Enzymes, substrates and ionic liquids as a triad for potential development of efficient industrial processes

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I hereby declare that the present thesis entitled “Enzymes, substrates and ionic liquids as a triad for potential development of efficient industrial processes” is the result of my own work, that all sources used or quoted have been indicated, and that I have not used any illegitimate means. I further declare that I have not submitted this thesis for a degree in some form or another.

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II. Publications

Part of this work has already been published or submitted for publication:

Antonovici M. M., Vlahovic S., Sandig B., Buchmeiser M. R. and Hauer B. „Enzymatic reactions in biphasic systems containing ionic liquids: a better understanding of the components interrelation". *Applied Biochemistry & Biotechnology*. In progress.

Sandig, B., Michalek, L., Vlahovic, S., Antonovici, M., Hauer, B., Buchmeiser, M. R. „A Monolithic Hybrid Cellulose-2.5-Acetate/Polymer Bioreactor for Biocatalysis under Continuous Liquid-Liquid Conditions Using a Supported Ionic Liquid Phase". *Chem. – A Eur. J.* **21** (44), 15835-42 (2015).

„Live as if you were to die tomorrow.

Learn as if you were to live forever.”

Mahatma Gandhi

All my thoughts and love to my kids,

Ana and Radu

III. Acknowledgements

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I have to say, I left on purpose Dr. Sandra Facey the last one on the list. Despite the fact that she was never my "legal" supervisor, she was my all-round support in everything, as a student and human being, till the very end.

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IV. TABLE OF CONTENTS

V. Abstract

Ionic liquids stand out for their unique physical-chemical properties that drive a large number of applications in different fields such as synthesis, catalysis, electrochemistry and nanotechnology. Their successful entrance in the enzymatic catalysis arena at the beginning of 2000, showed their potential as reaction medium. For several reactions the organic solvents were successfully replaced by pure ionic liquids generating higher yields and in some cases with proven increased enzymatic stability. This represents the starting point of the project that extended the usage of the ionic liquids for enzyme catalyzed reactions to biphasic systems, for possible new technological solutions, having SILP technologies as a model. The desired reaction takes place at the interface between an ionic liquid and an organic solvent as an immiscible mixture, being catalyzed by lipases, known to be active at the contact phase. The aim is to optimize the reaction system to favor the formation of the product mainly in the organic phase, in an attempt to avoid tedious separation steps, considered in many industrial processes as a bottle neck. The migration towards a particular phase is based on the partition of the compound of interest between the two corresponding solvents. Combination of several ionic liquids with organic solvents characterized by different polarities were set for analyzing the behavior of the substrates as well as for the products.

1-Phenylethanol and 1-methylpiperazine, targeted for the test reactions showed a strong correlation between their polarities and the system, being preferred the phase with the closes physical-chemical properties. The alcohol, as a polar compound was partition almost completely in the polar ionic liquids associated with hydrophobic solvents and shifted more towards the organic phase only when MTBE was part of the biphasic system. The amine, less polar than the alcohol displayed a different partition, to a lesser extend in ionic liquids, favoring the organic solvents. One ester, 1-phenylethyl acetate, as a possible reaction product was tested in the same conditions confirming the partition potential as a separation tool, being present in the organic solvent in larger quantities.

From a large pool of lipases, considered as interesting and robust enzymes, *Candida antarctica* lipase B was chosen for all the experiments. The activation of the lipases was accomplished using oleic acid that can mimic the hydrophobic interface necessary for this type of enzyme. This form of activated enzyme, despite the fact that doesn't reach the performance of an immobilized lipase fulfills the requirements for the later usage in a column.

Transesterification of 1-phenylethanol with different vinyl esters, butyrate, hexanoate and decanoate was chosen in order to proof the versatility of a biphasic system. Four imidazolium based ionic liquids [EMIM] PF_6, [BMIM] PF_6, [HMIM] PF_6 and [OMIM] PF_6 were combined with three organic solvents, MTBE, *n*-heptane and *n*-decane to determine the best conditions for the given product. 1-Phenylethyl butyrate was expected to favour a polar organic solvent, when paired with a more hydrophobic ionic liquid and vice-versa, 1-phenylethyl decanoate to be present in higher amounts in a hydrophobic solvent like *n*-decane, associated with a shorter alkyl chain ionic liquid. The same scenario was applied to the amidation of *α*-methylbenzylamine that clearly showed the potential of a tunable system, according to the chosen reaction. The impact of the cation/anion on the reaction outcome was investigated using 21 ionic liquids that shared the same cation or anion.

Candida antarctica lipase B was also tested for the development of potential intermediates for the pharma industry. Substrates based on piperazine and piperidine ring were investigated in amidation reaction using different acyl donors, like ethyl 3-phenylpropionate, ethyl cinnamate, benzyl acetate, butyl acetate, isopropenyl acetate, *tert*-butyl acetate and isopropylacetate. Best candidates were transferred in biphasic systems for improving product formation.

The last part of the study tried to bring in solutions for reactions that encounter technological challenges. Vitamin C, a strong polar substrate used in many industries as an ester is obtained using a fatty acid ester in the presence of a lipase. The solubility issues raised by the nature of the substrate and product can be solved using biphasic systems that could accommodate all the reactants requirements.

VI. Zusammenfassung

Ionische Flüssigkeiten zeichnen sich aufgrund ihrer einzigartigen physikalisch-chemischen Eigenschaften aus, welche in den verschiedensten Bereichen eine Anwendung finden, so zum Beispiel in der Synthese, Katalyse, Elektrochemie und der Nanotechnologie. Ihre erfolgreiche Einführung in die enzymatische Katalyse Anfangdes 21. Jahrhunderts zeigte ihr Potential als Reaktionsmedium. Bei mehreren Versuchen stellte sich der Ersatz von organischen Lösungsmitteln durch ionische Flüssigkeiten als ertragreicher heraus und teilweise zeigte sich, dass sogar die enzymatische Stabilität gesteigert wurde. Diese Erkenntnis wurde zur Grundlage dieses Projekts, welches die Erweiterung des Anwendungsgebietes von ionischen Flüssigkeiten für enzymkatalisierte Reaktionen zu Biphasischensystemen anstrebte um damit neue technische Lösungen zu finden. Dabei fungierten SILP Technologien als Modell. Die gewünschte Reaktion findet an der Phasengrenze des heterogenen Gemisches, bestehend aus der ionischen Flüssigkeit und dem organischen Lösungsmittel, statt, wobei Lipasen an dieser Stelle katalytisch wirksam sind.

Ziel ist die Optimierung des Reaktionssystems unter Bevorzugung der Produktsynthese in der organischen Phase, um aufwendinge Trennungsverfahren zu vermeiden, welche in industriellen Prozessen meist als limitierender Faktor gelten. Die Diffusion in Richtung einer bestimmten Phase hängt von der Verteilung des betreffenden Stoffes zwischen den zwei verwendeten Lösungsmitteln ab., Die Kombination mehrerer ionischer Flüssigkeiten mit organischen Lösungsmitteln verschiedener Ladungen wurde zur Analyse des Verhaltens sowohl der Substate als auch der Produkte verwendet. 1-Phenylethanol und 1-Methylpiperazine, speziell für Testreaktionen entwickelt, wiesen auf einen starken Zusammenhang zwischen ihrer Ladung und dem zugehörigen System hin, wobei die Phase mit den ähnlichsten physikalisch-chemischen Eigenschaften bevorzugt wurde. Der Alkohol als polarer Bestandteil wurde fast vollständig in den polaren ionischen Flüssigkeiten vermengt mit hydrophoben Lösungsmitteln diffundiert. Nur als MTBE dem zweiphasigen System hinzugefügt wurde, konnte der Alkohol vorwiegend in dem organischen Lösungsmittel nachgewiesen werden. Die Amine, welche weniger polar sind als der Alkohol, wiesen eine bevorzugte Verteilung in organischen Lösungsmittel auf und konnten seltener in ionischen Flüssigkeiten nachgewiesen werden. Der Ester 1-Phenylethyl acetat als mögliches Reaktionsprodukt wurde unter denselben Bedingungen getestet und in höheren Koncentrazionen in dem organischen Lösunsmittel nachgewiesen, was das Potential der ionischen Flüssigkeiten als Trennmittel bestätigt.

Aus einer großen Auswahl von Lipasen, welche als versuchsrelevant und stabiles Enzyme galten, wurde die *Candida antarctica* Lipase B als Standardenzym für alle Versuche gewählt. Die Aktivierung der Lipasen wurde mit Hilfe von Öleinsäure, welche die für dieser Art von Enzym benötige hydrophobische Oberfläche/Grenzfläche nachahmen kann, ermöglicht. Diese Form eines aktivierten Enzymes wird den Bedingungen gerecht, die für die spätere Nutzung in der Säule benötigt werden, obwohl es noch nicht die Leistung einer immobilisierten Lipase aufweist.

Die Umesterung von 1-Phenylethanol mit verschiedenen Estern, Butyrat, Hexanoat und Decanoat sollte die Vielseitigkeit des biphasischen Systems beweisen. Die vier ionische Flüssigkeiten [EMIM] PF_6, [BMIM] PF_6, [HMIM] PF_6 und [OMIM] PF_6 basierend auf Imidazolium wurden mit den drei organischen Lösungsmitteln MTBE, *n*-Heptan und *n*-Decan kombiniert, um die besten Bedingungen für das jeweilige Produkt zu ermitteln. Es war zu erwarten, dass 1-Phenylethylbutyrat in Kombination mit einer hydrophoberen ionischen Flüssigkeit ein polares organisches Lösungsmittel bevorzugt, und umgekehrt. Es wurde ebenso erwartet, dass 1-Phenylethyldecanoat hydrophobische Lösungsmittel wie *n*-Decan in Verbindung mit einer ionischen Flüssigkeit mit kürzerer Alkylkette bevorzugt. Dieselbe Herangehensweise wurde auf die Amidierung von α-Methylbenzylamin angewendet, was das Potential dieses in Bezug auf die gewählte Reaktion einstellbaren Systems klar zeigte. Der Einfluss der Kationen beziehungsweise Anionen auf die Reaktion wurde mit Hilfe von 21 ionischen Flüssigkeiten, welche dasselbe Kation oder Anion aufwiesen, ermittelt.

Candida antarctica lipase B wurde auch für die Entwicklung potentieller Zwischenprodukte für die Pharmaindustrie hin untersucht. Substrate basierend auf Piperazin- und Piperidinringen wurden bei der Amidierung mit verschiedenen Acyldonoren wie Ethyl-3-phenylpropionat, Zimtsäureethylester, Benzylacetat, Butylacetat, Isopropenylacetat, tert-Butylacetat und Isopropylacetat analysiert. Die Produkte, mit denen die besten Ergebnisse erzielt wurden, sind in das zweiphasige System übernommen worden um die Synthese zu verbessern.

Der letzte Teil dieser Studie hatte zum Ziel, sich mit Reaktionen zu befassen, welche technologische Herausforderungen darstellen. Vitamin C, ein stark polares Substrat, das vielfältige industrielle Verwendung als Ester findet, wird durch die Katalyse eines Fettsäureesters mittels einer Lipase gewonnen. Es konnte gezeigt werden, dass die mangelnde Löslichkeit aufgrund der Eigenschaften des Substrats und des Produktes, durch die Verwendung von zweiphasigen Systemen, welches alle Bedingungen der Reaktanten erfüllt, vermieden werden kann.

VII. Abbreviations

(v/v)	volume per volume
°C	degree Celsius
Å	Ångström
CaL B	*Candida antarctica* lipase B
DAD	diode array detector
DMSO	dimethyl sulfoxide
FID	flame ionization detector
g	gram
GC	gas chromatography
GC/MS	gas chromatography coupled to mass spectrometry
h	hour
HPLC	high performance liquid chromatography
IL	ionic liquid
LC/MS	liquid chromatography coupled to mass spectrometry
µl	microliter
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mm	millimeter
mmol	millimol
MTBE	*tert*-butyl methyl ether
nm	nanometer
t-BuOH	*tert*-butanol
U	units

1. Introduction

1.1 Enzymes

1.1.1 Historical background

en - zymē was defined in the Greek language as "in - sour dough" that sets back in time the awareness of an enzymatic process in living organisms. The leavening of bread by yeast wasn't the only one: wine making, vinegar and diary production and meat tenderizing were enzyme based processes known already around 2000 B.C.[1,2] The nineteenth century was full of new discoveries that brought up to life enzymes and their undeniable contribution. It was Edward Buchner's discovery in 1897 that showed, using "pressed juice" from rehydrated dried yeast that alcoholic fermentation could take place out of the living yeast cells.[3,4] However, Kühne in his comprehensive work on catalysis in yeast extracts was the first one to use the term of enzyme. The first purification of an enzyme, urease from jack beans was accomplished by James Sumner in a relentless work between 1917 and 1926. Noteworthy is, beside the isolation and crystallization of the urease, the proof that an enzyme is in fact a protein, a theory supported at that time by a series of chemical experiments.[5]

1.1.2 General consideration

Enzymes are usually globular proteins with a defined composition and structure originating from living cells that are capable to catalyze biochemical reactions in which a substrate undergoes chemical changes and a new compound is formed. As catalyzers are able to "accelerate the rate of chemical reactions by stabilizing the transition state of the reaction, hence lowering the activation energy barrier to product formation".[1] Their value to the reactions is reinforced by their unique properties such as substrate specificity, stereoselectivity, regioselectivity and chemoselectivity leaving behind organic chemistry helpless, but hopeful.[6]

1.1.3 Classification

The classification of approximately 75,000 known enzymes in the human body is not a trivial task. Currently, the International Union of Biochemistry and Molecular Biology (IUBMB) classified the enzymes in six major classes based on the type of catalyzed reactions:[7]

a. Oxidoreductases are responsible for oxidation and reduction reactions.
b. Transferases catalyze the transfer of functional groups.
c. Hydrolases catalyze in principle hydrolysis reactions.
d. Lyases are participating in the cleavage of C-C, C-O, C-S and C-N bonds by means of other reactions than hydrolysis or oxidation.
e. Isomerases are involved in the rearrangement of atoms in a molecule.
f. Ligases catalyze the joint of two molecules.

1.2 Lipases

Triacylglycerol ester hydrolases (EC 3.1.1.3), commonly known as lipases are part of the third class of enzymes. As their name reflects, they are enzymes responsible for the hydrolysis of triacylglycerides into free fatty acids and glycerol. In a very pragmatic way Robert Verger called them simply "fat-splitting ferments".[8] Their unique ability to act at an interface between water and fat make them distinguish. The lipolytic reaction is rather complex: the lipid substrate, insoluble in water creates a heterogeneous environment defined by a liquid-liquid interface. The enzyme present in the hydrating shell becomes active at the interface, where can access the substrate and catalyze the reaction.[9,10,11] Known as interfacial activation, this step is dependent on the nature of the interface, interfacial property and area. The phenomenon starts with the adsorption of the lipase at the interface that generates a sequence of events prior the completion of the reaction. The lid, a structural part of most lipases will open to give access for the substrate to the catalytic center, located generally under it. The product formed during the catalysis accumulates at the interface and reduces the interfacial pressure and sub sequentially increases the surface energy, effect tolerated by the lipases without denaturing.

This could describe a rather narrow reactivity window, which lipases had successfully contradicted, not in a punctual manner, but in many aspects. They are able to host a large and diverse palette of substrates in a chemo-, region- and stereoselective way that lead to alternative organic synthesis and industrial applications.[12] They are able to catalyze reactions

dissolved or as a suspension in other media than water (organic solvents and ionic liquids) and to sustain elevated temperatures.[13] Crystal structures for many lipase members were resolved facilitating considerably the evolution towards improved processes, task designed enzymes based on design of rational engineering strategies. Not to neglect the availability in large quantities once the sources of lipases extended to microbial organisms, namely fungi and bacteria. Some of the issues related to long term stability and recyclability were turned into successful stories by immobilization techniques.

It is generally accepted that lipases are ubiquitous enzymes and the short list below comprises the most common ones.

Table 1. Commercially available lipases and their origin[14,15]

Origin	Name	Abbreviation
Mammalian	Human pancreatic lipase	HPL
	Human gastric lipase	HGL
	Porcine pancreatic lipase	PPL
	Guinea pig pancreatic lipase	GPL-RP2
Fungal	*Candida rugosa*	CRL
	Candida antarctica lipase A	CaL A
	Candida antarctica lipase B	CaL B
	Geotrichum candidum	GCL
	Thermomyces lanuginosus	TLL
	Rhizomucor miehei	RML
	Aspergillus oryzae	AOL
	Aspergillus niger	ANL
	Penicillium camembertii	PEL
	Rhizopus delemar	RDL
	Rhizopus oryzae	ROL
	Rhizopusarrhizus	RAL
Bacterial	*Pseudomonas cepacia*	PCL
	Pseudomonas aeruginosa	PAL
	Pseudomonas fluorescens	PFL
	Pseudomonas mendocina	PML
	Burkholderia cepacia	PCL

	Chromobacterium viscosum	CVL
	Bacillus thermocatenulatus	BTL-2
	Fusarium solani	FSL
	Staphylococcus aereus	SAL

1.2.1 Structure and mechanism

Unrevealing the structure of the lipases, as for any living or non-living form is a natural and necessary step that contributes to the fundamental understanding of these enzymes. This will be the omnipresent information behind the agreement with the reaction mechanism, properties, stability in different environments which can lead to possible structural changes for a better catalyst.

The *α*/ß domain that characterizes this class of enzymes is preserved in lipases and is composed of central, parallel ß sheets surrounded by *α* helices. More precisely the canonical *α*/ß-hydrolase fold includes eight-stranded ß sheets enclosed in a variable number of *α* helices, according to the particular enzyme (Figure 1).[16,17]

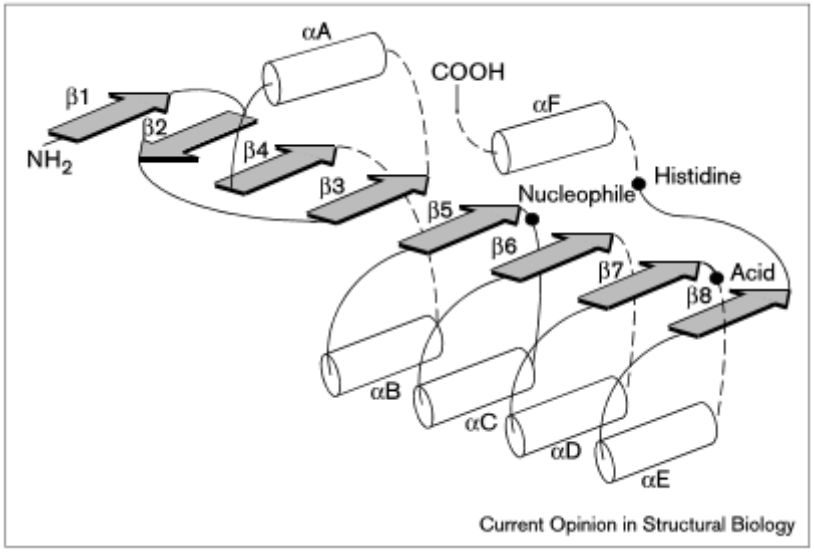


Figure 1. Secondary structure diagram of the canonical *α*/ß-hydrolase fold, including *α* helices and ß strands. The location of the catalytic triad is indicated by black dots.[16]

The arrangement and sometimes the number of the ß-strands can vary, but at least one is antiparallel.[18] The catalytic residues including a nucleophile (serine, cysteine or aspartate), an acidic moiety (glutamate or aspartate) and a conserved histidine form a catalytic triad that has a defined character, but adaptable capabilities and locations in a given frame.[19] Even though the acid is usually positioned after the ß7 strand, there are cases when the functional triad

could be constructed having it aligned with a previous strand. The site anatomy determines the function and mechanism of the enzyme and, as will be later underlined the possibility to accommodate multiple substrates in the active site.[20] As shown in the graphical representation the nucleophile has a particular position that has adopted the name: "nucleophile elbow". This strategic area is placed on a very sharp turn that could be identified by the consensus sequence G-X-Nu-X-G (X = any residue, Nu = nucleophile) and has the given role to expose the nucleophile in order to be easily approached by the substrate. This strand-turn-helix motif is a rigid construct that also energetically places the residue in a particular angle with steric restriction, valid also for the amino acids located in the proximity. The oxyanion-binding site, practically shaped by the geometry of the nucleophile elbow has the given function to stabilize the negatively charged tetrahedral intermediate that occurs during the transition state. Hydrogen bonds are formed with the amino acids responsible for the stabilization of the oxygen ion that are usually backbone nitrogen atoms.

The catalytic triad is buried under a secondary structure element, known as the lid or flap that is responsible, in the active form for the admittance of the substrate (Figure 2).

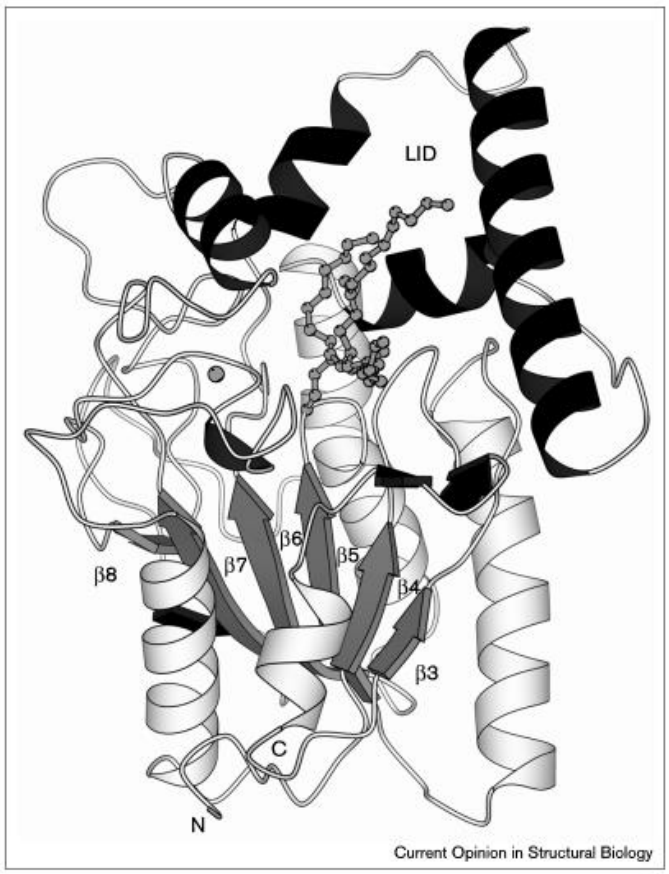


Figure 2. Position of the *α*-helical lid in an open conformation of *Pseudomonas aeruginosa* lipase (M. Nardini).[16]

The constituent α-helices vary in number and length and are flanked by hinge segments on both ends. This feature is typical only for a certain number of lipases like *Candida rugosa*, *Candida antarctica*, *Rhizomucor miehei*, *Pseudomonas aeruginosa* just to name a few of them. The suggested link between the lid positon (open or closed) and the interfacial activation was the topic of extended research, starting with the first two crystal structures solved in 1990.[21] From the X-ray structure it was possible, indirectly to prove that the lid undergoes conformational rearrangement towards an open stand, after a previous contact with a lipid-water interface.[15,10] From that point on, the statement related to this mechanism fluctuated and other hypotheses evolved through simulation studies. It was shown a strong correlation between the lid and parameters like working temperature, organic solvent, solution conditions and dielectric constant.[22,23] In 2015, a study performed on *Candida antarctica* lipase B at atomic resolution, highlights the protonation and the mechanism of interfacial activation that will be correlated with a section of the experimental part.[24]

The binding pocket of the lipases is positioned closed to the central ß sheet and can have different shapes (crevice, funnel-like and tunnel-like) that defines another way to classify lipases. A tridimensional structure of an enzyme, therefore of a protein could not be rounded up without the last bridge, the disulfide bonds. Lipases are cysteine rich proteins containing one to four disulfide bridges that brings stability by lowering the entropy. The ground mechanism on which the lipases perform their catalytic reactions is described in Figure 3.

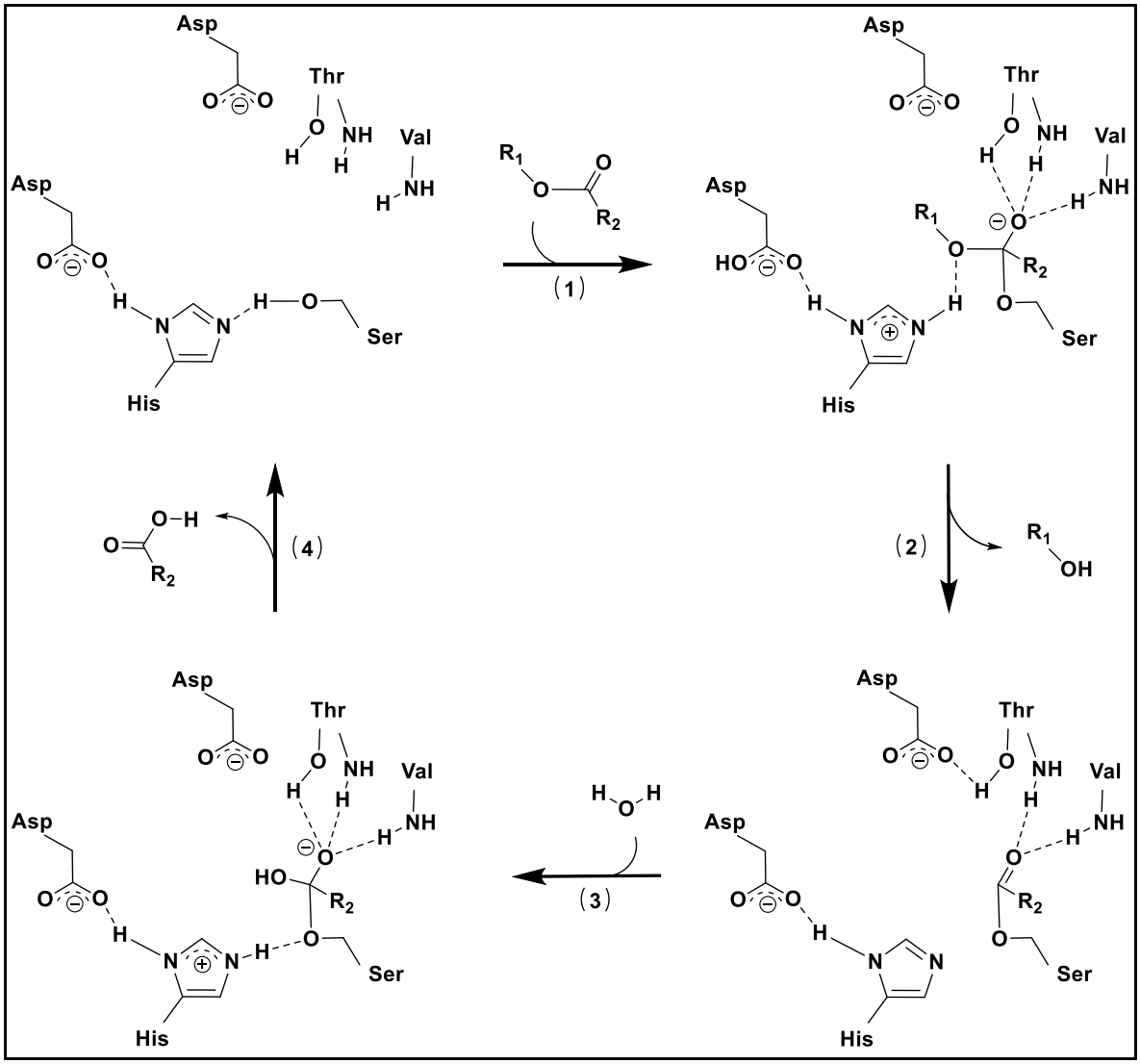


Figure 3. Proposed catalytic mechanism of lipase based on the serine, histidine, aspartate triad. The tetrahedral intermediate is stabilized by the oxyanion hole via hydrogen bonding (1), followed by the alcohol release (2). The addition of a water molecule generates a second complex (3), which will release the final product (4). Modified from [15] and [25].

The substrate, for example an ester in the active pocket is subjected to a nucleophilic attack generated by the serine residue, activated itself by the chain proton abstraction initiated by the other residues, histidine and aspartate.[15,25] The resulting tetrahedral intermediate is stabilized by the hydrogen bonding within the oxyanion hole. In the second step histidine transfers its proton to the alcohol product that is released. A water molecule enters and after being activated by histidine/aspartate pair, part of the former tetrahedral intermediate attacks the acyl enzyme construct to generate the second complex. This will eventually collapse and release the carboxylated product.

1.2.2 Classification of lipases

The clarification of the structure, in tandem with other elements provided a good start for a more comprehensive classification.

In 1999, Arpigny and Jaeger grouped the bacterial lipases and esterases in relationship to their conserved sequence motifs, biological properties and 3D structures.[26] This consists of eight families, in which six have the typical α/ß-hydrolase fold, the remaining two being characterized by an SGNH-hydrolase, respectively the ß-lactamase fold. The first group belongs to the so called true lipases and the first three subdivisions initially included the *Pseudomonas* lipases. The similarities within the first subfamily (I.1) are defined on the comparison of the amino acid sequence and their size range between 30-32 kDa. Once some of them were renamed as *Burkholderia* the classification was revised and formed a self-standing subfamily with a slightly larger molecular weight of 33 kDa (I.2). The residues involved in the catalytic triad, as well as two cysteines forming a disulphide bridge are conserved in the majority of the sequences belonging to these two subfamilies. The third one (I.3) includes lipases with higher molecular mass, in the range of 50 and 65 kDa and lack the N-terminal signal peptide and cysteine residues. The next two subdivisions are lipases from Gram-positive organisms, with alanine replacing the first glycine in the conserved pentapeptide (A-X-S-X-G, where X represents any amino acid). One of them has enclosed the smallest true lipases known (I.4) and the other is defined by higher masses and different activities (I.5). The last subgroup (I.6) includes two enzymes that have 39% identity and 50% similarity to each other, but not with other lipases.

The second family have a GDS(L) motif that allows the active site serine residue to be placed in the close vicinity of the N-terminus. The next family contains enzymes with the canonical fold of α/ß-hydrolase and possess the typical catalytic triad. The serine residue, identified by site-directed mutagenesis is not in the conventional position, showing one more time the functional versatility of the scaffold. The hormone-sensitive lipase family overlaps surprisingly the sequence of the mammalian HSL, but needs to be further investigated for other distinctive properties. The enzymes from the fifth family are deriving from cold or heat adapted organisms and mesophilic bacteria and have the α/ß-hydrolase fold as well as the catalytic triad conserved. The next family covers the smallest esterases known with the mass between 23 – 26 kDa and have about 40% sequence similarity to eukaryotic lysophospholipases. A group of rather large esterases (55 kDa) with 30% identity and 40%

similarity of amino acid sequence homology form the seventh family, followed by a small family of only three enzymes that resemble class C ß-lactamases.

The revisions of this classification, along with the derived improvements generated a broad database available on-line under the name ESTHER.[17] A more recent and simplified sequence based database was created in 2000 by the group of Pleiss, known as Lipase Engineering Database (LED) with an initial number of 92 microbial lipases and homologous serine hydrolases.[27] It was intended as a bioinformatics tool for the systematic analysis of the sequence-structure-function triad.[28] By 2009, the database extended to a number of 24783 sequence entries, corresponding to 18585 proteins out of which 656 would have a defined protein structure.[29] The classification criteria derived from sequence alignment and structure superposition is based on the conservation of the oxyanion hole, generating three classes of enzymes, GGGX, GX and Y and further superfamilies (Figure 4).

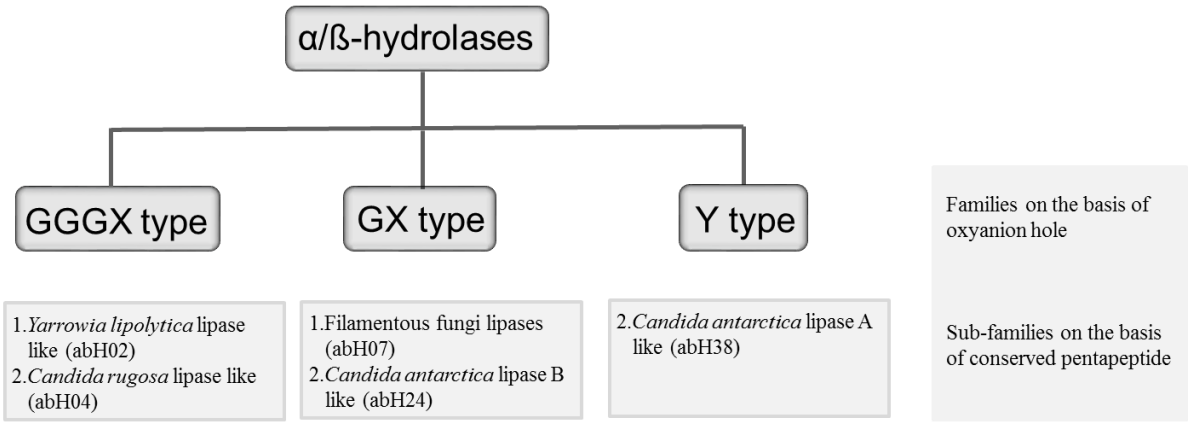


Figure 4. Classification of lipases, members of *α*/ß-hydrolase enzymes based on the Lipase Engineering Database (LED).[29]

Another classification of the lipases was performed by the same group taking in consideration the geometry of the binding site:[30]

a. Lipases with a hydrophobic, crevice-like binding site located near the protein surface (*Rhizomucor* and *Rhizopus*).
b. Lipases with a funnel-like binding site (*Candida antarctica*, *Pseudomonas* and mammalian pancreas).
c. Lipases with a tunnel-like binding site (*Candida rugosa*).

The structure of the lipases, reflected in the classification itself underlines their large variety and intrinsic their capability to accept structurally different substrates, leading to an extended

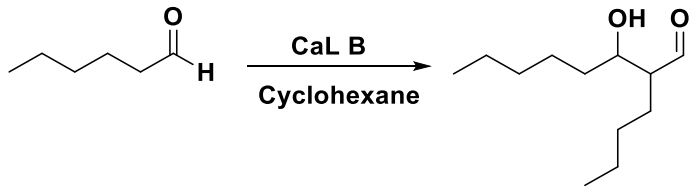
number of new possibilities. The ability to catalyze alternative reactions was extensively studied and the results revealed unexpected territories.

1.2.3 Catalytic abilities and accomplishments

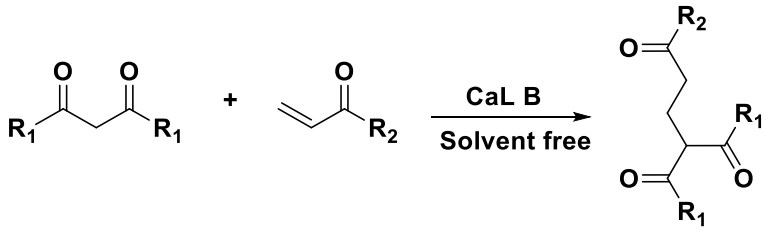
Even though Jensen introduced the idea in 1976, the term promiscuity didn't find the right outline or place for a long time in enzymology. A lot of ink has been spilled over the subject until a more coherent and sinful classification was done by Hult and Berglund in 2007.[31]
After they defined the promiscuous enzyme in a very straight forward way "*as one that does things it is not expected to do*", they draw the boundaries of the ongoing directions as: condition promiscuity (when the conditions differ from their natural ones); substrate promiscuity (poses a relaxed or broad substrate specificity) and catalytic promiscuity (for different chemical transformations with different transition states). Interestingly enough, lipases scored 37% from all the given examples of enzymatic promiscuity. And they have their own good reasons. From lipolysis, lipases proved an unprecedented expansion of reactions in organic synthesis based on a set of facts: [15,32]

a. As a first step of the enzymatic mechanism, the acyl lipase is formed and could be considered as an acylation agent. This explains the versatility of lipases to acylate other groups like amines, hydroperoxides and thiols (catalytic promiscuity).
b. They evolved unusually stable structures that were validated in organic solvents as reaction medium (condition promiscuity).
c. Lipases could accommodate a wide variety of non-natural substrates preserving the regio- and stereoselectivity (substrate promiscuity).
d. The thermodynamic equilibrium for the lipase catalyzed reactions is driven mostly by the reactant concentrations and so the ester hydrolysis in water could take a twist into ester synthesis and transesterifications once water is replaced with a solvent.

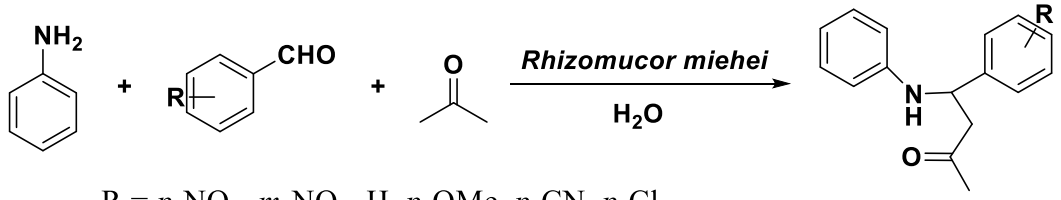
Without the intention to cover all the enzymatic capabilities of the lipases a few and hopefully relevant examples are discussed.
Catalytic promiscuity unleashed new prospects for lipases, like carbon-carbon, carbon-heteroatom, heteroatom-heteroatom bond formation being possible, beside oxidative processes.[33] Organic synthesizes rely a lot on the C-C bond formation, which enzymatically was performed by aldolases. Lipases joined the group, catalyzing aldol and Michael additions and Mannich reactions (Scheme 1).[34,35,36]



A.



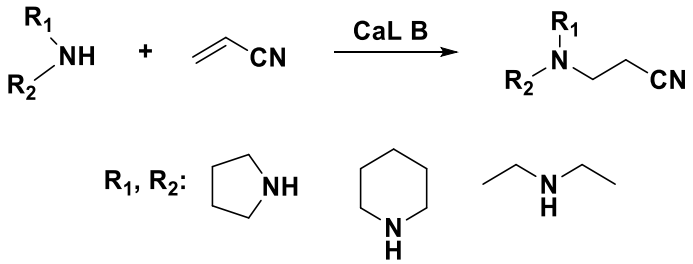
B. R_1 = Me, OMe; R_2 = H, Me, OMe



C. R = *p*-NO_2, *m*-NO_2, H, *p*-OMe, *p*-CN, *p*-Cl

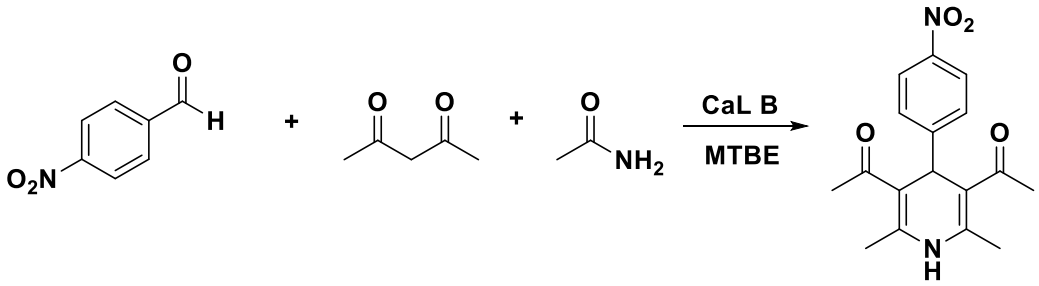
Scheme 1. Catalytic promiscuity displayed by lipases in C-C bond formation: Aldol addition (A); Michael addition (B); Mannich reaction (C). [34,35,36]

Carbon-heteroatom and heteroatom-heteroatom bond formation are possible to perform in the presence of lipases, through Michael type additions and Markovnikov and anti-Markovnikov additions (Scheme 2).[37]



Scheme 2. Catalytic promiscuity displayed by lipases in C-heteroatom bond formation: Michael addition.[37]

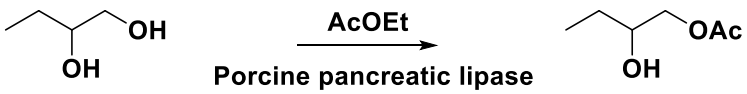
In terms of oxidative reactions lipases are able to catalyze perhydrolysis, direct epoxidation of alkenes and synthesis of heterocycles and Hantzsch-type reactions (last synthesis being represented in Scheme 3).



Scheme 3. Catalytic promiscuity displayed by lipases in oxidative processes like Hantzsch-type reactions.[37]

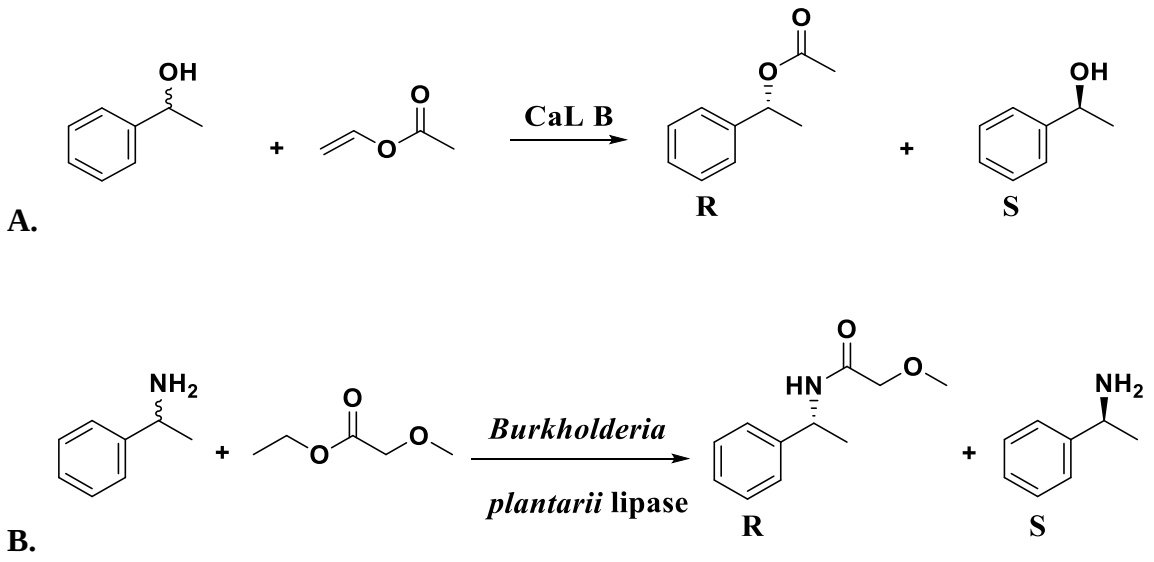
When we move towards the area of condition promiscuity, lipases excelled again being able to perform catalytic reactions in organic solvents. The group of Klibanov pursued a crucial direction in the development of enzymatic reactions, proving that lipases are stable and active in organic solvents.[38,39] When water was replaced by anhydrous solvents, hydrolysis was replaced by alternative reactions: esterification, transesterification, interesterification, acidolysis, alcoholysis, aminolysis and thiotransesterification.[37] Unprecedented high reaction temperatures were achieved in the new medium.[40] High salt concentrations, extreme pH, gaseous and solid phase, solvent free conditions, ionic liquids and at last but not at least, biphasic conditions (organic solvents – water or ionic liquids) are new possible choices for lipase catalyzed reactions.

As a start into the lipase reactivity spectrum, the specificity of lipases should be analyzed. They can catalyze triglycerides in a non-specific reaction, as glycerol and fatty acids are released or they can preferentially release the acids from positions 1 and 3.[37] It is also known that lipases would selectively hydrolyze unsaturated long chain fatty acids from oat seeds oil. Increased biodiesel yields were achieved during the 1,3 specific hydrolysis, once were associated with a typical acyl migration for lipase catalysis. For polyfunctional compounds, lipases proved their capability to generate regioselective transesterification like in the presence of porcine pancreatic lipase (Scheme 4).



Scheme 4. Regioselective transesterification with porcine pancreatic lipase.[15]

Lipases evolved towards large enzymatic domains necessary for the acyl group binding and are able to host beside triglycerides and aliphatic esters, other compounds like alicyclic, bicyclic, aromatic esters and thioesters. Regioselectivity was proven also for the amidation of benzyl esters in the presence of symmetrical or non-symmetrical diamines while *Pseudomonas cepacia* was the biocatalyzator.[41] The same reaction was possible with diesters in a one pot reaction (amidation and hydrolysis) that underlines the ability of the lipase. [42]
The selectivity of the lipases towards other substrates materialized in the resolution of racemic esters or amines.[43,44,45] The capability to recognize enantiomers led to kinetic resolutions with yields of maximum 50%. The acylation of a racemic alcohol will enable predominantly the (S) alcohol and the (R) ester as shown in Scheme 5. Same behavior is noticed for the acylation of amines.[46]



Scheme 5. Kinetic resolution of a racemic alcohol (A) or racemic amine (B) catalyzed by *Candida antarctica* respectively, *Burkholderia plantarii* lipases.[46]

Empirical rules for the enantiopreference of lipases, broadly accepted as "Kazlauskas rules" were published in 1997 in an attempt to explain how the enzymes "filter" the enantiomers based on the size of the substituents on secondary alcohols, a principle that is not generally accepted for carboxylic acids or primary alcohols (Figure 5).[47] The predictions were supported later on by crystallographic experiments.[48]

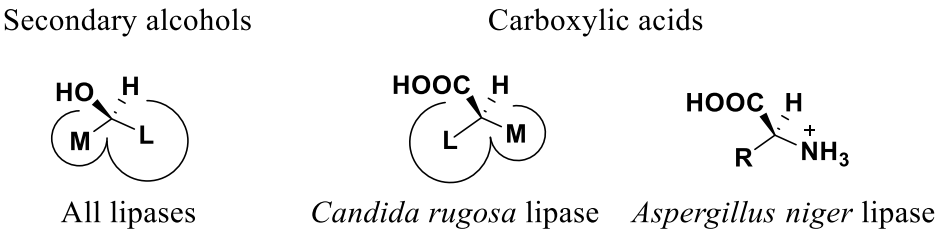


Figure 5. "Kazlauskas rules" (M = medium-sized substituent, L = large substituent).[47]

As depicted in Figure 5, when the hydroxyl group points out of the plane the particular enantiomer with the large substituent (phenyl) on the right side and the medium one on the left hand side is favored by the lipase. A test reaction involving secondary alcohols with both substituents similar in size, proved the concept while the enzyme didn't have the capability to resolve efficiently the racemic mixture.

In particular this type of reactions draw the attention, taken in consideration the large number of possible acyl donors. During this study the interest was shifted from the effectiveness in enantiomeric separations more towards the formation of new esters and amides with applicability in different areas of interest.

1.2.4 Immobilization

As the dominant performance of the lipases in the enzymatic world was presented, the issue related to their stability will be re-evaluated, from an immobilization perspective as a possible solution. Immobilization, as a method could be straightforward for an adsorption or a tedious multistep for an entrapment strategy. At the end of the procedure the enzyme should be in an active form, stable and in surroundings that do not alter the mass transfer. Once the step is successfully accomplished the benefits are evident:[49]

a. enhanced stability
b. multiple uses in batch or continuous processes
c. easy to recover from the reaction mass
d. possible diversification of the catalytic properties
e. clean product (without protein impurities)
f. avoid microbial contamination.

The immobilization methods can be organized, as shown in Figure 6 in four major groups: adsorption, immobilization via ionic interaction, covalent attachment and entrapment in a polymeric gel.[50]

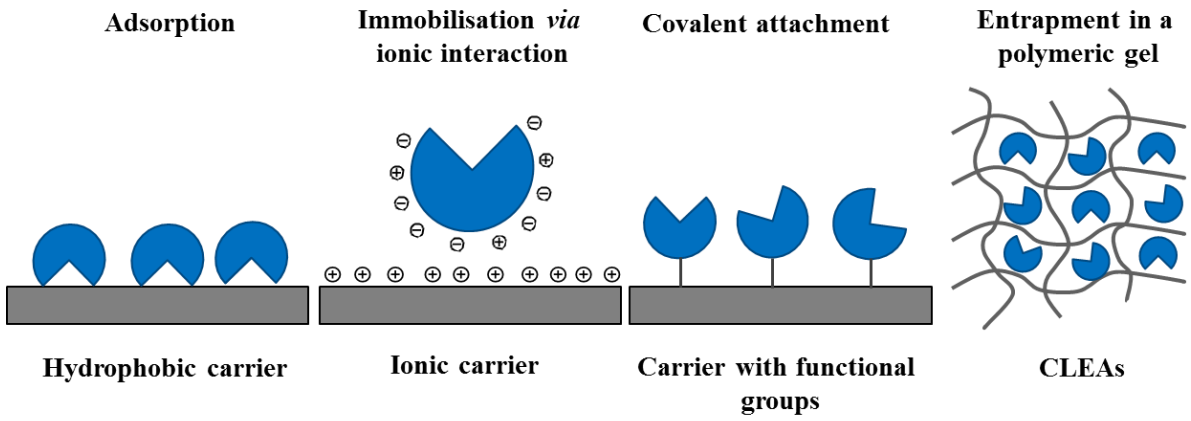


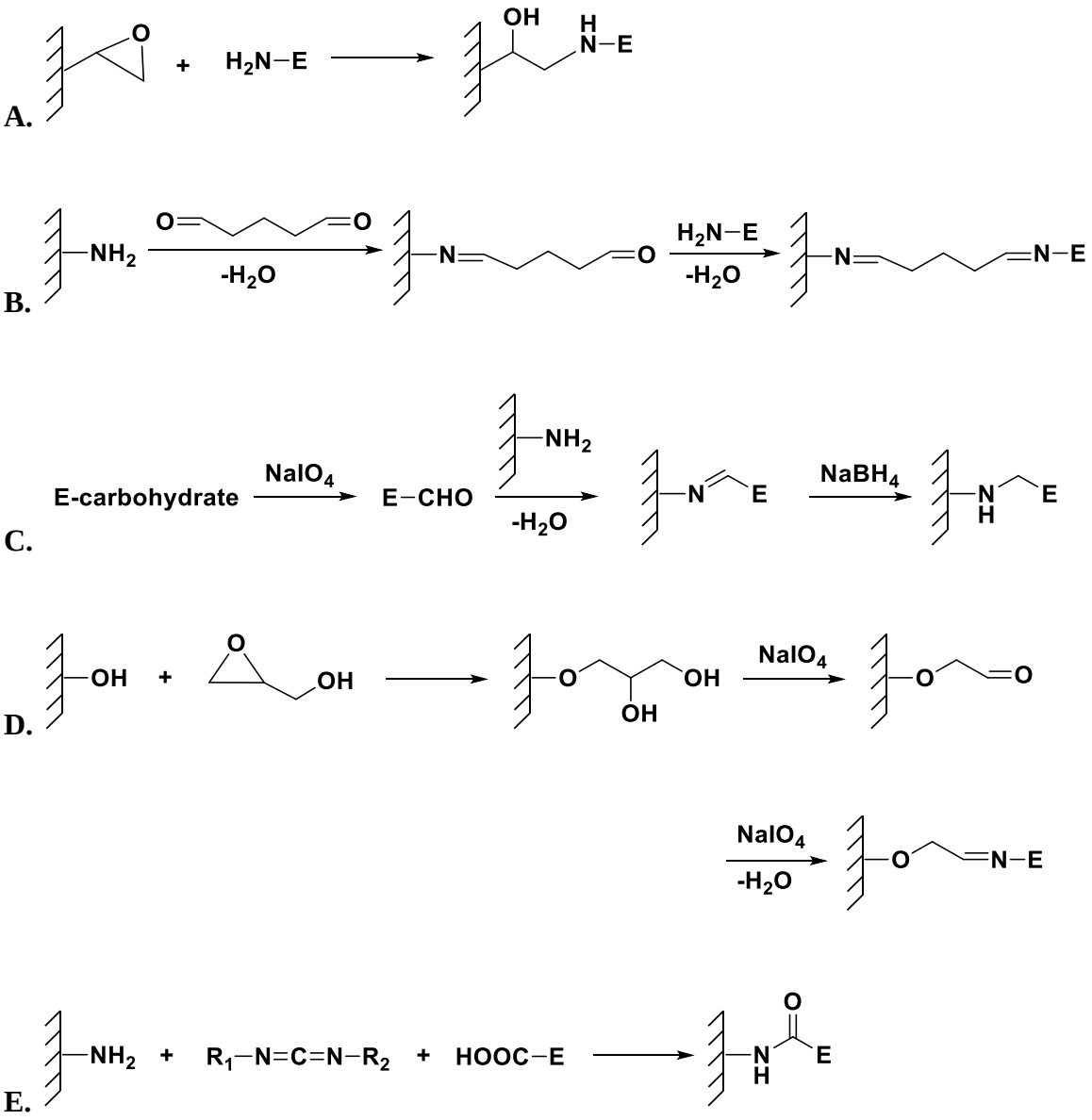
Figure 6. General enzyme immobilization techniques: adsorption, immobilization *via* ionic interaction, covalent attachment and entrapment in a polymeric gel. Modified from [50].

Adsorption occurs when a solution containing solubilized enzyme is brought in contact with a carrier in most cases, hydrophobic. At this point, clarifications related to the activation of some lipases containing a lid have to be taken in consideration. Adsorption could take place also on inorganic supports (Kieselguhr, hydroxyapatite or alumina), on mesoporous silica (with different pore size, porosity and particle size) or organic polymers (acrylate-based polymer, styrene-divinylbenzene, polypropene).[51,52,53,54,55] Enzyme coated microcrystals that belong to the same group were prepared by mixing an aqueous enzyme solution with a concentrated inorganic salt or optional a sugar or amino acid. The mixture will crystalize once in contact with a water miscible organic solvent. Hydrophobic or van der Waals interactions and hydrogen bonds are responsible for this type of immobilization.

For the case of ionic interactions, a variety of ion exchangers can be used according to the net surface charge of the enzyme.[56] Based on the correlation between pH of the solution used to dissolve the enzyme and the isoelectric point, the surface of the protein could bear a different charge.[57] A useful tool helping with the selection of the right support is available online and can provide values for surface charge and also the possible charge distribution. The carrier can have anchored carboxylated or protonated groups to match the charge on the enzyme. As an example polyethyleneimine (PEI) containing amino groups is used as ion exchanger for the immobilization of CaL B.

Covalent binding is the recommended method for working in aqueous solutions or when denaturing factors are involved in the reaction. This type of immobilization brings a set of factors, pro and contra that should be taken in account. One of the biggest advantage should be considered the stability of the enzyme that through multiple covalent bonds reduces the protein denaturation.[58,59] On the other hand there is the risk of enzyme inactivation, especially

when the multipoint attachment could not be delivered in controlled manner for all protein molecules.



Scheme 6. Enzyme covalent immobilization methods: (A) Direct coupling to epoxy-activated support; (B) Immobilization on a support with amino groups *via* glutaraldehyde; (C) Oxidation followed by coupling to a support containing amino groups; (D) Glyoxyl activation of a support containing hydroxyl groups and further immobilization of the enzyme; (E) Amide formation using amino groups on the support and carbodiimide .[60]

In Scheme 6 there are presented a few possibilities for covalent immobilization.[60] The particular case with glutaraldehyde evolved into a special immobilization pattern where the carrier was replaced by a crosslinked enzyme. The preparation of the so called CLEAs (Cross Linked Enzyme Aggregates) is basically done in two steps: first the enzyme precipitation

with the aim of a precipitant and the second step the crosslinking achieved commonly with glutaraldehyde (the crosslinker).[61,62]

The encapsulation of the enzyme, for example in a sol-gel network that is basically a chemical inert glass is able to preserve the structure of the protein in a thermally and mechanically stable architecture. The encapsulation procedure is rather easy and is based on the acid- or base-catalyzed hydrolysis of tetraalkoxysilanes $Si(OR)_4$.[63,64]

Lipases, in particular were associated with another immobilization method, microemulsions. This form emerged as a thermodynamically stable mixture of water, organic phase and surfactant that traps and concomitantly hosts the enzyme ready for catalytic reactions. Though, in some cases there is a risk that the enzymes could be inactivated by the surfactant in the microemulsions.

Immobilized lipases are commercially available on different supports by a significant number of manufacturers, for lipases, Novozyme 435 (on an acrylic resin carrier) being the most used form.

The evaluation of the immobilization is usually completed using standard reactions, like the hydrolysis of p-nitrophenyl esters for lipases. In some cases, where the distribution of the enzyme in/on a particular material has to be assessed this approach could not be used as a deciding criteria. Labelling the particular enzyme with a compound that could be optically visualized became a common procedure in particular using fluorophores.

The advantages are even more valuable in the context of the multiple industrial applications of the lipases. A whole research field is dedicated to this aspect that sometimes is the key of a successful reaction, on the way to a successful process.

1.2.5 Biotechnological Applications

Lipases represent about 5% of the enzyme market and the prediction for 2020 is to reach about $590.5 million worth that means about 6.5% of projected growth. Currently, over 500 products of the industrial market are enzymatic manufactured.[65] This could be explained by the incredible expansion of lipase applications in different sectors of the industry. [66,67]

Without doubt, the application summary has to refer first to the preparation of soaps and fatty acids. The hydrolysis of triglycerides in the presence of an enzyme, more precisely *Candida rugosa* lipase is able to produce in less than 48 h considerable amounts of soap and as a by-product a 20% glycerol/water mixture. Such a low cost process that requires only a stirred reactor as equipment is obviously preferred to the chemical one, plus the final product could

be defined by better colors.[15] The regioselectivity of the lipases towards the 1 and 3 position of natural triglycerides in interesterification reactions was considered of industrial interest able to generate products of commercial relevance. Margarines and shortenings are fats with lower melting point and improved spreadability that are prepared using this enzymatic capability, followed by hydrogenation when necessary.[68,69,70] Triglycerides are better absorbed when the sn-2-position is substituted with palmitic acid. In order to achieve this kind of easy to digest type of fats, lipases like *Rhizomucor miehei* are used to modify tripalmitin by interesterification with oleic acid.[71] Several companies used the same principle to replace the scarce production of highly demanding natural cocoa butter that contains mostly triglycerides with oleic acid in the sn-2-position and stearic and palmitic in the remaining positions (known as SOS or SOP). The chemical path was replaced by the transesterification of sunflower oil with stearic acid, again with the aid of *Rhizomucor miehei* lipase in a packed-bed reactor.

To remain in the food sector we could not neglect the impact of enzyme catalyzed processes in the dairy industry. Lipases and proteases replaced the usage of rennet and are able to accelerate the ripening of cheese or to enhance the cheese flavor. Depending on the chain length of the fatty acid released upon the enzymatic catalysis a sharp taste could be added to the cheese, for C_4-C_6 or a soapy flavor when the chain length is > C_{12}. The usage of Vitamin C as an oxidant in food industry was somehow limited by solubility issues in fatty products. The problem was overcome by the esterification of the ascorbic acid with fatty acids, a reaction that was initially performed chemically. The drawbacks were solved by introducing the enzymatic esterification catalyzed by *Candida antarctica* in solvents inexpensive and concomitant suitable for food manufacturing.[72, 73,74,75]

In leather manufacturing, the usage of lipases replaced the traditional methods, being able to eliminate the fats and grease from hides and skins that leads to an increase in product quality defined by a more uniform color and cleaner appearance.[76] In oil refining industry the lipase pre-hydrolysis is a prerequisite for the anaerobic biodigestion of the resulting soap stock as a waste in the manufacturing process, a step that covers only a 24 h time window.[77]

Biodiesel production is no longer only a bold idea, it is a necessity that absorbs a huge effort. Extra- and intracellular lipases are able to catalyze different sources of fat (vegetable oil, animal fat, used cooking oil, etc.) generating fatty acids esters with conversion rates over 90%.[78,79] There is an ongoing process for shortening the reaction time and the recycling of the enzyme for a cost effective application.

At last, but not at least the pharma industry could be considered the big winner of implementing the biocatalytical processes, with all the advantages already mentioned.[80,81] The stereoselectivity displayed by lipases provided the key to a fine tuning and never the less for the understanding of synthesis and mechanism of pharmaceutical compounds.[82] A long line of drugs could be listed, but once the reaction was previously sketched, the benefits of an industrial enzymatic process over the chemical one will be presented only for one product. The biocatalytic route of Pregabalin synthesis at Pfizer using *Thermomyces lanuginosus* and *Rhizopus delemar* lipases allows a low protein loading, resolution in the first step (wrong enantiomer recycled), high throughput, an E-factor improvement from 86 to 17, reduction in solvent usage and a reduced energy consumption with 83%.[83]

Two of the applications, the transesterification of vitamin C and synthesis of intermediates for pharma industries were reevaluated during this project from a technological perspective as well as an extension of the catalytic properties of the lipases.

Lipases, within the large enzyme family stand out, being capable to accept a large contingent of substrates, in conditions defined as rather harsh (for the other members of the family) gaining the reputation of "work horses". This reassures their place in the future of industrial development. For example, new materials like ionic liquids create new paths for lipase progress and expansion in biotechnological applications.

1.3 Ionic liquids

Probably we could not define ionic liquids before having an introspective look to their history. And that starts back with the middle of nineteenth century when it was for the first time described in a French scientific journal the reaction between aluminium chloride and amyl chloride that generates a two phase mixture.[84] Not too long after, Gabriel brought to the attention of the scientific world a protic ionic liquid that was characterized by a low melting point between 52 and 55° C. Nevertheless, the birth certificate was issued in 1914 and signed by Paul Weldon who is considered the architect of ionic liquids.[85] Wilkes was one of the pioneers who worked with the new class of room-temperature ionic liquids based on dialkylimidazolium chloroaluminates during the 1980s. This is considered more or less as the first generation of ionic liquids that were unfortunately oxygen sensitive and required special inert-gas atmosphere for their usage.[86] Beside imidazolium and pyridinium, new cations like ammonium and phosphonium were considered to create a new trend in the development of ionic liquids. The replacement of $AlCl_3$, a strong hygroscopic salt and other metal halides

involving Cl^-, Br^-, I^- with anions like BF_4^- and PF_6^- generated air and water-stable molten salts, believed to be the second generation. This wasn't probably possible without the particular input from Wilkes and Zaworotko. A major milestone in their progress is the decision of Eastman Chemical to produce in 1996 on industrial scale 2,5-dihydrofuran using tetraalkylphosphonium. More and more attention is given to the ionic liquids and the research is extended in various fields as reflected by the number of publications, which increased from about 150 in 2000 to over 1500 in only four years.

Despite their booming applications the issues related to their production costs and environmental concerns due to their toxicity led to a third generation of ionic liquids. Anions and cations with lower toxicity were targeted for the newly synthesized ionic liquids and also their biodegradability was reassessed. The costs were drastically reduced by simplifying the production steps and no tedious purification being required.

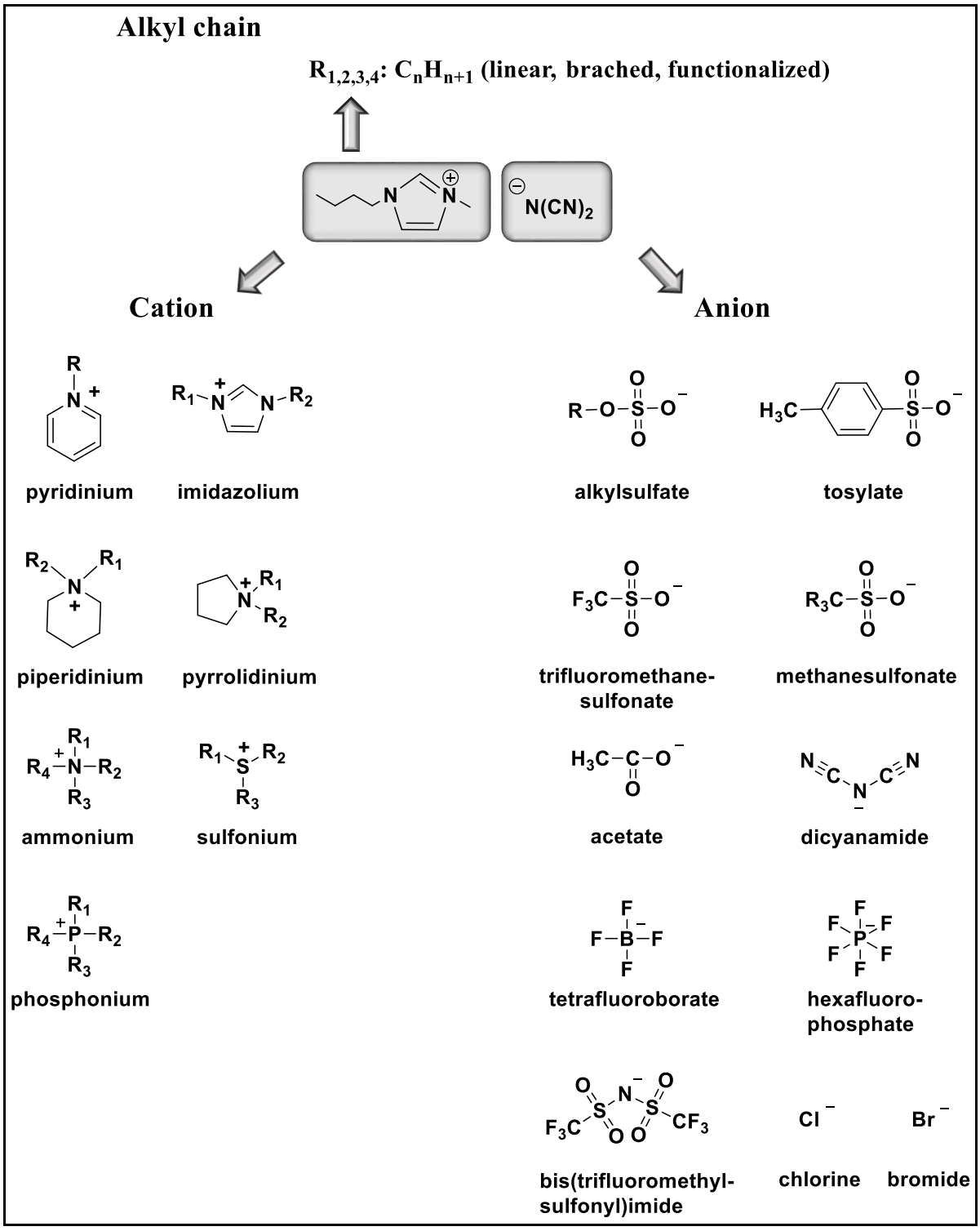
Through the years, the name and definition of the ionic liquids evolved adding more inside to the knowledge accumulated. Based on their structure and physical-chemical properties (liquid salts at temperatures below 100 °C) they appear in literature as room temperature molten salts or ionic liquids. While they replace the toxic organic solvents in synthesis, the name of green solvents was associated more often with ionic liquids, despite the toxic impurities derived from the raw materials that were slowly eliminated only with the third generation.[87,88] The considerable number of combinations between different anions and cations redefined them as tunable ionic liquids.[89] In recent years, the high demand of finding the right solvent for the right reaction has pushed the modeling of ionic liquids beyond to provide the task-specific ionic liquids.[90]

1.3.1 Structure and properties

The two components of the ILs are, as in any salt a cation and an anion. The singularity of the combination is given by the nature of the two ions: an organic cation and an organic or inorganic anion (Scheme 7). The Coulombic force that mainly governs the attraction between the two species is week and depends strictly on the selected type of constituents.

The first characteristic of the ionic liquids directly linked to the electrostatic bonding is their liquid form and range at room temperature. Furthermore, the melting point is strongly related to the size, charge and its distribution of the ions involved in the structure. Once larger anions are present, the Coulombic interaction is weaker and will decrease the melting point. For

example the sodium salts with Cl^- and $[AlCl_4]^-$ have melting points of 801 respectively 185°C as the thermochemical radius is higher.



Scheme 7. The structure of ionic liquids, including different cations and an anions.

More complex anions like $[(CF_3SO_2)_2N]^-$ would have a charge delocalization in the center of the ion, enforced by $–SO_2CF_3$. When we analyze the input of the cation, the shape will play a major role beside the size which has a similar influence like for anions. Other physical-

chemical properties that define the liquid salts have to be taken in the account. Ionic liquids are viscous and could record values on a quite broad range from 10 to 1000 cP. This parameter, known as dynamic viscosity or viscosity coefficient is dependent on temperature in a more complex manner than in organic solvents. The low vapor pressure of the ionic liquids, beside their high thermal stability creates a huge advantage over the organic solvents, and therefore increasing considerably their portfolio of applications. Finally, their density having values higher than water could be exploited as an advantage, according to the reaction involving ionic liquids.[91]

Their main structural characteristic defined by the rather large organic cation and inorganic or organic anion electrostatically bound will explain the impact on their polarity. When compared to organic solvents with well-defined polarities the ionic liquids fail to have an established classification. There were several attempts to quantify their polarity but the pool of ILs was rather small, so the relevance is reduced especially when the combinations of cations and anions are so diverse.[92,93,94,95,96] The number and length of the alkyl chains substituents on the cation have a big impact adjusting the charge of the IL.

Probably the absence of a general method for polarity quantification in case of ILs determined the ambiguities in their classification. Some measurements despite their accuracy were applied in order to measure the polarity: a) chromatographic measurements when the ionic liquids were used as stationary phases for gas chromatography to monitor the retention behaviour of different substances; b) absorption spectra based on solvatochromic shift; c) fluorescence spectra through π-π interactions generated by polycyclic aromatic hydrocarbons; d) FT-IR spectroscopy that employs $Fe(CO)_5$ and e) refractive index.[96,97,98,99,100] The most comprehensive work was probably done by C. Reichardt who used the negatively charged solvatochromic standard betaine dye no. 30 to quantify the polarity.[92] A normalized solvent polarity scale with $E^N_T = 1$ for water and $E^N_T = 0$ for tetramethylsilane will compare the ILs with some of the common organic solvents. The imidazolium based ILs which will be part of this work were evaluated as slightly less polar than short chain alcohols, beside pyridinium salts that are close to ethanol and methanol. Polarity, as a defining characteristic of the ILs will emphasize the importance in designing reactions in this type of solvent as part of a two-phase system.

1.4 Enzymatic reactions in ionic liquids

The year of 2000 represents the crossroad for the two emerging fields, enzymatic catalysis and ionic liquids, when the first biocatalytic reactions were hosted in the new liquid salts. At this point in time the ionic liquids were solely used as a replacement for organic solvents, considered toxic and demanding special requirements for handling. One of the first reactions the transesterification of ethyl butyrate with 1-butanol in [BMIM] BF_4 and [BMIM] PF_6 proved the potential of ionic liquids and generated higher yields in comparison with *t*-BuOH and 1-BuOH using immobilized CaL B.[101] In the same year the ionic liquids were used again as a replacement for molecular solvents but aiming a different task: the extraction of erythromycin-A after a biotransformation in a two phase liquid-liquid system.[87] Z-aspartame as another example was synthesized in a buffer-[BMIM] PF_6 mixture using thermolysin as a catalyzer.[102] With time the list of enzymes active in ionic liquids expanded, covering a whole range of enzymes from lipases, esterases, proteases to glycosidases and oxidoreductases. Obviously, beside the technological achievements the ionic liquids exhibit also the ability to preserve the enzymatic activity in the given reaction conditions. The isolated cases of contradictory results regarding the influence of some ionic liquids on the activity can be explained by the remaining impurities from their preparation, an issue currently solved by the analytical grade compounds that many companies could deliver.[103,104] Furthermore, this property was accompanied by the long term stability of the enzymes in ionic liquids, with or without additional water as a homogeneous mixture.[105,106] A half-life of over 240 h was possible for an esterase once it was immersed in [BMIM] PF_6 at 40°C that gives a 30 fold increase in comparison with organic solvents.[107] Other studies steered towards enzymatic thermostability proving the beneficial environment created by the ionic liquids at high temperature. It was shown for CaL B that activity could increase up to 120% at 80°C, in a time frame of 20 to 100 h as a free form and up to 350% once it was immobilized after 40 h reaction time.[108] A more comprehensive study using differential scanning calorimetry (DSC), fluorescence and circular dichroism (CD) showed that [EMIM] NTf_2 is capable to hold the native conformation of α-chymostrypsin at elevated temperatures up to 50°C.[109]

In order to create the proper environment for the synthesis of more challenging products, like glucose fatty acid esters in the presence of lipases, mixtures of hydrophobic and hydrophilic ionic liquids were used.[110] This setting brings in one more advantage related to their polarity range and consequently their ability to dissolve particular substrates, in the development of the reactions in ionic liquids. In many cases the impossibility to match all the requirements of

a reaction with one solvent that could solubilize substrates, acyl donors and not to forget the products, preserve the enzymatic activity and generate considerable yields at low costs gave the chance to the ionic liquids to proof their capacity. Concrete, substrates like cellulose, glucose, maltose and ascorbic acid with industrial potential are solubilized by some ionic liquids, facilitating the reaction. [104,110,111,112] The synthesis of glucose fatty acid esters in pure [BMIM] BF_4 and [BMIM] PF_6 or mixed with t-BuOH increased the conversion and generated higher yields in the presence of Cal B.[113] Vitamin C was esterified with oleic acid in [BMIM] BF_4 increasing the conversion with 33% in comparison with the organic solvent.

Concomitant to these clear advantages, the reactions in ionic liquids are targeted also for the ability to drive enantioselective synthesis.[114] CaL B, beside other lipases and esterases is able to generate enantiomers with industrial value in different sectors like food industry, pharma, surfactant production, etc. in the presence of ionic liquids.[95,105,115,116] Kinetic resolution studies were observed for 1-phenylethanol in several ionic liquids based on imidazolium and pyridinium salts using vinyl acetate as acyl donor.[117] A similar procedure was applied for the synthesis of racemic hydroxymethanephosphinates using *Pseudomonas fluorescens* where a six fold higher result was obtained for the E values.[118]

One technical challenge that has to be solved more on an individual approach refers to the product separation. Each process has its own particular conditions and each product its own properties. The easiest way is to evaporate the product out of the reaction mass, taking in consideration the low vapor pressure of the ionic liquids.[117,119] Supercritical carbon dioxide was associated sometimes with ionic liquids as a mobile phase for the extraction of the resulting compounds in a batch or continuous flow type of processes.[120,121,122] Furthermore, in some cases the ionic liquids were used for extraction of the product from the reaction medium.[96,123] Column chromatography, as a conventional technique in organic chemistry was used sometimes to generate a pure product.[115,124] The characteristic of some particular ionic liquids to form biphasic systems with water, acetone and *n*-heptane inoculated the idea of matching two phases for better technological solutions. [87,125,126,127] The potential of the lipases to work in a two phase system was proven and higher conversions were achieved.

CaL B, among a few other lipases is active and capable to produce higher yields when it is in an immobilized form. The large variety of ionic liquids and their derived properties instilled the idea of using them as a support for enzymes. The solutions are quite innovative and diverse. Coating enzymes with ionic liquids was one of the simplified answers to the technological quest, followed by microemulsions.[128,129,130,131] Before proceeding to a transesterification reaction several lipases were anchored directly on the chosen ionic liquids

and the recycling of the enzyme was possible.[132] Several tests showed that only a small loss in activity is observed after several cycles or longer time periods when enzymes are protected by ionic liquids.[116,133] This is associated with the constant effort put into technological solutions for cutting the production costs, for feasible and environmental friendly processes.
An assembly of all these ideas and conclusions related to the presence of ionic liquids in enzymatic reactions and processes could be considered the basis of Supported Ionic Liquid Phase technology, known as the short form SILP. A deeper look inside to the advantages of ionic liquids in industrial applications is required, due to the importance to the project.

1.4.1 SILP Technology

Supported Ionic Liquid Phase (SILP) technology made a breakthrough in 2003 as an original idea for reactions catalyzed by transition metal complexes. It found rapid applications in hydroformylation, hydrogenation, carbonylation and fine chemical synthesis.[134,135] Later on gas purification and separation completed the list of possible candidates for this methodology, beside biocatalysis, which is the main focus of this study. As the name reveals, ionic liquids supported by a solid structure are capable to host metal complexes or, as it was shown later, enzymes for different synthesis with improved technological solutions. The properties of the ionic liquids like low vapor pressure, liquid at room temperature as well as solvents with a large spectrum provide a good start for liquid-liquid catalysis as Chauvin and Wilkes initiated the idea.[136,137] The SILP particles of organic or inorganic origin were designed to create a pore structure with a large contact surface that will be later on covered with a thin film of ionic liquid containing the dissolved catalyst as shown in Figure 7.[138,139,140]

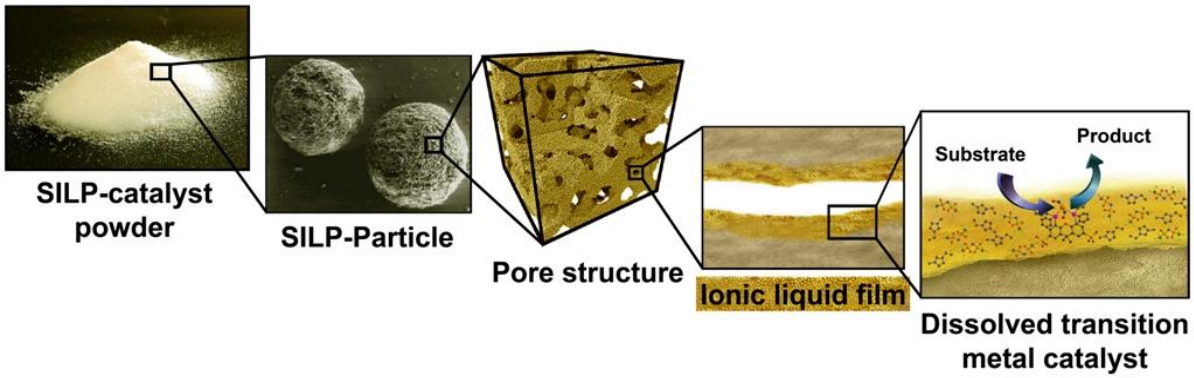


Figure 7. Schematic representation of Supported Ionic Liquid Phase (SILP) technology from particle preparation through ionic liquid coating, containing the catalyst till final reaction (from the webpage of the Institute of Chemical Reaction Engineering - Friedrich-Alexander-University Erlangen-Nuremberg).

This way also, the mass transfer limitations are overcome in a refined, simple manner. The narrow layer of ionic liquid would allow a quick diffusion of the substrate, brought in by a second immiscible solvent or gas phase to the catalytic center. The formation of a product, insoluble in the ionic liquid is accompanied by the immediate release in the second phase. This will finalize a homogeneous and efficient biphasic system that could be easily transferred in a continuous flow catalytic process.

One of the most common supports for this technology is silica, but other non-conventional materials are used (active carbon cloth, chitosan, polymers) or in development.[141,142,143] Part of the project, the configuration of new support materials was accomplished by the cooperation partner from the Institute of Polymer Chemistry, University of Stuttgart. A large surface porous material, capable to support ionic liquids was created using cellulose-2.5-acetate microbeads, incorporated in a polyurethane type of polymer.

1.4.2 Applications

The successful performance of SILP technology in the chemistry field made the biologists reconsider the technology, at a point in time when the first enzymatic reactions in ionic liquids were successful. One of the pioneers that blended the interest in industrial processes and enzyme catalyzed reactions was Pedro Lozano. He described a continuous process to synthesize short chain alkyl esters with the catalyzer, CaL B trapped in the ionic liquid dispersed on silica particles. [121] $scCO_2$, which has the ability to solubilize a wide range of compounds by adjusting the pressure and temperature conditions was used as solvent and carrier for the hydrophobic substrates in a fixed-bed reactor. The ionic liquids tested had a common anion bis(trifluoromethanesulfonyl) imide and different alkyl chains on the ammonium cation. Methylglucose fatty acid esters synthesis was subject of another SILP introspection aiming higher conversions.[144] CaL B, immobilized on macroporous acrylic beads generated the best results in the presence of a more polar type of ionic liquid, used to cover as a homogenous thin film. The ionic liquid used had a pyridine based cation associated with PF_6^- as anion.

A more advanced process that considered SILP technology as a possible host is the continuous dynamic kinetic resolution (DKR) of *rac*-1-phenylethanol in the presence of vinyl acetate.[145] The capability of ionic liquids to accommodate enantioselective reactions was already described. The racemization of the free alcohol formed in the solution can be carried out by an additional chemocatalyst, usually an acid or an organometallic catalyst. This

challenges the system, being known that these type of components would simply inactivate the enzyme. As in all standard settings CaL B was immobilized on acrylic beads, coated with different ionic liquids. The second catalyst was created by modifying silica with benzensulfonic acid, covered again in ionic liquid that could drastically reduce the site-site interactions between the two synergists. Furthermore, the fixed bed reactor was divided in compartments alternating the chemocatalyst with an enzymatic layer, separated by glass wool. Again $scCO_2$ guaranteed a constant flow of substrate and acyl donor mixture. The setup was successful in raising the yields up to 75%. The flexibility offered by the system was one more time exploited, by replacing the support with zeolites that could lower the acidity in the reactor and shelter the enzyme. In this case the yields reached almost 100%.

One more time, SILPs designs with their polymeric support were able to create an undeniable microenvironment for the biocatalyst and increase the mass transfer of the hydrophobic substrates from the $scCO_2$ carrier to fulfill the synthesis of citronellyl propionate.[146] The catalytic activity of CaL B was preserved for seven cycles.

Following these ideas, the replacement of the $scCO_2$ with an organic solvent comes as an extension that could open new avenues for improved processes. The efficiency would not target only increased yields and longer enzymatic life time, but better separation of the products based on their partitioning in the biphasic systems. Cal B, retained on the column in a slightly different form could prove, with similar or new substrates its catalytic power in a diverse microenvironment.

1.5 Aim of the project

The present work is focused on technological solutions for enzymatic reactions in an industrial context with the support of ionic liquids in combination with organic solvents. The core of the project was centered on three areas of interest:

I. Develop enzymatic reactions in biphasic systems for possible applications in continuous reactors using the Supported Ionic Liquid Phase model.

- Create biphasic systems using ionic liquids immiscible with organic solvent able to host and enhance reactions like the transesterification of 1-phenylethanol and amidation of α-methylbenzylamine with different vinyl esters.
- Taylor the reaction systems to favor the product formation mainly in the organic solvent phase, avoiding the separation step that is considered in many processes as a bottle neck. The physical-chemical properties of the ionic liquids coordinated with the ones of the molecular solvent will determine and enhance the partition of the product in the desired solvent layer.
- Analyze the impact of the ionic liquid structure on the enzymatic reactions through a large selection of ionic liquids structures, including imidazolium, pyrrolidinium, ammonium, pyridinium and phosphonium based cations paired with several anions like PF_6, BF_4, NTf_2, $MeSO_4$, $OctylSO_3$, OTf, $N(CN)_2$.

II. Extend the substrate promiscuity involving lipases, towards synthesis of potential pharmaceutical intermediates.

- Test piperazine and piperidine based substrates for acylation reactions in the presence of CaL B using targeted acyl donors capable to generate the desired compounds without by-products that could hinder the outcome of the reaction.
- Transfer and compare the successful acylation reactions in biphasic systems.

III. Improve existing processes with industrial value using the biphasic scheme.

- Establish a biphasic system for the transesterification of ascorbic acid with fatty acid esters, able to solubilize the strong hydrophilic substrate as well as the hydrophobic acyl donor. At the end of the reaction the product should be present in the organic phase in a soluble form.

2. Materials and methods

2.1 Materials

2.1.1 Chemicals

The chemicals were purchased from Sigma-Aldrich GmbH, Fluka Analytics, Alfa Aesar, Merk Chemicals GmbH, ABCR GmbH and Tokyo Chemical Industry Ltd in analytical grade. Fluorescamine was acquired from Alfa Aesar GmbH, Germany. The ILs bought from IoLiTec GmbH, Germany and Sigma-Aldrich GmbH with a purity of 98% or above are listed below in Table 2. Carl Roth GmbH was used as a source for the organic solvents, *n*-heptane, *n*-decane, MTBE and cyclohexane as Rotisolv grade for HPLC, ≥99%. DMSO and anhydrous *t*-BuOH were purchased from Sigma-Aldrich GmbH.

Table 2. List of ILs used and their abbreviation.

Full name	**Abbreviation**
1-Ethyl-3-methylimidazolium hexafluorophosphate	[EMIM] PF_6
1-Butyl-3-methylimidazolium hexafluorophosphate	[BMIM] PF_6
1-Hexyl-3-methylimidazolium hexafluorophosphate	[HMIM] PF_6
1-Octyl-3-methylimidazolium hexafluorophosphate	[OMIM] PF_6
1-Butyl-4-methylpyridinium hexafluorophosphate	[BMPy] PF_6
1-Ethyl-3-methylimidazolium tetrafluoroborate	[EMIM] BF_4
1-Butyl-3-methylimidazolium tetrafluoroborate	[BMIM] BF_4
1-Hexyl-3-methylimidazolium tetrafluoroborate	[HMIM] BF_4
1-Octyl-3-methylimidazolium tetrafluoroborate	[OMIM] BF_4
1-Ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide	[EMIM] NTf_2
1-Butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide	[BMIM] NTf_2
1-Hexyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide	[HMIM] NTf_2
1-Octyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide	[OMIM] NTf_2
1-Hexadecyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide	[HDMIM] NTf_2
Butyltrimethylammonium bis(trifluoromethylsulfonyl)imide	[BM_3N] NTf_2
Methyltrioctylammonium bis(trifluoromethylsulfonyl)imide	[MO_3N] NTf_2

Hexadecyltrimethylammonium bis(trifluoromethylsulfonyl)imide	[HDM_3N] NTf_2
Trihexyltetradecylphosphonium bis(trifluoromethylsulfonyl)imide	[H_3D_4P] NTf_2
1-Butyl-1-methylpyrroildinium bis(trifluormethylsulfonyl)imide	[BMPrl] NTf_2
1-Methyl-1-propylpyrrolidinium bis(trifluoromethylsulfonyl)imide	[MPPrl] NTf_2
1-Ethyl-3-methylimidazolium dicyanamide	[EMIM] $N(CN)_2$
1-Butyl-3-methylimidazolium dicyanamide	[BMIM] $N(CN)_2$
1-Butyl-1-methylpyrrolidinium dicyanamide	[BMPrl] $N(CN)_2$
1-Butyl-3-methylimidazolium trifluoromethanesulfonate	[BMIM] OTf
1-Hexyl-3-methylimidazolium trifluoromethanesulfonate	[HMIM] OTf
1-Octyl-3-methylimidazolium trifluoromethanesulfonate	[OMIM] OTf
1-Butyl-3-methylimidazolium octyl sulfate	[BMIM] $OctylSO_4$
1-Butyl-3-methylimidazolium methyl sulfate	[BMIM] $MeSO_4$

2.1.2 Enzymes

The lyophilized lipase B from *Candida antarctica*, recombinantly produced in yeast was purchased from ChiralVisions B. V., Holland having an activity of 40000 TBU/g and the immobilized form as recombinant in *Aspergillus oryzae* on Immobead 150 from Sigma-Aldrich GmbH with an activity of 1800 U/g. All other enzymes used for screening were purchased from Sigma-Aldrich with different enzymatic activity as it follows: *Aspergillus oryzae* - 20 U/mg, *Candida rugosa* 1176 U/mg, *Rhizopus arrhizus* 9.1 U/mg, *Rhizomucor miehei* 20 U/mg and *Pseudomonas cepacia* 37 U/mg. The enzymatic catalytic unit refers to 1 µmol of acid released per min/g of enzyme at a certain temperature and pH according to the particular lipase.

2.1.3 Column material

The mesoporous monolithic hybrid cellulose-2.5-acetate (CA)/polymer was provided by Prof. Michael Buchmeiser, the cooperation's partner from Institute of Polymer Chemistry, University of Stuttgart. The support material was prepared using cellulose-2.5-acetate microbeads with a diameter of 8 to 14 µm in THF and *n*-heptane as solvents. The monomeric mixture included 1.1.1-tris(hydroxymethyl)propane and 4.4'-methylenebis(phenylisocyanate) and was catalyzed by 4-(dimethylamino)pyridine and dibutyltin dilaurate (DBTDL).

2.2 Methods

2.2.1 Enzyme activation

For a successful enzymatic catalysis in the presence of lyophilized CaL B, the enzyme was activated using a long chain fatty acid. 3 mg of enzyme (approximately 120 U) were mixed with 3 µl oleic acid and 200 µl H_2O, followed by a 10 min incubation at room temperature. The suspension was deep-frozen for 30 min at -80°C and then lyophilized over-night in an Alpha 2-4 LD plus lyophilizer from Christ Gefriertrocknungsanlagen GmbH, Germany. The activated form was stored at 4°C until further usage.

2.2.2 Fluorescent assay using labelled enzyme

A stock solution of 0.6% fluorescamine was prepared by dissolving 3 mg of the substance in 500 µl acetonitrile and stored at room temperature in dark. 3 mg of lyophilized *Candida antarctica* lipase B were dissolved in 200 µl of 25 mM potassium phosphate buffer, pH 7 containing 10% of the fluorescamine stock solution, followed by an incubation at room temperature for 1h. The labelled enzyme was washed several times with the phosphate buffer only using a 30 kDa spin filter and brought up to a volume of 100 µl using the same buffer solution. The visualization was possible using a Cell Observer.Z1 confocal microscope from Carl Zeiss equipped with a microscopy camera AxioCam MRm. The light source on the microscope for fluorescent measurements was provided by a HBO mercury vapor short-arc lamp. The images were processed using the AxioVision Software. Fluorescamine has an excitation wavelength of 390 nm and an emission wavelength of 460 nm.

2.2.3 Enzyme binding

3 mg of lyophilized CaL B was labelled with fluorescamine as described above. The following step, the activation with oleic acid was carried out as presented previously. The sample was dried in the speed-vac (EZ-2 Series from GeneVac Ltd., England) and after that mixed with 100 µl of the corresponding IL. Pieces of column material (~30 mg) were immersed in the IL containing the labelled enzyme in an active form. The swelling of the column was achieved over a 24 h time period at room temperature. When the incubation step was finished the monolithic material was washed several with MTBE in order to remove the residual enzyme. The column containing enzyme was stored at 4°C.

2.2.4 Partition coefficient

Several compounds were investigated for their partition in biphasic systems, including 1-methylpiperazine, 1-phenylethanol, 1-phenylethyl acetate and vinyl acetate. The substance of interest (substrate or product) was dissolved in 750 ml of organic solvent to a final concentration of 1.33 M. 1 µl of the mixture was used as a reference and the remaining solution mixed with an equal volume of IL. The equilibration was achieved within 1 h at room temperature, under constant rotation. The partition for 1-methylpiperazine only was measured on GC using the organic phase. For all other substrates each phase was analyzed on HPLC using the corresponding method. The partition coefficients were calculated as a percentage using the integrated areas $P = C_{1 \text{ or } 2} / C_1+C_2 \times 100$, where C_1 and C_2 are the areas of the compound in organic phase respectively in IL. In case of 1-methylpiperazine $P = C / C_i \times 100$, where C is the concentration measured in the organic phase after partition and C_i is the reference.

2.2.5 Biochemical transformations

The reactions at 37°C were carried out in 2 ml Eppendorf Safe-Lock microcentrifuge tubes and for higher temperatures were used 2 ml standard opening glass vials with rubber lined screw caps from VWR International GmbH. The lyophilized enzyme already activated with oleic acid was resuspended in the particular IL. An equal volume of the corresponding organic solvent (MTBE, *t*-BuOH, *n*-heptane, *n*-decane and cyclohexane) was added to the reaction mass. 1 mmol of each substrate and analogous acyl donor were mixed in. The final volume for each sample was 1.5 ml. The vials were incubated for 24 h at the desired temperature under constant motion at 60 rpm on a Rotamix RM1 ELMI Ltd., Latvia placed in a thermostat from Edmund Bühler GmbH. For the one phase system, 3 mg of activated enzyme was resuspended in the organic solvent of choice, followed by the addition of 1 mmol of substrate and 1 mmol of corresponding acyl donor to the final volume of 1.5 ml. When the reactions were carried out in the presence of immobilized enzyme 5 mg of the biocatalyst were directly weighted in the vials.

2.2.6 Vitamin C stability test

Prior all the experiments, the vitamin C was dried in a desiccator containing phosphorus pentoxide. 0.1 mmol of the dried vitamin C was dissolved in 30 µl DMSO for 1 h at 50°C. Different amounts of immobilized CaL B were prepared for the test as follows: 5 mg, 6 mg, 8 mg and 10 mg. After the addition of 0.3 mmol of vinyl palmitate, the final volume was adjusted with anhydrous *t*-BuOH to 2 ml. Molecular sieves, 4 Å type from Sigma-Aldrich GmbH were included in the reaction mass for preventing the residual water to hydrolyze the product. The vials were incubated at 37°C with permanent rotation. After each 24 h, 60 µl of the mixture were sampled for further analysis and an additional 1 mmol of ascorbic acid, respectively 3 mmol of vinyl palmitate were added. For the analysis on the HPLC the samples were diluted 20x in methanol.

2.2.7 Evaluation of the biotransformation

The measurements for product formation were performed using directly the IL phase, as well as the organic phase, avoiding any extractions steps that could alter the accuracy of the determination.

2.2.7.1 HPLC and LC – MS analysis

The measurements were performed using a High Performance Liquid Chromatography (HPLC) system from Agilent Technologies (1200 Series) equipped with a diode-array detector (DAD). A long-life deuterium and tungsten lamps are used for the detection over a wavelength range of 190-950 nm and sampling interval < 1 nm. The identification of each product was carried out on an Agilent Technologies 1260 Infinity system, coupled with a 6130 Quadrupole LC/MS. This will include an electrospray ion source for the sample ionization. For the separation was used an Eclipse XDB-C18 column (Agilent, 5 µm, 4.6 x 150 mm) using an isocratic elution with 0.1% formic acid in MeOH/H_2O at a flow rate of 0.5 ml/min. The percentage of methanol in the mixture varied according to the substrate and acyl donor pair used: for 1-phenylethanol and vinyl butyrate or vinyl hexanoate was 80:20 MeOH/ H_2O (v/v) and 95:5 MeOH/ H_2O (v/v) respectively for vinyl decanoate at 210 nm. In case of *α*-methylbenzylamine and vinyl decanoate the mobile phase was 80:20 MeOH/ H_2O (v/v). All other reactions, using amines as substrates used the same solvents in a 70:30 (v/v) ration at a wavelength of 200 nm. The outcome from the transesterification of vitamin C was

investigated using 95:5:0.5 MeOH/ H_2O/ CH_3COOH (v/v) using 240 nm as a wavelength. Prior analysis, the samples were centrifuged for 1 min at 14 000 rpm and room temperature. 20 µl of each phase were diluted with 980 µl of MeOH for the IL phases, respectively MTBE for the organic phases. A volume of 1 µl for each sample was directly injected on the column. Benzyl benzoate was used as a reference for product evaluation after each peak area was integrated.

2.2.7.2 GC measurements

The GC evaluations were carried out on a GC-2010 Plus Shimadzu Gas Chromatograph system, equipped with an flame ionization detector (FID) or on a GC/MS – QP 2010 mass spectrometer for identification of the products. The Zebron ZB-5 fused silica column (Phenomenex, 30m x 0.25mm x 0.25µm) had a linear column temperature ramp from 70 to 300°C with 25°C/min and split ration of 20. Helium was used as a carrier gas, at a total flow of 5.8 ml/min and 0.94 ml/min column flow. The sample preparation followed the same procedure as above, using only the organic phase.

3. Results

3.1 Activation of *Candida antarctica* lipase B

In the following experiments, CaL B was used in a lyophilized form. The activation step, accomplished using oleic acid that mimics a hydrophobic carrier led to an active conformation of the enzyme and allows the substrates to access the catalytic pocket.[147,148] As a following step, the enzyme was resuspended in the particular ILs generating a suspension which doesn't alter the enzymatic activity and creates one of the layers of a two-phase system. [149,150,151,33]

To compare the activity of the lyophilized enzyme in the activated and non-activated form, the product formation was monitored for the reaction of 1-phenylethanol with various vinyl esters in different biphasic systems at 50°C as illustrated in Figure 8.

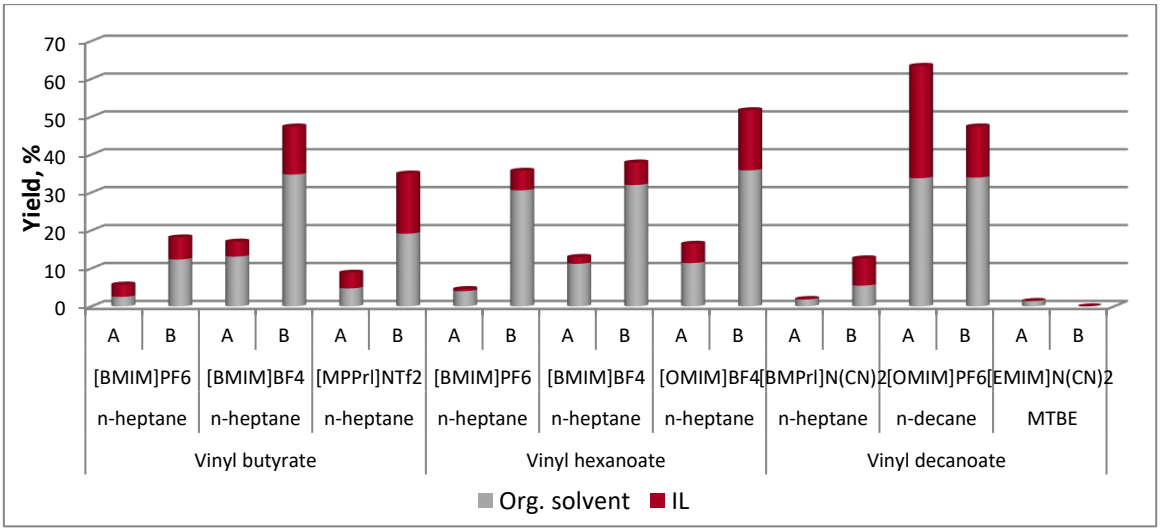


Figure 8. Comparison between the non-activated enzyme (A) and the activated form (B) for the transesterification of 1-phenylethanol with vinyl butyrate, vinyl hexanoate and vinyl decanoate in different biphasic systems.

Regardless of which IL was used, the activated form (B) shows a higher yield compared to the non-activated one. The shorter chain length fatty acids displayed a similar behavior in terms of yield and partition. For vinyl butyrate a 2.8 fold increase was detected in [BMIM] BF_4, while for vinyl hexanoate [BMIM] PF_6 showed the highest yield, with an almost 8 fold increase. The $N(CN)_2$ based ILs used for the last acyl donor followed the same trend when paired with [BMPrl] as cation, but no product was generated in association with [EMIM]. An exception was observed for vinyl decanoate, where the long alkyl chain on the cation created, beside the more hydrophobic organic solvent, the necessary microenvironment for the enzyme to generate better results without activation.

The water association with enzymes is believed to influence and preserve the conformation of the protein and so the properties and reactivity. Following this idea, the same experiment was repeated using a small amount of water 2.5% (v/v) in the reaction mixture (Figure 9). In case of vinyl decanoate the addition of water didn't bring any improvement, on contrary, for the [OMIM] PF_6/*n*-heptane pair the product formation considerably decreased. For the shorter chain vinyl donors, the discrepancy between the two forms was somehow reduced and moreover one ionic liquid, [BMIM] PF_6 in the presence of water increased significantly the yield. The hydrophobicity of the acyl donor will play a role in such systems. Also, the water responsible for the hydration of the lipase will interact with the ionic liquids involved, according to their miscibility with water that could lead to a complete different outcome.

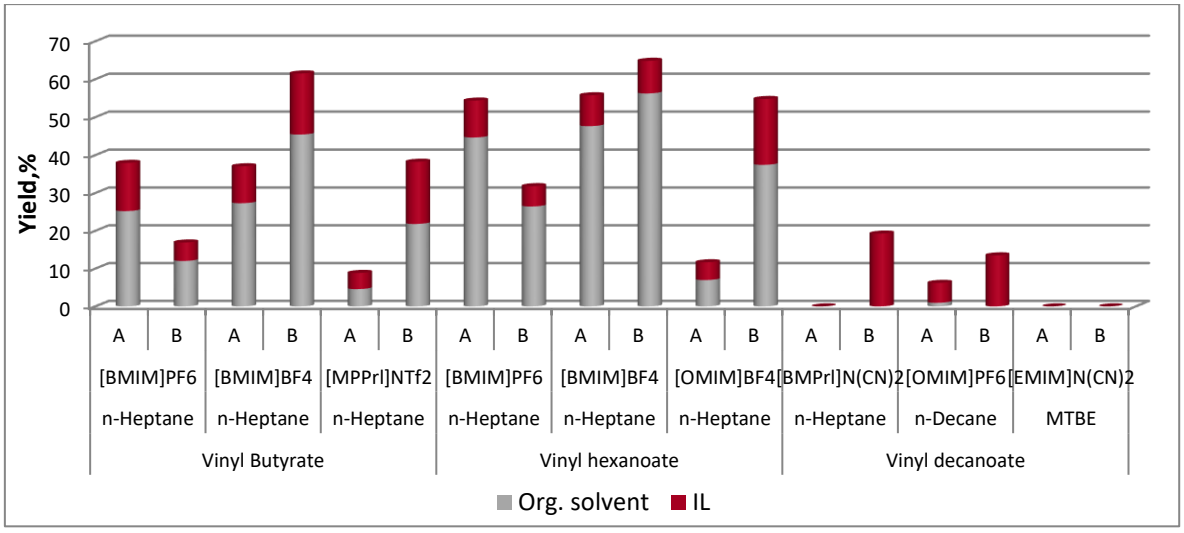


Figure 9. Comparison between the non-activated enzyme (A) and the activated form (B) for the transesterification of 1-phenylethanol with vinyl butyrate, vinyl hexanoate and vinyl decanoate in different biphasic systems with an additional 2.5% (v/v) H_2O.

3.2 Optimization of a biphasic system

SILP technology, developed initially for organic and inorganic chemistry for embedded catalysts in a thin layer of ionic liquid evolved as a possible alternative for bioenzymatic reactions. In this case a biocatalyst, the enzyme is trapped in the ionic liquid layer that will coat the column designed for a continuous flow. The second phase is an organic solvent immiscible with the ionic liquid containing the dissolved substrates and acyl donors. The reaction takes place at the interface between the two layers, being strongly influenced by the migration of the substrates and acyl donors in the ionic liquid phase that has incorporated the enzyme. Product formation will follow the same partition rules with the aim of being in a

higher percentage in the organic phase. These issues are critical points for an efficient process and are the subject of the coming experiments.

3.2.1 Partition

The logP known as partition coefficient is commonly measured for a compound by its distribution at equilibrium in two immiscible solvents (water-octanol as a standard system).[152] We are focused on ILs that are able to form biphasic systems with a certain range of organic solvents. In a biphasic system the tendency of a compound to migrate partially to one or the other solvent dramatically influences not only the separation of the product but also the outcome of the reaction. The chromatograms below (Figure 10) serve as an example for the separation of all the components involved in the reaction and for the partition between the two existing phases (organic solvent and IL).

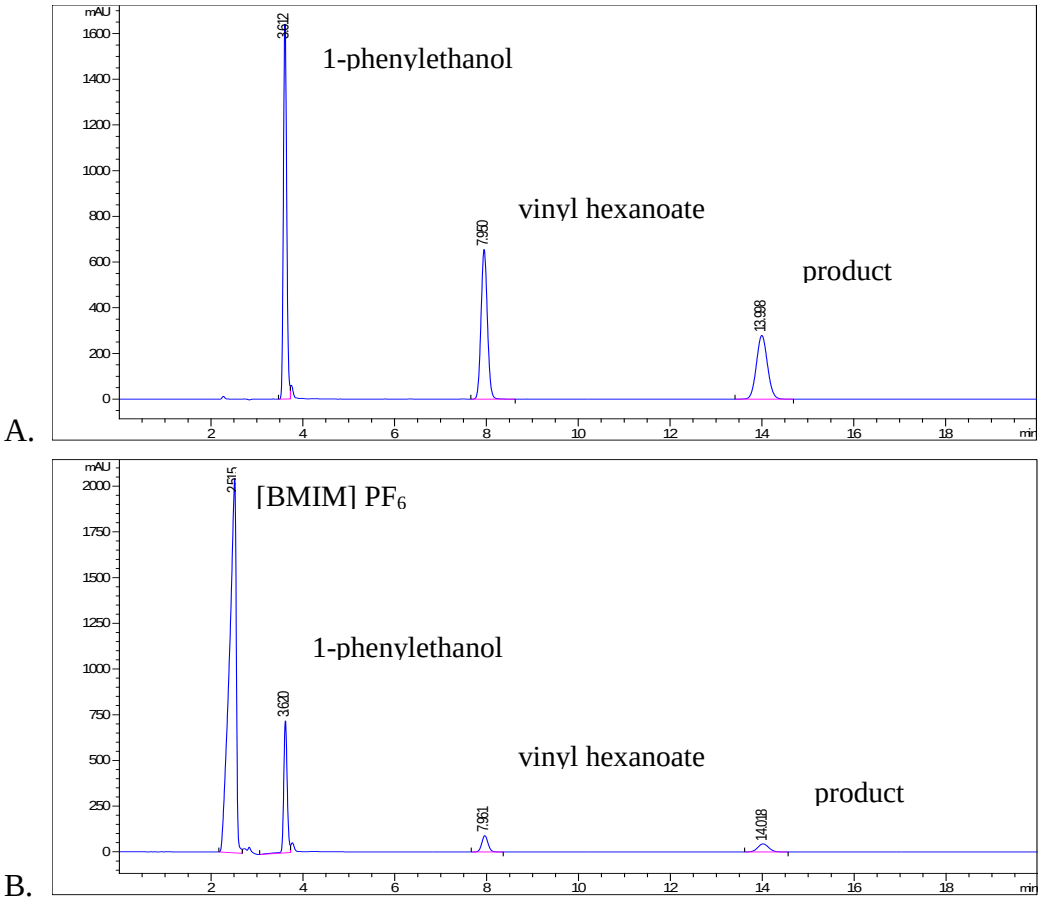


Figure 10. Transesterification of 1-phenylethanol with vinyl hexanoate in a biphasic system. (A) MTBE phase; (B) IL phase.

After a first round of substrate screening in a mixture of IL and organic solvent, the tendency towards the ILs was noticed, the degree of partition being influenced equally by the nature of

the solute and solution. The hydrophilic or hydrophobic character of the substrates, beside the polarity strength will decide the partition between the two layers. A set of three ionic liquids, [BMIM] CF_3SO_3, [HMIM] CF_3SO_3 and [OMIM] CF_3SO_3 were used in combination with three different organic solvents to configure a set of trial biphasic systems.

As shown in Figure 11, the first substrate 1-methylpiperazine, used only for partition tests is a cyclic amine that favors the ionic liquid phases when combined with two hydrophobic solvents like cyclohexane and heptane. While paired with MTBE, a polar solvent, the ratio was changed with about 20% towards the organic phase as expected. The structure of the ionic liquids used, more precisely the type of cation and anion or the length of the alkyl chain that vary from C4 to C8 didn't influence the outcome. When an alcohol, 1-phenylethanol was considered for the same study, the tendency to migrate almost completely in the IL was obvious, for the biphasic system containing hydrophobic solvents. This could be explained by the difference in polarity, alcohols being more polar than amines. Once the MTBE was used as organic phase the partition is shifted with about 25% towards the solvent because of the increased polarity. Next, the idea was extended to the acyl donor. As an ester, vinyl acetate will support the predictions related to the partition in the biphasic systems already tested. In the hydrophobic organic solvents the behavior of the ester is comparable to the amine. For a complete assessment, it was also evaluated the behavior of 1-phenylethyl acetate, the corresponding product from transesterification of 1-phenylethanol with vinyl acetate. The resulting ester, in comparison to its corresponding substrate is shifting as desired towards the organic phase because of its lower polarity. When MTBE was paired with the ILs, the trend was confirmed again with a 35% increase, especially for the IL with the shortest chain length. The missing combinations of MTBE with particular ILs for given substrates was due to the disturbance of the biphasic systems that generated a one phase mixture.

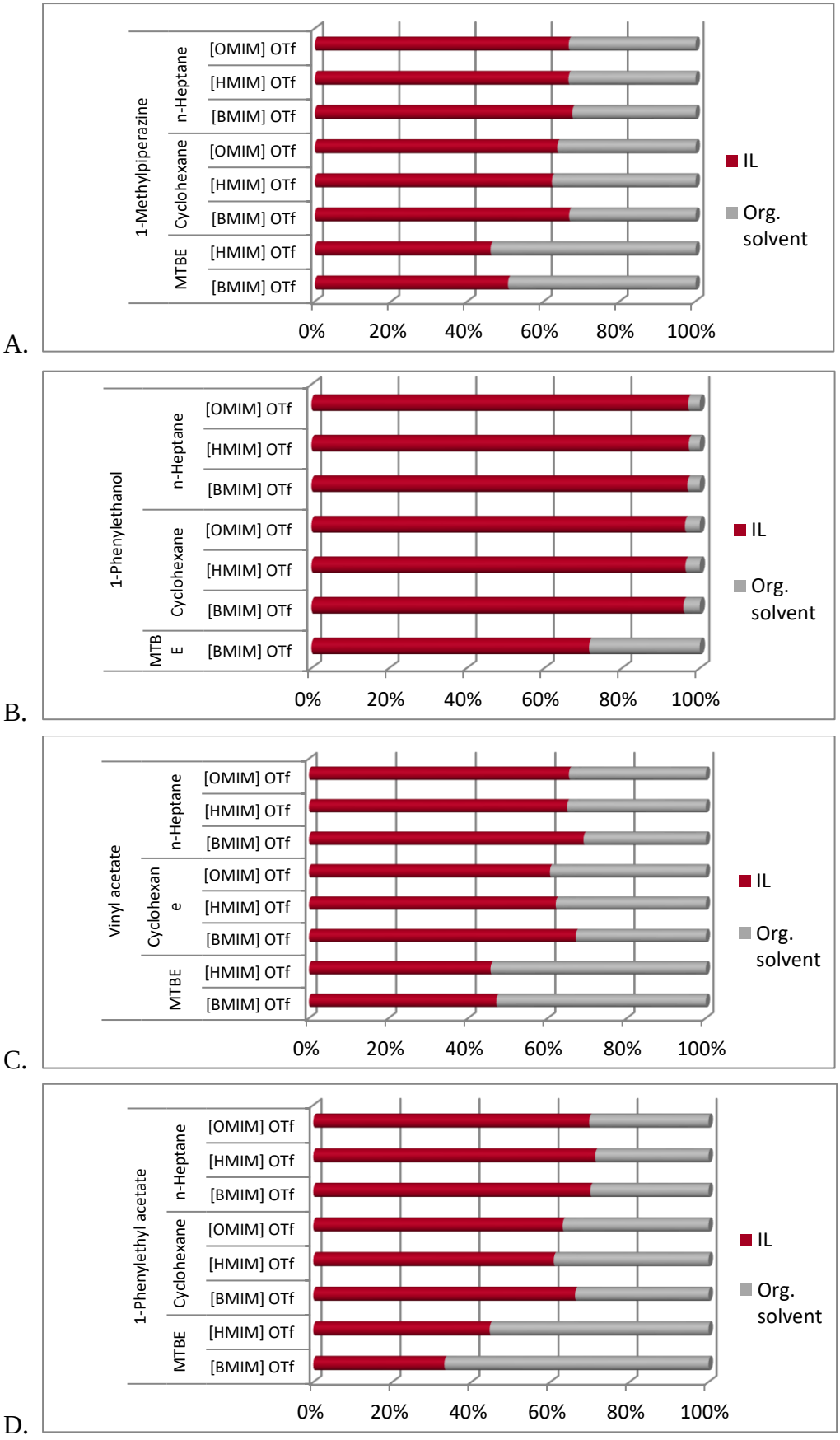
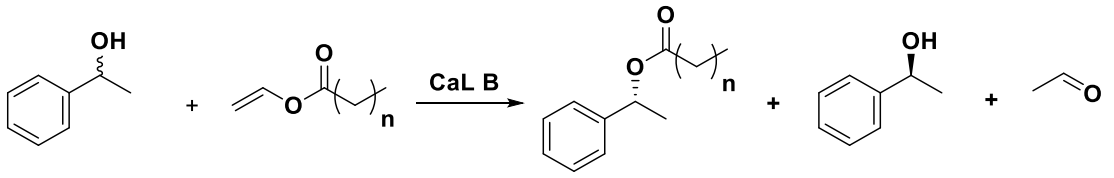


Figure 11. Partition coefficient of 1-methylpiperazine (A), 1-phenylethanol (B), 1-phenylethyl acetate (C) and vinyl acetate (D) in three organic solvents (MTBE, cyclohexane and *n*-heptane) combined with an equal number of imidazolium based ILs with different chain lengths.

3.2.2 Impact of the ionic liquid – organic solvent pair

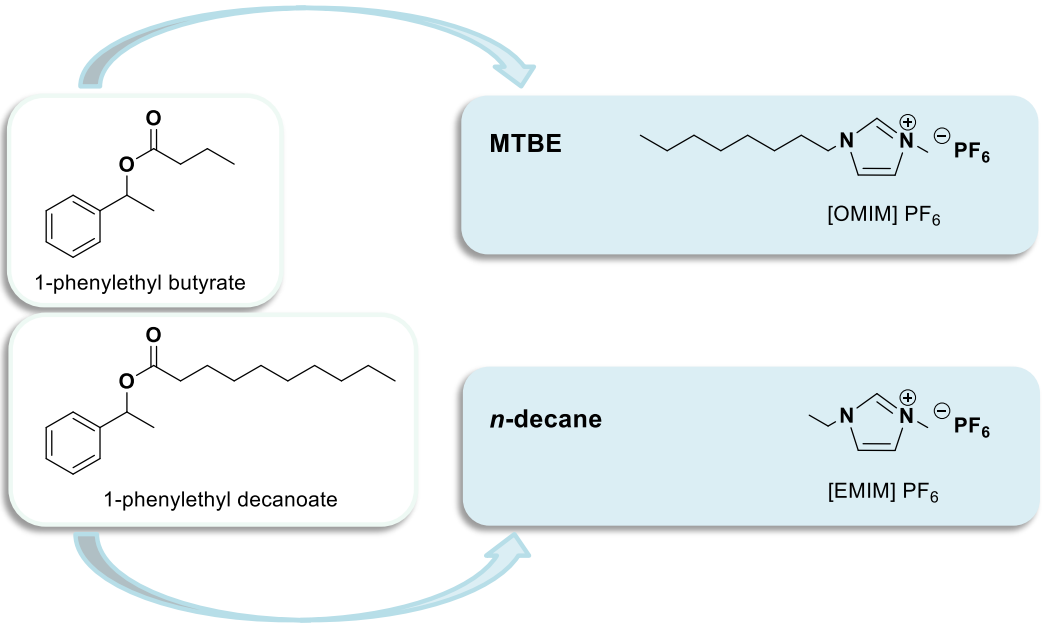
The knowledge gained during these tests was transferred to a set of different reactions were the conditions were modulated for a better partition of the product in the reaction mass, targeting the accumulation in the organic solvent. The transesterification of 1-phenylethanol with vinyl acetate is well known and was investigated in organic solvents, as well as in ILs for enhanced conversions and enantioselectivity.[101,95,117,115,43] For our experiments we extended the acyl donors to longer alkyl chains vinyl butyrate, vinyl hexanoate and vinyl decanoate (Scheme 8). The enantioselectivity of the products wasn't further investigated, the aim being mainly on the IL/organic solvent impact on the product separation.



(*R*,*S*) 1-phenylethanol vinyl ester derivatives (*R*)-1-phenylethyl ester (*S*)-1-phenylethanol acetaldehyde

Scheme 8. Transesterification of 1-phenylethanol with different alkyl chain vinyl esters in the presence of lyophilized CaL B activated with oleic acid.

The biphasic systems consisting of three organic solvents: MTBE, *n*-heptane and *n*-decane and four ILs [EMIM] PF_6, [BMIM] PF_6, [HMIM] PF_6 and [OMIM] PF_6, in all possible combinations were investigated for the correlation of the alkyl chain on the cation to yield and partition. For analogy reasons the reactions were also tested in the corresponding organic solvents only. The idea behind was to drive the partition, based on hydrophobicity of a short chain compound into a short chain organic solvent, when paired with a long chain ionic liquid and vice versa (Scheme 9).

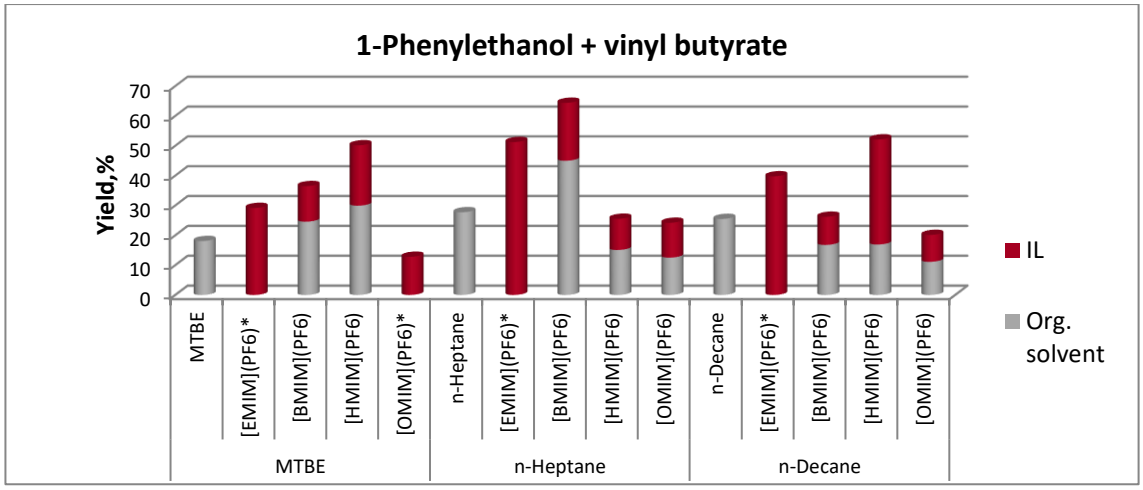


Scheme 9. Migration of products in biphasic systems containing ILs with opposite hydrophobicity that will favor the partition in the particular organic phase.

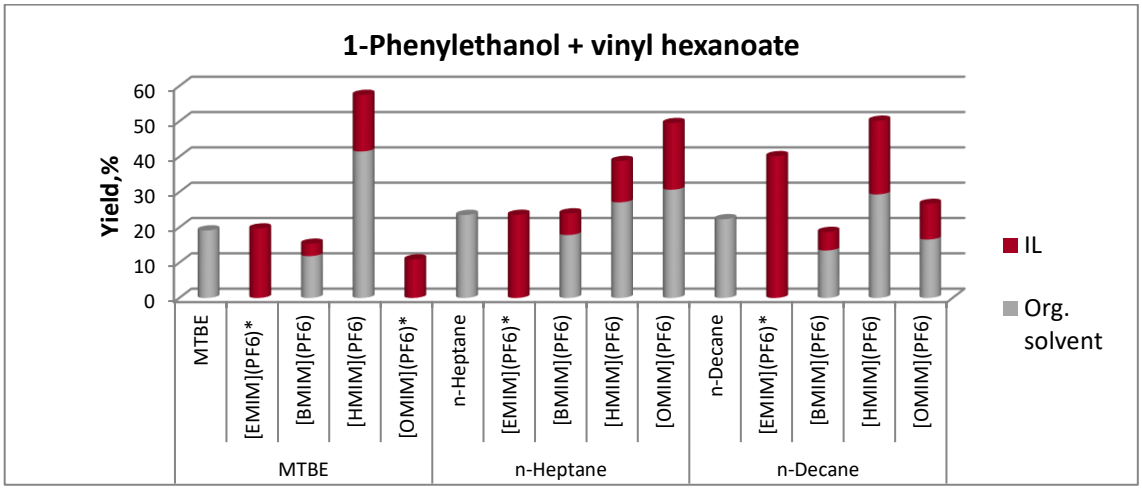
The results were first organized based on the ILs, which in principle carry the same anion PF_6^-. The imidazolium based cation is responsible for the graduate increase in hydrophobicity due to the alkyl chain substituent that varies from C_2 to C_8. The ILs, as a group were paired with each of the three organic solvents, which were arranged again as a hydrophobic scale. As references were used the organic solvents alone (Figure 12).

All reactions with [EMIM] PF_6, regardless of the organic solvent used, turned monophasic. For the reaction with vinyl butyrate, the yield and partition followed the expected trend and increased reaching a maximum for [HMIM] PF_6 when mixed with MTBE. With the more hydrophobic *n*-heptane the best conversion was achieved for [BMIM] PF_6 while decreased with the longer chain [HMIM] PF_6 and [OMIM] PF_6. The last organic solvent used, *n*-decane as the most hydrophobic, generally led to lower yields and partition. An exception was, in terms of yield, the pair [HMIM] PF_6 / *n*-decane. In case of vinyl hexanoate as predicted, *n*-heptane showed the best dependency increasing from short to long alkyl chain cations. For the last reaction, a reversed partition tendency was noticed decreasing from [BMIM] PF_6 to [OMIM] PF_6 in case of *n*-heptane and *n*-decane, to underline the preference of the product towards the long chain IL. In this case we could conclude that the less hydrophobic cation, [BMIM] didn't favor the formation of the product in IL, so it was shifted towards the organic solvent.

A.



B.



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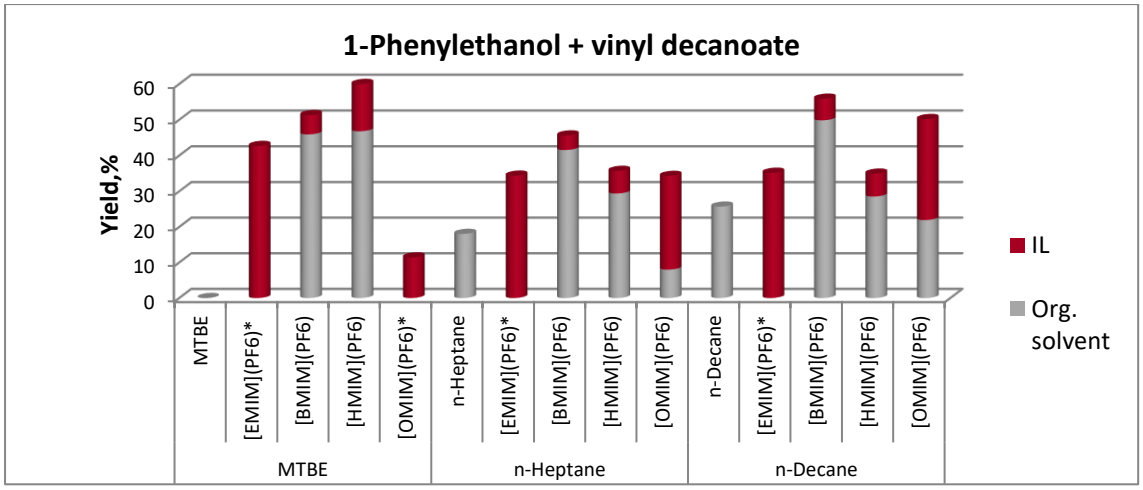


Figure 12. Comparison of the 1-phenylethanol reaction with vinyl butyrate (A), vinyl hexanoate (B) and vinyl decanoate (C) based on three organic solvents (MTBE, *n*-heptane and *n*-decane) paired with different chain length ionic liquids ([EMIM] PF_6, [BMIM] PF_6, [HMIM] PF_6 and [OMIM] PF_6.

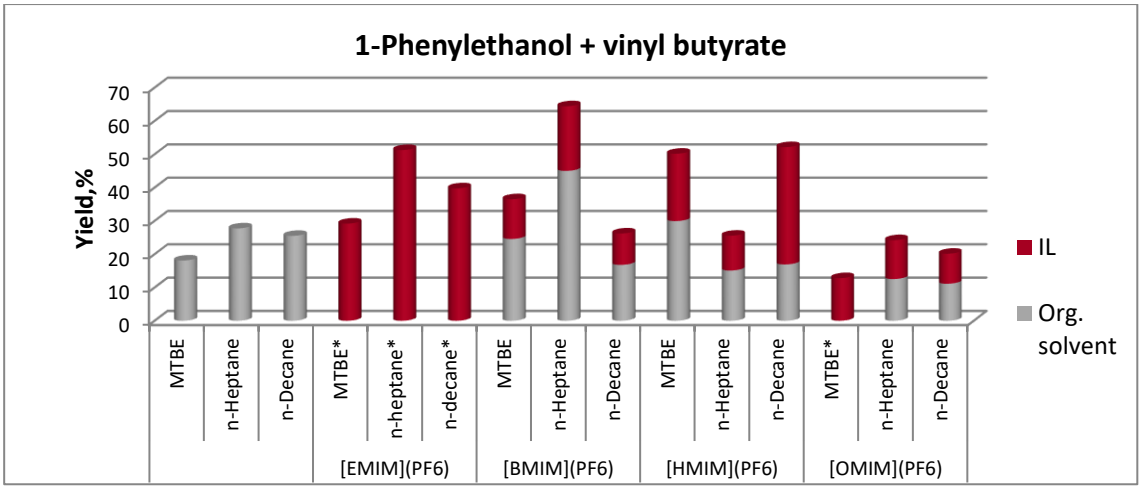
* describes a monophasic system after the reaction was completed.

MTBE, despite its polarity in combination with short chain ILs proved to be the best reaction medium for the product, despite the fact that the reaction didn't occur in the organic solvent alone.

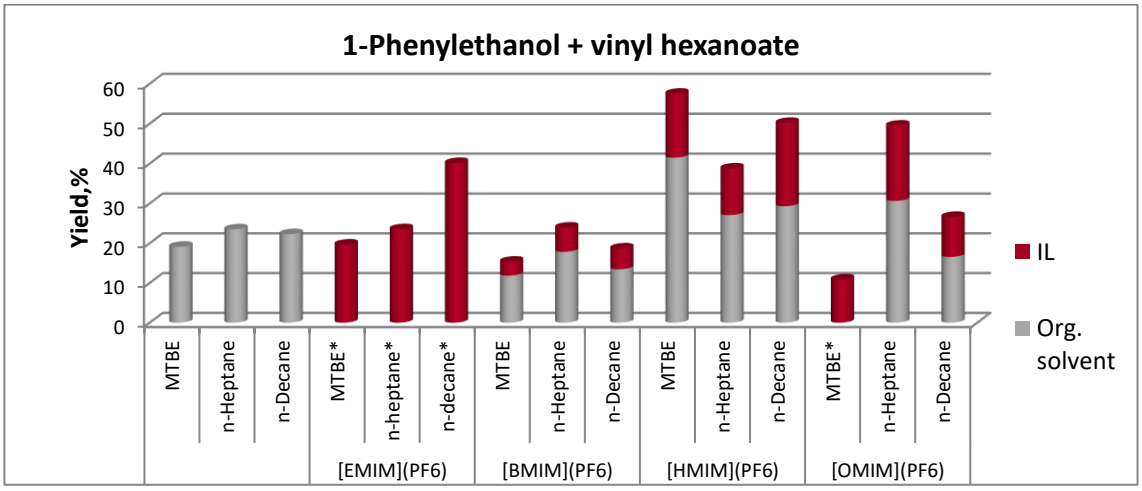
Regrouping the results, based this time on the polarity shift in the organic solvents paired with each of the four ILs, the partition tendency is emphasized again with some exceptions (Figure 13).

For vinyl butyrate the yield is increasing, reaching the maximum for [BMIM] PF_6 / *n*-heptane and decreasing towards [OMIM] PF_6 regardless the organic solvent used for this ionic liquid. [HMIM] PF_6 / *n*-heptane delivered surprisingly a low yield in comparison with MTBE and *n*-decane. Despite this oddity in this group of ionic liquids, the partition shows a nice trend from MTBE, where the product tends to accumulate, till decanoate, which favors the ionic liquid. Vinyl hexanoate reveals similar results with better yields in the longer alkyl ionic liquids [HMIM] and [OMIM]. The successful match of this acyl donor with [HMIM] PF_6 as ionic liquid, regardless the organic solvent is relevant for the proof of concept showing the balance between the participants in the reaction and the microenvironment. Furthermore, *n*-heptane as organic solvent fits in the frame with a good yield and partition when paired with [OMIM] PF_6. For vinyl decanoate, as mentioned before, the results are unexpected for the short and medium alkyl chains ILs. Despite that, [OMIM] PF_6 confirmed the initial hypothesis delivering a nice trend in all three organic solvents, with the yield increasing towards the more hydrophobic solvent, *n*-decane along with an improved partition which is directly linked to the properties of the product.

A.



B.



C.

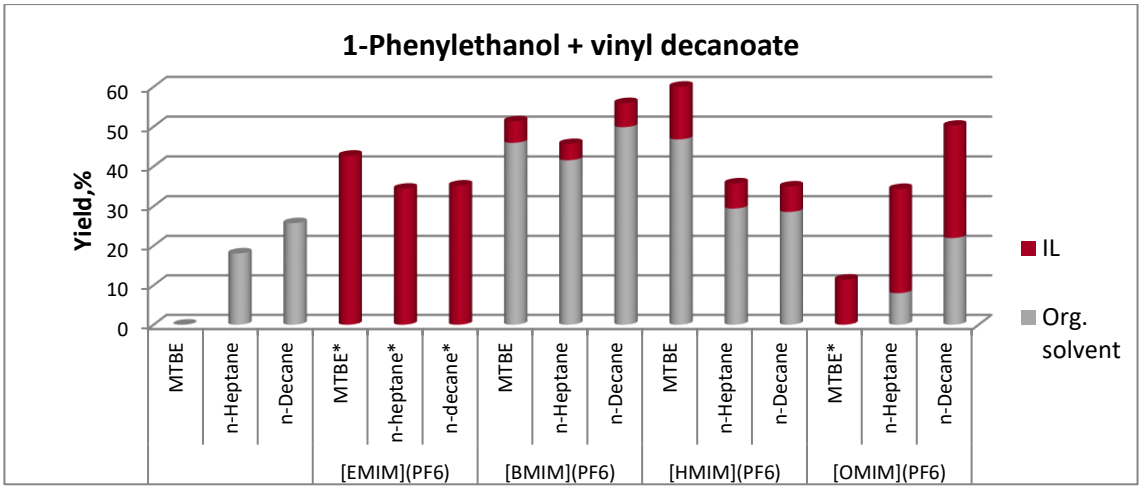
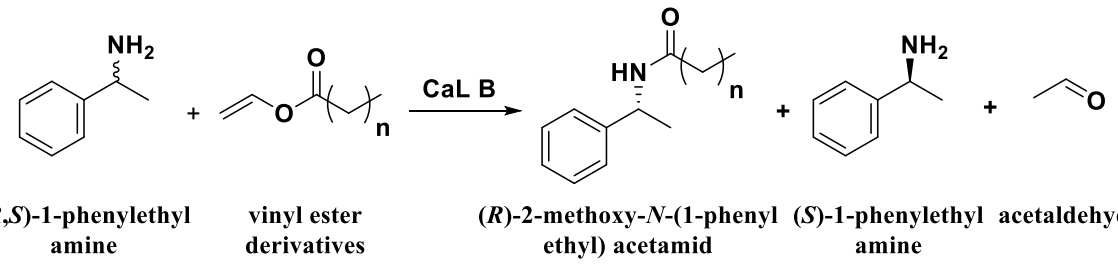


Figure 13. Comparison of the 1-phenylethanol reaction with vinyl butyrate (A), vinyl hexanoate (B) and vinyl decanoate (C) based on different chain length ionic liquids ([EMIM] PF_6, [BMIM] PF_6, [HMIM] PF_6 and [OMIM] PF_6) paired with three organic solvents (MTBE, *n*-heptane and *n*-decane).

* describes a monophasic system after the reaction was completed.

A second reaction, having *α*-methylbenzylamine as a substrate was explored in the same frame work and compared with the transesterification of 1-phenylethanol (Scheme 10).

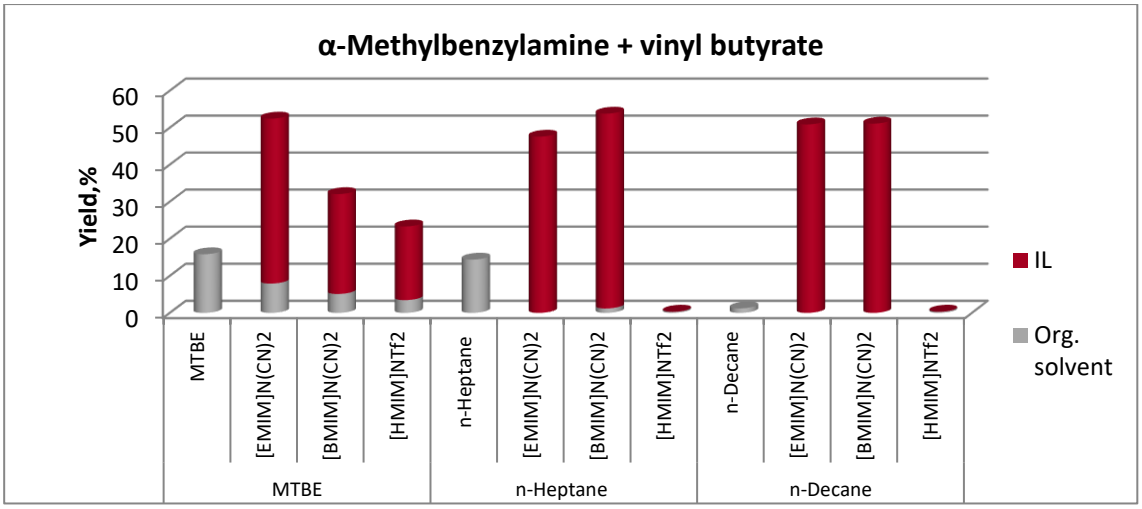


Scheme 10. Amidation of *α*-methylbenzylamine with different alkyl chain vinyl esters in the presence of lyophilized CaL B activated with oleic acid.

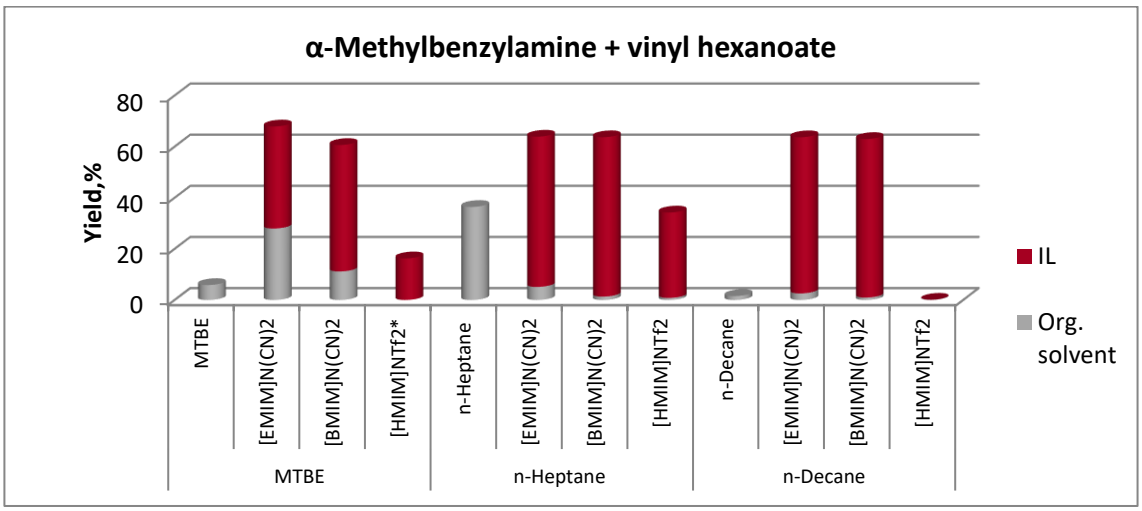
Despite the fact that the reaction could take place in the absence of the enzyme, generating small amounts of product, the experiments were conducted to evaluate the behavior of an amide, which is more polar than an ester, in a biphasic system for the proof of concept. The evaluation was performed using [EMIM] $N(CN)_2$, [BMIM] $N(CN)_2$ and [HMIM] NTf_2 as ILs and the same organic solvents (Figure 14). [HMIM] NTf_2 was chosen due to the unavailability of [HMIM] $N(CN)_2$. The presence of the ILs, as already discussed enhanced the reactions in comparison with single organic phase and dictate the partition according to the organic solvent used. The overall polarity of the product wasn't dramatically changed by the shorter chain substituent as it is shown by the acylation with vinyl butyrate in Figure 14 (A). This will explain the shift towards the ionic liquids, especially when hydrophobic organic solvents, like *n*-heptane and *n*-decane are part of the biphasic mix. For MTBE, the polarity tendency is changed and small amounts of product were found in the organic layer, inclining to an IL with shorter alkyl chain. In this case, the yield along with the partition will drop from [EMIM] $N(CN)_2$ to [HMIM] NTf_2. For vinyl hexanoate, the slightly increase in product hydrophobicity will induce more the product formation in the organic phase for MTBE and traces are noticeable in *n*-heptane and *n*-decane.

Once vinyl decanoate was used as acyl donor, the reaction was possible only in the hydrophobic solvents paired with short chain IL that created also the right conditions for a high percentage of the amide in the organic layer.

A.



B.



C.

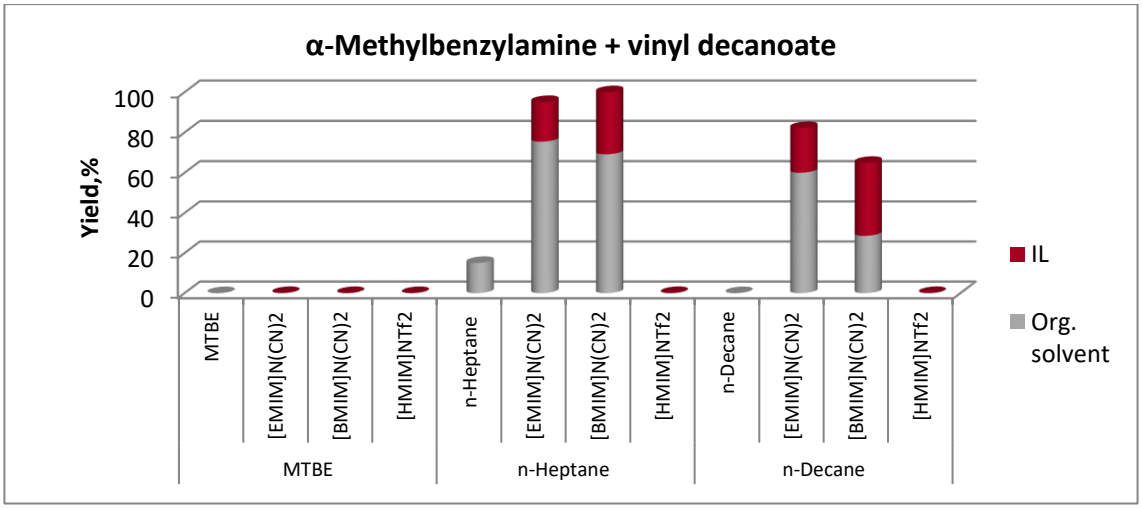


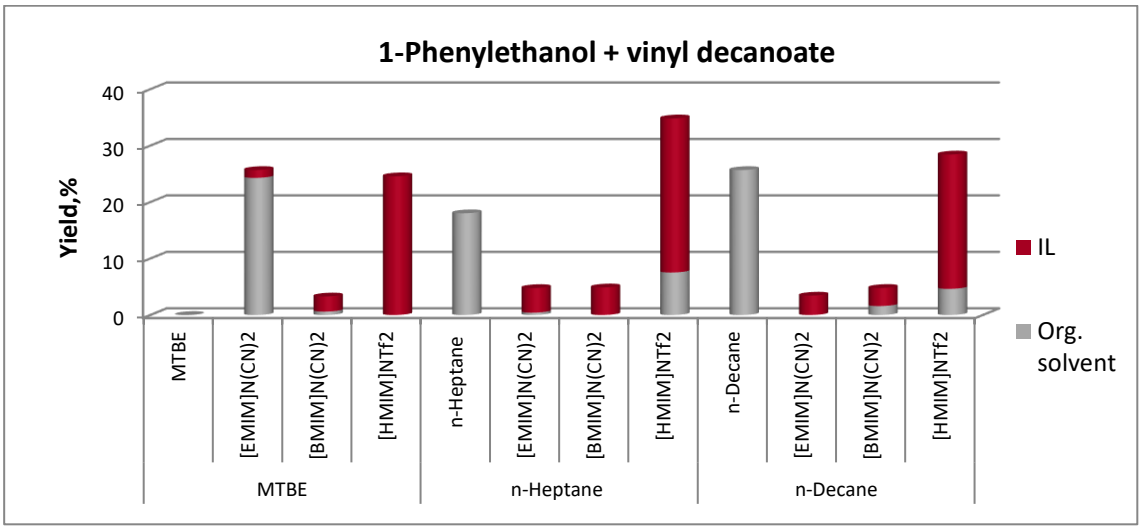
Figure 14. Comparison of the *α*-methylbenzylamine reaction with vinyl butyrate (A), vinyl hexanoate (B) and vinyl decanoate (C) based on three organic solvents (MTBE, *n*-heptane and *n*-decane) paired with different chain length ionic liquids ([EMIM] $N(CN)_2$, [BMIM] $N(CN)_2$ and [HMIM] NTf_2.

In all the reactions presented, the references in organic solvents only clearly reveal the benefit of the ionic liquids in the improvement of the reaction outcome (higher yields) and in parallel

of the biphasic system itself. In some particular cases, the presence of the ionic liquid will trigger the reaction that doesn't occur only in the monophasic solvent.

When we analyze the outcome given by the two substrates, 1-phenylethanol and *α*-methylbenzylamine only with vinyl decanoate in the same biphasic systems, it shows clearly that the tendency of the results is completely opposite for the two reactions, as expected (Figure 15).

A.



B.

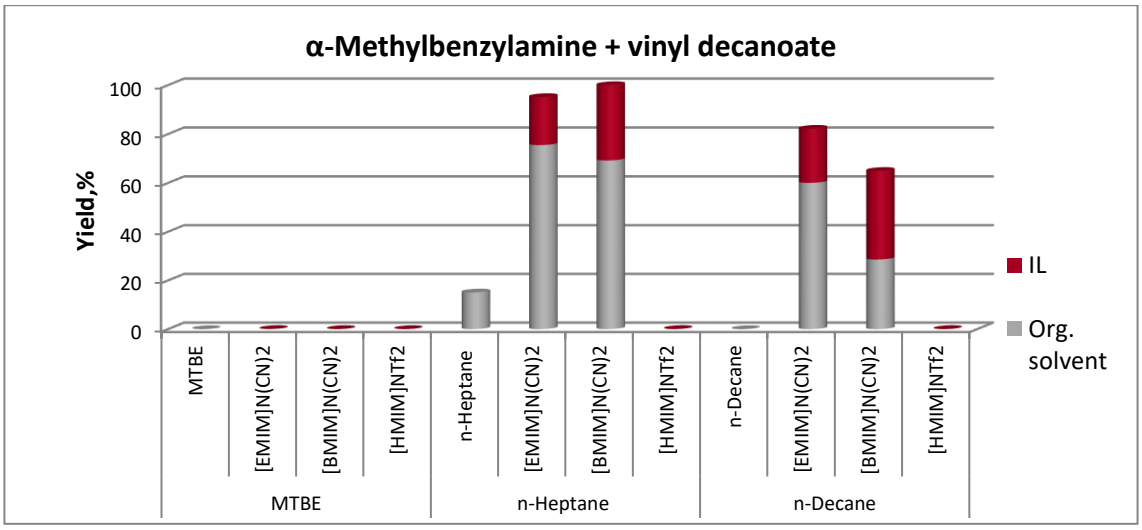


Figure 15. Comparison of the product behaviour in different biphasic systems of vinyl decanoate with 1-phenylethanol (A) *vs.* *α*-methylbenzylamine (B).

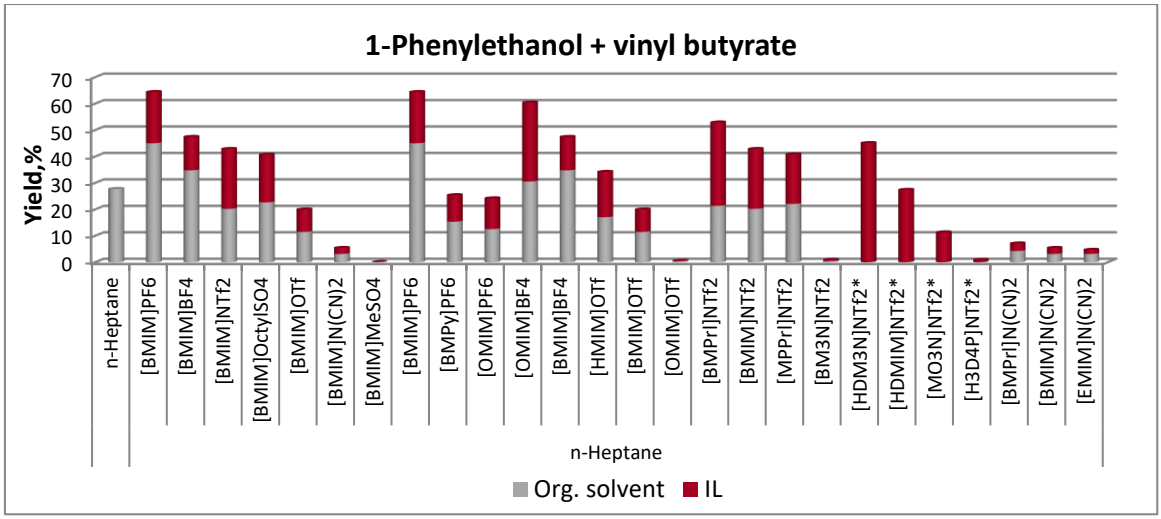
When *n*-heptane and *n*-decane were matched with [EMIM] $N(CN)_2$ and [BMIM] $N(CN)_2$ the amide formation recorded a substantial growth that is not observed for the ester, on contrary. MTBE, as a polar solvent wasn't able to host the reaction for *α*-methylbenzylamine in any combination. This behavior is defined mainly by the polarity of the product, which will directly correlate to the polarity of the biphasic systems and dictates the outcome of the

reaction and partition. This is in close relation to the hydrophobicity of the substituent on the final product.

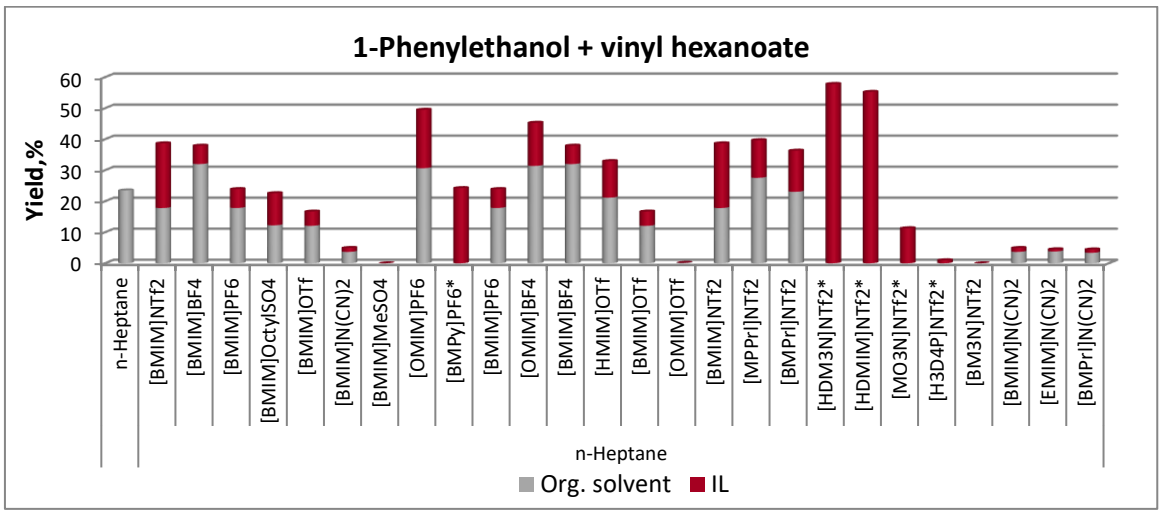
3.2.3 Impact of ionic liquid structure

The influence of the ionic liquid structure over the enzyme conformation and stability has been investigated.[153,154] Several attempts were done to gain the necessary knowledge regarding the impact on the reactions. In 2009, Yang *et al.* concluded in a review that ILs could have a similar influence on the enzymes as the organic solvents, including also the interaction with the substrates.[155] One major difference underlined by Yang *et al.* is the capacity of ILs to dissolve, dissociate and cluster in the reaction medium, interacting differently with all system components. For a more comprehensive analysis, we extended the list of ILs to 21 candidates using 1,3-imidazolium, pyrrolidinium, ammonium, pyridinium, phosphonium based cations and several anions PF_6, BF_4, NTf_2, $MeSO_4$, $OctylSO_3$, OTf, $N(CN)_2$ paired with *n*-heptane as organic phase (Figure 16).

The first cluster included ILs having the same cation [BMIM] and various anions. Further groups have the anion constant in combination with different cations. The comparison followed the same scheme as before, when we observed the changes in a left to right way, along a single reaction and a top to bottom approach to look at the modifications brought in by the vinyl donors. There was a significant increase in the overall yields when the reactions were switched from organic phase only to biphasic systems especially for imidazolium based ILs. In case of vinyl butyrate the best results were achieved for [BMIM] PF_6 followed by [OMIM] BF_4, [BMIM] BF_4 and [BMIM] NTf_2 (Figure 16 A). If we focus on the cluster having the common cation [BMIM] could be noticed a trend associated with the polarity of the ionic liquid, when the size of the anion is reduced and the charge distribution is more compact. For other ILs using PF_6 as an anion, the yields were lower, which could define a group of cations that generates poor results. However, when combining [OMIM], belonging to this category with BF_4 a completely different behavior can be observed giving the second best yield. Furthermore, to underline the importance of the mix and match of the ions for the best IL, no product was detected when [OMIM] was paired with OTf. Interestingly, for OTf the results were depending more on the alkyl chain length of the imidazolium salt, [HMIM] showing better yield than [BMIM] and [OMIM].



A.



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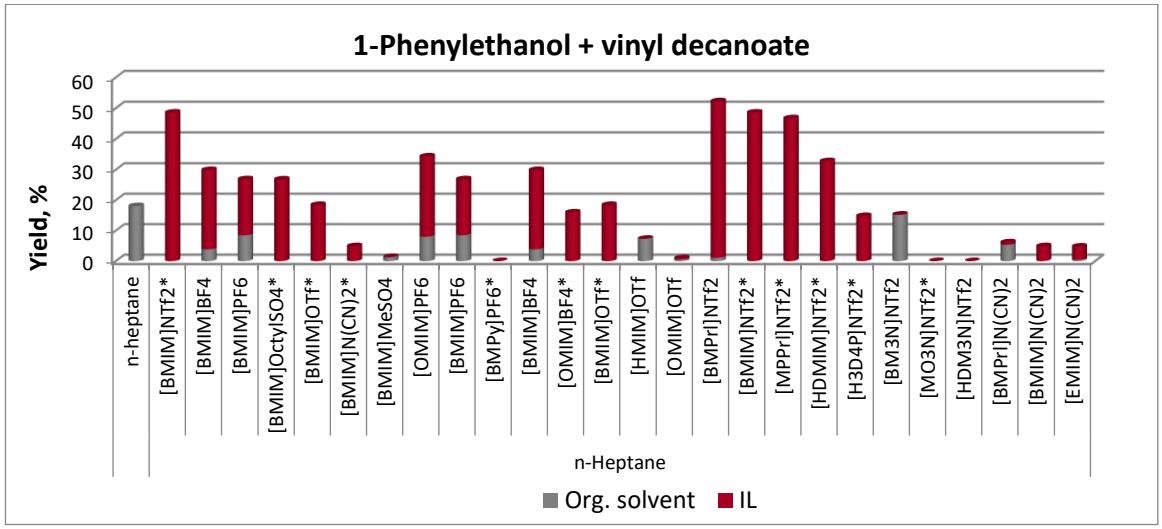


C.

Figure 16. Cation/anion impact on the transesterification of 1-phenylethanol with vinyl butyrate (A), vinyl hexanoate (B) and vinyl decanoate (C) in different ILs mixed with *n*-heptane.

* describes a monophasic system after the reaction was completed.

NTf_2 as an anion showed in most cases good conversions, but with a tendency to maintain a lower partition of the product towards the organic phase. All ILs containing $N(CN)_2$ generated low yields, while $MeSO_4$ inhibited the reaction in all cases.

Vinyl hexanoate as acyl donor showed similar behavior with vinyl butyrate, among the tested ILs, with good conversion and partition for the imidazolium based ILs [BMIM] BF_4 and [OMIM] PF_6. Cations containing hexadecyl alkyl chains provided the best results, but the biphasic system wasn't preserved.

For vinyl decanoate the yields followed the same pattern for the same group of anions or cations, though in most cases the biphasic system was disturbed probably due to the polarity changes initiated by product formation. Surprisingly, the decanoate derivatives favored the more polar ionic liquid phase despite their higher hydrophobicity.

The same study was extended to *α*-methylbenzylamine for the reaction with ethyl methoxyacetate (Figure 17). In this case MTBE was chosen along with 20 ILs. For the group of ILs sharing the same cation, only one system [HMIM] OTf / MTBE surpassed the yield generated by the organic solvent alone with about 20% forming a monophasic mixture. All pairs involving ILs with the common anion NTf_2, turned into one phase and, except [OMIM] NTf_2, the results were inferior to the reference in organic solvent only. Better results were recorded when anions like PF_6 and $N(CN)_2$ were involved in the reactions, but the partition towards MTBE was maintained under 20%. And the last anion BF_4 conserved the product formation in the same range of organic solvent.

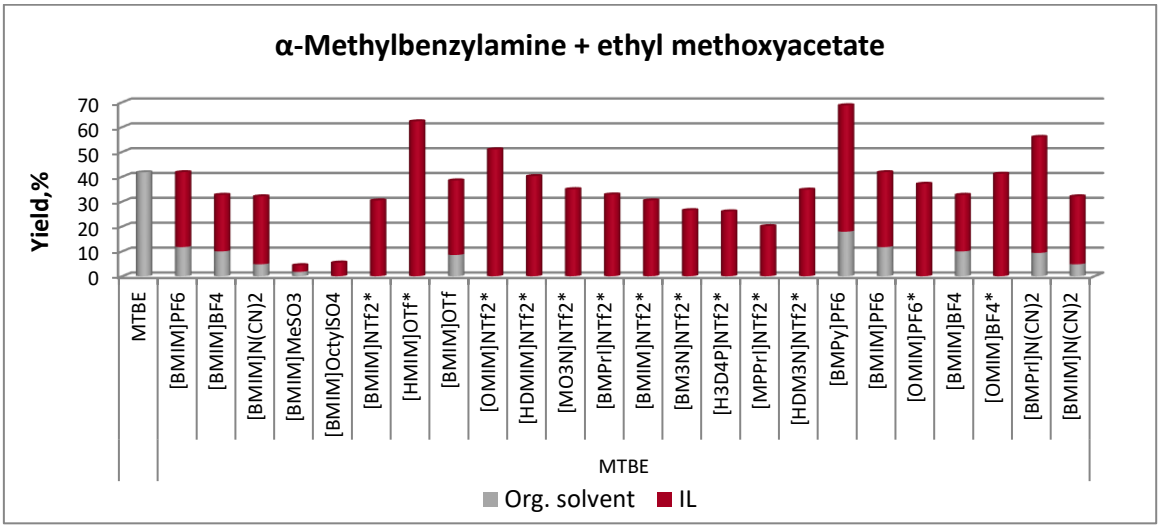


Figure 17. Cation/anion impact on the acylation of *α*-methylbenzylamine with ethyl methoxyacetate in different ILs mixed with MTBE.

* describes a monophasic system after the reaction was completed.

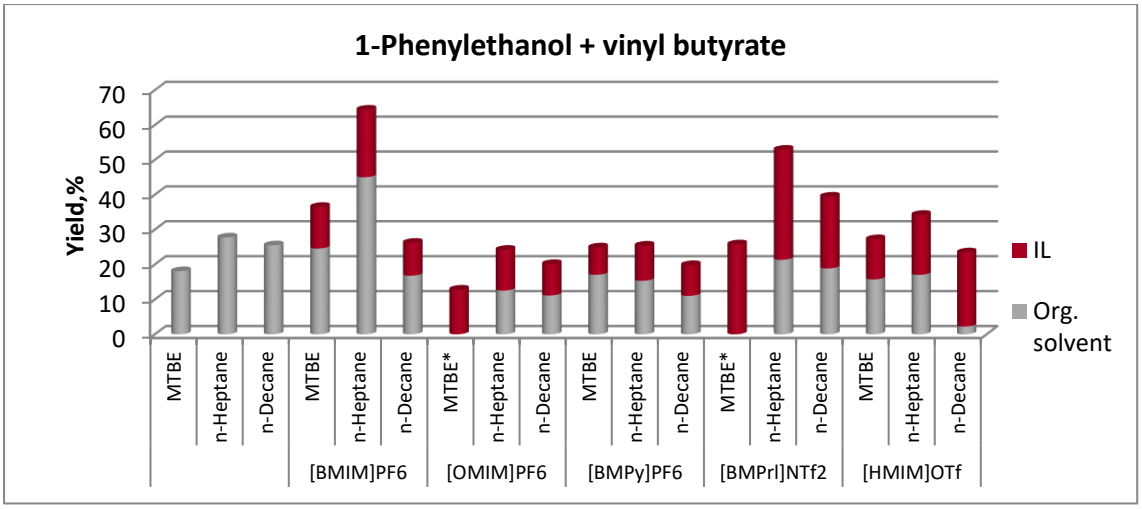
Based on this set of results an optimized and efficient system has to take in account every reaction as an individual case. These variations of the results are probably due to polarity changes associated with steric effects. The radii of the anions are slightly different and they induce changes in coordination with the particular cation which leads to other physical-chemical properties.[97] Welton *et al.* showed clearly that the influence of the anion or cation is strongly depended on the counter ion and the nature of the whole system.[96]

3.2.4 Organic solvent influence

The impact of the organic solvent, as part of the biphasic mixture needs to be also evaluated. For a whole range of ILs the reaction of 1-phenylethanol with vinyl esters were tested in three different organic solvents (MTBE, *n*-heptane and *n*-decane). The organic solvent dependency is shown in Figure 18.

n-Heptane showed a steady increase in yields for the short chain acyl donor, especially when it was paired with [BMIM] PF_6, with no major impact on the product partition. Unfortunately, the lack of physical-chemical properties of this set of ILs, which includes various cations and anions in a random selection could not bring more information for a comprehensive evaluation. In case of vinyl hexanoate, *n*-heptane was again a better partner in creating biphasic systems, except for [BMPrl] NTf_2 when associated with *n*-decane. For vinyl decanoate the shift in the product hydrophobicity was compensated by *n*-decane that changed the outcome of the reaction in terms of yield and partition.

A.



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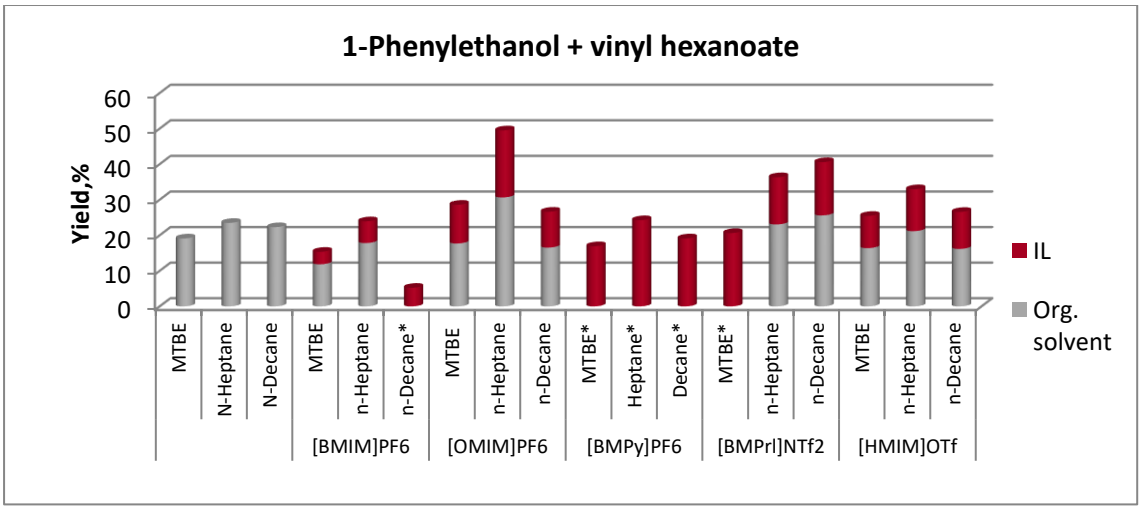

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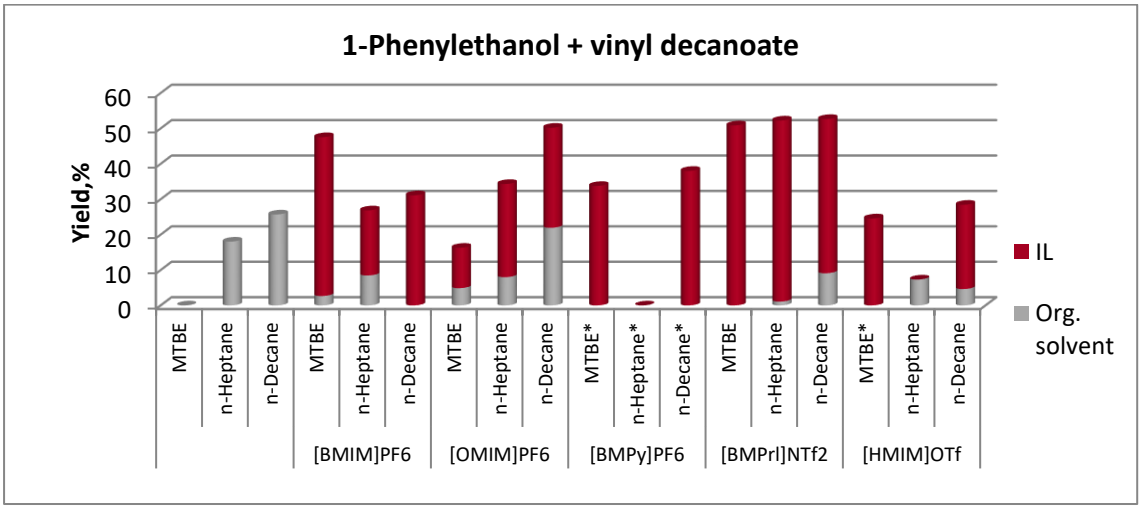


Figure 18. Solvent dependency for the transesterification of 1- phenylethanol with vinyl butyrate (A), vinyl hexanoate (B) and vinyl decanoate (C) using MTBE, *n*-heptane and *n*-decane combined with different ILs.

* describes a monophasic system after the reaction was completed.

Once a similar test was applied to the acylation of *α*-methylbenzylamine with ethyl methoxyacetate using MTBE, cyclohexane and *n*-heptane mixed with [BMIM] $N(CN)_2$, [HMIM] CF_3SO_3 and [OMIM] CF_3SO_3 the results revealed the importance of the organic solvent used in a biphasic system (Figure 19). *n*-Heptane generated the best yields from all three organic solvents and when associated with [BMIM] $N(CN)_2$ the product amount increased 2.5x in comparison with the same IL mixed with MTBE. Despite this gain, the partition wasn't optimal for the selected systems, with only 13% of the product formed in the organic phase, while in organic solvents the percentage varied from 23 to 37%.

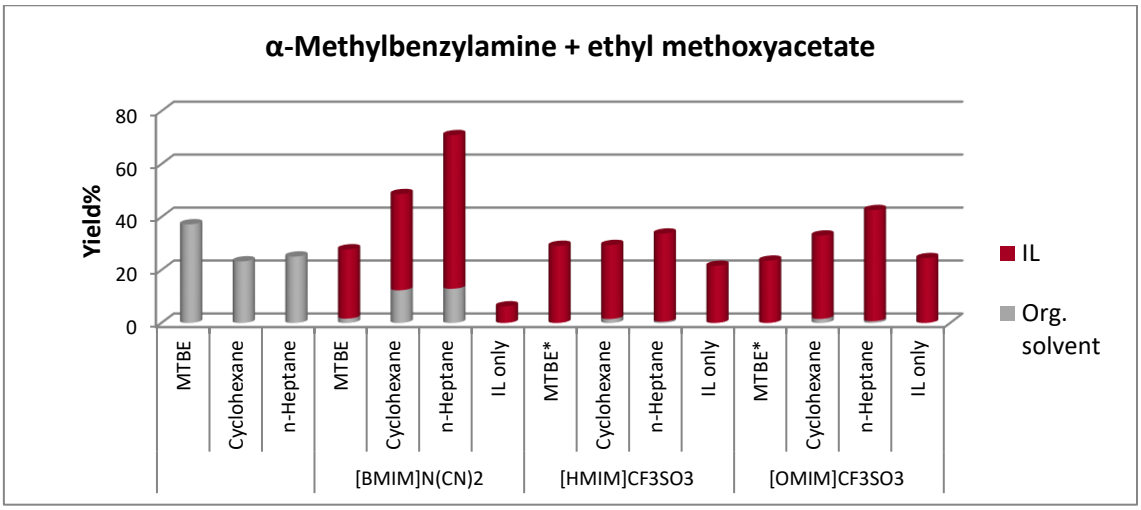


Figure 19. Solvent dependency for the acylation of *α*-methylbenzylamine with ethyl methoxyacetate in MTBE, cyclohexane and *n*-heptane mixed with [BMIM] $N(CN)_2$, [HMIM] CF_3SO_3 and [OMIM] CF_3SO_3.

* describes a monophasic system after the reaction was completed.

3.2.5 Temperature impact

Considering the viscosity of the ILs a critical point linked to mass transfer limitation and thus influencing the conversions, it was also investigated the temperature impact on the biphasic systems. All reactions using 1-phenylethanol as substrate were tested only in *n*-heptane in combination with the particular IL according to the acyl donor used. The temperature range varied from RT, 37°C, and 50°C up to 70°C.

Changes in temperature showed a great impact on the reaction conversions, as represented in Figure 20. For vinyl butyrate and vinyl hexanoate higher yields were generated at elevated temperatures using [BMIM] BF_4, respectively [BMIM] PF_6 as ILs, without major changes in the partition. For the longer chain vinyl decanoate, a significant increase in product formation was detected at 70°C showing a ~ 6 fold higher yield in the *n*-heptane phase.

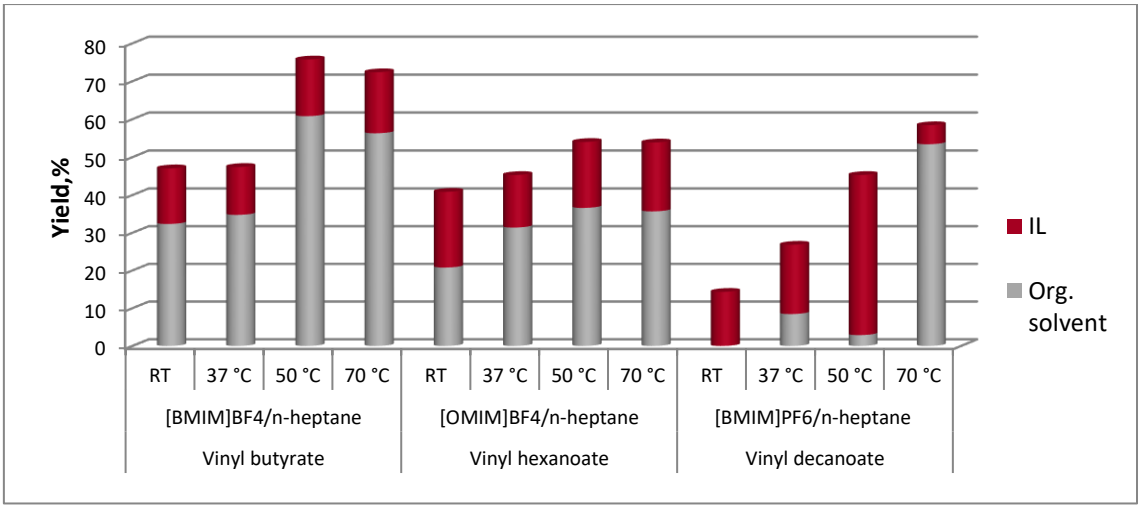


Figure 20. Temperature dependency for the transesterification of 1-phenylethanol with vinyl butyrate, vinyl hexanoate and vinyl decanoate in *n*-heptane mixed with [BMIM] BF_4, [OMIM] BF_4 and [BMIM] PF_6 respectively.

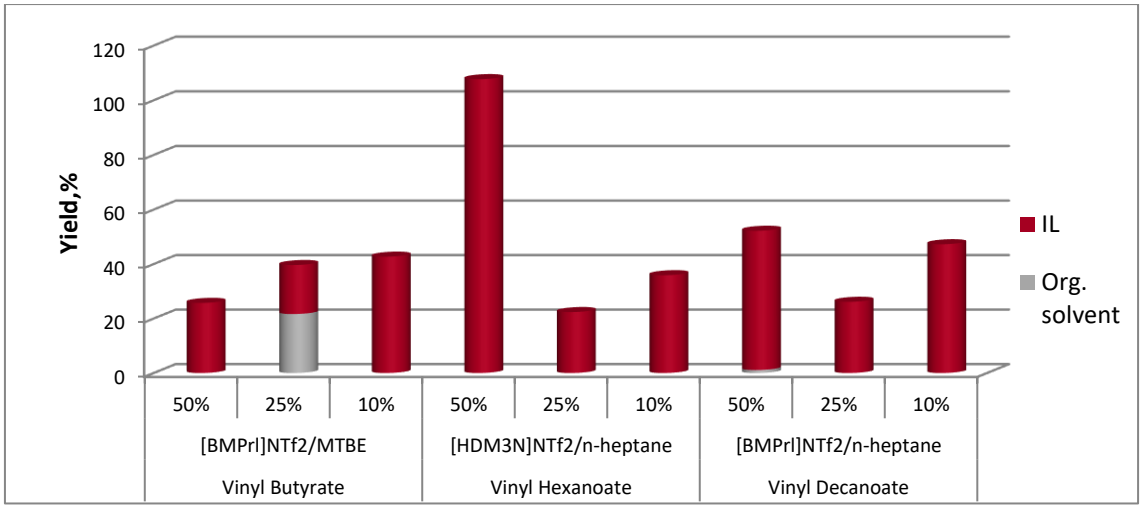
Furthermore, when compared to the shorter vinyl donors where no major partition differences occurred, a distinct product shift towards the organic phase was noticed.

According to these results, the performance of the enzyme was preserved at various temperatures, as well as the substrate behavior. When the viscosity values were compared in this context, there was no relation between viscosity and product formation at a particular temperature. The reaction outcome could be rather explained by the interactions between the corresponding IL and acyl donor or product at the given temperature than a viscosity issue.

3.2.6 Influence of ionic liquid/organic solvent ratio

One more factor that was studied to improve the partition of the product in the organic phase was the percentage of IL in the mixture (50%, 25% and 10%). The chosen systems were monophasic or had a low distribution of the product towards the organic phase (Figure 21). For the same group of reactions described previously, only in case of vinyl butyrate was noticed a recovery of about 50% in MTBE and higher yield when the volume of [BMPrl] NTf_2 was reduced to 25%. For the reaction of *α*-methylbenzylamine with ethyl methoxyacetate the yield raised when the IL percentage was only 25% for the following ILs, [BMIM] NTf_2, [BMIM] PF_6 and [BMIM] BF_4 mixed with MTBE. For [BMPy] PF_6 the product formation was reduced with the decreased amount of IL and the partition was no longer preserved.

A.



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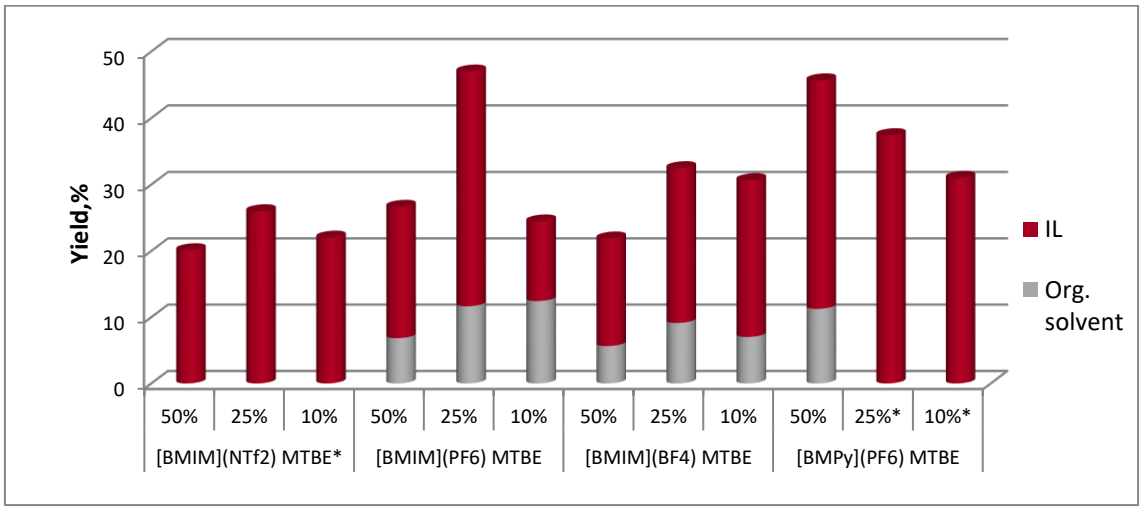


Figure 21. Impact of IL percentage on the (A) transesterification of 1-phenylethanol with vinyl butyrate, vinyl hexanoate and vinyl decanoate and (B) acylation of *α*-methylbenzylamine with ethyl methoxyacetate in MTBE and *n*-heptane mixed with a variety of ILs.

* describes a monophasic system after the reaction was completed.

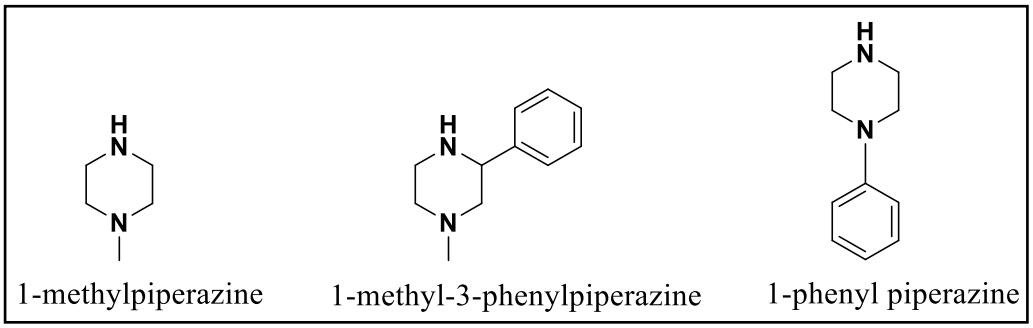
3.3 New biotransformations catalyzed by lipases

3.3.1 Selection of substrates for amidation reactions

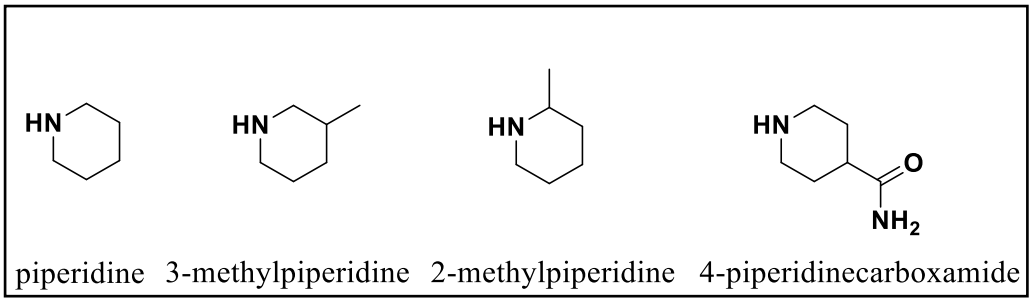
Lipases are known to catalyze amidation reactions as shown already, involving substrates like *α*-methylbenzylamine and 1-methylpiperazine. In this work, the focus was on synthesizing new amides or carbamates using CaL B and different acyl donors that could led to new intermediates for pharma industry. As targets were used piperazine ring substituents (1-methylpiperazine, 1-methyl-3-phenylpiperazine and 1-phenylpiperazine), piperidine and derivatives (3-methylpiperidine, 2-methylpiperidine and 4-piperidinecarboxamide) beside 1-phenylurea (Table 3). First the reactions were tested in organic solvents only, as a screening step and later on in biphasic systems for further development.

Table 3. Amines used for amidation reactions: (A) 1-methylpiperazine, 1-methyl-3-phenylpiperazine and 1-phenylpiperazine; (B) piperidine, 3-methylpiperidine, 2-methylpiperidine and 4-piperidinecarboxamide; (C) 1-phenylurea and *α*-methylbenzylamine.

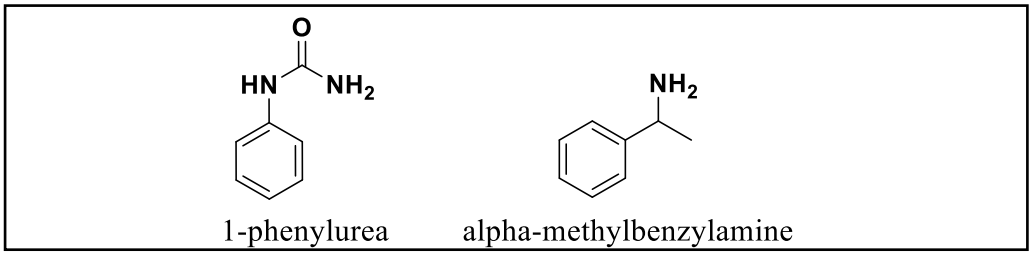
A.



B.



C.



The vinyl esters butyrate, hexanoate and decanoate already employed with *α*-methylbenzylamine were also tested with 1-methylpiperazine and 1-phenylpiperazine in MTBE. For 1-methylpiperazine only the chain length was extended to vinyl palmitate. The

experiments were carried on in the presence of lyophilized CaL B activated with oleic acid (Figure 22).

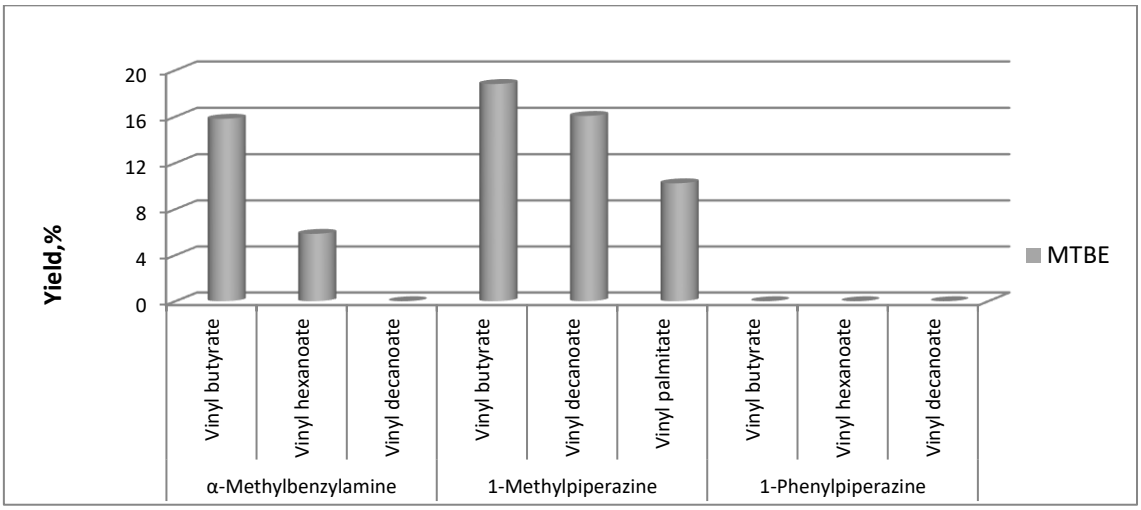


Figure 22. Amidation of *α*-methylbenzylamine, 1-methylpiperazine and 1-phenylpiperazine with three different vinyl esters butyrate, hexanoate and decanoate in MTBE at 37°C, using lyophilized activated CaL B.

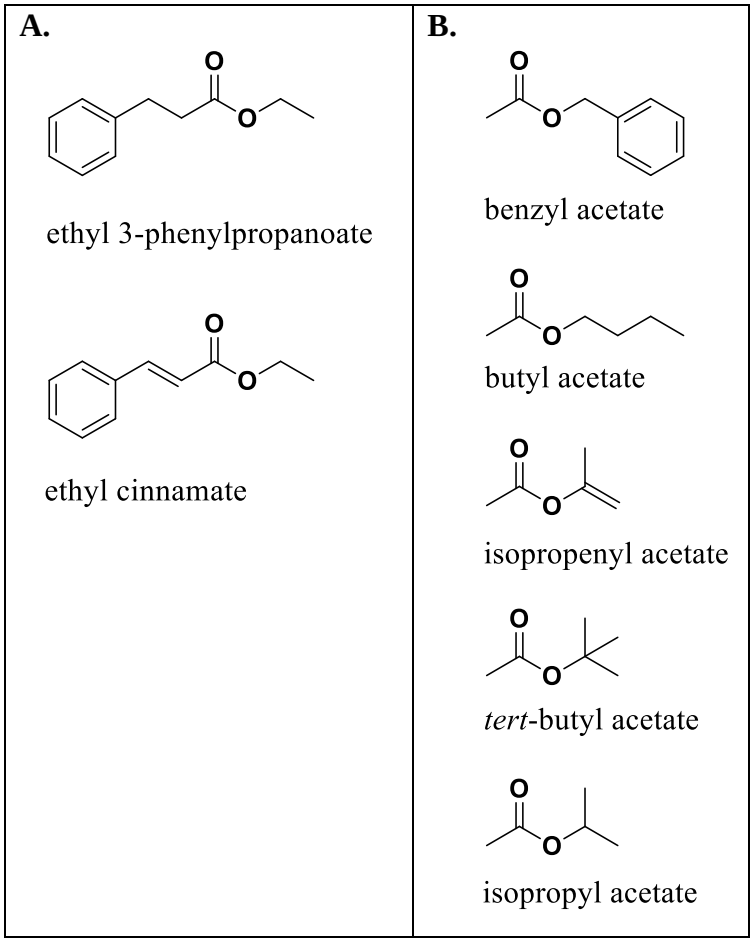
The reaction with 1-methylpiperazine gave the best results in comparison with the other substrates, with yields varying from 10% to 18%. This substrate was not only able to react with the longer alkyl vinyl esters, but generated in all three cases, more product. Despite that *α*-methylbenzylamine is a substrate well accepted by CaL B, in these conditions 1-methylpiperazine performed better, probably due to the fact that it is a secondary amine with increased reactivity. Once the methyl group was substituted with phenyl, that had an opposite effect on the amino group reducing the basicity, the reaction did not take place. On the other hand, this could be judged as a steric hindrance in the binding pocket of the enzyme. Despite this outcome, the reactions with vinyl esters were no longer investigated due to the spontaneous reaction in the organic solvent that generated small amounts of amides.

Along with the formation of the product, it was noticed a color development associated with these acyl donors. A vinyl ester involved in a transesterification will release an enol stabilized in the form of an aldehyde (keto-enol tautomerism), which could further react with lysine residues on the enzyme. This interaction could generally lead to the inactivation of some lipases, though CaL B is considered to be an exception. The acetaldehyde that is formed immediately in the reaction mass could react as well with the amines directly.

3.3.2 Selection of acyl donors

In order to avoid these eventual challenges, other alternatives were investigated, taking in account other types of acyl donors. On one hand, ethyl 3-phenylpropionate and ethyl cinnamate were considered for introducing new radicals on the amide group. A second thought was given to other acyl donors (ethyl 3-phenylpropionate, ethyl cinnamate, benzyl acetate, butyl acetate, isopropenyl acetate, *tert*-butyl acetate and isopropylacetate) that would generate the same product, but eventually form other by-products that will not interfere with the enzyme or with the substrate as listed in Table 4.

Table 4. Acyl donors used for amidation reactions: (A) ethyl 3-phenylpropionate and ethyl cinnamate; (B) benzyl acetate, butyl acetate, isopropenyl acetate, *tert*-butyl acetate and isopropylacetate.



It's important to mention that esters with other alkyl radicals were tested, like methyl or ethyl, without promising results. So, the new acyl donors would be less reactive than vinyl counter partners, but still believed to generate the product.

First screening was performed for *α*-methylbenzylamine in MTBE only, as shown in Figure 23.

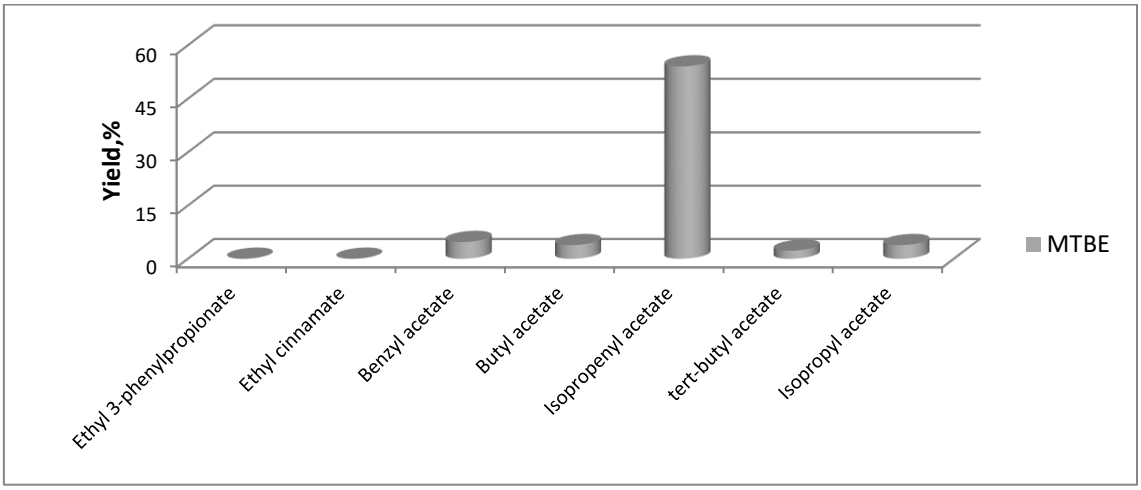


Figure 23. Amidation of *α*-methylbenzylamine with different acyl donors: ethyl 3-phenylpropionate, ethyl cinnamate, benzyl acetate, butyl acetate, isopropenyl acetate, *tert*-butyl acetate and isopropyl acetate in MTBE at 37°C, using lyophilized activated CaL B.

The first two substrates didn't produce any results and the negative control for isopropenyl acetate turned to be positive, so the reactions with the best three candidates benzyl acetate, butyl acetate and isopropyl acetate were transferred to biphasic systems using MTBE as organic solvent and several ILs [EMIM] $N(CN)_2$, [BMIM] BF_4, [BMIM] PF_6 and [BMIM] NTf_2. In all three cases the results showed an increase in product formation, but for butyl acetate and isopropyl acetate the yields were under 10% (Figure 24).

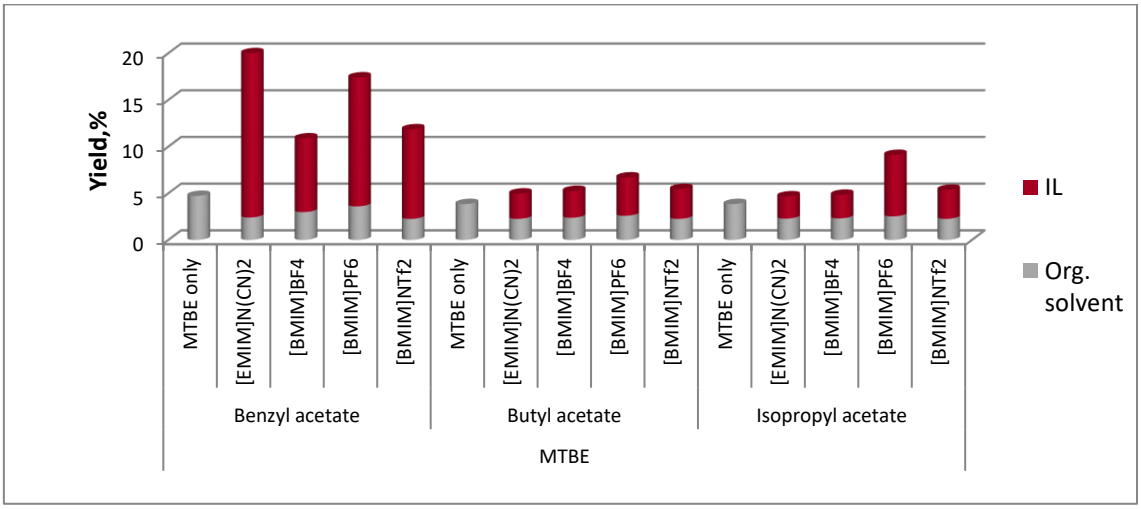
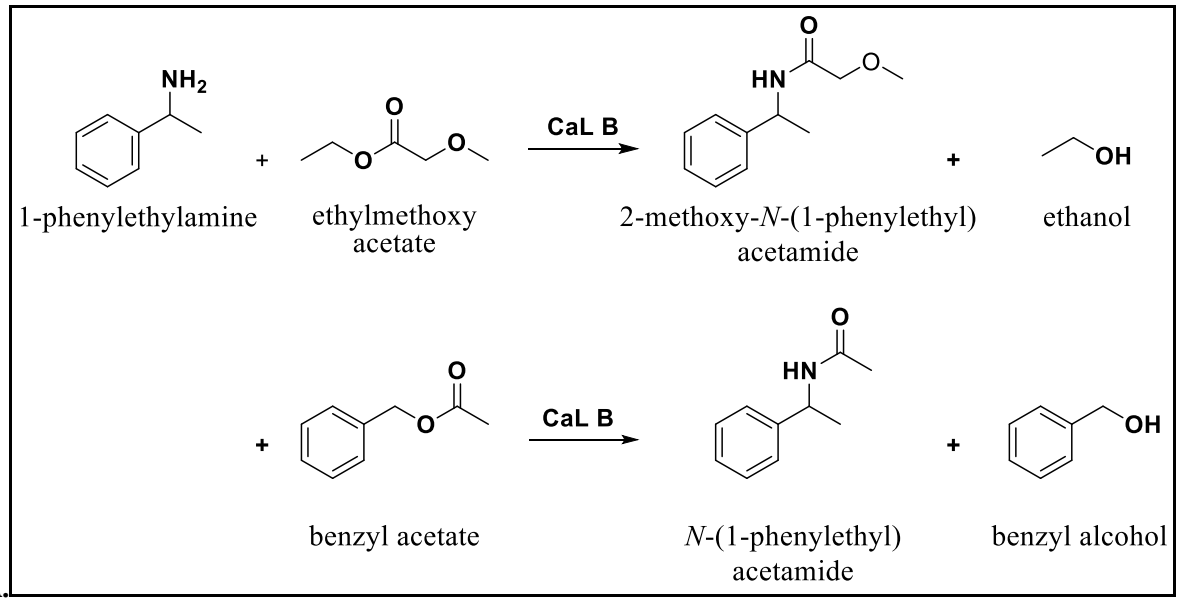


Figure 24. Amidation of *α*-methylbenzylamine with benzyl acetate, butyl acetate and isopropyl acetate in MTBE in comparison with biphasic systems using [EMIM] $N(CN)_2$, [BMIM] BF_4, [BMIM] PF_6 and [BMIM] NTf_2 as ILs at 37°C using lyophilized activated CaL B.

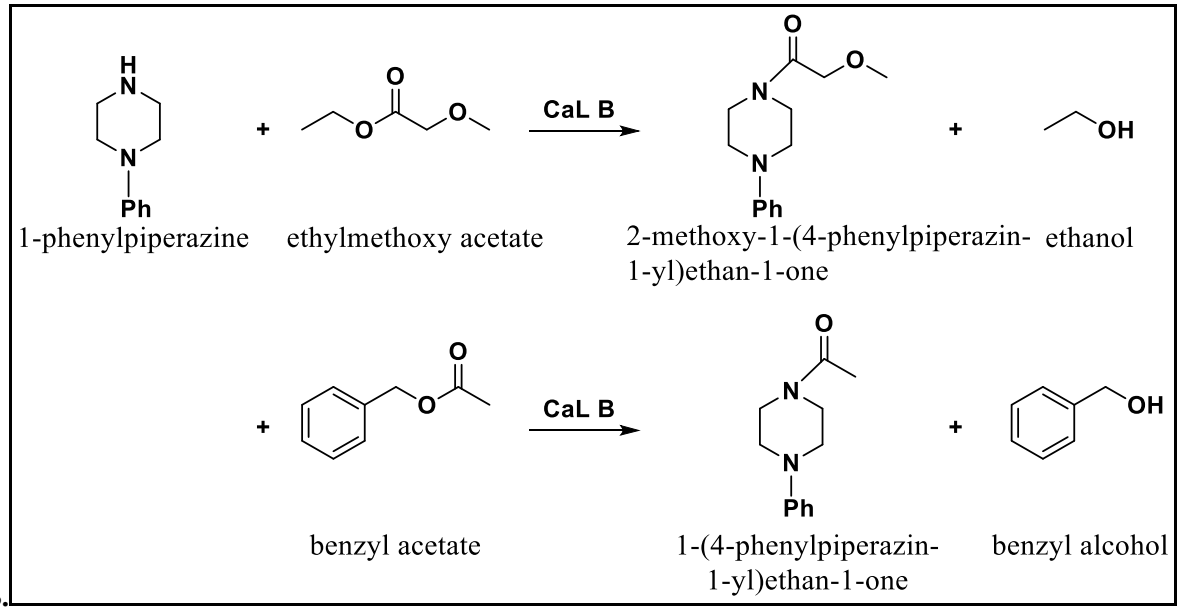
For all remaining experiments only ethyl methoxyacetate and benzyl acetate were systematically tested in combination with the substrates that created higher amounts of

product (α-methylbenzylamine, 1-phenylpiperazine, 1-methylpiperazine and piperidine) [Scheme 11].

A.



B.



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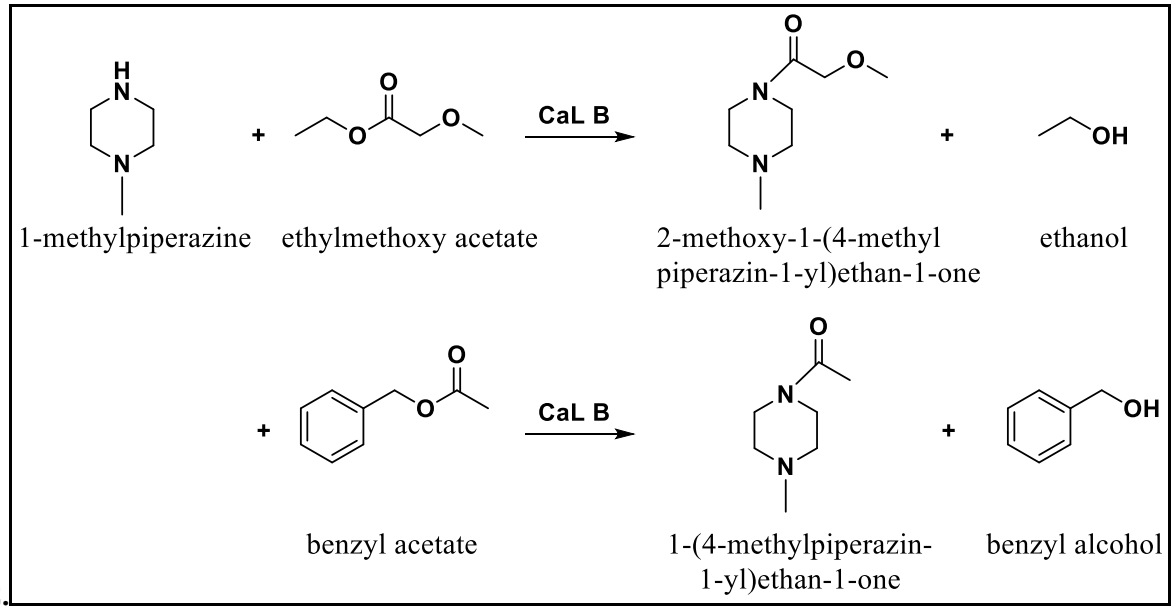
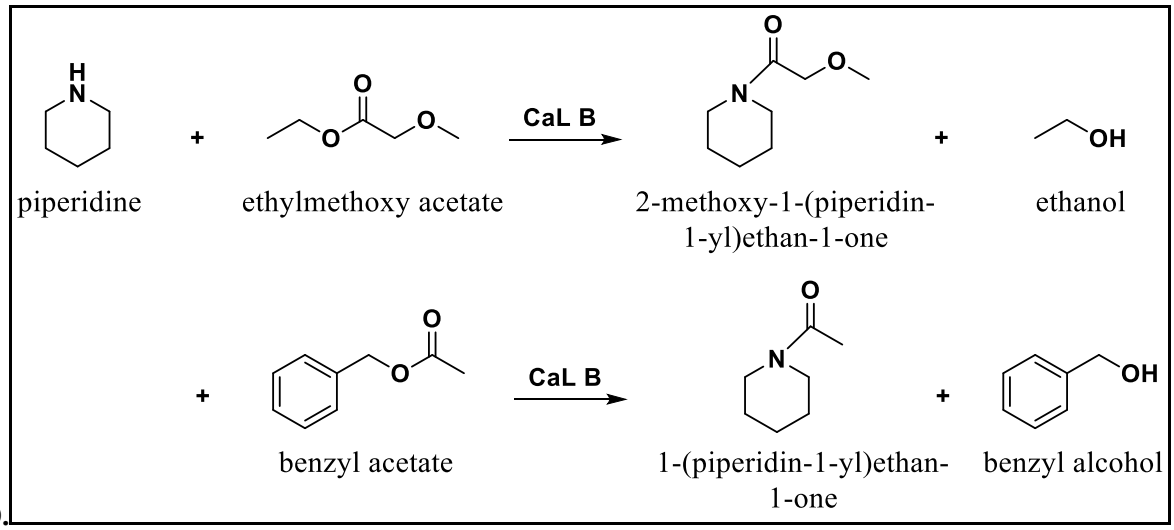


D.



Scheme 11. Amidation reactions for (A) *α*-methylbenzylamine, (B) 1-phenylpiperazine, (C) 1-methylpiperazine and (D) piperidine using ethylmethoxy acetate and benzyl acetate.

It has to be mentioned that all the other piperidine substituents were tested, as well as 1-phenylurea with yields under 5% (data not shown), which shifted the interest completely to the other amines mentioned above. All other substrates did not generated any product.

When we compared the results for all the substrates in the polar solvent, MTBE 1-phenylpiperazine, with both acyl donors generated yields over 40%, followed by *α*-methylbenzylamine with ethyl methoxyacetate and 1-methylpiperazine with benzyl acetate in the 30% range (Figure 25). The amidation product of piperidine and benzyl acetate generated a yield of ~ 24%. For cyclohexane the results registered a decrease, only *α*-methylbenzylamine with ethyl methoxyacetate gave a yield of about 22%. This shows one more time that a polar solvent would create a better environment for substrates like amines.

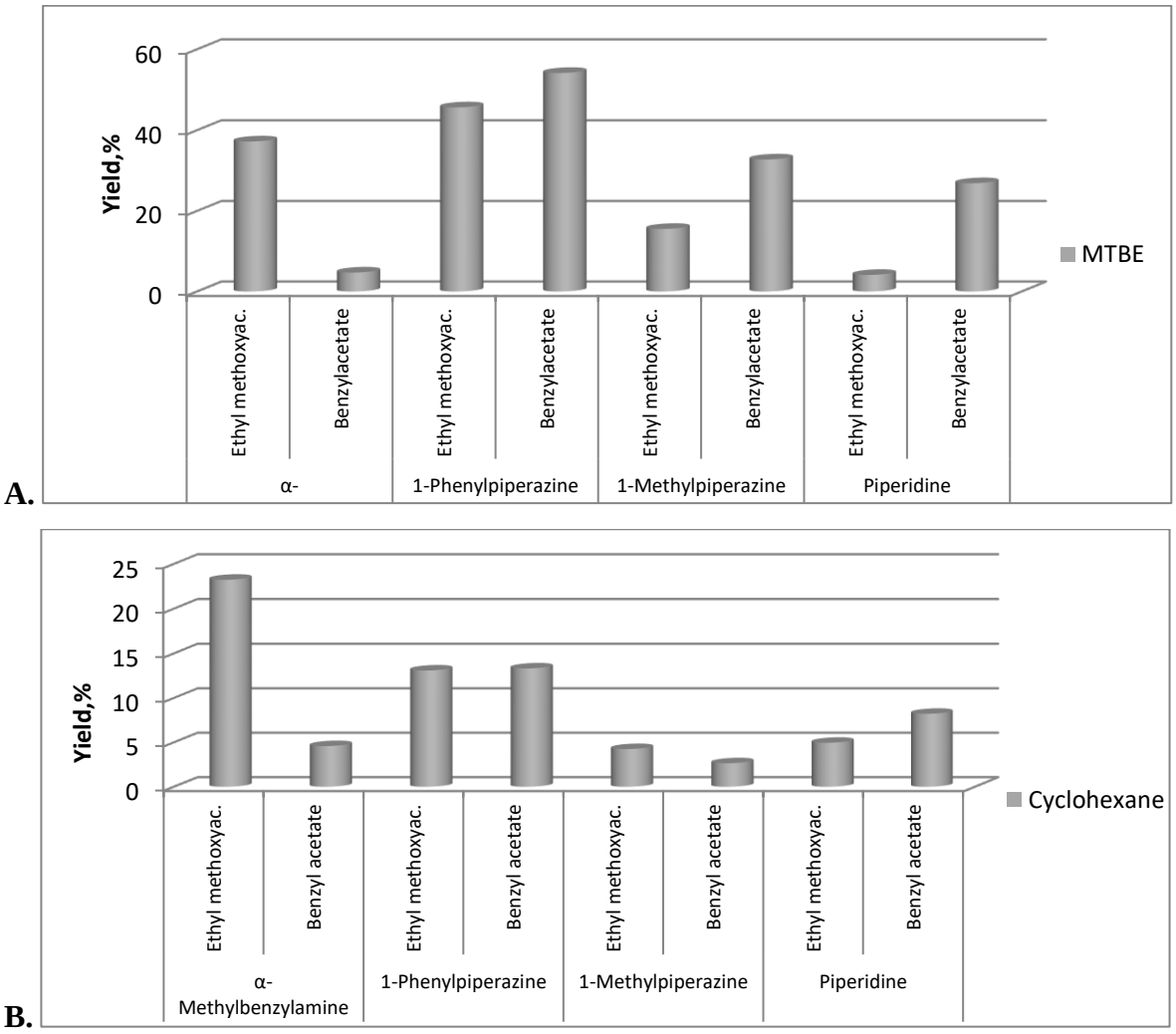


Figure 25. Amidation of *α*-methylbenzylamine 1-phenylpiperazine, 1-methylpiperazine and piperidine with ethyl methoxyacetate and benzyl acetate in (A) MTBE and (B) cyclohexane at 37°C using lyophilized activated CaL B.

3.3.3 Transfer of selected reactions in biphasic systems

Furthermore, each pair of acyl donors were studied with the corresponding substrate in biphasic condition. The comparison targeted one more time the behavior in systems where the polarity was tuned using different organic solvents and ILs.

For *α*-methylbenzylamine and ethyl methoxyacetate pair were selected MTBE, cyclohexane and *n*-heptane paired with [BMIM] $N(CN)_2$, [HMIM] CF_3SO_3 and [OMIM] CF_3SO_3. As shown in Figure 25, MTBE, as a single phase gave better results than cyclohexane, but the situation was completely reversed when the biphasic systems were introduced (Figure 26).

The reaction showed a steadily increase in product formation from MTBE to cyclohexane and *n*-heptane recording finally a 4x increase up to 70% when associated with [BMIM] $N(CN)_2$. The other ILs, with different anions and longer alkyl chains didn't surpass these results. The partition was also better for the first systems, while for the rest the immiscibility of the two solvents was lost in some cases and generated a homogenous phase after the completion of the reaction.

For the second acyl donor the trend was somehow preserved, but not at the same extend, increasing from 22% for MTBE / [EMIM] $N(CN)_2$ to 35% in case of cyclohexane / [EMIM] $N(CN)_2$ and comparable for *n*-heptane / [EMIM] $N(CN)_2$. The ILs used in this particular reaction were different ([EMIM] $N(CN)_2$, [BMIM] BF_4 and [BMIM] NTf_2), but the organic solvents were preserved. For the two hydrophobic organic solvents, the NTf_2 based ILs boosted the product formation. The partition towards the organic solvents was poor in all cases and constant under 3%. The biphasic approach for cyclohexane and *n*-heptane increased the performance in comparison with the sole usage of the corresponding organic solvent.

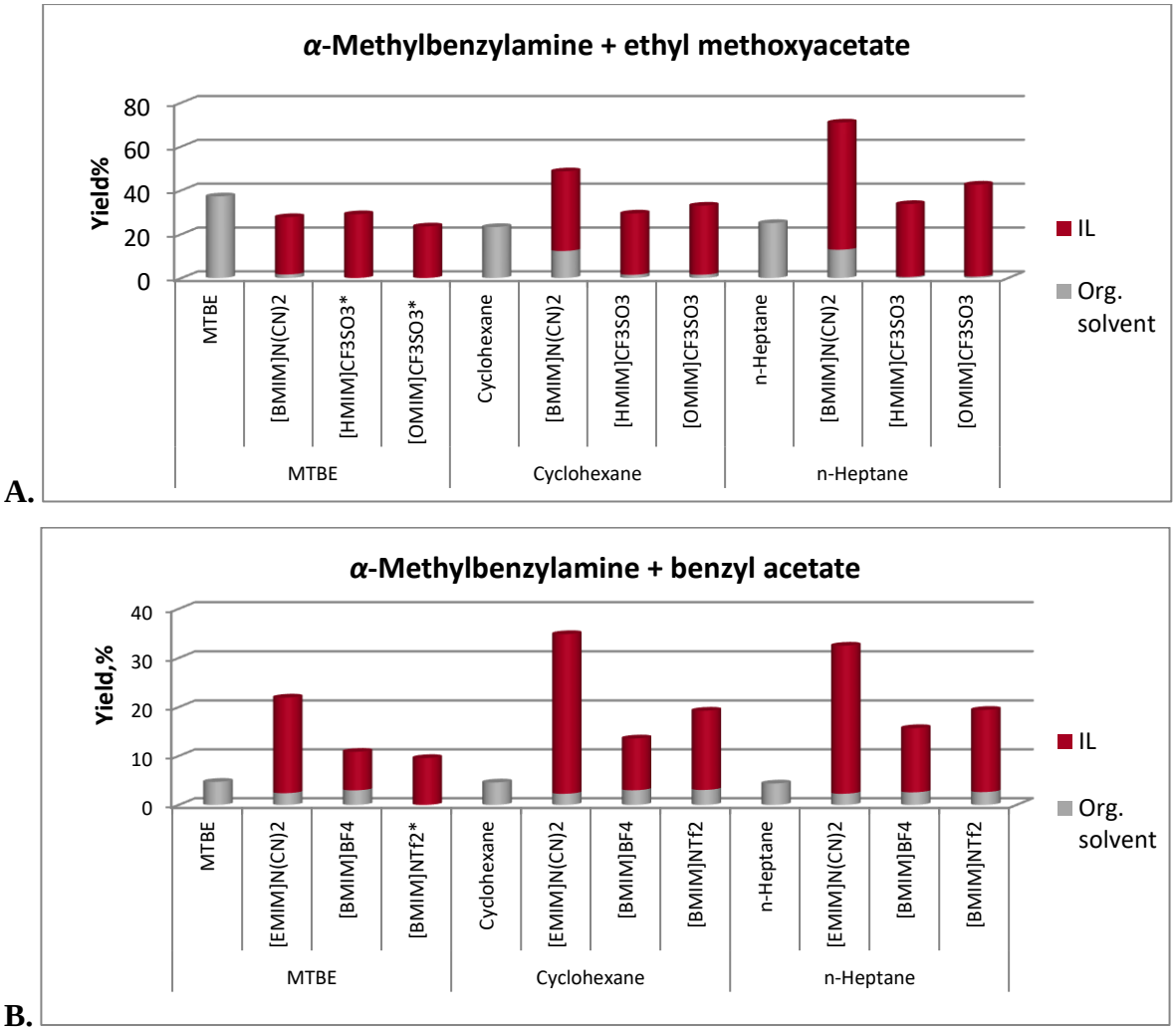


Figure 26. Amidation of *α*-methylbenzylamine with (A) ethyl methoxyacetate and (B) benzyl acetate in biphasic systems using MTBE, cyclohexane and *n*-heptane and different ILs, at 37°C using lyophilized activated CaL B.

* describes a monophasic system after the reaction was completed.

Once we switched to the next substrate 1-methylpiperazine, using the same conditions as for *α*-methylbenzylamine and ethyl methoxyacetate the yields dropped under 10% except for the pair *n*-heptane / [BMIM] $N(CN)_2$ that was slightly higher as showed in Figure 27.

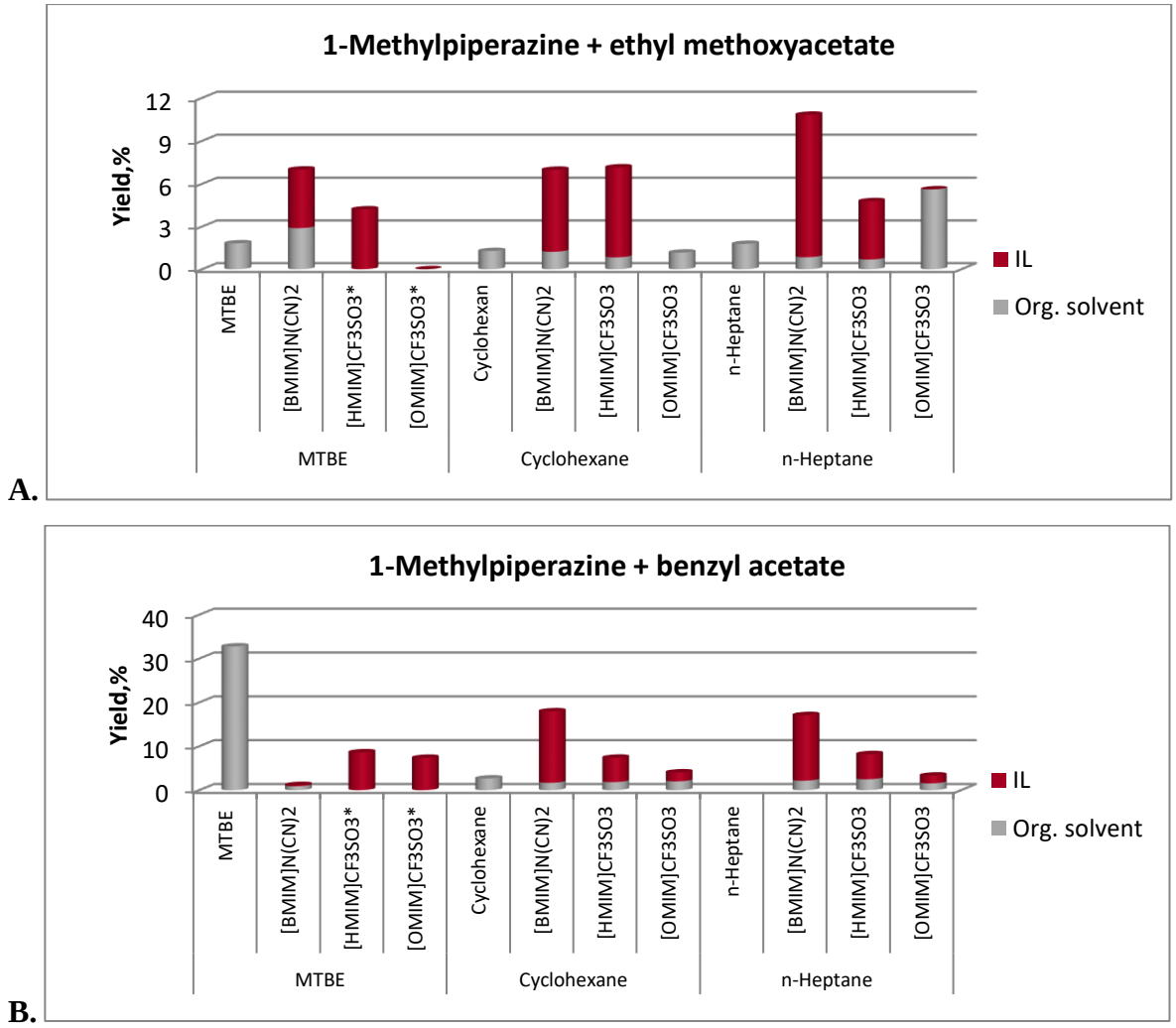


Figure 27. Amidation of 1-methylpiperazine with (A) ethyl methoxyacetate and (B) benzyl acetate in biphasic systems using MTBE, cyclohexane and *n*-heptane and different ILs, at 37°C using lyophilized activated CaL B.

* describes a monophasic system after the reaction was completed.

There is one interesting change noticed for the mixtures incorporating the hydrophobic organic solvents and [OMIM] CF_3SO_3 as IL, where the product was partitioned almost completely in the organic phase. When MTBE was employed the partition was better for the less hydrophobic IL respectively [BMIM] $N(CN)_2$. Looking again to the results generated by benzyl acetate, cyclohexane and n-heptane with all three ILs have an identical behavior and are proportional with the hydrophobicity of the IL, decreasing from [BMIM] towards the longer alkyl chain [OMIM]. Unfortunately, the yields in the biphasic systems were under 10%, far behind MTBE only. The product one more time was to a larger extend present in the IL phases.

For piperidine the same set of reactions and conditions were applied without any successful result (Figure 28). On one side the product with ethyl methoxyacetate wasn't detectable in the

chromatographic conditions employed for all the other samples, lacking the chromophore group. For benzyl acetate as donor, the yields were under 5% and way below the performance in MTBE only. Despite that the partition towards the organic phase was around 50% overall.

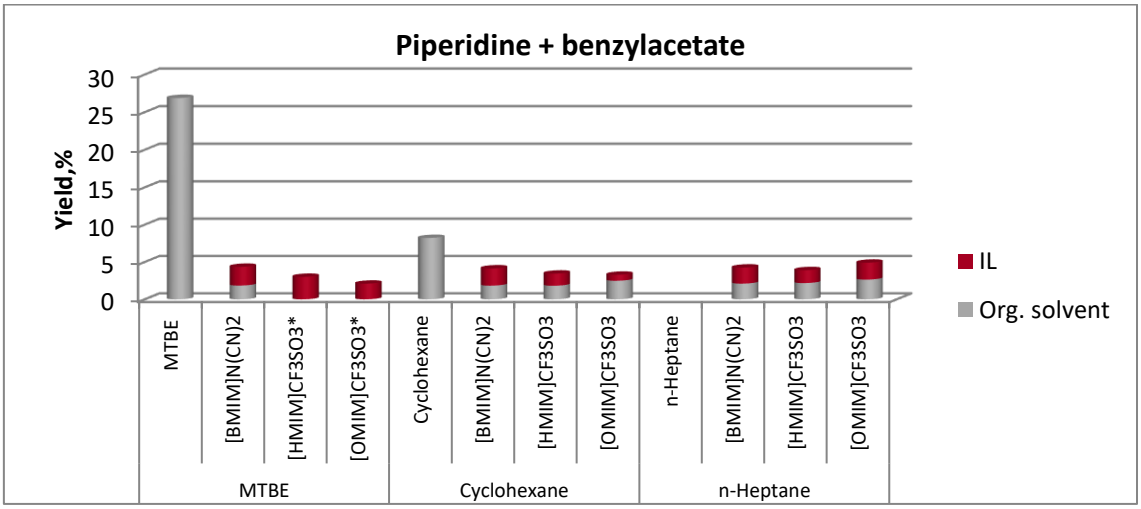
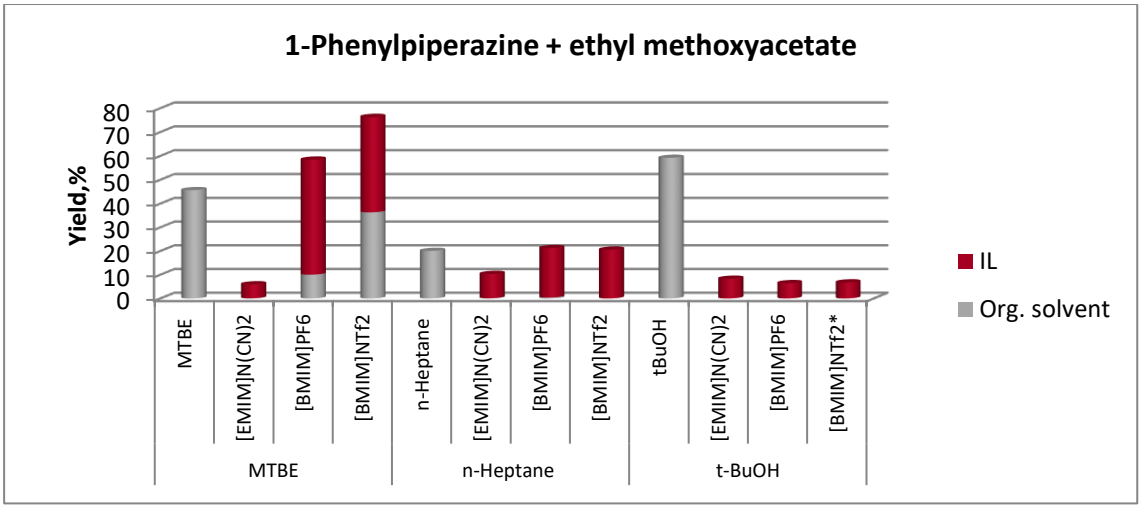


Figure 28. Amidation of piperidine with benzyl acetate in biphasic systems using MTBE, cyclohexane and *n*-heptane and different ILs, at 37°C using lyophilized activated CaL B.

* describes a monophasic system after the reaction was completed.

For the last substrate investigated, 1-phenylpiperazine the spectrum of organic solvent was changed to MTBE, *n*-heptane and *t*-BuOH for a better assessment with more polar solvents (Figure 29).

A.



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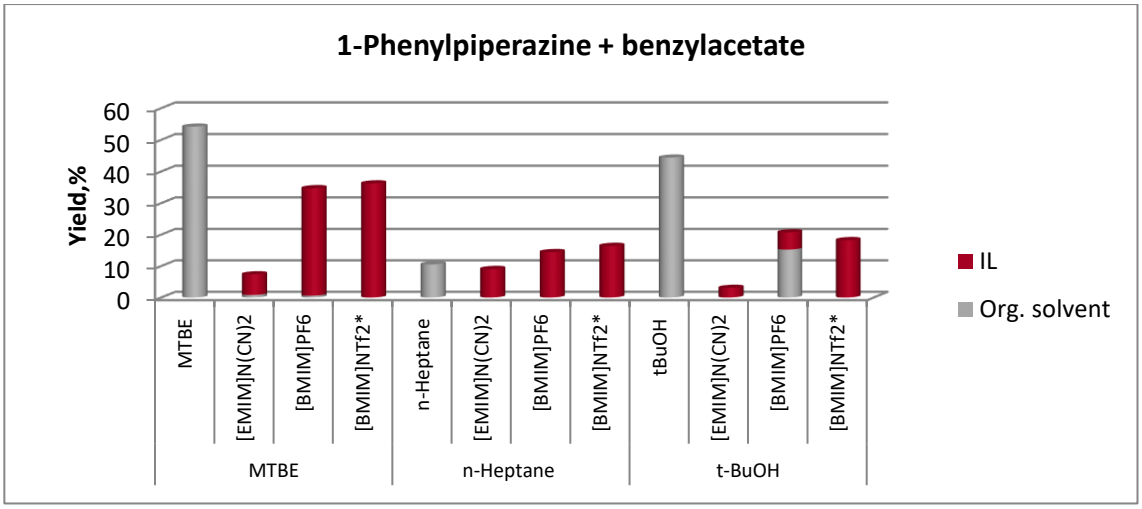


Figure 29. Amidation of 1-phenylpiperazine with (A) ethyl methoxyacetate and (B) benzyl acetate in biphasic systems using MTBE, *n*-heptane and *t*-BuOH and different ILs, at 37°C using lyophilized activated CaL B.

* describes a monophasic system after the reaction was completed.

The set of ILs was subject to a small change, [BMIM] BF_4 being replaced by [BMIM] PF_6. For the first reaction, MTBE associated with [BMIM] PF_6 and [BMIM] NTf_2 increased the yields substantially from 45% in MTBE only, to 60% respectively to 76%. The partition was also in the 50% range for the last IL. All the other biphasic systems were below 20%. Despite these results the reaction in pure *t*-BuOH generated returns of 60%. For benzyl acetate the product formation in organic solvents was in the range of 54% for MTBE and 44% for *t*-BuOH. Partition was about 75% towards *t*-BuOH when paired with [BMIM] PF_6, in all other systems being completely accumulated in the IL phase.

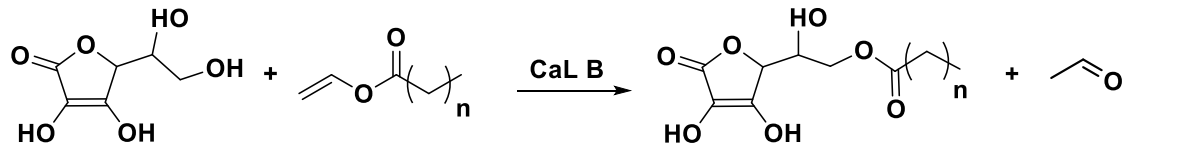
In terms of using new substrates for the developing of new products, CaL B proved one more time its versatility. Despite the lack of the right analytic method for product determination the overall results could be considered as a good starting platform for further improvements. The involvement of the ILs in current settings wasn't always a beneficial addition.

3.4 Industrial applications using SILP technology

The potential of the biphasic systems could be emphasized using reactions that are technically challenging due to solubility issues, especially when the substrate and the product have opposite physical-chemical properties. As mentioned before, ILs have the capability to dissolve compounds that are not soluble in some of the organic solvents that could host enzymatic reactions. Furthermore, when paired with particular organic solvents, as a system they could solubilize compounds (substrate and product) with diametrically opposite properties, being a perspective key to this type of problematic mixtures.

3.4.1 Transesterification of ascorbic acid as a model reaction for SILP technology

One reaction that could be used as a proof of principle is the esterification of ascorbic acid with fatty acids (Scheme 12). Ascorbic acid is a highly polar substance, commonly used as an antioxidant with applications in food industry and cosmetics. The limitation derives from the insolubility of vitamin C in the fatty products and could be solved by introducing a long alkyl chain in the molecule through an esterification reaction catalyzed by lipases. The hydrophobic substituent will change the polarity of the compound, without altering the initial properties. The enzymatic reaction, tested in organic solvents as well as in pure ILs is well known and applied on industrial scale. Despite the high conversions and yields already achieved, the process requires complicated steps for the extraction and purification of the product, which could be improved using SILP technology based on a biphasic IL/organic solvent system.



Scheme 12. Transesterification of ascorbic acid with fatty acids vinyl esters.

3.4.1.1 Evaluation of reaction conditions for the transesterification of ascorbic acid

As preliminary tests, the reaction was performed in the presence of various enzymes and different chain length fatty acids esters. For enzyme screening were used lipases from bacterial and fungal origin including *Aspergillus oryzae*, *Candida rugosa*, *Rhizopus arrhizus*,

Rhizomucor miehei, *Pseudomonas cepacia* and *Candida antarctica*. The reactions were tested using *t*-BuOH as organic solvent in the presence of vinyl palmitate, after the vitamin C was preliminary dissolved in DMSO (2% of the total volume). The best outcome was displayed by lipase B from *Candida antarctica* followed by *Pseudomonas cepacia* as shown in Figure 30. For the next experiments CaL B was used in an immobilized form.

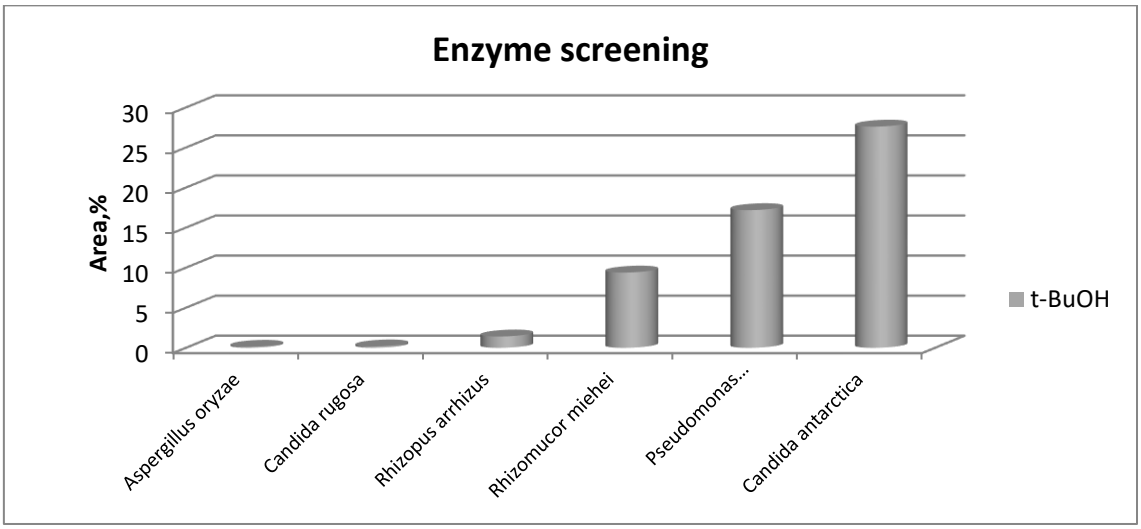


Figure 30. Transesterification of vitamin C with vinyl palmitate (ratio 1:3) in t-BuOH using lipase B from different bacterial and fungal sources (*Aspergillus oryzae*, *Candida rugosa*, *Rhizopus arrhizus*, *Rhizomucor miehei*, *Pseudomonas cepacia* and *Candida antarctica*).

The prescreening continued with a set of reactions for the best yield related to the chain length of the fatty acid. Using the same reaction conditions as described above, four vinyl esters were tested as it follows: vinyl caproate (C_6), vinyl laurate (C_{12}), vinyl palmitate (C_{16}) and vinyl stearate (C_{18}). As depicted in the graph (Figure 31) the efficiency of the reaction increased steadily with the longer chain acyl donors from 70% to 92% for vinyl palmitate and dropped significantly to 15% in the case of vinyl stearate. This change could be a consequence of the insolubility of the longer and more hydrophobic acyl donor in the organic phase. Based on these results vinyl palmitate was employed in the development of the reaction.

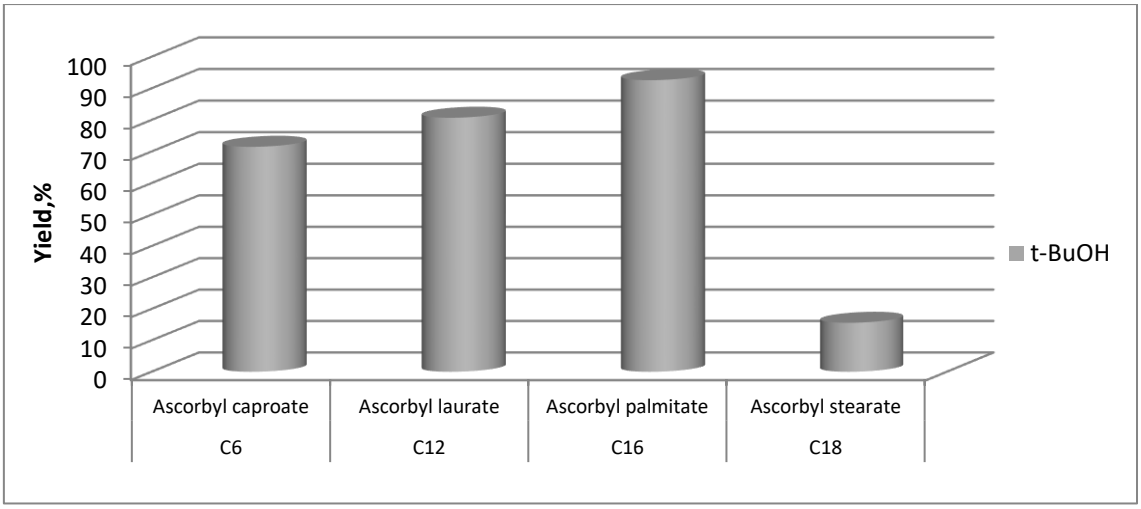


Figure 31. Transesterification of vitamin C with different chain length vinyl esters: vinyl caproate, vinyl laurate, vinyl palmitate and vinyl stearate (ratio 1:3) in t-BuOH using immobilized CaL B.

To ensure the stability and performance of the lipase during the esterification reaction in *t*-BuOH the yields were calculated on a course of 72 h, with the addition of substrate every 24 h in the reaction mass, as shown in Figure 32. Simultaneous was tested the influence of the amount of enzyme on the outcome of the process, using CaL B in different quantities, varying from 5 mg to 10 mg of immobilized biocatalyst. The results confirmed one more time that CaL B is a robust enzyme, stable in the reaction medium at 37°C for 72 h, without a significant decrease of the yield. For an amount of 5 mg enzyme the yield was 94% and the difference between 24 h and 72 h was about 10% giving the best results. The yield was slightly better for 8 mg CaL B in the first 24 h.

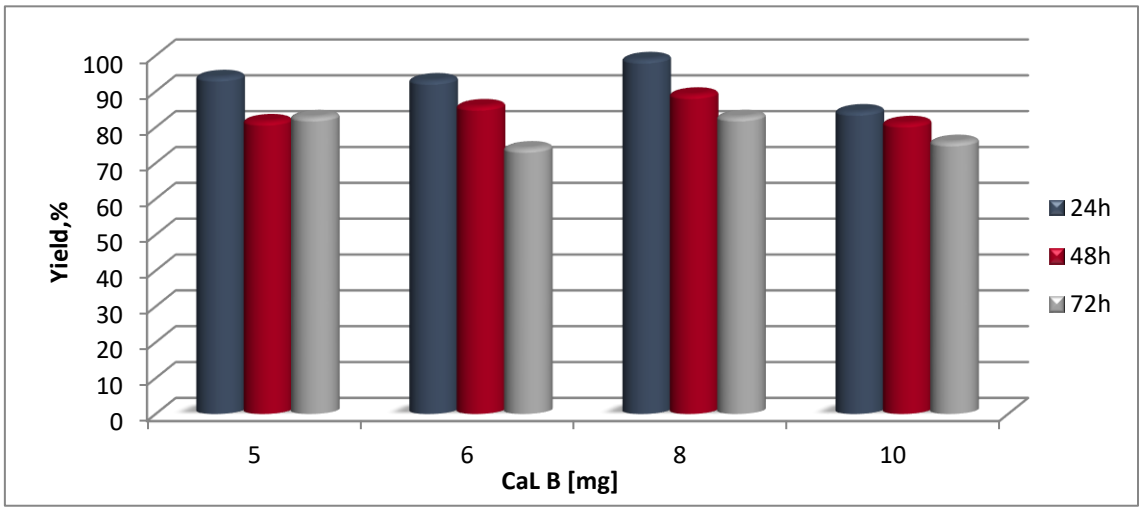


Figure 32. Transesterification of vitamin C with vinyl palmitate (ratio 1:3) in t-BuOH using immobilized CaL B in different quantities (5, 6, 8 and 10 mg) in a time frame from 24 h to 72 h.

3.4.1.2 Impact of the biphasic systems on the reaction of ascorbic acid with vinyl palmitate

Despite the fact that the reaction with the immobilized form of the enzyme gives better results, the following experiments were performed with the lyophilized CaL B, activated with oleic acid for further usage with SILP technology. As a first attempt, the reaction was transferred in an IL only, [BMIM] PF_6 and it was tested at two different temperatures to eliminate the drawback related to viscosity. In all the previous experiments DMSO was used to bring vitamin C in a soluble form, so for comparison reasons the transesterification in IL was completed in parallel using DMSO. Unfortunately, the reaction didn't take place regardless the usage of extra solvent or the yields were insignificant (Figure 33).

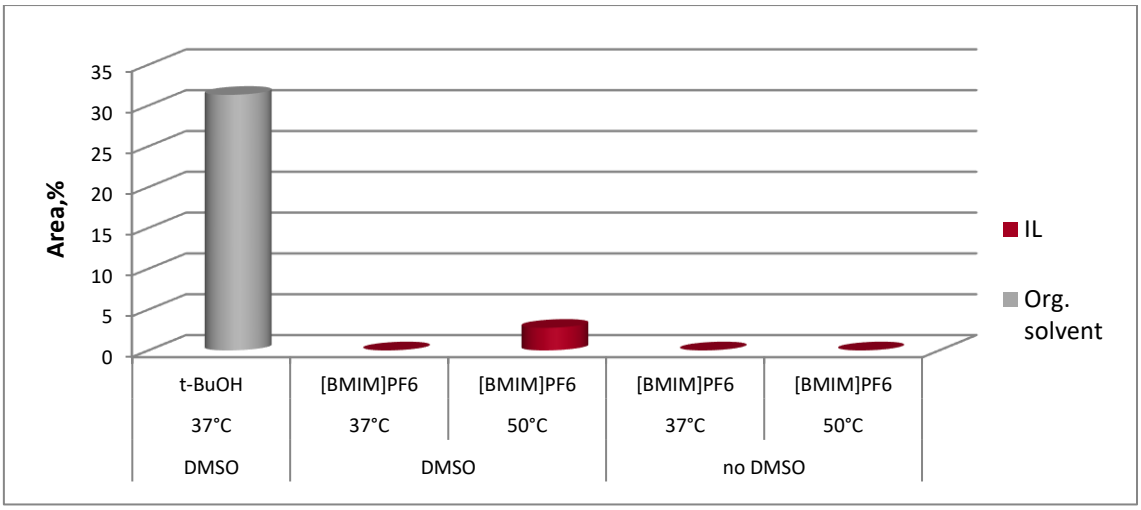


Figure 33. Comparison of the transesterification of vitamin C with vinyl palmitate (ratio 1:3) in t-BuOH vs. IL, [BMIM] PF_6 using the activated lyophilized form of CaL B at different temperatures (37° and 50° C).

Based on these data a more comprehensive test was set to identify the IL capable to improve the transesterification reaction. Vitamin C was mixed with six ILs ([EMIM] $N(CN)_2$, [BMIM] $N(CN)_2$, [BMIM] BF_4, [BMIM] NTf_2, [HMIM] CF_3SO_3, [OMIM] CF_3SO_3 and [OMIM] PF_6) for 1 h at 30°C. The substrate was completely dissolved by [EMIM] $N(CN)_2$ and [HMIM] CF_3SO_3, and partially for the other ILs. In this setting, the ILs should be responsible for the solubilization of the substrate and the organic phase, selected to form the biphasic system should provide the right environment for the acyl donor. Having this objective in mind *n*-heptane was paired with the ILs that dissolved the vitamin C. Beside these candidates, [OMIM] CF_3SO_3 and [OMIM] PF_6 were chosen to observe the impact of an IL with a hydrophobic substituent, as well as [BMIM] BF_4 and [BMIM] PF_6 as systems with

possible potential for comparison. *t*-BuOH was used in parallel as a reference. The immobilized form of the lipase in the biphasic system generated the highest amount of product in the presence of DMSO as shown in Figure 34.

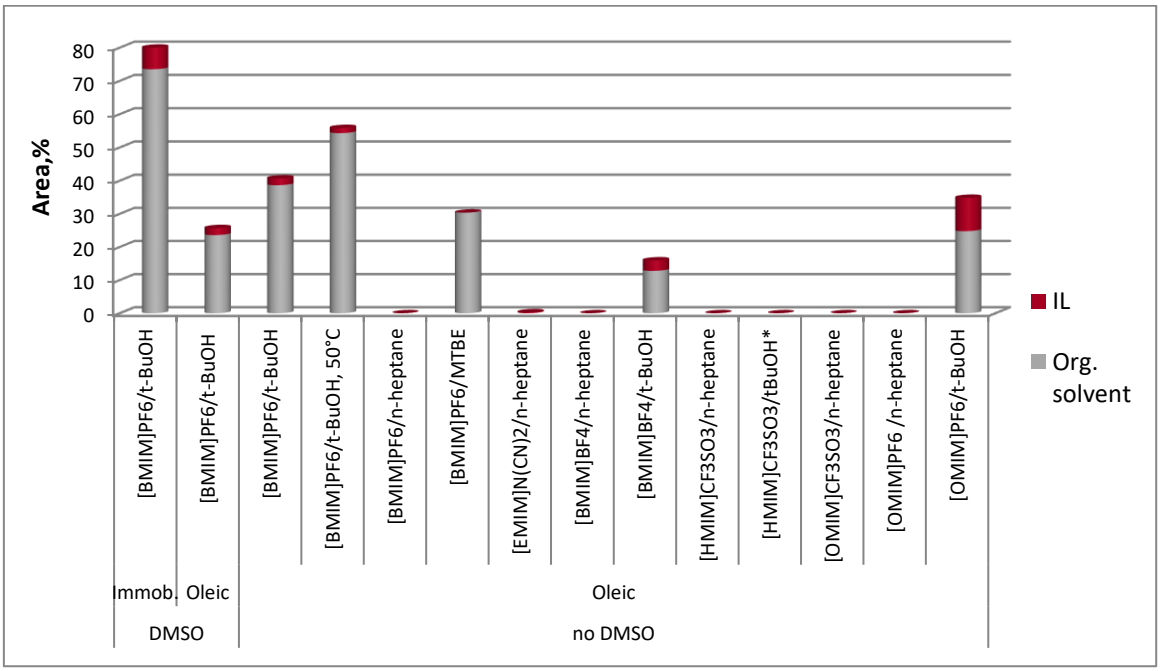


Figure 34. Transesterification of vitamin C with vinyl palmitate (ratio 1:3) in biphasic systems paired with *n*-heptane and *t*-BuOH without additional DMSO.

* describes a monophasic system after the reaction was completed.

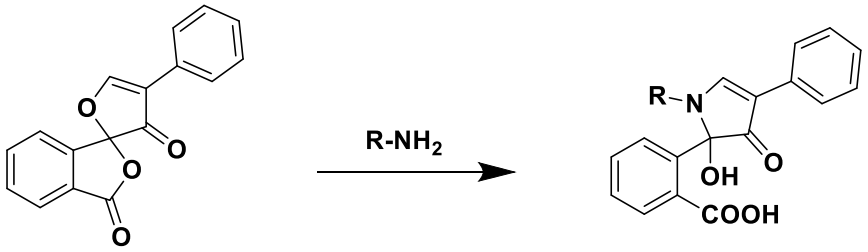
When we compared the transesterification in [BMIM] PF_6/ *t*-BuOH with and without the solubilizing organic solvent, it was clear that the mixture was able to accomplish the requirements necessary for the substrate and acyl donor without DMSO and raised the product formation with 15%. The elevated temperature of 50°C, for the pair [BMIM] PF_6/ *t*-BuOH increased the performance with 16% despite the fact that, for the same IL only didn't boost the reaction. Unfortunately, in all the biphasic systems containing *n*-heptane the reaction didn't take place. *t*-BuOH, also part of a biphasic system created the polar environment necessary for the reaction to take place, a fact supported also by the results with MTBE as an organic phase. Surprisingly, the biphasic system [HMIM] CF_3SO_3/ *t*-BuOH didn't generate any product, despite the fact that the IL solubilized completely the vitamin C and the organic solvent alone was able to host the reaction with good yields. Also the attempt to generate product using ILs with a more hydrophobic substituent on the cation like in the [OMIM] PF_6/ *t*-BuOH pair failed and the product partition towards the organic phase deteriorated.

[EMIM] $N(CN)_2$ and [OMIM] CF_3SO_3 systems with *t*-BuOH are missing from the graph due to the formation of a monophasic mixture. The area percent was used to express the obtained values in order to avoid any possible errors that could occur due to incomplete solvation of vitamin C in the corresponding IL. The possibility of using the biphasic system for the transesterification of ascorbic acid, without the aid of DMSO with a very good partition of the product in the organic phase was proven, even though the yields were reduced with about 20% in comparison to the immobilized form of CaL B in the same reaction conditions. Once the enzyme could be immobilized directly on the column material, as a transfer towards the SILP technology may be the efficiency of the process could be improved and surpass the existing methods.

3.4.2 Enzyme labelling and binding on the column material

The column material is a mesoporous monolithic hybrid cellulose-2.5-acetate polymer and was prepared by our cooperation partner at the Institute of Polymer Chemistry at University of Stuttgart. As a prerequisite step, the column provided in a dry form was immersed in several ILs that filled the porous structure and swelled the material to the initial form. This behavior instilled the idea of dispersing the catalyst in the pores by simply mixing the enzyme with the particular IL before loading it on the column. The swelling process will be simultaneously doubled by the distribution of the enzyme on the entire surface of the monolith for an improved column efficiency. The layer of IL, present on the material surface wasn't affected by the several washes performed with organic solvent.

A common way to monitor if the immobilization on a support material was successful is to label the enzyme with a fluorophore that could be easily visualized using fluorescence microscopy. Fluorescamine, a colorless reagent was chosen for the labelling due to its ability to react with primary amine on the surface of the enzyme generating a highly fluorescent compound visible at λ_{em} = 460 nm. The reaction that targets the primary amino groups present in the terminal amino acids and fluorescamine leads to a pyrrolinone type of structure (Scheme 13).



Fluorescamine **Fluorophore**

Scheme 13. Reaction between fluorescamine and primary amines generates a highly fluorescent compound.

One of the big advantages of this particular fluorophore is that after binding the solution turns yellow, as a proof that the reaction took place (fluorescamine is yellow only in an active form in normal light). The lyophilized form of the lipase was labeled in a phosphate buffer, pH 7 at room temperature followed by several washes. Fluorescent spots were detected once the sample was investigated using the fluorescent microscope confirming the labeling (Figure 35).

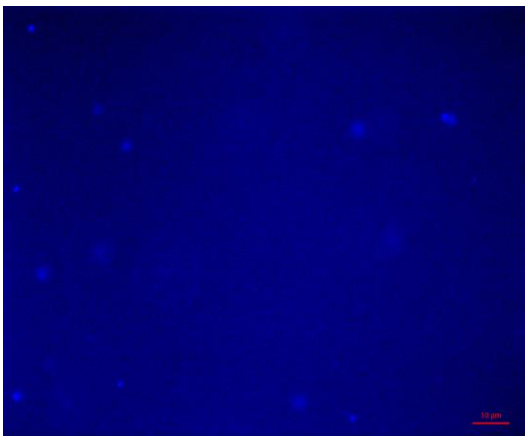


Figure 35. Lyophilized form of *Candida antarctica* lipase B labelled with fluorescamine visualized using a Cell Observer.Z1 confocal microscope from Zeiss at λ_{ex} = 390 nm, λ_{em} = 460 nm (20x magnification).

The knowledge accumulated was compiled to generate a column preparation flow: the lyophilized enzyme was labelled with fluorescamine as a first step, followed by the activation with oleic acid. This form of enzyme was mixed with the IL and loaded onto the polymeric column. The swelling step was accomplished overnight and completed by multiple washes with organic solvent. The amount of biocatalyst was determined by subtracting from the initial volume of IL, with a known amount of enzyme, the residual volume left after swelling and washes. The enzymatic stability and activity in ILs were previously tested and confirmed.

Several areas of the material were examined using the fluorescence microscope under the conditions already described.

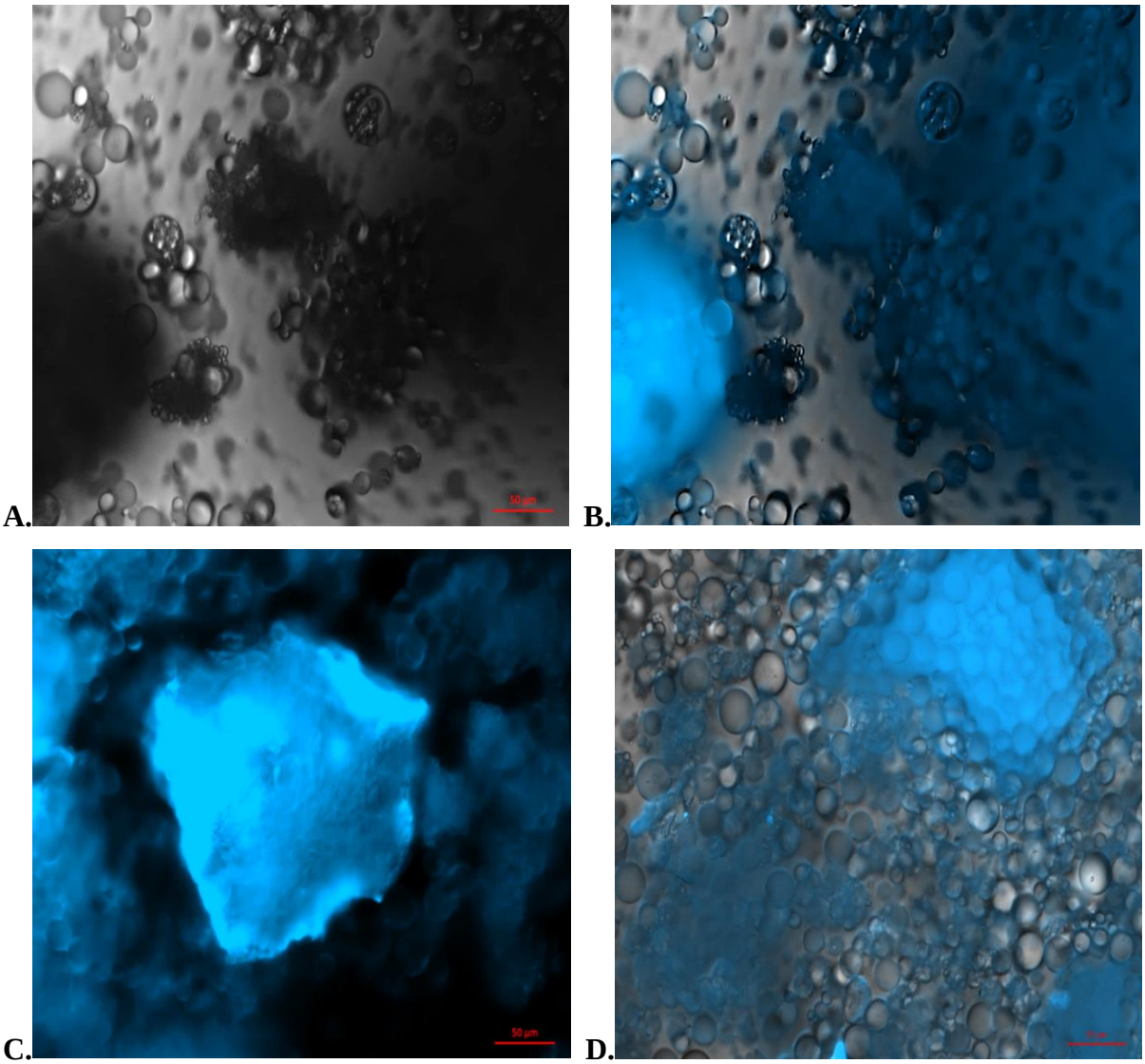


Figure 36. Fluorescent microscopy trial: comparison of transmitted (A) and fluorescence (B) light images of column material with bond enzyme, labelled with fluorescamine (λex = 390nm, λem = 460nm). Further examples of the same material under fluorescence light (C) and (D).

As shown in Figure 36, the enzyme was detected in the monolith structure when excited. The column material could be observed in transmitted light (A) and becomes fluorescent once it is illuminated (B). The other photos (C) and (D), which are the fluorescent and the normal light images overlapped further exemplified the entrapment of the enzyme in the monolith. Once the column pores were filled with the activated enzyme we could state that the preparative steps for performing reactions using SILP technology were fulfilled. The transesterification of vitamin C under these conditions were pursued by our cooperation partner in their facility.

4. Discussions

4.1 Enzyme activation and stability

In the following experiments, CaL B was used in a lyophilized form after an activation step, using oleic acid that mimics a hydrophobic carrier.[156] The active conformation will be preserved as an imprint during the lyophilisation process, allowing the substrates to access the catalytic pocket.[147,148] The enzyme was resuspended in the particular ionic liquids, as a following step generating a suspension, which doesn't alter the enzymatic activity and creates one of the layers of the two-phase system. [149,150,151,33] This particular form was chosen to match later on the requirements for a continuous reactor with a small pore type of support material. The ionic liquid containing the active lipase was applied to the column, as a thin film, coating the large contact surface to enhance the mass transfer along the column material.[142]

It is unanimously accepted that lipases, and moreover *Candida antarctica* lipase B have increased catalytic activity in an immobilized form, idea supported also by the present work.[157,107] Despite this clear advantage, the commercially available systems were not compatible with the actual targeted column design. The immobilization step, in the format of this project is an entrapment of the enzyme that takes place directly in the porous structure of the monolith when comes in direct contact with the enzyme/ionic liquid suspension. So, the activation of the enzyme should be performed prior to this step as described in paragraph 2.2.1 in "Methods".

The contact with a hydrophobic component, like oleic acid sets CaL B in "ready-to" form that will be preserved during the lyophilization step. If we do an analogy with the "molecular memory" effect described by Klibanov we may say that this is a similar situation, once the lyophilized lipase was resuspended in the non-aqueous biphasic system.[147] Wherever this activation phase is the result of a lid opening or not, is another issue, center of a long list of publications.[15,158,159,160] CaL B, a particular lipase that has no significant homology to the enzymes in the same group is a globular protein with a molecular mass of 33 kDa, only seven ß-sheet strands (last six parallel) and two helixes (α5 and α10) surrounding the active site. In a very informative paper, tackling the structure and the direct implications deriving from it, a presumptive lid formed by the two helixes is described with a resolution of 1.55 Å and 2.1 Å.[161] Despite the fact that they are flexible regions, a closed form of CaL B could not be related to their conformational changes and so the enzyme is placed at the boundary between

esterases and true interfacial activated lipase. Twenty one years later and with a 0.64 Å, respectively 1.19 Å drop in resolution it was shown that the interfacial activation shouldn't be correlated only to the presence of a lid, but rather to a more convoluted interaction between the amino acid composition of the lid regions and the dielectric value or ionic strength of the media.[162] If the contact with oleic acid in the present work guarantees the necessary conformational changes from a "molecular memory" point of view or from further interactions could not be defined (Figure 8). But definitely the case of transesterification of 1-phenylethanol with vinyl decanoate in the biphasic system [OMIM] PF_6/*n*-decane question the complex interactions between the structure of the enzyme, substrate properties and reaction media. When H_2O was introduced in the mixture, again the same idea could explain the behavior of the enzyme: for hydrophobic acyl donor the "matching" equilibrium was disturbed generating poor results, while for shorter vinyl esters the activation was influenced only by the presence of an anion PF_6 present in two ionic liquids and in the same organic solvent *n*-heptane (Figure 9).

The free enzyme activated or simply "marked" by the presence of oleic acid replaced the standard immobilized form of the lipase and was further mixed with the ionic liquids. The form in which the enzyme is presented in the ionic liquid, dissolved or as a suspension is not defining its catalytic capabilities. It was shown by other groups that the activity is preserved also as a powder within the ionic liquid.[33,104]

The stability of the enzymes in ionic liquids is probably one of the first issues examined as a potential step forward in the development of enzymatic catalysis. Substantial increase in synthetic activity was showed by CaL B in ionic liquids in comparison with molecular solvents.[104, 107,163] A clear cut of the direct influence was not defined, but it is believed that a more compact conformation of the enzyme is preserved. Hydrogen bonds and H_2O removal (in case of the ionic liquids that are miscible with H_2O) are other hypothesis regarded to contribute to the stability of the enzyme. Hydrophobic ionic liquids are believed, by some authors to decrease the stability of the enzyme, which wasn't confirmed by our results.[86]

Outlook

Prospectively, the non-activated form of the enzyme should be tested in ionic liquids with different alkyl chains in combination with multiple vinyl esters for comparison. In parallel, MTBE as a polar solvent should be involved in the test beside the hydrophobic solvent.

The stability of the enzyme in particular ionic liquids has to be investigated in a way that isolates this issue from the other aspects involved in the reaction, like solubility of the substrate and interactions with the solvent, impact of anion/cation. Correlating the data with CD spectra and may be with crystal structures in those particular ionic liquids for a more systematic approach would give a full inside of the matter.

4.2 Ionic liquids in biphasic systems

Lipases are stable and active in ionic liquids. It was shown that lipases could accomplish catalytic tasks in organic solvents. Lipases are interfacial active enzymes. Why not biphasic systems? The future of the biphasic systems was somehow foreseen in 2003 by the team of Rantwijk, Lau and Sheldon when they stated that "*the characteristic property of some ionic liquids to mix neither with water, nor with moderately non-polar organic solvents will revolutionize process design*".[149] Most of the attempts tried to enhance the performance of the process, from a technological point of view. Probably, supercritical carbon dioxide ($scCO_2$) was the most popular partner for ionic liquids, fulfilling the role of a carrier for continuous or batch wise flow processes and SILP approach as it's later on in detailed described.[120,164,165] This way the reaction and the separation of the products could be integrated in one step.[166,167] Liquid-liquid systems have made only timid pursuits to bring in technological solutions, water, acetone and *n*-heptane being the system partners.[87,125,126] The assets of such systems are the main theme of this project. One way to maximize the outcome is to modulate the microenvironment created by the pair ionic liquid/organic solvent for the reaction participants. As a novelty, matching a particular ionic liquid with a molecular solvent that cohere with the properties of the substrate/acyl donor to give a certain direction of the reaction was tested.

4.2.1 Partition in biphasic systems

A biphasic system means that two different substances, in our case two liquids defined by different properties are immiscible at equilibrium. Having distinctive and divergent characteristics the two components will be favored differently by a solute dissolved in the system. The partition of the substrate in the bilayers is expected to be unlike the product, which is defined by other physical chemical properties. This phenomenon could be exploited in connection with the diverse range of ionic liquids that are built on two ions that could be mixed and matched.

Partition, despite the fact that is a parameter that influences any extraction as part of any bio enzymatic catalysis, either from aqueous phases or ionic liquids is not a topic that is often discussed. Martinelle and Hult in 1995 measured the partition coefficients of their substrate and product in a biphasic mixture containing heptane and acetonitrile by analyzing both phases on GC.[168] Later on, Feher *et al*. calculated the distribution of their components in a biphasic system, this time involving an ionic liquid and moreover the partial solubility of the organic solvent in the particular ionic liquid.[169] In our case, due to the high amount of samples and also because of the impossibility to accurately measure the small variations in volume of the two phases, this behavior was considered constant along all experiments.

The primary factor driving the partition is the polarity of the solute that will migrate according to the properties of the solvents. The results clearly proved the strong connection and as a highlight the possibility to influence the outcome of this process by a careful selection of the hosting solvent pair. 1-Phenylethanol the most polar compound was completely shifted towards the polar ionic liquid when hydrophobic organic solvents were used. Once cyclohexane and *n*-heptane were interchanged with MTBE, known as a polar solvent, the outcome was substantially changed and more substrate was found in the organic phase. As a proof of concept 1-methylpiperazine, a structure presenting a secondary amine beside a tertiary one, less polar than primary ones and far less polar than an alcohol, changed the picture favoring to a higher degree the hydrophobic solvent (between 33 to 37%). Still, MTBE associated with the same ionic liquids was able to create the right medium for the amine. One these results were revealed, the partition of the product was questioned not only as a singular compound, but in the reaction context versus the substrates. The investigated reactions generated esters and amide that are characterized by different polarities: the first group less than alcohols and amides on the contrary. 1-Phenylethyl acetate was steered towards the organic phase in a higher percent (67%), once the MTBE was paired with [BMIM] OTf compared to the same ionic liquid mixed with *n*-heptane.

These promising results shaped the later ideas of technically favorable product separation that is the main interest of this research project.

Outlook

The list of ionic liquid has tremendously expended, but their characterization didn’t keep up the same paste. At this point, the physico chemical properties that define the ionic liquids would help the scientific world for a better understanding and usage in enzymatic reactions.

As pointed out, the availability of polarity values is a necessary condition and the only investment needed to create a database is the time for the measurements. Spectrophotometric methods using Reichardt's dye are generally used to generate this type of data.[93,92]

4.2.2 Tuning product partition in biphasic systems

In order to create a conclusive and representative data, a set of acyl donors with increasing hydrophobicity from vinyl butyrate to decanoate, as well as organic solvents and ionic liquids having the same tendency were selected. As solvents were used MTBE, *n*-heptane and *n*-decane associated with PF_6 based ionic liquids which have different alkyl chain cations, like [EMIM], [BMIM], [HMIM] and [OMIM]. The systems were set to cover all possible combinations of molecular solvents and ionic liquids for the reaction of 1-phenylethanol with the three acyl donors (Figure 12). The esters formed are less polar than the corresponding alcohol. The alkyl chains on the esters will influence the changes in polarity, implicit the behavior in the two phase mixtures. The whole set up targeted a certain type of separation: when the polarity of the organic solvent is the best match to the one held by the product and the ionic liquid exhibiting the exact opposite one, the product formation should be driven towards the organic solvent. An example of the variations brought in by all the participants in the reaction is schematically represented in Figure 37. Somehow, this was a theoretical arrangement, which hasn't always reflected the reality in terms of polarity. For butyrate derivatives, it was predicted to have a maximum partition and may be yield, for the heterogeneous system with a less hydrophobic organic solvent and an ionic liquid with longer chain substituents on the cation. The results are in a reasonable forecasted range, with best results for MTBE / [HMIM] PF_6 and a bit surprising for *n*-heptane / [BMIM] PF_6. Polarity is not the only determining factor or may be our predicted values are not always true. Despite that, a nice trend in terms of yields was noticed for the first two groups. On the contrary, for decanoate, the most hydrophobic organic solvent should host the product when paired with the shortest alkyl chain ionic liquid. In this case a more important role was definitely given to the ionic liquid side, with best results for [BMIM] and [HMIM]. As should be, the results for hexanoate products are placed in the middle range with one result for MTBE / [HMIM] PF_6 exceeding the expectations. Beside the C_6 substituent on the cation, *n*-heptane was able to generate good yields and to favor the partition of the product.

Once the hydrophobicity of the system components is a bit far away from recommended values then the system fails. The whole ensemble should match the polarity requirements, but

still create the right microenvironment for the reaction to take place. Here, we could not neglect the capabilities of the organic solvent itself on independent grounds.

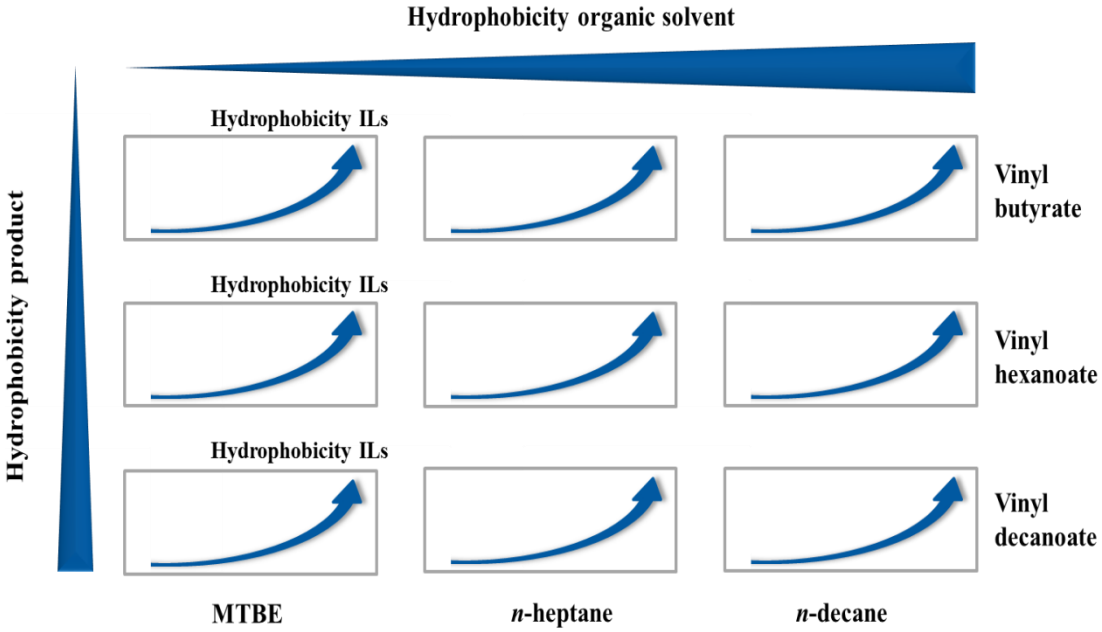


Figure 37 Schematic representation of hydrophobicity variation along a row (stands for organic solvents), along a column (stands for product) and within a box (stands for ionic liquids).

Having the results rearranged, with the ionic liquid as a reference and the solvent variable a different perspective is uncovered, giving more credit to the role of the ionic liquid in the design of the reaction (Figure 13). [BMIM] and [HMIM] cations were the best match for the products with vinyl butyrate respectively hexanoate, while the last acyl donor favored more or less both of them. *n*-Heptane, part of the heterogeneous system favors the reactions and partition, but not as a general all-round agent. While associated with [BMIM] PF_6 for the shortest acyl donor, produced the best result that wasn't replicated with [HMIM] PF_6. Although, this particular ionic liquid gave better results in the presence of MTBE and *n*-decane. These type of changes are noticeable also for the other products. So, the system has to be optimized for each reaction.

Once the physical properties of the product changed (the esters were replaced by the more polar amides) the impact on the partition is clearly reflected in the results Figure 14. The amides with shorter substituents (the case of butyrate) were almost completely driven in the direction of ionic liquids. Gradually, the polarity change (may be to higher degree than for the esters) forced the product to build up in the molecular solvent, until partition was

completely reversed for decanoate. For this particular case, the fact that no product was generated in MTBE is reflecting the switch in properties.

The weight of the relation polarity product *vs*. medium could not have been better wrapped-up than by the final comparison between the two reactions in the presence of vinyl decanoate (Figure 15). Opposite characteristics determine opposite outcomes.

Tuning the two components of a biphasic system, in a strong correlation with the product could lead to an improvement in the separation of the compounds of interest with technological applications.

Outlook

Choosing the solvents should be done in a way that describes a more even polarity increase among the selection. For example, between MTBE and *n*-decane there is a difference of 2.6 in polarity index, while between *n*-decane and *n*-heptane is only 0.3 (polarity index Interchim). The alkyl chain of the vinyl ester could have had, as starting point the shortest member of the series, vinyl acetate. Once the polarities of the ionic liquids would be available and also for the products, the empirical design could be replaced by solid predictions.

4.2.3 Cation/anion impact on enzymatic reactions

One of the biggest advantages of the "designer" solvents is the mobility in terms of combining the two structural components, according to the demands of a particular reaction. In terms of understanding the impact of such combinations, the things could get relatively complicated and this advantage is more speculated than understood. Once we look to the effects of the ionic liquids on a reaction, as a whole we could judge in a more simplistic way if the conversion or yield are enhanced. Though, all the participants involved in this fine and complex process have their own contribution, so could not be judged only on the basis of simple interactions with one partner, but rather described as a spider web. Where could we place each component in this little network is a relatively complex decision.

We have seen that CaL B is stable in ionic liquids with implications in the synthetic activity. The myth of polar solvents that generally inactivate enzymes was abolished.[104,170] The polarity of the ionic liquids that was initially accounted for the behavior of enzymes in this medium was usually evaluated, from all the methods already presented using solvatochromic scales, like Kamlet-Taft and E_T^N. Contradictory results from these scales, placed the ionic liquids in the range of lower alcohols or under and are defined by the H-bond basicity of the

cation, respectively by the coordination strength (nucleophilicity) of the anion.[93,105] Dielectric constants, keto-enol equilibrium, Hildebrand solubility and simple chemical predictions, also used to evaluate the polarity have not brought more light in the bioenzymatic catalysis. All these differences are structural related and so divers because of the charge distribution on both ions as presented in the previous chapter. The electrostatic interactions between the cation and anion, their size, the possible coordination are responsible for the polarity on one side, but they could change the rule of the game in the presence of an enzyme. One more example is the imidazolium type of ionic liquids that they possess in C(2) position a weak acidic hydrogen atom that could drop the polarity from ETN ~ 0.53 – 0.75 to ETN ~ 0.50 – 0.56 and could be responsible for the oligomerization of acetaldehyde in the enzymatic transesterification reactions.[92,133]

Hydrophobicity, quantified by log P follows in an effort to explain the interactions with the enzymes. It is universally accepted that immobilized CaL B has an increased activity with higher values of log P, in the presence of imidazolium and pyridinium based ionic liquids reaching a maximum for [EPy] NTf_2 (log P -2.57). Values higher than this lead to a decrease. In all our test reactions [BMIM] BF_4, considered hydrophilic have showed comparable results with [BMIM] PF_6 that is hydrophobic. [MMIM] $MeSO_4$ is somehow an oddity, as a hydrophilic ionic liquid that generated high enzymatic activity showed by other authors, but in our case inhibited the product formation (Figure 16). $MeSO_4$ as an anion was found responsible for the inactivation of CaL B in the transesterification of 1-phenylethanol in the work of Schöfer *et al*.[117] Generally, the ionic liquids having the imidazolium as cation found ionic partners able to generate good yields, with $N(CN)_2$ and $MeSO_4$ listed as exceptions. The cations with longer alkyl chain substituents are thought to block the non-polar active site of the enzyme, decreasing the activity, idea that was not supported by our findings.[171] Overall, the presence of the ionic liquids as a second phase increased the enzyme activity for the transesterification of 1-phenylethanol with the vinyl esters.

Once we extended the study to *α*-methylbenzylamine and ethyl methoxyacetate the situation changed, in the sense that the benefit of using biphasic systems wasn't proved anymore, except a few cases ([HMIM] OTf, [OMIM] NTf_2, [BMPy] PF_6). The reaction could be studied from the cation/anion perspective, but to overcome the high yields obtained in the organic solvent only was a hard task. MTBE, for the reaction itself including the solubility of the substrate, acyl donor and product is suitable and hard to overrule. Probably is unfair or risky to judge only for one organic solvent. As it was shown, other biphasic systems despite

the fact that they used organic solvent that weren't considered as first choices, delivered better results.

An overall comparison of the results with the existing literature is not straightforward. On one side, the ionic liquids tested don't overlap with the ones used in this project. The presence and influence of the organic solvent could not be estimated and the differences in the nature of the product add-on to the unknown.

A particular enzyme group or moreover a particular enzyme in that group, their tridimensional structure with the characteristic outer surface, their type of catalytic site and position, the presence of a lid or not are completely different aspects with undeniable significance for the synergy with the microenvironment, including the ionic liquids. Which element of the liquid salts has the most impact or how the whole molecule will interact with the enzyme has to be determined on single basis with a stronger background knowledge about the ionic liquids.[93,105] The general statements that were made earlier like "*enzymes are stable or not in ionic liquids*" should be avoided if not abolished. More and more voices in the scientific community started to refer to this aspect.[86,172]

Outlook

The assignment of solving the cation/anion influence on the reaction and its components, at experimental level has to be planned as a step-by-step approach. For the same enzyme, substrate and different types of acyl donors a more comprehensive set of ionic liquids combined with multiple organic solvents should be tested. That could be possible in the near future, taking in consideration the efforts of the ionic liquid manufacturers to extend the product choices at lower costs. Furthermore, for the same enzyme, other substrates should be submitted using the same biphasic systems. And interestingly enough would be, for a certain group of substrates to test enzymes from other microorganisms. Once a general overview could be outlined, the possible correlation with the physical chemical properties should be estimated.

4.2.4 Other factors influencing the reaction outcome

Parameters like temperature, amount of ionic liquid, beside the nature of the organic solvent could not be neglected and they were analyzed more from the perspective of yield increasing.

The role of the molecular solvents was already described and the extension of the reaction on a larger number of ionic liquids did not alter their impact. *n*-Heptane was in most cases the preferred candidate, boosting both the partition and yield.

Temperature, as a reaction parameter is normally explored for the improvement of the conversion. To add-on, in case of ionic liquids their viscosity and wide temperature tolerance requires and respectively, allows further experiments. The viscous form displayed by many ionic liquids raised often concerns related to mass transfer limitations.[103] Based on these factors, the temperature was increased from 37°C to 50°C and 70°C for the test reaction of 1-phenylethanol with the vinyl esters butyrate, hexanoate and decanoate.

According to these results, the performance of the enzyme was preserved at various temperatures and no impact on the substrate was noticed, which is in consensus with other authors for the same enzyme.[173] When the viscosity values were compared to the results, there was no relation between them and the product formation at any particular temperature.[93] For example another enzyme, penicillin G amidase wasn't influenced by the viscosities of [BMIM] PF_6 and [BMIM] BF_4 that hosted an amidation reaction.[174] In other cases, like the reaction catalyzed by *α*-chymotrypsin, the high viscosity of the ionic liquid (574 cP) determined a reduction of the enzymatic activity.[175] The relative small number of ionic liquids tested by the other authors could not be used as a representative batch for comparison. The reaction outcome in the current trials could be rather explained by the interactions between the corresponding ionic liquid and the acyl donor or product at the given temperature than a viscosity issue.

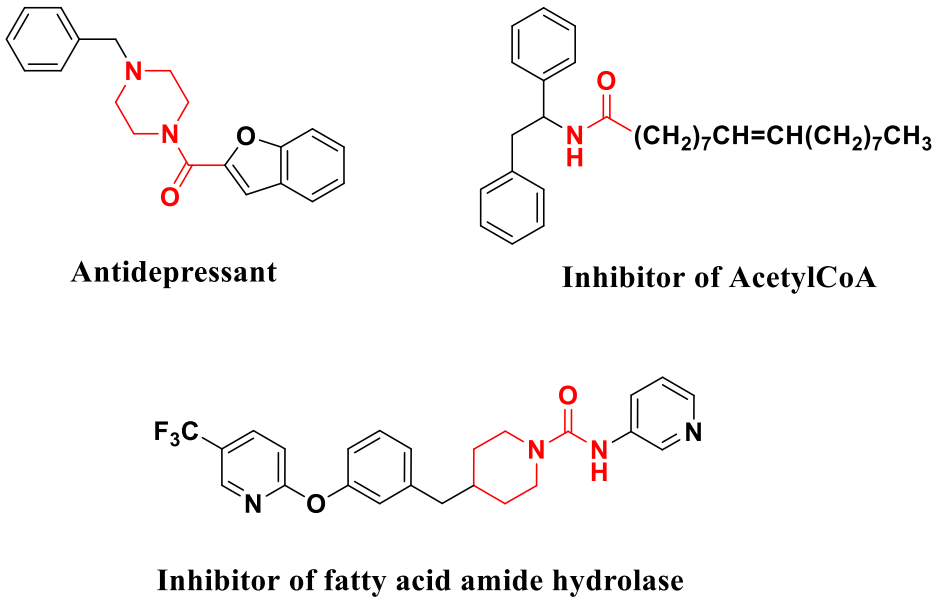
In terms of amount of ionic liquids the trend was somehow channeled into two clear directions, either the reaction was taking place only in pure ionic liquid or there were added to the reaction mass in small amounts.[157] For example in a study conducted by Ventura *et al*. up to 60mM of ionic liquids caused a decrease in the enzymatic activity of CaL B, defined by the capability to hydrolyze 1 μmol of *p*-nitrophenyl laurate per min.[171] In the present research, the impact was related more to the reaction itself, rather than defining a general trend. Only for *α*-methylbenzylamine the reduction of the ionic liquid amount to 25% generated for the [BMIM] based salts an increase in yields (Figure 21).

Outlook

Despite the few successful cases, each reaction has to be tested on individual basis for both temperature and amount of ionic liquid. Any correlation between these factors and the structure of the ionic liquid, would be beneficial. Viscosity, up to present time wasn't directly bound to any drawbacks despite all the speculations. The values for this property are available for most of the ionic liquids and a more thorough work should be established.

4.3 Potential new reactions for pharma industry

Amides beside carbamates and ureas are used as intermediates for natural products synthesis, enzyme inhibitors or building blocks for a large group of drugs as briefly exemplified in Scheme 14.[176,177,178,179,180]



Scheme 14. Examples of amides and ureas used in organic synthesis for medical treatment (antidepressives and inhibitors of different human enzymes involved in important biological pathways).[176,178,180]

Just to make more relevant the magnitude of amides involvement in current pharmaceutical industry, we have to refer to the Comprehensive Medicinal Chemistry (CMC) database, which includes 1752 compounds with carboxamide functionality, out of a 6454 total entries.

Despite the importance of the enantiomers and the factors that are involved in this reactions the focus of this thesis was channeled towards the possibility of synthesizing new compounds and better separations in the reactions with high conversions.

The *N*-acylation of amines for the synthesis of amides on industrial scale involves acyl chlorides, anhydrates or carboxylic acids, catalyzers and solvents.[181,182] There is a constant effort directed towards the improvement of these multistep processes that require toxic and corrosive reagents, purification of intermediates and occasionally building undesired by-products. Chemistry and biotechnology are looking for their own solutions.[182] Or maybe the future shapes a common path as in the one-pot chemoenzymatic aminolysis of propargyl amides, where CaL B catalyzed the aminolysis of carboxylates associated later on with a Sonogashira coupling to (hetero)aryliodides.[183]

In this frame, the capability of lipases was stretched to new substrates that might be considered as potential intermediates. That includes piperazine and piperidine rings as well as phenylurea. For the first group was chosen 1-methylpiperazine that has one of the amino functions further alkylated with a rather small substituent. The methyl group was then substitute with a larger moiety, phenyl to test how bulky substrates fit in the binding pocket and their input at the electronic level.[184] The idea was extended to 1-methyl-3-phenylpiperazine that has the phenyl in the close proximity to the targeted amino group and the possible steric hindrance was in focus. Following the same idea, piperidine itself was investigated along with methyl substituents in 2nd or 3rd positions and carboxamide in the 4th. As predicted no product was determined for 1-methyl-3-phenylpiperazine in all the attempts. With a different substituent in the α position CaL A, known to have a different configuration of the binding pockets was able to catalyze the kinetic resolution of piperazine-2-carboxylic acid.[185] If we remain to the same topic, it was shown that CaL B could accepted similar substrates, like for the synthesis of (-)-Paroxetime, an antidepressant.[81] In this case the target was a primary alcohol in the 2nd position of a pyridine ring ((4-(4-fluorophenyl)-1-Boc-piperidin-3-yl)methanol involved in an acylation reaction while the amine group was protected. Moreover, its remarkable how CaL B in a transesterification reaction for the production of (S)-(+)-Citalopram, a drug in the same category, was able to acetylate the primary benzylic alcohol at the quaternary chiral center. Our experiments confirmed also the ability to catalyze bulky substrates as long as the accessibility to the desired moiety was not blocked by a substituent in the proximal vicinity. 1-Phenylpiperazine was acylated using different acyl donors. Unfortunately the substituted piperidine, independently of the size and position of the substituent haven't deliver the expected results. 1-Phenylurea generated yields under 5% and so, was omitted from further studies, more from a lack of time for the development, rather than potential or interest. One more comment related to the piperidine outcome has to be done: some limitations were unfortunately connected to the analytics, more

precisely to the chromatographic conditions, where the detection mode failed to uncover the products lacking chromophores (as per piperidine compounds). The substrate spectrum would have been enlarged, if this could have been improved. The usage of GC was a more convenient analysis method for the organic phases but it wasn't meant for the ionic liquid phases.

The selection of the acyl donor, as part of the system, has to be tackled from the reactivity perspective, influence on the reaction equilibrium and by-products formed, especially when amines are involved.[186] Gotor, in a review related to amides and carbamates formation in the presence of lipases, pointed out the importance of the choice.[187] The reversibility of the reactions could be avoided using acyl donors that do not generate H_2O like activated esters, enol and vinyl esters, thioesters or oxime esters. However, when the employed substrates are amines, identified by a higher nucleophilicity in comparison to the analogous alcohols, there is a high probability that the reaction will take place in the absence of the enzyme. Fact that was unfortunately confirmed in the set of reactions between *α*-methylbenzylamine or 1-methylpiperazine with vinyl esters in organic solvent. Interestingly enough, the reaction with the same acyl donors and 1-phenylpiperazine didn't occur neither spontaneously, nor catalyzed by the lipase.

Vinyl esters, add on to the complexity of the reactions due to the formation of by-products with implications on the outcome. They are preferred to alkyl ones because they are more reactive and could generate better yields in most cases.[188] Along with the desired product, an unstable enol is generated that will form through tautomerization an aldehyde, in this case acetaldehyde and the reaction becomes irreversible. Normally the aldehyde could be eliminated from the reaction mass under vacuum which facilitates the formation of a pure product. When amines were used as substrates, a brown color was developed due to formation of by-products. It was described in literature the aldehydes form a Schiff's base with the lysine residues on the enzyme, the so called browning-type reaction and in some cases could lead to the deactivation of the lipase.[189] CaL B though, is one of the few enzymes that will tolerate the presence of the aldehyde and further catalyze the reactions. Despite the frequent usage of the vinyl esters, the problem is not often described in literature. Another possibility is the polycondensation of the aldehydes, but doesn't explain the lack of color formation in case of alcohol transesterification that leads again to the reaction of the aldehyde with the amine. The mechanism of aminolysis supports the idea through a succession of events: the tetrahedral intermediate is releasing the enol that is simultaneous generating the aldehyde, while the amine is available unreacted in the medium. For the reactions taking

place in ionic liquids containing imidazolium based cations the oligomerization of the acetaldehyde is hypothetically possible due to the presence of an acidic hydrogen in the 2-position of the ring.[133,170] Finally, all the roads lead to the direct interaction of the amines and aldehydes with imine formation. Negative controls containing the enzyme and the vinyl ester in organic solvent didn't develop any color. The color formation was initiated by the addition of the amine.

In an attempt to avoid all these consequences, the vinyl esters were replaced by other acyl donors more or less successful (Table 4). For group B, the same product (acetamide) is to be formed as a common ground and easiness to compare. The new compounds were thought to release by-products that do not further interfere with the outcome of the reaction or that could somehow attenuate it, being less reactive (acetone in comparison to acetaldehyde). For ethyl 3-phenylpropanoate and ethyl cinnamate no products were detected, while isopropenyl acetate reacted without the aim of the enzyme in the presence of *α*-methylbenzylamine. Benzyl-, butyl-, isopropyl- and *tert*-butyl acetates led to product formation in MTBE at 37°C, but the focus was maintained only on the first three acyl donors with better yields.

Following the project rational, the reactions were transferred to biphasic systems to fulfill a secondary trend, impact of ionic liquids in the amidation reactions.

The screening was again pursued with *α*-methylbenzylamine in several ionic liquids mixed with MTBE as a second phase. The best results were given by benzyl acetate that in all cases registered higher yields than in organic solvent only, while all other system were under 5% total yield. One downfall related to this acyl donor is the formation of benzyl alcohol due to the enzyme affinity to this ester as a substrate. The suppression of this side-reaction, a hydrolysis could be done by controlling the water content in the reaction mass. Ethyl methoxyacetate (that was previously tested and comes in concordance with other authors' work) and benzyl acetate were preserved as the selected substrates for the next experiments in biphasic systems.

The amidation of *α*-methylbenzylamine with ethyl methoxyacetate was already discussed but it was included for comparison with benzyl acetate. [BMIM] $N(CN)_2$ as an ionic liquid was the best choice for both reaction when was mixed with the hydrophobic organic solvents, cyclohexane giving higher yields for benzyl acetate and *n*-heptane for ethyl methoxyacetate. Partition was somehow constant under 5% for the second acyl donor.

For 1-methylpiperazine the reaction in biphasic systems surpassed all three organic solvents MTBE, cyclohexane and *n*-heptane used as references, but with yields under 11%. Only two systems, [BMIM] $N(CN)_2$/MTBE and [OMIM] CF_3SO_3/*n*-heptane were characterized by a

good partition towards the organic phase. The situation changed drastically when the second acyl donor was used: MTBE was able to provide the right environment and generate a way better yield than all other systems, including the ones with the same solvent paired with ionic liquids.

When we move on to 1-phenylpiperazine the yields for both acyl donors are recording a significant increase. Again, MTBE prove to be a better solvent for the reaction hosting benzyl acetate, followed by *t*-BuOH. The success of the reaction between 1-phenylpiperazine and ethyl methoxyacetate was based on [BMIM] ionic liquids only, when they were mixed with MTBE and almost 50% partition in this solvent when NTf_2 was the selected anion.

Because of product detection issues, in case of ethyl methoxyacetate, piperidine was evaluated only in the presence of benzyl acetate, with results that incline, without doubt towards MTBE alone as a single phase. Despite a general good partition, the yields were under 5%.

Outlook

The introspection of enantioselective processes in ionic liquids could be a valuable task if we consider asking:

- could the ionic liquids improve the enantioselectivity and to what extend;
- is it possible to drive the reaction towards a desired enantiomer;
- is it feasible to involve ionic liquids in dynamic kinetic resolution, while chemoenzymatic processes are in place.[186]

4.4 Industrial applications of Vitamin C and its derivatives

Ascorbyl fatty acids esters are widely accepted as natural antioxidants or surfactants in food industry, cosmetics etc. Currently, their chemical synthesis is a straight forward process based on the reaction of vitamin C with sulfuric acid followed by the esterification with palmitic or stearic acid.[72] Technological processes involving strong acids or purification steps with large amount of volatile organic solvents are constantly revised for improvement or for alternative solutions. Enzymatic methods are available since 1971 when first synthesis in aqueous solution was reported and in organic solvents in 1990.[74]

Already at the end of this study, the scrutiny of the lipases in the synthesis of ascorbic esters as a display of their high regioselectivity follows naturally. From a large pool of lipases tested by the scientific community, *Candida antarctica* lipase B was the favorite, fact proved by our experiments, as shown in Figure 30.[190,191] The association of the enzyme in the active form with the ionic liquid prior loading on the porous material proved to be beneficial for the technological development of the reaction in a column. Once the enzyme is uniformly dispersed in the porous support material in a form that allows a quick mass transfer the column preparation step was achieved. The mesh of ionic liquid containing the enzyme was visualized using the fluorophore labelling technique (Figure 36).

When the acyl donors were evaluated, the choices in terms of type and length varied, including palmitic, oleic, linoleic, linolenic acids, methyl and vinyl esters, just to mention a few of them. The activated acyl donors like vinyl palmitate will deliver higher rates and yields, which overlaps our results (Figure 31), with the only concern related to water amount present in the reaction mass, which could easily reverse the reaction. In all our experiments, the reagents were dried or purchased in an anhydrous form, accompanied by molecular sieves. The awareness of this problem initiated investigations having the focus on the effect of molecular sieves and water activity associated with the synthesis of vitamin C esters.[192]

After a few molecular solvents, like *t*-amyl alcohol, *t*-BuOH or acetone were tested with more or less success, the ionic liquids were explored for this particular reaction.[72,191,193] In our case the biphasic systems based on organic solvents mixed with ionic liquids were appealing, as a good compromise between the solubility issues raised by both the substrate, acyl donor and nevertheless the product, including separation and purification of the final ester. The ionic liquids as polar solvents were initially thought to be the best host for the problematic vitamin C. Unfortunately only a few of them were capable to dissolve the substrate and despite this, the reaction failed to take place in these two phases combinations.

From similar considerations, was not possible to determine the partition coefficient for vitamin C: the substrate is not soluble in most of the polar solvents used. For many biphasic systems were used hydrophobic solvents like *n*-heptane, *n*-decane and cyclohexane able to form biphasic systems, which would not match the physical chemical properties of vitamin C. When the substrate was dissolved in DMSO there was a restriction related to the immiscibility of the DMSO itself with the other organic solvents, so the partition test could not be performed.

Organic solvents like *n*-heptane or *n*-decane (data not shown) were selected in order to find a solution for the acyl donor and product, which should be soluble in the reaction mass. In all the cases using these solvents there was no product detected. One rational reason that has more to do with the way it was designed is the fact that the two components are more or less in separate phases with the enzyme present only in the ionic liquid mass. In this case, the reaction should take place at the interface.

Despite the failure in designing a biphasic system that will increase the yields to the levels in *t*-BuOH, the other task related to the possibility to avoid additional solvents for substrate solubilization was fulfilled. The reaction in the biphasic system [BMIM] PF_6 / *t*-BuOH in the absence of DMSO, generated better results than the case in which vitamin C was previously dissolved in DMSO (Figure 34). The two ionic liquids [EMIM] $N(CN)_2$ and [HMIM] CF_3SO_3 spotted as potential solution for the solubility problems of vitamin C, did not fulfill the further requirements for completing the transesterification. The other ionic liquids [BMIM] PF_6 and [OMIM] PF_6 alone were not considered as favorites at the starting point, but finally mixed with the right organic solvent changed the entire outcome. In terms of yields, our data is comparable or better than other groups that reached 72% using [BMIM] BF_4 in 24 h at 60°C, respectively 65% in 14 h at the same temperature.[191,193] We have to acknowledge, one more time that we focused mainly on developing systems with potential future in the industry, as a principle and the final adjustments to improve the yield (extended time, higher temperature or amount of enzyme) are missing, so the analogy to the others' results are hard to fulfill. For a fair comparison, the immobilized form of the enzyme was introduced in the graph and the increased performance *vs*. free form is also in agreement with the work of Adamczak *et al.*

The fact that certain ionic liquids are able to solubilize completely the substrates is a condition necessary, but not sufficient for the reaction to take place. Though, partition of the product in the organic phase, which is the highlight of such systems was accomplished. Here, the ideal case would have the substrate dissolved and retained in the ionic liquid and the final

product present in the opposite phase. This would confirm our targeted idea as a proof of principle.

Outlook

The knowledge related to the polarities of the ionic liquids would bring more solutions for this type of issues. The right ionic liquid for the right substrate. Following the same theory, a larger pool of organic solvents should be tested in combination with the ionic liquid partner. A more efficient mixing system would be required to overcome the low contact barrier of all reaction components. The development of reactions with similar difficulties should be in focus.

5. Conclusions

The demand of new or more efficient technologies is there. The need of protecting the environment creates in parallel an increasing pressure, making the two paths overlap. Biotechnology follows the trend, or better say takes a leadership in this area delivering green solutions to tedious and highly expensive chemical processes. Cosmetic and pharma industries, only to mention a few of them took advantage of the enzymatic reactions that could catalyze large amounts of raw materials, generating final products at lower prices. Bioengineering find its way to deliver the new generation of enzymes customized for very precise tasks according to the demand. The enzymatic reactions evolved to a different level of understanding that allowed the dissipation of lipases catalytic power, which were named "unfaithful enzymes".[194] May be this characteristic is not a desired one, but the consequences are highly regarded. Ionic liquids made their first timid steps in biotechnology and after about a decade they deliver a joint process, SILP technology.

The focus of the presented research was to create system models for new reactions or existing ones, based on a mixture of an organic solvent and an ionic liquid that will be able to dissolve the compounds involved in the reaction and to promote the catalytic properties of the enzyme. The match between the two immiscible solvents would constrain the formation of the product in the organic phase, while the substrate should be retained (in a perfect design) on the ionic liquid side. The major advantage of the ionic liquid that highlights the flexibility of the system is the large combinatorial choices between the two structural elements, the cation and anion.

This was first noticed when partition preliminary tests were performed on different substrates and their corresponding products. According to the pair of organic solvent/ionic liquid, the differences in polarity of the particular compound alcohol, ester or amide could be used to direct the partition.

For example an alcohol will prefer the polar organic solvent to the hydrophobic one, when the same ionic liquid is used. When an identical scenario was applied to the corresponding ester with a reduced polarity, the partition would reflect the change and a larger amount of compound will be found in the organic solvent. Partition is a valuable factor for enzymatic catalysis that should not be neglected for an accurate evaluation of the results.

Therefore, the idea of tuning the components to enhance the separation capabilities could open new doors in biotransformation reactions. Test reactions of 1-phenylethanol and α-methylbenzylamine with different chain length acyl donors, vinyl butyrate, hexanoate and

decanoate proved the applicability. Three organic solvents MTBE, *n*-heptane and *n*-decane were combined for this purpose with ionic liquids having the same cation with variable alkyl substituents and when possible a constant anion. The mix and match approach increased, in most cases the yields with partitions up to 90% in the organic phase.

The influence of the cations and anions on enzymes and their accomplishments is still a matter of debate. A better understanding of their role would make the reaction and process design more performant, also in the frame of better separation of the products. An attempt to sort the ionic liquids, based on their ionic contribution was performed using the same reactions. The relevance of the results wasn't as expected and a few factors might have been responsible: only one organic solvent was used; the pool of ionic liquids was rather limited and for some cations/anions the comparison was reduced to a very small number of entries; the selection of the products should have a better coverage in terms of physical chemical properties. Despite these limitations there were certain cations and anions that are strong candidates for these reactions and on the other hand combinations that should be avoided. The main conclusion of this part is, with certainty that the evaluation of a particular reaction has to be done on individual basis, along with all the reaction parameters.

In terms of unusual reactions the lipases established new frontiers for substrate spectrum with applicability in pharma industry. Piperazine and piperidine based homologues were screened in organic solvents. The promising reactions were also involved in the development of biphasic systems. The reactivity of the amine was somehow problematic and several other acyl donors were tested. Higher yields, but not always associated with good partitions were achieved.

At last, but not at least the perspective of biphasic systems applied directly to continuous flow technology, found in the transesterification of ascorbic acid a strong candidate for Supported Ionic Liquid Phase (SILP) technology. The capability of such a system to dissolve a polar substrate, along with a hydrophobic acyl donor and generate a soluble product in the reaction mass was demonstrated.

The take home message is that the ionic liquids are potential reaction partners for many processes and applications, regardless the system form. Their interchangeable structure giving them the ability to dissolve, host and drive the partition of all participants is a clear benefit that should shadow their intrinsic drawbacks (high viscosity and costs).

We could not talk about enzymes and their catalytic power in new fields. We could not talk about ionic liquids and their innovative power in various fields. We could not isolate technologies without giving them perspective. But we could build a puzzle that joins all the

pieces together for solutions. So, with full trust in lipases and ionic liquids and their capabilities we could say that the game is open.

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