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Switchable Solvents and Room-Temperature Solid Phase Ionic Liquids in Biocatalysis

MASTER'S THESIS

to achieve the university degree of

Diplom-Ingenieurin

Master's degree programme: Chemical and Pharmaceutical Engineering

submitted to

Graz University of Technology

Supervisor

Dipl.-Ing. Dr.techn. Heidrun Gruber-Wölfler

Institute of Process and Particle Engineering

Graz, 10/2015

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Abstract

The goal of this thesis is the development of alternative technologies for biocatalytic processes to make them more efficient and easier to process. Especially transformations of non-polar substrates in organic solvent, subsequent product recovery and enzyme recycling should be simplified.

A chemical and pharmaceutical industry without biocatalysis - inconceivable in the 21st century! Numerous processes made their way into industry, while some promising applications stuck in the research state. The enzymes stability under process conditions as well as the costly and time consuming product recovery pose two main challenges in biocatalysis. This thesis deals with this problems and shows new technologies which might help overcoming them.

The first half of the thesis deals with the usage of enzymes in organic solvents. The enzymatic amination of 4′-bromoacetophenone using the transaminases ATA-40 in methyl-tert-butyl-ether (MTBE) ran stable over 120 h after coating the enzyme with ionic liquids having a melting point close to room temperature. The cofactor pyridoxal 5′-phosphate (PLP) is protected by the coating and remains active. The reference reaction applying free enzyme and cofactor stops after less than 30 h due to cofactor breakdown.

In the second part of the thesis it is shown that enzymatic reactions can be run in switchable solvents. The polymerization of bovine serum albumin (BSA) using Transglutaminases was run in an amidine/ethanol system possessing interchangeable polarity. Besides the proof of feasibility, potential applications, challenges and benefits of reaction media possessing variable polarity and hydrophilicity are discussed.

Kurzfassung

Das Zeil dieser Arbeit ist die Entwicklung alternativer Technologien für biokatalystische Prozesse, um diese einfacher und effizienter zu gestalten. Besonders die Umsetzung von unpolaren Substraten in organischen Lösungsmitteln, die anschließende Produktgewinnung und das Enzymrecycling sollen vereinfacht werden.

Eine chemische und pharmazeutische Industrie ohne biokatalytische Prozesse scheint unvorstellbar im 21. Jahrhundert. Während schon zahlreiche Verfahren im industriellen Maßstab angewendet werden, bleiben einige vielversprechende Anwendungen im Entwicklungsstadium hängen. Wesentliche Probleme stellen die Stabilität der Enzyme unter Prozessbedingungen sowie das zeit- und kostenintensive Aufarbeiten der Produkte dar. Dieser beiden Probleme wird sich in dieser Arbeit angenommen. Die erste Hälfte der Arbeit beschäftigt sich mit der Anwendung von Enzymen in organischen Lösungsmitteln. Mittels Coating mit ionischen Flüssigkeiten deren Schmelzpunkt leicht über Raumtemperatur liegt konnte erreicht werden, dass die Aminierung von 4′-Bromoacetophenon mit der Transaminase ATA-40 in Methyl-tert-Butylether (MTBE) über 120 h stabil lief. Der Cofaktor Pyridoxal 5′-Phosphat (PLP) wurde durch die Ummantelung vor dem Lösungmittel geschützt. Bei der Verwendung von freien Enzymen und Cofaktor stoppte die Reaktion nach nur 30 h.

Im zweiten Teil wird gezeigt, dass enzymatische Reaktionen auch in Switchable Solvents durchgeführt werden können. Die Polymerisation von Rinderalbumin (BSA) wurde mittels Transglutaminasen in einer Amidine/Ethanol-Mischung mit veränderlicher Polarität durchgeführt. Zudem werden Anwendungsmöglichkeiten, Probleme und Potentiale von Reaktionsmedien mit variabler Polarität und Hydrophilie diskutiert.

Acknowledgment

First of all, I want to express my sincere gratitude to Professor John Woodley, who gave me the opportunity to work in his group. This opportunity allowed me to travel abroad and made me grow intellectually and personally - 6 months I would not want to have missed. He gave me the freedom to decide on my own on the research topic and enabled me to investigate this fascinating topic.

I would like to give a special thanks to Maria Gundersen Deslauriers for always taking time to give me support. She gave me the decisive advice when my ideas led to a dead end. Furthermore, she provided me with the required hardware and hence contributed directly to the successful outcome of my work.

Another big thanks goes to Heidrun Gruber-Wölfler for her support in the final phase of my studies.

I want to thank my dear friend Martin because he made my time as a student to five unforgettable years. It would not have been the same without him.

My deepest and sincere thanks goes to my boyfriend Albin. I am grateful to him because he supports me in everything I do and stands by me in rough times. You are the only motivation I need to master any challenge.

Of course, I would like to thank my mother Irene and the rest of my family for all their love and for being at my side when I needed.

Last but not least I want to thank all my friends I made in Denmark who spent many nights giving me strength to go on.

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A	Amidine
AA	Acidic anhydride
ACP	Acetophenone
Amim	1-Allyl-3-Methylimidazolium Cation
API	Active pharmaceutical ingredient
BA	Butylamine
BAP	4′-Bromoacetophenone
Bmim	1-Butyl-3-methylimidazolium Cation
BSA	Buvine serum albumin
DBU	Diazabicycloundecene (1,8-Diazabicyclo[5.4.0]undec-7-ene)
DMSO	Dimethylsulfoxid
DSP	Down stream processing
ee	Enantiomeric excess
Emim	1-Ethyl-3-Methylimidazolium Cation
EtOH	Ethanol
EWG	Electron-withdrawing group
FDA	Food and Drug Administration
Hmim	H-Methylimidazolium Cation
HPS	High polarity solvent
IL	Ionic liquid
ILCE	Ionic liquid-coated enzyme

IPA	Isopropylamine
LPS	Low polarity solvent
MBA	Methylbenzylamine
MBF	Methylbenzoylformate
MTBE	Methyl-tert-butyl-ether
mTGase	Microbial transglutaminase
PLP	Pyridoxal 5′-phosphate
PMP	Pyridoxamine 5′-phosphate
PPMIM	1-(3′-phenylpropyl)-3-methylimidazole
RT	Room temperature
RTSPIL	Room-temperature solid phase ionic liquid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHS	Switchable hydrophilicity solvent
SLIL	Sponge-like ionic liquid
SPS	Switchable polarity solvent
SSol	Switchable solvent
SW	Switchable water
TG2	Transglutaminase from mammalian tissue
TGase	Transglutaminase
TFAP	2,2,2-Trifluoroacetophenone
VOS	Volatile organic solvents

1 Introduction

1.1 Biocatalysis

Biocatalysis employs enzymes, isolated or in whole cells, to catalyze chemical transformations. Like other catalysts, enzymes accelerate reactions by lowering the activation energy by forming enzyme-substrate-complexes. (Figure 1.1)

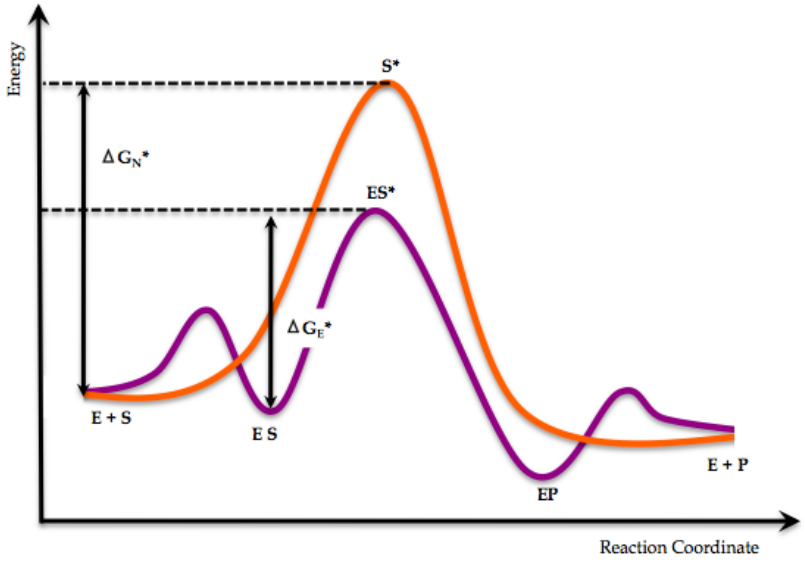
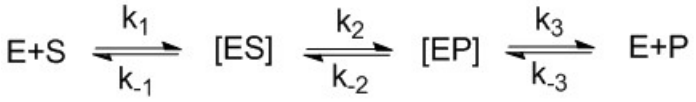


Figure 1.1: Mechanism of reducing the energy of activation by catalysis. ΔG_N^* and ΔG_E^* are the energies of activation of the uncatalyzed and catalyzed reaction[1].

The catalyst/enzyme itself remains unchanged after releasing the product. (Scheme 1.1)



Scheme 1.1: Reaction scheme of an enzymatic transformations. E=enzyme, S=substrate, P=product, ES=enzyme-substrate-complex, EP= enzyme-product-complex, k_x=reaction rate constant of respective step

Enzymes are proteins, natural catalysts, found in all types of organisms from microorganisms to animals, where each enzyme usually promotes one certain reaction. The selectivity to catalyze the reaction of one specific substrate is due to the step of binding the substrate to the active site of the enzyme according to the "lock and key principle". High substrate and reaction specificity as well as mild reaction conditions (pH, temperature) lead to a reduced byproduct and waste generation compared to traditional chemical processes[2,3].

Enzymes have already been used for thousands of years for fermentation to produce cheese, wine, vinegar and beer before Louis Pasteur in 1857 discovered that alcoholic fermentation is associated with a living organism, yeast[4]. Louis Pasteur also was the first one to observe the enantioselectivity of enzymes, when he treated a racemic tartaric acid ammonium salt solution with *Penicillium glaucum*. Only one enantiomer (+) was metabolized, while the other enantiomer (-) accumulated in the medium[5].

Benefits of biocatalysis

In a time where topics like global warming and the limited availability of fossil resources are more relevant than ever before, sustainable processes in industry are in demand. For chemical and pharmaceutical production biocatalysis offers a promising alternative to traditional chemistry. As biocatalysts possess high selectivity, additional chemical (de-)protection steps are circumvented reducing the necessary amount of chemicals. Biocatalytic processes can often be run in aqueous media at ambient conditions and hence constitute reduced risk for technical staff and do not attack the material of multipurpose equipment. Compared to organo- and metal catalysts enzymes are natural products and biodegradable. This aspect becomes important in food and pharmaceuti-

cal industry. Contaminations of toxic metal catalysts in the product stream are critical, while enzyme pose no risk.

The use of enzymes for organic synthesis further expands its outstanding role due to three main reasons[6,7].

Benefits of biocatalysis:[6]

Specificity and yield

- High chemo-, regio-, and stereo-selectivity
- Usually high yields
- Enzymes can work in aqueous media and organic solvents

Working safety and ecology

- Mild reaction conditions and biodegradable catalyst
- No runaway reaction possible
- Reduced health hazards for technical staff

Economic advantages

- No protection groups needed
- Heterologous protein expression allows competitive production of enzymes
- Low corrosive reaction media result in longer life-span of multipurpose installations

Due to these benefits biocatalysis has grown into an indispensable part of chemical industry in the last decade. Besides success stories in fine chemical and pharmaceutical production, also bulk chemical industry, such as the production of acrylamide on a large scale, benefits from biocatalysts. Acrylamide is the starting material for polyacrylamide, which is used in waste water treatment, soil erosion prevention and papermaking.

Chemically, acrylamide can be synthesized by oxidizing acrylonitrile using copper and sulfuric acid, causing environmental pollution. Today 650,000 t per year acrylamide are produced in Japan using nitrile hydratase to hydrate nitriles under mild conditions[8]. In pharmaceutical industry Atorvastatin, the active ingredient of Lipito®, has an outstanding position. The cholesterol-lowering drug exceeded $13 billion in sales in 2007. Chemical synthesis of the statin side chain of the final drug molecule requires four steps including Claisen condensation, borane reduction, a protection step and a following hydrogenation[9]. Hu et al invented a biocatalytic process using an aldolase followed by two chemical steps to circumvent the energy-intensive Claisen condensation and borane reduction both requiring -70°C reaction temperature[10]. This biocatalytic route leads to significant cost savings and waste reduction.

Challenges in biocatalysis

In contrast to the previously mentioned advantages, also several challenges are found with the use of enzymes in chemical synthesis. Enzymes were not developed to serve as catalysts in chemical industry and therefore cannot directly be transferred from the cell into a reactor. Issues like stability, throughput and substrate acceptance need to be adapted to the conditions of industrial processes[11,12]. Furthermore, inhibition can become critical at higher substrate or product concentration. On the other hand, low product concentration makes product recovery difficult because large quantities of solvent must be processed. Down stream processing (DSP) can therefore become the crucial part of the energy balance in the overall process.

Tasks in biocatalysis:

- Transform physiological catalysts into process catalysts able to perform under reaction conditions of an industrial process
- Adaption of the enzyme to unnatural substrates
- High cost properties of the current processes
- Challenging downstream processing due to low product concentration and large solvent quantities
- Susceptible to substrate and product inhibition

In the last 20 - 30 years new technological approaches employing various alternative media were presented aiming the enhancement of productivity and stability of biocatalysts. These novel techniques include very promising approaches like non-aqueous systems studied by Halling and co-workers[13,14]. Hobbs implements processes using supercritical fluids[15,16], while Eisenmenger studied the influence of high pressure on activity and stability of enzymes[17,18].

1.2 Trends in pharmaceutical applications

Besides others, two important trends merged in pharmaceutical drug development in the last decade. The first one is the trend towards complex structures and an increasing number of chiral centers in small-molecule drugs. Optically pure products are required to achieve the desired selectivity and minimize unwanted side effects[19]. Furthermore, the current regulation of the Food and Drug Administration (FDA) prescribe either to proof the non-teratogenity of the non-therapeutic isomer or to market only the optically pure product[20]. Biocatalysts possessing high regio- and enantio-selectivity are therefore the catalysts of choice in pharmaceutical industry to fulfill economical and regulatory requirements. Chemically synthesized molecules are often present in racemic mixtures, which must be resolved in an additional production step, causing extra costs and the loss of half of the product. Such as some chemical catalysts, enzymes are capable to produce the desired enantiomer with an excellent enantiomeric excess (ee) of 99.9% and up to almost 100% yield.

The second trend to be pointed out is the development towards biological products including peptide and protein drugs. In 2011 biopharmaceuticals made up 16% of the gross prescription drug sales[21]. Biological products are expected to grow into more than 50% of sales within the top 100 prescription drugs in 2020[22]. For solubility and stability reasons as well as drug targeting appropriate delivery systems need to be developed. Encapsulation and linkage to biodegradable polymers is a popular opportunity to stabilize biopharmaceuticals[23]. The harsh conditions of chemical processes for these linking reactions are barely sensitive enough to remain the protein in the active state. Biocatalysts may achieve the desired modifications selectively of the large molecule.

As this trend of synthesizing active pharmaceutical ingredients (API) by means of biocatalysis can pose some challenges, this work will present alternative technologies which may help overcoming these difficulties.

1.3 Scope

This work deals with two major problems in biocatalysis.

The first issue handled is the application of enzymes in organic solvents and their stability in this alternative media. As most enzymes are sensitive to environmental changes, reaction media different from aqueous buffer are often avoided. The class of transaminases (discussed later) displays a typical example for enzymes which are barely stable in organic solvents and hence hardly used in alternative media. In this thesis a new technology to enhance the long term stability of transaminases in organic solvent will be tested. By coating the enzyme with ionic liquids (ILs) a protection of the protein structure should be achieved. The enzyme possessing improved stability can be applied to process water insoluble substrates in non-polar solvents. Based on an exemplary reaction two different coating methods will be compared to the reference reaction using free enzyme. In addition, the effect of the amount of coating material and the cofactor location will be examined.

The second part of the thesis handles down stream processing (DSP) of biocatalytic reactions. Product isolation and purification often pose the most cost and time consuming process step because biocatalytic synthesis usually run in low concentration and the separation of enzyme, product, substrates and co-product can be challenging. Solvents possessing two different characters can help in this case. Switchable solvents (SSol) having interchangeable polarity or hydrophilicity may simplify the separation of the product from the reaction mixture. In this work SSol will be used as medium for biocatalysis for the very first time. Finally, the feasibility of this technology and possible applications, limits and opportunities will be discussed.

2 Part I: Ionic Liquids as a Tool in Biocatalysis

The potential of enzymes to catalyze complex industrially relevant reactions in big scale has already been proved. Two examples for employing enzymes successfully in industry are given in the introduction. Both processes acrylamide and Atorvastatin production are carried out in aqueous solution like the majority of biocatalytic reactions. One main reason is the enzymes sensitivity towards deviations from their natural optimal environmental conditions. Nature evolved enzymes to work in diluted aqueous solutions. However, against the still wide spread presumption enzymatic applications are not limited to aqueous media. Alternative media, like organic solvents, are often criticized to be environmental unfriendly and to inactivate enzymes rapidly. These critics underestimate the reduction of solvent due to the enhanced solubility of hydrophobic substrates in non-polar solvents. Processing hydrophobic substrates in very low concentrations in aqueous media generates large quantities of waste water and hampers down stream processing (DSP)[24]. These and further advantages of employing enzymes in organic solvents are already known since 1990[25].

Potential advantages of biocatalysis in organic solvent

- Increased solubility of non-polar substrates
- Shifting thermodynamic equilibrium to favor synthesis over hydrolysis
- Suppression of water-dependent side-reactions
- Alteration in substrate- and enantioselectivity
- Immobilization is often unnecessary because enzymes are insoluble in organic solvents
- Enzymes can be recovered by simple filtration
- Ease of product recovery from low boiling solvents
- Enhanced thermostability
- Elimination of microbial contamination
- Potential for enzymes to be used directly within a new or existing chemical process

On the other hand, high inactivation rates of free enzymes display a great disadvantage leading to elevated expenditure. The inactivation process can occur due to interactions of the solvent molecules with the protein or interfacial inactivation in two-phase systems. Organic solvent can occupy the active site or interfere with amino acids altering the structure of the enzyme leading to conformational changes. While hydrophobic solvents show little intention to interact with the hydrophilic enzyme surface, solvents with log P lower than 2 tend to replace the tightly bound water on the enzyme surface, which is essential for the catalytic activity[26,27]. Strategies to overcome the problem of activity loss can be grouped into three categories: (1) isolation of novel enzymes that can function under extreme conditions, (2) modification of enzyme structures to increase

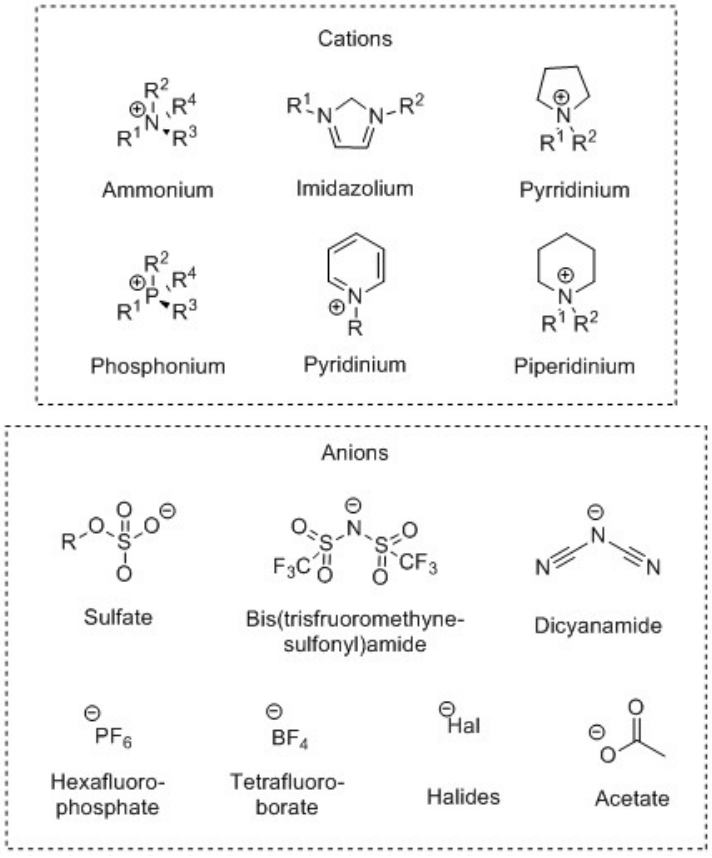
their resistance toward non-conventional media and (3) modification of the solvent environment to decrease its denaturing effect on enzymes[28]. The first category includes a laborious and time-consuming screening and potential risks and problems when producing the enzyme in different hosts. The latter strategy may be difficult to implement in existing processes, because altering the solvent characteristics may require adjusted reaction conditions and DSP.

Immobilization is a fast and usually simple method to stabilize enzymes not only towards solvent and thermal effect, but also mechanical forces. Immobilization gives enzymes more robustness and resistance to environmental changes. In addition, heterogeneous catalysts enable easy recovery of both enzyme and product as well as uncomplicated multiple reuse of the catalyst, what favors continuous processes. A drawback on the other hand is the reduced activity immobilized enzymes often show. In literature countless protocols on various immobilization methods are available. Most frequently used techniques are binding onto an inert matrix or entrapment within an inert matrix. Supporting materials for covalent coupling and adsorption can be inorganic polymers, synthetic resins or biopolymers, while entrapment encloses enzymes into fiber or gel networks. Each of the method has its benefits and drawbacks, allowing the design of a immobilization system matching the process requirements[29–31].

Beside all these immobilization strategies coating enzymes with ILs display a new possibility to stabilize enzymes and enable catalyst recycling. Up to now only few reports are available applying this novel stabilization technique. To expand the area of application, in this work enzymes will be coated with ILs to examine the stability enhancement toward organic solvent. The target is to develop a simple stabilization method to prolong the enzyme life time in organic solvent. This would enable biocatalytic transformations in non-aqueous media with enhanced substrate solubility widening the substrate spectrum of enzymatic reactions to hydrophobic molecules.

2.1 Introduction

Ionic liquids (ILs) are salts which are liquid at or close to room-temperature. Due to their near-zero vapor pressure ILs are considered as possible green replacements for volatile organic compounds (VOC)[32]. Furthermore, their thermal stability and widely tunable properties regarding hydrophilicity and solvent miscibility makes them interesting for usage as solvent[33]. The first IL was invented in 1914 by Walden, when he synthesized ethyl ammonium nitrate[34]. Today more than 1000 ILs are described in literature and over 300 are commercially available[35]. Compared to the 600 commercially used organic solvents in industry, theoretically about one million (10^6) simple ILs can be synthesized. Combinations of various ILs can make up one trillion (10^{12}) of ternary systems[36]. Most commonly Pyrrimidinium, Imidazolium, Pyrrolidinium as well as Ammonium- and Phosphonium-compounds are used[37]. (Figure 2.1)



Scheme 2.1: Most commonly used cations and anions for ILs[38].

Using ILs makes it easy to adjust the critical solvent properties to a particular process. Melting point, miscibility with water and organic solvents, stability towards moisture, viscosity and polarity can be adapted by the appropriate selection of the cation and/or the anion component. Therefore, ILs are also known as "designer solvents" and make them attractive as media for traditional chemical and biocatalytic processes[39].

2.1.1 Ionic liquids in biocatalysis

The first paper on enzyme catalysis in ILs was published in 2000. Erbeldinger et al reported the thermolysin-catalyzed synthesis of Z-aspartame in ILs containing only 5% water[40]. In the same year the group of R. Sheldon published the first enzymatic process in water-free ILs[41]. The used lipase showed comparable or better reaction rate in the IL than in conventional organic solvents. Cull et al employed ILs for whole cell processes. [Bmim][PF_6] was used as a solvent for a biphasic system with water for the hydration of 1,3-dicyanobenzene using *Rhodococcus 312*[42]. These first successful reports propagated intensive investigations in biocatalysis in ILs in the following years although the roots of using ILs to enhance enzymatic activity go back to the 1980s[43]. To not lose the overview of this plenty of new information, the technology of using ILs for enzymatic processes has been reviewed extensively[32,33,44–46].

Furthermore, Lozano recently invented a process using sponge-like ionic liquids (SLIL)[47]. Therefore, he used ILs which are solid at room temperature, but melt below the process temperature of the enzymatic step. In the described biodiesel production step the IL enables miscibility between the substrates methanol and triolein. This system facilitates a homogeneous reaction mixture resulting in close to 100% conversion as well as easy product removal. In this process the SLIL represents kind of supporting phase for biocatalyst immobilization. This makes the enzyme easy to reuse.

Room-temperature solid phase ionic liquids

Lee was the first to use room-temperature solid phase ionic liquids (RTSPIL) in biocatalysis[48]. [PPMIM] [PF_6] with a melting point of 53°C was used to coat enzyme powder to overcome limitations like reduced selectivity and stability in non-aqueous media. The coating was prepared by melting the RTSPIL, adding 0.1 mass equivalent of enzyme powder, stirring the mixture to a heterogeneous solution and cool it to RT. The solidified mixture was then broken into small particles and used without further treatment. The coated lipase was tested for its selectivity and activity for the transesterification reaction of secondary alcohols in organic solvent. It has been shown that the enantioselectivity could be enhanced by coating the lipase with ILs without losing any significant activity. Furthermore, the coated enzyme is easy to reuse without any loss of activity. The IL used in this example is water immiscible. Nevertheless, it can absorb a few percent of water. The water may cause hydrolysis of anions ([PF_6], [BF_4]) leading to the formation of HF, what deactivates enzymes[33].

Eight years after their first successful application of IL coated enzymes (ILCE) Lee and Kim published a slightly altered approach of using RTSPIL in biocatalysis[49]. *Burkholderia cepacia* lipase was co-lyophilized with ILs composed from imidazolium or pyridinium cation and hexafluorophosphate anion. Compared to KCl salts co-lyophilized lipase displaying 40-fold enhanced activity in relation to the salt-free enzyme, IL co-lyophilizied lipase showed up to 663-fold enhanced activity. In enantioselectivity an enhancement of about 2.5-fold was observed with RTSPIL compared to KCl and salt-free enzymes.

Inspired by this inventions other scientists applied coated lipases for various processes. Mutschler compared four processing methods using Novozyme 435 for the esterification of methylglucose with a fatty acid[50]. The method with ionic liquid-film coated lipase beads appeared to be the preferred process compared to transformation in organic solvent, solvent free conditions and IL as solvent. The ILCE resulted in a 4.5 times higher efficiency than under solvent free conditions. 1-Butyl-methylpyridine hexafluorophosphate [4bmpy][PF_6] as coating material led to a superior efficiency and broader substrate tolerance.

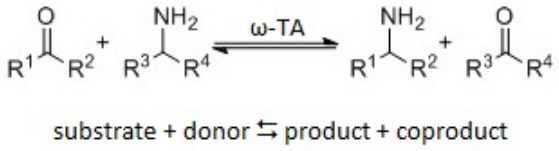
Three years later a lipase from *Candida rugosa* was coated with a novel tetraethylammonium amino acid IL for the esterification of fatty acid esters. Rahman found enhanced conversion for all tested substrates[51].

Dong implemented ILCE in polymer chemistry[52]. Novozyme 435 was coated with [BMIM][PF_6] by pre-incubation for 6h to catalyze the ring-opening polymerization (ROP) of 1,4-dioxan-2-one (PDO). The coated enzyme yield an enhanced molecular weight compared to free Novozyme 435 in IL. Poly(caprolactone) can be synthesized analogous to PDO by a ROP. Wu investigated coated and free Novozyme 435 for this reaction[53]. The ILCE was observed to result in enhanced molecular weight and in most cases generated superior yield, while the amount of used IL for coating is only a moiety of that for ILs as a solvent.

For all these processes lipases have been coated. Protocols using other enzymes have not, to the best of my knowledge, been reported so far. For this work transaminases will be carried out using IL coated ω-TAs with the objective of expanding the application spectrum of ILCE.

2.1.2 ω-transaminase

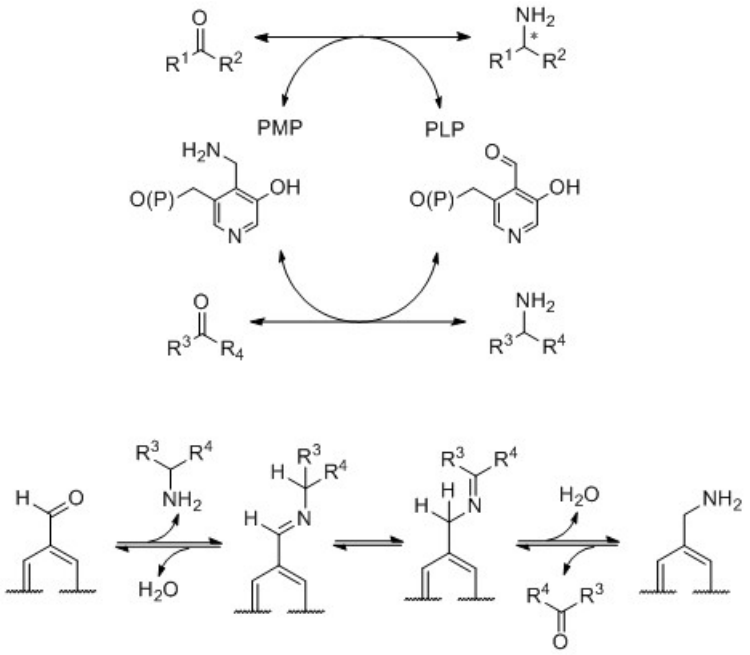
Aminotransferases (EC 2.6.1.x) catalyze the redox-neutral transfer of an amino group from a donor molecule to a carbonyl function of an acceptor molecule. (Scheme 2.2)



Scheme 2.2: Transaminase catalyzed reaction: The amino group it transferred from the amino donor to the ketone (amino acceptor). The donor is oxidized to the corresponding ketone. The acceptor goes through a reduction step from the ketone to the corresponding primary amine.

Each transaminase can be assigned to one of four subgroups. The enzymes in the subgroups I, III and IV only transfer amino groups bonded to an α-carbon of amino

acids. Subgroup II is ω-TAs. The ω represents all non-α positions. For the reaction mechanism water is essential and pyridoxal 5′-phosphate (PLP), a derivative of vitamin B6, is necessary as a cofactor[54]. The proposed reaction mechanism is depicted in Scheme 2.3.



Scheme 2.3: Transaminase reaction mechanism: In the initial step PLP forms a Schiff base with the active site lysine. In the first half of the reaction the amino group of the donor is transferred to the E-PLP-complex, giving a E-PMP-complex and the ketone co-product. In the following step the E-PMP-complex is recycled to the E-PLP-complex by transferring the amino group to the acceptor ketone[54].

The reaction mechanism consists of two half-reactions, the oxidative deamination of the donor followed by the reductive amination of the acceptor. In the initial step PLP forms a Schiff base with the active site lysine. In the first half of the reaction the amino group of the donor is transferred to the E-PLP-complex, giving a E-PMP-complex and the ketone co-product. In the following step the E-PMP-complex is recycled to the E-PLP-complex by transferring the amino group to the acceptor ketone[55]. Donor amines are often either derived from the α-amino acid pool (alanine, phenylalanine, glutamic acid, aspartic acid) or they are simple amines like isopropylamine, cyclohexylamine, 3-amino-1-phenylbutan, 1-phenylenthylamine (Figure 2.1).

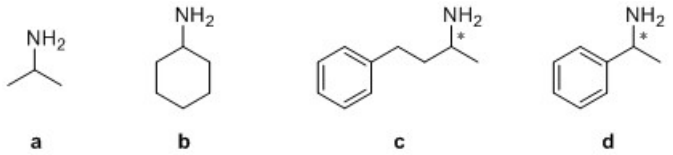


Figure 2.1: Selection of typical amino donors: isopropylamine **a**, cyclohexylamine *b*, 3-amino-1-phenylbutan **c**, 1-phenylenthylamine **d**

In this work small chemical compounds are used because they are inexpensive and have the advantage, that the co-product may be highly volatile. The corresponding ketone to isopropylamine is acetone, which has a boiling point of 56°C[56] and therefore can be removed from the reaction mixture by mild heat. In-situ product removal (ISPR) of the co-product prevents enzyme inhibition and favors the reaction to run to higher yield. Without ISPR enzymatic transamination is often quite unfavorable. The equilibrium constant of the reductive amination of (*R*)-PAC (Phenylacetylcarbinol) to (1*R*,2*S*)-Norephedrine with alanine was determined to be 2.31×10^{-3}. With equimolar substrate concentration the low equilibrium constant leads to a theoretical conversion of 5%. A theoretical conversion of 90% can only be achieved by an 5.000-fold excess of alanine in this reaction[57].

ω-TAs in industry

The importance of enantioselective amine synthesis can be observed when taking a look at the structure of small molecule drugs. 40% of active pharmaceutical ingredients (APIs) contain a chiral amine in their structure. The most famous and successful example for employing an ω-TA to synthesize an API is sitagliptin phosphate, the active substance in Januvia®[58]. Januvia® is within the top 20 best selling drugs world wide (top 10 small molecule drugs) in the last 3 years (2012-2014) reaching sales of 4 billion dollar each year[59,60]. The traditional synthesis of sitagliptin phosphate is a three step process including an asymmetric hydrogenation of the enamine intermediate using a rhodium-based catalyst[61,62]. All the three steps were replaced by one enzymatic step.

2.2 RTSPIL for coating transaminases

Coating transaminases with ILs has not been reported yet, to the best of my knowledge. All the enzymes coated so far follow a reaction mechanism which does not require water or a cofactor as a reactant. Therefore, hydrophobic ILs were used.

The requirements for ILs for coating enzymes that **do not require water** in the reaction mechanism:

- Solid at room (and operation temperature)
- Melting point low enough to not inactivate the enzymes while coating
- Insoluble in the used solvent

To apply this technique to transaminases, some factors need to be considered. As the transaminase mechanism requires water, it is essential to use water miscible or at least water insensitive ILs with anions different from PF_6. Further, the melting point must be high enough to result in a SPIL at RT, but low enough to melt the IL and mix it with the enzyme powder without denaturation of the enzyme.

The requirements for ILs for coating enzymes that **do require water** in the reaction mechanism:

- Solid at room (and operation temperature)
- Melting point low enough to not inactivate the enzymes while coating
- Water miscible in liquid state
- Insoluble in the used solvent

There are few ionic liquids fulfilling all these requirements. Table 2.1 shows a selection of potential ILs for the coating process.

Table 2.1: Selection of possible ionic liquids for the coating of enzymes

Entry	CAS	Product	mp [°C]
1	65039-08-9	[Emim] Br	53
2	85100-77-2	[Bmim] Br	60
3	65039-10-3	[Amim] Cl	65
4	79917-90-1	[Bmim] Cl	73
5	35487-17-3	[Hmim] Cl	75
6	342789-81-5	[Bmim] CH_3SO_3	75 - 80

The ionic liquid-coated enzymes (ILCE) can either be prepared from the lyophilized enzyme or from an enzyme solution. In the first case only the enzyme is immobilized in the IL, while cofactors and water, need to be in the liquid phase together with the substrates. In the latter case when solubilized enzymes are coated, the water and cofactor are enclosed in the coating with the enzyme and the surrounding liquid phase only contains the substrates.

Co-lyophilization with ILs is another method for enhancing stability and selectivity of enzymes[49]. Due to the lyophilization the result is a dried enzyme/IL mixture. In this case water and cofactor would need to be in surrounding liquid phase.

Depending on the solubility and miscibility of the selected IL, either organic or aqueous reaction media can be used and hence water-soluble and water-insoluble substrates can be processed.

The ILs selected for this work is [Emin]Br. Further product information is available in Appendix B - Product information [Emim]Br. As this IL is highly hygroscopic and water-soluble dry tert-butyl-methyl-ether (MTBE) was used as reaction media. The coatings were all prepared from lyophilized enzymes.

2.2.1 Coating process

For this work two different coating methods will be carried out. In the first method melted IL is used to coat enzymes[48]. For the second procedure the enzymes are coated by precipitating the IL from a concentrated solution. In literature enzyme-coating ratios from 0.1 : 1 to 1 : 1 (w/w) can be found.

Coating with melt

The solid IL is melted in a water bath. Enzyme and cofactor are added and the mixture is stirred to a heterogeneous solution. Afterwards the mixture is allowed to cool down and solidify. The received coated enzymes are then used to catalyze the transamination of water insoluble substrates in organic solvents. The necessary amount of water for the reaction is provided by the hygroscopicity of the IL. When using this coating method for non-hygroscopic coating materials the required water must be supplied by the organic solvent. Therefore, the reaction media must not be dried.

Coating by precipitation

The aqueous IL solution is mixed with the lyophilized enzyme and the cofactor. Most of the contained water is evaporated. Afterwards ice cooled and dried organic solvent the IL must not be soluble in (acetone, toluene, hexane) is added to precipitate the IL enclosing the enzyme, cofactor and water. The received coated enzymes are then used to catalyze the transamination of water insoluble substrates in organic solvents.

2.2.2 Reaction setup and benefits of coated enzymes

The coating is hoped to aid in:

- protecting the enzyme from losing activity by denaturing (unfolding) in the solvent
- retain the cofactor (PLP)
- ease enzyme retention in downstream processing

The final reaction setup is a two-phase system with a solid phase of IL, enzyme and cofactor and the solvent with the substrates. (Figure 2.2)

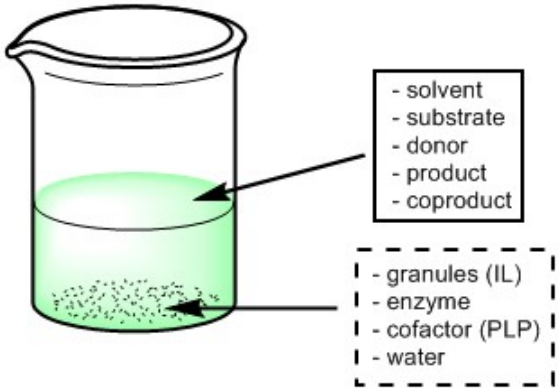


Figure 2.2: Reaction setup for biocatalytic transamination in organic solvent employing ILCE. The coating encloses the enzyme, the cofactor and water. The organic media contains the substrate, donor, product and co-product.

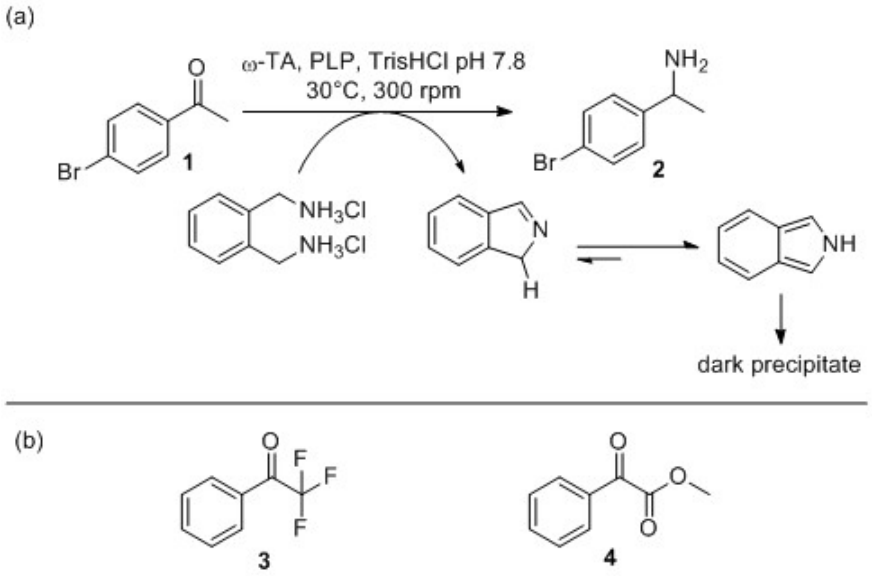
The method of coating enzymes can be used to protect the catalyst from shearing strength in a stirred tank reactor. Further the enzyme is immobilized in the ionic liquids and therefore can be recycled.

If there was a possibility to produce a defined pore size in the coating using polymerisable ILs, two reactions could be run in one reactor. The biocatalytic step takes place in the net of polymerized IL and a following reaction with another enzyme or a metal catalyst can happen in the surrounding liquid. As enzymes do not dissolve in ILs, but remain as a suspended powder[63], the coating material can be recycled when the enzyme has lost its activity. The used IL is not only soluble in water, but also in methanol and dichlormethane. Thus, the IL can be dissolved in the solvent, while the enzyme remains solid. The enzyme can be removed by simple filtration and the coating material can be recycled after evaporating the solvent.

2.3 Results

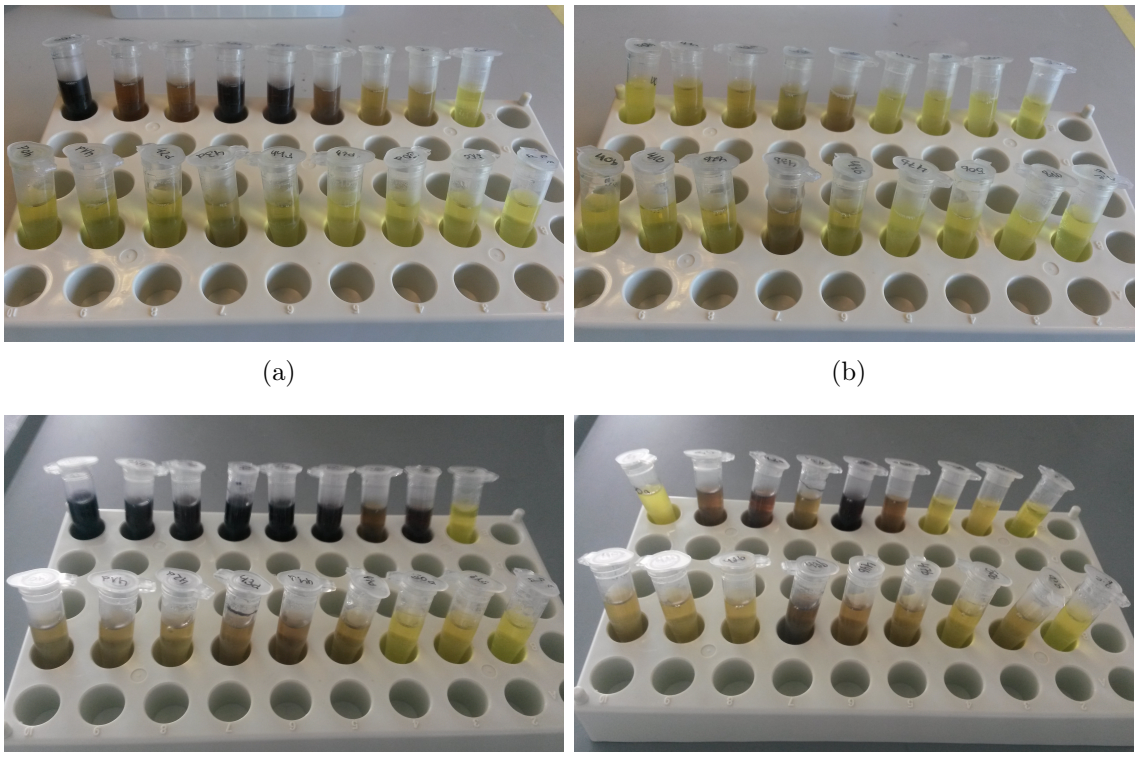
2.3.1 Screening

Eight transaminases were tested for their ability to convert 4′-bromoacetophenone (BAP) **1**, 2,2,2-trifluoroacetopheone (TFA) **3** and methylbenzoylformate (MBF) **4** to the corresponding amines. The screening was done as described by Green[64]. (Scheme 2.4)



Scheme 2.4: **a)** Reaction scheme of screening: Reductive amination of BAP **1** with o-xylylendiamine dihydrochloride as donor to 1-(4′-bromophenyl)ethylamine (BPEA) **2**. One amino group of o-xylylendiamine dihydrochloride is transferred to the keto-substrate. The keto-co-product undergoes a spontaneous ring-closing reaction forming a cyclic imine. 1,5-hydride shift or tautomerization convert the imine into the more stable aromatic isoindole, which forms a dark-colored precipitate. **b)** Other substrates: 2,2,2-trifluoroacetopheone (TFA) **3** and methylbenzoylformate (MBF) **4**

The screening showed that all the tested enzymes are active on **1**, but the aminotransaminase ATA 50 shows the lowest activity. ATA 40, 82 and 44 show the fasted reaction. ATA 44 has the highest activity on **3**. ATA 41, 42, 43 and 47 show low activity, while ATA 40, 50 and 81 do not accept **3** at all. For **4** ATA 82 is the only enzyme accepting the substrate, but shows very low activity. All the other enzymes did not show any activity. (Figure 2.3)

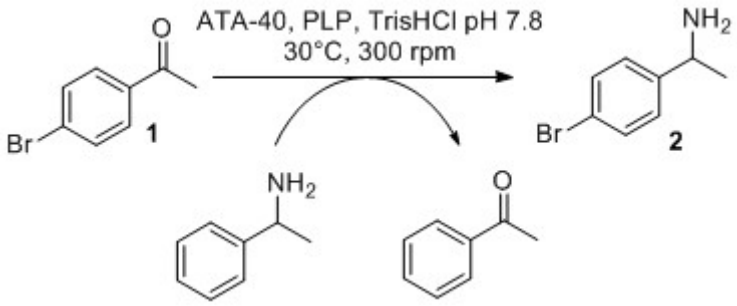


(a) (b)

(c) (d)

Figure 2.3: Screening of ATA 40, 41, 42, 82, 44, 47, 50, 81 with o-xylylendiamine. **(a) 1** (top) and blank (bottom) after 6h reaction time, **(b) 3** (top) and **4** (bottom) after 6h reaction time, **(c) 1** (top) and blank (bottom) after 24h reaction time, **(d) 3** (top) and **4** (bottom) after 24h reaction time

For all the further work ATA-40 was used. **1** was chosen as substrate and MBA was used as donor. (Scheme 2.5)



Scheme 2.5: Enzymatic amination of BAP with ATA-40 to the corresponding amine. The donor MBA is oxidated to ACP. This reaction was used for all further experiments. The catalyst was pretreated in various ways to examine the effects of coating method and thickness.

2.3.2 Long-term stability

To asses the stabilizing effect of the IL coating two reactions were run, one with free enzyme and one with [Emim]Br coated enzyme. The reactions using ATA 40 for the amination of 4′-bromoacetophenone (BAP) with MBA as donor forming the co-product ACP ran for 120 h. Samples were taken over time and analyzed by means of GC-FID to observe the formation of co-product, representing the enzyme activity. (Figure 2.4 and 2.5) The co-product peak was chosen for the evaluation because the product peak appears very late in the chromatogram (14.0 min) and hence is broad and difficult to analyze. As the substrate and donor peaks were much higher than the co-product peak, changes were difficult to detect because the reaction happened very slow.

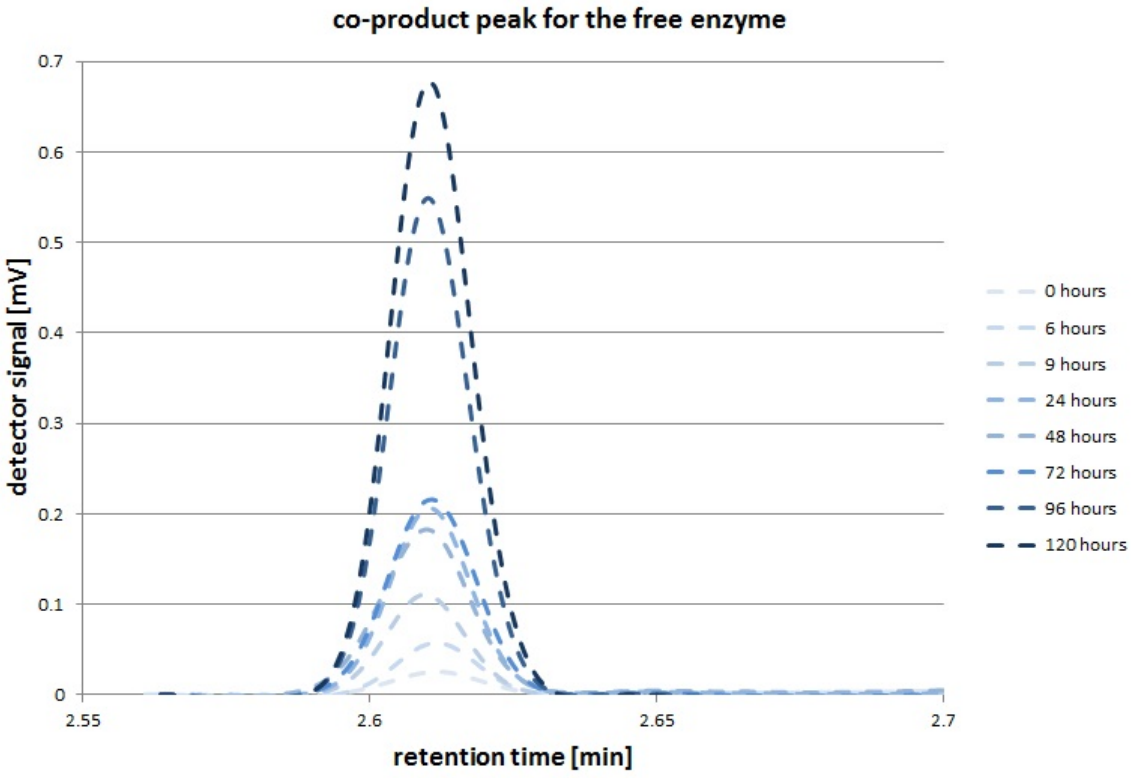


Figure 2.4: Co-product peak of the reaction run with free enzymes. The peak grows to the 24 h sample, representing the turnover. After one day the reaction stops and continues after adding new PLP.

In the reference reaction with free enzyme the co-product peak grows significantly within the first 24 hours reaction time. The peak at 2.61 min possesses almost the same area for 24, 48 and 72 hours. This observation led to the conclusion that the enzyme is inactivated after 24 hours reaction time due to solvent interaction. Surprisingly, the ACP peak continued growing rapidly after adding further cofactor PLP showing that the reaction is limited by the cofactors stability in the organic solvent. For the last two samples it can already be recognized, that the concentration of cofactor in the reaction mixture is getting lower again. It can be assumed that the reaction would stop soon due to a lack of PLP.

The reaction mixture with the coated catalyst shows steady and continuous co-product formation over 120 hours. As the cofactor is also enclosed in the coating with the enzyme PLP is protected from the solvent and hence stable over a long time.

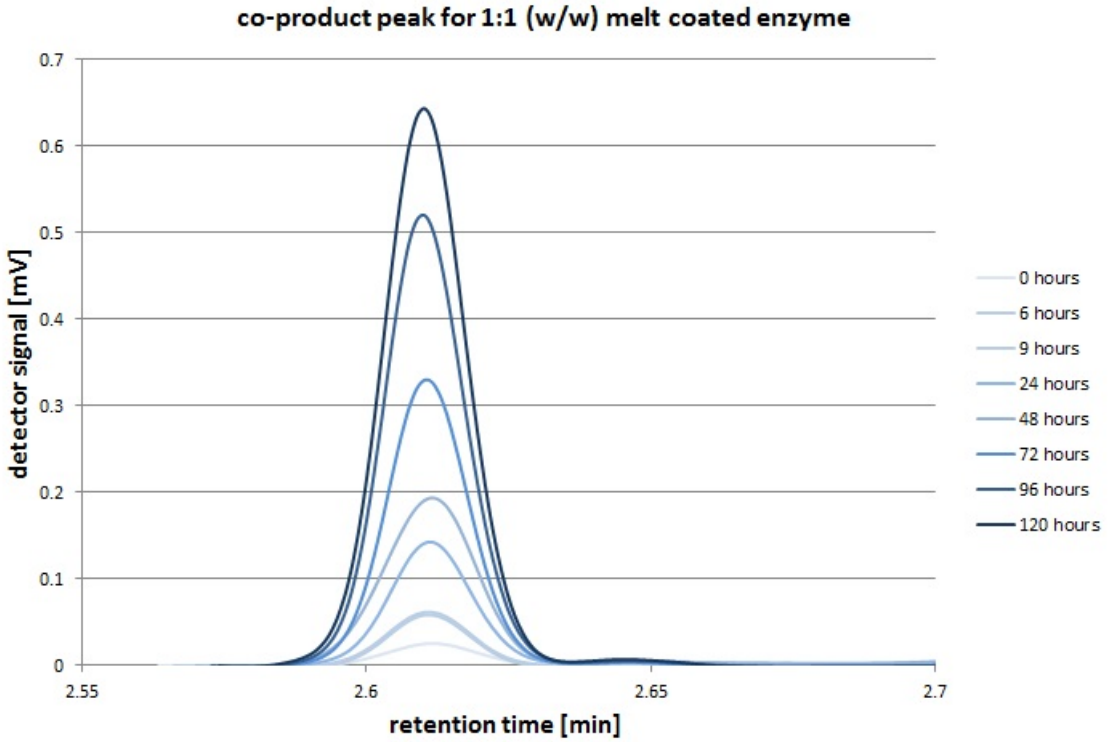


Figure 2.5: Co-product peak of the reaction run with 1:1 (w/w) melt coated enzymes. The peak grows continuously representing constant reactions rate during the whole experiment (120 h).

To simplify the comparison of the turnover in both reaction mixtures, the peaks for six time point are depicted in separate diagrams. (Figure 2.6)

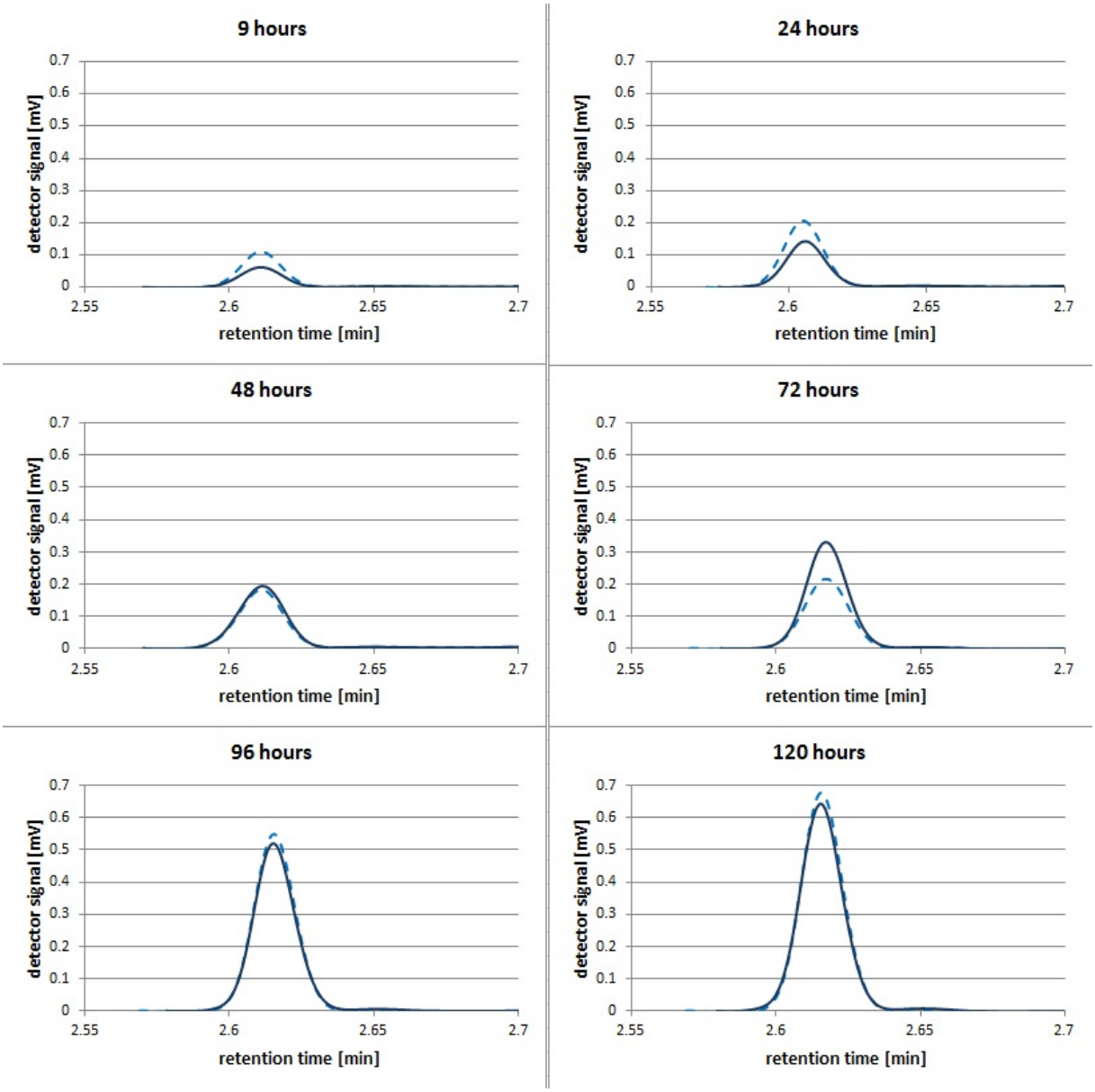


Figure 2.6: Comparison of co-product formation of the reaction run with free (dashed line) and melt coated (solid line) enzymes at each time point. Until 24 h reaction time the free enzyme shows higher activity than the coated. The reaction with free enzyme and PLP stops after less than 48 h. After adding further PLP the reaction continues until the end of the experiment. The coated catalyst works with a constant reaction rate over 120 h.

As expected the reaction rate of the coated catalyst is lower than the rate of the free

enzyme. This is either due to activity loss owing to the coating process or the mass transfer limits because of the IL cover the substrates need to pass.
The leading of the free enzyme lasts until the 24 h peak. After this sample the starting mixture is not working anymore, the coated enzyme catches up within 48 h form the start and clearly leaves the free enzyme behind after 72 h.
After PLP addition to the free enzyme reaction the enzyme is reactivated and the co-product peak quickly increases to the double of size. Finally, both reactions show the same co-product concentration after 120 h.

The co-product concentration over time (Figure 2.7) shows constant slope for the coated enzyme. The graph of the free enzyme has a curvy shape starting steep, but flattening after less than 30 h reaction time. After the hillock due to PLP addition at the end of the experiment after 120 h the reaction rate is low again and the reaction will soon stop again, if no further cofactor is added.

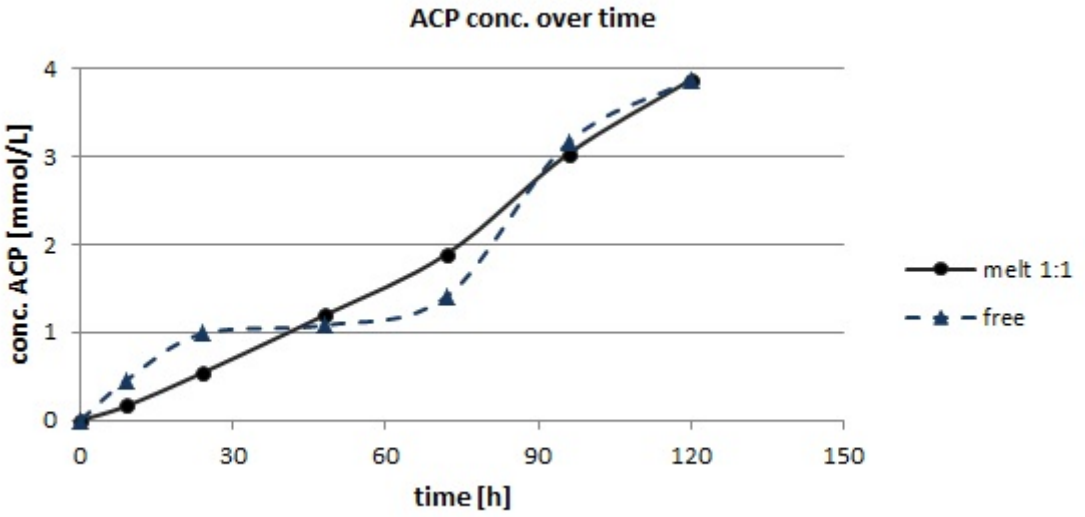


Figure 2.7: Co-product formation of free (dashed line) and 1:1 (w/w) melt coated (solid line) enzymes over time. The coated enzyme works with constant reaction rate throughout the whole experiment for 120 h. The free enzyme shows a higher reaction rate at the beginning but slows down and stops working after one day. After addition of further PLP the reaction continues rapidly but slow down again at the end of the experiment due to PLP inactivation.

If one assumes that the cofactor would be stable in the organic solvent and the reaction

with the free enzyme would continue with its initial rate, the yield after 120 h would reach 6.1 mmol/L. (Figure 2.8) The yield of the coated enzyme after 120 h amounts 3.9 mmol/L. This makes a difference of 2.2 mmol/L

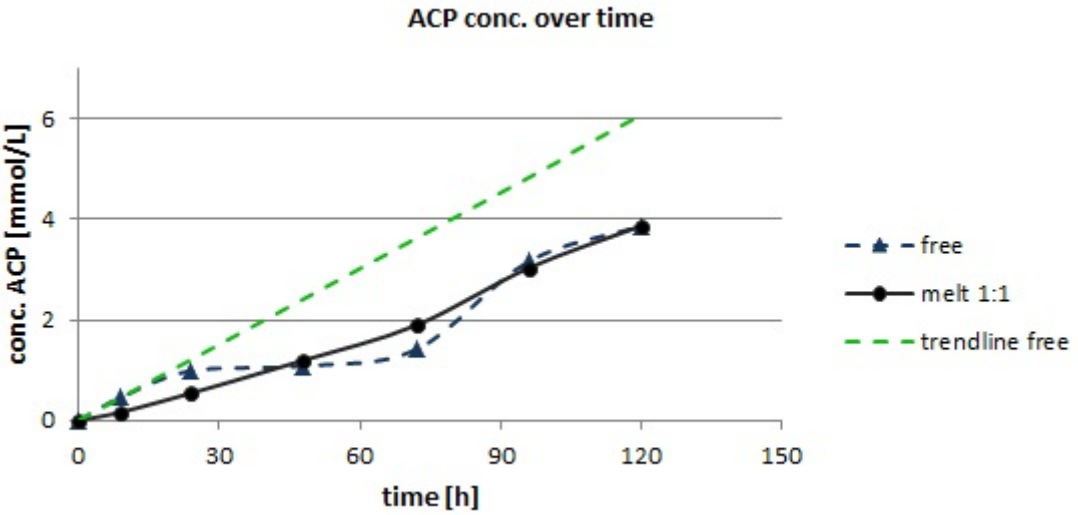


Figure 2.8: The trend of the free enzyme reaction if there was no cofactor inactivation: The green dashed line shows the amount of co-product the free enzyme yield if the reaction would run with the initial rate for 120 h.

Assuming that the cofactor is inactivated within 30 h the 4-fold amount of PLP is necessary for running 120 h of reaction with the free enzyme. To achieve a concentration of 1 mmol PLP per liter reaction medium (MW: 247.14 g/mol), 0.25 g PLP are required for the coated enzyme are standing against 1.00 g PLP for the free enzyme.

2.3.3 Effect of coating method

The coating method using the melted IL should be compared with a new technique, where the enzyme is coated by precipitation of the IL in an organic solvent the salt is not soluble in. Therefore, enzyme were coated with the precipitation method in a 1:1 (w/w) enzyme-IL-ratio and employed under the same conditions as the melt coated and the free enzyme. (Figure 2.9)

The observation was almost the same as with the melt coated catalyst. The reaction rate is approximately constant during the whole experiment of 120 h. Like with the melt coated catalyst, the reaction does not stop due to PLP inactivation. However, compared to the melt coated enzyme the reaction is running slower. The reaction rate is reduced by more than the half.

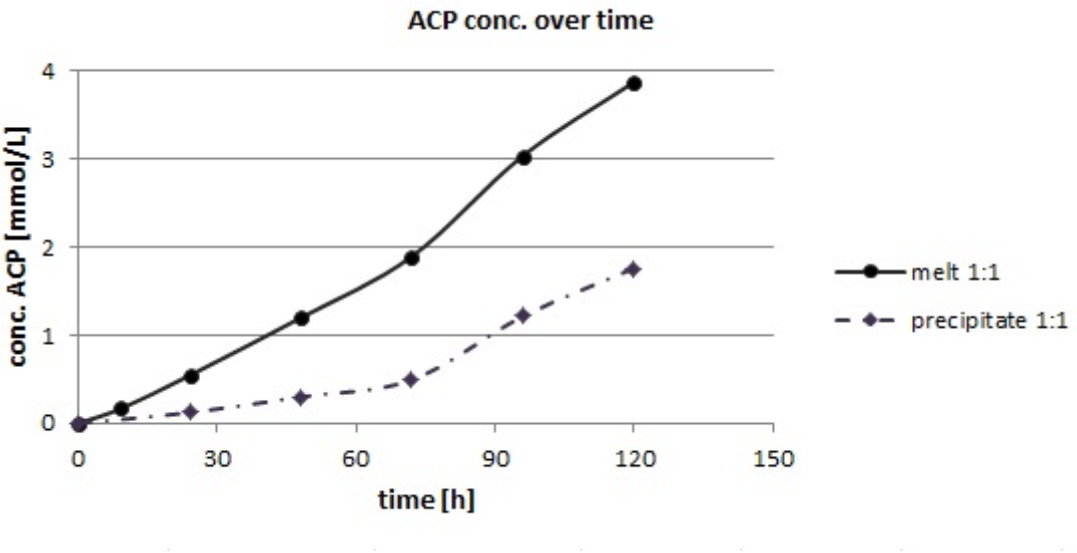


Figure 2.9: Co-product formation of melt coated enzyme (solid line) and precipitate coated enzyme (dot-dashed line). Both reactions run with an approximately constant reaction rate throughout the whole experiment showing that the cofactor is not inactivated. The reaction rate of the precipitate coated enzyme is lower that the rate of the melt coated enzyme.

2.3.4 Effect of coating thickness

To find out how the coating thickness effects the reaction rate enzymes were coated in various enzyme-IL-ratios (1:1, 1:2, 1:5 and 1:10). The formation of co-product of each catalyst are shown in Figure 2.10 and 2.11)

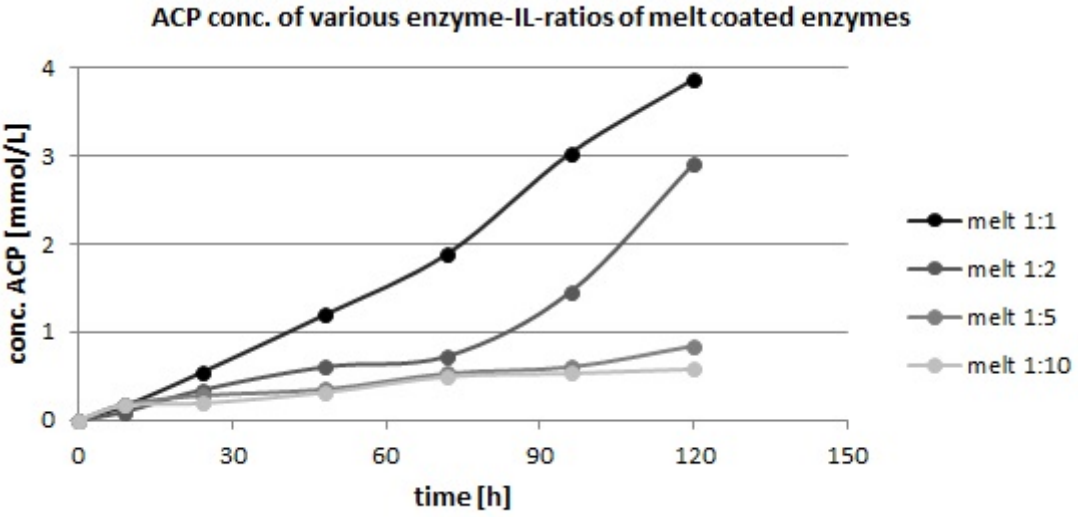


Figure 2.10: Co-product formation of four different coating ratios for melt coating. The reaction rate decreases with increasing amount of IL per mass enzyme. 1:5 and 1:10 show barely any activity.

Using the double amount of IL for the melt coating process lowers the reaction rate. It is likely that this effect is due to a more difficult mass transport through the coating. The reaction rate for the thicker coating is constant at the beginning, but shows a flat area at approximately 60 h. This low in the graph is probably caused by mass transfer limitations around the catalyst due to too little shaking. For higher amounts of coating material almost no conversion was observed. The covering might get too thick for the substrate to permeate.

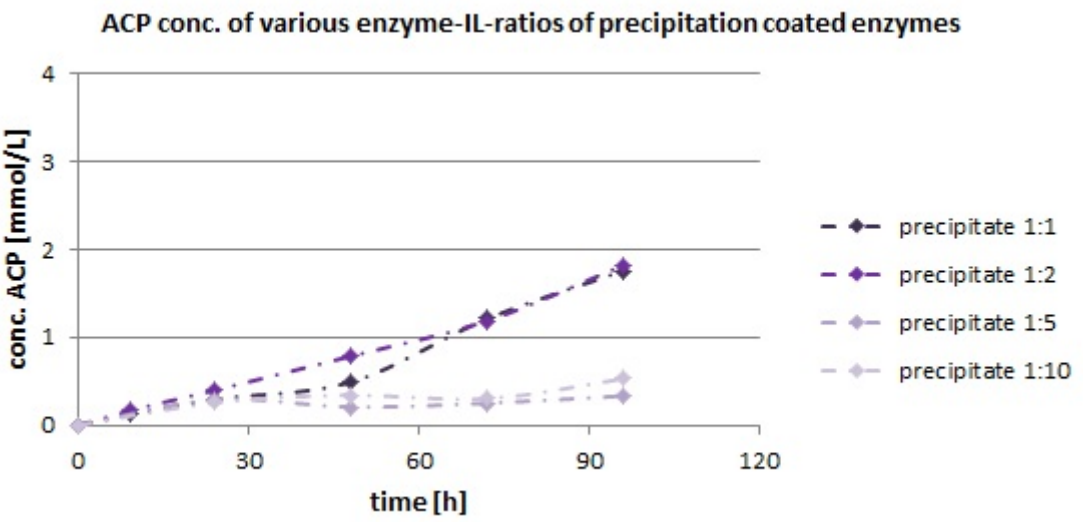


Figure 2.11: Co-product formation of four different coating ratios for precipitate coated enzymes. The reaction rate is hardly influence when the coating thickness is doubled. For 5- and 10-fold coating thicknesses hardly any conversion is observable anymore.

Coating the enzyme with the double amount of IL with the precipitation method hardly effects the initial reaction rate. Like the 1:2 melt coated catalyst also the precipitate coated enzyme shows a lower activity in the middle of the experiment (40 h), but in the end catches up with the 1:1 coated enzyme regarding the total turnover after 96 h. Thicker coatings (1:5 and 1:10) result in very low activity.

2.3.5 Effect of cofactor location

To determine the effect of the proximity of the cofactor to the enzyme on the activity two reactions (one with melt coated and one with precipitate coated catalyst) were run, where PLP was not enclosed in the coating, but present in the organic solvent together with the substrate and the donor.

For this reaction mixtures no turnover was observed. This observation can either be due to the inability of the cofactor to permeate through the coating or the low probability of a substrate and PLP molecule to permeate and bind to the enzyme simultaneously.

2.3.6 Leaching study

The strength of immobilization was assessed by the Bradford Assay[65]. After extracting the probably leached enzymes from the organic reaction medium into water, this assay was used to measure the amount of proteins in the sample. The Bradford Assay can detect active and inactivated enzymes.

The samples from the extraction show the same absorption as the blank. Therefore, no enzyme dissolved in the reaction medium, the enzyme is strongly bound to the IL. (Figure 2.12)

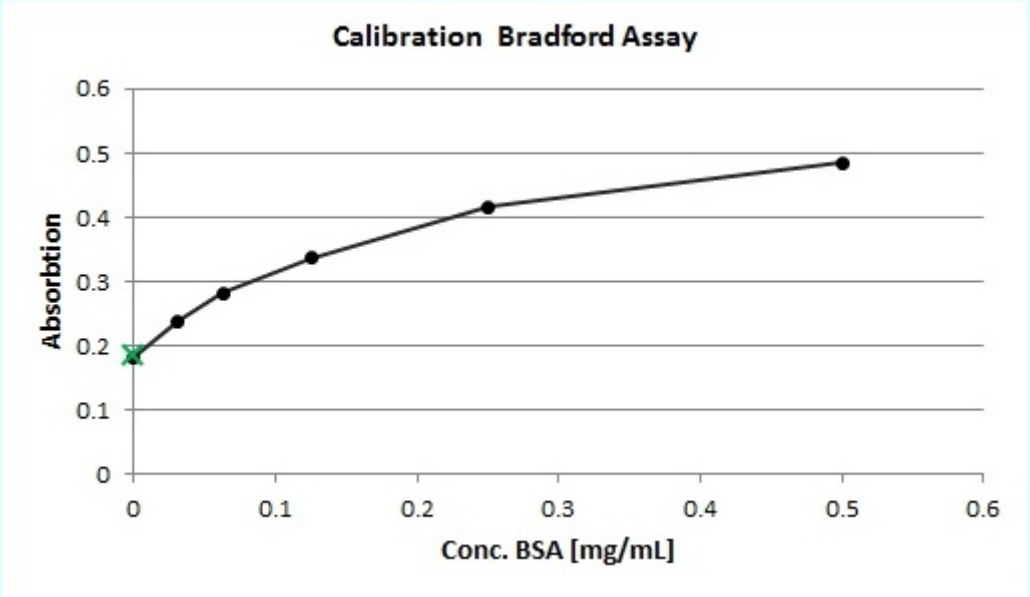


Figure 2.12: Bradford Assay calibration curve (black) and the result of the leaching study (green cross). There is no protein in the samples hence no enzyme dissolved from the IL into the solvent

2.4 Conclusion

The goal of stabilizing the enzymatic amination in organic solvents was met. It was shown that sensitive cofactor dependent enzymes like transaminases can be coated with RTSPIL and applied in organic solvent remaining active. ATA-40 was coated with [Emim]Br and employed for the enzymatic amination of 4′-bromoacetophenone in MTBE. The reaction was run over 120 h with constant reaction rate. On the other hand, the reference reaction containing free enzyme stopped within 30 h. The end of the reaction is due to cofactor decomposition, but not enzyme inactivation. It was further observed, that the reaction rate is reduced to approximately the half when the enzymes is coated.

The coating method and the amount of coating material have significant influence on the reaction rate. Enzymes coated by the precipitation method show half less activity than those coated with melted ILs. Moreover, a thicker coating reduces the reaction rate due to the increased barrier the substate needs to overcome to reach the active site. While coating up to a ratio of 1:2 (enzyme:coating w/w) show activity, ratios higher than 1:5 hardly show any conversion.

Another crucial factor is the cofactor. If PLP is located with the enzyme within the coating a reaction can occur. Placing the cofactor outside, in the solvent, prevents the reaction from taking place.

In the next step it is necessary to find an optimal ratio of enzyme and coating material to increase the stability but minimize the reduction of the reaction rate. Furthermore, it is important to study other enzymes, ionic liquids and solvents to broaden the field of application. Moreover, the recycling process for the IL and renewing the enzyme and the cofactor within need to be developed. In addition, it should be tested, if the coating can reduce inhibition due to high substrate concentration and the effect of the coating thickness in a concentration range, where usually inhibition happens.

This technology can broaden the substrate spectrum for enzymes which could not be employed in organic solvent so far because the enzyme and/or the cofactor inactivates rapidly. Coating the catalyst and the cofactor enables processing of water insoluble substrates.

2.5 Experimental

2.5.1 Material

The IL was obtained from Solvionic (Toulouse, France). [Emim]Br was delivered in a 66.0wt% H_2O solution. The amino-transaminases were received from c-LEcta (Leipzig, Germany). Phosphoxal-5′-phosphate ≥97.0% (PLP), 4′-bromoacetophenone 98% (BAP), isopropylamine ≥99.5% (IPA), cyclohexanone ≥99.5% (CK), hexane 97%, acetone ≥99.5% and o-xylylendiamine dihydrochloride 95% were from Sigma-Aldrich. DL(±)α-methylbenzylamine 98% (MBA), cyclohexylamine 99.5% (CA), tert-butyl-methyl-ether 98.0%, acetophenone 99.0% (ACP) were purchased from Fluka Analytical. Triethylamine 99% (TEA) and acetic anhydride 97+% (AA) were from Acros organics. Sodium-Sulfate 99% was from Riedel-de Haën.

2.5.2 Screening of ω-transaminases with o-xylylendiamine

A spatula tip of the enzyme (∼1 mg) with 1.3 mM PLP and 4 mM o-xylylendiamine as amino donor was incubated in 1 mL TrisHCl buffer pH 7.8 in a 1.5 mL Eppendorf tube for 10 min at 30°C in the thermoshaker. After adding 3.3 mM of the substrates the samples were shaken for 24 h at 37°C with 300 rpm. The activity was determined by the color change due to the dark colored co-product.

2.5.3 Amination of BAP in buffer

The amination of **1** was done in aqueous media to synthesize the corresponding amine **2** as reference material for later analysis. 2 mg ATA-40 with 1 mM PLP and 3.7 mg MBA (3.9 mg, 30 mM) were incubated in a total volume of 1 mL Tris-HCl buffer pH 7.8 in an 1.5 mL Eppendorf tube in the thermoshaker. 2 mg BAP (10 mM) were added and the reaction mixture was shaken for 12 h at 250 rpm and 30°C. The product was extracted in 500 μL MTBE. After drying over Na_2SO_4 and spinning down the drying agent (75 s, 13500 rpm) 150 μL of the supernatant were transferred into a GC vial.

50 μL of an 8 mM cyclohexylamine were added as an internal standard (IS). The amines were derivatized by adding 15 μL TEA and 10 μL AA. The sample was analyzed by means of gas chromatography. See Chapter 2.5.8

2.5.4 Coating

Coating with melt

The IL solution was heated to 150°C and N_2 was blown over it to get rid of the water. The remaining melt was precipitated by adding ice cooled dried acetone, resulting in the formation of white crystals. The solid IL was stored in acetone in a tightly sealed container in the fridge. For the coating the necessary amount of solid IL was weighed in with acetone to protect the IL from moisture. After evaporating the acetone with an N_2 stream, the remaining crystals were melted in a water bath at 60°C. The enzyme (5 mg) (and the cofactor (0.1 mg)) were added and the mixture was stirred to a heterogeneous solution. The mixture was allowed to cool down in the fridge. As the ILs are very hydrophilic, all the steps carried out in atmosphere must be done as fast as possible. Otherwise the IL soaks water from the air and forms a solution. This procedure was adopted from literature[48].

Coating by precipitation

The required amount aqueous IL solution was weighed into a 1.5 mL Eppendorf tube mixed with the enzyme (5 mg) (and the cofactor (50μL of 20 mM stock, 1 mM for 1 mL reaction volume)). The water was evaporated by blowing N_2 on the mixture and mild heating (<50°C). The remaining concentrated solution was mixed with 100μL ice cooled dried acetone to precipitate the salt with the enzyme and cofactor in light yellow crystals. The acetone was evaporated afterwards.

2.5.5 Aminination of BAP with ILCE in MTBE

The prepared coated enzymes were placed in an 1.5 mL Eppendorf tube. A total volume of 1 mL MTBE containing the substrate **1** (10 mM) and a racemic mixture of the donor amine MBA (30 mM) was added. The reaction mixtures were shaken at 30°C at 300 rpm. Samples were taken over the time (0h, 9h, 24h, 48h, 72h, 96h, 120h). 40 μL of the supernatant were transferred in an Eppendorf tube and diluted with 260 μL MTBE. After drying over Na_2SO_4 and spinning down the drying agent (75s, 13,500 rpm) 150 μL of the supernatant were transferred into a GC vial. 50 μL of a 4 mM cyclohexylamine in MTBE were added as an internal standard. The amines were derivatized by adding 15 μL triethylamine (TEA) and 10 μL acetic anhydride (AA). The sample was analyzed by means of gas chromatography. See Chapter 2.5.8

2.5.6 Effect of coating thickness

Coatings for the transamination in MTBE were preferred in various enzyme-IL-ratios. ATA-40 was coated with both coating methods in the ratios 1:1, 1:2, 1:5, 1:10 (w/w). Reactions were run under standard conditions with these ILCEs (see 2.5.5) and samples were taken over time to determine the influence of the coating thickness and method on reaction rate and enzyme stability.

2.5.7 Effect of cofactor location

5 mg ATA-40 were coated with 10 mg IL. Once with melt and once with the precipitation method. The coated enzyme was placed in a 1.5 mL Eppendorf tube. 1 mL MTBE containing the substrate **1** (10 mM), MBA (30 mM) and PLP (1 mM) was added. The reaction mixture was shaken at 30°C with 300 rpm. Samples were taken over time to follow the conversion (0h, 9h, 24h, 48h).

2.5.8 Analysis

All the samples from the transamination were analyzed my means of gas chromatography (Clarus 5000, Perkin Elmer, Shelton, CT, USA with a CP-ChiralSil-DEX CB 25x0.25 column Agilent Technologies, Santa Clara, CA, USA). The heating program started at 120°C. With a rate of 7.0°C/min the oven was heated to 170°C where it was held for 5.00 min. The heating continued with a rate of 12.0°C/min to the final temperature of 200°C. The whole method took 14.64 min. The retention time of each substrate and product are shown in Table 2.2.

Table 2.2: Retention times of the substrates and products of the transamination of BAP with MBA

Compound	Retention time [min]
Substrate (BAP)	5.7
Product (BPEA)	14.0
Donor (MBA)	6.8 + 7.0
Co-product (ACP)	2.6
Internal Standard	5.4

2.5.9 Leaching Study

A Bradford Assay was done to detect enzyme detachment from the IL and dissolving in the solvent. Therefore the organic phase of both, the 1:1 (w/w) melt coated and the 1:1 (w/w) precipitate coated enzyme was extracted with water. The samples were analyzed according to the standard procedure[65].

50 mL of with distilled water 1:4 diluted dye reagent were prepared. Five dilutions of the BSA were prepared with concentrations of 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL and 0.0625 mg/mL were prepared. 20 μL of each standard were mixed with 1.0 mL diluted dye reagent. For the sample 10, 15 and 20μL of the extract were mixed with the diluted dye reagent. Further one blank with pure water and one blank with MTBE saturated water were prepared. All the samples were incubated for 5 min at RT. The absorption was measured at 595 nm.

3 Part II: Switchable Solvents as a Tool in Biocatalysis

In the previous chapter the challenges and benefits of running an enzymatic reaction in organic solvent were discussed. The IL coating facilitates the continuous and stable run for at least 120 h in MTBE. As a result of immobilizing the catalysts and the cofactor the separation of the reaction medium is already simplified. Further steps to purify the product are usually distillation to remove the solvent and highly volatile co-products and chromatography to ensure the required purity. These techniques are energy intense and solvent consuming. To reduce the afford for DSP new technologies are in development. Processes generating a separate phase containing the product display the ideal process. Ulijn worked on solid-to-solid reactions[66,67]. These processes do not only ease product recovery, but also positively influence the thermodynamic equilibrium. Unfortunately, solid-to-solid reactions are only applicable to certain processes with restricted solubility for the product.

Solvents having interchangeable properties can be a first approach to simplify DSP. Switchable solvents (SSol) are liquids which can change their properties depending on the atmosphere. This useful characteristic of certain liquids was discovered 10 years ago and found its first application in few chemical processes. This work will be the first to discuss potential applications in biocatalysis to ease product recovery.

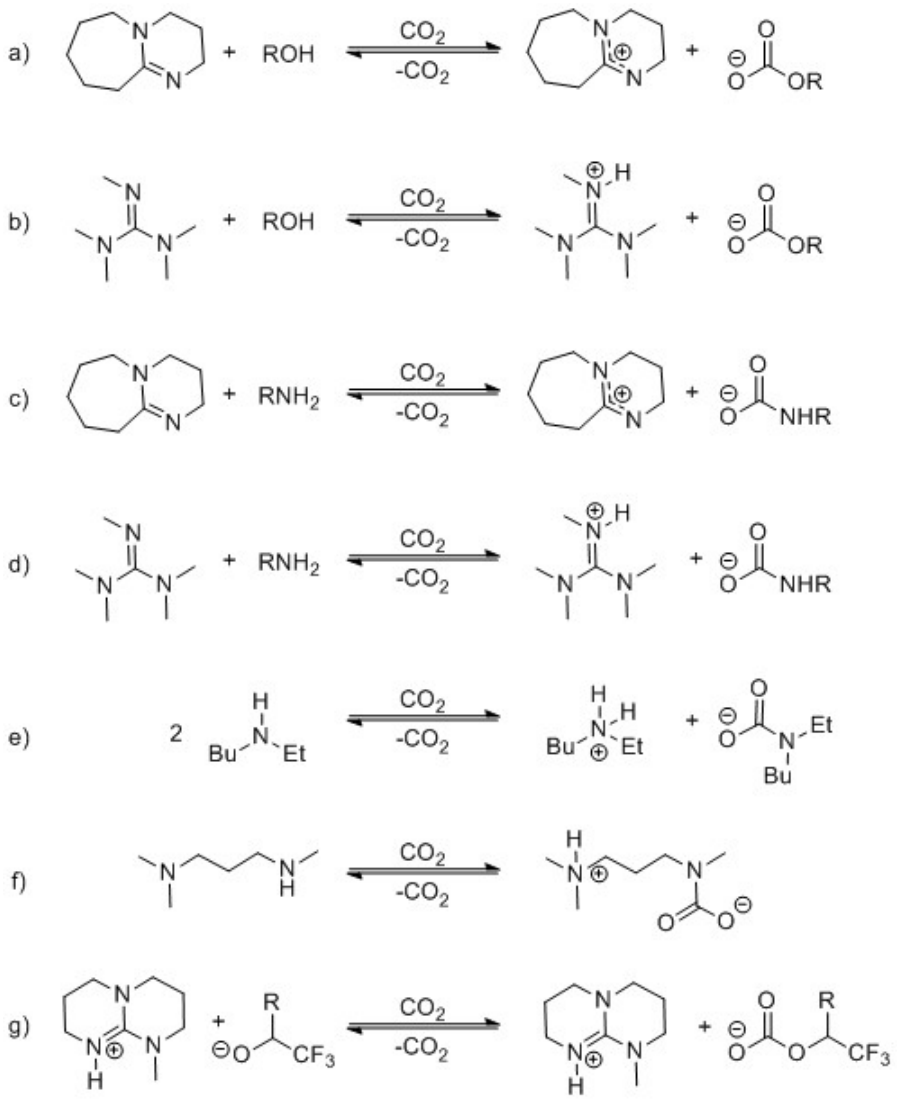
3.1 Introduction

Switchable solvents (SSol) are compounds or mixtures, which can abruptly change their physical properties, like hydrophilicity or polarity[68]. The switching is induced by bubbling CO_2 at atmospheric pressure through the system. The CO_2 reacts with a compound in the mixture leading to the formation of an ionic liquid (IL) with different properties than the initial molecular liquid. The process can be reversed by removing the CO_2 by applying mild heat (50-60°C) or bubbling inert gas (N_2) through the liquid. There are various other materials which can switch their properties, like surfactants, solutes and polymers[69]. With the help of some appropriate organic bases also water can be switched from low to high ionic strength[70]. SSol are promising for processes which require different solvent properties in consecutive steps, like extraction or product recovery. Using a solvent with switchable properties reduces the amount of solvent of a chemical process.

3.1.1 Switchable Polarity Solvents (SPS)

Switchable Polarity Solvents (SPS) are solvents, which can change from low to high polarity when treated with CO_2. This type of solvents was first described by Jessop in 2005[71]. SPS can either contain only one component, secondary amines[72], or consist of two components. Two component SPS mostly consist of either an amidine or a guanidine and either an alcohol[73] or a primary amine[74–76]. Nevertheless, there are some more examples of SPS, like diamines or guanidine/acidic alcohol mixtures. (Scheme 3.1)

Each SSol system has its characteristics. These properties must be considered, when choosing a SSol for a certain reaction. While systems containing the amidine 1,8-diazabicyclo-[5.4.0]-undec-7-ene (DBU) (Scheme 3.1 **a** and **c**) are very sensitive to moisture, secondary amine SPS and other amidines show less water sensitivity. Another advantage of single component SPS is the fact that they do not require the operator to monitor the molar ratio of the two liquids. In addition to that, secondary amines are available for much lower prices than DBU or amidines. On the other hand, due to their reactivity amines are not suitable as solvent for reactions involving acidic or alkylating



Scheme 3.1: Selection of compounds and mixtures possessing switchable polarity: amidine/alcohol **a**, guanidine/alcohol **b**, amidine/primary amine **c**, guanidine/primary amine **d**, secondary amine **e**, diamine **f**, amidine/acidic alcohol **g**[69]

agents[72].

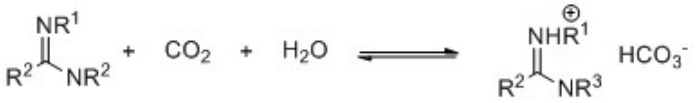
The changes of polarity through treatment with CO_2 can be shown by mixing and separating the SPS and a non-polar VOC. Depending on the SPS system ILs with different polarity are received. While short alkyl chains on alcohols and amines yield highly polar ILs, comparable with methanol, long alkyl chains reveal minor changes in polarity and yield less polar ILs. This fact influences the miscibility of the ILs with other solvents. Phan et al showed that the IL received from a DBU/ethyl alcohol mixture is

immiscible with hexane, toluene and ethyl acetate[73]. Alcohols with longer carbohydrate chains only separated from hexane. DBU and 1-decyl alcohole was miscible with all the tested organic solvents. Interestingly, the length of the alkyl chain on the cationic position (amidine) does not influence the polarity of the IL, like it is observed in most ILs[76].

The chain length also influences the viscosity and melting point of the obtained IL. ILs prepared from DBU and methanol or ethanol are solid at RT, while mixtures of alcohols with longer chains result in salts with melting points around room temperature[73].

3.1.2 Switchable Hydrophilicity Solvents (SHS)

Switchable Hydrophilicity Solvents (SHS) can switch from hydrophobic to water miscible solvents. In the presence of water some amidines can react with CO_2 yielding a water-soluble bicarbonate salt[77]. (Scheme 3.2)



Scheme 3.2: Reaction scheme of amidine as SHS reacting with CO_2 and water yielding a water soluble bicarbonate salt.

Besides amidines, tertiary amines and bulky secondary amines possess the capability to switch their hydrophilicity. In contrast to primary and non-bulky amines, which can form bicarbonate and carbamate salts, tertiary and bulky secondary amines can only produce bicarbamates[78]. (Figure 3.1). This phase behaviour can be applied for extraction and subsequent product recovery.

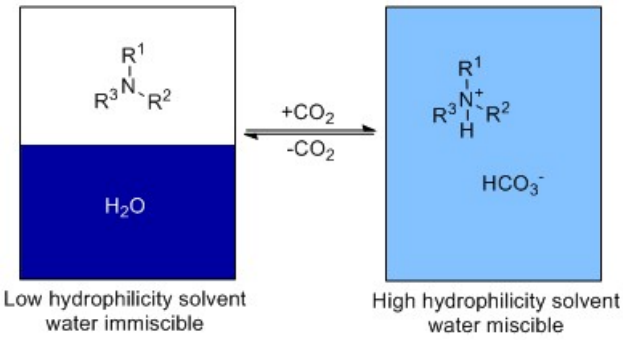


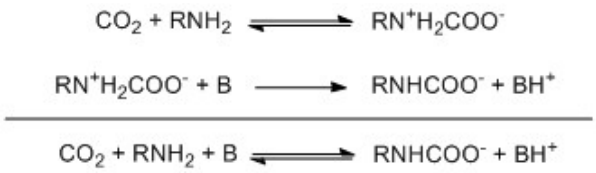
Figure 3.1: Tertiary amines which can switch their hydrophilicity by reversibly adding and removing CO_2 [79].

3.1.3 The switching reaction

The formation of the covalent bond between the reactant and CO_2 is an exothermic reaction. Depending of the reacting amine or alcohol and hence the strength of the formed bond, already small amounts of the reaction mixture can heat to approximately 60°C. For reversing the reaction and switching the IL back to a molecular liquid, the previously released amount of energy must be provided by the surrounding.

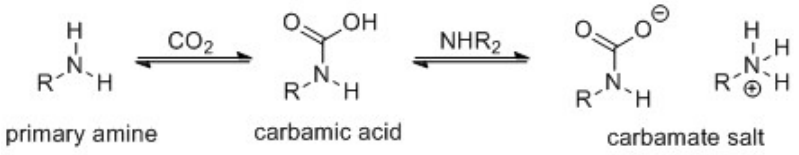
Amine reacting with CO_2

Two reaction mechanism are widely used to describe the kinetic of CO_2 reacting with an amine[80]. The first mechanism described by Caplow and reintroduced by Danckwerts, is the zwitterion mechanism. In the first step in this two-step mechanism the amine and CO_2 form a molecule with two charged functional groups, the zwitterion. In the consecutive step, an amine-proton of the zwitterion is transmitted to a second molecule, a base like another amine, amidine or guanidine. The overall product is a carbamic salt (carbamate anion + ammonium cation)[81,82]. The two steps of the reaction are depicted in Scheme 3.3.



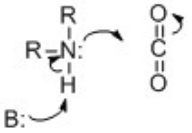
Scheme 3.3: Zwitterion mechanism of an amine reacting with CO_2. Step 1: Amine and CO_2 form zwitterion. Step 2: Amine-proton of the zwitterion is transferred to a second molecule, a base like another amine, amidine or guanidine.

If the amine-proton of the intermediate is transferred to another primary amine, the reaction results in a solid salt. (Scheme 3.4)



Scheme 3.4: Reaction scheme for a primary amine reacting with CO_2 resulting in a solid carbamate salt. One molecule harboring a primary amine binds one molecule CO_2 covalently forming carbamic acid. The carbamic acid reacts with another primary amine resulting in a carbamic salt (carbamate anion + ammonium cation).

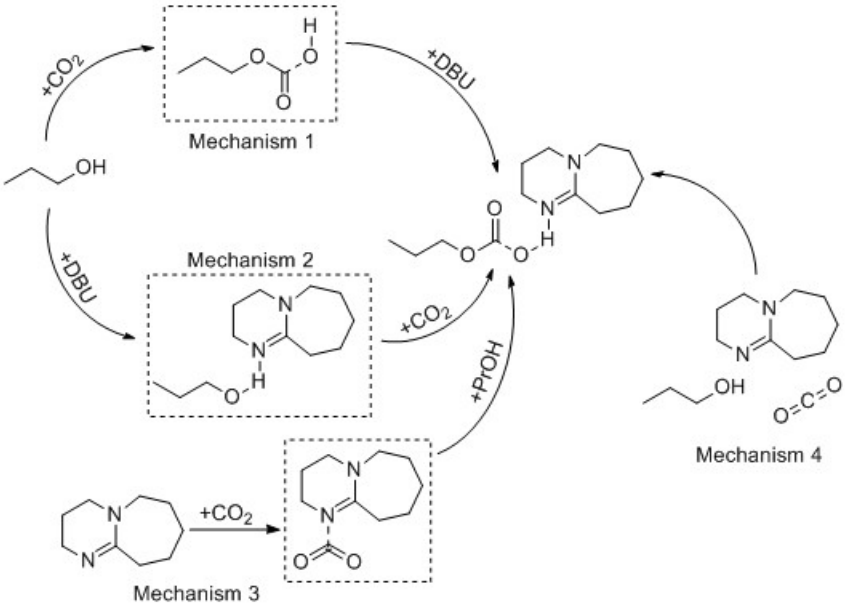
The second mechanism is a single-step mechanism, called the termolecular or 3-molecular reaction mechanism, where all reactants react concerted. (Scheme 3.5)



Scheme 3.5: Termolecular or 3-molecular mechanism of an amine reacting with CO_2. All three reactants react concerted in a single step[83].

Alcohol reacting with CO_2

The reaction mechanism of CO_2 binding to an alcohol has not been fully investigated yet. There are a few suggested mechanism, two-step bimolecular mechanisms as well as one-step trimolecular reactions[84]. Possible mechanisms are shown in Scheme 3.6.



Scheme 3.6: Suggested reaction mechanisms for alcohols reacting with CO_2 in a two-component SSol with an amidine. Mechanism 1, 2 and 3 are two-step bimolecular reactions with the intermediates in dashed boxes. Mechanism 4 shows a the one-step trimolecular reaction [84].

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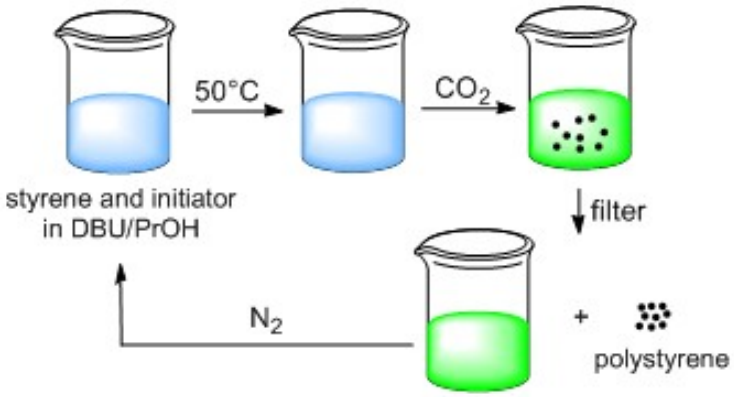
3.1.4 Switchable Solvents as reaction media

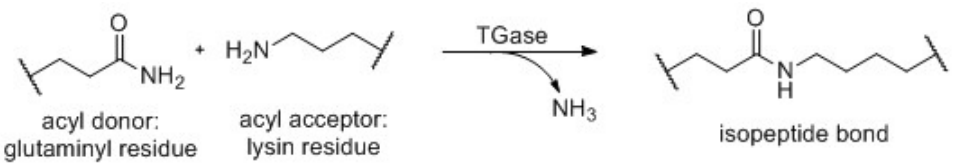
So far switchable solvents have already been applied as reaction media for chemical reactions. Phan et al ran a polymerization of styrene in a SPS of a 1:2.5 mixture of DBU and 1-propanol receiving 97% overall yield in four cycles [73]. (Scheme 3.7) Hart et al carried out a Claison-Schmidt condensation and a Heck reaction in RevILs [85]. Mercer et al used SW for recycling homogeneous catalysts [86]. Furthermore, Xue et al successfully transersterified soybean oil to produce biodiesel in SPS [87].



Scheme 3.7: Example of using SSol as reaction media for chemical synthesis. SPS for styrene polymerization: All components (styrene, initiator, SPS) are mixed and heated to 50°C to start the polymerization. After the reaction the SPS is switched by bubbling CO_2 through the system to precipitate the polymerized product. After product recovery by filtration, the solvent is switched back to reuse it [73].

3.1.5 Transglutaminases

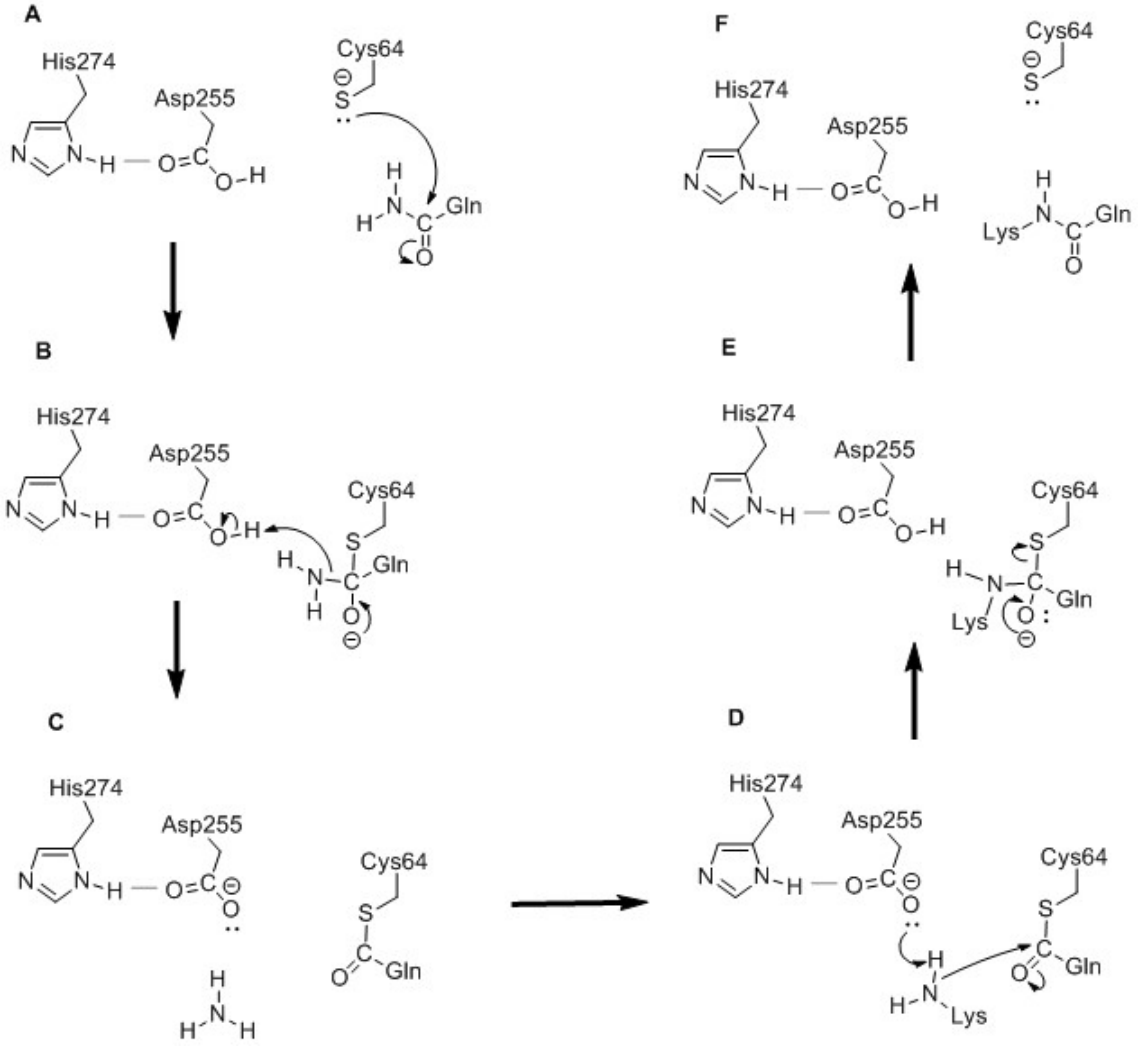
Transglutaminase (TGase; protein-glutamine γ-glutamyl-transferase, EC 2.3.2.13) catalyzes the acyl transfer between a γ-carboxyamide group of a peptide-bound glutaminyl residue (acyl donor) to a primary amine (acyl acceptor). The result is a isopeptide bond. (Scheme 3.8) In the natural reaction of TGases the ϵ-amino group of lysine represents the acyl acceptor [88]. These enzymes naturally catalyze the cross-linking of proteins to form insoluble protein aggregates. In food and textile industry transglutaminases are commercially used to alter the texture and properties of products by cross-linking of proteins [89].



Scheme 3.8: Isopeptide bond formation catalyzed by TGase. The acyl residue of glutamine is transferred to the primary amine of lysine releasing NH_3.

The ping-pong mechanism of the acyl transfer underlies a catalytic triade of histidine, asparagine and cysteine. The mechanism consists of three phases. In the first step cys-

teine binds the glutamine substrate. With the formation of an acyl-enzyme intermediate an ammonia molecule dissociates. In the final phase the acyl-enzyme intermediate reacts with an acyl acceptor, in most cases a primary amine, and forms the γ-glutamyl-amine product[90]. (Scheme 3.9)



Scheme 3.9: MTGase reaction mechanism: Step 1: Cysteine binds glutamine substrate **(A)**. Step 2: Formation of acyl-enzyme intermediate and release of ammonia molecule **(B+C)**. Step 3: Acyl-enzyme intermediate reacts with acyl acceptor (primary amine of lysine residue) and forms the γ-glutamyl-amine product **(D+E)**. Release of the final product **(F)**[91].

TGases can be found in microorganisms, plants as well as in mammals. In animals and human a calcium-dependent TGase has been identified, referred to as TGase 2 (TG2). The microbial TGase (mTGase) on the other hand is calcium-independent. The

smaller, more robust mTGase is employed in industry, while TG2 has greater medical importance[92].

TGase in industry

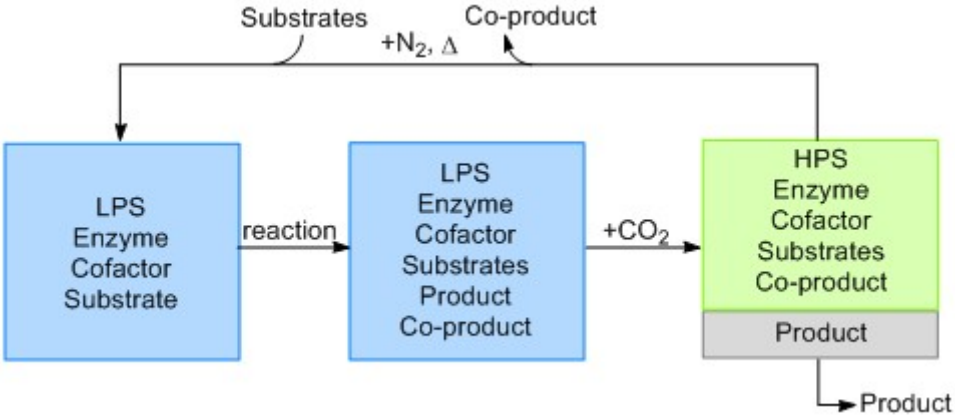
Microbial TGase are not only imperative for food technology[93], but also gaining more and more importance in pharmaceutical industry. Due to the increasing use of biologicals in medical treatment, new production technologies are required. As protein drugs possess shortcomings limiting their use, like high susceptibility to proteolysis, short circulating half-life in vivo and rapid kidney clearance, strategies are sought to overcome these problems[94]. Nowadays, conjugation of protein drugs with natural or synthetic polymers is considered as a practical methodology[95]. One method for chemical protein conjugation is the covalent linkage of polyethylen glycol (PEG) to the protein. However, this methodology is limited due to not site-specific conjugation and hence heterogeneity of the products[96]. Biocatalysis can help linking polymers and proteins specifically.

3.2 Switchable Solvents for biocatalysis

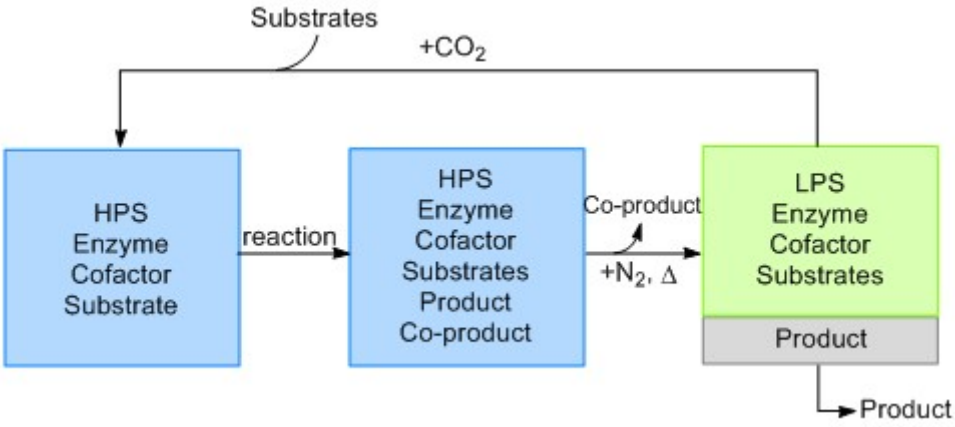
3.2.1 SPS in biocatalysis

The variable polarity of SSol can be employed to simplify product recovery after synthesis like described by Phan et al for styrene polymerization[73]. If the solubility of the product is greatly different in both states of polarity, like in that process, the product can be separated from the reaction mixture by switching the solvent. This theory cannot only be applied to traditional chemical synthesis, but also to biocatalytic processes. In the perfect case the product separation during switching would happen by precipitation in a pure form. The general overall principle for product recovery in a biocatalytic process by switching the solvent from low to high polarity is shown in Scheme 3.10, the principle for switching from high to low polarity is shown in Scheme 3.11. For both schemes it is assumed, that almost all the substrate is transformed into product. The small amount left after the reaction is assumed to be soluble in the switched solvent, while the product is present in too high concentration exceeding the solubility limit, precipitates and hence can be removed by filtration. Co-product removal is sketched parallel to the switching to low polarity like it can be carried out for a highly volatile co-product (bp$<$60°C).

The principle of switching from low to high polarity can be applied for processes where two molecules are linked together like a polymerization. The product, bigger than the substrates, is often less polar and hence less soluble in polar solvents.



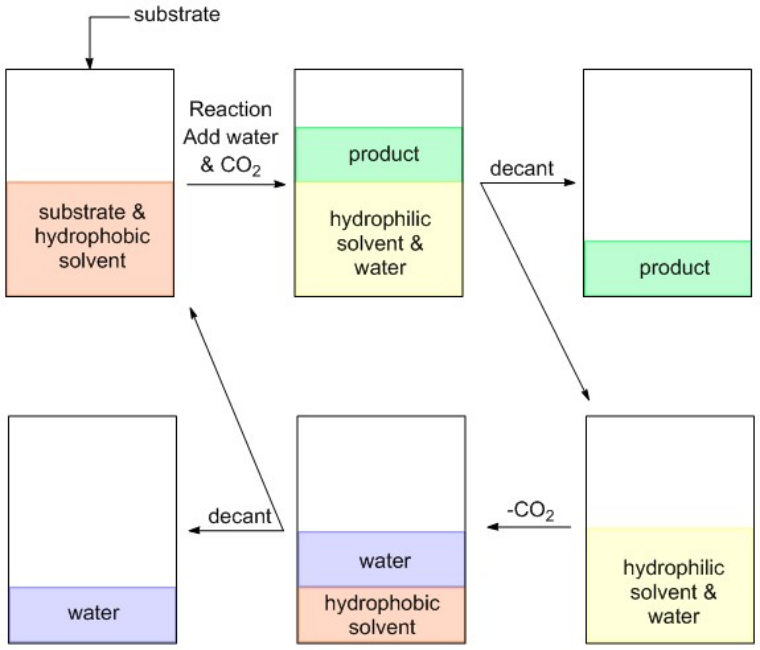
Scheme 3.10: Principle of using SPS switched from low polarity solvent (LPS) to high polarity solvent (HPS) for product recovery. Assumptions: Substrates is almost entirely converted to the product. The small amount left is soluble in the switched solvent. Product concentration exceeds solubility limit, precipitates and can be removed by filtration. The co-product is highly volatile and can be evaporated during the second switching step.



Scheme 3.11: Principle of using SPS switched from high polarity solvent (HPS) to low polarity solvent (LPS) for product recovery. Assumptions: Substrates is almost entirely converted to the product. The small amount left is soluble in the switched sovent. Product concentration exceeds solubility limit, precipitates and can be removed by filtration. The co-product is highly volatile and can be evaporated during the second switching step.

3.2.2 SHS in biocatalysis

The principle of using SHS for extraction was described by Jessop and coworkers[77]. The changeable hydrophilicity provides the ability to reversibly mix and demix the solvent with water. This property can be used to process hydropholic compounds. The switchable water miscibility of the SHS simplifies the product recovery. The water insoluble substrate can be solubilized in the hydrophobic SSol agent to run the reaction. For product recovery water is added and the SSol is switched. Thus, the switchable agent amidine dissolves in the aqueous phase and the reaction product as well as the substrate remain in a water immiscible phase. After product recovery the SHS can be switched back, demixes from the aqueous phase and can be recycled. (Figure 3.12) This strategy for product recovery has already been described[69]. For this process the used enzymes should be immobilized to remove it before adding water. Otherwise it would be taken out of the process with the water in the last step and would get lost.



Scheme 3.12: Principle of using SHS in biocatalysis: Hydrophobic substrates can be dissolved and processed in the hydrophobic solvent. Afterwards water is added and the SHS is switched to hydrophilic, whereby it mixes with the aqueous phase and leaves the product in a separate phase. After product recovery the solvent is switched back and hence separates from the water, which can be removed. The SHS can now be used for the next process cycle[69].

3.2.3 Challenges utilizing SSol in biocatalysis

At first glance a changeable polarity opens up new opportunities considering catalyst recycling and product recovery. A non-polar substrate can be dissolved in the low polarity form and after running the reaction the non-polar product can be separated from the mixture by switching the solvent to higher polarity. But the application of this technology is not simple. The process must be well designed and solvent, substrate and product properties need to be fully understood. Due to the reactivity of SPS components and the desired separation during the switching reaction, SPS media can only be used for a limited number of applications fulfilling the following criteria.

Criteria an enzymatic reaction mixture needs to meet to enable the use of SPS as reaction media:

- High substrate solubility in the initial polarity state
- Low product solubility in the switched polarity state
- No functional group which reacts with the switching agent CO_2 (amine, alcohol)
- Inability of substrates, products and catalyst to react with a component of the SPS system spontaneously
- Stability of SPS components under reaction conditions

These challenges and possible solutions for various biocatalytic processes will be discussed in the following section.

3.2.4 Potential applications of SSol in biocatalysis

SSol are potential reaction media for various enzymes and substrates. This section will give a few examples. The application of SPS using a solvent system containing a primary amine will be discussed first. Then the use of SPS for enzymatic polymerization with transglutaminases and SHS for processing hydrophobic substrates with lipases will be described briefly.

Transaminases

Transaminases (see Part I) catalyze a highly relevant reaction for synthesizing chiral amines in fine chemical and pharmaceutical industry. In this reaction an amino group is transferred to a ketone resulting in the formation of the corresponding amine, while the donor amine is oxidized to a ketone. As donors usually small, inexpensive amines like IPA, MBA or BA are used. However, the transfer of the amino group from these donors to more complex acceptors is often thermodynamically not favored. One possibility to shift the equilibrium to the product side is in-situ product removal (ISPR) where the ketone co-product is removed from the reaction mixture during the process. Another way to push the equilibrium towards the product side is the excessive use of donor.

As mentioned in the introduction some SSol systems contain primary amines. These amines are usually small molecules like those used as donors in transamination reactions. If one of these solvent systems was used for transamination, the donor would automatically be present in a desired excess to increase the conversion and additionally, the switchable properties of the solvent might simplify product recovery.

Substrate solubility and concentration

To significantly influence the conversion of a thermodynamically not favored transamination reaction and raise the yield from 5% to 90% an 5000-fold excess of the amino donor is necessary[57], as already mentioned in the introduction 2.1.2. This means that for converting 1 mmol of a substrate (4′-bromoacetophenone, MW: 199.04 g/mol) 5 moles amino donor (IPA, MW: 59.11 g/mol, density: 0.69 g/mL) are necessary. Thus, 199 mg

of substrate would be dissolved in 300 mL of IPA. As the amine is only one component in the SPS system, additional 5 moles of amidine (butyl-dimethylacetimidamide, MW: 142.15 g/mol, density: 0.855 g/mL) would be present resulting in a solvent volume of more than 1 L and a substrate concentration of 1 mM.

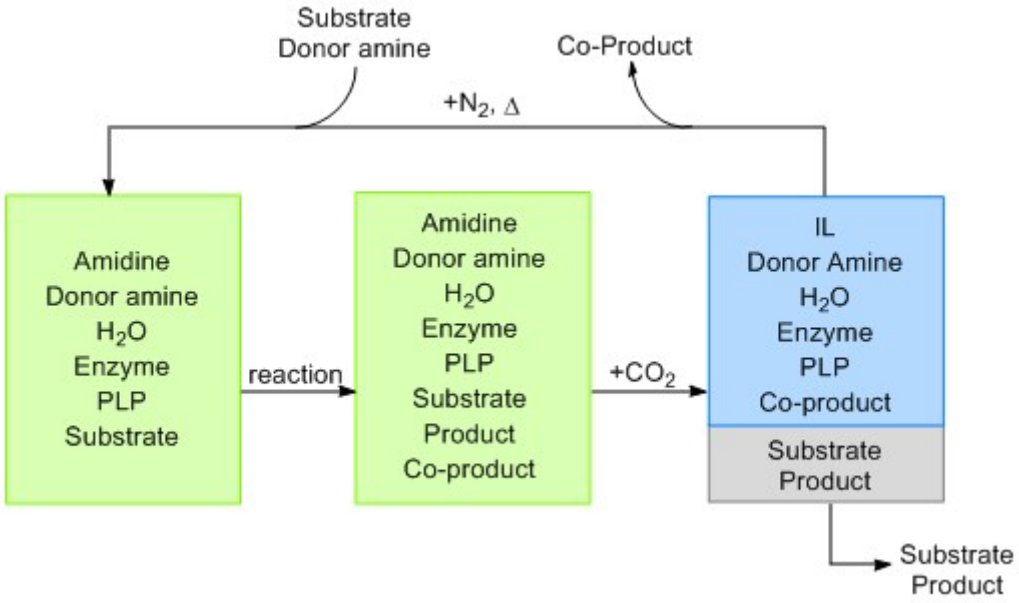
For such low substrate concentrations it is unnecessary to run the reaction in SSol. On the one hand such low concentrated reactions are undesirable due to low space-time yield and high energetic effort to process the large quantities of solvent. Moreover, transformations in this concentration range can be run in environmentally friendly buffer systems. The last point speaking against low substrate concentrations in SPS systems is the switching and separation step following the reaction. If the substrate concentration is very low, the product will also appear in a diluted solution, what hinders the separation by precipitation.

Nevertheless, the excess of donor can effect the thermodynamic equilibrium, even for higher substrate concentrations.

The substrate needs a solubility high enough to exceed the product solubility in the switched solvent taking the percentage of turnover into account. This point is closely related to the solubility problem in solid-to-solid reactions[66,67].

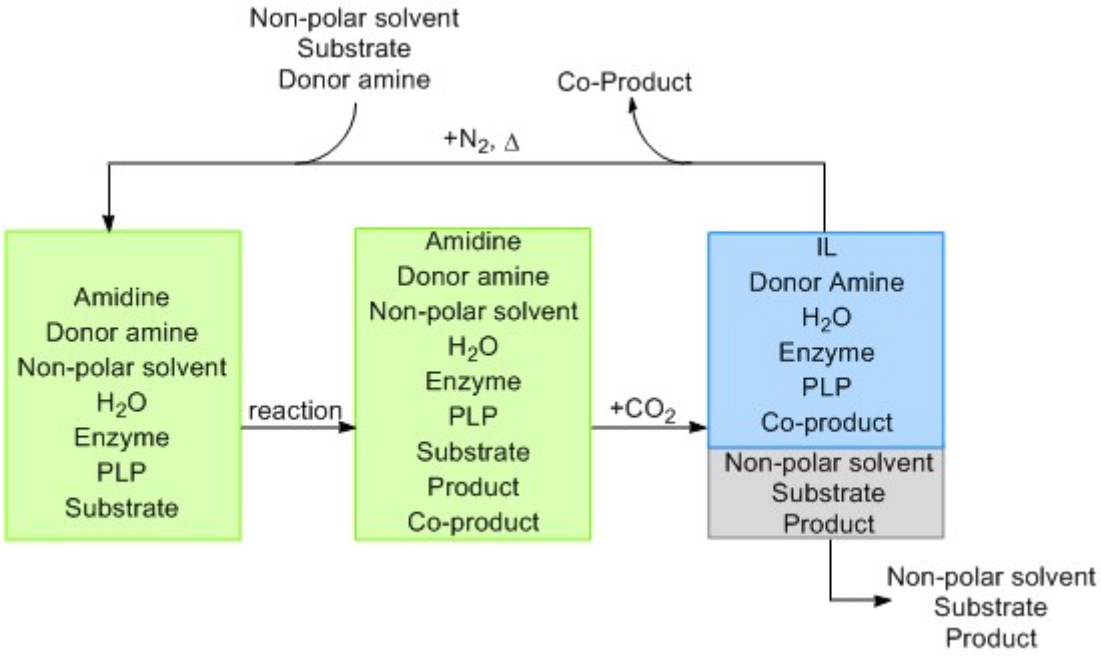
Product solubility in switched state

The product solubility should be as low as possible in the high polarity state. In the ideal case the product concentration in low polarity solvent (LPS) exceeds the solubility in the high polarity solvent (HPS) leading to the precipitation of the product during the switching process. Due to the fact, that the ketone has a lower logP than the corresponding amine, the substrate would also precipitate. (Scheme 3.13)



Scheme 3.13: Application of SPS for transaminases: The reaction runs in LPS. Afterwards the solvent is switched to HPS. The substrate and product precipitate due to low solubility and can be separated by filtration, while enzyme and the cofactor remain in the SPS. During switching back to the LPS heat is applied and the highly volatile co-product can be removed. New substrate and donor amine are added before another reaction cycle can be started.

If the product is too soluble in the HPS product recovery can the achieved by using a non-polar organic solvent which separates readily from the switched solvent but is miscible with the LPS. This process would be like an extraction. Substrate and product need to be separated in an additional step. (Scheme 3.14)



Scheme 3.14: Application of SPS for transaminases: The reaction runs in LPS mixed with a non-polar solvent. Afterwards the solvent is switched to a HPS and the non-polar solvent forms a separate phase containing the product. The phases are separated and the SSol is switched back to LPS. During switching back the highly volatile co-product evaporates due to the applied heat. After adding new substrates and non-polar solvent a new reaction cycle can be started.

Functional group reacting with CO_2

As transaminases transfer the amino group from donor to the ketone, not only the donor, which is part of the SSol is present in the mixture, but also the product, a primary amine able to react with CO_2 and become a part of the ionic liquid. According to Pollet one-component reversible ionic liquids (RevIL) "employ solely a neutral molecule containing at least one basic nitrogen functionality"[68]. It has also been shown that most amines which show switchable hydrophilicity behavior have a pKa above 9.5[97]. Further it is reported that only aliphatic amines can react with CO_2 but aromatic amines with pKa values less than 5 cannot[98]. With the knowledge that amines with higher pKa values will react more readily with CO_2 than amines with a lower pKa, one can clearly define the substrate properties. The donor should have a high pKa so it readily reacts with CO_2. However, the product must have a low pKa to protect it from the reaction. The pKa of

an amine is influenced by the nearby atoms in the molecule. An electron withdrawing group (EWG) near the amino group can lower the pKa, while electron donating groups like aliphatic chains have hardly no effect on the pKa, but revoke the pKa lowering effect of an EWG. EWGs are for example halides, keto-, ester-, acid- and azo-groups as well as nitro-groups. With that knowledge it is possible to design a product molecule with an adequate pKa. With the software ACD/Labs the prediction of the pKa values of various molecules is possible. Figure 3.2 shows a selection of molecules with the predicted pKa to demonstrate the effect of the various EWGs.

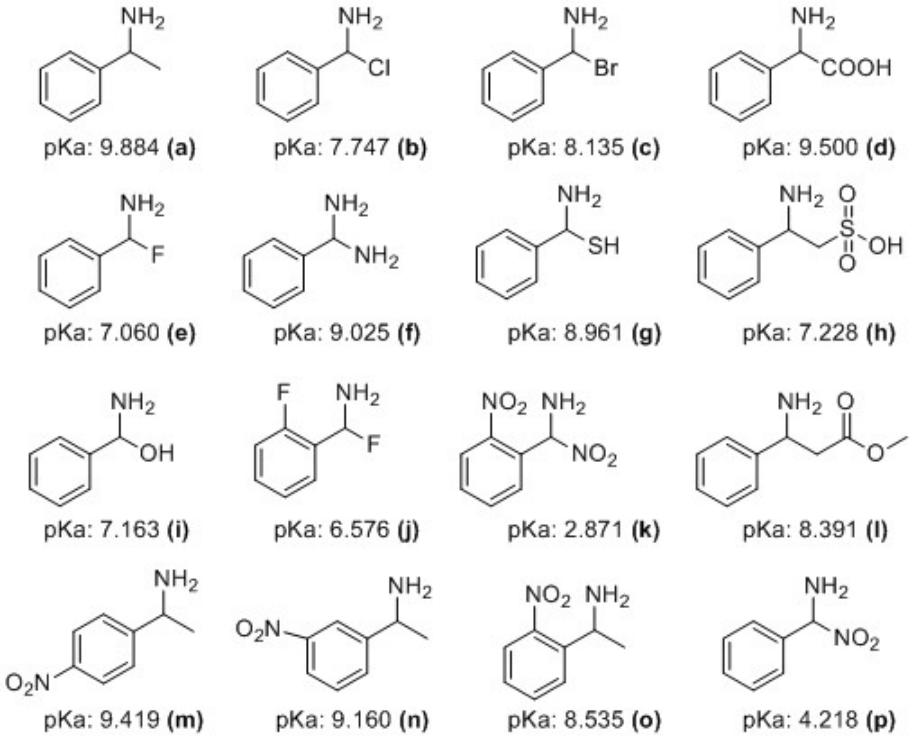


Figure 3.2: Influence of different EWGs on the pKa of a primary amine: Selection of various functional EWGs in various positions on the molecule to show their influence on the pKa-value of the primary amine (pKa-values predicted via ACD/Labs)

It is clearly visible that the nitro group in α (g) has the greatest effect on the pKa value leading to a pKa of 4.2 in comparison to a pKa of 9.9 without an EWG, followed by the fluorid (d). Another amino group and bromine hardly have any influence. Two EWGs in the molecule can lower the pKa even to 3.7 (j) or 2.9 (k). According to the predicted pKa values some molecules (d, g, j-l) should react slow or even not react with

CO_2.

In the next step various substrates were designed and their properties were predicted. (Table 3.1)

According to Penny the formation of carbamate from CO_2 and primary amines follows a second order reaction; first order in CO_2 and amines, respectively[99]. It was also shown, that the second-order rate constant is directly dependent on the pKa of the amine, following the Brønsted relation in equation 3.1

$$log_{10}k = 0.34pK_a + 0.45 \tag{3.1}$$

Starting from this equation one can estimate the difference in the reaction rate for the product and the donor amine, leading to a rate constant of k_7=524.81 for the product and k_{IPA}=11,482 for IPA.

Table 3.2: Calculated rate constants for carbamate formation according to the equation of Penny[99]

Substrate	pK_a	$log_{10}k$	k $[dm^3mol^{-1}s^{-1}]$
6	6.67	2.72	525
7	6.60	2.69	494
IPA	10.63	4.06	11,482

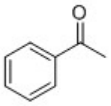
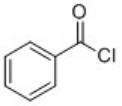
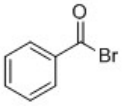
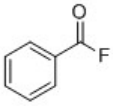
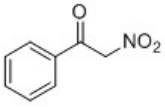
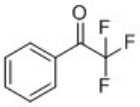
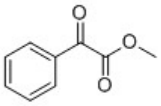
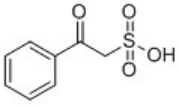
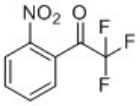
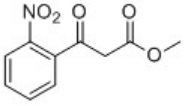
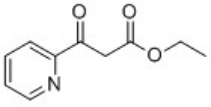
If the product does not possess a pKa value low enough to react slower than the donor amine or to react not at all, the amino function can be protected by in-situ derivatization. This can for instance be done by another enzymatic reaction. Ideally the derivatization is enantioselective and only the product amine is converted, but not the donor.

Stability of SPS under process conditions

Switchable solvents containing DBU are very water sensitive and hence cannot be used for transaminases because a small amount of water is necessary for the mechanism. (Scheme 2.3) Nevertheless, aliphatic amidines can contain up to 3 wt% water but still form a long persisting ionic liquid when CO_2 is bubbled through[74].

The used amidines are rarely available from general suppliers, so it has to be synthe-

Table 3.1: Selection of prochiral ketone substrates for transamination in SPS. Prediction of logP, Product pKa and logP by ACD/Labs

Entry	CAS	Substrate	MW [g/mol]	logP	Product pKa	logP
1	98-86-2		120.15	1.63	9.04	1.35
2	98-88-4		140.57	2.25	6.24	1.16
3	618-32-6		185.02	2.36	6.24	1.56
4	455-32-3		124.11	1.63	6.13	1.04
5	614-21-1		165.15	1.35	6.41	0.89
6	434-45-7		174.12	2.40	6.15	1.40
7	15206-55-0		164.16	1.42	6.70	0.98
8	4496-92-8		200.21	-0.68	7.94	-1.23
9	17408-17-2		219.12	1.95	4.91	1.61
10	61158-02-9		223.18	1.37	6.44	1.10
11	26510-52-1		193.20	1.71	6.51	1.40

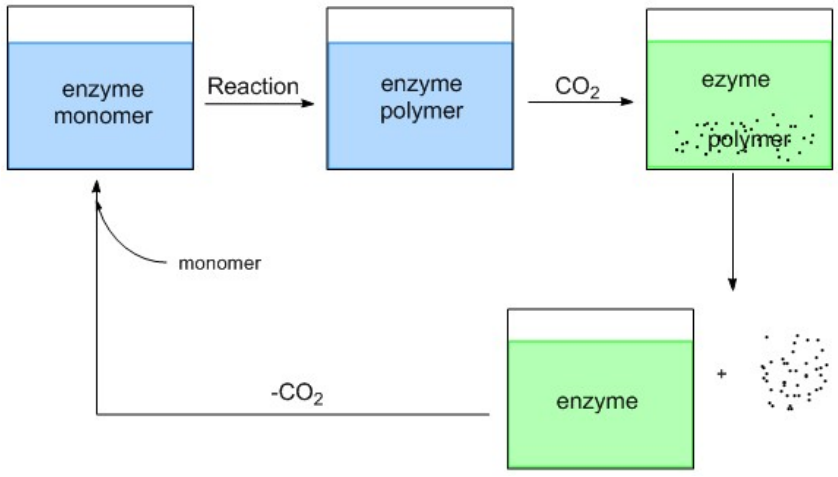
sized on one′s own. Depending on the desired molecule the chemical synthesis is quite simple[100].

From the last few pages it is apparent that designing a reaction in a SSol is not easy. Many factors must be considered and a lot of knowledge about the reaction mechanism, the reactivity of the substrates and the reaction medium need to be gained beforehand.

All the factors which need to be considered and the mentioned challenges from this section are also valid for the following examples.

Transglutaminases

The principle of this reaction system for enzymatic polymerization founds on the chemical analogue[73]. The substrate monomer and the initiator, in this case the enzyme, are placed in the one polarity form to run the reaction. Afterwards the medium is switched to the other polarity state to seperate the polymer. The solvent is recycled. (Scheme 3.15)



Scheme 3.15: Enzymatic BSA polymerization with mTGase in SPS: The enzyme and the monomer are mixed with the LPS. After running the reaction the solvent is switched to the HPS. The polymer is not soluble in the HPS and precipitates. Simple filtration separates the polymer from the solvent. The solvent is switched back to the LPS and a new cycle can be started by adding new monomer.

3.3 Results

The very first idea was the application of transaminases in SSol in the way it is described in 3.2.4. Throughout the planning process of the reaction more and more challenges appeared. After a comprehensive analysis of the reaction system and the limits it is clear that developing this transaminase process would exceed the temporal and financial resources of this thesis. For this reason the experimental work was restricted to the investigation of the feasibility of applying enzymes in SSol considering the catalysts stability.

The example reaction chosen is the polymerization of proteins using tranglutaminase because transgluaminases are relatively stable enzymes which do not need any cofactor and the polymer product might differ from the substrate in a way it is possible to separate them by switching the solvent.

As a reference and to determine the best enzyme-substrate ratio the trials where carried out in an aqueous buffer system. After 72 h reaction time the reaction mixture was analyzed by means of SDS-PAGE to determine the progress. (Figure 3.3)

On the left hand side of the gel samples from the casein polymerization are shown. The first sample containing only the substrate shows that the pure casein is not visible on the gel. In the other four casein samples the polymerization product is on the top of the gel. On the right hand site the product of the BSA polymerization is visible. Again, the band at the very top stems from the polymer, while all the bands below come from the substrate. As the reaction worked fine in the buffer the medium was switched to a SSol system consisting of the synthesized amidine and ethanol.

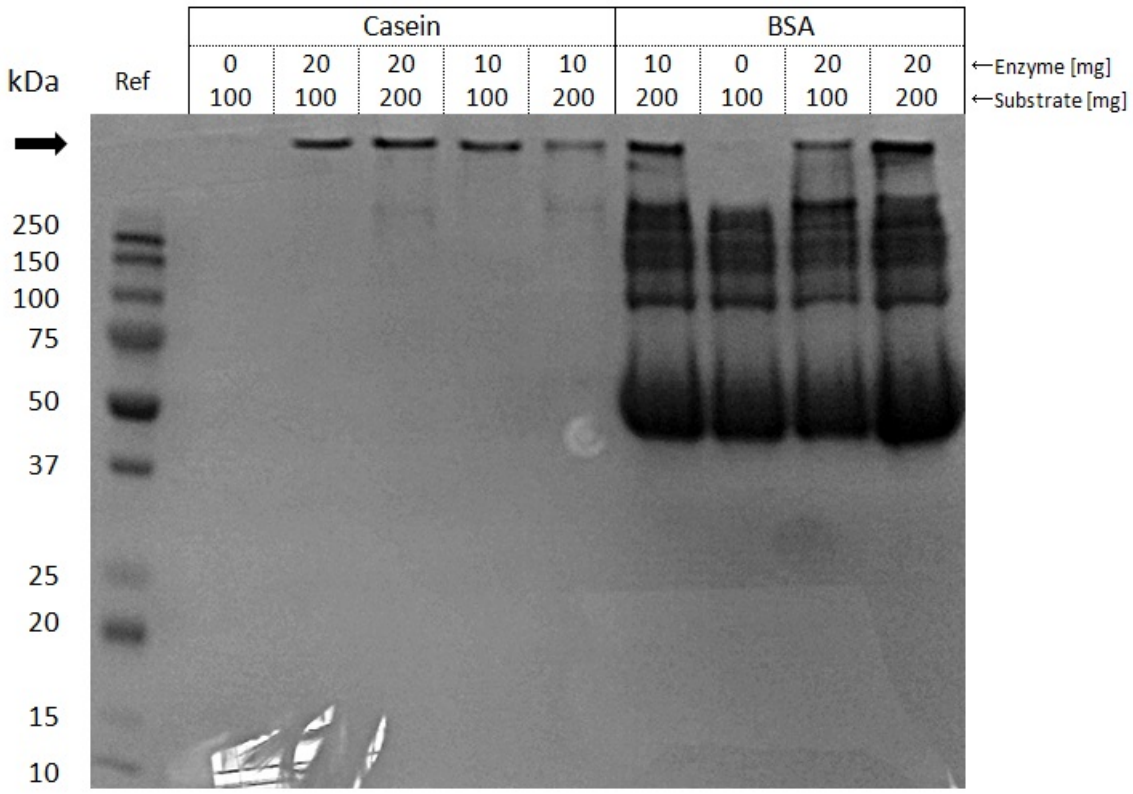


Figure 3.3: SDS-PAGE analysis of Casein and BSA Polymerization in buffer

For the first reactions in a SSol a variety of systems was chosen to find out which works best. For both substrates (Casein and BSA) two reactions were carried out, one in a low polarity solvent (amidine + EtOH 1:1 n/n not switched) and one high polarity solvent system (amidine + EtOH 1:1 n/n switched with CO_2. (Figure 3.4)

The analysis revealed that only one reaction set-up led to a reaction with detectable yield. The sample of the high polarity system with BSA shows the polymer band at the top of the gel. However, both reaction with casein, in high and low polarity solvent as well as the LPS reaction with BSA did not yield any product. After 76 h reaction time each reaction mixtures were switched to the other polarity state, respectively. No changes were observed while switching process. The change in polarity is too little to have an influence on solubility on the substrate or the product.
For all the following reactions BSA was used as substrate because it is easier to evaluate

the reaction progress if the substrate is also visible on the gel.

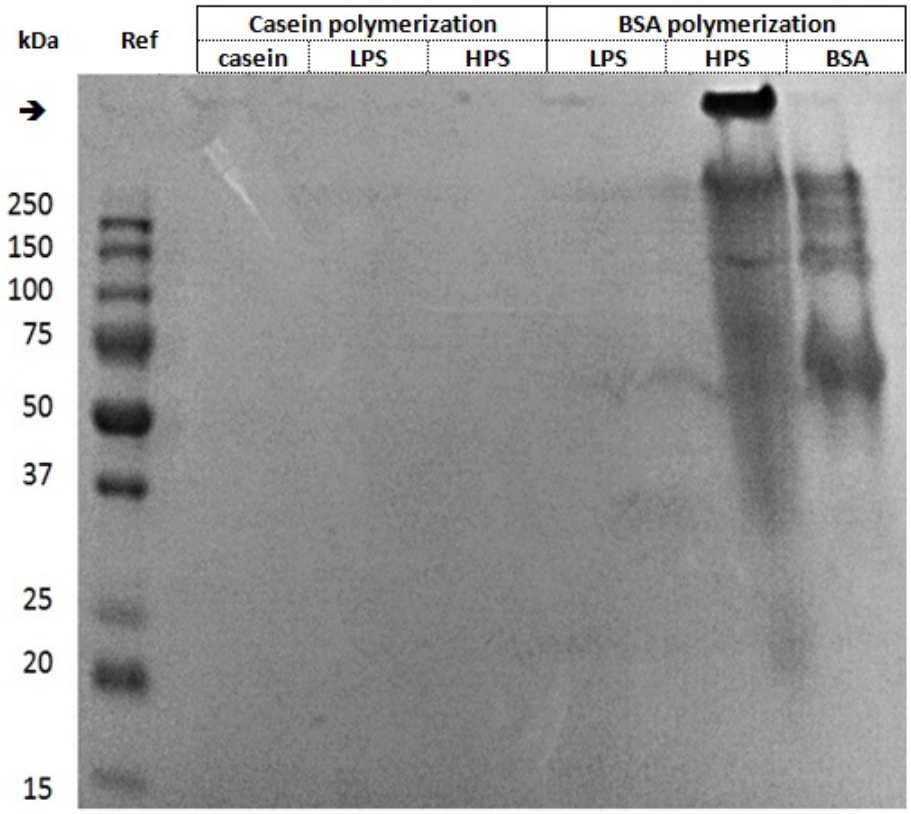


Figure 3.4: SDS-PAGE analysis of samples of the polymerization with of Casein and BSA with mTGase in various SSol systems. Left hand side: samples from the polymerization of casein; the substrate, in low polarity solvent (LPS), in high polarity solvent (HPS). Right hand side: samples from the polymerization of BSA; in LPS, in HPS, substrate.

To investigate the long term stability of the enzyme in the SSol three reactions were run for 96 h and samples were taken over time. For the first reaction a aqueous buffer was used as reaction medium. The samples taken over time were analyzed by means of SDS-PAGE. (Figure 3.5)

In the first few samples (t=0h to t=9h) there is no product band visible, but there is a slight band from the substrate in the middle of the gel. This lower band disappears with progressing reaction time and the intensity of the product band increases with each sample. After 24 h reaction time the reaction mixture became gelatinous indicating the

growth of protein chains and network formation.

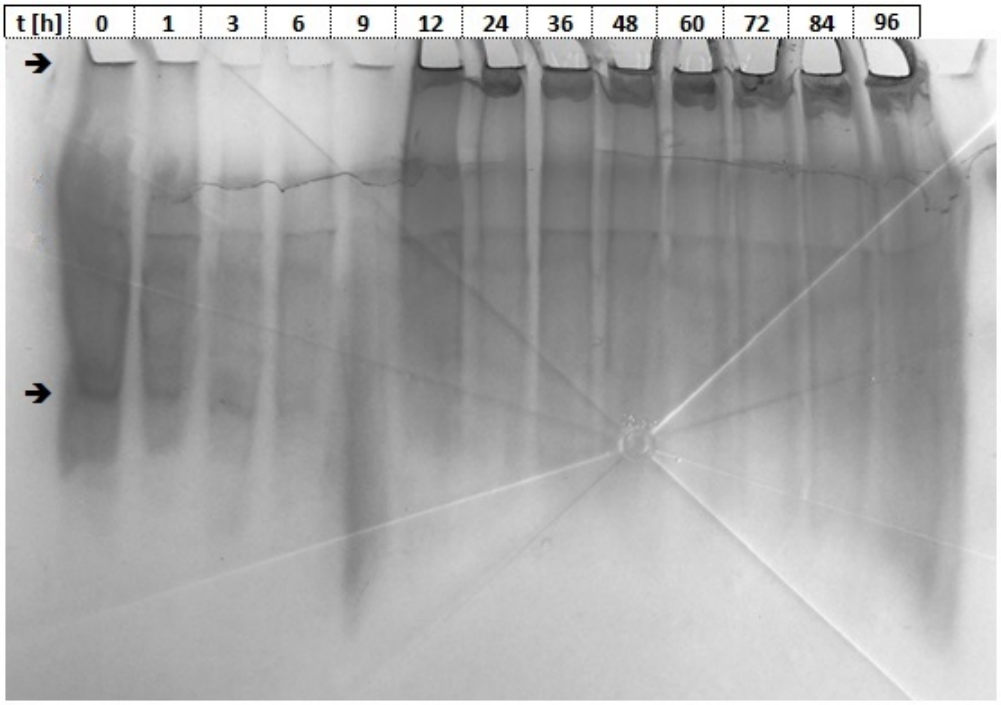


Figure 3.5: SDS-PAGE analysis of samples of the polymerization of BSA with mTGase in the aqueous buffer system over time

For the second reaction set-up the SSol system amidine combined with ethanol in the high polarity form (switched with CO_2) was used as reaction medium. (Figure 3.6)

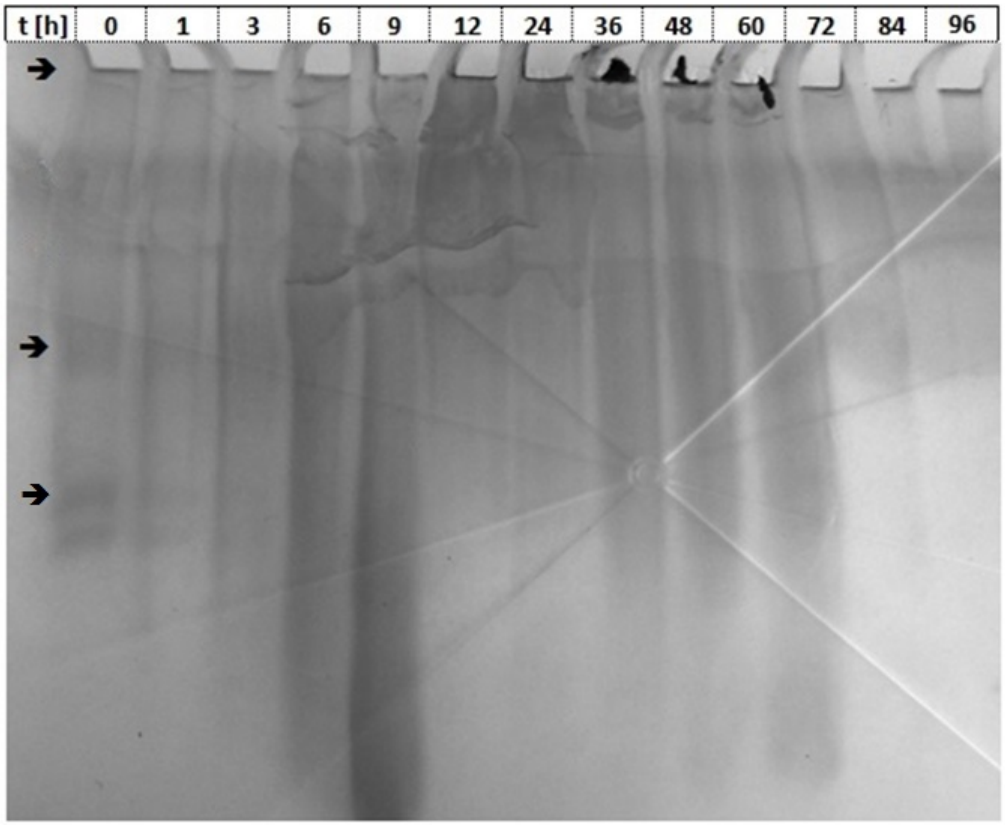


Figure 3.6: SDS-PAGE analysis of samples of the polymerization of BSA with mTGase in the SSol system composed of amidine and ethanol over time.

The observations in this reaction system are similar to those in the aqueous medium. The substrate band in the middle disappears over the time and seems to move to the upper part of the gel. Within 36 h reaction time the polymerization product became too big to dissolve in the electophoresis buffer. Hence, the product formed a solid in top of the gel. Like for the aqueous system the reaction solvent started forming a gel after 24 h reaction time indicating a high polymerization degree. After 72 h the polymerization degree was too high to take a sample. The bands here are caused by impurities in the multiple used buffer.

For the third reaction the SSol system of amidine combined with ethanol in the high polarity form (switched with CO_2) was used as reaction medium. (Figure 3.7)

The reaction system containing butylamine shows different behavior than the two previous systems. Hardly any changes can be observed over the whole reaction time of 96 h. The top band becomes a bit more intense, but cannot unequivocally be put down to product formation because for the substrate band no decrease in intensity can be observed. The enzyme might be inactivated by a functional group on reaction mixture. The only difference from the SSol with ethanol is the amine in this system. The carbamate anion might not be compatible with free enzymes.

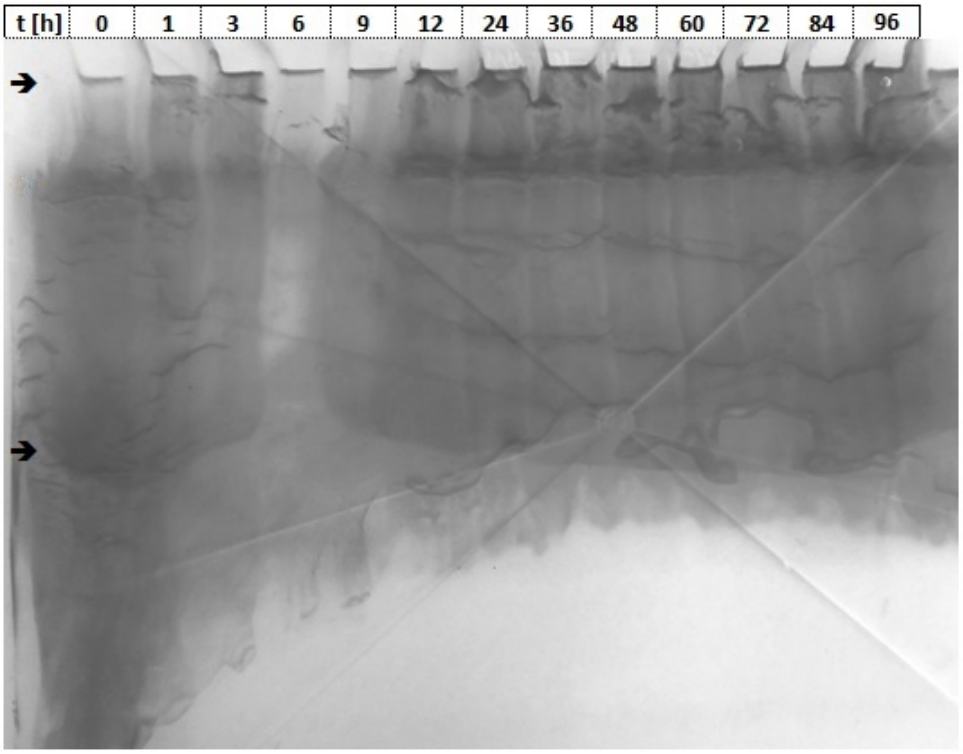


Figure 3.7: SDS-PAGE analysis of samples of the polymerization of BSA with mTGase in the SSol system composed of amidine and butylamine over time

To ease the comparison of the different solvent systems two samples of each reaction were run on one gel together with the obtained polymer and BSA. (Figure 3.8)

In the samples of the SSol containing butylamine only a pale band at the top, which is also visible in the reference. It can be assumed that no reaction happened in this mixture because there is the substrate band at 55 kDa visible although the sample is

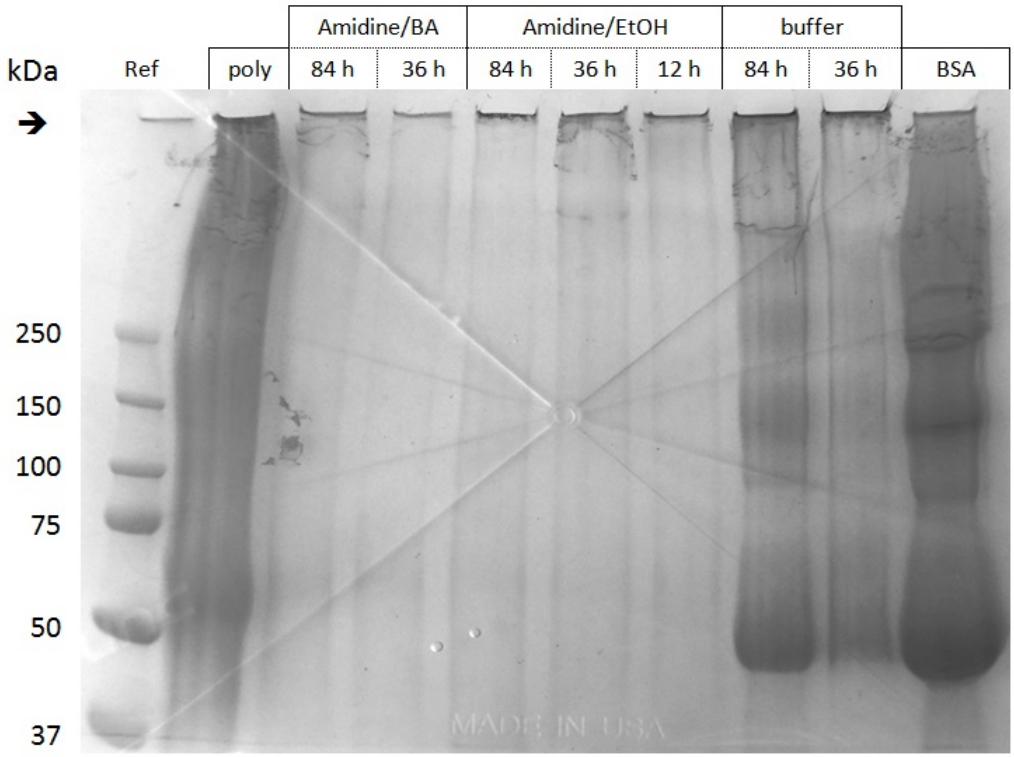


Figure 3.8: Comparison of the polymerization of BSA with mTGase in various solvent systems by means of SDS-PAGE analysis. poly=product polymer obtained from the Amidine/Ethanol system after 96 h; Amidine/Butylamine system after 36 and 84 h; Amidine/Ethanol system after 12, 36 and 84 h; aqueous buffer system after 36 and 84 h; BSA=substrate

very diluted. Compared to this, the top band in the sample of the SSol system with ethanol are much more intense and the substrate band are hardly detectable. The intensity of the product band increases with time. The samples of the aqueous reaction medium are both higher but differently concentrated. In both samples there is polymer and substrate detectable. The polymer sample on the left hand side was isolated from the reaction mixture of the SSol system with ethanol and is a prove for the feasibility of the enzymatic polymerization in this solvent. The bands from the substrate almost disappeared and a continuous smear was formed with the highest intensity at the top.

The feasibility of the biocatalytic process in the switchable solvent medium is shown. The enzyme remains active in SSol. To make a clear statement of the activity on the enzyme and the reaction happening in the solvent, further analysis would be necessary.

3.4 Conclusion

The objective of this second part of the thesis was to prove the feasibility of running a enzymatic reaction in a SSol system and simplify the separation step due to interchangeable solvent characteristics. Half of this target could be reached, while the switching and product recovery is more challenging and needs more prior knowledge of the reaction system.

It was shown that it is possible to run an enzymatic reaction in a SSol system. The enzyme can remain active if the solvent system is mild enough. The amidine/ethanol system did not affect the transaminase activity and some product polymer could be isolated. The amidine/butylamine system on the other hand inactivated the enzyme and no product could be detected.

However, it was not possible to separate the product while switching. This in on the one hand due to the difficulties because of gel formation with ongoing reaction. On the other hand far more knowledge about the SSol system needs to be gained. It is necessary to determine the exact polarities of the low and high polarity state and the solubility of the substrate and the product to be able to design the process.

The discussion in the previous section (Switchable Solvents for biocatalysis) already demonstrated that for the design of a reaction in SSol many factors need to be considered and is more complicated than it seems at a glance. Some of the required data is not available yet and hence the next step needs to be the further investigation of the solvent systems and the switching reaction.

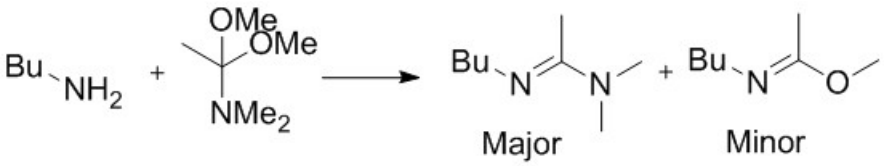
3.5 Experimental

3.5.1 Material

All the chemicals are commercially available. 1,1-dimethoxyethyl(dimethyl)amine, butylamine, isopropylamine, ethanol, heptane, hexane, cyclohexane were purchased from Sigma Aldrich. CO_2 was purchased from Messer. The TGase (Meat Glue 100 g) was bought from Special Ingedients. BSA was purchased from Sigma Aldrich. Pancreatic Digest of Casein was purchased from Becton, Dickinson and Company. All the equipment for SDS-PAGE was obtained from Bio-Rad (Mini.PROTEAN® TGX^{TM} precast gels, 12%; XT Sample buffer 4x, Precision Plus Protein^{TM} Standards Dual Color; 10 mL; 10x Tris/Glycine/SDS Buffer for SDS-PAGE 5L; Bio-Safe^{TM} Coomassie G-250 Stain 5L).

3.5.2 Synthesis of N′-butyl-N,N-dimethyl-ethanimidamide

The amidine was synthesised as described in Methode 2 by Harjani et al[100]. The reaction equation in shown in Scheme 3.16. Butylamine (63.6 mmol, 4.65 g) is dropwise added to 1,1-dimethoxyethyl(dimethyl)amine (76.4 mmol, 10.17 g) over 5 min while stirring. The mixture was stirred for 10 min at RT. The reaction mixture was allowed to stand for 18h at RT with slow stirring. The co-product was evaporated on a rotary evaporator for 8 h at 60°C and 30 mbar. The product appeared as an intense orange liquid. Yield: 7.98 g (56.1 mmol, 88.2%) ^{1}H NMR (400 MHz,MeOD): δ=0.9 (t, 3H, CH_2-CH_3), 1.4 (m, 2H, $CH_2-CH_2-CH_2$), 1.5 (m, 2H, $CH_2-CH_2-CH_2$), 2.35 (s, 3H, $N-CH_3$), 2.95 (t, 2H, CH_2-CH_2-N), 3.4 (s, 6H, $N-(CH_3)_2$); ^{13}C NMR (100 MHz, MeOD): δ=13.0 (CH_2-CH_3), 17.4 ($C-CH_3$), 20.3 ($CH_2-CH_2-CH_2$), 33.5 ($CH_2-CH_2-CH_2$), 37.5 ($N-(CH_3)_2$), 48.6 (CH_2-N), 161.4 (N=C−N).



Scheme 3.16: Synthesis of N′-butyl-N,N-dimethylacetimidamide: The primary amine of butylamine condensates with N,N-dimethylacetamide dimethyl acetal yielding the desired acetamidine in majority and an imidate ester as by-product.

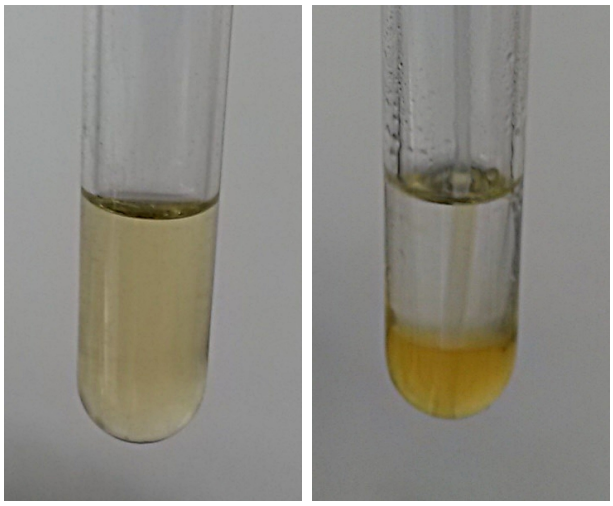
3.5.3 Switchability tests

The synthesized amidine was tested for its switchability in a two-component SPS. An equimolar mixture (2 mmol) of amidine and the second compound (primary amine or alcohol) as well as 500 μL non-polar organic solvent were charged in a test tube and formed a homogeneous, light yellow one-phase system. CO_2 was bubbled through the liquid to switch the solvent to higher polarity and separate the mixture into a highly viscous orange IL at the bottom and the clear organic solvent as supernatant. To reverse the reaction N_2 was bubbled through the two-phase system and it was heated in a water bath.

It was observed that primary amines react more readily than alcohol. While the primary amine switches within 2 min, it takes 5 min to separate the mixture of amidine with alcohol from the organic solvent. For switching back the observations were contrariwise. The alcohol switches back as fast as it reacts to the ionic liquid, while the amine must be heated and treated with N_2 for at least 30 min at appropriate temperature (80°C)

SPS Amidine/Primary amines

An equimolar (2 mmol) mixture of butylamine (BA) and amidine was mixed with 1 mL of heptane to give a homogeneous solution. When bubbling CO_2 through the mixture, the solution heated to approximately 60°C, became cloudy and a the IL settled directly to the bottom. Finally it resulted in an orange IL at the bottom and a colorless supernatant after only 2 min of CO_2 bubbling. (Figure 3.9) After 2 h bubbling N_2 and heating in a water bath to 60°C a single clear light yellow phase appeared. Adding 0.5 mL of



(a) before switching (b) after switching

Figure 3.9: Switching with CO_2 from low polarity (a) to high polarity (b) with IL at the bottom and the non-polar organic solvent in the supernatant

heptane and shaking led to an emulsion and finally to separation again. Bubbling N_2 again renewed the single phase system.

An equimolar (1 mmol) mixture of 2-propylamine (IPA) and A was mixed with 1 mL of heptane to give a homogeneous solution. Due to the low boiling point of IPA (31.8°C)[56] some of it evaporated and mist up the tube during the exothermic reaction of the switching process. The switching process was finished after 2 min. Switching back with bubbling N_2 and heating to 80°C was possible within 1 h. IPA evaporated during this process leaving back the amidine and some heptane in a homogeneous mixture. Adding heptane did not affect the homogeneous solution. After adding 300 μL IPA switching to the high polarity form was possible again.

Both SPS systems also separated from hexane and cyclohexane.

SPS Amidine/Ethanol

An equimolar (1 mmol) mixture of amidine and ethanol (EtOH) was mixed with 500 μL hexane. When bubbling CO_2 through the system, the solution slightly warmed up to approximately 40°C. The components separated within 5 min, but the phase border was

not as sharp as in the amidine/amine system. Switching back was possible within 5 min (far faster, than with amines). Separation was also possible with cyclohexane.

3.5.4 Polymerization Casein and BSA with mTGase in buffer

100/200 mg of substrate (Casein, Bovine Serum Albumin (BSA)) and 10-20 mg of enzyme in 0.1 M Tris-HCl buffer pH 7.8 were dissolved in a final volume of 1 mL. The reaction was incubated for 72 h at 37°C in a closed 1.5 mL Eppendorf tube. A control reaction without mTGase was run for both substrates. The samples were analyzed by means of SDS polyacrylamide gel electrophoresis (SDS-PAGE).

3.5.5 Polymerization Casein and BSA with mTGase in SSol

50/100 mg of substrate (Casein, BSA) and 20 mg of enzyme were dissolved in a final volume 540 μL of SSol (A/EtOH 1:1 n/n) to give a saturated solution with some solid residue. For each substrate one reaction in low polarity solvent (not switched) and one reaction in high polarity solvent (switched) was run. The switching process was carried out before enzyme and substrate were added. The reaction was incubated for 72 h at 37°C in a closed 1.5 mL Eppendorf tube. A control reaction without mTGase was run for BSA in a low polarity solvent. The samples were analyzed by means of SDS-PAGE. After taking the samples, the supernatant was transferred into a new tube and switched to the other polarity, respectively.

3.5.6 Stability test

Three reactions were run, two in SSol (A/EtOH 1:1 n/n and A/BA 1:1 n/n), both in high polarity form, and one in aqueous media. Each sample contained 100 mg BSA and 20 mg TGA in a final volume of 500 mL. The reactions were incubated for 96 h at 37°C in a closed 1.5 mL Eppendorf tube. Samples were taken after 0, 1, 3, 6, 9, 12, 24, 36, 48, 60, 72, 84 and 96 h. The samples were denatured with SDS sample buffer and stored in the freezer to be analyzed at once by means of SDS-PAGE. From the A/EtOH

system the produced polymer was isolated by precipitating in methanol. The polymer was solubilized in DMSO for SDS-PAGE analysis.

3.5.7 SDS-PAGE

10 μL of sample were added to the same volume of SDS sample buffer and heated to 95°C for 5 min. The total volume of the samples and 10 μL of the standard were loaded on the gel. The gel was run for 55 min at 150 V. After washing with water, the gel was stained with Coomassie blue and again washed with water to destain it.

4 Future Work

With this work the first step was done towards new technologies for biocatalytic processing. As both technologies are in a very early stage of development, not only the final application but also fundamental understanding of the underlying mechanisms need to be explored.

For the coated enzymes it has been shown that sensitive cofactor dependent enzymes can be coated with ILs to stabilize the reaction system and safe cofactor when running a reaction in organic solvents. The reduced reaction rate due to the additional barrier is a problem that needs be overcome by finding the optimal coating method and amount of coating material. A compromise between reaction rate and stabilization must be found. The next step can be the expansion of the IL coating to other enzyme classes.

The application of SSol is a more challenging topic. As SSol are a little investigated systems it is imperative to get familiar with the characteristics of the solvent system and the switching mechanism. Knowledge about the reactivity of the components, polarity of the solvent in both states as well as kinetics and thermodynamics of the switching process are crucial for designing an actually applicable process in a SSol medium.

The final step could be the combination of both of the above mentioned methods. The final process might consist of immobilized enzymes in RTSPIL in a solvent having switchable polarity. For the example of the transaminase the solvent system can be a mixture of an amidine and the amino donor. In the low polarity form of the solvent

the substrate is dissolved to run the reaction. Afterwards the heterogeneous catalyst is removed by filtration and the solvent is switched to its higher polarity state by adding CO_2. The substrate as well as the product precipitates from the mixture and can be separated. While switching back to lower polarity by heating and/or bubbling N_2 through the solvent the highly volatile co-product (acetone) is removed from the solvent. The solvent can now be brought back to beginning of the process.

However, it will take a few more master students before any of these techniques can be implemented in industry.

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Appendix

Appendix A - Calibarion curve ACP

Table 4.1: Concentrations and Areas for GC Calibration for acetophenone

Conc. [mM]	Area(ACP)	Area(IS)	Area(ACP)/Area(IS)
0.1	148.91	6372.36	0.024
0.2	268.87	6942.20	0.039
0.5	682.99	6876.99	0.099
1.0	1380.58	6973.53	0.198
2.0	2919.75	7416.61	0.394
5.0	7089.23	7449.52	0.952
10.0	15766.92	6972.95	2.261

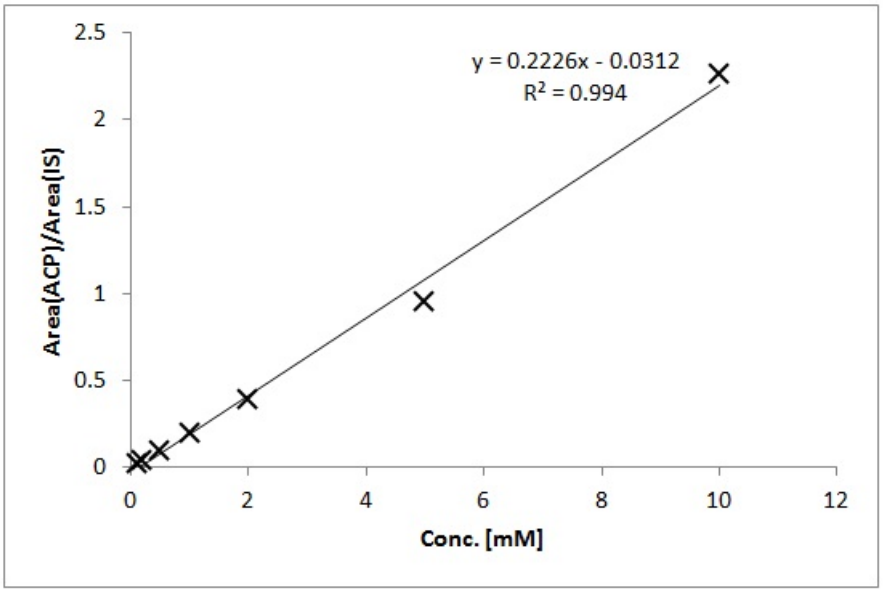
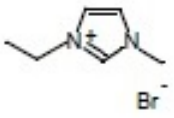


Figure A.1: Calibration curve for acetophenone

Appendix B - Product information [Emim]Br

Description

Product:	**1-Ethyl-3-Methylimidazolium Bromide**
Synonyms:	[Emim] Br
Purity:	98%
CAS Number:	[65039-08-9]
Molecular Formula:	$C_6H_{11}BrN_2$



Properties

Appearance:	White solid
Molecular Weight (g/mol):	191,09
Melting point (°C):	53
Decomposition temperature (°C):	

Solubility

Miscibility with water:	Yes
Miscibility with methanol:	Yes
Miscibility with acetone:	No
Miscibility with dichloromethane:	Yes
Miscibility with toluene:	No
Miscibility with ethyl acetate:	No
Miscibility with butyl acetate:	No
Miscibility with diethyl ether:	No

Specification

	Unit	Specification
Water content (Karl-Fisher)	%	≤ 1
Methylimidazole	%	≤ 0,3
Spectroscopy Infrared		Corresponds to standard spectrum

Applications

- Synthesis

Appendix C - NMR amidine

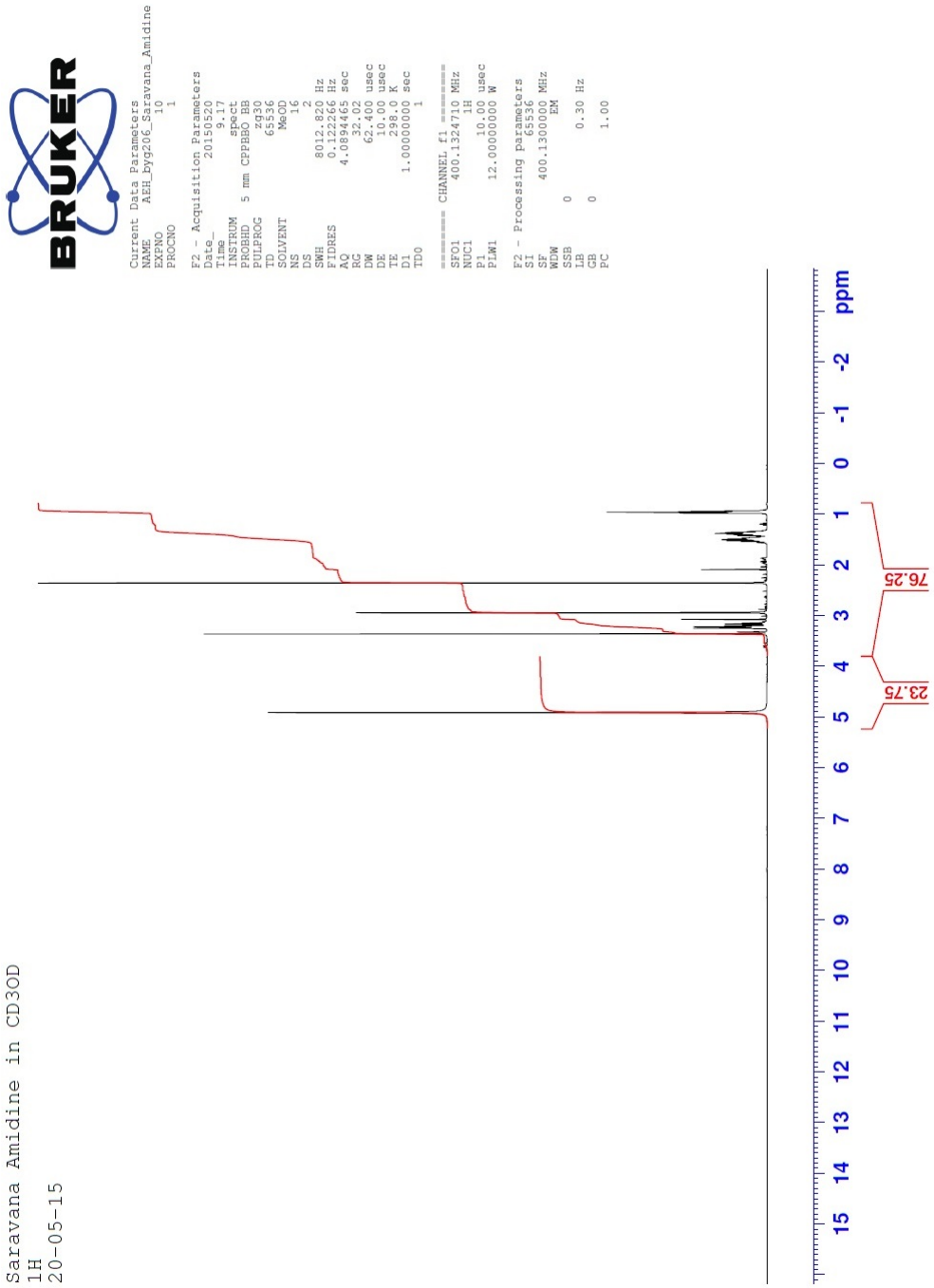


Figure A.2: ^{1}H NMR of amidine

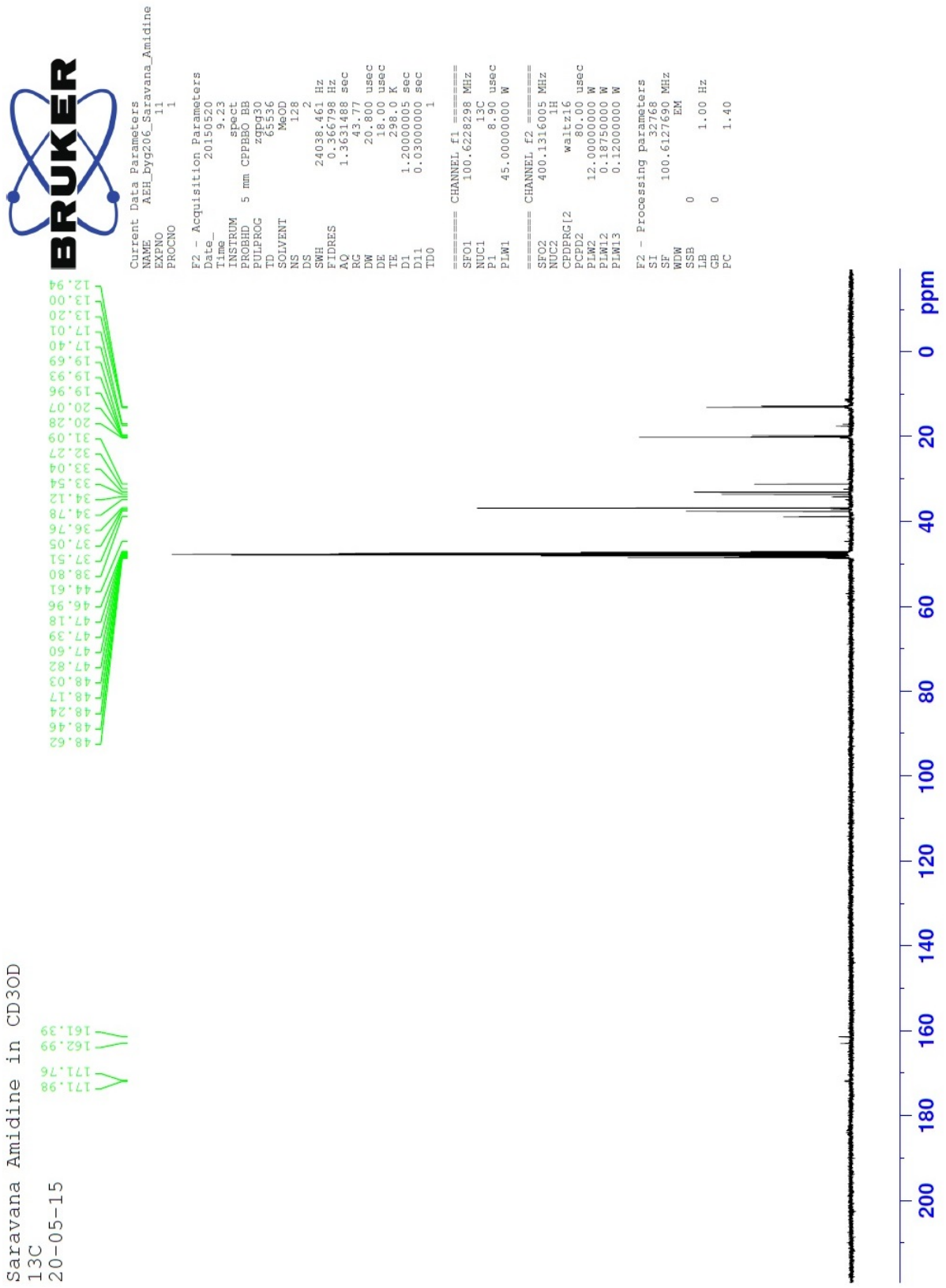


Figure A.3: ^{13}C NMR of amidine