive cycles, an almost full enzyme deactivation was observed. Taking into consideration that the enzyme was not immobilized, it is reasonable to expect better results when good immobilization methods are applied, leading to a more robust biocatalyst. Overall, the free enzyme already showed promising stability in DESs.

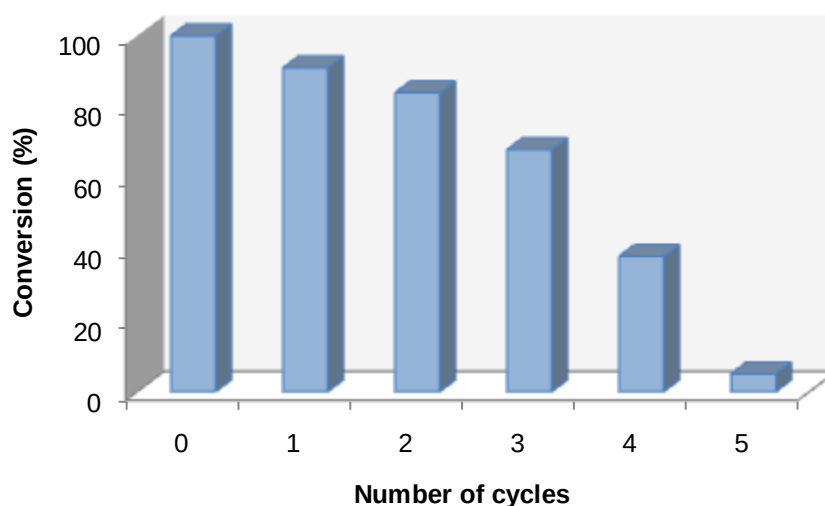


Figure 25. Enzyme recycling in the APG synthesis. Reaction conditions: APEE 100 mM, GH 100 mM, TEA 200 mM, G-DES (10 vol.% water), α -chymotrypsin 20 mg·mL⁻¹, RT, 2 hours. Conversions determined by ¹H-NMR spectroscopy.

Proteases are industrially used for a broad range of substrates, such as different amino acids (free or protected), or oligopeptides. Since each substrate has its own solubility in a given solvent, DESs with different HBDs, such as urea, glycerol, isosorbide and xylitol were studied for the prototypical reaction (Figure 26). Good results were obtained when glycerol or isosorbide were used as HBDs, leading in both cases to full conversion, with complete selectivity towards the desired product APG. Despite the denaturing effect of urea, U-DES provided high conversions as well. Interestingly, X-DES led to a much lower dipeptide formation, together with significant amounts of the hydrolysis product.

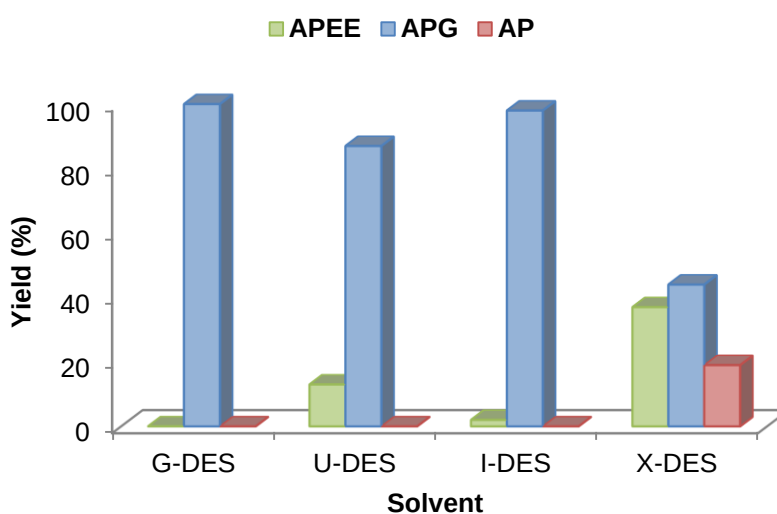


Figure 26. DESs screening for the APG synthesis. Reaction conditions: APEE 100 mM, GH 100 mM, TEA 200 mM, DES (10 vol.% water), α -chymotrypsin 40 mg·mL⁻¹, RT, 4 hours. Conversions determined by ¹H-NMR spectroscopy.

3.2.4. Benzaldehyde lyase (BAL)-catalyzed synthesis of α -hydroxy ketones in DESs

Thiamine-diphosphate dependent lyases (ThDP-lyases) are a versatile group of enzymes that catalyze asymmetric carboligations of two aldehydes (e.g. benzoin condensation) with high regio- and stereocontrol, to afford chiral α -hydroxy ketones, useful building blocks for pharmaceutical (stimulants, anti-depressants, fungicides, anti-cancer drugs) and fine chemical applications.^[211–217] A core step is the reaction of the cofactor (ThDP) with the “donor” aldehyde, to form an active enamine carbanion. Subsequently, the carbanion attacks the “acceptor” aldehyde to form the α -hydroxy ketone (Figure 27). Due to the broad acceptance of donor and acceptor aldehydes, many different optically active products can be obtained. To stabilize the holoenzyme Mg^{2+} must be added to the reaction. While the thiamine interacts via hydrophobic and ionic interactions with the protein side chain, the diphosphate part interacts with the protein through Mg^{2+} .^[218]

An outstanding example of ThDP-lyases is benzaldehyde lyase from *Pseudomonas fluorescens* (BAL, EC.4.1.2.38).^[213,214,219–223] Naturally, BAL catalyzes the cleavage of benzoin but in organic synthesis it can be used for several enantioselective carboligations of both aromatic and aliphatic aldehydes.

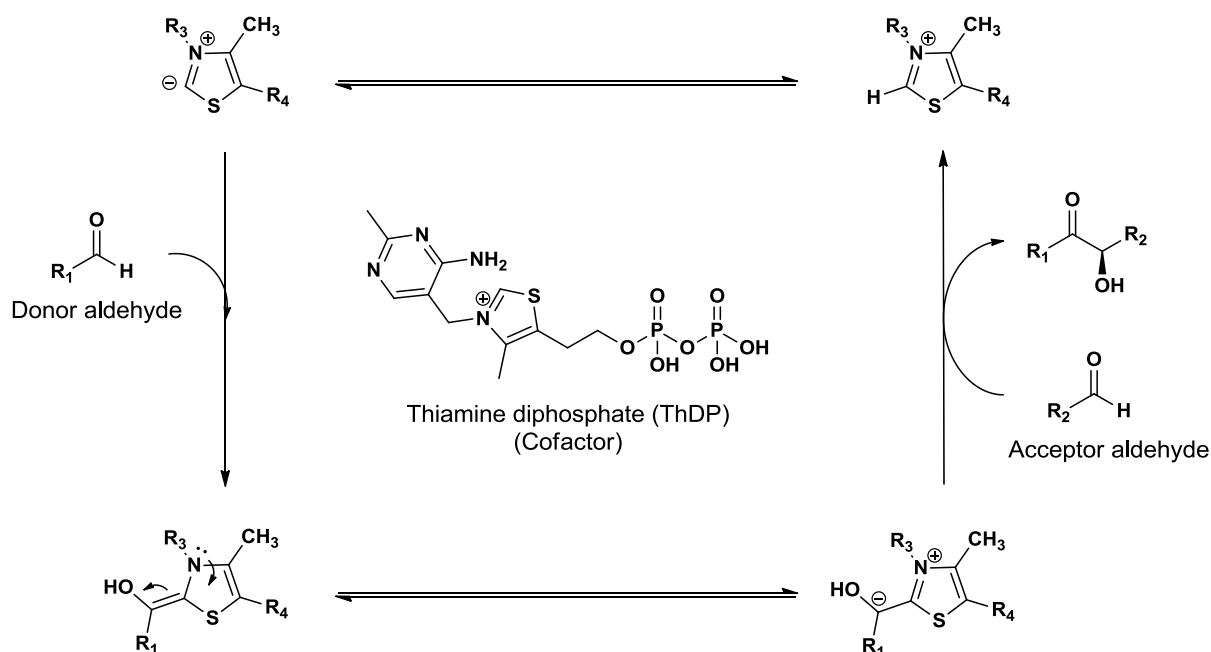


Figure 27. Mechanism for the ThDP-lyase-catalyzed carbonyl-alkylation of aldehydes (adapted from Hoyos *et al.*^[213]).

Several process-development studies concerning BAL-catalyzed synthetic systems (e.g. biphasic media, whole-cell approaches, solid/gas phase bioreactors etc.)^[224–229] demonstrated the potential of this enzyme at industrial scale as well. Most research on BAL is carried out in aqueous media, being the natural milieu for enzymes. Using this approach, low synthetic productivities are often obtained due to the poor solubility of the substrates and products in aqueous media. To overcome these issues, several strategies are widely employed, such as the use of co-solvents (e.g. DMSO, 2-propanol, *tert*-butanol, 2-MTHF, etc.) or a second phase (toluene, MTBE, etc.). Depending on the organic solvent and its proportion significant influence on enzyme activity, stability and selectivity may be observed. Alternative media such as organic solvents^[216,230], ionic liquids^[216], gases and supercritical fluids^[229] could be promising and some investigations have been carried out with lyases in these areas as well.

Given the excellent results obtained in the peptide synthesis reaction (previous section) in enzyme-friendly DESs, derived from the great ability of these solvents to dissolve highly functionalized molecules, carboligations reactions catalyzed by BAL were assessed in these media as well representing the first example of lyases in DESs (Figure 28).

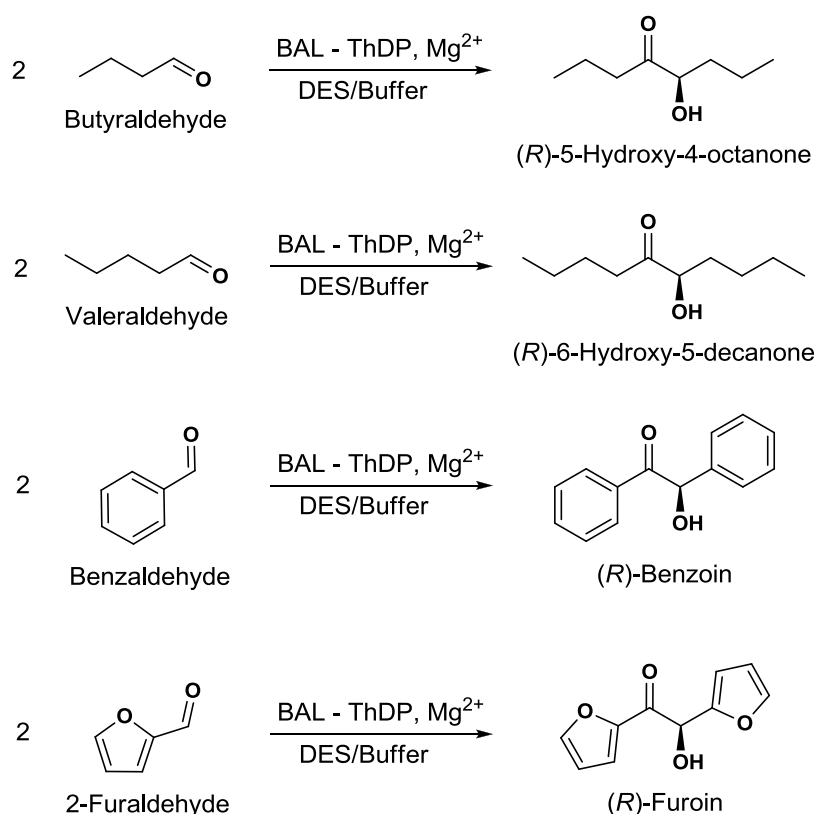


Figure 28. Synthesis of α -hydroxy ketones catalyzed by BAL in DES.

BAL was used in a lyophilized form and in a first set of experiments the effect of water was investigated (Figure 29). The enzyme resulted to be more sensitive compared to α -chymotrypsin showing no activity at 10 vol.% water. Conversely, when higher water proportions were added (30 vol.%), the enzyme catalyzed the reaction, with moderate conversions for all substrates. Upon addition of 40 vol.% water virtually full conversions were reached and enantioselectivities calculated (shown in Table 18).

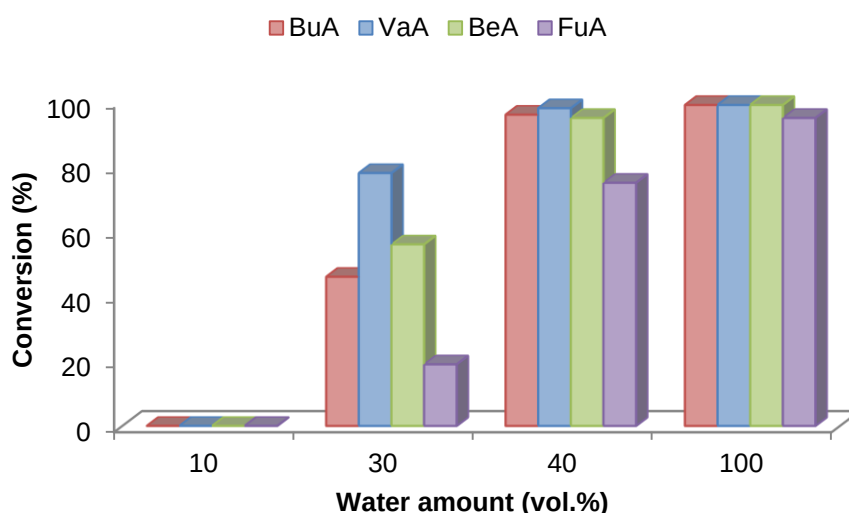


Figure 29. BAL-catalyzed carboligation of butyraldehyde (BuA), valeraldehyde (VaA), benzaldehyde (BeA) and 2-furaldehyde (FuA) in G-DES (10-100 vol.% phosphate buffer). Reaction conditions: aldehyde 50 mM, BAL 2 mg·mL⁻¹, ThDP 0.05 mg·mL⁻¹, phosphate buffer 50 mM pH 8 with 2.5 mM MgSO₄, RT, 24 hours. Conversions determined by ¹H-NMR spectroscopy.

Table 18. BAL-catalyzed carboligation of butyraldehyde (BuA), valeraldehyde (VaA), benzaldehyde (BeA) and 2-furaldehyde (FuA) in G-DES (40 vol.% phosphate buffer). Conversions determined by ¹H-NMR spectroscopy and *ee* by GC.

Substrate	Conversion (%)	<i>ee</i> %
Butyraldehyde	96	52 (R)
Valeraldehyde	98	27 (R)
Benzaldehyde	95	> 99 (R)
2-Furaldehyde	75	63 (S)

The obtained values were similar to those ones previously reported in literature^[219,228], except for the furoin where a lower enantiomeric excess was obtained.

To verify whether the enzyme is stable in the DES:water mixture of 70:30 vol./vol., further reactions were conducted at longer reaction times (> 24 h) (Figure 30). As observed, full conversions were not achievable even after 72 hours reaction time, clearly suggesting the enzyme denaturation in these DES-water mixture media.

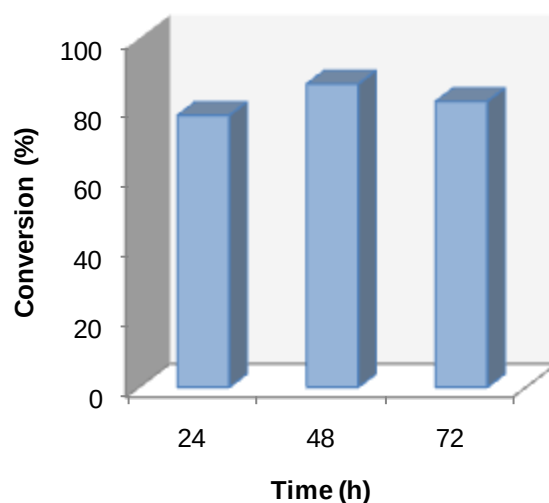


Figure 30. BAL-catalyzed carboligation of valeraldehyde in G-DES (30 vol.% phosphate buffer). Reaction conditions: aldehyde 50 mM, BAL 2 mg·mL⁻¹, ThDP 0.05 mg·mL⁻¹, phosphate buffer 50 mM pH 8 with 2.5 mM MgSO₄, RT. Conversions determined by ¹H-NMR spectroscopy.

From the data obtained working with reaction mixture of DES:water 60:40 vol./vol. may result as an adequate compromise, allowing the dissolution of a broad range of substrates and assuring, at the same time, a good enzymatic activity.

Later on, X-DES and U-DES were assessed using the 30 vol.% water (Figure 31). While X-DES led to a similar result in comparison to the one found for G-DES (ca. 80% conversion), using U-DES full conversion was achieved suggesting that the enzyme may actually be more stable in this solvent. However further investigations are needed to support this hypothesis.

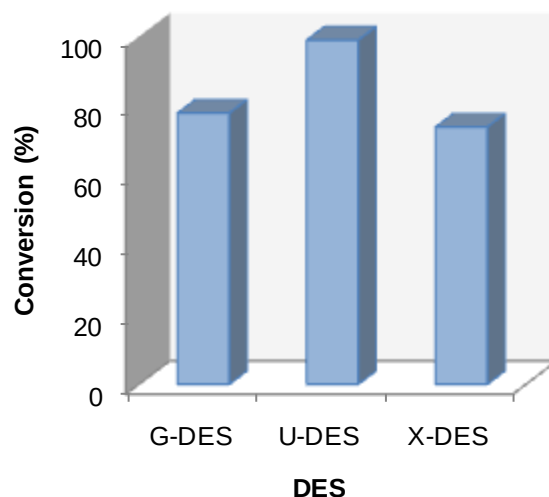


Figure 31. BAL-catalyzed carboligation of valeraldehyde in DES (30 vol.% phosphate buffer). Reaction conditions: aldehyde 50 mM, BAL 2 mg·mL⁻¹, ThDP 0.05 mg·mL⁻¹, phosphate buffer 50 mM pH 8 with 2.5 mM MgSO₄, RT, 24 h. Conversions determined by ¹H-NMR spectroscopy.

3.2.5. Whole cell biocatalysis in DESs

When conducting biocatalytic reactions in aqueous media – either using isolated enzymes or whole cells –, the low solubility of organic compounds may represent a drawback, together with the probability of other (enzymatically-catalyzed) undesired reactions, and cost-intensive downstream processing. To overcome these problems, the possibility to exploit non-conventional media (organic solvents, biphasic system, ILs, etc.) has been widely investigated.^[59,206,231–242] In this context, DESs might represent a valid alternative in virtue of their features (see previous sections). With regard to DESs and microorganisms in general, Gutiérrez *et al.* reported on the incorporation of bacteria in ChCl:glycerol DES in its pure state by a freeze-drying process showing that their integrity and viability was preserved^[47]. Later on, Hayyan *et al.* performed some experiments concerning the toxicity and cytotoxicity of ChCl-based DESs using two Gram positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and two Gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*).^[11] Results showed that there was no toxic effect for the tested DESs on all of the studied bacteria confirming their benign effects on these microorganisms. These promising findings concerning DESs and bacteria encouraged us to investigate the possibility of using whole cells of baker's yeast to perform asymmetric reactions in these media.

Baker's yeast (*Saccharomyces cerevisiae*) is an eukaryotic microorganism classified in the kingdom fungi, well known since a long time for being applied e.g. in beer and bread

production, performing the alcoholic fermentation of different natural resources (starch, potato, wheat mill, etc.). Its first utilization in organic synthesis was reported at the beginning of the 20th century, when Neuberg *et al.* described the oxidation of *n*-pentanal and the enantioselective reduction of some prochiral ketones.^[243,244] Owing to its enormous substrate acceptance (including both aliphatic and aromatic substrates), baker's yeast was used afterwards for a much broader extent, often providing high enantioselectivities.^[245–247] Yet, a common issue derived by using the commercial baker's yeast is the difficulty to get reproducible results. The reason for that was clear when the genome of baker's yeast was totally elucidated, showing that approximately 50 different redox enzymes coexist in that yeast, each with its particular specificity and enantioselectivity.^[248,249] To control the enantioselectivity of some of these enzymes toward a certain substrate, keeping the activity of the desired biocatalysts, several strategies were employed, such as modification of the substrate structure^[250,251], control of the substrate concentration^[236,250,252], use of different growth condition in baker's yeast^[253] or inclusion of enzyme inhibitors in the culture medium.^[254–257] The subsequent step was – as it is nowadays – the metabolic engineering of baker's yeast cells, in order to knock-out the expression of some oxidoreductases while overexpressing others, more active and/or selective for the target substrate. Many successful results were obtained with this approach.^[258–262] For modern practical purposes the cloning and overexpression of enzymes from baker's yeast in heterologous hosts seems to be the most reproducible and solid approach.^[245] Herein, commercially available baker's yeast (easily accessible at food delivery shops) was chosen as model case to avoid regulatory restrictions on using recombinant microorganisms while validating whole-cell biotransformations in DESs at the same time.

3.2.5.1. Baker's yeast catalyzed reduction of a β -keto-ester

The benchmark reaction was the enantioselective reduction of β -keto-ester ethyl acetoacetate in ethyl-3-hydroxybutyrate (Figure 32), as this substrate has been reported to be converted successfully by baker's yeast.^[59,233,235,245,247,253]

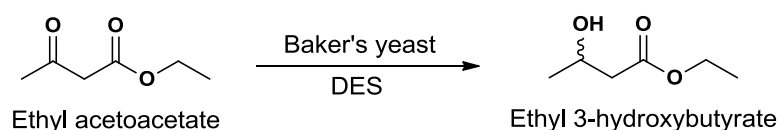


Figure 32. Baker's yeast catalyzed reduction of β -keto-ester ethyl acetoacetate in DESs.

First investigations were performed varying the amount of water in the mixture, using G-DES as reaction media (Figure 33). As observed, the conversion increased proportionally with the added water amount. Longer time reactions were performed in presence of 10 and 50 vol.% water and, as shown, improved conversions were obtained after 6 days (144 hours). However, only a small increase was observed after 12 days (288 hours).

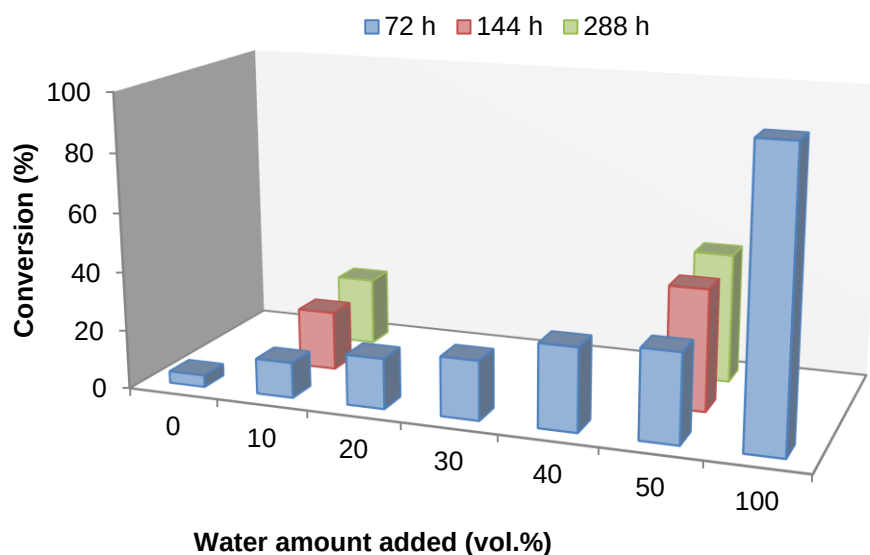


Figure 33. Baker's yeast catalyzed reduction of ethyl acetoacetate in G-DES. Reaction conditions: ethyl acetoacetate 50 mM, yeast 200 mg·mL⁻¹, RT. Conversions determined by ¹H-NMR spectroscopy.

The water content in pure G-DES, previously determined by Karl Fischer titration, was ca. 1 wt%. This amount (value corresponding to 0% of added water, Figure 34), together with the one contained in the baker's yeast as well, was enough to catalyze the reaction, albeit at low conversions.

An interesting effect on the enantioselectivity was observed (Figure 34). At water amounts lower than 30 vol.% the (*R*)-ethyl-3-hydroxybutyrate was the preferred synthesized enantiomer. Conversely, when higher amounts of water were added to the reaction mixture the enantioselectivity was inverted and the *S* enantiomer was predominantly formed. The effect gives support to the presence of numerous oxidoreductases in baker's yeast.^[245] Presumably at low water amounts some *S* enantioselective enzymes were inhibited favoring the formation of the other enantiomer. When higher water amounts are present in the reaction mixture, small amounts of the *R* enantiomer are formed as well, due to the fact that both *R* and *S* selective enzymes are active, although with different rates.

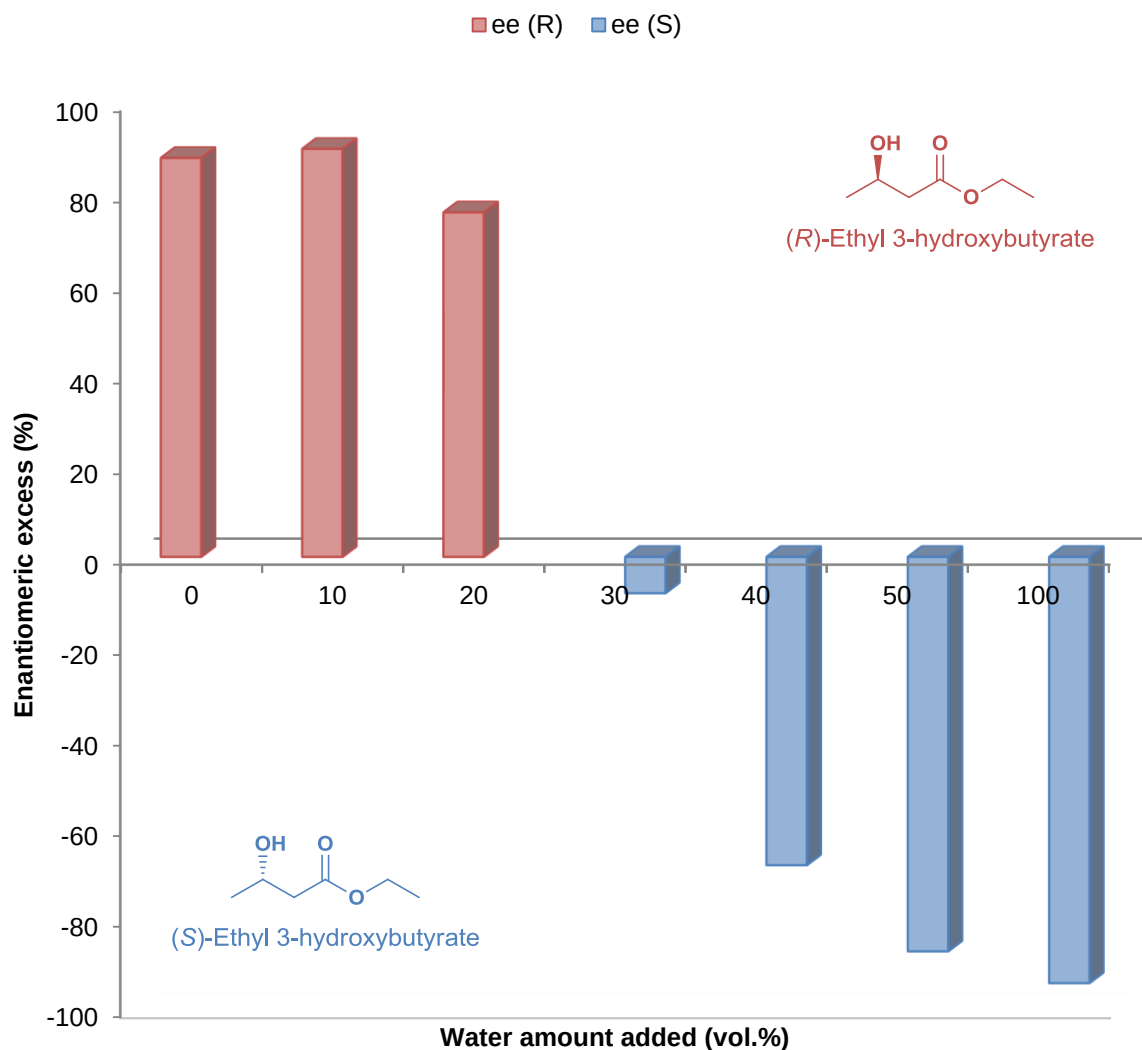


Figure 34. Influence of the water amount on the enantioselectivity in the baker's yeast catalyzed reduction of ethyl acetoacetate, in G-DES. Reaction conditions: ethyl acetoacetate 50 mM, yeast 200 mg·mL⁻¹, RT, 72 h. Enantioselectivities determined by GC.

These preliminary experiments carried out in DESs showed how a whole microorganism is actually able to catalyze organic reactions in these solvents. Conversions and enantioselectivities may be certainly improved by using recombinant whole-cells overexpressing the desired enzymes (leading to designer bugs and high catalyst loadings). More research is needed to further determine whether the low activities in the pure DES are caused by enzyme denaturation, by whole-cell damage, or by both effects concomitantly.

3.2.5.2. Baker's yeast catalyzed reduction of a β -carboline imine to amine

Considering the results obtained with baker's yeast in DESs, a more structurally complex compound was afterwards considered. As the great pharmacological importance of β -carbolines^[263–265], the baker's yeast catalyzed asymmetric reduction of 1-methyl-4,9-dihydro-3H-pyrido[3,4-*b*]indole to its correspondent amine was taken in consideration (Figure 35). The reaction has been scarcely investigated in whole cells transformations and some examples were described by using recombinant cells of *Saccharomyces cerevisiae* and *Saccharomyces bayanus*.^[266]

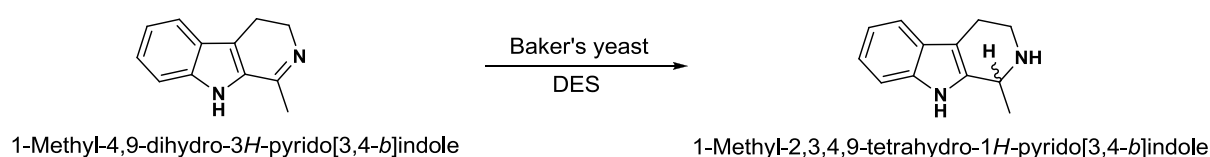


Figure 35. Baker's yeast catalyzed reduction of a β -carboline imine to its correspondent amine in DESs.

The β -carboline imine was not commercially available and therefore it was first synthesized from tryptamine and acetic acid by coupling them with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and hydroxybenzotriazole (HOBt) to afford the amide.^[267–270] The latter was then subjected to a Bischler-Napieralsky cyclization and the correspondent imine was obtained (reaction scheme showed in Figure 36).^[269,271]

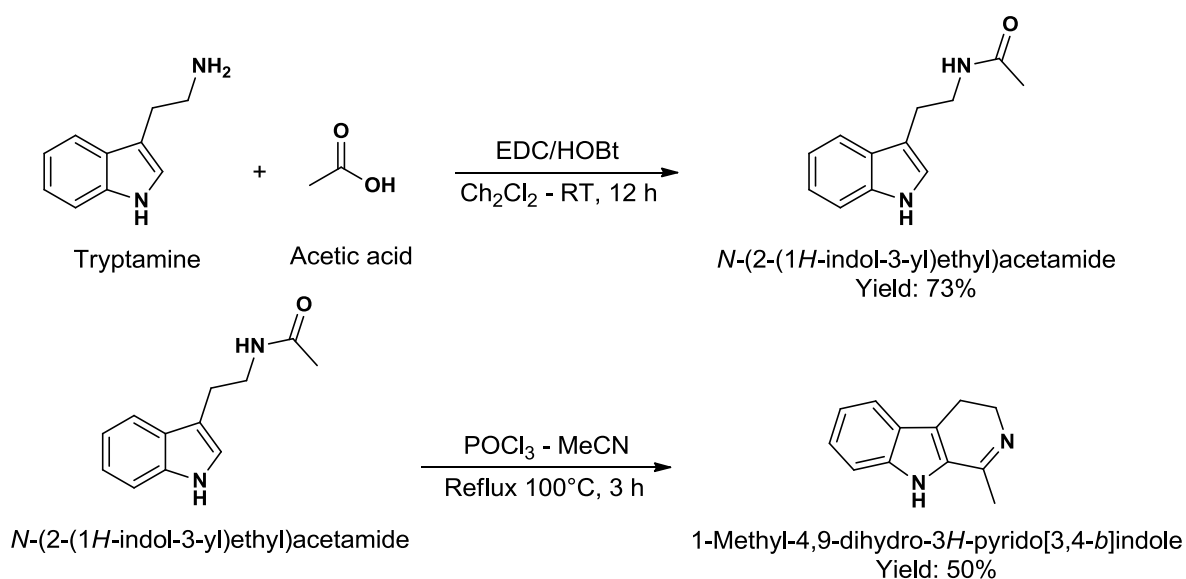


Figure 36. Reaction scheme of the β -carboline imine's synthesis.

Once the β -carboline imine was synthesized, first attempts were done in G-DES. The water amount in the mixture was varied within a range of 0-100 vol.%. Unfortunately no traces of product were observed in all reactions, after stirring them at room temperature for 3 days.

4. CONCLUSIONS AND FUTURE PERSPECTIVES

DESs represent novel solvents from which many innovative applications can be envisaged. Considering their importance, this PhD Thesis has focused on several relevant aspects of DESs with an interdisciplinary conceptual approach ranging from the production of novel biomass-derived DESs and their physical-chemical characterization, to their application in different biocatalytic reactions, either as separative agents or as (co)solvents.

In the first part of this PhD Thesis twelve novel DESs were synthesized combining inexpensive and biodegradable choline chloride (ChCl) with biomass-derived hydrogen bond donors (HBDs) such as carboxylic acids and polyols, with emphasis on delivering bio-based solvents. Chiral DESs were obtained employing D-sorbitol, D-isosorbide and L-tartaric acid as HBDs showing the possibility to synthesize renewable and chiral solvents in a straightforward way. All novel DESs exhibited melting points below than 100°C; four of them, when xylitol, D-sorbitol, D-isosorbide and levulinic acid were used as HBDs, were liquid at room temperature. Variable viscosities were observed, depending on the HBD used. In particular low viscous DESs were obtained with levulinic acid, xylitol, D-sorbitol and D-isosorbide. Ternary DESs were afterwards synthesized by adding glycerol (up to 25 vol.%) to the novel DESs. In all cases solutions with lower viscosities and melting points were obtained leading to liquid DESs at room temperature also when itaconic, L-tartaric and caffeic acids were used as HBDs. Following these thoughts the number of achievable combinations – and consequently properties – might be significantly broaden the field of DESs.

ChCl:levulinic acid 1:1 mixture, solid at room temperature, absorbing water from the air humidity, turned reversibly into a liquid when the water amount of 9 wt% was incorporated. Water absorption kinetic experiments were performed on ChCl:levulinic acid DESs with the molar ratios of 1:1 and 1:2, displaying similar and measurable trends, reaching a plateau with a number of water molecules of 219 and 276 respectively.

The solubilities of the novel DESs were tested with some protic and aprotic solvents and a classification of “weak” and “strong” DESs was proposed considering the interaction strengths involved among the DES’s components. When the addition of an aprotic solvent led to ChCl precipitation the DES was defined as “weak”. Conversely, if a biphasic system was formed the DES was considered as “strong”. A systematic study was performed on twenty different ChCl-based DESs using HBDs bearing one or more hydroxyl or carboxyl groups.

From this study it was possible to observe that when HBDs bearing hydroxyl groups were used, the presence of three or more functional groups in the HBD was necessary to form a strong DES while when carboxylic acids were used as HBDs, the presence of only 2 groups was already sufficient. Based on this property a novel method to separate alcohol-ester, alcohol-ketone, amine-ketone mixtures was established, enabling to recover 70-90% of the separated compound at a purity of 90-99%.

In the second part of this PhD Thesis DESs were used as non-conventional media for several biocatalyzed reactions. Firstly, the well-known kinetic resolution of *rac*-1-phenylethanol, catalyzed by immobilized lipase from *Candida antarctica* B, was assessed, providing consistent data with those reported in previous literature, and confirming that when urea was used as HBD – despite the denaturing effect of this compound on proteins - no deactivation of the enzyme was observed. This suggests that urea, being involved in the interactions with ChCl, does not interact with the enzyme, and the latter retains its catalytic conformation. However, when using other HBDs such as glycerol, xylitol or isosorbide, undesired reactions occurred among the DESs' components and the acyl donor, leading to overall lower conversions. More research is needed to explain why in some cases HBDs seem to not influence the enzymatic performance whereas in other cases the occurrence of secondary reactions suggests that the interaction of HBDs with choline chloride is not sufficiently strong to avoid the use of HBDs as enzyme substrates.

Likewise, the lipase-mediated epoxidation of styrene *via in situ* peracid formation was assessed. Chiral DESs were used as reaction media to investigate whether a chiral environment could influence the enantioselectivity of the reaction. In all cases low values of conversions were obtained and for this reason it resulted difficult to understand whether the observed enantioselectivities were really influenced by the chosen solvent or just due to instrumental errors and traces of impurities. More investigations are still needed, considering as well the possibility of changing one or both components of the DESs and working with more hindered ones.

The synthesis of the protected dipeptide N-acetyl-phenylalanine-glycinamide catalyzed by α -chymotrypsin was afterwards studied. A minimal amount of water (10-20 vol.%) in the DES was needed to keep the enzyme in its catalytically active conformation. Once this value was fixed, assuring high activity and selectivity for the peptide synthesis, the reaction was

optimized and further investigations were undertaken concerning the enzyme loading, time, substrate concentration, DESs screening and recycling of the biocatalyst. The optimized conditions led to productivities of ca. $20 \text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ with complete selectivity towards the peptide synthesis. Once the possibility to carry out dipeptide synthesis reactions in DESs was established with successful results, the synthesis of longer peptide chains, as oligo- and polypeptides, should be addressed.

Furthermore, DESs were assessed in the carbonylation reaction of aliphatic and aromatic aldehydes, catalyzed by benzaldehyde lyase from *Pseudomonas fluorescens* (BAL). In this reaction the amount of water present in the reaction media turned out to be more critical than for α -chymotrypsin, and investigations suggested that the enzyme does not actually survive in DES for long times, being quickly denaturated. Those results confirmed that BAL is more sensitive to other organic (co)solvents than other enzymes.

Finally, a whole microorganism such as baker's yeast (*Saccharomyces cerevisiae*) was tested in DESs for the reduction of the β -keto-ester ethyl acetoacetate. An interesting effect, concerning the enantioselectivity, was displayed when the water amount in the reaction mixture was varied. Inverted enantioselectivity (from *R* to *S*) was observed when water amounts increased. Presumably, DES would inhibit/denature some of the oxidoreductases present in the yeast, whereas other enzymes would remain active.

In conclusion, the great versatility of the DESs has been shown, as well as the possibility of modulate their properties (such as the viscosities and the melting points) by varying the composition of the mixture and adding a second HBD (e.g. glycerol). The diverse solubilities found for the DESs with protic and aprotic compound have been applied in separation achieving satisfying results. DESs showed to be potential (co)solvents in enzyme-catalyzed reactions, being able to enhance the solubilities of the substrates in comparison to the natural aqueous media. However, for each enzyme and reaction the amount of water must be adjusted to guarantee the survival of the biocatalyst, and the DES carefully chosen to avoid side reactions occur.

5. EXPERIMENTAL

5.1. Preparation of novel DESs from biorenewable resources. Properties and applications

5.1.1. Chemicals

All chemicals were used without any further purification. Choline chloride, D-sorbitol, L-tartaric, caffeic, malonic and gallic acid, glycerol, n-butanol, 1,2-propanediol, benzyl alcohol, 1,4-butanediol, 2,3-butanediol, 2-methyltetrahydrofuran, ethyl acetate and acetone were purchased from Sigma; *p*-coumaric, formic, propionic, butanoic and succinic acid, 1-butanol, acetophenone, ethanol, n-propanol, 2-propanol, ethylene glycol, 2-butanol from Fluka; *trans*-cinnamic, suberic, levulinic and itaconic acid, xylitol, 4-hydroxybenzoic, D-isosorbide, vinyl acetate, isopropenyl acetate, 1-phenylethyl acetate, *rac*-1-phenylethanol, 1-phenylethyl butyrate, benzyl acetate, benzyl butyrate, butyl acetate, 1,3-propanediol, 1-phenyl-1,2-ethanediol, 1-phenylethylamine from Aldrich; acetic acid from Merck; glucose, methanol from Roth; urea from KMF Laborchemie Handels GmbH. Immobilized form lipase from *Candida antarctica* B was a kind gift from c-LEcta GmbH.

5.1.2. Synthesis of novel DESs from bionewable resources

In a 20 mL vial the quaternary ammonium salt choline chloride and the correspondent hydrogen bond donor were mixed together and heated at the temperature of 100°C until a clear and homogenous liquid was formed (time variable within a range of 15-120 minutes). Melting points were measured afterwards only for the solid DESs at room temperature. They were heated at a rate of 1°C/min and the melting point was taken as the temperature at which the first liquid began to form. Table 7 and 8 summarized the synthesized DESs, the correspondent ChCl:HBD molar ratios, and melting points.

5.1.3. Viscosities' measurement

The effect of glycerol on some saccharides- and polyols-based DESs was studied by measuring the viscosities at the temperatures of 30, 50, 70 and 100°C, using an MCR 301 rheometer from Anton Paar with a thermostated jacket (viscosity range 130–34400 cP). Data are reported in Table 19 and depicted in Figure 6. From the obtained values, the activation energies for viscous flow E_η were determined for each DES by fitting the equation (4) (see section 3.1.2.) to the data using the software Origin.

Table 19. Viscosities of saccharides- and polyols-based DESs and effect of glycerol addition (25 mol%). n.d. = not determined. For comparison the viscosity of glycerol at 20°C is ca. 1200 cP.

DES	ChCl:HBD:Gly molar ratio	Viscosity (cP)			
		30°C	50°C	70°C	100°C
ChCl:glucose	1:1	n.d.	34400	4470	560
ChCl:glucose:glycerol	1:0.5:0.5	4430	930	280	n.d.
ChCl:xylitol	1:1	5230	860	250	n.d.
ChCl:xylitol:glycerol	1:0.5:0.5	1420	370	130	n.d.
ChCl:sorbitol	1:1	12730	2000	480	n.d.
ChCl:sorbitol:glycerol	1:0.5:0.5	1710	560	180	n.d.

5.1.4. Water absorption determination of levulinic acid-based DESs

Gravimetric determinations of water absorption kinetics were performed on the ChCl:levulinic acid DESs with two different compositions (1:1 and 1:2 molar ratios). The initial water contained in the DES was measured by Karl Fischer titration and the obtained value (ca. 1.2 wt%) was converted into the molar ratio and used as starting point. 1.7 g of DES was placed in a closed glass Petri dish (having a diameter of 32 mm) at a specific humidity level ($50 \pm 3\%$) and the water sorption was determined by measuring the weight variation (0.1 mg precision) over the time. After 6 days the equilibrium was reached and no more weight variation was observed (data shown in Table 20). The equation (5) (see section 3.1.3.) was fitted to the obtained data and kinetic parameters were calculated by using the software Origin.

Table 20. Data from Figure 8.

Time (min)	Molar ratio of water (%) ChCl:levulinic acid (1:1 molar ratio)	Molar ratio of water (%) ChCl:levulinic acid (1:2 molar ratio)
0	14.7	24.6
10	16.8	27.7
20	17.3	28.7
30	18.1	30.5
40	18.6	31.8
50	19.5	33.2
60	20.0	34.5
90	22.3	38.3
120	24.0	42.3
200	28.9	50.9
390	42.1	74.6
480	47.6	83.3
540	51.3	89.5
1680	101.0	169.4
4240	187.9	265.0
4450	191.5	267.3
4780	195.3	267.3
5620	209.2	278.5
6220	214.8	278.5
7480	220.0	278.5
8890	220.0	278.5

5.1.5. ChCl's recovery of the levulinic acid-based DES

ChCl:levulinic acid DES (1:2 molar ratio) was synthesized by mixing 5.52 g of ChCl and 9.28 g of levulinic acid in a 20 mL vial and stirring them at the temperature of 100°C until a clear and homogenous liquid was formed (ca. 20 minutes). The liquid was afterwards cooled down to the room temperature and a precise amount of acetone (2.9, 5.9, 11.8, 17.6 or 29.4 mL) was added (data reported in Table 21). The precipitated ChCl was filtered, dried under reduced pressure and analyzed by ¹H-NMR. The spectrum showed no traces of impurities.

Table 21. Data from Figure 9.

Acetone (equivalent)	2	4	8	12	20
Amount of ChCl recovered (%)	68	77	87	92	93

5.1.6. Miscibility of DESs with protic and aprotic solvents

The study on the miscibility with protic and aprotic solvents was first performed on the DESs liquid at room temperature, reported in Table 7. To 1 mL of DES 5 mL of water or organic solvent (methanol ethanol, 2-methyltetrahydrofuran, ethyl acetate and acetone) were added, mixed and then let them decanted. After few minutes three scenarios were observed: the formation of a biphasic system, the dissolution of the DES into the organic solvent, or the formation of a biphasic system with the precipitation of ChCl. Further systematic studies were afterwards performed on other DESs made by ChCl and HBDs bearing one or more hydroxyl or carboxyl groups. The same synthetic procedure, described in the section 5.1.2., was used. In Table 10 the synthesized DESs with the correspondent ChCl:HBD molar ratios are shown.

5.1.7. Kinetic resolution of *rac*-1-phenylethanol catalyzed by iCALB in neat substrates

The procedure for *rac*-1-phenylethanol:acyl donor molar ratio of 1:3 is exemplified. 286 μ L of *rac*-1-phenylethanol, 726 μ L of vinyl acetate (or 783 μ L of isopropenyl acetate) and variable amount of iCALB (10, 20 or 30 mg) were mixed together and stirred at 300 rpm in G8 vial (8 mL volume) at 60°C. After defined intervals of time, the samples were taken, diluted with ethyl acetate and analyzed by GC equipped with the chiral column CP Chirasil Dex (250 μ m $\times$ 0.250 μ m $\times$ 25 m) and a flame ionization detector. The following temperature program was used for the analysis: 80°C (0 min) - 3°C/min - 160°C (10 min). The temperature of the injector and split ratio were 250°C and 35:1 respectively. The carrier gas was hydrogen and the flow rate was 2.0 mL/min. The recycle of the iCALB was done by filtering it off, washing it with 2-propanol and directly using it in the next catalytic cycle. All the results are reported in Table 22, 23 and 24.

Table 22. Data from Figure 13.

<i>rac</i> -1-phenylethanol:vinyl acetate (molar ratio)	Conversion (%)	ee (product) (%)	ee (substrate) (%)
1:2	46	99	83
1:3	48	99	92
1:4	50	99	97

Table 23. Data from Figure 14.

Enzyme loading (mg·mL ⁻¹)	Conversion (%)			
	12 hours	16 hours	20 hours	22 hours
10	39	43	45	48
20	49	50	50	50
30	50	50	50	50

Table 24. Data from Figure 15.

Cycles	0	1	2	3	4	5	6	7	8
Obtained conversion with IPA (%)	100	92	81	70	59	49	23	25	23
Obtained conversion with VA (%)	100	82	72	57	48	40	38	34	30

5.1.8. DESs as separating agents for alcohol-ester mixtures

As a representative example the mixture of 1-phenylethanol and 1-phenylethanol acetate was selected. 0.877 g of 1-phenylethanol (0.0072 mol) was first mixed with 1.178 g of 1-phenylethanol-acetate (0.0072 mol). 10 mL of ChCl:glycerol DES (1:2 molar ratio) was added to this equimolar mixture, then mixed and centrifuged at 4000 rpm for 4 minutes (by using the centrifuge EBA 20 from Hettich). The upper phase was recovered and analyzed by ¹H-NMR. To reach a specific level of purity this phase was subjected to the procedure 4 times and finally 0.8 g of were obtained containing almost exclusively 1-phenylethanol acetate (purity > 99%).

After removal of the upper phase, the DES mixture was subjected to 3 extractions using 1 mL of ethyl acetate or 0.75 mL of ethyl acetate:water mixture (2:1 vol./vol.). The organic phases were collected and the ethyl acetate was evaporated under reduced pressure. The remnant mixture, mainly composed of 1-phenylethanol, was then analyzed by ¹H-NMR.

5.2. Assessment of DESs in biocatalysis

5.2.1. Chemicals

All chemicals were used without any further purification. Choline chloride, D-sorbitol, glycerol, 2-methyltetrahydrofuran, toluene, isooctane, acetonitrile, styrene, hydrogen peroxide 30 wt%, triethylamine, α -chymotrypsin from bovine pancreas (Type II, lyophilized, powder, ≥ 40 units/mg protein; 1.0 U hydrolyzes 1.0 μ mol of N-benzoyl-L-tyrosine ethyl ester per min

at pH 7.8 and 25°C), thiamine-diphosphate, magnesium sulfate, butyraldehyde, valeraldehyde, benzaldehyde, furaldehyde, ethyl acetoacetate, ethyl acetate were purchased from Sigma; from Fluka; xylitol, D-isosorbide, vinyl acetate, isopropenyl acetate, *rac*-1-phenylethanol, glycine hydrochloride, octanoic acid from Aldrich; glucose, methanol, monopotassium phosphate and dipotassium phosphate from Roth; urea from KMF Laborchemie Handels GmbH; N-acetyl-phenylalanine ethyl ester was purchased from Bachem AG. Immobilized form lipase from *Candida antarctica* B was a kind gift from C-LEcta. Benzaldehyde lyase from *Pseudomonas fluorescens* was cloned in *E. coli*, overexpressed, and produced by fermentation. After fermentation and purification, BAL was lyophilized and stored at -20°C until use. Baker's yeast (from Deutsche Hefewerke GmbH) was purchased in the supermarket.

5.2.2. DESs' synthesis

In a 20 mL vial the quaternary ammonium salt choline chloride and the correspondent hydrogen bond donor were mixed together and heated at the temperature of 100°C until a clear and homogenous liquid was formed (time variable within a range of 15-120 minutes). In Table 14 the synthesized DESs and the correspondent ChCl:HBD molar ratios are shown.

5.2.3. Kinetic resolution of *rac*-1-phenylethanol

Conditions for the 6 mL reaction volume: 29 μ L of *rac*-1-phenylethanol and 79 μ L of isopropenyl acetate were mixed together and added to 6 mL of organic solvent or DES containing 60 mg of iCALB. The reaction mixtures were stirred at 300 rpm in G8 vials at 60°C. Conditions for the 1 mL reaction volume: 4.8 μ L of *rac*-1-phenylethanol and 13.2 μ L of isopropenyl acetate were mixed together and added to 1 mL of organic solvent or DES containing 10 mg of iCALB. After 24 hours, in case of organic solvents the samples were directly analyzed; when DESs were used, the reaction mixtures were first extracted with ethyl acetate and then analyzed. The analyses were performed by using a GC equipped with the chiral column CP Chirasil Dex (250 μ m $\times$ 0.250 μ m $\times$ 25 m) and a flame ionization detector. The following temperature program was used for the analysis: 80°C (0 min) - 3°C/min - 160°C (10 min). The temperature of the injector and split ratio were 250°C and 35:1 respectively. The carrier gas was hydrogen and the flow rate was 2.0 mL/min. In Table 25 the results shown in Figure 19 are reported.

Table 25. Data from Figure 19.

Organic solvent	Conversion (%)	ee ₁ (%)	ee ₂ (%)	E
U-DES	41	68	99	> 200
G-DES	2	1	99	> 200
I-DES	16	18	99	> 200
S-DES	13	15	99	> 200
X-DES	11	12	99	> 200

5.2.4. Epoxidation of styrene via peracid formation

In a G8 vial 10 mg of iCALB were weighted and 1 mL of organic solvent or DES was added. To this solution 11 μ L styrene, 1 μ L of octanoic acid and 3 μ L of H₂O₂ 30% were mixed together and then the reaction was stirred at room temperature at 300 rpm. After 1, 2 and 3 hours 3 μ L of H₂O₂ 30% were added. After 24 hours, in case of organic solvents the samples were directly analyzed; when DESs were used, 2 mL of water were added to the reaction mixtures and then extracted with ethyl acetate (3 x 2 mL). The organic phases were collected and the ethyl acetate was evaporated under reduced pressure. The samples were analyzed by using a GC equipped with the chiral column Ivdex-7 (250 μ m $\times$ 0.250 μ m $\times$ 25 m) and a flame ionization detector. The following temperature program was used for the analysis: 50°C (0 min) - 3°C/min - 160°C (10 min). The temperature of the injector and split ratio were 250°C and 40:1 respectively. The carrier gas was hydrogen and the flow rate was 2.0 mL/min.

5.2.5. Dipeptide synthesis catalyzed by α -chymotrypsin

As example the reaction with 10 vol.% water is described. 0.5 mL of DES and 5.6 mg of glycineamide hydrochloride (dissolved in 55 μ L of distilled water) were added to 11.8 mg of N-acetyl-phenylalanine ethyl ester previously weighted in a G8 vial. 14 μ L of triethylamine and variable amount of α -chymotrypsin (within a range of 2-20 mg) were finally added to the reaction mixture and stirred at room temperature at 300 rpm. After a defined interval of time the reaction was stopped, 2 mL of water were added and then the mixture was extracted with ethyl acetate (3 x 2 mL). The organic phases were collected and the ethyl acetate was evaporated under reduced pressure. The solid was then analyzed by ¹H-NMR. The results are reported in Table 26, 27, 28, 29 and 30.

Table 26. Data from Figure 22.

Water amount (vol.%)	APG (yield %)	AP (yield %)
0	0	0
4	4	0
10	91	0
25	90	0
50	55	30

Table 27. Data from Figure 23.

Time (h)		Yield (%)	
		Enzyme loading 10 mg·mL ⁻¹	Enzyme loading 20 mg·mL ⁻¹
APEE	4	11	0
	8	3	0
	24	1	0
APG	4	89	100
	8	97	80
	24	61	47
AP	4	0	0
	8	0	20
	24	38	53

Table 28. Data from Figure 24.

APEE (g·L ⁻¹)	Conversion (%)
20	100
50	98
80	96
110	81
140	61
170	47

Table 29. Data from Figure 25.

Cycle	Conversion (%)
0	100
1	91
2	84
3	68
4	38
5	5

Table 30. Data from Figure 26.

DES	APEE (yield %)	APG (yield %)	AP (yield %)
G-DES	0	100	0
U-DES	13	87	0
I-DES	2	98	0
X-DES	37	44	19

5.2.6. Benzaldehyde lyase (BAL)-catalyzed synthesis of α -hydroxy ketones in DESs

The phosphate buffer (pH 8) was prepared by dissolving 7.49 g of K_2HPO_4 with 0.95 g of KH_2PO_4 and 300 mg of $MgSO_4$ in 1 L water. The pH was adjusted to 8.00 with phosphoric acid or 2.0 M potassium hydroxide solution. As example the reaction with 30 vol.% phosphate buffer is considered. A solution of ThDP was prepared by dissolving 2 mg of ThDP in 16 mL of phosphate buffer. 2 mg of BAL were dissolved in 400 μ L of phosphate buffer containing ThDP and then this mixture was added to 600 μ L of DES in a G8 vial. The aldehyde (4.5 μ L of butyraldehyde, 5.3 μ L of valeraldehyde, 5.1 μ L of benzaldehyde or 4.1 μ L of 2-furaldehyde) was finally added to the solution and stirred at room temperature at 300 rpm. After a precise interval of time the reaction was stopped, 2 mL of water were added and then the mixture was extracted with ethyl acetate (3 x 2 mL). The organic phases were collected and the ethyl acetate was evaporated under reduced pressure. The product was then analyzed by 1H -NMR. The enantiomeric excesses for the 5-hydroxy-4octanone and 6-hydroxy-5-decanone were determined by a GC equipped with the chiral column CP Chirasil Dex (250 μ m $\times$ 0.250 μ m $\times$ 25 m) and a flame ionization detector. The following temperature program was used for the analysis: 50°C (0 min) - 3°C/min - 160°C (10 min). The temperature of the injector and split ratio were 250°C and 40:1 respectively. The carrier gas was hydrogen and the flow rate was 2.0 mL/min. The enantiomeric excesses of the benzoin and furoin were determined by HPLC equipped with a Chiralpak AD-H column (n-heptane:2-propanol = 90:10, λ = 240 nm), 0.5 mL/min. The results are reported in Table 31, 32 and 33.

Table 31. Data from Figure 29.

Aldehyde	Conversion (%)			
	10 vol.% water	30 vol.% water	40 vol.% water	100 vol.% water
Butyraldehyde	0	46	96	99
Valeraldehyde	0	78	98	99
Benzaldehyde	0	56	95	99
2-Furaldehyde	0	19	75	95

Table 32. Data from Figure 30.

Time (h)	Conversion (%)
24	78
48	87
72	82

Table 33. Data from Figure 31.

DES	Conversion (%)
G-DES	78
U-DES	99
X-DES	74

5.2.7. Baker's yeast catalyzed reduction of a β -keto-ester

200 mg of baker's yeast were weighted in a G8 vial and dissolved in variable amounts of water (within a range of 0.1-1 mL). The DES was added to the solution until a total volume of 1 mL was reached. Finally 6.4 μ L of ethyl acetoacetate were added to the mixture and stirred at room temperature at 300 rpm. After a defined interval of time the reaction was stopped, 2 mL of water were added and then the mixture was extracted with ethyl acetate (3 x 2 mL). The organic phases were collected and the ethyl acetate was evaporated under reduced pressure. The product was then analyzed by ^1H -NMR. The enantiomeric excesses for the ethyl 3-hydroxybutyrate was determined by a GC equipped with the chiral column Hydrodex (250 $\mu\text{m} \times 0.250 \mu\text{m} \times 25 \text{ m}$) and a flame ionization detector. The following temperature program was used for the analysis: 50°C (0 min) - 3°C/min - 160°C (10 min). The temperature of the injector and split ratio were 250°C and 40:1 respectively. The carrier gas was hydrogen and the flow rate was 2.0 mL/min. All the results are reported in Table 34.

Table 34. Data from Figure 33 and 34.

Water amount added (vol.%)	Conversion (%)			ee %
	72 h	144 h	288 h	
0	4	n.d.	n.d.	88 (R)
10	12	20	23	90 (R)
20	17	n.d.	n.d.	76 (R)
30	20	n.d.	n.d.	8 (S)
40	28	n.d.	n.d.	68 (S)
50	30	41	44	87 (S)
100	97	n.d.	n.d.	94 (S)

5.2.8. Synthesis of the β -carboline imine

To a solution of 382 mg of tryptamine (2.38 mmol) and 136 μ L of acetic acid (2.38 mmol) in 24 mL of CH_2Cl_2 were added 464 μ L of EDC (2.62 mmol) and 354 mg of HOBT (2.62 mmol) at 0°C. The reaction mixture was stirred at room temperature for 12 hours, then washed with 5% aqueous HCl (30 mL), 5% aqueous NaHCO_3 (3x30 mL), 40 mL of water, 40 mL of brine and then finally dried with Na_2SO_4 . The organic solvent was evaporated under reduced pressure and then the solid characterized by ^1H -NMR (300 MHz, DMSO-d_6): δ = 1.81 (s, 3H), 2.81 (t, J = 7.28, 2H), 3.30 (t, J = 7.51, 2H), 6.98-7.52 (m, 5H), 7.94 (t, J = 5.27, 1H), 10.81 (br s, 1H). The amide was obtained in 73% yield. 100 mg of the β -carboline amide (0.5 mmol) were dissolved in 6 mL of dry CH_3CN and then 300 μ L of POCl_3 (3 mmol) were added to the solution. The reaction mixture was heated at reflux (100°C) for 3 hours and cooled to room temperature. Placed in an ice-bath it was carefully treated with 1 mL of water, then the CH_3CN evaporated under reduced pressure. The solution was washed with Et_2O (2x3 mL), then 4M aqueous NaOH was added until pH 11 was reached and finally it was extracted with CH_2Cl_2 (3x15 mL). The organic phases were collected, washed with brine and dried with Na_2SO_4 . The solvent was evaporated under reduced pressure and then the solid characterized by ^1H -NMR (300 MHz, DMSO-d_6): δ = 2.27 (s, 3H), 2.74 (t, J = 8.24, 2H), 3.68 (t, J = 8.04, 2H), 7.04-7.55 (m, 5H), 11.32 (br s, 1H). The imine was obtained in 50% yield.

5.2.9. Baker's yeast catalyzed reduction of a β -carboline imine to amine

100 mg of baker's yeast were loaded in a G8 vial and dissolved in variable amounts of water (within a range of 0-500 μ L). The DES was added to the solution until a total volume of 0.5 mL was reached. 4 mg of β -carboline imine were added to the mixture and this one was stirred at room temperature at 300 rpm. After 72 hours the reaction was stopped, 2 mL of water were added and then the mixture was extracted with ethyl acetate (3 x 2 mL). The organic phases were collected and the ethyl acetate was evaporated under reduced pressure. The product was then analyzed by ^1H -NMR.

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LIST OF PUBLICATIONS

- [1] Z. Maugeri, P. Domínguez de María, *J. Mol. Catal B-Enzym.* **2014**, 107, 120-123, *Benzaldehyde lyase (BAL)-catalyzed enantioselective C-C bond formation in deep-eutectic-solvents–buffer mixtures.*
- [2] Z. Maugeri, P. Domínguez de María, *ChemCatChem.* **2014**, 6, 1535-1537, *Whole-cell Biocatalysis in Deep-Eutectic-Solvents-Aqueous Mixtures.*
- [3] Z. Maugeri, W. Leitner, P. Domínguez de María, *Eur. J. Org. Chem.* **2013**, 2013, 4223-4228, *Chymotrypsin-Catalyzed Peptide Synthesis in Deep Eutectic Solvents.*
- [4] C. Lehmann, F. Sibilla, Z. Maugeri, W. R. Streit, P. Domínguez de María, R. Martinez, U. Schwaneberg, *Green Chem.* **2012**, 14, 2719-2726, *Reengineering CelA2 cellulase for hydrolysis in aqueous solutions of deep eutectic solvents and concentrated seawater.*
- [5] Z. Maugeri, P. Domínguez de María, *RSC Adv.* **2012**, 2, 421–425, *Novel choline-chloride-based deep-eutectic-solvents with renewable hydrogen bond donors: levulinic acid and sugar-based polyols.*
- [6] Z. Maugeri, W. Leitner, P. Domínguez de María, *Tetrahedron Lett.* **2012**, 53, 6968–6971, *Practical separation of alcohol–ester mixtures using Deep-Eutectic-Solvents.*
- [7] P. Domínguez de María, Z. Maugeri, *Curr. Opin. Chem. Biol.* **2011**, 15, 220–225, *Ionic liquids in biotransformations: from proof-of-concept to emerging deep-eutectic-solvents.*

CURRICULUM VITAE

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EDUCATION

September 2010 – March 2014: **PhD in Chemistry** at the Institut für Technische und Makromolekulare Chemie (ITMC) within the group of Prof. Dr. W. Leitner, supervised by Dr. P. Domínguez de María. RWTH Aachen University, Germany.
Thesis's title: Deep Eutectic Solvents: Properties and Biocatalytic Applications.

October 2007 – November 2009: **Second degree - Master in Organic and Bioorganic Chemistry**, University of Catania, Italy.
Thesis's title: New Biocatalyzed Procedure to Esterify Polyunsaturated Fatty Acids.
Graduate with 110/110 cum laude.

October 2004 – November 2007: **First degree - Bachelor in Chemistry**, University of Catania, Italy.
Thesis's title: Molecular Recognition in Aqueous Solution of Organic Anions and Amino Acids by a Tetracationic Calix[4]Arene Receptor.
Graduate with 106/110.

WORK EXPERIENCES

April 2011 – July 2013: **Laboratory assistant for the practicum “Biocatalysis”**. Institut für Technische und Makromolekulare Chemie (ITMC), RWTH Aachen University, Germany.

April – October 2009: **Internship at CNR** Istituto di chimica biomolecolare (NRC Institute of biomolecular chemistry) within the group of Prof. Dr. G. Nicolosi, supervised by Dr. R. Morrone. Catania, Italy.

April – October 2007: **Research student** at the Organic Department within the group of Prof. Dr. D. Sciotto, supervised by Dr. C. Bonaccorso. University of Catania, Italy.

SCHOLARSHIP

September 2010 – August 2013: PhD Scholarship “Biocatalysis using Non-Conventional Media (BioNoCo)” – DFG Research training group at RWTH Aachen University, Germany.

LANGUAGES

Mother tongue: Italian

Other languages: English (fluent)
German (intermediate)

TECHNICAL SKILLS

Characterization/Separation techniques: Gas Chromatography (GC), High Pressure Liquid Chromatography (HPLC), UV-Vis Spectroscopy, Nuclear Magnetic Resonance (NMR), Microwave Reactor. (Common)-chemistry laboratory techniques.

Computer Skills: Windows OS, Microsoft Office (Word, Power Point, Excel, Publisher), Origin, ChemBioOffice, ISIS Draw, ChemSketch, On-line Databases (e.g. SciFinder).

COMPETENCES

Organic chemistry (synthesis of calixarenes, esters, amides, amines, α -hydroxy ketones).
Biocatalysis (experience with lipases, ThDP-dependent enzymes, proteases, alcohol dehydrogenases and whole cells of baker's yeast).
Ionic liquids - Deep eutectic solvents.
Project management and student supervision.

SOFT-SKILL AND EXTENTION COURSES

- Business and Etiquette
- Project and Self-management for your PhD
- Voice and Presentation Training
- GMP (Good Manufacturing Practices)
- Enzyme Immobilization
- Computational Biology
- Biocatalysis
- Bioanalytics

WORKSHOPS AND SUMMER SCHOOL

Green Chemistry Workshop at DSM. Geleen, Netherlands.
BioNoCo Workshops. Dechema Summer School “Biocatalysis using non-conventional media”. RWTH Aachen University, Germany.

CONFERENCES CONTRIBUTIONS

- 1) Maugeri Z, Domínguez de María P. 2011. Frontiers in white technology, Delft, Netherlands, 20.06-22.06.2011.
- 2) Maugeri Z, Domínguez de María P. 2011. Biotrans 2011, Giardini Naxos, Italy, 02.-06.10.2011.
- 3) Domínguez de María P, Maugeri Z. 2011. Biotrans 2011, Giardini Naxos, Italy, 02.-06.10.2011.
- 4) Lehmann C, Sibilla F, Maugeri Z, Domínguez de María P, Streit W, Schwaneberg U. 2011. Biotrans 2011, Giardini Naxos, Italy, 02.-06.10.2011.
- 5) Maugeri Z, Domínguez de María P. 2012. Biocat 2012, Hamburg, Germany, 02.-06.09.2012.
- 6) Maugeri Z, Domínguez de María P. 2012. Green Solvents for Synthesis, Boppard, Germany, 08.-10.10.2012.
- 7) Maugeri Z, Domínguez de María P. 2012. Biotrans 2013, Manchester, UK, 21.07-24.07.2013.