Universität Rostock Traditio et Innovatio

Improving biocatalytic reactions – Immobilization *via* spray congealing & promising coupling reactions

Dissertation

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Declaration of independence

I, Claudia Engelmann (matriculation number: 8200434), hereby declare, that this thesis was written on my own and I did not use any unnamed sources or aid.

Hamburg, 25.04.2018

Claudia Engelmann

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Abbreviations

ABTS	–	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
BDE	–	Bond dissociation energy
BSA	–	Bovine serum albumin
BW	–	Beewax
CA	–	Cetyl alcohol
CHA	–	Cyclohexylamine
CIE	–	Covalently immobilized enzyme
CLEA	–	Cross-linked enzyme aggregates
CLEC	–	Cross-linked enzyme crystals
CP	–	Cetyl palmitate
CW	–	Carnauba wax
DMP	–	Dimethylphenol
DMSO	–	Dimethyl sulfoxide
DSC	–	Differential Scanning calorimetry
EC	–	Enzyme class
EPR	–	Electron paramagnetic resonance spectroscopy
Fac	–	Formic acid
FT-IR	–	Fourier transform infrared spectroscopy
GABA	–	γ-aminobutyric acid
Gel	–	Gelucire
GRAS	–	Generally recognized as safe
GTB	–	Glyceryl tribenzoate
GTP	–	Glyceryl tripalmitate
GTS	–	Glyceryl tristearate
HFCS	–	High fructose corn syrup
HIHB	–	Cleavage enthalpy
HLB	–	Hydrophilic lipophilic balance
IHB	–	Intramolecular hydrogen bonds
+I	–	Positive inductive effect
-I	–	Negative inductive effect
IL	–	Ionic liquids
IP	–	Ionization potential

IUBMB	–	International Union of Biochemistry and Molecular Biology Enzyme Commission
IW	–	Immobilized whole cells
LAc	–	Lauric acid
LC/MS	–	Liquid chromatography/ mass spectrometry
+M	–	Positive mesomeric effect
MA	–	Myristyl alcohol
MAc	–	Myristic acid
MC	–	Methylcatechol
MeOH	–	Methanol
MOAT	–	Methyl trioctylammonium triflate
Mp	–	Melting point
MtL	–	Myceliophthora thermophile
NCIE	–	Non-covalently immobilized enzyme
NI	–	Native intermediate
NLC	–	Nanostructured lipid carriers
p-NPA	–	*p*-nitrophenyl acetate
TvL	–	Trametes versicolor
PAc	–	Palmitic acid
PDB	–	Protein data bank
PEG	–	Polyethylene glycol
PFR	–	Plug flow reactor
PI	–	Peroxy intermediate
PI+ e-	–	One-electron-reduced form of PI
PLE	–	Pig liver esterase
Pre	–	Precirol
PSD	–	Particle size distribution
SA	–	Stearyl alcohol
SEM	–	Scanning electron microscopy
SLN	–	Solid lipid nanoparticles
T	–	Copper type
TIC	–	Total ion currents
WPN	–	Wide pneumatic nozzle

Publications & Presentations

C. Engelmann, U. Kragl. „Spray congealing as innovative technique for enzyme encapsulation". *Journal of Chemical Technology and Biotechnology.* **2017**, *93*, 191.

C. Engelmann, S. Illner, U. Kragl. „Laccase initiated C-C couplings: Various techniques for reaction monitoring". *Process Biochemistry.* **2015**, *50*, 1591.

C. Engelmann, S. Illner, U. Kragl. „Laccase catalyzed couplings: Tools to achieve a multigram scale", Poster presentation at the „10th European Congress of Chemical Engineering", **2015**, Nizza.

C. Engelmann, U. Kragl „Spray congealing: An immobilization method for biocatalysts". Poster presentation at the „7th International Congress on Biocatalysis", **2014**, Hamburg.

C. Engelmann, S. Illner, U. Kragl. „*In situ* FT-IR reaction monitoring of laccase catalyzed C-C- and C-N-coupling reactions", Oral presentation at the PhD Workshop „Catalysis for Sustainable Synthesis" (CaSuS), **2013**, Rostock.

Abstract

Nowadays biocatalysts are promising alternatives to conventional chemical homogenous or heterogeneous catalysts, because they are able to realize reactions under ambient conditions. Since these enzymes show often comparable low process stabilities, immobilization is highly interesting to increase stability and productivity. During this study, spray congealing was investigated as new immobilization technique, whereby two different set-ups were compared and the immobilized enzymes characterized. Thereby, spray congealing with a modified spray dryer showed beside smaller particles, higher residual activities and furthermore a better recyclability compared to a self-developed spray congealing apparatus. Furthermore, an ionic liquid was found to be an interesting new carrier for this application, due to 30% residual activity of the immobilized esterase after 5 consecutive reactions. In addition a laccase was immobilized with this method and tested for C-N coupling reactions of catechols and amines. These reactions were comparable unselective, nevertheless the selectivity could be increased with the usage of a fed-batch reactor and slow substrate addition at pH 5. With these conditions a compromise between amine availability and pH optimum of the enzyme was found and the coupling of different amines could be realized.

1. Introduction

Nowadays the chemical industry is focused on production of new compounds and optimization of existing processes with regard to increasing revenues. Nevertheless, companies are obliged to optimize processes, which include using and production of non-hazardous substances leading to the development of green processes. As a result, 12 principles for green chemistry were proposed by Anastas:

1. *Prevention*
2. *Atom economy*
3. *Less hazardous chemical synthesis*
4. *Designing safer chemicals*
5. *Safer solvents & auxiliaries*
6. *Design for energy efficiency*
7. *Use of renewable feedstocks*
8. *Reduce derivatives*
9. *Catalysis*
10. *Design for degradation*
11. *Real-time analysis for pollution prevention*
12. *Inherently safer chemistry for accident prevention*

These principles were extended by 12 principles of chemical engineering as a framework for scientists and engineers for consideration of environmental, economic, and social factors. Based on these principles, catalysis provides the best solution for complying social requirements. [Anastas *et al.*, 1998; Wenda *et al.*, 2011] Especially biocatalysis is gaining more and more interest for industrial applications, due to very promising features in the context of green chemistry. These catalysts are eco-friendly and offer interesting opportunities improving chemical reactions and realizing new synthetic routes. Furthermore, they exhibit great biocompatibilities and biodegradabilities constituting a favorable alternative to conventional homo- or heterogeneous catalysts. The main applications for biocatalysts in industry are fields of pharmaceutical industry, medicine, food and fine chemicals as well as substitution of traditional chemical processes, e.g the synthesis of pregabalin, a lipophilic γ-aminobutyric acid (GABA) analogue, at Pfitzer. [Dunn, 2012; Schmid *et al.*, 2001; Alcalde *et al.*; Liese *et al.*, 2006]

Nevertheless, the reuse and recovery of biocatalysts as well as low long-term stabilities, with regard to temperature, co-solvents and pH values, still limit industrial applications. [Liese *et al.*, 2013; Sheldon, 2007; Liese *et al.*, 2006] To overcome these drawbacks there are different possibilities. Thereby changing the molecular structure of the enzyme by protein engineering or a physical stabilization by immobilization are playing important roles in the biocatalytic process, which is represented in figure 1-1. [Liese *et al.*, 2013; Lee *et al.*, 2017] After the fermentation and harvesting of the biocatalyst, it can be introduced to biocatalytic reactions as whole cells, purified or non-purified enzyme.

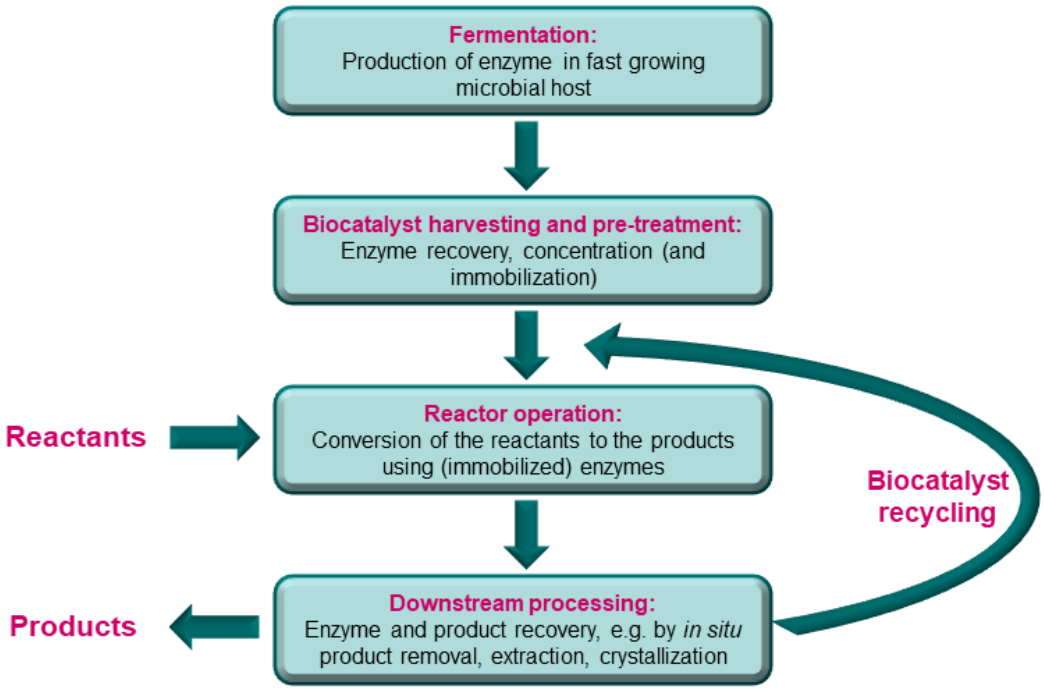

Figure 1-1. Scheme of a biocatalytic process. (adapted from [Lee *et al.*, 2017])

The obtained biocatalyst can be immobilized to improve the reactor operation and the subsequent downstream processing. In addition, immobilization of the biocatalyst allows the recycling of the enzyme in consecutive reactions enabling a superior increase of productivity for industrial processes. Different methods were developed for immobilization, e.g. covalent binding or cross-linking; however, no concept is given for an immobilization method of biocatalysts. The demand of immobilization techniques is present and it is still challenging to find suitable methods for optimal reaction performance. [Liese *et al.*, 2006; Dunn, 2012; Choi *et al.*, 2015]

2. Scope of the work

The aim of this thesis was the evaluation of spray congealing as new immobilization technique for application in biocatalytic processes. Thereby the active compound, in this case the biocatalyst, is emulsified or suspended in a lipid matrix and sprayed with subsequent re-solidification of the mixture (figure 2-1). In the end fine particles should be obtained with the encapsulated active compound. This technique is well-known for encapsulation of pharmaceuticals or food ingredients; however, no work is reported based on immobilization of biocatalysts and only a few publications described the encapsulation of proteins. For our investigations two different set ups should be used and various carriers with different properties should be tested with regard to their suitability for spray congealing and enzyme immobilization. The obtained encapsulated enzymes should be characterized in detail with regard to occurrence, release, water content, as well as retained activity and further characteristics. The most interesting property was thereby the recovery of the immobilized biocatalyst and the re-usage in further biocatalytic reactions. For the investigation of the feasibility of spray congealing for enzyme immobilization two different laccases, from *Myceliophthora thermophila* (*MtL*) and from *Trametes versicolor (TvL),* as well as a Pig liver esterase (PLE) will be used.

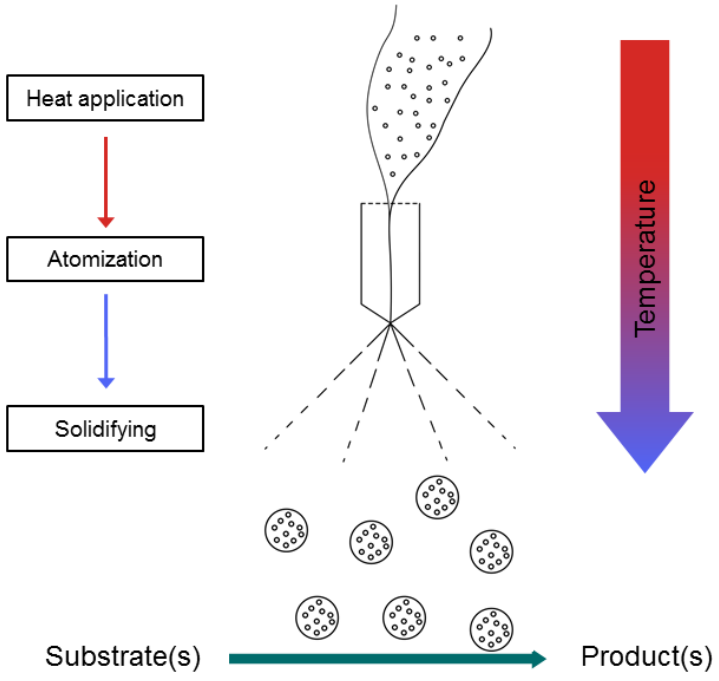

Figure 2-1. Spray congealing process.

After a successful immobilization and characterization of the immobilized enzymes, laccase initiated C-N coupling reactions of phenolic compounds with amines should be investigated. Thereby selected substrates had to be screened and should be used for C-N coupling reactions with amines showing often bioactive properties. Starting from this functionalized products should be obtained, which could be interesting compounds for pharmaceutical applications. These C-N coupling reactions are often in competition with C-C coupling reactions, like dimerization, oligomerization or polymerization of the oxidized phenolic compound, based on the radical mechanism of this reaction. [Engelmann *et al.*, 2015; Constantin, 2012; Mogharabi *et al.*, 2014] To increase the selectivity, the coupling reaction should be examined in detail, to realize optimal reaction conditions regarding to pH value as well as ideal reaction mode, like batch or fed-batch. In addition, the immobilized laccase should be studied in detail with regard to C-N coupling reactions using a fixed-bed reactor set up. Based on the results, the feasibility of the spray congealing as immobilization technique should be discussed and rated. Furthermore the substrate scope of the laccase using different amines as C-N coupling reagents should be investigated to analyze the accessibility for further highly interesting products.

3. Theoretical background

3.1. Biocatalysis – An alternative for classical catalysis?

Biocatalysts are the most important catalysts in nature, with good biocompatibilities and biodegradabilities. These enzymes as proteins are catalyzing reactions in living organisms efficiently for their survival and reproduction. They consist of polypeptide chains forming a complex structure with a special activity, whereby the structure is divided in different levels:

- *Primary structure:* Different amino acids connected *via* peptide bonds
- *Secondary structure:* Chains of amino acids are folded based on hydrogen bonds (α-helices and ß-folds)
- *Tertiary structure:* Fold of the protein chain, based on disulfide, hydrogen, ionic and hydrophobic interactions
- *Quarternary structure:* Forming of subunits through combination of protein chains

Biocatalysts are able to catalyze chemical reactions, and became an important field for process optimization in industry during the last five decades, based on remarkable advantages in comparison to conventional catalysts. [Buchholz *et al.*, 2012; Liese *et al.*, 2006] They can catalyze a broad range of specific reactions, even from complex mixtures, with high regio- and stereoselectivites. This results in less by-product formation, which is often a problem for chemically catalyzed reactions. The application of biocatalysts presents a good alternative for hetero- and homogenous catalysts, due to more environmentally friendly processes. Furthermore, biocatalytic processes are performed under mild conditions, e.g. in aqueous solutions, at ambient temperature and under atmospheric pressure and exhibiting increased reaction rates of up to 10^8 - 10^{10} in comparison to non-catalyzed reactions. [Sheldon *et al.*, 2013; Schmid *et al.*, 2001; Bornscheuer *et al.*, 2012; Faber, 1992]

Enzymes are divided in different enzyme classes by the type of catalyzed reaction. In figure 3-1 the six main classes of biocatalysts are illustrated developed by the International Union of Biochemistry and Molecular Biology (IUBMB) and the Enzyme Commission. These main classes (i) are further divided in different subclasses and subgroups by a numerical system (EC i.ii.iii.iv) according to type of substrate or transferred group (ii), nature of the co-substrate (iii) and furthermore, an individual enzyme number. [Liese *et al.*, 2006; Drauz, 2012; Buchholz *et al.*, 2012]

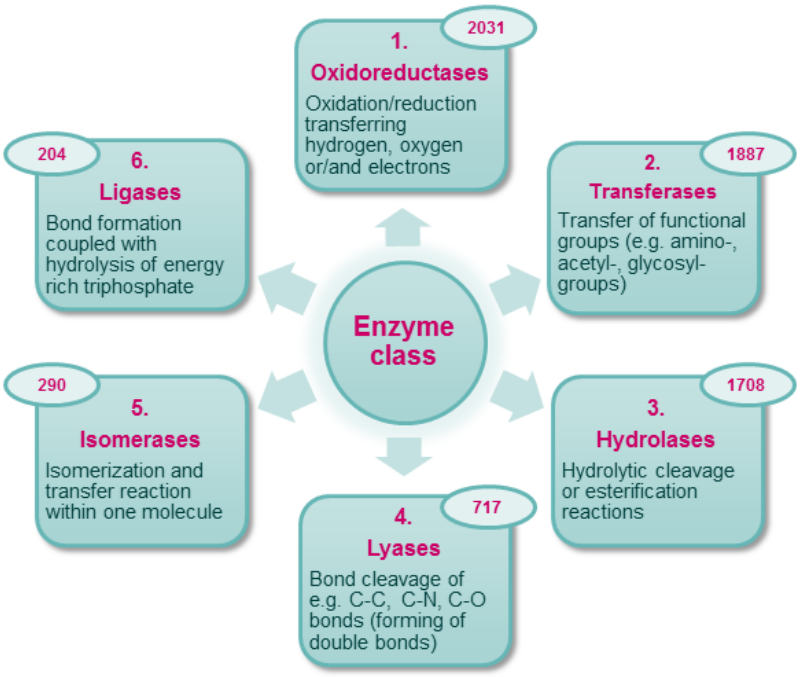

Figure 3-1. The main enzyme classes with their catalyzed reactions and number of listed enzymes in BRENDA. [Schomburg, 2018]

Biocatalysis underwent great developments during the last decades and became an important alternative to classical catalysts. Bornscheuer *et al.* divided the evolution of biocatalysis for chemical applications in three waves. During the first wave the catalyzing properties of parts of living cells were discovered and used in different biotransformations, as well as in detergents. The second wave was characterized by the development of new enzymes *via* protein engineering enhancing substrate ranges and the synthesis of new chemical compounds. In the end of 1990s the third wave of biocatalysis was initiated by the development of directed evolution to modify proteins effectively. The improved enzyme engineering technology during the third wave enabled the generation of custom designed biocatalysts. [Bornscheuer *et al.*, 2012] This continuing production of new biocatalysts according to the requirements results in more than 6800 listed enzymes, whereby the majority are recombinant biocatalysts, which were developed by different research groups. In figure 3-1 it can be seen, that over 80% of the listed enzymes belong to the first three main classes of enzymes, whereby 95% are commercial available. [Robinson, 2015; Schomburg, 2018] However, the majority of applied biocatalysts in industry belong to the hydrolases (80%), due to their broad application in detergents and food industry. In contrast, Straathof *et al.* investigated 134 industrial processes, regarding to the application in biotransformations, in detail and found out that hydrolases (44%) and redox biocatalysts

(30%) are dominating the sector of biotransformations. [Straathof *et al.*, 2002; Robinson, 2015; Liese *et al.*, 2006] Due to the promising properties and various applications of laccases, which belong to the enzyme class of the oxidoreductases, and an esterase as a hydrolase were chosen for the investigations during this study.

3.1.1. Laccases – Promising oxidative enzymes

Laccases (*p*-benzodiol:oxygen oxidoreductase EC 1.10.3.2.) belong to the group of multicopper oxidases, and are investigated since the nineteenth century. The first laccase was described by Yoshida, who discovered this enzyme in the Japanese lacquer tree *Rhus vernicifera*. [Morozova *et al.*, 2007; Madhavi *et al.*, 2009] This enzyme with molecular weights of 50 – 130 kDa is occurring in funghi, higher plants, as well as insects, whereby the natural role of laccases are e.g. syntheses of lignin, as well as degradation of humic acid and lignin. Laccases are glycoproteins with covalently linked carbohydrate moieties of up to 45%, which are supposed to stabilize the enzyme. These biocatalysts are oxidizing a broad range of substrates, like phenolic compounds, aromatic and alkylamines, benzothiols and some metal ions, forming the corresponding radicals. [Kudanga *et al.*, 2017; Morozova *et al.*, 2007; Claus, 2004; Madhavi *et al.*, 2009] Figure 3-2 depicts the global structure of the fungal laccase from *Trametes versicolor* in the oxidized form with the copper containing active center. It can be seen, that the active center contains four copper (Cu) atoms, which are classified in three types, depending on their spectroscopic properties. [Jones *et al.*, 2015]
The Cu(1) atom belongs to type 1 absorbing at 600 nm, which results in a blue occurrence and is detectable with electron paramagnetic resonance spectroscopy (EPR). The Cu(1) atom is located at the substrate binding site and is coordinated trigonal with a cysteine residue and two further histidine residues. [Strong *et al.*, 2011] These amino acids enable an electron transfer to a tri-nuclear copper cluster containing the other three copper atoms. One histidine residue of the amino acid bridge between the two sites of the active center is coordinated to a copper atom type 3, respectively, which is illustrated as Cu(2) and Cu(3) in figure 3-2. This bi-nuclear copper center is connected *via* a hydroxyl group resulting antiferromagnetic properties and therefore no detection with EPR is possible, however, this type is absorbing at 330 nm. The last atom Cu(4) is a type 2 copper, which shows no significant absorbance, but a hyperfine coupling constant during EPR investigations. [Morozova *et al.*, 2007; Jones *et al.*, 2015; Rulíšek *et al.*, 2005]

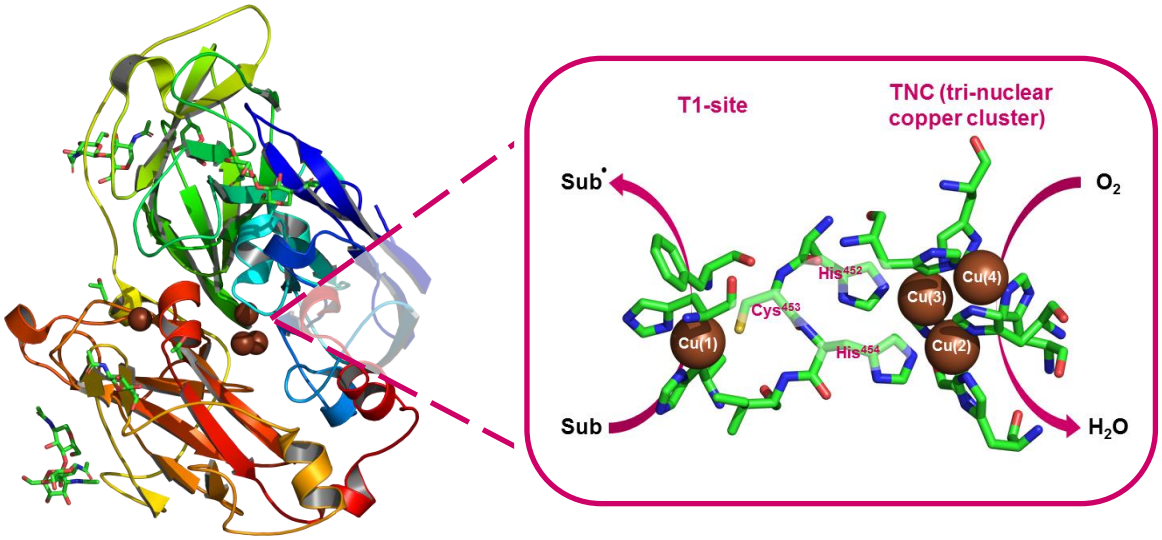

Figure 3-2. Active center of the laccase from *Trametes versicolor*. PDB 1GYC [Piontek *et al.*, 2002; Rulíšek *et al.*, 2013]

Some intensive theoretical studies of the active site structure of multicopper oxidases and the catalytic mechanism were performed. [Rulíšek *et al.*, 2013; Rulíšek *et al.*, 2005; Enguita *et al.*, 2004; Augustine *et al.*, 2010; Li *et al.*, 2015] Thereby, different intermediates in the tri-nuclear copper cluster could be observed, shown in figure 3-3. Firstly, the substrate is oxidized at the T1 site to the radical compound, with an electron transfer over the amino acid residues, while oxygen is reduced to water at the tri-nuclear copper cluster *via* a four electron reduction. [Kudanga *et al.*, 2017; Claus, 2004] After the electron transfer from the T1-site, the tri-nuclear copper cluster is in the ***reduced state*** with no connection to the type 2 atom. The enzyme is now able to bind the oxygen molecule with two electrons, forming the ***peroxy intermediate*** (PI), whereby the peroxide is strongly bonded to all three copper atoms. The cleavage of O – O is realized by another electron transfer from the T1 copper to the PI resulting to a ***one-electron-reduced form of PI*** ($PI + e^-$). Due to a low barrier a fast cleavage is occurring and the copper cluster is reaching the ***native intermediate*** (NI). The dissociation of water enables the forming of the ***reduced form*** of the tri-nuclear copper cluster completing the catalytic cycle. [Rulíšek *et al.*, 2005; Augustine *et al.*, 2010]

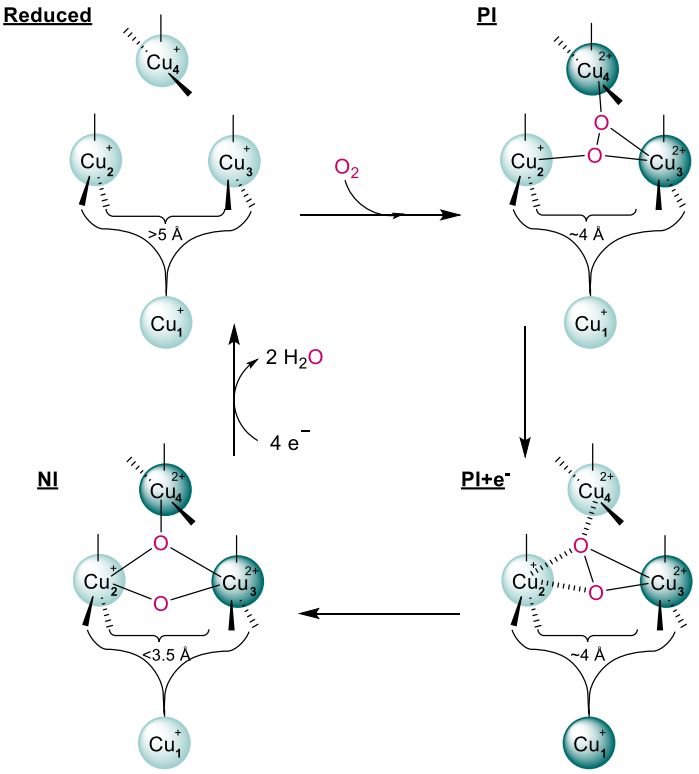

Figure 3-3. Suggested catalytic mechanism of the laccase. (PI – Peroxy intermediate, PI + e^- - One-electron-reduced form of PI, NI – Native intermediate, T – Copper atom) [Rulíšek *et al.*, 2005; Augustine *et al.*, 2010]

This catalytic mechanism is only possible, if the potential of the substrate is lower than that of the laccase. Nevertheless, involving low-molecular mediators can also enable the electron-transfer for non-laccase substrates. [Božič *et al.*, 2013; Yang *et al.*, 2013] After oxidation of the phenolic compounds the formed substrate radical is able to undergo various coupling reactions with each other to dimers, oligomers or polymers *via* C-C and/or C-O coupling reactions and in addition, coupling reactions with non-laccase substrates (figure 3-4). [Claus, 2004; Engelmann *et al.*, 2015; Herter *et al.*, 2013; Herter *et al.*, 2011; Constantin *et al.*, 2013] The substrates are described as natural compounds for radical scavenger, which bioactive properties can be enhanced by laccase catalyzed coupling. Furthermore an increase of their antioxidant capacity can occur. [Adelakun *et al.*, 2012; Riebel *et al.*, 2015]

Laccases are already applied in industrial processes like wastewater treatment and textile industry for degradation of dyes or for bleaching of pulp and paper *via* delignification. [Kim *et al.*; Engelmann *et al.*, 2015; Kudanga *et al.*, 2011] This application is mainly based on a low

selectivity linked with a broad substrate range of these enzymes. Today the research focus is shifting and laccases are used for several applications, like synthesis of organic compounds, biooxidation, or even implementation in biosensors. Especially, syntheses of different fine chemicals catalyzed through laccases are very interesting as a green alternative to transition metal catalysis. Various research groups are using laccases for coupling reactions with non-laccase substrates, e.g. in synthesis of many alkaloids or other natural substances. [Constantin *et al.*, 2012a; Claus, 2004; Engelmann *et al.*, 2015; Kudanga *et al.*, 2017] For example, Wellington *et al.* realized the laccase catalyzed synthesis of compounds with anticancer activity and the research group of Beifuss used these enzymes for formation of new interesting compounds. [Wellington *et al.*, 2013; Wellington *et al.*, 2012; Constantin *et al.*, 2012a; Constantin *et al.*, 2012b]

Due to the feasibility of biocatalytic coupling reactions without expensive co-factor requirements, the laccase seems to be a promising candidate for the investigation and evaluation of spray congealing as immobilization method for enzymes.

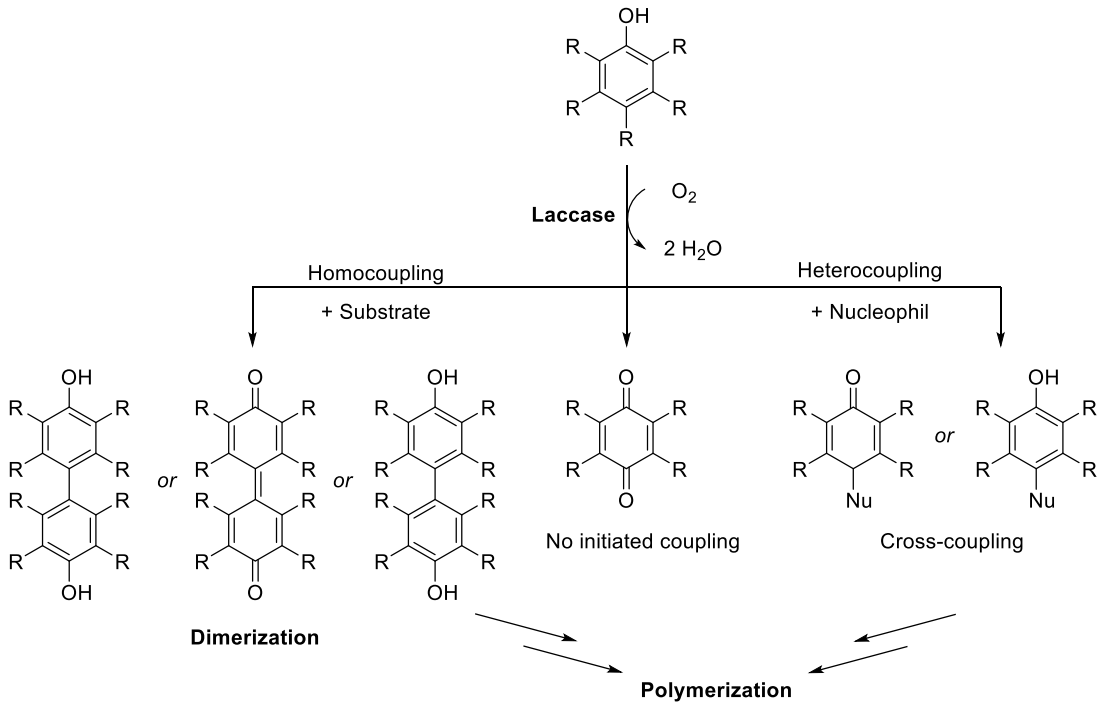

Figure 3-4. Laccase initiated oxidation of phenolic compounds and possible coupling reactions.

3.1.2. Esterases – Good old hydrolases

Esterases (EC 3.1.1.x.) belong to the group of hydrolases and catalyze the cleavage or forming of ester bonds. This enzyme is ubiquitous in life and can be isolated from plants, animals and microorganisms where they are a part of the digestion processes. [Bornscheuer, 2002; Bornscheuer *et al.*, 1999; Chen *et al.*, 2016] Most of these enzymes are active in non-aqueous solvents and show a high substrate tolerance, including simple esters and short chain triglycerides. In addition, esterases are metal and co-factor free enzymes and show a high stereo- and regioselectivity. These facts suggest that esterases are highly interesting for the synthesis of organic compounds, e.g. the production of chiral synthons.

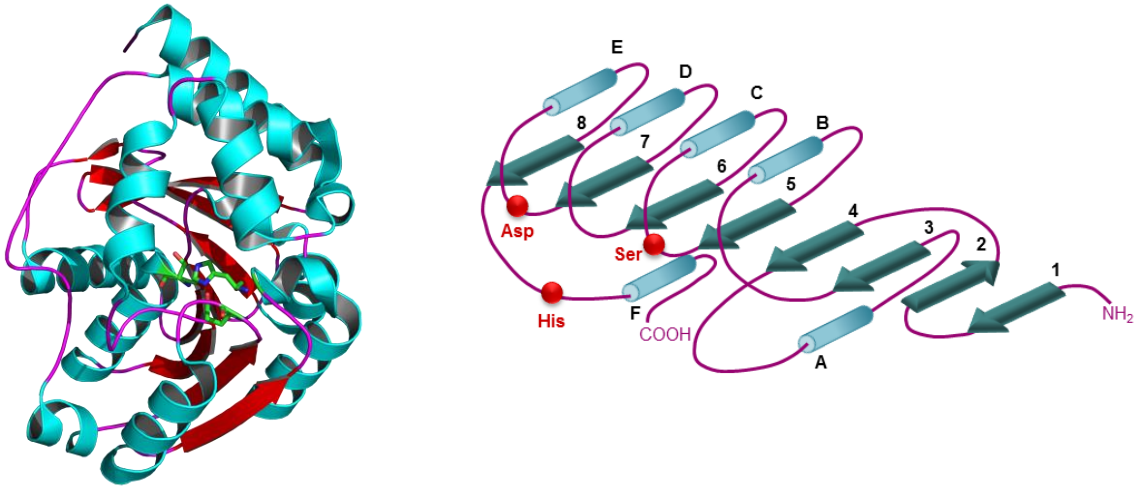

Figure 3-5. *Left:* Crystal structure of a carboxyl esterase from *Bacillus subtilis* (PDB: 4CCW), catalytic triad is shown as sticks. *Right:* Schematic structure of the α/ß-fold. The α-helices are presented as columns and the ß-sheets as narrows. The catalytic triad is illustrated as red spheres. [Bornscheuer, 2002; Ollis *et al.*, 1992; Rozeboom *et al.*, 2014]

The structure and properties of these enzymes are similar to the lipases. They show a characteristic α/ß-hydrolase fold for the active center, whereby the catalytic triad is formed, shown in figure 3-5. The protein core is surrounded by eight ß-folds (1-8) connected by six α-helices (A-E), which form a special arrangement of the amino acid residues responsible for the catalytic activity. [Bornscheuer, 2002; Ollis *et al.*, 1992; Romano *et al.*, 2015; Drauz, 2012] The hydrolysis of esters is not only based on the catalytic triad, also the possibility of charge delocalization *via* the amino acid residues and the oxyanion stabilization by three additional amino acid residues are playing an important role for the catalytic mechanism,

shown in figure 3-6. The catalytic triad is formed by serine, histidine and asparagine (or glutamine) residues *via* hydrogen bonds. In the first step an acylation by a nucleophilic attack of the serin residue at the carbonyl carbon of the ester is occurring, while a parallel proton transfer of the serin residue to the histidin residue is forming a tetrahedral intermediate. This intermediate is stabilized by charge delocalization *via* an oxyanion hole and the reactive part of the enzyme through electrostatic interactions. Subsequently, the alcohol is eliminated and an acyl-enzyme complex is formed, whereby the acid is covalently bound to the hydrolase. The imidazolium intermediate is now activating water, which attacks the complex resulting in a second tetrahedral complex and is stabilized again by the oxyanion hole. The release of the acid results in the native intermediate of the catalytic triad and a new catalytic cycle can be performed. [Drauz, 2012; Zhang *et al.*, 2002]

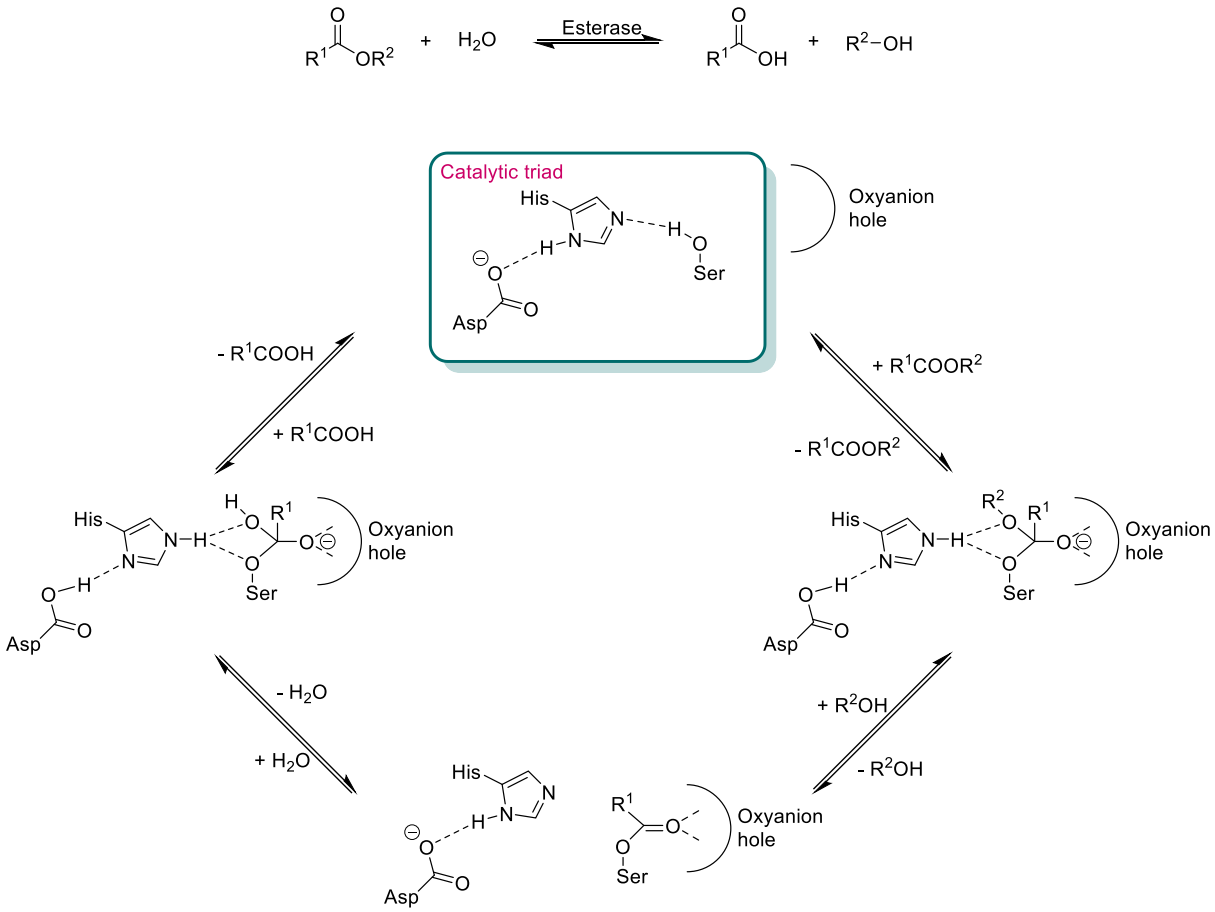

Figure 3-6. Catalytic reaction mechanism of esterases. [Drauz, 2012]

In this study the pig liver esterase (PLE, 3.1.1.1.) was investigated, which can be categorized into the group of the carboxylic hydrolases. This enzyme hydrolyzes a broad range of esters, in particular methyl esters of carboxylic acids or acetates of alcohols and is a commercial available low cost enzyme. [Bornscheuer *et al.*, 1999; Süss *et al.*, 2014] The PLE is frequently applied in kinetic resolutions and desymmetrizations of prochiral compounds. The first asymmetric reaction was carried out in the early 20th century. However, since Huang *et al.* in 1975 employed this enzyme for organic synthesis of *(R)*-mevalonic acid, it moved into the research focus of many scientists. [Huang *et al.*, 1975; Dakin, 1903; Dakin, 1905; de María *et al.*, 2005; de Maria *et al.*, 2007] The PLE is gained from animal tissue by extraction and comprises several isoenzymes. Despite only slight structural differences, the properties like pH and temperature dependence, as well as substrate scope and selectivity show significant variations. As a result the research is nowadays focused on the functional expressions of the isoenzymes resulting in increased selectivities, higher yields and reproducible purities of the product. Especially the research group of Bornscheuer has to be mentioned for intensive investigation of expressions and characterizations of PLE isoenzymes with regard to increasing selectivties, yields and stabilities. [Böttcher *et al.*, 2007; Süss *et al.*, 2014; Süss *et al.*, 2015; Hinze *et al.*, 2016] Since 2001, where the first isoenzyme was produced, the isoenzymes became commercial available and therefore easily accessible for organic synthesis resulting in synthesis of over 400 compounds with superior enantioselectivities. [Süss *et al.*, 2014]

3.2. Immobilization of biocatalysts – An alternative for native enzymes?

Biocatalysts exhibit several advantages for highly selective and specific synthesis. However, implementation of water soluble enzyme into a process can be hampered by low operational stability or process applicability. To avoid these disadvantages, immobilization of biocatalysts is a key prerequisite for industrial application resulting in an enhanced economic efficiency of large-scale processes. [Bisswanger, 2013; Garcia-Galan *et al.*, 2011] The main reasons represent the recovery and re-usage of the catalyst, whereby continuous processes can easily be realized with improved enzyme stabilization and simpler product purification. This leads to higher productivities and furthermore more economic implementation. [Buchholz *et al.*, 2012; Whittall *et al.*, 2009; Mateo *et al.*, 2007] Other applications for immobilized enzymes are controlled protein release or usage as analytical device, as well as selective adsorbent for purification. [Cao, 2006]

Table 3-1. Advantages & limitations of immobilized biocatalysts. [Buchholz *et al.*, 2012; Eş *et al.*, 2015]

Reason	Advantage	Limitation
Reuse/ recovery	Separation and re-usage of valuable biocatalyst	Cost of immobilization technique and carrier
Continuous processing	Facilitating process control Low residence time High volumetric activity Higher productivity	Mass transfer limitations Co-factors problems Biocatalyst separation & regeneration Difficulty with multi-enzyme systems
Downstream processing	Easy product separation & recovery Reduction of product contamination	Specific reactor system & high engineering design
Stabilization	Enhanced operational stability against e.g. pH, temperature, solvents	Activity loss during immobilization Depending on immobilization technique

The advantages of immobilized biocatalysts are presented in table 3-1. Here it also has to be highlighted that the stability is enhanced against various influences during the process and productivity is increased, due to a simplification of the downstream process. In addition, a simple recycling of the biocatalyst is highly interesting for the industry. However, immobilization is comparable expensive and often not worthwhile for cheap enzymes.

Nevertheless, there are several advantages: For example the simplified downstream processing to obtain an enzyme free product and circumventing toxicological risks of enzymes, such as allergic reactions. Further aspects in this context are the operational stability against temperature and pH. [Katchalski-Katzir, 1993; Mohamad *et al.*, 2015]

In contrast, immobilization changes the microenvironment of the enzyme, which can also induce inactivation of the biocatalyst by changing the spatial structure itself or blocking of the active center. Complex systems, as well as mass transfer limitations are still challenges for application of immobilized enzymes. More than 5000 available immobilization techniques are described for several hundred different enzymes. However, no procedure is available to choose the right immobilization technique for special biocatalysts until today. [Cao, 2005; Eş *et al.*, 2015; Liese *et al.*, 2013]

3.2.1. Types of immobilization

Various factors and numerous procedures have to be considered establishing a suitable immobilization method for the desired biocatalyst. The immobilization techniques can be divided into three different main classes: non-covalent binding, covalent binding and the entrapment of the enzyme, demonstrated in figure 3-7. Thereby the choice of supports is of great interest for the effectiveness of enzyme immobilization. For example, the need to be inert against the reaction conditions and mechanical stability is important allowing the usage e.g. in a fixed-bed reactor. In addition, a compromise between enzyme and carrier costs, as well as cost of preparation including additional chemicals, has to be found. Based on this the application of the immobilized enzyme constitutes higher productivities and therefore an increased profit. Furthermore, it should exhibit a high affinity for the enzyme, a good biocompatibility and biodegradability, as well as a good regeneration feasibility. [Foresti *et al.*, 2007; Mohamad *et al.*, 2015; Brena *et al.*, 2006] The different properties of the supports are the basis for different immobilization techniques and therefore different enzymes. In the following section the different immobilization methods are described.

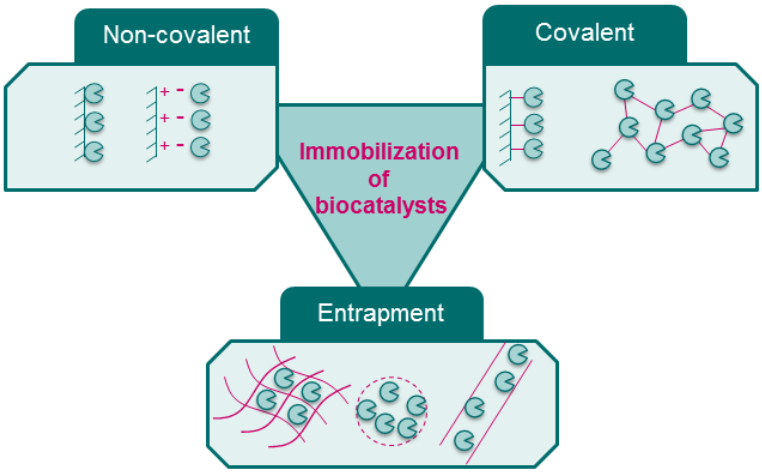

Figure 3-7. Types of immobilization.

Non-covalent binding

Non-covalent attachment of an enzyme onto a support can be realized *via* physical binding, which is based on hydrophobic or van-der-Waals interactions. In addition this immobilization can be performed by hydrogen bonds or ionic bonding. This is a physical and therefore reversible bonding and can be comparable weak. The immobilized enzyme can easily leach from the support under operating conditions, like continuous processes and high reactant and product concentrations. In addition, a contamination of the product can occur, as well as less efficient recycling as a result of the leaching. [Sheldon, 2007; Whittall *et al.*, 2009] This is important, if the enzyme activity decreases and the support has to be regenerated with fresh and active enzyme. Nevertheless, the adsorption onto supports is widely used in reactions with water-immiscible organic solvents, as well as for formulations of enzymes in detergent powders. In particular, the immobilization of lipases is performed successfully, due to their catalytic activity in organic solvents. As a result several immobilized lipases are commercial available. [Mohamad *et al.*, 2015; Sheldon, 2007; Tischer *et al.*, 1999; Whittall *et al.*, 2009] Various studies showed an enhanced activity of the lipase, when a more hydrophobic support is used. For example, Cunha *et al.* compared different immobilized lipases, whereby the ionic bonded enzyme exhibited a higher activity. In contrast the adsorptive bonding on a more hydrophobic carrier resulted in a higher thermal stability for pH 5 and pH 7 over 20 h. [Cunha *et al.*, 2008] The hydrophobic nature is supposed to open the hydrophobic lid of the lipase, resulting in an hyperactivation of the enzyme. [Sheldon, 2007; Cunha *et al.*, 2008; Petkar *et al.*, 2006] Thereby Novozym®435 has to be highlighted as a commonly used

immobilized lipase in industrial processes. This is a *Candida antarctica lipase B* adsorbed on a macroporous acrylic resin. This special lipase has in contrast to others no lid which protects the active site, but retains its activity in organic solvents and shows a great thermostability. [Branco *et al.*, 2009; Sheldon, 2007; Eigtved, 1987]

Covalent binding

This immobilization technique is widely used and can be performed with the linkage to a support or with a cross-linker resulting in cross-linked enzymes. A covalent bonding exhibits less leaching of the enzyme enabling a better reusability and an increased stability.

For the immobilization on a support a linkage of a nucleophilic group of the enzyme with the electrophilic groups of the support is performed, e.g. with epoxy or amino groups [Datta *et al.*, 2013; Whittall *et al.*, 2009; Purolite, 2015] If the linking group of the enzyme is near to the active center, whether to a support or another enzyme molecule, this immobilization method can also lead to a deactivation of the biocatalyst. However, this method was carried out for several industrial applications with the immobilization on acrylic resins, e.g. the immobilization of penicillin G acylase onto the hydrophilic support Eupergit®C with a retained activity of over 60% after 800 cycles. This carrier exhibits a high density of oxirane groups, whereby the enzyme is able to bind on various sites resulting in a “multi-point-attachement”. [Sheldon, 2007; Whittall *et al.*, 2009; Katchalski-Katzir, 1993]

Another possibility for covalent immobilization is the cross-linking of single enzyme molecules. Thereby a bifunctional reagent such as glutaraldehyde, is used to prepare cross-linked enzyme crystals (CLECs) or cross-linked enzyme aggregates (CLEAs) without an additional carrier. Despite comparable low mechanical and hydrodynamic stability, high catalyst loadings and volumetric productivities of cross-linked enzymes are promising for enzyme immobilization. Especially CLEAs, due to an easier preparation have been intensively investigated by Sheldon and co-workers. For example, the immobilization of a Penicillin G acylase was performed as CLEAs for the synthesis of antibiotics with different precipitants. The retained activities of the enzyme after immobilization reached 100% compared to the enzyme solution with a specific activity of 214 µmol g^{-1} min^{-1}. [Tischer *et al.*, 1999; Sheldon *et al.*, 2013; Sheldon, 2007; Cao *et al.*, 2000]

Entrapment

Entrapment is usually a non-reversible method, whereby the enzymes can be immobilized in a polymeric network, like organic polymers or silica sol-gel, encapsulation in spheres or in membranes, like hollow fibers. For example, for the entrapment of the enzyme in a sol-gel network of tetraalkoxysilanes are hydrolyzed by acids or bases. Thereby compounds of low molecular weights can diffuse through the polymeric network to the immobilized biocatalyst. Despite an often low enzyme loading and comparable high mass transfer limitations, the biocatalyst is protected by the surrounded network and the cell density can be maintained during continuous processing. In addition, no pretreatment is necessary, but a leaching of the enzyme is often observed if a non-covalent entrapment is used. This effect can be inhibited by the combination with covalent binding of the enzyme at the matrix resulting in increased activity retention. [Eş *et al.*, 2015; Brena *et al.*, 2006; Whittall *et al.*, 2009; Luckarift *et al.*, 2004] A common method is the entrapment using alginate found to prevent enzyme leaching and increase the stability. Thereby alginate is modified with different additives such as calcium, copper or chitosan to improve immobilization efficiency. [Taqieddin *et al.*, 2004; Palmieri *et al.*, 1994; Datta *et al.*, 2013] Membrane technology, as reversible method, is a promising field for the enzyme retention *via* entrapment, due to separation through the different molecular weights between the enzyme (10 - 150 kDA) and the substrates. Enzyme membrane reactors are well established for the continuously production of enantiomerically pure amino acids, like L-methionine (>300 t a^{-1}) at Evonik. [Drauz, 2012; Wöltinger *et al.*, 2001]

3.2.2. Industrial application of immobilized enzymes

Several immobilized enzymes are nowadays applied in chemical industries, due to replacement of classical chemical reaction or completely new syntheses of different compounds. In table 3-2 different enzymes with their industrial application are shown.

Table 3-2. Immobilized enzymes in industry. (IW – Immobilized whole cells, CIE – Covalently immobilized enzyme, NCIE – Non-covalently immobilized enzyme) [DiCosimo *et al.*, 2013; Liese *et al.*, 2006; Krajewska, 2004]

Enzyme	Immobilization	Substrate	Product	Product scale (t per a)
Glucose isomerase (5.3.1.5.)	IW	Glucose	High fructose corn syrup (HFCS)	10^7
Nitrile hydratase (4.2.1.84)	IW, CIE	Acrylonitrile	Acrylamide	10^5
Penicillin G acylase (3.5.1.11.)	NCIE	Penicillin G	Semi-synthetic ß-lactam antibiotics	10^4
Lipase (3.1.1.3.)	NCIE	Triglycerides Alcohols, amines	Biodiesel Esterification	10^4 10^3
Thermolysin (3.4.24.27)	NCIE	Peptides	Aspartam	10^4

Thereby lipases are frequently used for many different reactions, due to their high activity and selectivity in organic solvents, which is of great importance for several esterification reactions. However, most industrial enzymes are used especially for one reaction, due to the high selectivity of the biocatalysts. For example, the glucose isomerase enables the production of the sweetener high fructose corn syrup (HFCS) with a product scale of over 10^7 t per year and is used especially for this reaction in industry. With regard to costs for purification whole cells are used for this biotransformation, which are immobilized by different techniques depending on supplier and strain. The reactions are carried out in 20 individual reactors, whereby the enzyme has a half-live time of up to 100 days, enabling the production of 1000 t HFCS per day. [Liese *et al.*, 2006; DiCosimo *et al.*, 2013]

For the enzyme entrapment, which was investigated in this study, the synthesis of acrylamide is one of the most important and well-established examples in industrial-scale biocatalysis. During this process acrylonitrile is hydrolyzed to acrylamide. This compound consists two functional groups, a vinyl and an amino group, which enable polymerization reactions representing a building block for industry e.g. for waste water and paper treatment, as well as in the coating industry. [Rothenberg, 2008; Gong *et al.*, 2017]

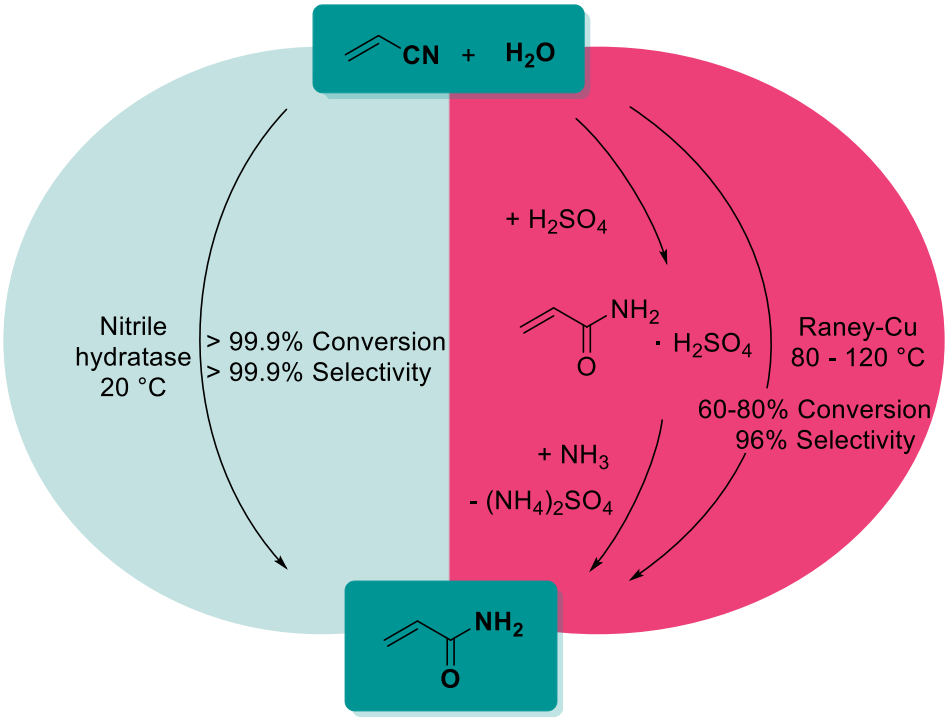

Figure 3-8. Biocatalytic (left) and chemical synthesis (right) of acrylamide. [Rothenberg, 2008]

The biocatalytic and the chemical pathways for the production of acrylamide are shown in figure 3-8. The biocatalytic synthesis can be performed in a one-step reaction at 20 °C and even if the chemical pathway can be realized also in one-step using Raney-Cu, the temperatures are with up to 120 °C still high. The chemical pathways reached a selectivity of 96% with yields of 60 – 80%. However, due to harsher reaction conditions, more energy input is required leading to a higher CO_2-emission. Furthermore, side product formation, as well as toxic waste is occurring based on e.g. high temperatures and the copper-based catalyst. The impurities may disturb the subsequent polymerization, so that the biocatalytic route is favored for the production of acrylamide. The enzymatic production of acrylamide is based on the Mitsubishi Rayon process and runs up to 50,000 tons per year worldwide. During this process acrylonitrile is hydrolyzed by a nitrile hydratase from *Rhodococcus*

rhodochrous in aqueous solution with a selectivity and yield over 99.9%. For this process the nitrile hydratase is immobilized as whole cells in a polyacrylamide matrix and enables an increased stability of the enzyme with a residual activity of 50% after 13 cycles. Nowadays, this reaction has been transferred to further productions of several amines and acids in an industrial scale by different companies (Lonza, BASF, Dow, DSM, DuPont) and represents the promising implementation of immobilized enzymes in industry and especially for the biosynthesis of bulk chemicals. [Gong *et al.*, 2017; Rothenberg, 2008; Yuryev *et al.*, 2010; Development, 2001]

This example shows the importance of biocatalysis in industry today, which is often named as “White biotechnology”. Around 200 processes are already implemented in chemical industry, however, new biocatalytic processes still have to compete with well-established chemical processes often optimized over decades. This implementation of biocatalytic processes in industry is still challenging resulting in several research in academia as well as industry with interdisciplinary teams including engineers, biologist, biochemists and chemists. [Woodley *et al.*, 2013]

3.2.3. Spraying techniques for encapsulation

Spraying techniques represent highly interesting methods for enzyme encapsulation. Mostly a narrow size distribution is obtained with a spherical shape of the particles, whereby the method itself shows an easy performance with good reproducibilities. Thereby three different forms can be categorized: spray drying, electrospraying and spray congealing.

Spray drying is an extensively used technique for encapsulation processes. Thereby a solution or emulsion of the dissolved carrier and the compound to be immobilized are atomized through a nozzle in a hot chamber forming solidified particles due to a rapid evaporation of the solvent (e.g. water). [Vega *et al.*, 2006] For this method numerous equipment is available and the processing costs are comparable low. In industry it is mostly used for the production of dry powder to enhance the stability of the compounds e.g. eggs, milk or pharmaceuticals. Also encapsulation of biocatalysts in different matrices, like chitosan or other polymers has been well investigated by different research groups. [Kašpar *et al.*, 2013; Chranioti *et al.*, 2016; Dahili *et al.*, 2015; González Siso *et al.*, 1997; Mucha, 1997; Johnson *et al.*, 2009] In comparison to spray drying electrospraying belongs to the electro-hydrodynamic processes. Thereby a polymer solution or a melted polymer is used and atomized by an external high potential electric field (1-30 kV) applied between the nozzle and earth or charged substrate. [Enayati *et al.*, 2011] The solution is fed by a syringe pump,

whereby at the end of the capillary the droplet is distorted into a Taylor cone, due to the charged droplet.

However, the aim of this study was the immobilization of biocatalysts *via* spray congealing. Spray congealing, also known as spray chilling, spray cooling or prilling, is a carrier based immobilization technique combining spray drying and a hot melt technology. A molten mixture, containing the carrier and the active compound (here an enzyme), is atomized and the formed droplets re-solidify in a cooling chamber forming solid microparticles, due to temperature decrease (figure 3-9). [Rosiaux *et al.*, 2014]

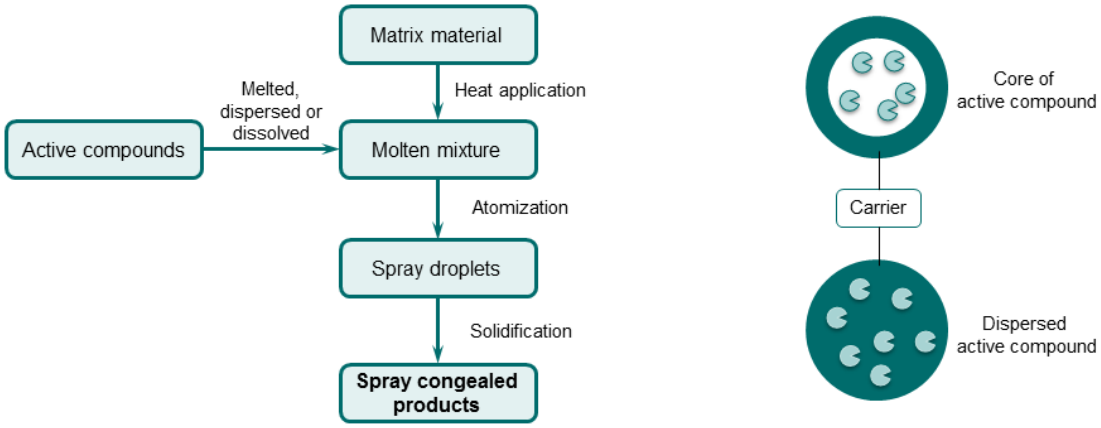

Figure 3-9. Spray congealing process and possible distribution of active compounds. (adapted from [Oh *et al.*, 2014])

For the dispersion of the active ingredient two possibilities can occur. One possibility is the forming of an active core surrounded by the carrier, like a membrane and the other is the forming of a compact particle, where the separated molecules are dispersed. However, the carrier should melt at comparatively low melting temperatures, between 45 – 100 °C, depending on the immobilized compound and should be solid at room temperature. [Albertini *et al.*, 2008; Oh *et al.*, 2014] For this technique no organic solvent is necessary as well as no downstreaming, due to a single-step process. The prepared particles are with a size in the nano- or micrometer range, depending on the set-up, very small and can provide less diffusion limitation in comparison to greater particles, due to an increasing of the surface-to-volume ratio. [Yonekura *et al.*, 2014] Spray congealed particles showed in most studies a spherical shape with a high density, in comparison to spray dried particles, which are influenced by the mass transfer due to a crumpled shape. [Favaro-Trindade *et al.*, 2015] For

spray congealing carriers with a comparable low melting point are necessary, which are mostly long chain alcohols, fatty acids or triglycerides. These compounds are furthermore environmentally friendly and cheap in comparison to carriers used for other immobilization techniques, like encapsulation in a hydrogel. [Okuro *et al.*, 2013] The properties of the obtained particles can be customized to a certain extent by different set ups, as well as different operating conditions. For example, the particle size can be affected by the viscosity and therefore temperature, loading of the carrier and the chosen carrier itself. The size is increasing with an increasing viscosity, a lower atomizing pressure and a greater orifice opening, resulting in a changing of the diffusion and release of the immobilized mixture. [Albertini *et al.*, 2008; Oh *et al.*, 2014] A big advantage of this technique is that nowadays a spray dryer is often used in industry, in particular in the food industry. With a liquefying bath, as an additional modification of the apparatus, it can be easily applied for spray congealing processes. Therefore it is highly interesting to use this modified system for spray congealing in comparison to a self-developed one, such as in the research group of Nadia Passerini. [Di Sabatino *et al.*, 2012; Albertini *et al.*, 2009; Passerini *et al.*, 2009; Passerini *et al.*, 2006] Furthermore, this method is easy to scale up and the carriers are easy to handle due to transportation in powder or tablet form. [Favaro-Trindade *et al.*, 2015] All in all, spray congealing is deemed to be an affordable process for encapsulation in an industrial scale. [Pedroso *et al.*, 2013]

4. Methods

4.1. Immobilization techniques

The immobilization of the biocatalysts was performed using two different set-ups. The first technique was the immobilization with a modified spray dryer B-290 (Büchi) and the second a spray congealing system developed by the research group of Prof. Nadia Passerini (Bologna, Italy).

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4.1.1. Determination of the biocatalytic activity

For the immobilization three enzymes were used. With respect to further reactions two different laccases and an esterase were used: the crude extract from *MtL* and the lyophilisates from *TvL* and PLE were chosen. The activities were measured spectrophotometrically in buffered systems at 25 °C. All samples were measured with a standard assay against a reference using the reactions shown in figure 4-1. [Bourbonnais *et al.*, 1990; Schmidt *et al.*, 2005]

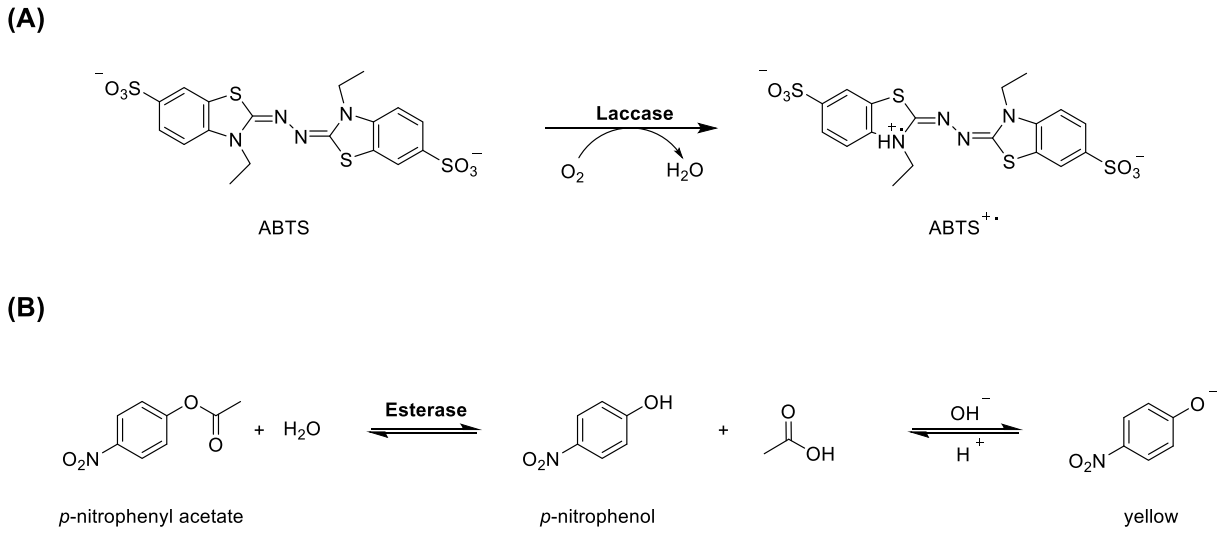


Figure 4-1. Corresponding reactions for measurement of biocatalytic activities. (A) Oxidation of ABTS to the radical form by the laccase. (B) Hydrolytic cleavage of *p*-nitrophenyl acetate by the esterase.

As substrate for the laccases 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, 5 mM) was used, which was oxidized during the reaction to the radical form absorbing at 420 nm. For the activity measurement of the PLE *p*-nitrophenyl acetate (*p*-NPA, 10 mM) dissolved in the solubilizer dimethyl sulfoxide (DMSO) was used. The substrate was hydrolyzed to *p*-nitrophenol and under basic conditions it was changing to the deprotonated form absorbing at 401 nm. The corresponding enzymatic assays are presented in table 4-1.

Table 4-1. Reaction conditions for the biocatalytic assays.

	TvL	***MtL***	**PLE**
Substrate	ABTS	ABTS	*p*-NPA (in DMSO)
Wavelength	420 nm	420 nm	401 nm
Buffer	Citrate-phosphate buffer (pH 4)	Phosphate buffer (pH 6, 0.1 M)	Phosphate buffer (pH 7.5, 0.1 M)
Extinction coefficient	36,000 1/(M·cm)	36,000 1/(M·cm)	13,9365 1/(M·cm)

[1]Measured at 30 °C (Data not shown).

The corresponding activities of the enzymes were calculated *via* equation 1. The specific activity A described the conversion of substrate per minute depending on the used amount of enzyme. The changing of the product concentration was measured by the linearic slope of the extinction ΔE at the specific wavelength in the corresponding buffer (table 4-1).

$$A = \frac{\Delta E \cdot V_{cuvette} \cdot F}{\varepsilon_{product} \cdot d_{cuvette} \cdot V_{enzyme} \cdot c_{enzyme}} \qquad (1)$$

Furthermore $V_{cuvette}$ presented the reaction volume, $d_{cuvette}$ the diameter of the cuvette, V_{enzyme} the added volume of the enzyme solution and c_{enzyme} the concentration of the enzyme solution. The F value constituted the dilution of the enzyme solution, which was essential to obtain extinctions within the validity of the Lambert-Beers law.

4.1.2. Enzyme encapsulation with a spray dryer

Immobilization. The commercial available spray dryer (B-290) from the company Büchi was modified with an additional liquefying bath. In figure 4-2 the set-up is shown, whereby the liquefying bath was added on the top of system controlled with an additional transformer, so that the nozzle could be still heated by the spray dryer itself.

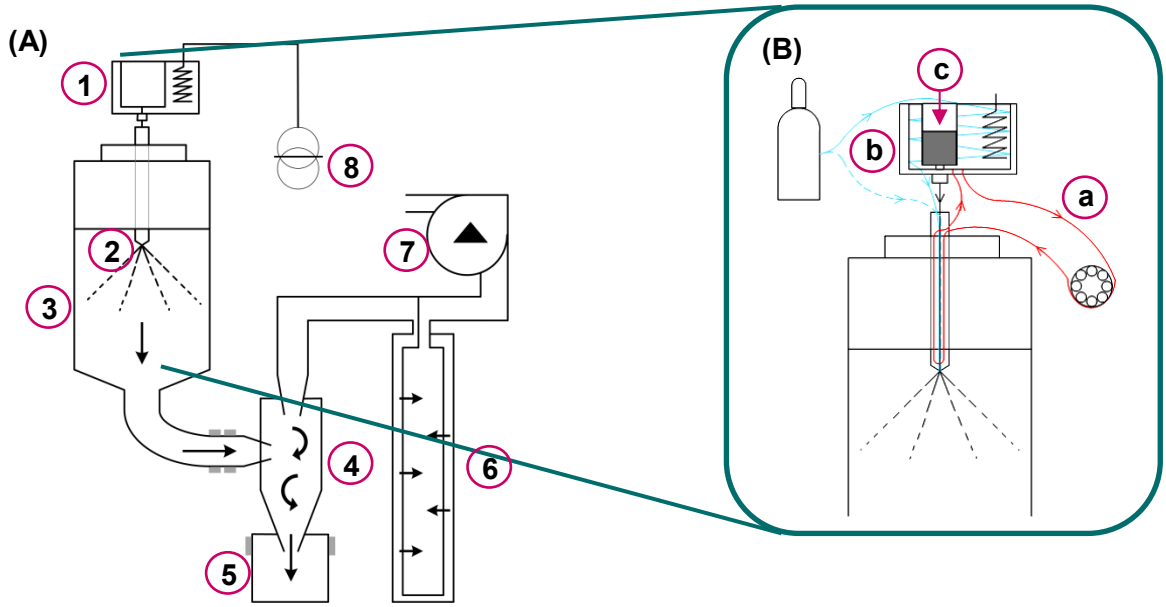


Figure 4-2. Modified set-up of the spray dryer: **(*A*)** 1 – Liquefying bath, 2 - Nozzle, 3 – Spray cylinder, 4 - Cyclone, 5 – Collection vessel, 6 – Outlet filter, 7 – Aspirator, 8 – Transformator; **(*B*)** a – Heating fluid, b - Pressured air, c - Mixture of carrier & enzyme.

The liquefying bath and the nozzle were heated 10 °C above the melting temperature of the used carrier. The carrier itself was heated external in a beaker up to the same temperature. For enzyme encapsulation the two-fluid nozzle was used, whereby the complete carrier-enzyme mixture had to be prepared external. After melting the carrier the enzyme solution was emulsified. The mixture was filled into the liquefying bath, wherefrom it flowed through the nozzle and atomized into the spray cylinder. The particles re-solidified in the spray cylinder and were transported into the collection vessel by the aspirator. For spraying the maximum aspirator rate of 100% (800 L/h) was used to obtain the main product in the collection vessel. Furthermore, 100% (25 mL/min) of the pumping rate for the heating

solution through the nozzle was chosen to ensure the same temperature for nozzle and liquefying bath. The resulting product was collected and stored in the freezer until further investigations.

Activity measurements. The activity measurements were conducted with a suspension of 10 mg microcapsules in 100 mL buffer. Samples were taken over time and filtered with a syringe filter before analysis.

Release experiments. The protein release was detected spectrophotometrically by the method of Whitaker and Granum. [Whitaker *et al.*, 1980] Thereby the protein concentration $c_{protein}$ was determined of two different absorptions *A* (235 nm and 280 nm):

$$c_{protein} = \frac{A_{235} - A_{280}}{2.51} \qquad (2)$$

All release measurements were performed in 50 mL phosphate buffer (pH 6, 0.1 M) with 10 mg of the microparticles. The samples were taken over the time and filtered by a syringe filter before measurement.

Recycling experiments. The recycling measurements were performed in a fixed-bed reactor. 500 mg of the immobilized enzyme were filled in the reactor and retained with a filter (5 µm). The reaction was performed in a continuous mode with a flux of 10 mL/min and 100 mL of the substrate solution. For the laccases a modified activity assay was used with 2.2 mL of a 5 mM ABTS solution, whereby for the esterase the standard assay with a 10 mM *p*-nitrophenyl acetate solution was used. The samples were taken over defined time intervals and measured spectrophotometrically.

Scanning electron microscopy (SEM). The analysis of the shape and surface was performed with scanning electron microscopy using a Supra™ 25 FESEM (Carl Zeiss AG, Oberkochen, Germany). Before analysis no further sample pretreatment was conducted.

Viscosity measurements. The viscosity of the carriers was measured with a rheometer MCR 302 (Anton Paar, Austria). The samples were placed in the rheometer and viscosity was measured at different temperatures. Due to limitations of the measurements, the points were fitted and the viscosity extrapolated at 10 °C above the melting point.

Measurements of the water content. To determine the water content Karl Fischer titration of Metrohm (Gallen, Switzerland) was used. Therefore 15 - 25 mg of the sample were suspended in the coulometric solution. The water content of the microparticles with immobilized enzyme $\theta_{calculated}$ was determined by equation 3.

$$\theta_{calculated} = \frac{\theta_{enzyme\ solution}}{m_{carrier}} + \theta_{carrier} \qquad (3)$$

The content was calculated by the water content of the enzyme solution $\theta_{enzyme\ solution}$, the carrier mass $m_{carrier}$ and the water content of the carrier without enzyme $\theta_{carrier}$.

Particle size distribution (PSD). The particle sizes were measured by the Chemical Process Engineering of the Max Planck Institute for Dynamics of Complex Technical Systems (Magdeburg, Germany).

4.1.3. Spray congealing system

The investigated spray congealing system was developed by the research group of Prof. Dr. Nadia Passerini. The set-up is shown in figure 4-3 containing mainly a nozzle and a cooling chamber.

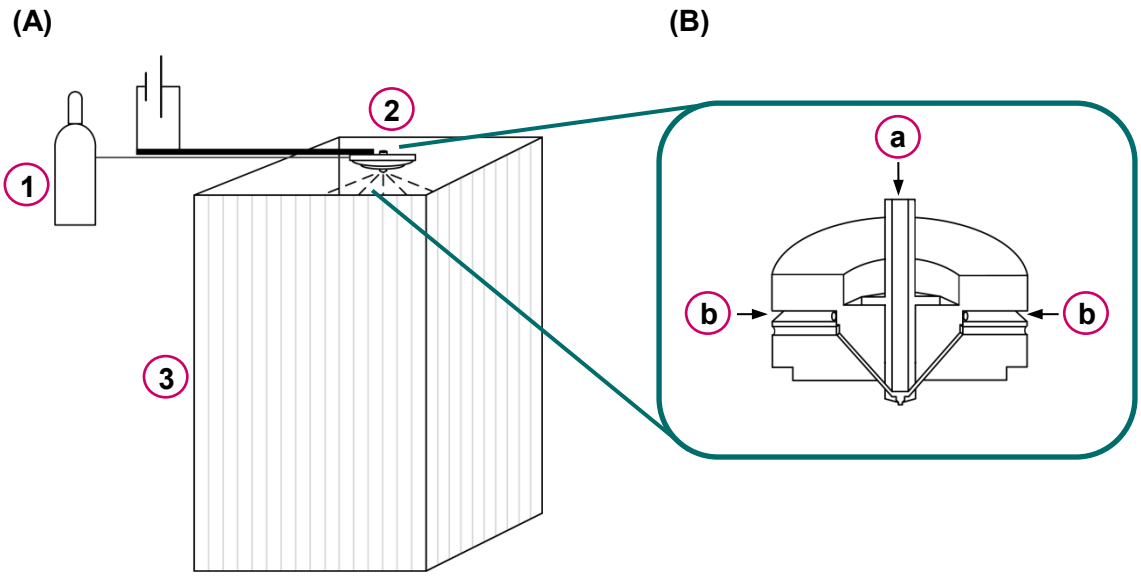


Figure 4-3. Set-up of the spray congealing system (*A*) 1 – Pressured air, 2 - Nozzle, 3 – Cooling chamber; (*B*) a - Feed, b – Gas inlet.

The nozzle provided a big orifice diameter of 4.5 mm and was heated 10 °C above the melting point of the used carrier by voltage adjustment. Another special feature of this system was the radially influxing pressured air through the nozzle, which was set at 3.0 bar. For this system the carrier-enzyme mixture had to be prepared before and was purred manually into the nozzle, which controlled the velocity of the atomization. The mixture was flowing through the nozzle until the end, where it was atomized and re-solidified in the cooling chamber. The produced microparticles were collected on the bottom of the cooling chamber and stored in the freezer for further investigations.

Activity measurements. The activities of the enzymes were measured spectrophotometrically with a flow-through cell. Therefore 100 mL substrate solution was used and 100 mg of the produced microparticles were added. A modified assay for the laccase was developed with 1 mM ABTS solution.

During the recycling experiments a fixed-bed reactor filled with 250 mg of the produced microparticles was prepared and 100 mL substrate solution. The reactor was washed with 3 mL buffer before measurement and between the batches. After every batch a new substrate solution was used and the residual activity was measured and calculated.

Particle size distribution (PSD). The particle size was measured by sieve analysis, using a vibrational shaker (Octagon Digital, Endecotts, London, UK). For our investigations standard sieves with sizes of 50, 75, 100, 150, 250, 355 and 500 µm were used. The frequencies were calculated by mass fractions.

Differential scanning calorimetry (DSC). DSC measurements were performed with a Perkin-Elmer DSC 6 (Perkin-Elmer, Beaconfield, UK) and the software Pyris. The samples with a mass between 10 – 12 mg were placed into the DSC under nitrogen with a flow of 20 mL/min. They were scanned with a temperature range from 30 – 100 °C and a rate of 10 °C/min.

Viscosity measurements. The viscosity of the carriers were determined with a viscosimeter (Visco Star-R, Fungilab S.A., Barcelona, Spain). Therefore 100 g of the carrier were heated 10 °C above the melting point and after some preliminary tests, the spindle TR9, was selected and the spindle rotating speed was set to 200 rpm.

Recycling measurements. The recycling measurements were performed as before described with minor modifications. The fixed-bed reactor was filled with 250 mg and a tubing pump was set-up for continuous flow. Furthermore, the spectrophotometrical measurements were performed continuously.

4.2. Laccase initiated coupling reactions

Substrate screening. The substrate screening of the *TvL* was performed in a reaction volume of 10 mL with an enzyme concentration of 1 U/mL. In addition, a citrate phosphate buffer (pH 4) was used with a substrate concentration of 5 mM and 20% methanol as co-solvent. During the oxidation of the substrates oxygen was consumed by the laccase and reduced to water, which could be observed with an online oxygen measurement system (PreSens, Regensburg, Germany). Therefore an oxygen sensor spot was placed at the reactor and *via* an optical fiber cable the oxygen content could be measured *in situ*. The device was calibrated with argon to a content of 0% oxygen and with pressured air to 100%. After calibration the solution was aerated up to an oxygen content of 100% and the different slopes of the oxygen consumption could be compared regarding the activity of the laccase towards different substrates.

Coupling reactions. For the laccase initiated C-N coupling reactions the *TvL* was used and the reactions were carried out in an sodium acetic acid buffer at 30 °C and different pH values. The reaction volume was 20 mL with a substrate concentration of 5 mM**,** an amine concentration of 5 mM and an enzyme activity of 5 U/mL. The reaction solution was aerated with pressured air continuously to reduce the limitation of the reaction by oxygen content. Beside batch reactions, this reaction was also carried out as fed-batch with a syringe pump, shown in figure 4-4. Therefore the amine and enzyme solution (V = 10 mL) were placed in the reactor and the substrate solution was added continuously with different pump rates.

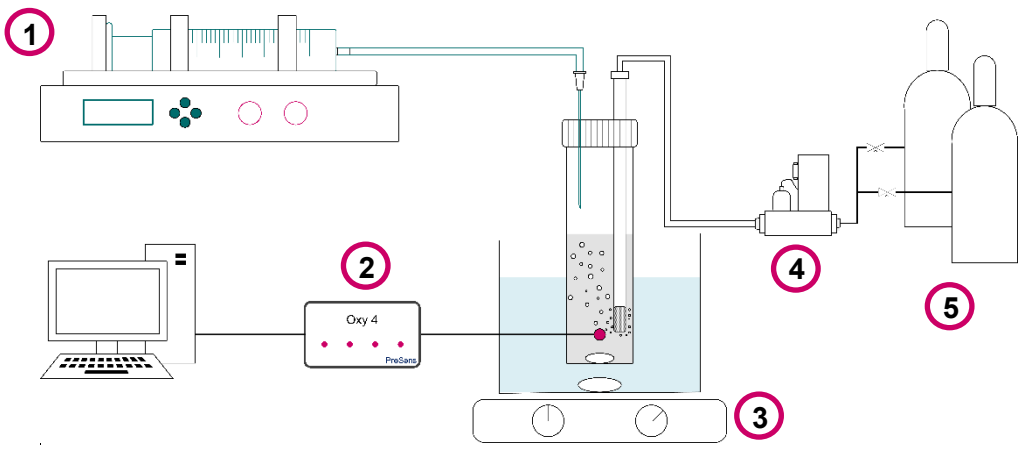


Figure 4-4. Set-up of the C-N coupling reaction *via* fed-batch. 1 – Syringe pump, 2 – Online oxygen measurement *via* Oxy 4 and sensor foil at the reactor, 3 – Magnetic stirrer, 4 – Mass flow controller, 5 – Aeration of the reaction solution.

LC/MS measurements. The obtained reaction solutions were analyzed *via* LC/MS measurements. Therefore a device from Thermo Fisher Scientific was used with an ion trap mass analyzer. For the measurements a Kinetex Biphenyl column (150 mm x 2.1 mm, 2.6 µm), an oven temperature of 35 °C and a partial loop of 2 µL were applied. As eluent water and methanol with 0.1% formic acid (Fac) with a flow rate of 0.2 mL/min was used with the gradient, shown in table 4-2.

Table 4-2. Gradient used during LC/MS measurements.

Time [min]	Water with 0.1% Fac [%]	Water with 0.1% Fac [%]
0	90	10
5	90	10
20	20	80
30	80	20
50	80	20

The obtained data were evaluated with Xcalibur™ in the positive ion mode *via* peak integration. The area ratios were compared, because no downstream processing could be realized and therefore no pure product could be obtained for calibration.

5. Results & discussion

5.1. Spray congealing – A new possibility for enzyme immobilization?

Efficient and cheap immobilization methods for biocatalysts are highly interesting especially for industrial applications. Spray congealing as innovative immobilization technique is a new strategy to encapsulate biocatalysts, due to the usage of environmentally friendly and cheap carriers. Within this work two different set-ups for spray congealing were evaluated for enzyme immobilization. The first system was a commercial available spray dryer (Büchi B-290) modified with an additional liquefying bath, whereby the second system presents a self-developed system. In the end the results were compared and evaluated with regard to feasibility of the application of the methods.

5.1.1. 1st system: The modified spray dryer

For the first system the commercially available spray dryer B-290 of the company Büchi was modified with an additional liquefying bath. This was added on the top of the spray dryer to keep the temperature of the mixture 10 °C above the melting point of the carrier (cf. figure 4-2). The common set-up tempered the heating solution in the liquefying bath, which heated the nozzle. This heating procedure revealed often in nozzle clogging during the investigations and therefore no enzyme immobilization could be realized. To minimize the risk of nozzle clogging a transformer was added to adjust the temperature of the liquefying bath realizing a parallel heating of the mixture and the nozzle. The temperature of the melting bath was investigated with regard to applied voltage by the transformer, whereby the lowest possible voltage of around 83 V resulted in the desired temperatures of 10 °C above the melting point of the carriers (cf. appendix). The results of the enzyme immobilization with this modified spray dryer are already published.[1]

[1]C. Engelmann, U. Kragl. „Spray congealing as innovative technique for enzyme encapsulation". Journal of Chemical Technology and Biotechnology. 2017, 93, 191.

5.1.1.1. Carrier investigation for enzyme immobilization

For spray congealing it was very important to find an appropriate carrier. It was essential that the carrier was solid at room temperature with a comparable low melting point, but still able to re-solidify during the spraying process. Commonly used compounds were different lipids, e.g. glyceryl derivatives, waxes, as well as fully or partially hydrogenated vegetable oils and fats. [Becker *et al.*, 2015] To find a suitable system, different carriers were chosen and tested with respect to spraying behavior and retained activity of the immobilized biocatalyst. Table 5-1 lists the tested carriers with chosen properties. Usually carriers with a melting point between 50 – 100 °C were used for spray congealing, but due to the sensitivity of enzymes, compounds with a lower melting point between 35 – 86 °C were chosen for the investigations in this study (table 5-1). [Oh *et al.*, 2014; Wong *et al.*, 2016]

Table 5-1. Properties and suitability of the investigated carriers.

Matrix	Structure	Mp [°C]	Price [€/kg][a]	Spraying
Myristyl alcohol	$C_{14}H_{29}OH$	35 – 39	52.00	✓
Cetyl alcohol	$C_{16}H_{33}OH$	48 – 50	38.00	✓
Stearyl alcohol	$C_{18}H_{37}OH$	56 – 59	54.00	✓
Lauric acid	$C_{11}H_{23}COOH$	44 – 46	42.00	✓
Myristic acid	$C_{13}H_{27}COOH$	52 – 54	44.00	✓
Palmitic acid	$C_{15}H_{31}COOH$	61 – 63	42.00	✓
Cetyl palmitate	$C_{32}H_{64}O_2$	55 – 56	100.00	✓
Glyceryl tripalmitate	$C_{51}H_{98}O_6$	66 – 67	2380.00	✓
Glyceryl tristearate	$C_{57}H_{11}O_6$	72	180.00	✓
Glyceryl tribenzoate	$C_{14}H_{29}OH$	68 – 72	84.00	×
Beeswax	Mixture (acids, alcohols, etc.)	61 – 65	150.00	×
Carnabua wax	Mixture (acids, alcohols, etc.)	82 – 86	112.00	×

[a] Sigma-Aldrich online on 09.11.2016.

Spraying of the tested carnauba and beeswax was not possible, due to their sticky behavior and an immediately clogging of the nozzle. For all other carriers it could be noticed, that the spraying was possible, except of glyceryl tribenzoate. For this carrier no re-solidification of the droplets directly after the atomization could be observed, which might be influenced by benzoate residue as an unsaturated aromatic residue. The viscosity was another criterion selected for the feasibility of the spray congealing. Figure 5-1 presents the calculated viscosities from extrapolation of the rheometer measurements over various temperatures 10 °C above the melting point of the corresponding carrier. The viscosities had to be calculated, due to limitations by the rheometer itself. These measurements showed a viscosity temperature relationship according to the structure of the compounds. The viscosity-temperature relationship was also investigated by Wong *et al.* who tested lipid-based materials such as cetyl, stearyl alcohol and further compounds with a long alkyl chain and a hydrophobic nature. They found out that these materials showed a biexponential viscosity-temperature relationship, depending on e.g. hydroxyl groups, suggesting complex phenomena at the molecular level. Firstly, the temperature debilitates the weak intermolecular interactions resulted in a viscosity decrease. After complete alignment of the long chain a minimum is reached with no further significant change in viscosity. [Wong *et al.*, 2015] The profiles found in this study showed a similar progress (cf. appendix). Carnauba wax showed by far the highest viscosity of all tested compounds with 121.5 mPa·s, followed by glyceryl tribenzoate (69.6 mPa·s) and beeswax (55.3 mPa·s). The higher viscosity was influencing the size of the formed droplet while atomization resulting in greater microparticles. [Wong *et al.*, 2015; Albertini *et al.*, 2008] However, the investigated waxes showed no suitability for spray congealing with the modified spray dryer, due to an immediately nozzle clogging. Waxes presented nature-based mixtures of long-chain fatty acids and alcohols and were well investigated for several encapsulations. Savolainen *et al.* prepared microparticles based on a formulation of felodipine with carnauba wax as carrier. In contrast to our set-up they used a different construction and higher temperatures up to 120 °C. This higher temperature resulted in a lower viscosity, which could avoid the clogging of the nozzle. Furthermore the capillary length was with 5 cm shorter than the capillary from the spray dryer (15 cm), which was advantageous for usage of this highly viscous carrier. [Albertini *et al.*, 2008; Savolainen *et al.*, 2002] Due to the long capillary of the spray dryer and the aim of immobilization of the heat sensitive enzymes, the unsuited waxes were not further investigated. In contrast, the spraying of glyceryl tripalmitate enabled an easy spraying, however, this carrier showed a high electrostatic charge, despite grounding of the system.

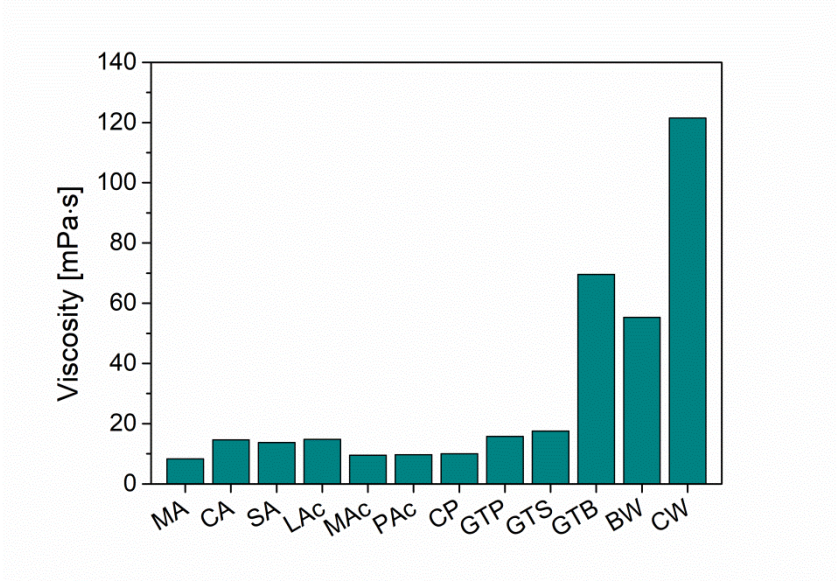


Figure 5-1. Extrapolated viscosities 10 °C above the melting point of the tested carriers. (cf. appendix) MA - Myristyl alcohol, CA – Cetyl alchol, SA – Stearyl alcohol, LAc – Lauric acid, MAc – Myristic acid, Pac - Palmitic acid, CP - Cetyl palmitate, GTS – Glyceryl tristearate, GTB – Glyceryl tribenzoate, GTP - Glyceryl tripalmitate, BW - Beewax, CW – Carnauba wax.

This resulted in a difficult handling before and after spraying. Consequently, glyceryl tripalmitate as a comparable expensive compound was not suitable for our application. The other investigated carriers, with a comparable low viscosity and low melting points were promising for the application in spray congealing for enzyme immobilization. These compounds were tested in the following section for the immobilization of a laccase regarding retained activities.

5.1.1.2. Activity of the immobilized laccase

The laccase from *MtL* was chosen for a first evaluation of enzyme immobilization *via* spray congealing. Different carriers were chosen and the retained activities of the immobilized enzyme as well as the yield of immobilization were investigated.

In figure 5-2 the retained activities, as well as the spraying yields are shown, depending on the used carrier. For the investigation of the retained activity, great differences became visible. The shorter the chain length of the lipids, the lower was the melting point and the easier the spraying. However, during the activity measurements a slight abrasion of these compounds while suspending in the reaction solution was visible. This disruption of the particles could lead to a higher loss of the immobilized biocatalyst, which was highly disadvantageous for separation, as well as the recovery of the enzyme. In addition, a short chain length resulted in a softer structure of the particles. Based on this property the carriers could lead to a higher compression while using in e.g. a fixed-bed reactor revealing in a pressure drop. In contrast, an increasing chain length of the compounds, e.g. stearyl alcohol or glyceryl tripalmitate, resulted in more stable particles. Due to the possibility of comparable stable particles and the proof of concept during this study, no other reactor concept was taken into account for the screening.

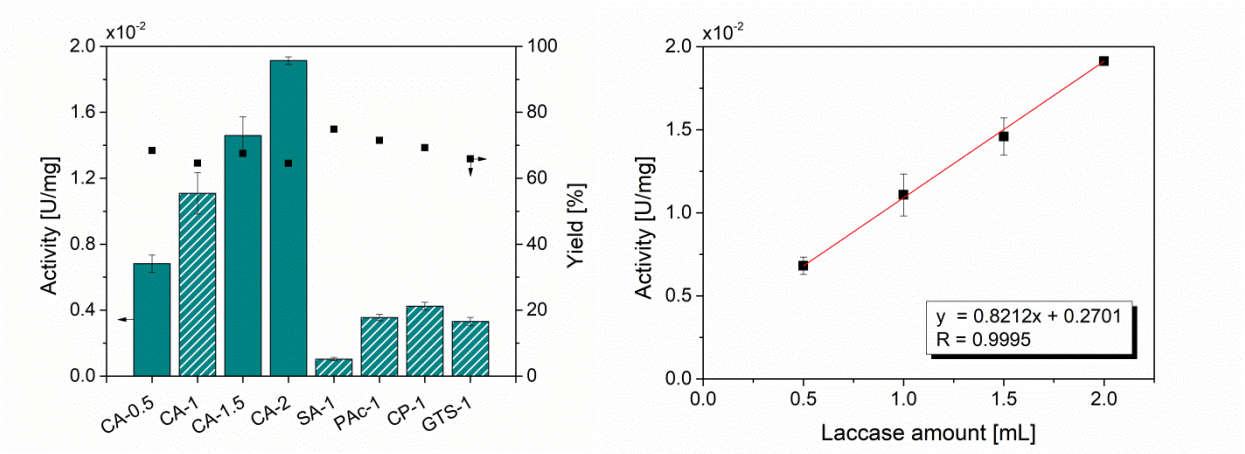


Figure 5-2. Activities of the immobilized enzyme and yields of microparticles. *Left:* Bars: Retained activities depending on the carrier. Dashed bars: Different carriers with 1 mL enzyme (490 U). Rectangles: Spraying yields depending on the carrier. *Right:* Linear regression of the activity using different enzyme amounts. Conditions: *Activity:* $m_{capules}$ = 10 mg, V_{buffer} = 100 mL, c_{ABTS} = 5 mM, λ = 420 nm, n = 3; *Spray congealing*: m_{matrix} = 20 g, $A_{laccase}$ = 490 - 980 U, n = 3. (CA – Cetyl alcohol, SA – Stearyl alcohol, PAc – Palmitic acid, CP – Cetyl palmitate, GTS - Glycerol tristearate, 0.5 - 2 – 0.5 - 2 mL *MtL*).

The microparticles made of these lipids were located on the surface of the solution, due to the higher hydrophobicity and density resulting in an improper suspension behavior. The

different structures of the used carriers and the corresponding properties had also a significant influence on the activity of the encapsulated laccase. The dashed bars in figure 5-2 demonstrate the influence of the selected carrier for the enzyme activity. The found activity for stearyl alcohol was with 1.0 mU/mg the lowest for all tested materials with only 4% residual activity of the encapsulated enzyme. The activities for the encapsulated enzyme in palmitic acid, cetyl palmitate and glyceryl tristearate were slightly higher with values up to 4.2 mU/mg (17.1% residual activity). Nevertheless, cetyl alcohol exhibited the best retained activity of 44.9% (1.0 mU/mg) representing a ten times higher activity than immobilization in stearyl alcohol. One reason for this high activity could be the low melting point of 48 – 50 °C which could result in less denaturation of the enzyme. However, during these measurements it became visible that the immobilizations of the biocatalyst in carriers with higher melting points were not always leading to a lower activity. For example, glyceryl tristearate exhibited a 10 °C higher melting point than stearyl alcohol (56 – 59 °C) and the retained activity was determined four times higher with 4.2 mU/mg. This indicated that other parameters, like lipid structure, influenced the spray congealing process as well as the enzyme itself. Palmitic acid, the corresponding acid to cetyl alcohol, and the esters cetyl palmitate and glyceryl tristearate showed also better suitabilities for enzyme immobilization than stearyl alcohol with regard to the retained activity.

Nevertheless, cetyl alcohol showed the best activities (44.9%) and therefore the best compromise due to activity, handling, spraying behavior and costs. This carrier was chosen for further immobilizations of *MtL* and the immobilization was tested with different amounts of the enzyme. In figure 5-2 it can be seen, that the higher the enzyme amount, the higher was also the retained activity. In particular, we found a linear correlation for the activity depending on the immobilized enzyme amount. The immobilization of higher enzyme amounts than the investigated maximum of 2 mL was only limited by the method itself. During this study the enzyme was used as a liquid, a high viscous crude extract, which had to be emulsified in the molten carrier before spraying. The enzyme had to be stored in the freezer and was warmed up to room temperature before using. This resulted in a comparable high temperature difference of the crude extract (room temperature) and the melted carrier (10 °C above the melting point) and therefore in a partly re-solidification of the lipid compound during the emulsification process. For the homogenous emulsification this mixture should have been heated for a longer time or the enzyme solution should have been heated before filling into the molten carrier. Both possibilities would have been resulted in an additional heating and therefore an increasing denaturation of the enzyme. To prevent a greater loss of activity based on enzyme denaturation 2 mL enzyme amount was used as maximum. However, the higher amount of laccase (2 mL, 980 U) resulted with 38.8% in less residual activity than with

a lower laccase amount of 0.5 mL (245 U), which resulted in 55.5% residual activity. This could be based on the fact, that the higher enzyme amount could result in more enzyme sticking in the spray congealing system and therefore lower activities. In comparison, Wang *et al.* encapsulated a laccase from *Trametes versicolor* in calcium alginate with a retained activity of 29.3% and in novel core-shell capsules with aginate/ protamine/ silca hybrid membranes resulting in only 13.9% activity. [Wang *et al.*, 2013] With our method we could reach a higher residual activity of over 50% with the laccase originating from the thermophilic fungus *Myceliophthora thermophila*. For further investigations, 1 mL (490 U) of the laccase was chosen, as a compromise to realize a comparable high activity of the immobilized enzyme and good retained activity.

During these investigations high yields up to 80% could be reached, shown in figure 5-2. Only slight differences were found for all spraying experiments and no significant dependence on the used carrier as well as the amount of enzyme could be observed. These good spraying yields were also obtained in prior studies of other research groups with different set-ups and applications. Gavory *et al.* obtained yields of 89% using a spray dryer (B-191, Büchi) for encapsulation of an adhesive in a hydrogenated palm oil. They used an external heating bath, whereby the mixture was pumped by a peristaltic pump, while in this study an equipped melting bath on the top of the spray dryer was used. [Gavory *et al.*, 2014] The set-up used during the investigations in this study provided the great advantage of heating the liquefying bath and the nozzle with only one heating solution. No additional use of a peristaltic pump for feeding the mixture was needed, so that the solidification of the mixture in the tubes could be avoided. In contrast, the group of Passerini developed a special spray congealing system for immobilization of pharmaceuticals reaching yields up to 92%. [Passerini *et al.*, 2009] Despite external melting of the carriers, all studies of immobilization *via* spray congealing offered excellent spraying yields and differed only slightly due to the procedure, like temperature or pressure. Furthermore enzyme encapsulation with this method could be realized for the first time with a moderate residual activity. This easy feasibility, as well as effective and reproductive immobilization of the laccase, are great advantages of the spray congealing for enzyme immobilization, so that this technique represents a good alternative to other immobilization methods, especially for industry.

5.1.1.3. Water content

The water content of the prepared microcapsules has a significant impact on the enzyme stability. A low water content reveals a higher stability of the biocatalyst, due to a lower availability of water for biochemical reactions. [Bampi *et al.*, 2016] Nevertheless, water is essential for the activity of biocatalysts, depending on the nature of the used enzyme. Laccases need a certain degree of water to realize a biocatalytic activity, even if the used *MtL* is active at high amounts of water-miscible organic solvents such as methanol. [Illner *et al.*, 2014]

$$\theta_{calculated} = \frac{\theta_{enzyme\ solution}}{m_{carrier}} + \theta_{carrier} \qquad (3)$$

Despite a better evaluation by the water activity, which is describing the partial pressure of water vapor in equilibrium, the water content of the microparticles was investigated by Karl-Fischer titration, due to easier feasibility. [Clark, 1994] The calculated water contents, based on the used enzyme solution (equation 3), of immobilized *MtL* reached values between 3.2 and 3.7%. However, the measurement revealed that the residual water content of the microparticles with encapsulated enzyme was significantly lower than the calculated ones (figure 5-3). Especially palmitic acid showed a strong decrease up to 0.1% in comparison to the other tested carriers.

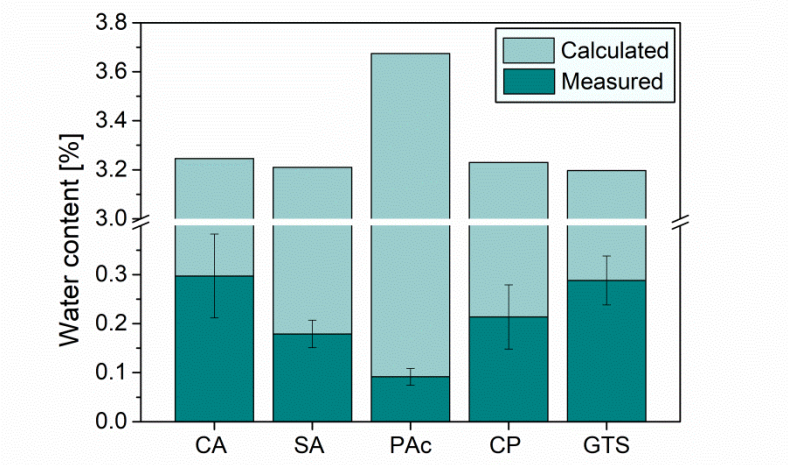


Figure 5-3. Calculated and measured water contents of microparticles made of different carriers with 1 mL enzyme. Cond.: $m_{carrier}$ = 20 g, $V_{laccase}$ = 1 mL (CA – Cetyl alcohol, SA – Stearyl alcohol, PAc – Palmitic acid, CP - Cetyl palmitate, GTS – Glycerol tristearate)

The highest water content was found for glycerol tristearate and cetyl alcohol with up to 0.3%. However, a high water loss was found for the used laccase, which was an aqueous crude extract. This loss could result on the one hand from the heating of the mixture before and during the spraying and on the other hand from evaporation of water, which occurred during the spraying. A reason for this observation could be that the activity measurements were performed in an aqueous solution and thereby enough water was present to receive a high enzymatic activity. In particular, the encapsulated stabilizer and buffer salts in the particles among the protein compounds were substantial for the retained activity. However, the low water content could support a higher shelf life of the biocatalyst, due to less availability of water for biochemical reactions resulting in a more stable biocatalyst. [Bampi *et al.*, 2016] This fact could lead to higher activities of the immobilized enzyme after a first incubation time before starting the reaction.

5.1.1.4. Protein release

For the investigation of the efficiency of enzyme immobilization, the protein release was of great interest regarding to recyclability. Therefore the method of Whitaker and Granum was used, whereby the protein content was calculated from measurement of two wavelength *via* equation 2 (chapter 4.1.2.), determined from amino acids of the enzyme. [Whitaker *et al.*, 1980] Microcapsules of immobilized laccase in cetyl alcohol were suspended in an aqueous solution and the protein content was determined as function of time. Thereby 100% presented the amount of protein, which was used for immobilization. Figure 5-4 shows the protein concentration in the solution depending on the pumping rates, whereby only slight differences occurred. However, for both samples a rise of the protein concentration was visible remaining stable after 30 min. The concentration in the solution reached a maximum of 71% for a pumping rate of 10 mL min^{-1} and 45% for 20 mL min^{-1}. This first increase of the concentration presents a washing out of the enzyme from the particles. After the immobilization of the enzyme, a certain quantity of the protein was sticking on the surface and was not completely immobilized. This was washed from the surface resulting in a higher protein concentration in the solution. Nevertheless, after this washing the concentration in the solution remained stable with only slight fluctuations based on the method itself. This indicates that no significant amount of additional enzyme was washed out after the first increase and a recycling could be possible.

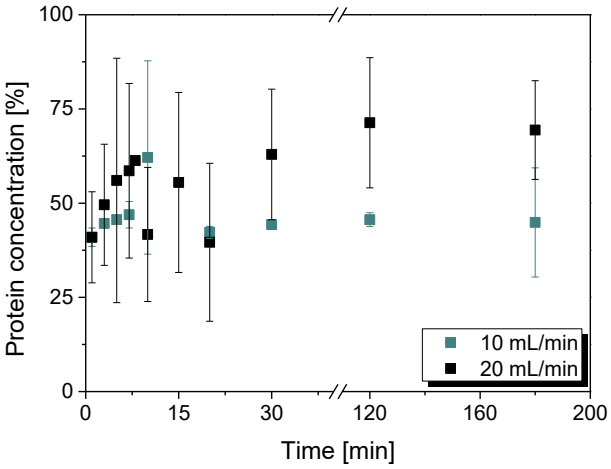


Figure 5-4. Protein release of laccase encapsulated in cetyl alcohol depending on the pumping rate in a fixed bed reactor concept. Conditions: *Protein content*: $m_{capules}$ = 500 mg, V_{buffer} = 100 mL, λ = 235 nm and 280 nm; *Spray congealing*: $m_{carrier}$ = 20 g, $V_{laccase}$ = 1 mL.

Nevertheless, an equilibrium between the particles and the solution could be reached as well. The lower leaching effect with a higher rate, as well as the high standard deviations, could result from increasing compression of the microparticles and therefore non optimal and different flow pattern in the fixed-bed reactor for the different measurements. In literature the release of the active compounds from microparticles made by spray congealing were mostly pharmaceuticals aiming at drug delivery, as well as taste masking. [Sohi *et al.*, 2004; Albertini *et al.*, 2009] This technique enabled the immobilization and slower release of pharmaceuticals as well as improving the dissolution rate of e.g. praziquantel, depending on the solubility of the compound. Furthermore, in several studies a release of the active ingredient over the time was reported, whereby no stable concentration was reached in the end of the measurement, indicating an equilibrium as well. [Albertini *et al.*, 2008; Passerini *et al.*, 2009; Passerini *et al.*, 2006] The measurements suggest the immobilization of the biocatalysts with no significantly destruction of the particles and a further release. A low protein release was a prerequisite for the recovery and re-use of immobilized enzyme and a first hint for the applicability in further reactions.

5.1.1.5. Morphology of the particles

The morphology of the particles was highly interesting, due to e.g. diffusion limitations and reactor operation. The immobilized enzyme was obtained as a white powder and in figure 5-5 SEM images of different particles are shown.

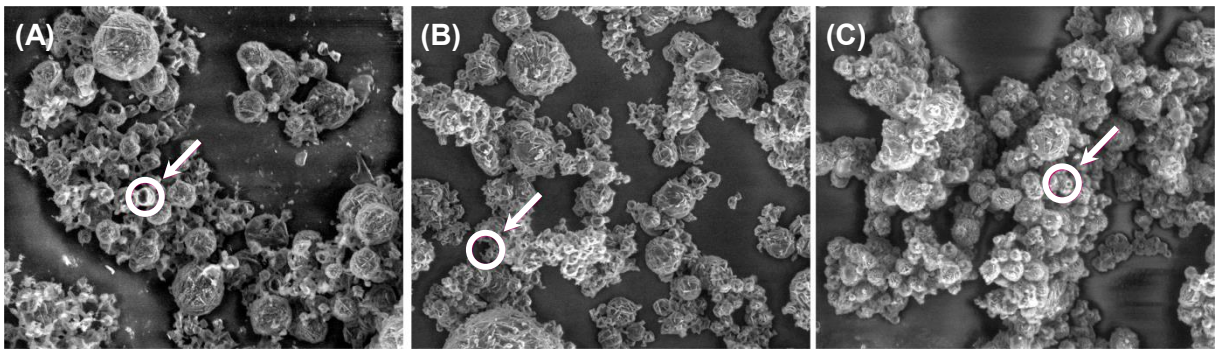


Figure 5-5. SEM images of the produced particles. Imperfections highlighted with circles. (A) Cetyl alcohol, (B) Stearyl alcohol, (C) Cetyl palmitate. Cond.: $m_{carrier}$ = 20 g, $V_{laccase}$ = 1 mL.

For all particles a spherical shape was obtained. This shape exhibited the best form for particles, due to a superior surface-to-volume ratio and therefore an optimal shape for immobilization. The images clarified that the particles appear in different sizes, as well as different surfaces depending on the used carrier. For particles made of cetyl palmitate a slightly more sealed and smooth surface was obtained (figure 5-5 A), in contrast to stearyl alcohol or the ester cetyl palmitate, which yielded in spheres with more structured surfaces (figure 5-5). The already mentioned poor suspension behavior of stearyl alcohol and cetyl palmitate particles could be based also on the structure of the surface, beside the more lipophilic properties of these compounds. Furthermore, the more structured surface could result in a greater release of the enzyme. In the SEM images it became obvious, that the particles tended to agglomerate, especially visible for cetyl palmitate (figure 5-5 C). This was also investigated for the measurements with the self-developed set-up, shown in chapter 5.1.3, whereby other methods were used and similar results could be observed. This indicated the influence from the spray congealing and not from the handling of the particles or measurements for characterization. Ilìc *et al.* observed also an agglomeration for the microencapsulation of glimepiride, an antidiabetic agent, in hydrophilic polymers indicating that spray congealing technique is the main factor for this effect. [Ilić *et al.*, 2009]

Nevertheless, the comparison of particles with respect to the presence of enzyme revealed a significant influence in agglomeration. Figure 5-6 illustrates that a higher agglomeration of the particles appeared with immobilized laccase. In contrast, atomized carriers without enzyme exhibited more separated microparticles. This effect resulted from the influence of the viscous crude extract containing additional mediators, stabilizers and proteins favoring the sticking of the particles. The formation of agglomerates was on the one hand undesirable, due to a decreasing of the surface-to-volume ratio; on the other hand it could contribute to a greater stability of the particles. This effect was used for the immobilization as CLEAs, where agglomerates were formed *via* enzyme precipitation and non-covalent bonding without denaturation. [Sheldon *et al.*, 2013]

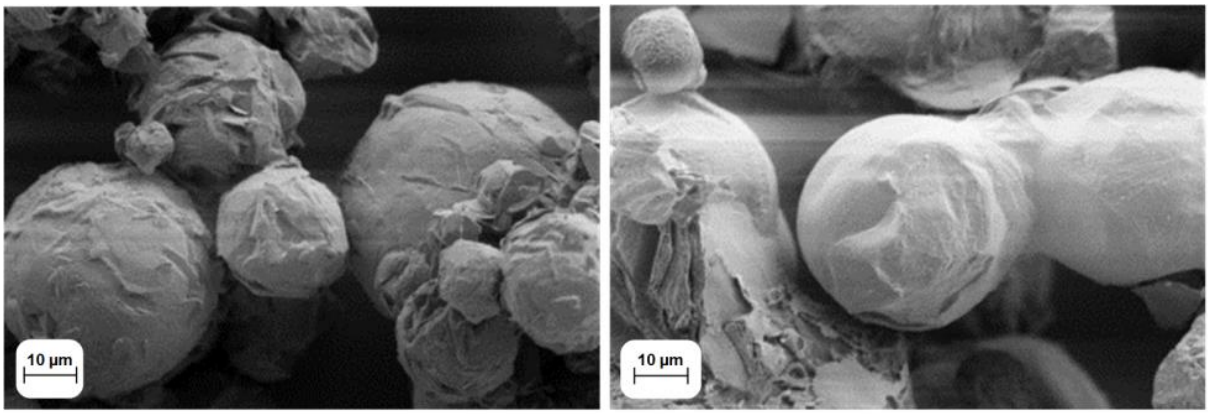


Figure 5-6. Microparticles made of cetyl alcohol. *Left:* Without laccase. *Right:* With laccase. Cond.: $m_{carrier}$ = 20 g, $V_{laccase}$ = 1 mL.

All produced microparticles exhibited imperfections based on the used set-up, the modified spray dryer (figure 5-5, circles). It is known, that the encapsulation with the spray dryer could result in open particles due to e.g. bubbles in the mixture. [Consoli *et al.*, 2016] This could lead to an easier release of the enzyme, but also in decreasing of the diffusion limitation, due to a more porous structure and therefore an easier availability of the substrate for the enzyme and an increasing activity.

As discussed before, in the SEM images different sizes of the particles were visible and the particle sizes were investigated *via* laser diffraction measurements and plotted with the number density of emulsified particles, shown in figure 5-7. The particle size distribution of immobilized laccase in cetyl alcohol, stearyl alcohol and cetyl palmitate showed for all carriers a similar shape and a comparable narrow distribution. The low number density

during the measurements of immobilized laccase in stearyl alcholol could be based on the difficult suspension of the microparticles in the aqueous environment, despite using of an emulsifier. Nevertheless, the increase of the stirring velocities was not influencing the particle size distribution and also the same shape could be observed.

These results showed that the size is mainly depending on the used set-up. The average size of the obtained microparticles was 40 - 55 µm, while the mean size was found between 25 - 35 µm. The research group of Passerini obtained comparable big microparticles with a mean diameter of 150 - 300 µm. [Di Sabatino *et al.*, 2012] The greater particle size was mainly based on the different diameter of the spraying nozzle, which was 4.5 mm, whereby the used nozzle in this study was with 2.0 mm much smaller. Furthermore a higher atomization pressure was exhibiting a smaller particle size, as well as a slower liquid feed. [Ilić *et al.*, 2009]

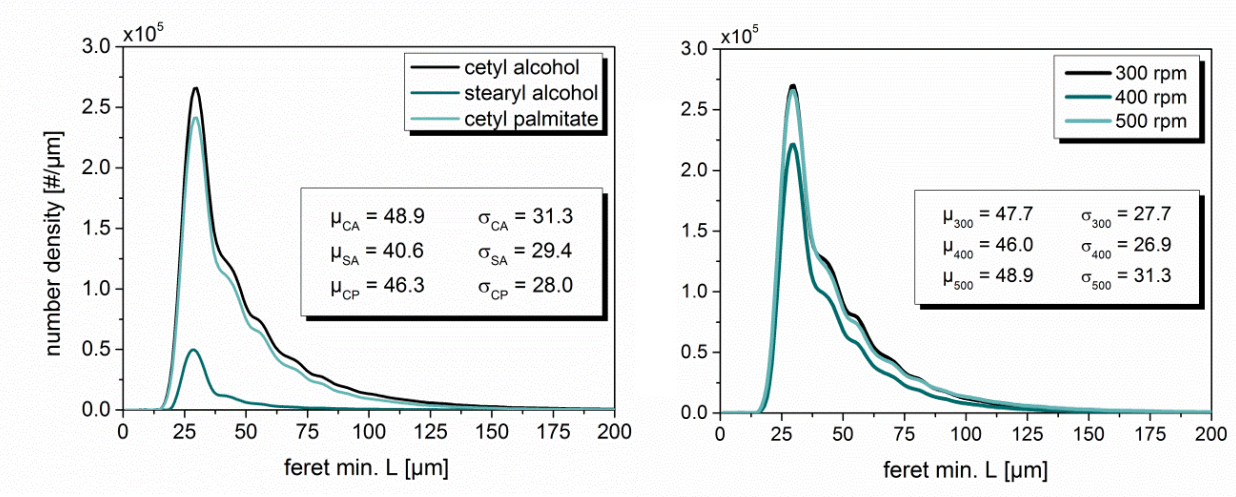


Figure 5-7. Particle size distribution of microparticles with immobilized laccase. *Left:* Comparison of microparticles made of different carriers at 500 rpm. *Right:* Comparison of different velocities of microparticles made of cetyl alcohol. Cond.: $m_{carrier}$ = 20 g, $V_{laccase}$ = 1 mL.

5.1.1.6. Recycling

As described before a main advantage of immobilization is the possibility to recycle the biocatalyst and therefore to increase the productivity. In figure 5-8 the re-use of microcapsules prepared with cetyl alcohol depending on the encapsulated enzyme amount is shown. Due to high retained activities with higher enzyme amounts the immobilization was investigated with 490 U (1 mL) and 980 U (2 mL) of the *MtL*, whereby the activity obtained in the first reaction was set to 100%. It can be seen, that for both amounts of immobilized enzyme a high decreasing was obtained after the first batch. After immobilization, a certain amount of the enzyme is located on the surface of the particle and is rinsed during the reaction, despite a previous washing. This activity loss is also visible for the consecutive reactions, whereby the decreasing of the activity was much lower. Nevertheless, in figure 5-8 differences in residual activities for different enzyme loadings became visible. In the second batch with 2 mL of the enzyme the microparticles exhibited 22% residual activity and therefore 5% more than the particles with less enzyme amount.

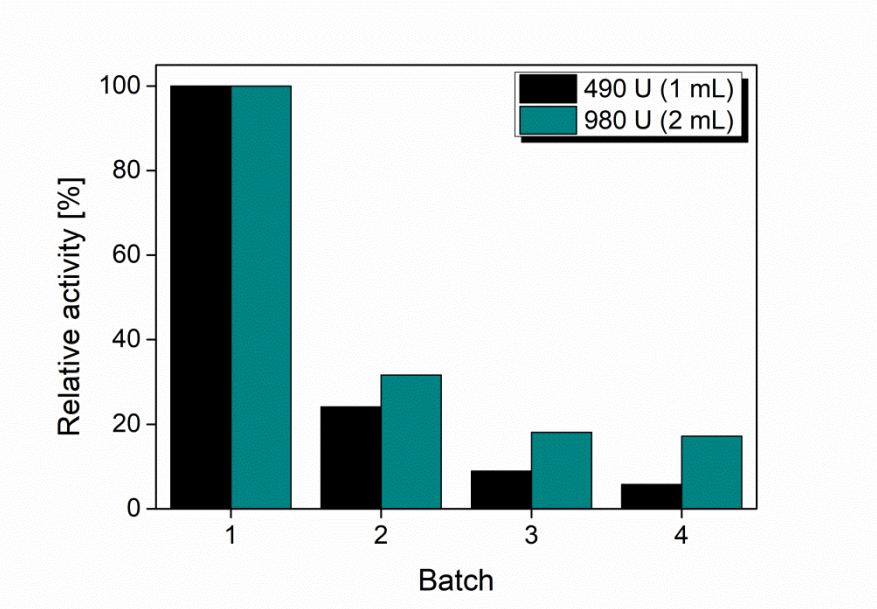


Figure 5-8. Residual activity of the laccase after 4 batches depending on the enzyme amount. Cond.: *Spray congealing:* $m_{mixture}$ = 20 g, V_{enzyme} = 1 – 2 mL. *Recycling:* Phosphate buffer pH 6, 0.1 M, V = 100 mL, c_{ABTS} = 5 mM, V_{ABTS} = 2.2 mL, $m_{capsules}$ = 500 mg, Q = 10 mL/min.

This difference in activity loss was also found for the following batches; however, in the fourth batch the retained activity of the capsules for 1 mL enzyme loading was only 5%. In contrast, a higher enzyme amount resulted in a more than three times higher activity of over 17% in the fourth batch. This indicated that a higher laccase amount in the immobilization process resulted in more stable enzyme preparation. These recycling measurements showed a loss of activity, which confirmed the protein release studies of the particles before. The reaction and the continuous flow resulted in a further release of the biocatalyst and therefore in a loss of activity. Consequently, the main influence on the residual activity was the release of the enzyme from the particle, beside the deactivation of the biocatalyst during the reactions. Despite the entrapping of the enzyme, this long chain alcohol was not providing a solid structure and a not visible abrasion resulting in the observed activity loss. Nevertheless, the usage of more crude extract would suggest more imperfections of the microparticles and in an increasing activity loss. However, this could not be observed and the immobilization efficiency remained stable with the investigated compositions. An additional possibility of activity loss was observed by Luckarift *et al.*, who obtained a decreasing conversion rate during usage of fixed-bed reactors. They described a plugging of the material, which formed channels through the particles resulted in a lower residence time of the solution. They were able to retain the starting conversion by re-suspending of the particles in the reactor. [Luckarift *et al.*, 2004]

5.1.1.7. Comparison of different enzymes

For a further evaluation of the technique, additional biocatalysts were immobilized by spray congealing. For comparison with the *MtL*, a crude extract, two lyophilisates were investigated: a second laccase, from *Trametes versicolor* (*TvL*), and a pig liver esterase (PLE). These enzymes were dissolved in 2 mL of the corresponding buffer and encapsulated in cetyl alcohol. In table 6 the residual activities are shown and it can be seen, that the *MtL* exhibited with 39% the highest activity after immobilization. Nevertheless, also the laccase *TvL* showed a more than twice higher activity in comparison to the immobilized esterase with 11% residual activity.

Table 5-2. Residual activities of immobilized enzymes in cetyl alcohol.

Enzyme	Activity of the capsules 10^{-3} [U/mg]	Residual activity [%]
MtL	19	39
TvL	2.6	26
PLE	1.1	11

Cond.: $m_{carrier}$ = 20 g, V_{enzyme} = 2 mL, $A_{TvL,\ PLE}$ = 200 U, A_{MtL} = 980 U

This tendency towards the highest activity of *MtL* and the low activity of the PLE was also visible for the recycling investigations, shown in figure 5-9. The encapsulated *MtL* exhibited a residual activity of 22% after the first reaction, whereby the laccase *TvL* reached an activity of 8% and the PLE 6%. The investigation of the re-usage of the immobilized enzyme presented also differences between the enzymes. For all biocatalysts a comparable activity loss was obtained after the first reaction followed by a slightly decreasing over five consecutive batches. It has to be pointed out, that the best retained activity during the second reaction of 17% was found with the *MtL* and only for this enzyme five batches could be realized. The immobilized *TvL* was able to be reused four times, whereby a comparable residual activity to *MtL* was visible during the fourth reaction. In contrast, during the third reaction the PLE presented the lowest activity of only 2.4%, which was illustrating the influence of the nature of the biocatalyst, beside the deactivation based on immobilization and the reaction.

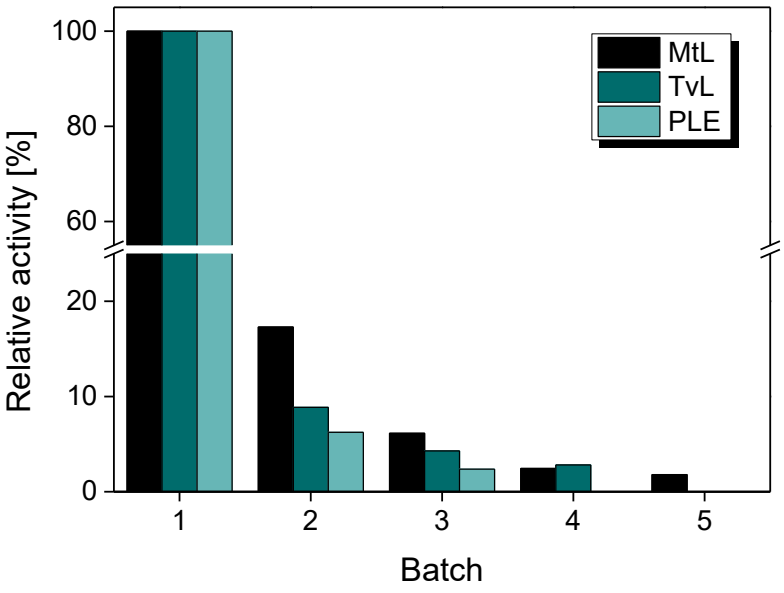


Figure 5-9. Comparison of residual activities depending on the used enzyme. Cond.: *TvL*: phosphate-citrate buffer pH 4; *MtL*: 0.1 M phosphate buffer pH 6; PLE: 0.1 M phosphate buffer pH 7.5. *Spray congealing:* $m_{mixture}$ = 20 g, V_{enzyme} = 2 mL $A_{TvL,\ PLE}$ = 200 U, A_{MtL} = 980 U. *Recycling:* 0.1 M phosphate buffer pH 6, V = 100 mL, $c_{ABTS,\ p}$ = 5 mM, $c_{p\text{-}NPA}$ = 10 mM $m_{capsules}$ = 500 mg, Q = 10 mL/min.

The laccase *MtL* was produced from the thermophilic fungus *Myceliophthora thermophila* resulting in a more thermostable biocatalyst, than the laccase from *Trametes versicolor*. During the measurements of the temperature dependency of the biocatalyst (cf. appendix) for *MtL* an increase of the activity could be observed after 30 min incubation time with temperatures up to 40 °C, followed by a stable activity over 3 h. In addition, at 50 °C the activity was not increasing, but showed still a very good stability during the measurements. Berka *et al.* found that this enzyme retained an activity of around 75% at an incubation time of 1 h at 60 °C, comparable to our investigations with a retained activity of 72%. [Berka *et al.*, 1997] In contrast to the significant increase of the activities of the *MtL* at different temperatures, only a slight increasing for the PLE could be found. For the *TvL* only a decreasing of the activity could be observed, presenting a denaturation during incubation at higher temperatures. The optimum temperatures for the *MtL* and PLE were 35 °C and for the *TvL* 30 °C. Nevertheless, for the immobilization in cetyl alcohol temperatures of 60 °C were used and for the PLE and *TvL* no activity was found after an incubation at higher temperatures than 45 °C. Due to the fast loss of activity the retained activities of these two

immobilized enzymes were highly depending on the velocity of the procedure. However, the thermostable *MtL* showed the best heat stability (table 5-2). The high thermal stress during the immobilization *via* spray congealing on heat sensitive enzymes had a big influence on the structure and therefore the residual activities. The enzymes were exposed to higher temperatures during the external mixing with the carrier, in the liquefying bath before spraying and while flowing through the nozzle. Despite usage of carriers with comparable low melting points and a fast procedure of around 15 min, a deactivation of the enzymes was still occurring.

The catalytic activities of enzymes are strongly dependent on the structure of the active center. The here used different enzymes –laccases and esterases– have significant differences in their active center. The laccase belongs, as described in chapter 3.1.1, to the group of oxidoreductases. The active center consists of four copper atoms, whereby one atom is near to the binding pocket and the substrate is oxidized *via* long-range intermolecular electron transfer between the copper atoms. During the oxidation a His-Cys-His tripeptide is part of the electron pathway from the tri-nuclear-copper cluster to the T1-site. These enzymes are known to exhibit a high stability, which is potentially based on the high level of glycosylation of 10 to 50%. [Claus, 2004; Kunamneni *et al.*, 2008] In contrast, the active center of the PLE, belonging to the hydrolases, contains only amino residues and a typical catalytic triade. [Bornscheuer, 2002; Ollis *et al.*, 1992; Romano *et al.*, 2015] The activity of the esterase is depending on special α/ß-fold, whereby the catalytic triad with Ser-Asp-His is formed in the protein core. This conformation could be destroyed by heat treatment during the immobilization. For both enzymes the stability of the structure of the active center is necessary for a high catalytic activity. Nevertheless, the great heat influence can destroy this important part of the biocatalyst, resulting in a high activity loss.

5.1.1.8. Conclusion of the 1st system

In this study spray congealing as immobilization technique for enzymes with a modified spray dryer was evaluated. For the characterization of the immobilized enzyme the following results were obtained:

- Highest residual activity of 44.9% with *MtL* in cetyl alcohol.
- Increasing residual activity of up to 55.5% with decreasing enzyme amount.
- High water loss up to 0.1% after immobilization.
- High protein release of up to 71%.
- Spherical shape of the particles with imperfections and agglomerations.
- Narrow particle size distribution with an average size of 40 – 55 µm.
- Higher residual activities with higher enzyme amounts.
- Laccase *MtL* showed the highest residual activity after immobilization.
- Recycling of immobilized *MtL* during 5 consecutive reactions.

These results showed that this technique is highly interesting for the immobilization of biocatalysts. Despite several advantages, some deeper investigations have to be performed to improve the residual activities, especially during the recycling of the enzyme. This was investigated in the following section using a second set-up, the self-developed spray congealing system.

5.1.2. 2nd System: Improving of the immobilization with an optimized set-up

For a further optimization of the immobilization *via* spray congealing a second set-up was tested. Therefore the spray congealing apparatus of the research group of Prof. Passerini at the University of Bologna was used, which was especially developed for the atomizing of highly viscous systems (cf. chapter 4.1.3.)[1]. This set-up contained a wide pneumatic nozzle (WPN) with a comparable big orifice opening of 4.5 mm and a radially entering of the pressured air, while axially flowing of the melted mixture (cf. figure 4-3). As described before, spray congealing is usually used for the immobilization of solid pharmaceuticals or liquid compounds, which are miscible with the carrier. [Di Sabatino *et al.*, 2012; Albertini *et al.*, 2008; Maschke *et al.*, 2007] Immobilization of an emulsion was not investigated yet with the set-up and should be realized *via* encapsulation of enzyme solutions compared to the previous investigations with the modified spray dryer. The dissolved enzyme and therefore the water amount was highly interesting, as e.g. water could act as protection against heat and shear stress during atomization.

For the investigation of the system two enzymes were used for immobilization. Firstly the laccase *TvL*, which demonstrated promising results during the first investigations and secondly the PLE. Thereby the immobilization was tested with dissolved enzymes and the system was evaluated with regard to different water amounts, particle sizes, shapes as well as differential scanning calorimetry (DSC) and recycling behavior.

5.1.2.1. Evaluation of the system

The pre-investigations were performed with the laccase *TvL*, which was immobilized in cetearyl alcohol, a mixture of cetyl and stearyl alcohol. This compound seemed to be interesting, due to prior investigations in the research group of Prof. Passerini and as a compromise between the good retained activities with cetyl alcohol and possible stability increase of particles based on stearyl alcohol. Figure 5-10 illustrates the applicability for the second system with superior spraying yields of 70 – 90%. The system exhibited a comparable large orifice nozzle (4.5 mm) with an inner diameter of 1 mm preventing nozzle clogging by high viscous mixture and enabled an easy spraying. In contrast, the orifice opening for the spray dryer was only 2.5 mm with an inner diameter of 0.5 mm.

[1]The results described in this chapter were performed at the research group of Prof. Nadia Passerini, Università di Bologna, Italien.

For evaluation of the system the *TvL* was dissolved in different water amounts and encapsulated. Higher activities up to 54% could be obtained with this set-up (figure 5-10) compared to the results obtained with the modified spray dryer of only 26% activity, which presented the high efficiency of this system. These high activities could be explained by faster spraying, due to the greater orifice opening and increased velocity through the radially entering of the pressured air. The inclination of the pressured air and the melted mixture was 45° benefiting the atomization and the contact of the air with an angle resulted in kind of a venturi tube, due to the narrowing of the mixture before atomizing. In addition, the particles were sprayed in an angle of 90°, which is symmetrically to the cone angle of the nozzle, resulting in a uniform distribution of the microparticles. [Albertini *et al.*, 2008]

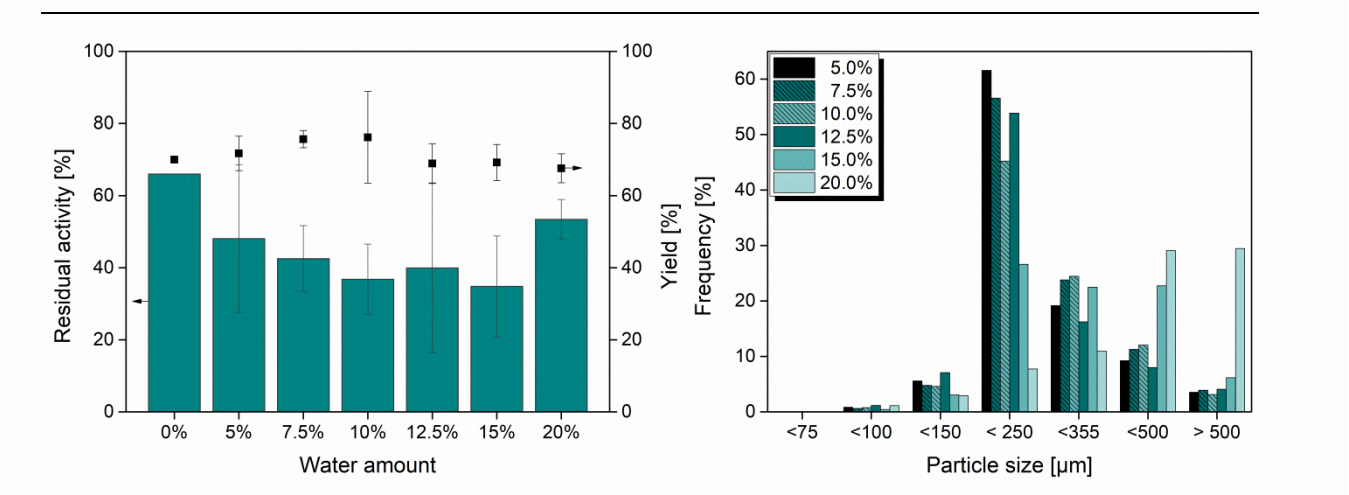


Figure 5-10. Properties of the microparticles with immobilized *TvL* depending on the water content. Cond.: $m_{carrier}$ = 10 g, θ = 5 – 7%, citrate phosphate buffer pH 4, A_{TvL} = 100 U, p = 3 bar, n = 3. *Left:* Residual activity and spraying yield. *Right:* Particle size distribution.

However, with regard to the used water amount it became obvious, that no significant differences for the activities and yields were found (figure 5-10). Especially the high standard deviations presented the problem in reproducibility for the encapsulation with this technique. The immobilization was strictly depending on the performance during atomization. The carrier-enzyme mixture was prepared before the spraying and poured manually into the nozzle resulting in different spraying velocities for each experiment with significant differences in enzyme activities.

In contrast, a significant influence of the water amount for the particle size distribution was visible (figure 5-10). Water amounts of up to 12.5% showed a narrow particle size distribution with a main particle sizes between 150 – 250 µm, whereby for higher water amounts a

shifting to larger particle size could be observed. An increase of the water amount led to an agglomeration of the microparticles, which was observed before using the modified spray dryer. Furthermore, with 15% water the size distribution was broader and mean sizes between 150 and 500 µm occurred. This effect increased with 20% water resulting in sizes of over 355 µm for most of the particles. These investigations showed the influence of water during enzyme immobilization to the particle structure. The formation of agglomerates based on higher contents could also be preferable for the stabilization of the particles, as discussed before. The microparticles prepared with this spray congealing system are up to ten times larger than the particles obtained with the modified spray dryer (around 40 µm). The main reason for this increase was the larger orifice opening of 4.5 mm, as well as the twice as big nozzle diameter. No significant difference for the activity depending on the water amount was detectable, however, during the particle size measurements particles with 10% water amount showed the best properties for enzyme immobilization. The microparticles exhibited good retained activities, superior spraying yields and a narrow particle size distribution. These results confirmed the investigations with the spray dryer in previous studies, despite the usage of another laccase (*MtL*) with a higher viscosity.

5.1.2.2. Formulations of the carriers

For the investigation of the second spray congealing system additional carriers, as well as formulations were studied. Table 5-3 summarizes the carriers, whereby also emulsifiers were included. These compounds were able to contribute a better distribution of the aqueous enzyme solution in the hydrophilic carrier. For the evaluation of formulations as carriers the emulsification behavior of the emulsifiers was tested, which was highly depending on the hydrophilic lipophilic balance (HLB). The HLB concept was developed by Griffin, who described the experimental determination of HLB values of compounds containing hydrophilic and lipophilic groups. Surfactants with a lower HLB are more lipophilic and compounds with an HLB higher than 10 are more hydrophilic. For example, low values from 4 – 6 represent W/O emulsifiers and compounds with a high HLB value from 15 – 18 act as solubilizer. [Griffin, 1949; Pasquali *et al.*, 2008; Macedo *et al.*, 2006] Between HLB and logP, introduced before, a reverse relationship is present: the higher the HLB value, the lower logP. [Kronberg *et al.*, 2014] The second part of table 5-3 presents commercially available formulations, exhibiting different HLB values. It became visible, that long chain alcohols and fatty acids showed a comparable poor emulsification behavior, comparable to previous investigations. Only cetyl alcohol, cetearyl alcohol and stearic acid showed a moderate emulsification, whereby for the other carriers big aqueous droplets were found on the bottom of the beaker despite stirring. These carriers offered lipophilic and hydrophilic groups, but the lipophilic amount in these compounds was too high resulting in unstable emulsions. The commercial available formulations exhibited emulsifying properties, due to their chemical structure. The more hydrophobic formulation Precirol ATO 5 with an HLB of 2 made of glyceryl distearate and glyceryl palmitostearate offering a better emulsification than e.g. cetearyl alcohol. In contrast, Compritol contained glyceryl derivatives esterified with behenic acid, whereby Compritol 888 ATO formed foam during emulsification in water. This foam would lead to a decomposition of the microparticles, so that this compound was not further investigated. In contrast carriers named Gelucire were made of polyethylene glycol (PEG) esters, which were more hydrophilic depending on the length of the ester. Due to high HLB values these compounds were not interesting as carrier itself, but they could act as emulsifier and were highly interesting for usage in a formulation. However, for the immobilization of the laccase in ceterayl alcohol the highest activity of 37% was reached, which was slightly higher than for Precirol ATO 5 (32%) and stearic acid (30%). With Compritol HD5 the lowest activity was obtained with only 23%. During these investigations no significant differences could be observed, due to the fluctuation of the activities based on the method, as described before.

Nevertheless, with Precirol ATO 5 and cetearyl alcohol good retained activities of the used laccase could be realized as well as an easy spraying, so that these compounds were chosen for further investigations.

Table 5-3. Properties of the investigated carriers.

Carrier	Mp [°C]	HLB[1]	Emulsification[2]	Residual activity[3] / Comments
Cetyl alcohol	49	n.d.	✓	26%
Stearyl alcohol	59	n.d.	×	-
Cetearyl alcohol	50	4.7	✓	37%
Lauric acid	69	n.d.	×	-
Palmitic acid	63	n.d.	×	-
Stearic acid	69	n.d.	✓	30.3%
Trimyristin (Dynasan 114)	56	n.d.	×	-
Tristearin (Dynasan 118)	54-72.5	n.d.	×	-
Precirol ATO 5	56	2	✓	31.8%
Gelucire 44[d]/14[e]	44	14	-	Hydrophilic
Gelucire 50[d]/13[e]	48.5	13	✓	Hydrophilic
Gelucire 55[d]/18[e]	55	18	×	Hydrophilic
Gelucire 62[d]/05[e]	62	5	✓	Hydrophilic
Compritol 888 ATO	70	2	✓	Foam forming
Comprotil HD5	60-67	5	✓	22.9%

[1][Karatas *et al.*, 2005; Aburahma *et al.*, 2014; Albertini *et al.*, 2009], [2]Cond.: $m_{carrier}$ = 10 g, V_{water} = 1 mL. [3]Cond.: $m_{carrier}$ = 9 g, $A_{laccase}$ = 100 U, citrate phosphate buffer pH 4, $V_{enzyme\ solution}$ = 1 mL, V_{buffer} = 100 mL, $m_{capsules}$ = 100 mg, c_{ABTS} = 1 mM. (Mp – Melting point, HLB – Hydrophilic-lipophilic balance).

Beside well known carriers for spray congealing and immobilization of active ingredients, during this study new interesting carriers should be investigated as well. Ionic liquids (IL) were highly interesting as carrier, based on special properties of these compounds. Ionic liquids are salts with a melting point below 100 °C, good solvation abilities and high viscosities. In addition they have a negligible vapor pressure and high thermal stabilities, which could be highly advantageous for the usage as carrier system for spray congealing. [Joshi *et al.*, 2012] For our investigations melting points between 35 – 80 °C were necessary, whereby the commercial available ionic liquid methyltrioctylammonium triflate (MOAT), shown in figure 5-11, with a melting point of 55 - 56 °C exhibited suitable properties as carrier for enzyme immobilization. The structures of chosen compounds for further investigations are presented in figure 5-11.

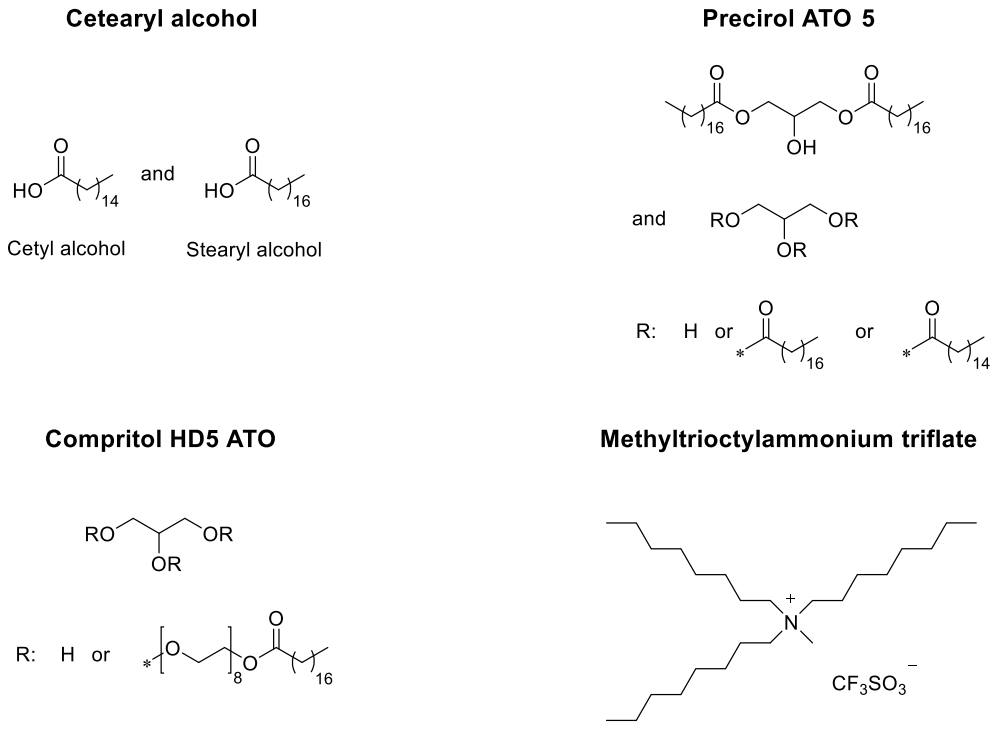


Figure 5-11. Structures of chosen carriers.

Beside the *TvL*, the PLE should be immobilized with this method as well, whereby the immobilization efficiency of PLE with the modified spray dryer was comparable low. The developed spray apparatus in addition with other carriers should enhance the retained activity of this enzyme, as well as the recyclability. Despite good results of the *TvL* in cetearyl alcohol with a residual activity of up to 37%, for the esterase an activity of only 8.6% was obtained. However, PLE encapsulated in Precirol ATO 5 exhibited an activity of 12.1% and with cetyl alcohol as carrier it increased up to 35.5%. The retained activity in cetyl alcohol is over three times higher than for prepared microparticles with the previous set-up (11%) and elucidates again the immobilization dependency on the set-up. Nevertheless, the carrier influences the biocatalyst activity as well, due to the nature of the enzyme.

5.1.2.3. Encapsulation of PLE and activity enhancement

The characterization of the microparticles with differential scanning calorimetry (DSC) was performed to investigate the thermal properties of the prepared microparticles. The specific crystallization of the carriers could reveal in polymorphism or amorphous states and interactions between carrier and immobilized biocatalyst. In addition, carrier properties were influenced by the temperature during solidification and spraying method. [Di Sabatino *et al.*, 2012; Hamdani *et al.*, 2003; Windbergs *et al.*, 2009] In figure 5-12 the difference of the melting behavior between crude carrier and atomized mixture with immobilized enzyme are presented.

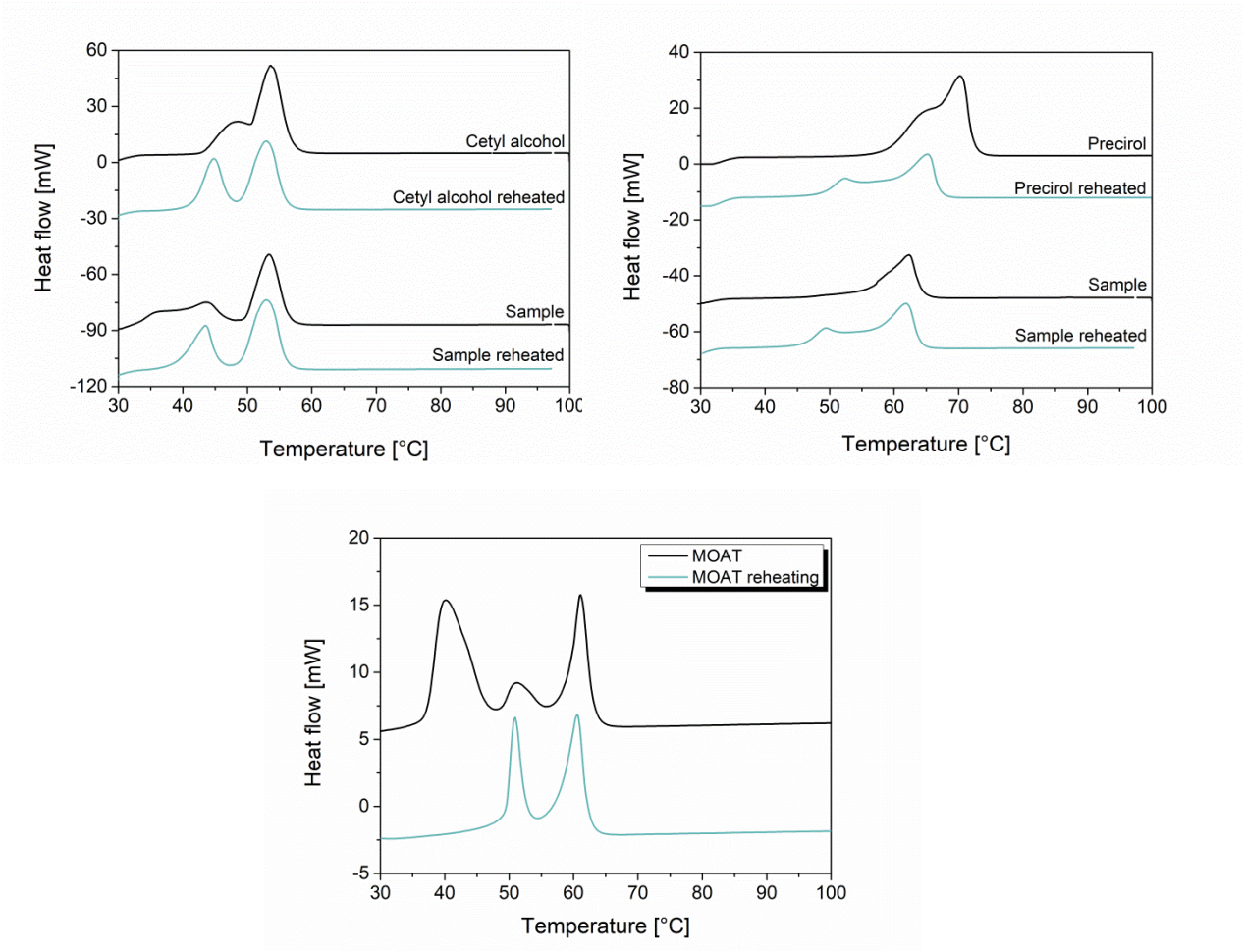


Figure 5-12. DSC curves of the carrier and a sample with immobilized esterase with an additional reheating.

The DSC traces for cetyl alcohol exhibited a main peak at 53 °C for both, crude carrier and the encapsulated enzyme. However, the crude carrier showed a second shoulder at 43.5 °C, which shifted slightly during the second heating to lower temperatures forming separated peaks. These additional peaks indicated additional species, which might result from an impurity of cetyl alcohol, e.g. by stearyl alcohol from the production process. The trace for microparticles with encapsulated esterase showed an additional peak at 39 °C during the first heating with a broad peak between 35 and 43 °C, which seemed to contain two peaks. The reheating of the sample resulted in a similar trace as for the crude carrier with two separated peaks. These shoulders indicated different crystalline forms of the carrier such as the already mentioned polymorph or amorphous states. This effect was characteristically for spray congealing, due to a fast solidification of the carrier after spraying. Also for the carrier Precirol ATO 5 a main peak at 70 °C with a shoulder around 62 °C could be observed, which were separated after reheating to 65 °C and 52 °C, respectively. These two endothermic peaks for Precirol presented two polymorphic forms, which were usually occurring using fresh Precirol ATO 5. These different forms were found to be instable, so that the lower endotherm peak was shifting to the higher peak during storage. [Hamdani *et al.*, 2003] For the immobilized enzyme this shoulder was not visible after reheating, however, a similar trace as for the crude carrier was found with a peak separation after reheating. In contrast, Sabatino *et al.* found significant differences for trace of raw Precirol and Precirol with immobilized protein (bovine serum albumin - BSA). For the immobilized protein they found only one peak for the stable ß-form, whereas the raw material showed two not separated peaks. They suggested a stabilization of the carrier by protein inclusion, which was not influenced by protein loadings. High loadings of the protein hindered the crystallization in more stable forms. [Di Sabatino *et al.*, 2012] In addition, the shifting of the melting points showed that the impurity of the carrier, here the enzyme solution, resulted in a low melting-point depression.

In contrast, MOAT exhibited a significant polymorphism before and after reheating with completely separated endothermic peaks. In every case ionic liquids have a comparable complex thermal behavior. Typically these compounds show a glass transition, which is followed in some cases by crystallization and an endothermic peak for the melting point. [Wasserscheid *et al.*, 2008; Kärnä *et al.*, 2009] However, for some ionic liquids multiple solid-solid transitions could be observed. The investigations of [BMIM]Cl for example showed two endothermic peaks identified as a monoclinic and a orthorhombic polymorph, which present a plastic crystal transition. [Holbrey *et al.*, 2003] In contrast, some 1-alkyl-3-methylimidazolium tetrachloropalladate(II) salts exhibiting low melting points and similar structures as ionic liquids showed also polymorphism with crystal-crystal and crystal-liquid transitions. [Hardacre *et al.*, 2001] The used ionic liquid in our study showed three separated

endothermic peaks (figure 5-12). The additional reheating resulted in a shifting of the peak from 40 °C to 51 °C, whereby the peak around 61 °C remained stable. The trace was comparable to a similar palladium containing IL, due to the more complex cation than for ILs with chloride as anion. In contrast, DSC measurements of ILs containing cations with a similar complexity showed a completely different endothermic behavior. These investigations indicated that the polymorphism of the IL was based on the complex anion, beside the ionic structure, which exhibited many vibrations and rotations of this molecule and an increased number of conformational modes. [Wasserscheid *et al.*, 2008; Kärnä *et al.*, 2009] However, the degree of the lipid crystallinity and modification of the crystal lattice were important parameters influencing the enzyme inclusion, as well as release behavior. The polymorphic rearrangement from the unstable α- and ß'-formation to the stable ß-formation resulted in an expulsion and an increased release of the enzyme. The density of the thermodynamically less stable forms was lower, which resulted in an increasing mobility of the entrapped compound and differences in release behavior and immobilization efficiency. [Scalia *et al.*, 2015] However, in Precirol encapsulated esterase presented only the stable ß-form, in comparison to cetyl alcohol illustrating an advantage for immobilization efficiency.

Table 5-4. Residual activities for the immobilized esterase.

Carrier	**Residual activity 10^{-3} [U/mg]**	**Residual activity [%]**
Cetyl alcohol	5.2	26%
Precirol	1.6	8%
Precirol / Gelucire 10%	2.2	11%
Precirol / Gelucire 15%	3.9	19%
Precirol / Gelucire 20%	4.3	22%
MOAT	0.5	3%

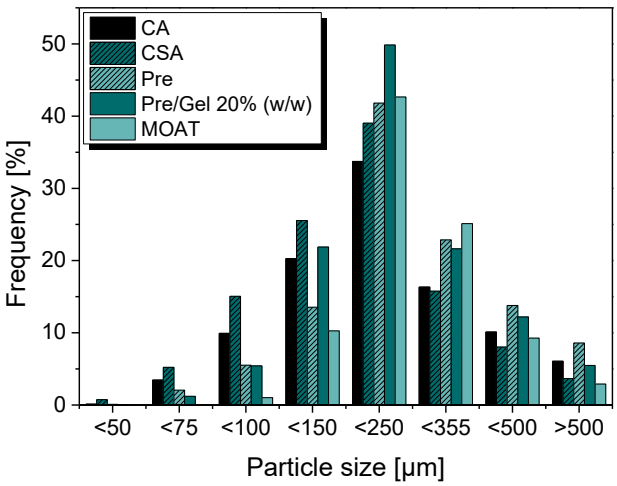
Cond.: Phosphate buffer pH 7.5, 0.1 M. *Spray congealing:* $m_{carrier}$ = 9 g, $A_{esterase}$ = 200 U, $V_{enzyme\ solution}$ = 1 mL. *Recycling:* $m_{capsules}$ = 250 mg, Q = 2.5 – 3 mL/min, $V_{substrate\ solution}$ = 100 mL, $c_{p\text{-}NPA}$ = 10 mM.

For the evaluation of this set-up the activity of the encapsulated enzyme was important and tested for the chosen carriers (cf. figure 5-1). Due to the usage of lyophilisates and the common usage of spray congealing for immobilization of solid pharmaceuticals, the lyophilisates without dissolving in buffer were tested as well. Despite milling of the biocatalyst before atomizing and a high retained activity of up to 66%, it was not embedded in the carrier. The lyophilisate was still visible after atomization between the particles made of the carrier, which presented no immobilization and an easy wash out of the enzyme. Table 5-4

demonstrates the activity of encapsulated esterase in different carriers, as well as formulations. It can be seen, that the highest initial activity of $5.2 \cdot 10^{-3}$ U/mg was obtained with cetyl alcohol, which showed 26% residual activity of the immobilized esterase. This result confirmed the investigations with the modified spray dryer, where also the highest residual activities were found using this carrier. In contrast, the investigated carrier Precirol made of different glyceryl derivatives presented a comparable low residual activity of only 8% ($1.6 \cdot 10^{-3}$ U/mg). Precirol ATO 5 was more hydrophobic than cetyl alcohol and cetearyl alchol (HLB 4.7), due to the long chain glyceryl derivatives. Therefore this compound was not able to emulsify the aqueous solution sufficient for a high retention of the enzyme. This led to the suggestion that an emulsifier was necessary to realize better activity retention. For enzyme immobilization the preparation of a fine emulsion of the aqueous enzyme solution in a carrier melt was needed. The droplets of enzyme solution were comparable large and difficult to emulsify in the carrier resulting in an inhomogenous dispersion. Wehrung *et al.* investigated nano emulsions of a formulation made of cetyl alcohol and Gelucire 44/14 as drug delivery systems. The addition of Gelucire resulted in formation of stable emulsions with a decreasing size of the droplets in emulsions of up to 20%. However, no significant differences in various investigated amounts of Gelucire were found. [Wehrung *et al.*, 2012] In addition, Date *et al.* found out that the addition of Gelucire 50/14 to Precirol ATO 5 could act as stabilizer for lipid nano carriers like solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC). This could be also a great advantage for our investigations, due to the emulsification of the aqueous enzyme solution in the lipid carrier. Additionally, this compound had the GRAS (generally recognized as safe) status and was already used in solid dispersions to enhance the solubility in aqueous solutions for hydrophobic drugs. [Vippagunta *et al.*, 2002; El-Badry *et al.*, 2009; Date *et al.*, 2011] Gelucire 50/13 with an HLB value of 13 was introduced as an emulsifier for the aqueous enzyme solution. A great advantage of Gelucire was that no foam formation could be observed, which is typically occurring while using emulsifiers, e.g. with Compritol 888 ATO (cf. table 5-3). In this study different formulations of Precirol and Gelucire were tested with regard to the influence on the immobilization of the enzyme solution. This formulation formed a homogenous solution after melting of both compounds. The subsequent addition of the aqueous enzyme solution formed a fine dispersion, despite the first miscibility.

The residual activities were investigated with regard to the amount of Gelucire, shown in table 5-4. The initial activity of the immobilized esterase was increasing with an increasing Gelucire amount. With 20% (w/w) Gelucire in Precirol the activity was $4.3 \cdot 10^{-3}$ U/mg, which was three times higher than without Gelucire. This could be explained by the formation of nanoemulsions, which was investigated by Wehrung *et al.* [Wehrung *et al.*, 2012]. The

addition of a higher amount than 20% led to a disruption of the particles, so that it was not considered further. The higher activity during our investigation with the emulsifier Gelucire could be explained with the formation of fine emulsions and therefore reduction of remaining droplets in the beaker. In addition, imperfections had a comparable low influence of the enzyme leaching with this fine dispersion.



Carrier	Viscosity [mPa·s]
CA	32
CSA	37
Pre	49
MOAT	356

Figure 5-13. Particle size distribution of encapsulated esterase in different carriers and the viscosities 10 °C above their melting point. Cond.: $m_{carrier}$ = 10 g, A_{PLE} = 200 U, $V_{enzyme\ solution}$ = 1 mL, phosphate buffer pH 7.5, 0.1 M.

The microparticles were further investigated with regard to their particle size, depending on the used carrier, and their occurrence with SEM imaging. For all tested carriers a narrow particle size distribution with a main size of 150 – 250 µm was found, presented in figure 5-13, comparable to the results with different water amounts (cf. figure 5-10). A slight difference of the size was visible between the carriers. Microparticles prepared with cetearyl alcohol and cetyl alcohol resulted in majority of particles with sizes below 250 µm, whereas Precirol and MOAT formed a majority of particles with 250 µm and higher. In addition, a formulation of Precirol with 20% Gelucire 50/13 was investigated to obtain a more homogenous emulsification of the dissolved enzyme in the carrier. This formulation showed the most narrowed distribution with the same main size between 150 – 250 µm, which could result from the fine distribution of the aqueous phase in the lipid carrier by the emulsifier. The main impact on the size constituted the viscosity. MOAT showed a comparable high viscosity of 356 mPa·s, which was more than seven times higher than the viscosity of Precirol. However, the immobilized esterase in MOAT showed a very sticky behavior after spraying

and only the fine powder could be used for measurements of the particle size distribution. The increased viscosity might lead to larger particles with a preferred agglomeration of greater particles.

Based on this, also SEM images were highly interesting, due to the usage of the additive Gelucire and for comparison with the microparticles prepared with the modified spray dryer. In figure 5-14, images of the esterase (solid and liquid) encapsulated in Precirol with 10 and 20% (w/w) Gelucire are shown. It can be seen, that the particles showed a spherical shape with a smooth surface compared to the particles prepared with the spray dryer. This effect was mainly based on the particle size and therefore the used set-up. The greater the particles, the greater are also the stabilities during spraying. The immobilization of the enzyme resulted in imperfections of the capsule based on the enzyme solution and during the spray congealing the heat influenced the liquid with a partly evaporation of the solution. This led to a collapse of the particle, which could sometimes be seen for the particles prepared with the spray dryer (cf. figure 5-5). However, in the SEM images of the particles prepared with the wide pneumatic nozzle no significant imperfections were visible. In figure 5-14, the contrast of solid and dissolved encapsulated enzyme, as well as the usage of an emulsifier was visible. The immobilization of solid enzymes resulted in a rougher surface and it seemed that the particles exhibited small holes. These holes were based on the use of the lyophilisate resulting in a two-phase carrier made of Precirol and Gelucire. In contrast, the usage of dissolved enzyme showed a significantly smoother surface and only some darker spots, as well as some imperfections indicated the immobilized enzyme solution (figure 5-14, C). The usage of less Gelucire (10% (w/w), figure 5-14 B) showed a higher collapsing of the particle, as well as more holes. This demonstrated the great advantage of using an enzyme solution and an additional emulsifier for a homogenous distribution of the enzyme in the lipophilic carrier.

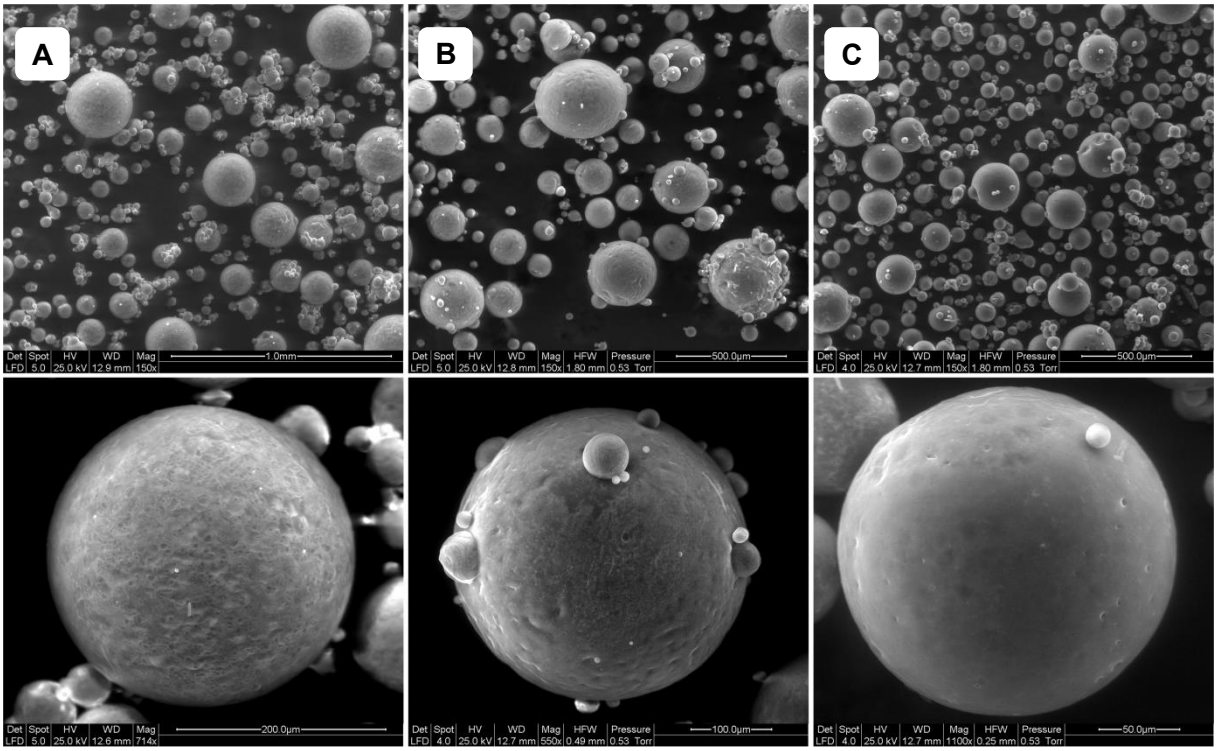


Figure 5-14. SEM images of encapsulated esterase. (A) Solid in Precirol with 20% Gelucire, (B) Liquid in Precirol with 10% Gelucire, (C) Liquid in Precirol with 20% Gelucire. Cond.: $m_{carrier}$ = 10 g, $A_{esterase}$ = 200 U, phosphate buffer pH 7.5, 0.1 M, $V_{solution}$ = 1 mL.

Also for the immobilized enzyme prepared with this second set-up recycling measurements of the investigated carriers were performed. Results are shown for 5 batches in figure 5-15. Despite good retained activities with esterase immobilized in cetearyl alcohol no recycling of these microparticles could be realized. After the first reaction no flow could be realized using the tubing pump, due to a too high back pressure. Cetearyl alcohol contained mainly cetyl and steryl alcohol, but furthermore laurylic and myristic alcohols, which exhibited shorter chain lengths. [Albertini *et al.*, 2009] This could result in softer particles and furthermore in an increasing of sticking and compressed particles in the fixed-bed reactor. Subsequently a pressure drop occurred, so that no second reaction could be realized leading to an unsuitability of this compound for this application. However, for the other carrier systems a significant decrease after the first batch is occurring based on the wash out of non-immobilized enzyme, which was comparable to the experiments with the spray dryer before (cf. figure 5-8 and 5-9).

The activity yield of immobilized PLE in cetyl alcohol revealed values of 5.2 mU/mg, which were the highest compared to other used carriers. The high immobilization efficiency for cetyl alcohol could be explained by less enzyme release based on the formation of larger particles and therefore a lower surface-to-volume ratio. Precirol showed with 1.6 mU/mg more than a 3-fold less activity compared to cetyl alcohol. However, with addition of Gelucire the residual activity increased significantly up to 4.3 mU/mg, which was in the same range as observed for cetyl alcohol. This result demonstrates the importance of an emulsifier for encapsulating aqueous solutions causing a reduction in enzyme loss and thereby increasing of the productivity, due to fine dispersion. The lowest obtained activity was found with the IL MOAT. Only 3% of the used enzyme activity were received after immobilization, which showed an additional influence of this compound beside the heat influence while spray congealing. In contrast, Wallert *et al.* found similar conversions and enantioselectivities with addition of up to 5% of different ILs, based also on a quaternary ammonium cation. The anions of these molten salts were highly important as well and higher concentrations of these compounds were found to have also a negative influence on the enzyme activity suggesting the comparable low activity yield after immobilization in this study. [Sheldon *et al.*, 2002]

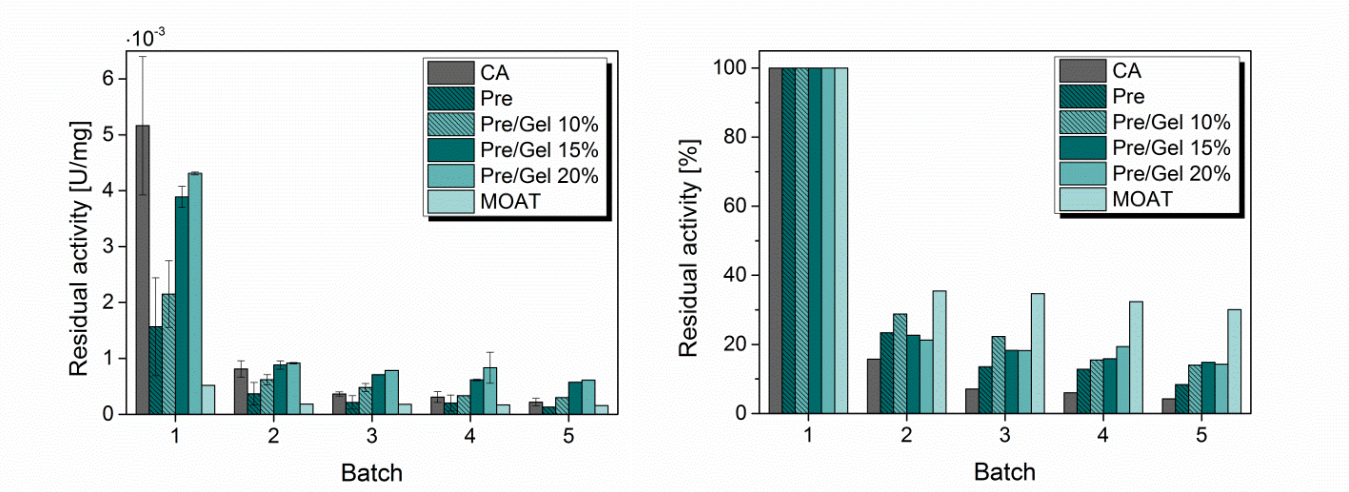


Figure 5-15. Residual activities of immobilized esterase depending on the carrier. *Left:* Absolute residual activities. *Right:* Relative residual activities (1. Batch set 100%). Cond.: Phosphate buffer pH 7.5, 0.1 M. *Spray congealing:* $m_{carrier}$ = 9 g, $A_{esterase}$ = 200 U, $V_{enzyme\ solution}$ = 1 mL. *Recycling:* $m_{capsules}$ = 250 mg, Q = 2.5 - 3 mL/min, $V_{substrate\ solution}$ = 100 mL, $c_{p\text{-}NPA}$ = 10 mM.

Nevertheless, for all tested carrier materials with immobilized esterase high activity losses were detected after the first batch of the immobilized enzyme, comparable to the results of the modified spray dryer. An explanation therefore could be the enzyme loss located on the particle surfaces, as discussed before. The enzyme was dispersed in the whole carrier after

the immobilization, due to the emulsification of the aqueous solution before atomizing. An amount of esterase was located on the surface of the particles and despite washing, the enzyme on the surface could be lost during the reaction.
Although the highest activities after immobilization were detected with cetyl alcohol as carrier, here the highest loss during the recycling studies was found as well (figure 5-15, right). After the significant high activity decrease during the first batch, this formulation revealed the highest activity losses during the enzyme recycling with only 4.2% remaining activity in the first batch. A similar loss was obtained with the carrier Precirol exhibiting a twice as high activity of 8.4%. With Gelucire 50/13 also a slight deactivation of the enzyme based on the release could be observed, but in the end a higher residual activity of up to 15% was obtained. However, in the fifth batch no significant difference could be observed for all stabilizer contents. This result was consistent with the investigations of Wehrung *et al.*, who found higher activities for the first reaction, based on a certain enzyme amount on the surface of the particle. [Wehrung et al., 2012] Nevertheless, the particle sizes of the droplets in the lipid carrier were the same with the same amount of enzyme, so that the release behavior of the microparticles was similar.

Here it has to be highlighted, that the best residual activity over all batches was found with the ionic liquid MOAT, despite a comparable low activity yield after immobilization. In the fifth batch the residual activity was with 1.6% comparable with the activity in the second batch with 1.8%, whereby no auto-catalyzed reactwion based on the ionic liquid could be found (data not shown). A possible reason for this effect could be a lower enzyme release during the consecutive reactions based on the nature of the carrier. MOAT was as ionic liquid a salt containing an anion and a cation, whereby the other carriers are long chain fatty alcohols or glyceryl derivatives. Ionic liquids are known to have special properties like a high thermal stability or low vapor pressure. The thermal stability could be an important factor for the good residual activities after five batches, due to a protection of the enzyme against the thermal stress during immobilization. Nevertheless, the addition of an emulsifier like Gelucire could be very promising with regard to an increasing activity yield, as well as high residual activities.

5.1.2.4. Conclusion of the 2nd system

In this chapter the enzyme immobilization via spray congealing was investigated further and optimized with regard to retained activity and recycling. The optimized immobilization with the self-developed system led to the following results:

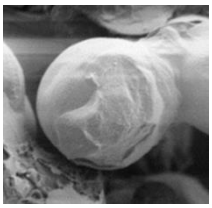
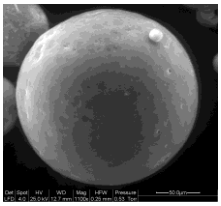
- Highest residual activity of 26% with cetyl alcohol.
- Increasing activity for the PLE immobilized in Precirol ATO 5 with emulsifier.
- No influence of the water amount on activity or yield.
- Higher water amounts lead to greater particles.
- DSC curves showed polymorphic transitions of the carrier.
- Narrow particle size distribution with an average size of 150 – 250 µm.
- Spherical shape of the particles with some imperfections.
- Highest leaching during recycling measurements with cetyl alcohol.
- Highest residual activity with IL MOAT of 30% after 5 batches.

With these investigations the immobilization could be improved significantly. Nevertheless, with the modified spray dryer the immobilization efficiency was higher and should be investigated further with the carriers found during the study with the self-developed apparatus.

5.1.3. Critical evaluation of the spray congealing processes

During this study two different set-ups for enzyme immobilization *via* spray congealing were investigated and compared, shown in table 5-5. One great difference between the systems was the handling. The spray dryer Büchi B-290 was usually used for spray drying of different compounds, like eggs, milk or pharmaceuticals. The advantage of this system was the commercial availability and the closed set-up avoiding the inhalation of fine particles or the contact with toxic compounds. The spray dryer was modified with a liquefying bath and an additional heating for optimization of the immobilization with respect to optimal temperature and heating times. After optimizing of the procedure, it was still complicated to spray without nozzle clogging based on a comparable long and thin nozzle. In contrast, the second system was especially developed for spray congealing and provided a short way of the molten mixture through a wide pneumatic nozzle avoiding a nozzle clogging. Unfortunately, this was an open system, so a possible inhalation of the sprayed particles and the contact with toxic compounds could occur, as well as a contamination of the microparticles. Nevertheless, this second system provided a fast and easy spraying resulting in higher yields of up to 80 % in comparison to the modified spray dryer. The obtained particles showed for both systems a narrow particle size distribution, whereby the second system produced particles with a greater main particle size of 150 – 255 µm, in comparison to the first system with 40 - 45 µm. The particle diameter played a crucial role in minimization of diffusion limitation. In view of applicability the particle diameter for usage in packed-bed reactors should be ideally between 200 – 400 µm to ensure low back pressures. For applications in stirred tank reactors the diameter could be reduced up to 10 µm still enabling separation by filtration or sedimentation. [Liese *et al.*, 2013] With regard to the different systems, the particles prepared with the spray dryer could be used for reactions in e.g. a continuous stirred tank reactor and the immobilized enzyme produced by the second system are of high interest for the application in a packed-bed reactor enabling an easy recycling and high productivities.

Table 5-5. Comparison of the two used methods for enzyme encapsulation *via* spray congealing.

	1st system	**2nd system**
Equipment	Spray dryer 290	Spray congealing apparatus
Set-up characteristics		
Orifice opening	2 mm	4.5 mm
Nozzle length	150 mm	50 mm
Air flow	Axially	Radially
Fluid flow	Axially	Axially
Cooling chamber	0.33 m x Ø 0.17 m	1.80 m x 0.75 m x 0.75 m
Yield	40 – 66%	70 – 80%
Particle size	40 – 45 µm[1]	150 - 255 µm[2]
Activity yield	50%[3]	24%[4]
Retained activity after 1st batch	32%[3]	16%[4]
SEM		
Advantage	Closed system Commercially available Automatically heating	Easy handling, cleaning No nozzle clogging
Disadvantages	Difficulty handling, cleaning Nozzle clogging	Self-constructed Open system Heat control by voltage adjustment

Cond.: $m_{cetyl\ alcohol}$ = 10 g, $A_{laccase}$ = , Immobilization was carried out in cetyl alcohol and under optimized conditions for each system. [1]Determined by Max Planck Institute for Dynamics of Complex Technical Systems. [2]Determined by sieves. [3]Phosphate buffer pH 6, 0.1 M, V = 100 mL, c_{ABTS} = 5 mM, V_{ABTS} = 2.2 mL, $m_{capsules}$ = 500 mg, Q = 10 mL/min. [4]$m_{capsules}$ = 250 mg, Q = 2.5 – 3 mL/min, $V_{substrate\ solution}$ = 100 mL, $c_{p\text{-}NPA}$ = 10 mM.

However, the residual activity of the immobilized laccase was twice as high when the particles were prepared with the first spray congealing system. The reason for this might be the distribution of the enzyme solution in the matrix. Figure 5-16 illustrates the different distribution possibilities for an encapsulated active ingredient in a matrix. Thereby figure 5-16, a and b, are presenting layered cells enclosing a core, solid core or an open void, which contain the active ingredient. This could be realized when the used technique would produce a droplet of the enzyme solution and enclosed it with the melted matrix. A possibility for generating such a particle is the three-fluid nozzle, also available for the spray dryer. Thereby the solution of the ingredient (first fluid) and the matrix (second fluid) are separately delivered to the end of the nozzle and atomized by pressured air (third fluid). With this set-up it could be possible to realize an enclosed core. Other possibilities are, that cells could be formed during immobilization with closed shell (figure 5-16, c), as well as an open shell (figure 5-16, e), which would result in an increased release of the immobilized ingredient. Furthermore, the nanoparticle itself could be embedded and dispersed in a capsule or directly as a shell (figure 5-16, d or f). In figure 5-16, g, an irregular non spherical form of the particle is presented which could exhibit voids and an internal composition gradient. [Dixit *et al.*, 2011] However, in the case of this study spherical shapes could be obtained. The ideal form would be a core of the enzyme surrounded by a shell exhibiting less diffusion limitation to a comparable thin membrane of the carrier.

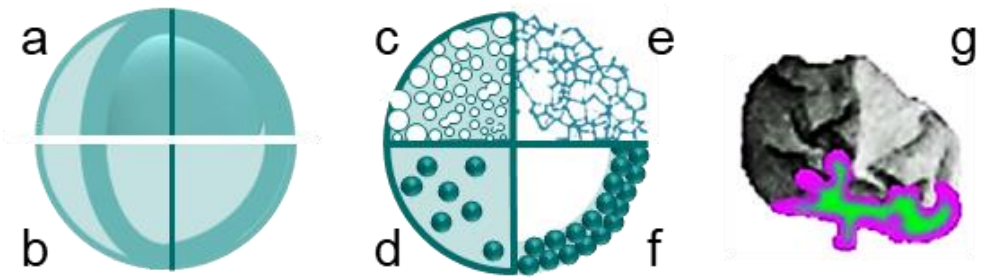


Figure 5-16. Possible particle morphologies. a) Layered particle with solid core. b) Layered particle with central void. c) Solid foam, closed cell. d) Particle with embedded nanoparticles. e) Solid foam, open cell. f) Composite shell. g) Irregular particle with external voids and internal concentration gradients. [adapted from [Dixit *et al.*, 2011]]

Based on the performance of the technique, the mixing of the carrier and the enzyme before spraying, only a dispersed active ingredient in the carrier was possible using the spray congealing for immobilization. The usage of solid enzyme resulted in a low encapsulation efficiency, due to comparable big enzyme particles. With smaller particles an embedding would be conceivable, however, because of the sensitivity of the biocatalyst a milling of the

enzyme before adding to the molten carrier was not considered in this study. The usage of dissolved enzyme could result in particles with solid foam morphologies. For all investigations a considerable release of the protein content, as well as a significant activity loss could be found after the first batch. This effect suggested that an open shell (figure 5-16, e) was obtained for the immobilized enzyme prepared with the spray congealing technique.

Despite the advantages of the spray congealing process, the enzyme showed a loss of the residual activity after immobilization, presenting a common problem for enzyme immobilization. [Homaei *et al.*, 2013; Dwevedi, 2016] The main effects during spray congealing were e.g. a loss of the enzyme in the beaker during the preparation of the mixture and the subsequent purring in the spray congealing system. The enzyme could stick on the bottom of the beaker, as well as inside the heating bath (first system) or in the nozzle. The heat influence, as well as the shear stress during atomization can highly affect the enzyme activity and can introduce polymorphic transitions of the carriers, influencing the retention of the enzyme. [Wong *et al.*, 2016] The main influence, which represented the disadvantage of this method, was a loss of activity during the heating in the beaker and in the system. The structure of the enzyme (cf. chapter 3.1.) was the reason for temperature sensitivity, which could lead to an inactivation of the enzyme by heating of the enzyme mixture. Two different possibilities for inactivation are described. A reversible deactivation could occur while cleaving weak bonds, which would destroy the tertiary (and often secondary) structure. A cleavage of covalent bonds would destroy the primary structure and would inactivate the enzyme irreversible. [Daniel et al., 1996] However, the retained activity after the first batch was for the first system more than twice as high as for the second system with the same carrier. This effect occurred also for the activity obtained in the second batch after recycling of the particles. A main reason for this result could be the different analytical measurements. These measurements were performed in different institutes so that the same equipment was not available and different set-ups had to be used. In addition, the smaller particles prepared by the modified spray dryer resulted in an increased pressure during the measurements. Based on this a different flow behavior occurred and was also influenced by different reactors. Nevertheless, the investigation of further carriers with the second system resulted in a retained activity of 30% with the ionic liquid MOAT presenting a highly interesting carrier for this application.

An interesting overview for the preparation of microparticles was given by Rosiaux *et al.* [Rosiaux *et al.*, 2014] They described the influences of the process with regard to the wettability, porosity and release behavior of the microparticles, shown in table 5-6. The

release behavior of the encapsulated ingredient exhibits a direct correlation to porosity of the particle. However, for biocatalytic reactions the release of the enzyme should be completely avoided, contrary to the diffusion of the substrate and product, which has to be ensured. The higher the porosity of the capsules, the better is the diffusion of the substrates and products through the particle, and the better the availability for the enzyme. Thereby a compromise has to be found, so that small molecules, like substrate and product, can diffuse through the membrane and the enzyme as a comparable big molecule is retained in the capsule for an adequate separation. In table 5-6 it can be seen that with optimization of lipid carrier and the dimension of the particles the porosity and therefore the release behavior could be influenced in the given system of this study. Thereby the arrows indicate a decreasing or an increasing of the corresponding properties by changing reaction conditions. Interesting properties for enzyme immobilization are highlighted in pink.

Table 5-6. Effect of formulation parameters for prepared capsules. (Desired effects for enzyme immobilization highlighted in pink) [Rosiaux *et al.*, 2014]

		Wettability	Porosity	Release
Drug				
Water solubility	↑	↑	↑	↑
Dose	↑	↑	↑	↑
Lipid matrix				
Melting point	↑	↑	↓	↓
Concentration	↑	↑	↓	↓
HLB	↑	↓	↑	↑
Other excipients				
Water solubility	↑	↑	↑	↑
Concentration	↑	↑	↑	↑
Surfactants	↑	↑	↑	↑
Disintegrants	↑	↑	↑	↑
Geometry				
Dimension	↑	↓	↓	↓
Radial:axial	↑	↑	↑	↑

This overview confirmed the results for immobilized enzymes *via* spray congealing. An increasing of the melting point, resulted in a higher stability and lower abrasion of the particles, but due to the temperature sensitivity these investigations were limited to a maximum melting point of the carrier. Furthermore, a higher HLB led to a higher release of the enzyme, so that only a limited amount of emulsifier could be used. However, the addition

of an emulsifier increased the recyclability of the immobilized enzyme. The increasing of the particle size was also accompanied with lower release of the immobilized ingredient confirming the investigations in this study. Here, the studies with larger particles prepared by the second system demonstrated a re-usage in up to five batches without complete activity loss.

The investigations of these two spray congealing systems showed a new promising and suitable possibility for enzyme immobilization. With the second system increased residual activities of the immobilized enzyme could be realized using different carriers, as well as a better recyclability. However, since the activities obtained with the modified spray dryer were higher, the here investigated carriers has to be tested for the first system as well. For further optimizations the heat influence for the enzyme has to be reduced, so that more temperature sensitive enzymes can be immobilized by this promising cheap technique and re-used in several applications, as well as in industry.

5.2. Laccase initiated C-N coupling reactions – The “green” way

Cross-coupling reaction products are highly interesting, due to an application as building blocks for active pharmaceutical ingredients (API). Especially nitrogen containing species are present in natural products as well as synthesized compounds which show biological activities. Today different routes for the synthesis of C-N coupling products are available, summarized in figure 5-17. The first synthesis using copper salts was developed by Ullmann and Goldberg in 1903, improved continuously over the following years. [Bariwal *et al.*, 2013; Ullmann, 1903; Monnier *et al.*, 2008] Buchwald and Hartwig established palladium and copper catalyzed cross-coupling reactions with diamine or phosphine ligands in 1930. Nowadays palladium catalyzed syntheses of C-N coupling products are extensively used in academia and industry. [Bariwal *et al.*, 2013; Jiang *et al.*, 2004; Muci *et al.*, 2002; Blaser *et al.*, 2004] In most of the used reactions metal catalysts and pre-activated starting materials like (hetero)aryl (pseudo)halides are necessary and despite a lot of possibilities C-N cross-coupling reactions under mild and inexpensive conditions are still challenging. For these reactions biocatalysis is a promising field, whereby laccases enable a green alternative to classical metal catalyzed reactions. [Bariwal *et al.*, 2013]

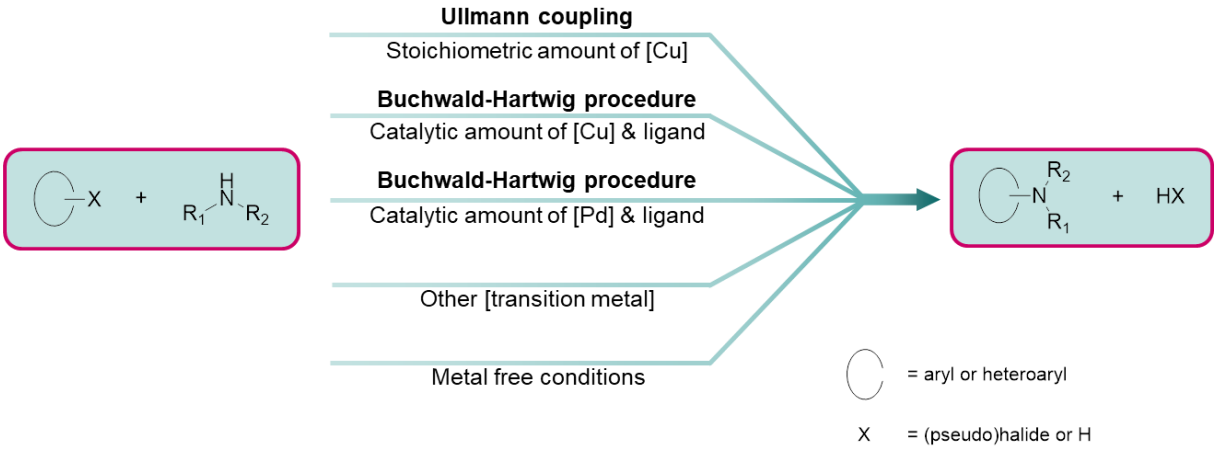


Figure 5-17. Currently available C-N bond formation strategies. [Bariwal *et al.*, 2013]

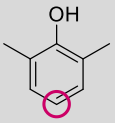
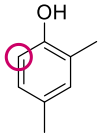
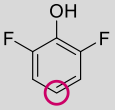
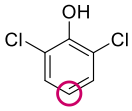
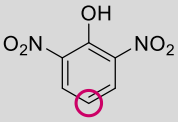
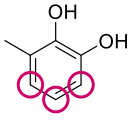
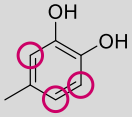
Beside the immobilization of biocatalysts described in the first part of the thesis, in this part the immobilized laccase should be tested for coupling reactions and new laccase initiated coupling reactions should be evaluated. Due to no need of un-activated aromatic rings or any co-factor and the possible usage of different nucleophiles as coupling agent these reactions are highly interesting. Furthermore, oxygen is the only co-substrate needed with water as by-product. [Cannatelli *et al.*, 2013] Beside a lot of advantages and interesting properties these enzymes have one disadvantage: Since the catalytic mechanism is based on a radical mechanism, the reaction is very unselective and results in different coupling products, like mono-, di- and oligomers, as well as different cross-coupling products (cf. figure 3-4). In earlier studies several analytical methods were investigated to get an insight into laccase initiated C-C coupling reaction mechanisms. Thereby it became obvious that the positions of residues at the aromatic ring played a crucial role for the selectivity, published during the thesis.[1] The aim of these investigations was the coupling with different amines, whereby glucosamine as highly functionalized compound represented the most interesting coupling reagent. This amine could enable further reactions resulting in complex molecules with potential bioactive properties. Due to several investigations of this enzyme aiming coupling reactions and the good immobilization results in this study, the commercial available *TvL* was used for these investigations. This enzyme showed the highest activity at pH 4 (cf. appendix) and as a side effect the polymerization of the phenolic compounds was avoided, which is promoted by a basic environment based on proton abstraction. [Eickhoff *et al.*, 2001]

[1]C. Engelmann, S. Illner, U. Kragl. „Laccase initiated C-C couplings: Various techniques for reaction monitoring". Process Biochemistry. 2015, 50, 1591.

5.2.1. Substrate activation by laccase oxidation

Different mono- and bi-phenolic compounds were chosen as possible substrates for the coupling system based on previous work. The reactions were carried out with 20% methanol to ensure dissolving of less soluble compounds. This presented a compromise to the stability of the enzyme, which is around 60% with this methanol content (cf. appendix).

Table 5-7. Substrate screening *via* measurement of the oxygen consumption. (Pink circles: Possible coupling positions.)

Chemical name	Structure	Possible coupling positions	Initial oxygen consumption [mg · L^{-1} · min^{-1}]	Conversion rate[1] [$mmol_{substrate}$ U^{-1} min^{-1}]
2,6-Dimethylphenol		1	0.954	0.477
2,4-Dimethylphenol		1	1.145	0.573
2,6-Difluorophenol		1	0.097	0.049
2,6-Dichlorophenol		1	0.226	0.113
2,6-Dinitrophenol		1	0	0
3-Methylcatechol		3	0.213	0.053
4-Methylcatechol		3	0.609	0.152

Cond.: V = 30 mL, $c_{substrate}$ = 5 mM, $A_{laccase}$ = 0.5 U/mL, 20 vol.-% MeOH, citrate phosphate buffer pH 4.

[1]Substrate conversion was calculated on the basis of reduction of 1 mol oxygen while oxidation of two or four substrates (depending on number of OH groups) and used enzyme amount.

For the estimation of the substrate suitability, the oxygen consumption was determined during the biocatalytic oxidation (table 5-7). The highest oxygen consumptions were obtained with 2,6-dimethylphenol (2,6-DMP), 2,4-dimethylphenol (2,4-DMP) and 4-methylcatechol (4-MC), whereby 3-methylcatechol (3-MC) showed only a slightly lower value. In contrast, the reaction rates of 2,6-dichlorophenol and 2,6-difluorophenol were significantly lower, while 2,6-dinitrophenol showed no conversion. For the oxygen consumption the number of hydroxyl groups had to be taken into account. The laccase catalyzed a four electron oxidation of the substrate with a four electron transfer of one molecule oxygen. Thereby one mol oxygen was consumed for oxidation of two substrate molecules concerning bi-phenolic compounds or four substrates considering mono-phenolic compounds. This revealed in a higher oxygen demand for 3-methylcatechol and 4-methylcatechol and increasing oxygen consumptions, due to a faster availability of a further oxidizable compound. In figure 5-18 the mesomeric structure of phenolic compounds and possible coupling reactions are illustrated.

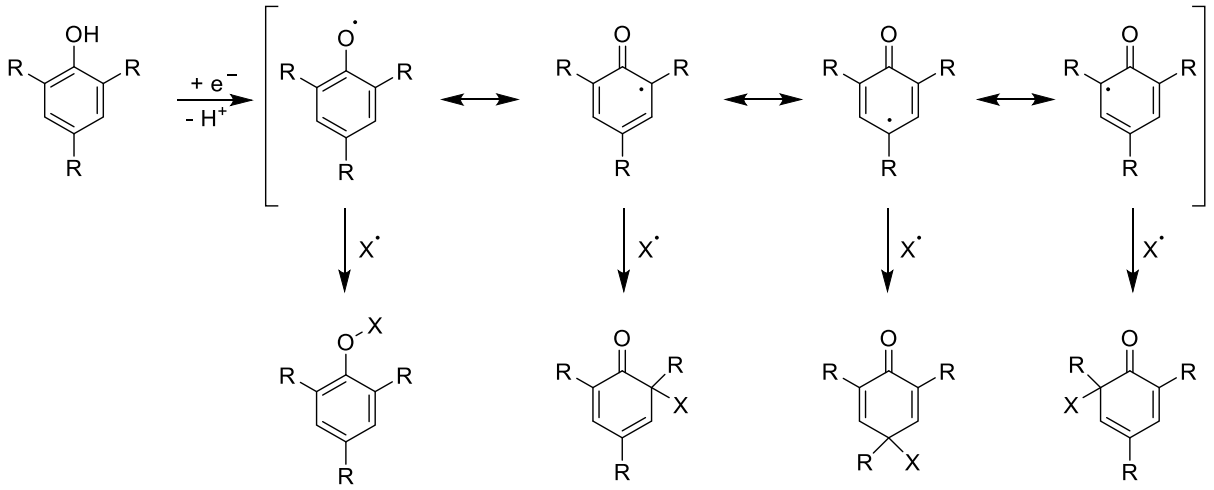


Figure 5-18. Resonance structure of oxidized phenolic compounds and suggested possible coupling reactions. [Eickhoff *et al.*, 2001; Engelmann *et al.*, 2015]

The π-system of the benzolic part of the phenol was overlapping with an occupied *p*-orbital of the oxygen atom resulting in a delocalization of the electrons. [Vollhardt *et al.*, 2011] This positive mesomeric effect (+M) led to highly activating electrons which directed the coupling in *para*- and *ortho*-position forming different coupling products. The negative inductive (-I) effect of OH-groups was too low for a deactivation of the ring. For the substitution in *meta*-position no stabilization of the oxygen radical could be obtained, which led to an unfavorable position and no coupling. Nevertheless, the coupling positions of the phenolic compounds were further influenced by additional substituted groups. The investigated methyl

catechols exhibited two OH-group shifting electrons also into the ring and enabled more coupling positions, as illustrated in table 5-7, and therefore more possible reaction products. The methyl group itself was not exhibiting a mesomeric effect, however, it implied a positive inductive effect (+I). This could result in a weak activation and stabilization effect, if the second substitution took place in *para*- or *ortho*-position of this group. Substrates containing halogens, like 2,6-difluorophenol and 2,6-dichlorophenol, showed a poor reactivity based on weak deactivation of the phenolic compound by these atoms. This effect was highly increased by the introduction of NO-groups exhibiting a strong deactivation of the ring, so that no coupling reaction occurred. [Hädener *et al.*, 2006; Solomons, 2011]

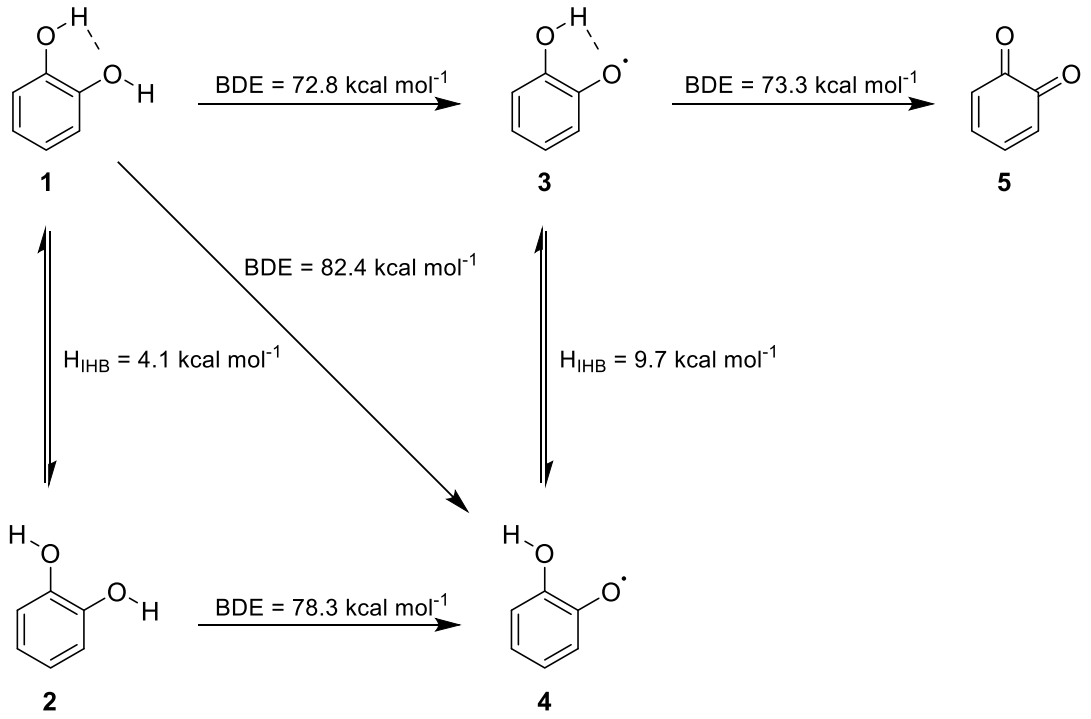


Figure 5-19. Energy scheme for catechol. (BDE – bond dissociation engery, H_{IHB} – intramolecular hydrogen bond enthalpies) [Zhang *et al.*, 2003]

Another important factor for the oxidizability is the bond dissociation energy (BDE). Lower BDE values enable an easier H-abstraction and therefore the formation of the radical. In figure 5-19 the thermodynamic scheme for catechol is shown, whereby intramolecular hydrogen bonds (IHB) are illustrated stabilizing catecholic compounds. On the one hand, the cleavage enthalpy (H_{IHB}) for these bonds was higher when the second hydroxyl group was already oxidized. This fact is based on the longer hydrogen bond of 2.124 Å for **1** than for **3** with 1.974 Å presenting a stronger bond. On the other hand, the bond dissociation energy for the H-abstraction for **1** was lower than for **2**, based on the stabilized structure of **1** by the

IHB. Otherwise BDEs were different depending on the IHBs of the intermediates. The BDE of intermediate **1** was 5.5 kcal mol^{-1} lower than for **2**, due to an easier abstraction of the proton based on the stabilization *via* IHB. However, the necessary BDE to obtain compound **4**, was with 82.4 kcal mol^{-1} the same constituting the sum of the BDE and H_{IHB}. Both cleavages of the IHBs were reversible and the oxidation of the second hydroxyl group was only possible with the stabilized form **3**. All in all, with every pathway this would result in intermediate **3**. Due to a higher stability based on the radical, a slightly higher BDE of 73.3 kcal mol^{-1} was needed for the abstraction of the proton in comparison to compound **1**. [Zhang et al., 2003]

Zhang *et al.* described also the BDEs and H_{IHB} for mono-phenolic compounds, as well as substituted phenolic compounds. Thereby it became obvious that the net value of the BDE of mono-phenolic compounds was always higher than for bi-phenolic ones. In addition, electron donating groups, which were e.g. chloride, fluoride or methyl groups, showed a decreasing of the BDEs, which was also true for the ionization potential (IP) reducing the electron transfer between the catecholic compound and also the pro-oxidative potency. [Zhang *et al.*, 2003; Quideau *et al.*, 2011]

The oxidizabilities showed differences between catecholic compounds with different residues. The highest oxygen consumptions were obtained with 2,4-DMP and 2,6-DMP exhibiting only activating groups such as 3-MC and 4-MC. Nevertheless, CH_3-groups were only weakly activating in comparison to OH-groups. This suggested that the affinity of this compound to the enzyme, which was derived from the structure of the substrate and the nature of the residual groups, played the most important role for oxidation. One reason could be the different coupling reactions of the mono- and bi-phenols. 2,4-DMP and 2,6-DMP had only one position where the coupling of a second compound could take place. This was based on the non-activation of *meta*-positions, as discussed before, as well as the steric hindrance by the CH_3-groups in *ortho*-position. As a result, only a coupling in *para*-position was possible. This led to the formation of dimers confirmed in earlier investigations. As described elsewhere 3-MC and 4-MC had two oxidizable OH-groups resulting in various possible coupling products and reactions. On the one hand there were two possibilities for the oxidation of the compound; on the other hand after the first oxidation these species could undergo beside dimerizations additional oligo- and polymerizations forming greater molecules, which were not fitting into binding pocket of the enzyme. The oxygen consumption was compared in the beginning of the reaction, due to no possible oxidizabilty of greater molecules. Based on different oxygen demands, the conversion rates for the phenolic compounds were calculated on the number of OH groups, presented in table 5-7. It can be seen that the highest conversion could be reached for 2,4-DMP and 2,6-DMP, which confirmed the oxygen measurements. In contrast the conversion of the bi-phenolic

compound 3-MC was comparable to 2,6-dichlorophenol and significant lower than for the other mono-phenolic compounds. Earlier investigations showed, that 2,6-substituted compounds could be oxidized very easily. However, these compounds underwent very fast dimerizations. This could be explained by a specific coupling position and an over oxidation, based on a keto-enol tautomerism as illustrated in figure 5-20. Based on these fast laccase initiated dimeric coupling reactions the tested mono-phenolic compounds were not investigated for the C-N coupling reactions.

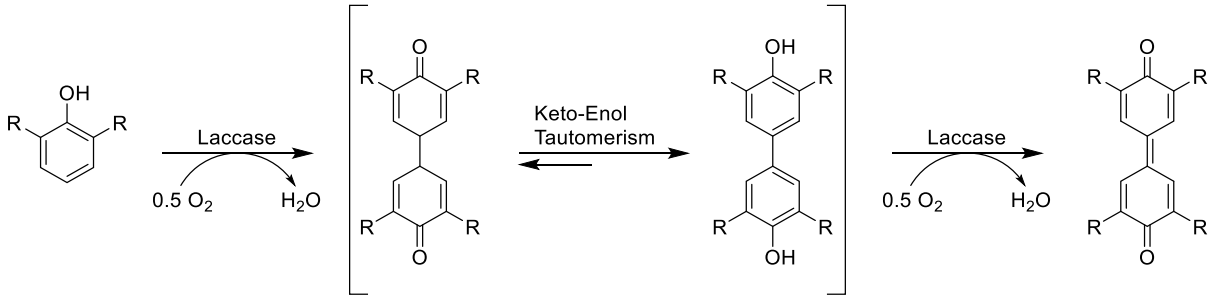


Figure 5-20. Laccase initiated oxidation of 2,6-substituted phenolic compounds.

Nevertheless, 3-MC and 4-MC were highly interesting for the C-N coupling reactions, due to good reactions rates and furthermore various laccase based coupling reactions were performed in previous investigations. [Illner *et al.*, 2014; Engelmann *et al.*, 2015; Herter *et al.*, 2011] 4-MC as bi-phenolic compound showed thereby the third highest conversion rate of all tested substrates and three possible coupling positions in comparison for 2,6-substituted mono-phenolic compounds (only one coupling position). The chance for the desired C-N coupling could be increased, due to additional coupling positions for the amine competing with a second catecholic compound. For further investigations of laccase initiated C-N coupling reactions during this study 4-MC was chosen as substrate. This catechol exhibited higher steric hindrance by the CH_3-group suggesting higher selectivities.

5.2.2. Coupling of 4-methylcatechol and cyclohexylamine

The laccase initiated coupling reaction of 4-MC was investigated with cyclohexylamine as coupling reagent based on previous studies, where hydrophobic amines, e.g. *n*-hexylamine, were used. [Herter *et al.*, 2011; Illner *et al.*, 2014] As discussed before, the coupling of 4-methylcatechol and cyclohexylamine could result in various products, like C-C, C-O or C-N coupling products (cf. figure 3-4). The complex downstream processing and the isolation of the products were comparable difficult, so that the analytical investigations were performed *via* mass spectrometry. Thereby the positive ion modes were evaluated, due to detection of both coupling reagents.

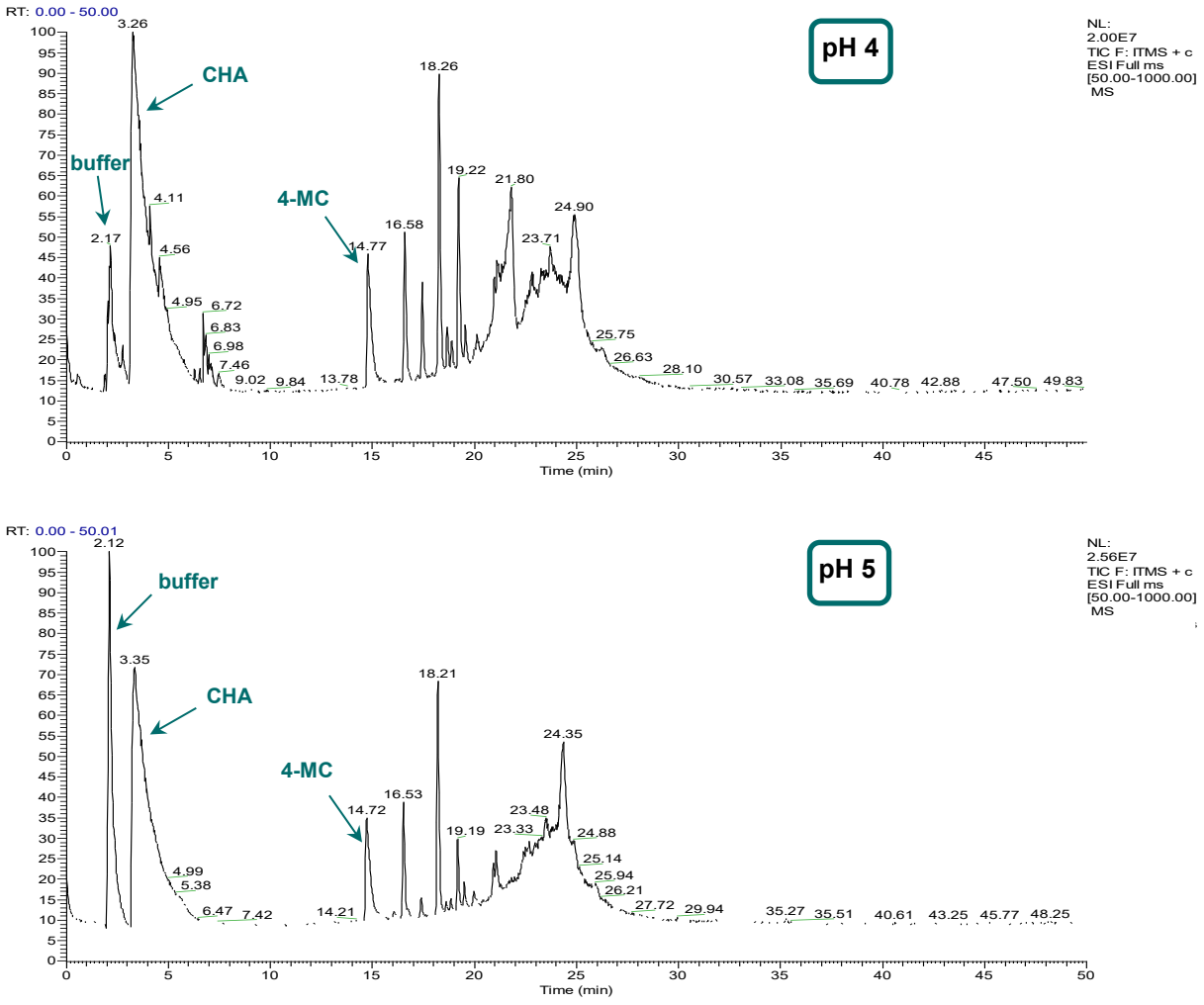


Figure 5-21. TIC chromatogram for the coupling reaction of 4-methylcatechol and cyclohexylamine. Cond.: V = 20 mL, $c_{4\text{-MC}}$ = 5 mM, c_{CHA} = 5 mM, $A_{laccase}$ = 5 U/mL, sodium acetic acid buffer.

Figure 5-21 showed the total ion current (TIC) chromatogram of the batch reaction after two hours, whereby significant differences in comparison to the chromatogram without amine as coupling agent could be seen (cf. appendix). It was visible that after 2 hours 4-methylcatechol (14.77 min) and cyclohexylamine (3.26 min), with the typical tailing for amines, were not converted completely and could still be detected in the reaction solution. Various products were occurring, however, the peaks with the retention time of 17.44 min and 19.22 min showed a m/z ratio of 245 in the negative mode indicating the dimer of 4-MC in the phenolic form, also occurring at 21.8 min. This implied more possible coupling positions (C-C or C-O, cf. figure 3-4) for a second 4-MC molecule resulting in different retention times based on their hydrophobicity. The suggested structures of possible reaction products were illustrated in figure 5-22. The main coupling products were the dimeric forms of 4-methylcatechol. In addition, the stabilization of phenolic compounds by water could be observed at 18.26 min presenting an oxidized dimer with a coordinated water molecule and a m/z ratio of 261. Nevertheless, the peak at 16.58 min showed also the presence of the completely oxidized C-N coupling product with a mass of 219 g/mol.

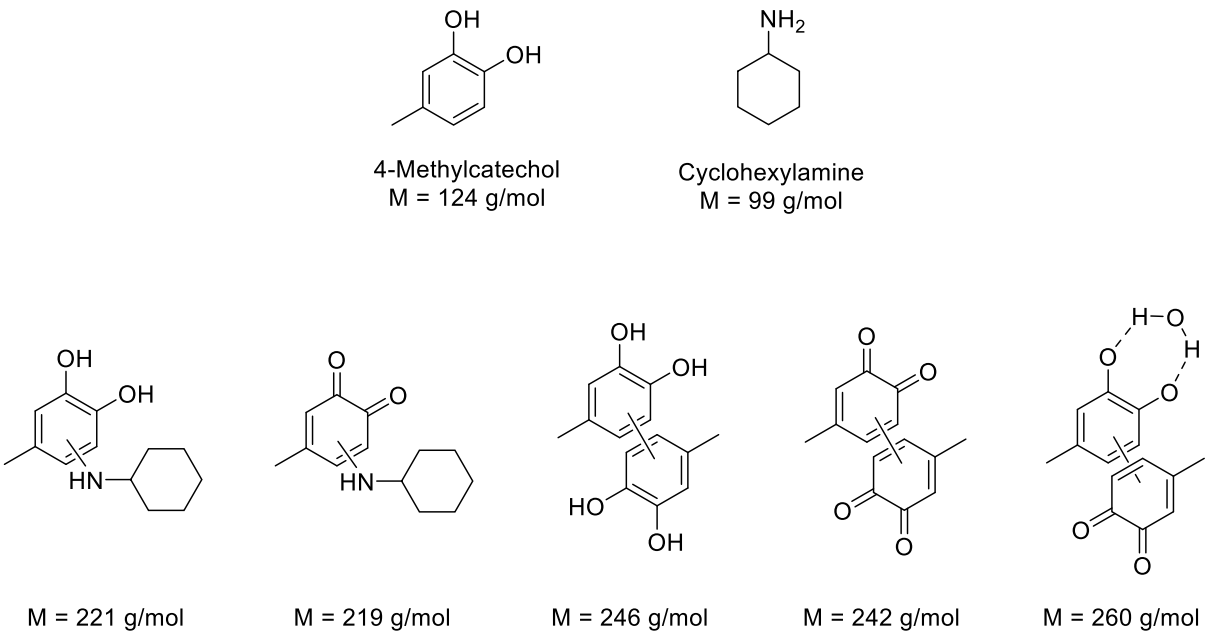


Figure 5-22. Suggested coupling products based on the observed masses during the laccase initiated coupling reaction of 4-methylcatechol and cyclohexylamine.

In the negative mode the m/z of 138, resulting from the defragmentation of the CH_3-group, and 181, presenting the product without the ring of the amine, were visible. However, the poor selectivity was based on the low regioselectivity for coupling reactions of phenolic compounds resulting in a small amount of desired C-N coupling products. [Davin *et al.*, 1997] For increasing of the selectivity the two parts of the reaction had to be considered and therefore different reaction conditions. On the one hand, the enzyme had a pH optimum at a comparable acidic value of pH 4 for the oxidation of the phenolic compound (cf. appendix). On the other hand, the amine as coupling agent was also pH dependent. The orbitals of the amine were almost sp^3-hybridizated and form a tetraedrical configuration, whereby three points were occupied by residues and one by the free electron pair. Due to this electron pair the amine could react as nucleophile and realized C-N couplings. If the pH value would be to acidic the electron pair could be occupied by hydrogen and no coupling reaction would take place. [Vollhardt *et al.*, 2011] In figure 5-21, the TIC chromatogram of a reaction at pH 4, enzyme optimum, and an increased pH of 5 is shown and it became visible that the increasing pH resulted in less product peaks. This lower amount of products suggested that an increasing pH value seemed to increase the selectivity of the coupling reaction. With this slightly higher pH value the C-N coupling product at 16.5 min and a dimeric form of the phenolic compound with a corresponding water molecule were obtained as main products. Nevertheless, the reaction solution contained still various coupling products, due to the unfavorable homo-couplings of 4-MC.

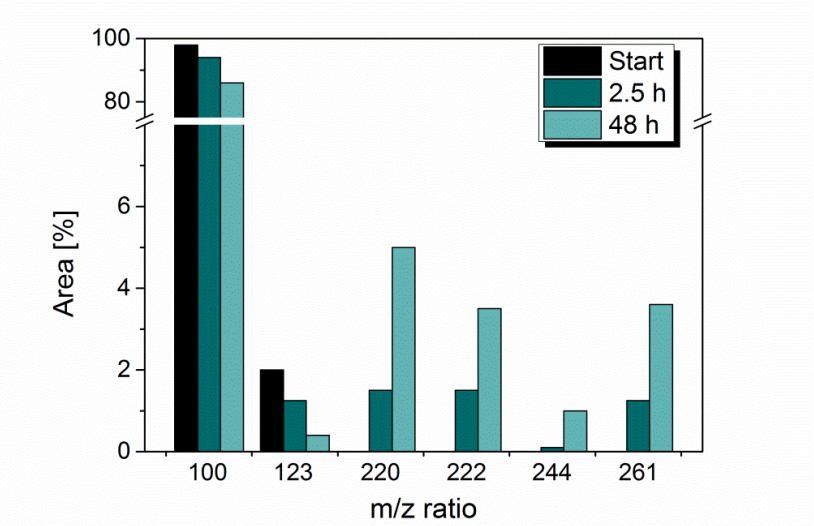


Figure 5-23. Reaction monitoring *via* LC/MS in the pos. mode of the laccase initiated coupling reaction of cyclohexylamine and 4-methylcatechol. Cond.: Sodium acetic acid buffer pH 5, V = 10 mL, c_{CHA} = 10 mM, $c_{4\text{-}MC}$ = 10 mM, $A_{laccase}$ = 5 U/mL.

In figure 5-23 the reaction products over time are shown, whereby the formation of the products and the conversion of the reactants could be observed. The coupling products could not be isolated, so that the peak areas were used instead for investigations of the reaction in the following considerations. These areas could not be compared directly, due to different ionization potentials and detection with LC/MS of the molecules, however, trends for each compound could be observed by evaluating the ratios. During these investigations the positive mode was used, so that the detection of all substrates and products was possible. Thereby the excess of the amine and the good detectability resulted in the largest area during the measurements. Nevertheless, the consumptions of the substrate and the amine were visible and also the trend for the product formation during the reaction. After 2.5 h the amounts of 4-MC (m/z 123), the both C-N coupling products (m/z 220, 222) and the dimeric C-C coupling product with a coordinated water molecule had a similar area ratio. However, after 48 h the ratio of the oxidized C-N coupling product (m/z 220) increased the most. Also the amount of the hydroxyl product increased, which showed that the C-N coupling products exhibited a higher production rate in comparison to the dimeric C-C coupling products. This illustrated the favored coupling with the amine after a longer reaction time during this batch reaction. A reason for the favored C-N coupling after longer reactions could be based on the less availability of the substrate and therefore an increasing excess of the amine for the proceeded reaction. After all it could be concluded that increasing times were favorable for C-N coupling in batch reactions.

An aim of this study was to investigate also the immobilized laccase for C-N coupling reactions prepared *via* spray congealing (cf. chapter 5.1.). Therefore the same set-up as for the recycling measurements was used and the reaction products were analyzed *via* LC/MS. The main difference was the substrate availability for the enzyme and the different set-up: Batch or plug flow reactor (PFR). In figure 5-24 concentration profiles of an ideal batch reaction in comparison to an ideal plug flow reactor against time and location are illustrated. In theory for the batch reaction the concentration of the substrate [A] is decreasing over the reaction time, while the product [B] is increasing. Nevertheless, the reaction in a batch reactor is only dependent on the time; however, the concentration is always the same for every location of the reactor. If a PFR is used this effect is contrary; the reaction is not dependent on the time, but on the location. The reaction solution is flowing through the reactor and the enzyme and the substrate could react over the reactor length L_R and the product concentration is increasing.

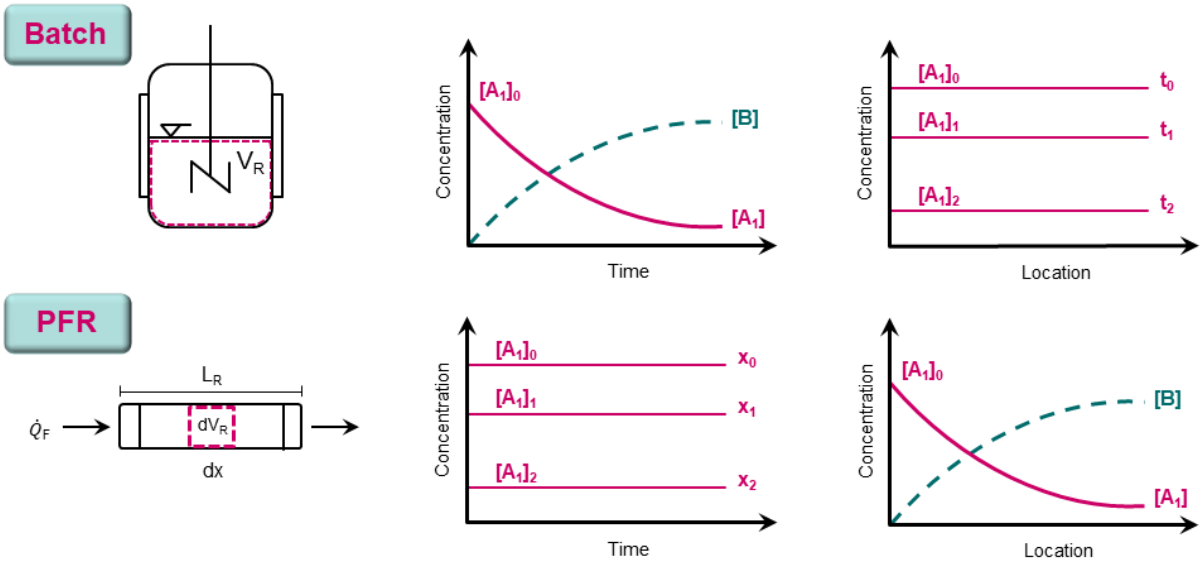


Figure 5-24. Symbolic illustration of a batch and plug flow reactor (PFR) with illustration of the corresponding ideal concentration profiles depending of time and location. The volume of the PFR dV_R is a small batch reactor moving through the PFR with a defined velocity v_x. (adapted from [Chmiel, 2011])

The great advantage of the immobilized laccase used in a PFR was the easy separation of the enzyme from substrates and products. However, in comparison to the C-N coupling reaction in a batch reactor it could be seen that the reaction solution contained various different species, especially between 20 and 28 min, presented in figure 5-25. This unselective reaction was based on the combination of the reactor type and the used laccase. The reaction mechanism of this enzyme was based on a radical mechanism, as described in chapter 3.1.1. The laccase was initiating the reaction, due to an oxidation of the substrate forming a radical, which was able to couple with various compounds. In a batch reactor the substrate was oxidized over a certain time (figure 5-24), based on the almost homogenously allocated molecules of the reaction mixture. In a PFR the enzyme was concentrated and therefore the probability of substrate and enzyme contact increased, so that the oxidation rate might be higher. This resulted in a decreasing of amine excess and increasing formation of coupling partners. In addition, the possible oxidation of a second substrate led to an easy electron transfer so that C-C couplings were preferred and many different compounds based on several di-, oligo- and polymerizations were produced.

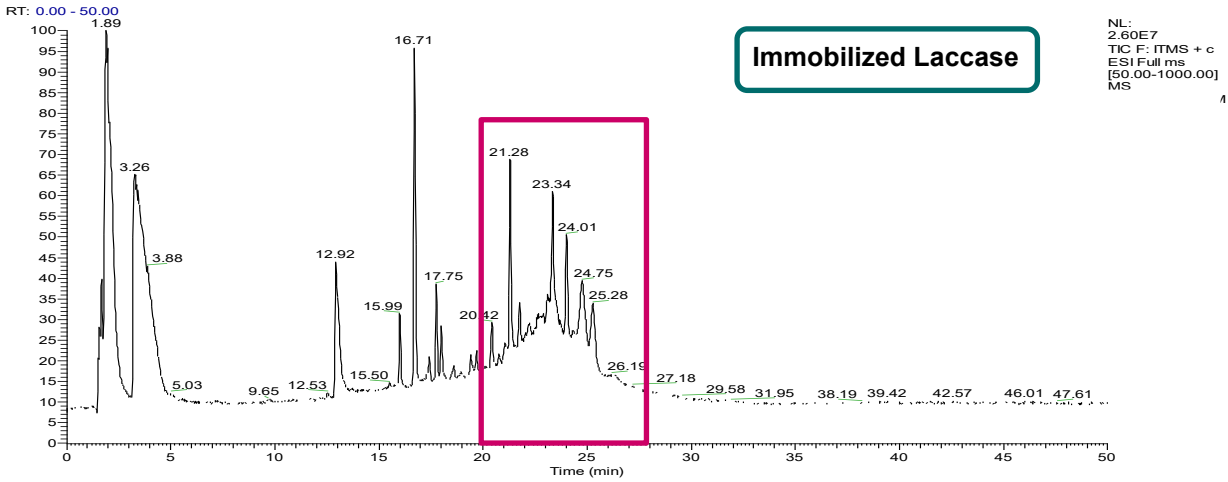


Figure 5-25. TIC chromatogram (pos. Mode) of the coupling reaction with immobilized laccase. Cond.: V = 100 mL, $c_{4\text{-MC}}$ = 5 mM, c_{CHA} = 5 mM, $m_{laccase}$ = 500 mg, Sodium acetic acid buffer pH 5.

These results showed that the laccase with their radical reaction mechanism was not the ideal enzyme for the evaluation of this immobilization method and their usage in a PFR reactor. PFR reactor is also a batch reactor, so that no difference in selectivity should occur. This might be based on a higher amount of laccase immobilized in the particles. In earlier investigations it could be observed, that a higher enzyme amount led to a decreasing selectivity of laccase catalyzed coupling reactions [Engelmann et al., 2015]. Due to an easier adjusting of the used enzyme amount the reaction was investigated in detail using the batch reactor. The reactions performed in batch reactor showed a competition of homo- and hetero-couplings. An interesting alternative reactor type for this reaction was a fed-batch reactor. This enabled the coupling with the available amount of coupling reagent, which was present in excess. For the reduction of catechol homocouplings, a stepwise addition of the substrate to a mixture of enzyme and amine was used to enable an amine excess during the laccase initiated reaction. In figure 5-26 it became apparent, that this reactor operation resulted in a superior increasing selectivity for the laccase initiated coupling reactions. The main product was the oxidized form of the C-N coupling product at 21.0 min, in addition, the phenolic form could be found at 18.0 min. In comparison to the batch mode, the intensity of the coupling products increased significantly and no additional fragmentations were visible.

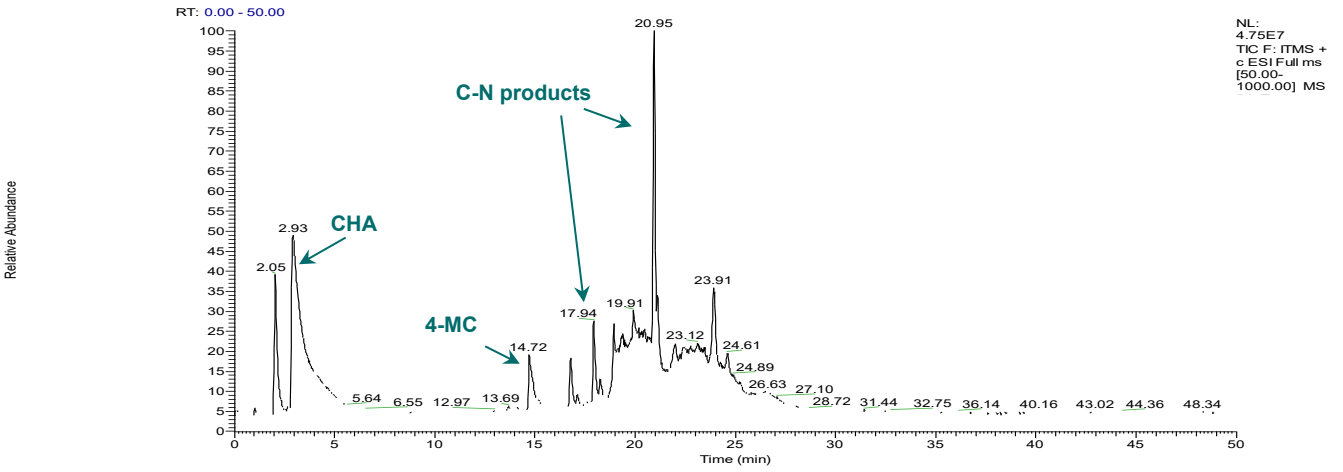


Figure 5-26. TIC chromatogram (pos. Mode) of the coupling reaction with a fed-batch reactor. Cond.: $V_{4\text{-MC}}$ = 12.7 mL, $c_{4\text{-MC}}$ = 5 mM, $V_{laccase+CHA}$ = 10 mL, c_{CHA} = 5 mM, $A_{laccase}$ = 5 U/mL, v_{pump} = 0.09 mL/min, Sodium acetic acid buffer pH 5.

During this measurement additional products with m/z ratios of 257 and 259 appeared in the positive ionization mode. These masses might refer to a completely or partially oxidized dimeric coupling product with an additional methyl group. However, using this set-up the enzyme oxidized immediately 4-MC after addition, so that the substrate could not be observed in the reaction during LC/MS measurements. With the slow addition of the catechol a large excess of the amine could be realized resulting in a preferred coupling of the oxidized 4-MC with cyclohexylamine, due to easier availability. The excess of the laccase favored also the activation of substrate molecules, so that more radical forms of 4-MC were able to perform the C-N coupling. In addition, the half continuous reactor could reduce a possible inhibition of the enzyme by the substrate and furthermore it realized higher conversion rates of the substrate. This confirmed the advantage of this reaction operation for laccase catalyzed reaction, based on a radical reaction mechanism.

In general, the investigated coupling reactions produced especially in the oxidized forms of the products. These results confirmed earlier studies were the laccase initiated C-C couplings were investigated with *in situ* IR spectroscopy, as well as studies of Wellington *et al.*, which showed that the laccase was oxidizing phenolic compounds in two steps: After a first oxidation the formed radical could couple with the second substrate molecule or the here used amine. This intermediate is subjected to a keto-enol tautomerism forming the hydroxyl compound and is oxidized again by the laccase resulting in the completely oxidized form of the coupling product (cf. figure 5-20). [Wellington *et al.*, 2013; Engelmann *et al.*, 2015] These results indicated the found hydroxyl form of the coupling products could be also an intermediate.

For a further increasing of the selectivity the C-N coupling *via* fed-batch reaction should be optimized. In figure 5-27 the area ratios for the different products were shown depending on different pumping rates and varying the feeding rate of the substrate. It could be seen, that after 30 min of complete substrate addition no 4-MC could be found in the reaction solution for every rate. The substrate was able to be oxidized completely by the laccase, so that enough free enzyme was available for initiation of the coupling reactions. Furthermore, the measurements showed a significant increasing of the selectivity of the coupling reaction. The main product for all pumping rates was the oxidized C-N coupling product, whereby the hydroxyl form was only visible with a rate of 0.03 mL/min. The comparison of the different pumping rates showed only slight differences. The highest amount of the C-N coupling product with over 30% was reached with a pumping rate of 0.03 mL/min and 0.13 mL/min. The standard deviation of 0.13 mL/min pumping rate was comparable high within the range of the other rates, so that it was not considered further.

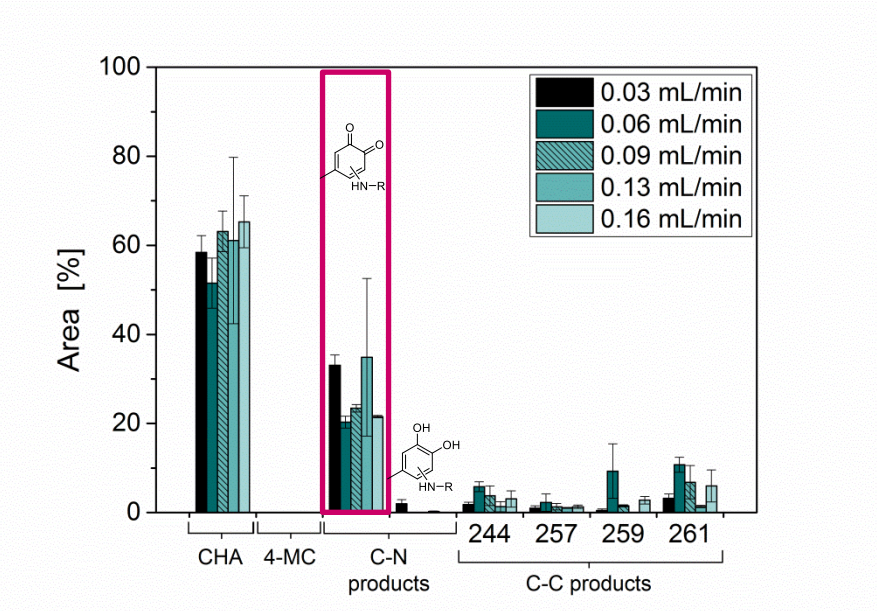


Figure 5-27. Product formation of the laccase initiated coupling reactions of 4-methylcatechol (m/z 123) and cyclohexylamine (m/z 100) at different pumping rates. Samples were taken 30 min after complete addition of the substrate. Cond.: Sodium acetic acid buffer pH 5, V = 20 mL, c_{CHA} = 5 mM, $c_{4\text{-}MC}$ = 5 mM, $A_{laccase}$ = 5 U/mL.

The lowest pumping rate of 0.03 mL/min illustrated the improvement of the selectivity of this heterogeneous coupling reaction. Due to a less substrate availability the enzyme was able to oxidize all substrate molecules rapidly and the high excess of the amine resulted in the higher coupling rates with this compound instead of the reaction with a second substrate

molecule. Especially for these fast radical reactions with subsequent heterogeneous couplings the fed-batch with a low feeding rate of the substrate of 0.03 mL/min was highly advantageous.

For a further optimization the best pumping rate of 0.03 mL/min was chosen and different pH values were tested based on the positive effect of higher values in batch experiments (cf. figure 5-21). In figure 5-28 it could be seen clearly, that the pH was highly influencing the coupling behavior of the oxidized substrate. The amount of 4-MC was increasing with higher pH values, which indicated a decreasing activity of the enzyme. The laccase from *Trametes versicolor* showed the highest activity at pH 4 (cf. appendix) resulting in less oxidation potential of the substrate and therefore less coupling products, neither C-N nor C-C couplings. In contrast, the amounts of C-N coupling products increased with an increasing pH value and also the hydroxyl form was formed. Nevertheless with a higher value of pH 5 the amount started to decrease again. This was based on the free electron pair at a higher pH value and therefore the ability of the amine to react as nucleophile, as discussed before. The easier availability of the amine for coupling reactions was contrary to the enzyme activity, so that the amount of product was decreasing again at higher values. The C-C coupling products showed a different behavior, due to the competing reaction. However, the amount was much lower than the oxidized C-N coupling product and pH 5 exhibited the best conditions for this C-N coupling reaction.

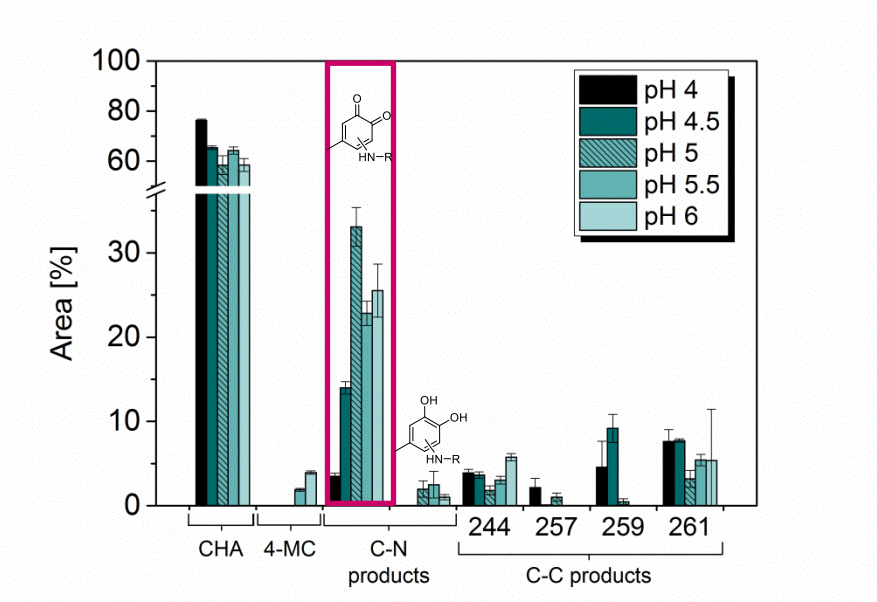


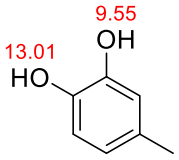
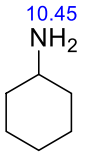
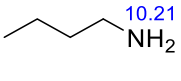
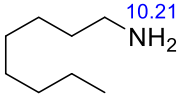
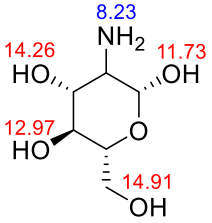
Figure 5-28. Product ratios of the laccase initiated coupling reactions of 4-methylcatechol (m/z 123) and cyclohexylamine (m/z 100) at different pH values. Cond.: Sodium acetic acid buffer pH 4-6, Q = 0.03 mL/min, c_{CHA} = 5 mM, $c_{4\text{-}MC}$ = 5 mM, $A_{laccase}$ = 5 U/mL.

A comparable effect was found by Illner *et al.*, who used a laccase from *Myceliophthora thermophila* for the coupling of 3-methylcatechol and *n*-hexylamine reaching higher concentrations of the desired product with higher pH values. [Illner *et al.*, 2014] Also Wellington *et al.* chose a higher pH value for C-C coupling reactions to enable the deprotonation of the reactant. [Wellington *et al.*, 2013] In contrast, in other studies often a pH value was used which exhibited the highest enzyme activity, e.g. pH 5 or 4.5. [Wellington *et al.*, 2012; Herter *et al.*, 2013] However, all these reactions were carried out in batch systems and a fed-batch could be a possibility to increase the selectivity of all these already investigated laccase initiated coupling reactions.

5.2.3. Coupling reactions with other amines

After the successful optimization of the laccase initiated coupling reaction of 4-MC with cyclohexylamine other amines should be investigated with regard to the biocatalytic synthesis of interesting building blocks. Beside the already investigated cyclohexylamine, *n*-butylamine and *n*-octylamine as additional hydrophobic coupling agents were taken into account. As further highly interesting compound D-glucosamine was chosen for the investigation of the coupling behavior. In contrast to the other reagents, this amine was highly hydrophilic and offered several functional groups, which could enable subsequent reactions after the laccase initiated coupling reaction with a catecholic compound. This could result in the formation of different active pharmaceutical ingredients. In table 5-8 the chosen amines are shown with selected properties, which might influence the C-N coupling reaction.

Table 5-8. Investigated amines for C-N coupling reactions.

Structure (incl. pK_a values[1])					
Name	4-Methylcatechol	Cyclohexylamine	*n*-Butylamine	*n*-Octylamine	D-Glucosamine
logP[a]	1.88	1.17	0.70	2.48	-3.04
Solubility[a]	-1.06	-0.97	-0.31	-2.48	0.25 (-0.39[2])

[1]Predicted by Chemicalize.com [Chemicalize, 2018], [2]Solubility for glucosamine hydrochloride.

All amines were investigated under optimized conditions and compared in figure 5-29. For all amines the complete conversion of the substrate 4-MC could be observed. This showed that the laccase was not influenced by the different amines. In contrast, the coupling of 4-MC with the amines showed big differences (figure 5-31). The ratios of cyclohexylamine and octylamine to the other compounds in the reaction mixture were still high after the reaction, which indicated a low reaction rate with these coupling agents. In contrast, butylamine and D-glucosamine could not be found in the solution assuming high coupling rates with these compounds. The reason for the low coupling rates with octylamine could be the poor

solubility accompany with a high logP value. This could result in a low availability for the coupling with 4-MC and almost no product formation. The observation for cyclohexylamine with a logP of 1.17 and higher solubility could also describe the lower amine to product value. With regard to the conversion of 4-MC there should be at least C-C coupling products, even if no C-N coupling products were formed. A possible reason for the low detection of the products could be the analytical method itself. Based on the assumption of the C-N coupling reaction with the hydrophobic amine, the formed C-N coupling product would show also a high hydrophobicity resulting in a bad solubility and therefore the LC/MS would eventually not be able to detect these compounds. A possible method to detect these compounds would be an extraction and measurement with e.g. gas chromatography or if these are stable species analyzing with nuclear magnetic resonance would be a possibility.

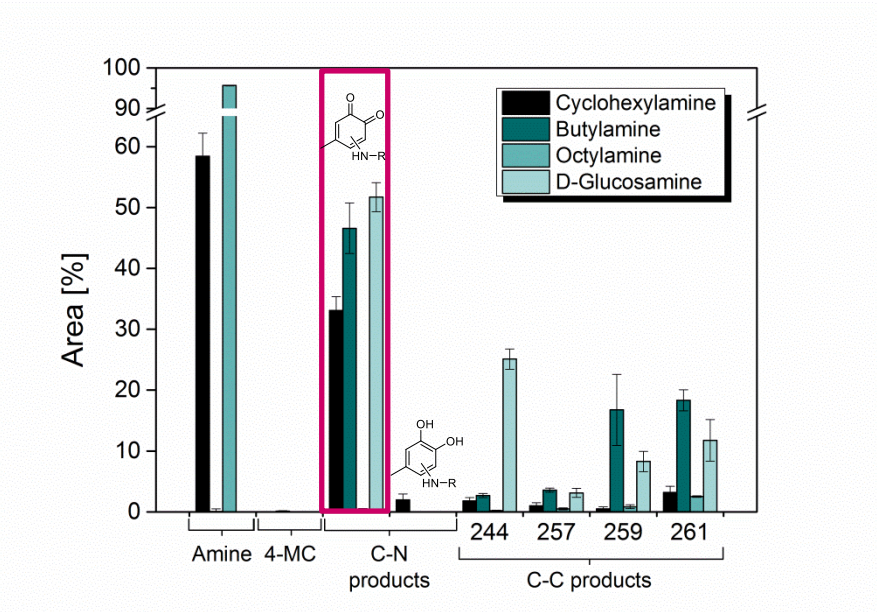


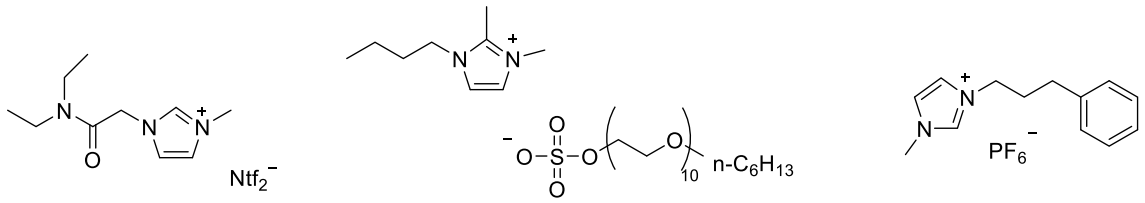
Figure 5-29. Comparison of the product ratios of chosen amines as reagents for C-N coupling reactions. Cond.: Sodium acetic acid buffer pH 5, Q = 0.03 mL/min, c_{Amine} =5 mM, $c_{4\text{-}MC}$ = 5 mM, $A_{laccase}$ = 5 U/mL.

Nevertheless, for the other amines the highest ratio of the products was found for the oxidized C-N coupling product. Thereby the ratios were increasing with decreasing of the logP value of the amines, which showed the high influence of the hydrophobicity and in addition, the availability of the amine during the C-N coupling reaction. Only for cyclohexylamine also the hydroxyl form of the C-N coupling product was found, which could be based on the low solubility on the C-N coupling product. The hydroxyl form was consequently less available for the laccase and therefore it was slower re-oxidized by the laccase in comparison to the products of butylamine and D-glucosamine. Another reason

could be that this intermediate was more stable than the other intermediates or it was no suitable substrate for the laccase. For cyclohexylamine as coupling agent the main product was the oxidized form of the C-N coupling product, comparable to D-glucose- and butylamine as reagents. However, for this compound no complete conversion was found, but in addition lower amounts of all products could be detected. With D-glucosamine the highest ratio of the C-N coupling product occurred, which was on the one hand based on the hydrophilicity and a better availability. On the other hand the pK_a values were very important for the coupling reactions and D-glucosamine contained five functional groups, which were all possible coupling positions. The other amines exhibited only the amine group as possible coupling position and therefore less possible products. However, the amine group showed the lowest pK_a value of the functional groups in this molecule. This resulted in an easier deprotonation on the amine position than for the hydroxyl groups obtaining a negative loading of the nitrogen atom. The negative loaded atom was able to react with the oxidized 4-MC, which exhibited in the radical form a partial positive loading at the radical position. Due to the similarity in their structure the other amines had pKa values in the same range (cf. table 5-8). Although the pKa value had an influence in the reaction behavior, the investigated reactions were especially dependent on the logP values and therefore the solubility of the compounds. In comparison to the other amines, the coupling reaction with D-glucosamine enabled the highest amount of C-N products and also the best properties to realize highly interesting building blocks. Unfortunately the isolation of the obtained C-N coupling products led to several problems. The purification was possible by solid phase extraction, however, the products were highly unstable and no further isolation could be realized. This would be a highly interesting topic for further investigations of C-N coupling reactions. Therefore the lyophilization or a subsequent reaction in the aqueous phase would be conceivable approaches to obtain interesting products.

6. Conclusion and outlook

Spray congealing is highly interesting for enzyme immobilization, however, the heat sensitivity of the enzymes is still challenging. Therefore carriers with comparable low melting points have to be found and at the same time they have to be stable against e.g. abrasion. Especially the good retained activities with MOAT showed the promising usage of ionic liquids as carriers. Therefore possible ionic liquids have to be synthesized and evaluated, whereby interesting compounds are already described in literature, some of them are shown in figure 6-1. [Gathergood *et al.*, 2004] In addition, the comparable high residual activities found with this compound could be further increased with additives, already investigated with other carriers.



3-Methyl-1-(N,N-diethyl carbamoylmethyl)imidazolium
mp.: 43-45°C

1-(3´-Phenylpropyl)-3-methylimidazolium PF_6
mp.: 53°C

1-Butyl-2,3-dimethylimidazolium poly(oxyethylene) alkylsulfate
mp.: 35-37°C

Figure 6-1. Further possible ionic liquids as carriers for enzyme immobilization *via* spray congealing. [Gathergood *et al.*, 2004]

Another possibility to increase residual activities would be the modification of the set-up and to keep the heat influence as low as possible. Therefore a three fluid-nozzle, which is also provided by e.g. Büchi, could be used. This nozzle allows the separation of the already heated and melted carrier solution and the enzyme solution avoiding a comparable long heat influence on the enzyme. With an additional shortening of the nozzle length, as well as a cooling immediately after spraying the residual activity could be increased significantly.

During this study, the application of laccases as green catalyst for C-N coupling reactions could be realized with an increased selectivity and also the coupling with the interesting coupling agent D-glucosamine. However, the isolation of the products was not possible. The separation of the different products could be performed easily by chromatography; nevertheless, a lyophilization of the sensitive product would be necessary. After product

isolation the exact structure, especially the coupling position, could further be analyzed by e.g. 2D-NMR. Based on this knowledge and a deeper insight in the reaction mechanism of the coupling, the selectivity might be improved further with an optimization of the reaction conditions. Subsequent further coupling reactions would be possible, either bio- or chemo-catalytically and new interesting APIs could be synthesized.

The investigation of these promising biocatalytic cross-coupling reactions brings the research and also todays industry a big step closer to transform conventional chemical reactions to biocatalytic green alternatives.

7. Summary

This study evaluated the spray congealing as a new promising technique for the immobilization of biocatalysts and investigated three different enzymes for the characterization and efficiency of this method. In addition, laccase initiated coupling reactions were investigated with the usage of different reactor types and further improved with regard to a high selectivity.

For the immobilization two different set-ups were investigated in detail: a modified commercially available spray dryer and a self-developed spray congealing system. The main differences influencing the particles were the nozzles, whereby the self-developed system exhibited a shorter one with a wider orifice opening. Various carriers were evaluated for enzyme immobilization, whereby cetyl alcohol showed promising properties. Further carrier evaluation and addition of non-foam forming emulsifiers of up to 20% resulted in an increasing activity yield and an improved retained activity during consecutive reactions. The investigated ionic liquid MOAT showed a great retained activity of over 30% after five batch reactions in comparison to common carriers exhibiting a great potential for this technique despite the high viscosity. The results of the particles are summarized in table 7-1.

Table 7-1. Comparison of the produced particles.

	Modified spray dryer	**Spray congealing apparatus**
Yield	40 – 66%	70 – 80%
Particle size	40 – 45 µm	150 - 255 µm
Activity yield	50%	24%
Retained activity after 1st batch	32%	16%

Despite a higher spraying yield, as well as a greater particle size, the spray congealing apparatus showed lower activities and also consecutive reactions in a plug-flow reactor resulted in lower retained activities. Due to the heat influence on the biocatalyst before and during the atomization process this immobilization technique is only suitable for heat sensitive enzymes if a low melting carrier is used, which should exhibit a great stability.

The immobilized laccase was afterwards tested for C-N coupling reactions. Firstly, various substrates were investigated and 4-methylcatachol was chosen for coupling investigations with cyclohexylamine. For this laccase initiated radical reaction different homo- and hetero-coupling products could be found partially or completely oxidized by the enzyme, as well as further products formed by e.g. the stabilization through water molecules. The usage of a batch and PFR reactor led to a low selectivity of the immobilized enzyme, so that a fed-batch reactor was evaluated to improve the reaction. With a pumping rate of 0.03 mL/min and pH 5 the selectivity could be increased significantly and the oxidized form of the C-N coupling product was obtained as main product. Under these optimized conditions three further amines were tested for the coupling with 4-methylcatechol. Thereby it became obvious that the solubility of the coupling reagent was mainly influencing the formation of C-N coupling products. D-glucosamine with its low logP value showed the best selectivity and was furthermore the most interesting coupling reagent, due to the exhibition of several functional groups enabling further possible coupling reactions.

In conclusion, it could be shown that the immobilization *via* spray congealing was a promising technique for enzymes especially with the ionic liquid MOAT. Even if the immobilized laccase was not the optimal choice for the application in C-N coupling reactions, also these reactions could be improved with the optimal reactor choice and reaction conditions, so that the selectivity could be increased significantly.

8. Appendix

8.1. References

ABURAHMA, M. H., BADR-ELDIN, S. M.: Compritol 888 ATO: a multifunctional lipid excipient in drug delivery systems and nanopharmaceuticals. *Expert Opinion on Drug Delivery,* 11**,** 12**, 2014**, 1865-1883.

ADELAKUN, O. E., KUDANGA, T., GREEN, I. R., LE ROES-HILL, M., BURTON, S. G.: Enzymatic modification of 2,6-dimethoxyphenol for the synthesis of dimers with high antioxidant capacity. *Process Biochemistry,* 47**,** 12**, 2012**, 1926-1932.

ALBERTINI, B., PASSERINI, N., DI SABATINO, M., VITALI, B., BRIGIDI, P., RODRIGUEZ, L.: Polymer–lipid based mucoadhesive microspheres prepared by spray-congealing for the vaginal delivery of econazole nitrate. *European Journal of Pharmaceutical Sciences,* 36**,** 4–5**, 2009**, 591-601.

ALBERTINI, B., PASSERINI, N., PATTARINO, F., RODRIGUEZ, L.: New spray congealing atomizer for the microencapsulation of highly concentrated solid and liquid substances. *European Journal of Pharmaceutics and Biopharmaceutics,* 69**,** 1**, 2008**, 348-357.

ALCALDE, M., FERRER, M., PLOU, F. J., BALLESTEROS, A.: Environmental biocatalysis: from remediation with enzymes to novel green processes. *Trends in Biotechnology,* 24, 6, **2006,** 281-287.

ANASTAS, P. T., WARNER, J. C.: Principles of green chemistry. *Green chemistry: Theory and practice***, 1998**, 29-56.

AUGUSTINE, A. J., KJAERGAARD, C., QAYYUM, M., ZIEGLER, L., KOSMAN, D. J., HODGSON, K. O., HEDMAN, B., SOLOMON, E. I.: Systematic Perturbation of the Trinuclear Copper Cluster in the Multicopper Oxidases: The Role of Active Site Asymmetry in Its Reduction of O2 to H2O. *Journal of the American Chemical Society,* 132**,** 17**, 2010**, 6057-6067.

BAMPI, G. B., BACKES, G. T., CANSIAN, R. R. L., DE MATOS, F. E. Q., ANSOLIN, I. M. A., POLETO, B. C., COREZZOLLA, L. R., FAVARO-TRINDADE, C. S.: Spray Chilling Microencapsulation of Lactobacillus acidophilus and Bifidobacterium animalis subsp. lactis and Its Use in the Preparation of Savory Probiotic Cereal Bars. *Food and Bioprocess Technology,* 9**,** 8**, 2016**, 1422-1428.

BARIWAL, J., VAN DER EYCKEN, E.: C-N bond forming cross-coupling reactions: an overview. *Chemical Society Reviews,* 42**,** 24**, 2013**, 9283-9303.

BECKER, K., SALAR-BEHZADI, S., ZIMMER, A.: Solvent-Free Melting Techniques for the Preparation of Lipid-Based Solid Oral Formulations. *Pharmaceutical Research,* 32**,** 5**, 2015**, 1519-1545.

BERKA, R. M., SCHNEIDER, P., GOLIGHTLY, E. J., BROWN, S. H., MADDEN, M., BROWN, K. M., HALKIER, T., MONDORF, K., XU, F.: Characterization of the gene encoding an extracellular laccase of Myceliophthora thermophila and analysis of the recombinant enzyme expressed in Aspergillus oryzae. *Applied and Environmental Microbiology,* 63**,** 8**, 1997**, 3151-3157.

BISSWANGER, H.: *Practical enzymology.* John Wiley & Sons. **2013**.

BLASER, H.-U., INDOLESE, A., NAUD, F., NETTEKOVEN, U., SCHNYDER, A.: Industrial R&D on Catalytic C-C and C-N Coupling Reactions: A Personal Account on Goals, Approaches and Results. *Advanced Synthesis & Catalysis,* 346**,** 13-15**, 2004**, 1583-1598.

BORNSCHEUER, U. T.: Microbial carboxyl esterases: classification, properties and application in biocatalysis. *FEMS Microbiology Reviews,* 26**,** 1**, 2002**, 73-81.

BORNSCHEUER, U. T., HUISMAN, G. W., KAZLAUSKAS, R. J., LUTZ, S., MOORE, J. C., ROBINS, K.: Engineering the third wave of biocatalysis. *Nature,* 485**,** 7397**, 2012**, 185-194.

BORNSCHEUER, U. T., KAZLAUSKAS, R. J., BRAKMANN, S., JOHNSSON, K.: Hydrolases in Organic Synthesis. **1999**.

BÖTTCHER, D., BRÜSEHABER, E., DODERER, K., BORNSCHEUER, U. T.: Functional expression of the γ-isoenzyme of pig liver carboxyl esterase in Escherichia coli. *Applied Microbiology and Biotechnology,* 73**,** 6**, 2007**, 1282-1289.

BOURBONNAIS, R., PAICE, M. G.: Oxidation of non-phenolic substrates: An expanded role for laccase in lignin biodegradation. *FEBS Letters,* 267**,** 1**, 1990**, 99-102.

BOŽIČ, M., ŠTRANCAR, J., KOKOL, V.: Laccase-initiated reaction between phenolic acids and chitosan. *Reactive and Functional Polymers,* 73**,** 10**, 2013**, 1377-1383.

BRANCO, R. J., GRABER, M., DENIS, V., PLEISS, J.: Molecular mechanism of the hydration of Candida antarctica lipase B in the gas phase: Water adsorption isotherms and molecular dynamics simulations. *ChemBioChem,* 10**,** 18**, 2009**, 2913-2919.

BRENA, B. M., BATISTA-VIERA, F.: Immobilization of enzymes. In *Immobilization of enzymes and cells.* **2006**, pp. 15-30.

BUCHHOLZ, K., KASCHE, V., BORNSCHEUER, U. T.: *Biocatalysts and enzyme technology.* John Wiley & Sons. **2012**.

CANNATELLI, M., RAGAUSKAS, A.: Laccase-Catalyzed CC Bond Forming Reactions. *Organic Chemistry: Current Research,* 2**,** 3**, 2013**, 1.

CAO, L.: Immobilised enzymes: science or art? *Current Opinion in Chemical Biology,* 9**,** 2**, 2005**, 217-226.

CAO, L.: *Carrier-bound immobilized enzymes: principles, application and design.* John Wiley & Sons. **2006**.

CAO, L., VAN RANTWIJK, F., SHELDON, R. A.: Cross-Linked Enzyme Aggregates: A Simple and Effective Method for the Immobilization of Penicillin Acylase. *Organic Letters,* 2**,** 10**, 2000**, 1361-1364.

CHEMICALIZE: Chemicalize. **2018**, vol. 2017-2018.

CHEN, Y., BLACK, D. S., REILLY, P. J.: Carboxylic ester hydrolases: Classification and database derived from their primary, secondary, and tertiary structures. *Protein Science,* 25**,** 11**, 2016**, 1942-1953.

CHMIEL, H.: *Bioprozesstechnik.* Springer-Verlag. **2011**.

CHOI, J.-M., HAN, S.-S., KIM, H.-S.: Industrial applications of enzyme biocatalysis: current status and future aspects. *Biotechnology advances,* 33**,** 7**, 2015**, 1443-1454.

CHRANIOTI, C., CHANIOTI, S., TZIA, C.: Comparison of spray, freeze and oven drying as a means of reducing bitter aftertaste of steviol glycosides (derived from Stevia rebaudiana Bertoni plant)–Evaluation of the final products. *Food chemistry,* 190**, 2016**, 1151-1158.

CLARK, D. S.: Can immobilization be exploited to modify enzyme activity? *Trends in Biotechnology,* 12**,** 11**, 1994**, 439-443.

CLAUS, H.: Laccases: structure, reactions, distribution. *Micron,* 35**,** 1–2**, 2004**, 93-96.

CONSOLI, L., GRIMALDI, R., SARTORI, T., MENEGALLI, F. C., HUBINGER, M. R. D.: Gallic acid microparticles produced by spray chilling technique: Production and characterization. *LWT - Food Science and Technology,* 65**, 2016**, 79-87.

CONSTANTIN, M.-A.: Selektive und effiziente Laccase-katalysierte oxidative Phenolkupplungen. *Dissertation***, 2012**

CONSTANTIN, M.-A., CONRAD, J., BEIFUSS, U.: An unprecedented oxidative trimerization of sesamol catalyzed by laccases. *Tetrahedron Letters,* 53**,** 26**, 2012a**, 3254-3258.

CONSTANTIN, M.-A., CONRAD, J., MERISOR, E., KOSCHORRECK, K., URLACHER, V. B., BEIFUSS, U.: Oxidative Dimerization of (E)- and (Z)-2-Propenylsesamol with O2 in the Presence and Absence of Laccases and Other Catalysts: Selective Formation of Carpanones and Benzopyrans under Different Reaction Conditions. *The Journal of Organic Chemistry,* 77**,** 10**, 2013**, 4528-4543.

CONSTANTIN, M.-A., CONRAD, J., MERIŞOR, E., KOSCHORRECK, K., URLACHER, V. B., BEIFUSS, U.: Oxidative Dimerization of (E)- and (Z)-2-Propenylsesamol with O2 in the Presence and Absence of Laccases and Other Catalysts: Selective Formation of Carpanones and Benzopyrans under Different Reaction Conditions. *The Journal of Organic Chemistry,* 77**,** 10**, 2012b**, 4528-4543.

CUNHA, A. G., FERNÁNDEZ-LORENTE, G., BEVILAQUA, J. V., DESTAIN, J., PAIVA, L. M. C., FREIRE, D. M. G., FERNÁNDEZ-LAFUENTE, R., GUISÁN, J. M.: Immobilization of Yarrowia lipolytica Lipase—a Comparison of Stability of Physical Adsorption and Covalent Attachment Techniques. *Applied Biochemistry and Biotechnology,* 146**,** 1**, 2008**, 49-56.

DAHILI, L. A., KELEMEN-HORVÁTH, I., FECZKÓ, T.: 2, 4-Dichlorophenol removal by purified horseradish peroxidase enzyme and crude extract from horseradish immobilized to nano spray dried ethyl cellulose particles. *Process Biochemistry,* 50**,** 11**, 2015**, 1835-1842.

DAKIN, H.: The hydrolysis of optically inactive esters by means of enzymes: Part I. The action of lipase upon esters of mandelic acid. The resolution of inactive mandelic acid. *The Journal of physiology,* 30**,** 3-4**, 1903**, 253.

DAKIN, H.: The fractional hydrolysis of optically inactive esters by lipase: Part II. *The Journal of physiology,* 32**,** 3-4**, 1905**, 199.

DANIEL, R. M., DINES, M., PETACH, H. H.: The denaturation and degradation of stable enzymes at high temperatures. *Biochemical Journal,* 317**,** 1**, 1996**, 1-11.

DATE, A. A., VADOR, N., JAGTAP, A., NAGARSENKER, M. S.: Lipid nanocarriers (GeluPearl) containing amphiphilic lipid Gelucire 50/13 as a novel stabilizer: fabrication, characterization and evaluation for oral drug delivery. *Nanotechnology,* 22**,** 27**, 2011**, 275102.

DATTA, S., CHRISTENA, L. R., RAJARAM, Y. R. S.: Enzyme immobilization: an overview on techniques and support materials. *3 Biotech,* 3**,** 1**, 2013**, 1-9.

DAVIN, L. B., WANG, H.-B., CROWELL, A. L., BEDGAR, D. L., MARTIN, D. M., SARKANEN, S., LEWIS, N. G.: Stereoselective Bimolecular Phenoxy Radical Coupling by an Auxiliary (Dirigent) Protein Without an Active Center. *Science,* 275**,** 5298**, 1997**, 362-367.

DE MARIA, P. D., GARCIA-BURGOS, C. A., BARGEMAN, G., VAN GEMERT, R. W.: Pig liver esterase (PLE) as biocatalyst in organic synthesis: From nature to cloning and to practical applications. *Synthesis,* 2007**, 2007**, 1439-1452.

DE MARÍA, P. D., KOSSMANN, B., POTGRAVE, N., BUCHHOLZ, S., TRAUTHWEIN, H., MAY, O., GRÖGER, H.: Improved process for the enantioselective hydrolysis of prochiral diethyl malonates catalyzed by pig liver esterase. *Synlett,* 2005**,** 11**, 2005**, 1746-1748.

DEVELOPMENT, O. F. E. C.-O.: *The application of biotechnology to industrial sustainability.* OECD Publishing. **2001**.

DI SABATINO, M., ALBERTINI, B., KETT, V. L., PASSERINI, N.: Spray congealed lipid microparticles with high protein loading: Preparation and solid state characterisation. *European Journal of Pharmaceutical Sciences,* 46**,** 5**, 2012**, 346-356.

DICOSIMO, R., MCAULIFFE, J., POULOSE, A. J., BOHLMANN, G.: Industrial use of immobilized enzymes. *Chemical Society Reviews,* 42**,** 15**, 2013**, 6437-6474.

DIXIT, M., KULKARNI, P., ASHWINI, G. K., SHIVAKUMAR, H.: Spray drying: A crystallization technique: A review. *International Journal,* 1**,** 2**, 2011**

DRAUZ, K.: *Enzyme catalysis in organic synthesis: a comprehensive handbook.* John Wiley & Sons. 2012.

DUNN, P. J.: The importance of Green Chemistry in Process Research and Development. *Chemical Society Reviews,* 41**,** 4**, 2012**, 1452-1461.

DWEVEDI, A.: Basics of Enzyme Immobilization. In *Enzyme Immobilization.* **2016**, pp. 21-44.

EICKHOFF, H., JUNG, G., RIEKER, A.: Oxidative phenol coupling—tyrosine dimers and libraries containing tyrosyl peptide dimers. *Tetrahedron,* 57**,** 2**, 2001**, 353-364.

EIGTVED, P.: Method for immobilizing lipase **1987**, vol. WO8902916 (A1).

EL-BADRY, M., FETIH, G., FATHY, M.: Improvement of solubility and dissolution rate of indomethacin by solid dispersions in Gelucire 50/13 and PEG4000. *Saudi Pharmaceutical Journal,* 17**,** 3**, 2009**, 217-225.

ENAYATI, M., CHANG, M.-W., BRAGMAN, F., EDIRISINGHE, M., STRIDE, E.: Electrohydrodynamic preparation of particles, capsules and bubbles for biomedical engineering applications. *Colloids and Surfaces A: Physicochemical and Engineering Aspects,* 382**,** 1-3**, 2011**, 154-164.

ENGELMANN, C., ILLNER, S., KRAGL, U.: Laccase initiated CC couplings: Various techniques for reaction monitoring. *Process Biochemistry,* 50**,** 10**, 2015**, 1591-1599.

ENGUITA, F. J., MARÇAL, D., MARTINS, L. O., GRENHA, R., HENRIQUES, A. O., LINDLEY, P. F., CARRONDO, M. A.: Substrate and dioxygen binding to the endospore coat laccase from Bacillus subtilis. *Journal of Biological Chemistry,* 279**,** 22**, 2004**, 23472-23476.

EŞ, I., VIEIRA, J. D. G., AMARAL, A. C.: Principles, techniques, and applications of biocatalyst immobilization for industrial application. *Applied Microbiology and Biotechnology,* 99**,** 5**, 2015**, 2065-2082.

FABER, K.: *Biotransformations in organic chemistry.* Springer. **1992**.

FAVARO-TRINDADE, C. S., OKURO, P. K., DE MATOS JR, F. E.: Encapsulation via Spray Chilling/Cooling/Congealing. *Handbook of encapsulation and controlled release***, 2015**, 71.

FORESTI, M., PEDERNERA, M., BUCALÁ, V., FERREIRA, M.: Multiple effects of water on solvent-free enzymatic esterifications. *Enzyme and Microbial Technology,* 41**,** 1-2**, 2007**, 62-70.

GARCIA-GALAN, C., BERENGUER-MURCIA, Á., FERNANDEZ-LAFUENTE, R., RODRIGUES, R. C.: Potential of different enzyme immobilization strategies to improve enzyme performance. *Advanced Synthesis & Catalysis,* 353**,** 16**, 2011**, 2885-2904.

GATHERGOOD, N., GARCIA, M. T., SCAMMELLS, P. J.: Biodegradable ionic liquids: Part I. Concept, preliminary targets and evaluation. *Green Chemistry,* 6**,** 3**, 2004**, 166-175.

GAVORY, C., ABDERRAHMEN, R., BORDES, C., CHAUSSY, D., BELGACEM, M. N., FESSI, H., BRIANCON, S.: Encapsulation of a pressure sensitive adhesive by spray-cooling: optimum formulation and processing conditions. *Advanced Powder Technology,* 25**,** 1**, 2014**, 292-300.

GONG, J.-S., SHI, J.-S., LU, Z.-M., LI, H., ZHOU, Z.-M., XU, Z.-H.: Nitrile-converting enzymes as a tool to improve biocatalysis in organic synthesis: recent insights and promises. *Critical Reviews in Biotechnology,* 37**,** 1**, 2017**, 69-81.

GONZÁLEZ SISO, M. I., LANG, E., CARRENÕ-GÓMEZ, B., BECERRA, M., OTERO ESPINAR, F., BLANCO MÉNDEZ, J.: Enzyme encapsulation on chitosan microbeads. *Process Biochemistry,* 32**,** 3**, 1997**, 211-216.

GRIFFIN, W. C.: Classification of surface-active agents by "HLB". *Journal of Cosmetic Science* 1**,** 5**, 1949**, 311-326.

HÄDENER, A., KAUFMANN, H.: *Grundlagen der organischen Chemie.* Springer-Verlag. **2006**.

HAMDANI, J., MOËS, A. J., AMIGHI, K.: Physical and thermal characterisation of Precirol® and Compritol® as lipophilic glycerides used for the preparation of controlled-release matrix pellets. *International Journal of Pharmaceutics,* 260**,** 1**, 2003**, 47-57.

HARDACRE, C., HOLBREY, J. D., MCCORMAC, P. B., MCMATH, S. E. J., NIEUWENHUYZEN, M., SEDDON, K. R.: Crystal and liquid crystalline polymorphism in 1-alkyl-3-methylimidazolium tetrachloropalladate(ii) salts. *Journal of Materials Chemistry,* 11**,** 2**, 2001**, 346-350.

HERTER, S., MICHALIK, D., MIKOLASCH, A., SCHMIDT, M., WOHLGEMUTH, R., BORNSCHEUER, U., SCHAUER, F.: Laccase-mediated synthesis of 2-methoxy-3-methyl-5-(alkylamino)- and 3-methyl-2,5-bis(alkylamino)-[1,4]-benzoquinones. *Journal of Molecular Catalysis B: Enzymatic,* 90**,** 0**, 2013**, 91-97.

HERTER, S., MIKOLASCH, A., MICHALIK, D., HAMMER, E., SCHAUER, F., BORNSCHEUER, U., SCHMIDT, M.: C–N coupling of 3-methylcatechol with primary amines using native and recombinant laccases from Trametes versicolor and Pycnoporus cinnabarinus. *Tetrahedron,* 67**,** 48**, 2011**, 9311-9321.

HINZE, J., SÜSS, P., STROHMAIER, S., BORNSCHEUER, U. T., WARDENGA, R., VON LANGERMANN, J.: Recombinant Pig Liver Esterase-Catalyzed Synthesis of (1 S, 4 R)-4-Hydroxy-2-cyclopentenyl Acetate Combined with Subsequent Enantioselective Crystallization. *Organic Process Research & Development,* 20**,** 7**, 2016**, 1258-1264.

HOLBREY, J. D., REICHERT, W. M., NIEUWENHUYZEN, M., JOHNSON, S., SEDDON, K. R., ROGERS, R. D.: Crystal polymorphism in 1-butyl-3-methylimidazolium halides: supporting ionic liquid formation by inhibition of crystallization. *Chemical Communications***,** 14**, 2003**, 1636-1637.

HOMAEI, A. A., SARIRI, R., VIANELLO, F., STEVANATO, R.: Enzyme immobilization: an update. *Journal of chemical biology,* 6**,** 4**, 2013**, 185-205.

HUANG, F.-C., LEE, L. H., MITTAL, R., RAVIKUMAR, P., CHAN, J. A., SIH, C. J., CASPI, E., ECK, C. R.: Preparation of (R)-and (S)-mevalonic acids. *Journal of the American Chemical Society,* 97**,** 14**, 1975**, 4144-4145.

ILIĆ, I., DREU, R., BURJAK, M., HOMAR, M., KERČ, J., SRČIČ, S.: Microparticle size control and glimepiride microencapsulation using spray congealing technology. *International journal of pharmaceutics,* 381**,** 2**, 2009**, 176-183.

ILLNER, S., PLAGEMANN, R., SALING, P., KRAGL, U.: Eco-efficiency analysis as a reaction-engineering tool - Case study of a laccase-initiated oxidative C-N coupling. *Journal of Molecular Catalysis B: Enzymatic,* 102**,** 0**, 2014**, 106-114.

JIANG, L., BUCHWALD, S. L.: Palladium-Catalyzed Aromatic Carbon-Nitrogen Bond Formation. *Metal-Catalyzed Cross-Coupling Reactions, Second Edition***, 2004**, 699-760.

JOHNSON, L. M., FAIRBANKS, B. D., ANSETH, K. S., BOWMAN, C. N.: Enzyme-mediated redox initiation for hydrogel generation and cellular encapsulation. *Biomacromolecules,* 10**,** 11**, 2009**, 3114-3121.

JONES, S. M., SOLOMON, E. I.: Electron transfer and reaction mechanism of laccases. *Cellular and Molecular Life Sciences,* 72**,** 5**, 2015**, 869-883.

JOSHI, M. D., ANDERSON, J. L.: Recent advances of ionic liquids in separation science and mass spectrometry. *RSC Advances,* 2**,** 13**, 2012**, 5470-5484.

KARATAS, A., YÜKSEL, N., BAYKARA, T.: Improved solubility and dissolution rate of piroxicam using gelucire 44/14 and labrasol. *Il Farmaco,* 60**,** 9**, 2005**, 777-782.

KÄRNÄ, M., LAHTINEN, M., VALKONEN, J.: Preparation and characterization of new low melting ammonium-based ionic liquids with ether functionality. *Journal of Molecular Structure,* 922**,** 1–3**, 2009**, 64-76.

KAŠPAR, O., TOKÁROVÁ, V., NYANHONGO, G. S., GÜBITZ, G., ŠTĚPÁNEK, F.: Effect of cross-linking method on the activity of spray-dried chitosan microparticles with immobilized laccase. *Food and bioproducts processing,* 91**,** 4**, 2013**, 525-533.

KATCHALSKI-KATZIR, E.: Immobilized enzymes—learning from past successes and failures. *Trends in biotechnology,* 11**,** 11**, 1993**, 471-478.

KIM, S., REQUEJO, K. I., NAKAMATSU, J., GONZALES, K. N., TORRES, F. G., CAVACO-PAULO, A.: Modulating antioxidant activity and the controlled release capability of laccase mediated catechin grafting of chitosan. *Process Biochemistry***,**

KRAJEWSKA, B.: Application of chitin- and chitosan-based materials for enzyme immobilizations: a review. *Enzyme and Microbial Technology,* 35**,** 2–3**, 2004**, 126-139.

KRONBERG, B., HOLMBERG, K., LINDMAN, B.: *Surface chemistry of surfactants and polymers.* John Wiley & Sons. **2014**.

KUDANGA, T., NEMADZIVA, B., LE ROES-HILL, M.: Laccase catalysis for the synthesis of bioactive compounds. *Applied Microbiology and Biotechnology,* 101**,** 1**, 2017**, 13-33.

KUDANGA, T., NYANHONGO, G. S., GUEBITZ, G. M., BURTON, S.: Potential applications of laccase-mediated coupling and grafting reactions: A review. *Enzyme and Microbial Technology,* 48**,** 3**, 2011**, 195-208.

KUNAMNENI, A., CAMARERO, S., GARCÍA-BURGOS, C., PLOU, F. J., BALLESTEROS, A., ALCALDE, M.: Engineering and applications of fungal laccases for organic synthesis. *Microbial cell factories,* 7**,** 1**, 2008**, 32.

LEE, S. Y., NIELSEN, J., STEPHANOPOULOS, G., YOSHIDA, T.: *Applied Bioengineering: Innovations and Future Directions.* John Wiley & Sons. **2017**.

LI, J., FARROKHNIA, M., RULÍŠEK, L., RYDE, U.: Catalytic Cycle of Multicopper Oxidases Studied by Combined Quantum- and Molecular-Mechanical Free-Energy Perturbation Methods. *The Journal of Physical Chemistry B,* 119**,** 26**, 2015**, 8268-8284.

LIESE, A., HILTERHAUS, L.: Evaluation of immobilized enzymes for industrial applications. *Chemical Society Reviews,* 42**,** 15**, 2013**, 6236-6249.

LIESE, A., SEELBACH, K., WANDREY, C.: *Industrial biotransformations.* John Wiley & Sons. **2006**.

LUCKARIFT, H. R., SPAIN, J. C., NAIK, R. R., STONE, M. O.: Enzyme immobilization in a biomimetic silica support. *Nature biotechnology,* 22**,** 2**, 2004**, 211.

MACEDO, J. P., FERNANDES, L. L., FORMIGA, F. B. R., REIS, M. F., JÃºNIOR, T. N., SOARES, L. A., EGITO, E. S. T.: Micro-emultocrit technique: a valuable tool for determination of critical HLB value of emulsions. *Aaps Pharmscitech,* 7**,** 1**, 2006**, E146-E152.

MADHAVI, V., LELE, S. S.: Laccase: Properties and Applications*.* **2009**.

MASCHKE, A., BECKER, C., EYRICH, D., KIERMAIER, J., BLUNK, T., GÖPFERICH, A.: Development of a spray congealing process for the preparation of insulin-loaded lipid microparticles and characterization thereof. *European Journal of Pharmaceutics and Biopharmaceutics,* 65**,** 2**, 2007**, 175-187.

MATEO, C., PALOMO, J. M., FERNANDEZ-LORENTE, G., GUISAN, J. M., FERNANDEZ-LAFUENTE, R.: Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme and Microbial Technology,* 40**,** 6**, 2007**, 1451-1463.

MOGHARABI, M., FARAMARZI, M. A.: Laccase and Laccase-Mediated Systems in the Synthesis of Organic Compounds. *Advanced Synthesis & Catalysis,* 356**,** 5**, 2014**, 897-927.

MOHAMAD, N. R., MARZUKI, N. H. C., BUANG, N. A., HUYOP, F., WAHAB, R. A.: An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. *Biotechnology & Biotechnological Equipment,* 29**,** 2**, 2015**, 205-220.

MONNIER, F., TAILLEFER, M.: Catalytic C-C, C-N, and C-O Ullmann-Type Coupling Reactions: Copper Makes a Difference. *Angewandte Chemie International Edition,* 47**,** 17**, 2008**, 3096-3099.

MOROZOVA, O. V., SHUMAKOVICH, G. P., SHLEEV, S. V., YAROPOLOV, Y. I.: Laccase-mediator systems and their applications: A review. *Applied Biochemistry and Microbiology,* 43**,** 5**, 2007**, 523-535.

MUCHA, M.: Rheological characteristics of semi-dilute chitosan solutions. *Macromolecular Chemistry and Physics,* 198**,** 2**, 1997**, 471-484.

MUCI, A. R., BUCHWALD, S. L.: Practical palladium catalysts for CN and CO bond formation. In *Cross-Coupling Reactions.* **2002**, pp. 131-209.

OH, C. M., GUO, Q., WAN SIA HENG, P., CHAN, L. W.: Spray-congealed microparticles for drug delivery – an overview of factors influencing their production and characteristics. *Expert Opinion on Drug Delivery,* 11**,** 7**, 2014**, 1047-1060.

OKURO, P. K., DE MATOS JUNIOR, F. E., FAVARO-TRINDADE, C. S.: Technological challenges for spray chilling encapsulation of functional food ingredients. *Food Technology and Biotechnology,* 51**,** 2**, 2013**, 171-182.

OLLIS, D. L., CHEAH, E., CYGLER, M., DIJKSTRA, B., FROLOW, F., FRANKEN, S. M., HAREL, M., REMINGTON, S. J., SILMAN, I., SCHRAG, J.: The α/β hydrolase fold. *Protein Engineering, Design and Selection,* 5**,** 3**, 1992**, 197-211.

PALMIERI, G., GIARDINA, P., DESIDERIO, B., MARZULLO, L., GIAMBERINI, M., SANNIA, G.: A new enzyme immobilization procedure using copper alginate gel: Application to a fungal phenol oxidase. *Enzyme and Microbial Technology,* 16**,** 2**, 1994**, 151-158.

PASQUALI, R. C., TAUROZZI, M. P., BREGNI, C.: Some considerations about the hydrophilic–lipophilic balance system. *International Journal of Pharmaceutics,* 356**,** 1–2**, 2008**, 44-51.

PASSERINI, N., ALBERTINI, B., PERISSUTTI, B., RODRIGUEZ, L.: Evaluation of melt granulation and ultrasonic spray congealing as techniques to enhance the dissolution of praziquantel. *International Journal of Pharmaceutics,* 318**,** 1-2**, 2006**, 92-102.

PASSERINI, N., GAVINI, E., ALBERTINI, B., RASSU, G., SABATINO, M., SANNA, V., GIUNCHEDI, P., RODRIGUEZ, L.: Evaluation of solid lipid microparticles produced by spray congealing for topical application of econazole nitrate. *Journal of Pharmacy and Pharmacology,* 61**,** 5**, 2009**, 559-567.

PEDROSO, D. L., DOGENSKI, M., THOMAZINI, M., HEINEMANN, R. J. B., FAVARO-TRINDADE, C. S.: Microencapsulation of Bifidobacterium animalis subsp. lactis and Lactobacillus acidophilus in cocoa butter using spray chilling technology. *Brazilian Journal of Microbiology,* 44**, 2013**, 777-783.

PETKAR, M., LALI, A., CAIMI, P., DAMINATI, M.: Immobilization of lipases for non-aqueous synthesis. *Journal of Molecular Catalysis B: Enzymatic,* 39**,** 1–4**, 2006**, 83-90.

PIONTEK, K., ANTORINI, M., CHOINOWSKI, T.: Crystal structure of a laccase from the fungusTrametes versicolor at 1.90-Å resolution containing a full complement of coppers. *Journal of Biological Chemistry,* 277**,** 40**, 2002**, 37663-37669.

PUROLITE: Application Guide - Lifetech™ ECR Enzyme Immobilization Procedures. **2015**.

QUIDEAU, S., DEFFIEUX, D., DOUAT-CASASSUS, C., POUYSEGU, L.: Plant polyphenols: chemical properties, biological activities, and synthesis. *Angewandte Chemie International Edition,* 50**,** 3**, 2011**, 586-621.

RIEBEL, M., SABEL, A., CLAUS, H., FRONK, P., XIA, N., LI, H., KÖNIG, H., DECKER, H.: Influence of Laccase and Tyrosinase on the Antioxidant Capacity of Selected Phenolic Compounds on Human Cell Lines. *Molecules,* 20**,** 9**, 2015**

ROBINSON, P. K.: Enzymes: principles and biotechnological applications. *Essays In Biochemistry,* 59**, 2015**, 1-41.

ROMANO, D., BONOMI, F., DE MATTOS, M. C., DE SOUSA FONSECA, T., DE OLIVEIRA, M. D. C. F., MOLINARI, F.: Esterases as stereoselective biocatalysts. *Biotechnology Advances,* 33**,** 5**, 2015**, 547-565.

ROSIAUX, Y., JANNIN, V., HUGHES, S., MARCHAUD, D.: Solid lipid excipients - Matrix agents for sustained drug delivery. *Journal of Controlled Release,* 188**, 2014**, 18-30.

ROTHENBERG, G.: Biocatalysis. In *Catalysis.* **2008**, pp. 189-229.

ROZEBOOM, H. J., GODINHO, L. F., NARDINI, M., QUAX, W. J., DIJKSTRA, B. W.: Crystal structures of two Bacillus carboxylesterases with different enantioselectivities. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics,* 1844**,** 3**, 2014**, 567-575.

RULÍŠEK, L., RYDE, U.: Theoretical studies of the active-site structure, spectroscopic and thermodynamic properties, and reaction mechanism of multicopper oxidases. *Coordination Chemistry Reviews,* 257**,** 2**, 2013**, 445-458.

RULÍŠEK, L., SOLOMON, E. I., RYDE, U.: A Combined Quantum and Molecular Mechanical Study of the O2 Reductive Cleavage in the Catalytic Cycle of Multicopper Oxidases. *Inorganic Chemistry,* 44**,** 16**, 2005**, 5612-5628.

SAVOLAINEN, M., KHOO, C., GLAD, H., DAHLQVIST, C., JUPPO, A. M.: Evaluation of controlled-release polar lipid microparticles. *International Journal of Pharmaceutics,* 244**,** 1-2**, 2002**, 151-161.

SCALIA, S., YOUNG, P. M., TRAINI, D.: Solid lipid microparticles as an approach to drug delivery. *Expert opinion on drug delivery,* 12**,** 4**, 2015**, 583-599.

SCHMID, A., DORDICK, J. S., HAUER, B., KIENER, A., WUBBOLTS, M., WITHOLT, B.: Industrial biocatalysis today and tomorrow. *Nature,* 409**,** 6817**, 2001**, 258-268.

SCHMIDT, M., BORNSCHEUER, U. T.: High-throughput assays for lipases and esterases. *Biomolecular Engineering,* 22**,** 1**, 2005**, 51-56.

SCHOMBURG, D.: Brenda - The Comprehensive Enzyme Information System. **2018**.

SHELDON, R. A.: Enzyme Immobilization: The Quest for Optimum Performance. *Advanced Synthesis & Catalysis,* 349**,** 8-9**, 2007**, 1289-1307.

SHELDON, R. A., LAU, R. M., SORGEDRAGER, M. J., VAN RANTWIJK, F., SEDDON, K. R.: Biocatalysis in ionic liquids. *Green Chemistry,* 4**,** 2**, 2002**, 147-151.

SHELDON, R. A., VAN PELT, S.: Enzyme immobilisation in biocatalysis: why, what and how. *Chemical Society Reviews,* 42**,** 15**, 2013**, 6223-6235.

SOHI, H., SULTANA, Y., KHAR, R. K.: Taste Masking Technologies in Oral Pharmaceuticals: Recent Developments and Approaches. *Drug Development and Industrial Pharmacy,* 30**,** 5**, 2004**, 429-448.

SOLOMONS, T. W.: *Organic Chemistry.* John Wiley&Sons. **2011**.

STRAATHOF, A. J. J., PANKE, S., SCHMID, A.: The production of fine chemicals by biotransformations. *Current Opinion in Biotechnology,* 13**,** 6**, 2002**, 548-556.

STRONG, P. J., CLAUS, H.: Laccase: A Review of Its Past and Its Future in Bioremediation. *Critical Reviews in Environmental Science and Technology,* 41**,** 4**, 2011**, 373-434.

SÜSS, P., BORCHERT, S., HINZE, J., ILLNER, S., VON LANGERMANN, J., KRAGL, U., BORNSCHEUER, U. T., WARDENGA, R.: Chemoenzymatic Sequential Multistep One-Pot Reaction for the Synthesis of (1S,2R)-1-(Methoxycarbonyl)cyclohex-4-ene-2-carboxylic Acid with Recombinant Pig Liver Esterase. *Organic Process Research & Development,* 19**,** 12**, 2015**, 2034-2038.

SÜSS, P., ILLNER, S., VON LANGERMANN, J., BORCHERT, S., BORNSCHEUER, U. T., WARDENGA, R., KRAGL, U.: Scale-Up of a Recombinant Pig Liver Esterase-Catalyzed Desymmetrization of Dimethyl Cyclohex-4-ene-cis-1,2-dicarboxylate. *Organic Process Research & Development,* 18**,** 7**, 2014**, 897-903.

TAQIEDDIN, E., AMIJI, M.: Enzyme immobilization in novel alginate–chitosan core-shell microcapsules. *Biomaterials,* 25**,** 10**, 2004**, 1937-1945.

TISCHER, W., WEDEKIND, F.: Immobilized enzymes: methods and applications. In *Biocatalysis-from discovery to application.* **1999**, pp. 95-126.

ULLMANN, F.: Ueber eine neue bildungsweise von diphenylaminderivaten. *European Journal of Inorganic Chemistry,* 36**,** 2**, 1903**, 2382-2384.

VEGA, C., ROOS, Y. H.: Spray-Dried Dairy and Dairy-Like Emulsions - Compositional Considerations. *Journal of Dairy Science,* 89**,** 2**, 2006**, 383-401.

VIPPAGUNTA, S. R., MAUL, K. A., TALLAVAJHALA, S., GRANT, D. J. W.: Solid-state characterization of nifedipine solid dispersions. *International Journal of Pharmaceutics,* 236**,** 1–2**, 2002**, 111-123.

VOLLHARDT, K. P. C., SCHORE, N. E.: *Organische Chemie.* John Wiley & Sons. **2011**.

WANG, J.-Y., YU, H.-R., XIE, R., JU, X.-J., YU, Y.-L., CHU, L.-Y., ZHANG, Z.: Alginate/protamine/silica hybrid capsules with ultrathin membranes for laccase immobilization. *AIChE Journal,* 59**,** 2**, 2013**, 380-389.

WASSERSCHEID, P., WELTON, T.: *Ionic liquids in synthesis.* John Wiley & Sons. **2008**.

WEHRUNG, D., GELDENHUYS, W. J., OYEWUMI, M. O.: Effects of gelucire content on stability, macrophage interaction and blood circulation of nanoparticles engineered from nanoemulsions. *Colloids and Surfaces B: Biointerfaces,* 94**, 2012**, 259-265.

WELLINGTON, K. W., KOLESNIKOVA, N. I.: A laccase-catalysed one-pot synthesis of aminonaphthoquinones and their anticancer activity. *Bioorganic & Medicinal Chemistry,* 20**,** 14**, 2012**, 4472-4481.

WELLINGTON, K. W., QWEBANI-OGUNLEYE, T., KOLESNIKOVA, N. I., BRADY, D., DE KONING, C. B.: One-Pot Laccase-Catalysed Synthesis of 5,6-Dihydroxylated Benzo[b]furans and Catechol Derivatives, and Their Anticancer Activity. *Archiv Der Pharmazie,* 346**,** 4**, 2013**, 266-277.

WENDA, S., ILLNER, S., MELL, A., KRAGL, U.: Industrial biotechnology-the future of green chemistry? *Green Chemistry,* 13**,** 11**, 2011**, 3007-3047.

WHITAKER, J. R., GRANUM, P. E.: An absolute method for protein determination based on difference in absorbance at 235 and 280 nm. *Analytical biochemistry,* 109**,** 1**, 1980**, 156-159.

WHITTALL, J., SUTTON, P. W.: *Practical methods for biocatalysis and biotransformations.* John Wiley & Sons. **2009**.

WINDBERGS, M., STRACHAN, C. J., KLEINEBUDDE, P.: Understanding the solid-state behaviour of triglyceride solid lipid extrudates and its influence on dissolution. *European Journal of Pharmaceutics and Biopharmaceutics,* 71**,** 1**, 2009**, 80-87.

WÖLTINGER, J., BOMMARIUS, A. S., DRAUZ, K., WANDREY, C.: The Chemzyme Membrane Reactor in the Fine Chemicals Industry. *Organic Process Research & Development,* 5**,** 3**, 2001**, 241-248.

WONG, P. C. H., HENG, P. W. S., CHAN, L. W.: Viscosity–temperature relationship of lipid-based excipients amenable for spray congealing: Derivation of a rheological parameter with good correlation to particle size. *European Journal of Lipid Science and Technology***, 2015**, n/a-n/a.

WONG, P. C. H., WAN SIA HENG, P., CHAN, L. W.: A study on the solid state characteristics of spray-congealed glyceryl dibehenate solid lipid microparticles containing ibuprofen. *Drug Development and Industrial Pharmacy,* 42**,** 3**, 2016**, 364-377.

WOODLEY, J. M., BREUER, M., MINK, D.: A future perspective on the role of industrial biotechnology for chemicals production. *Chemical Engineering Research and Design,* 91**,** 10**, 2013**, 2029-2036.

YANG, S.-T., EL-ENSASHY, H., THONGCHUL, N.: *Bioprocessing technologies in biorefinery for sustainable production of fuels, chemicals, and polymers.* John Wiley & Sons. **2013**.

YONEKURA, L., SUN, H., SOUKOULIS, C., FISK, I.: Microencapsulation of Lactobacillus acidophilus NCIMB 701748 in matrices containing soluble fibre by spray drying: Technological characterization, storage stability and survival after in vitro digestion. *Journal of Functional Foods,* 6**, 2014**, 205-214.

YURYEV, R., LIESE, A.: Biocatalysis: the outcast. *ChemCatChem,* 2**,** 1**, 2010**, 103-107.

ZHANG, H.-Y., SUN, Y.-M., WANG, X.-L.: Substituent Effects on O-H Bond Dissociation Enthalpies and Ionization Potentials of Catechols: A DFT Study and Its Implications in the Rational Design of Phenolic Antioxidants and Elucidation of Structure–Activity Relationships for Flavonoid Antioxidants. *Chemistry – A European Journal,* 9**,** 2**, 2003**, 502-508.

ZHANG, Y., KUA, J., MCCAMMON, J. A.: Role of the Catalytic Triad and Oxyanion Hole in Acetylcholinesterase Catalysis: An ab initio QM/MM Study. *Journal of the American Chemical Society,* 124**,** 35**, 2002**, 10572-10577.

8.2. Supporting data

Temperature-current dependency of the spray dryer

The temperature of the liquefying bath was regulated by a transformator. For an estimation of the suitable melting temperature the temperature-current dependency was determined over the time. A region between 60 and 90 °C (highlighted in the diagram) was chosen as a compromise between temperature sensitivity of the enzyme and the melting of the carrier. A current of arount 83 V was chosen to be the best for our investigations.

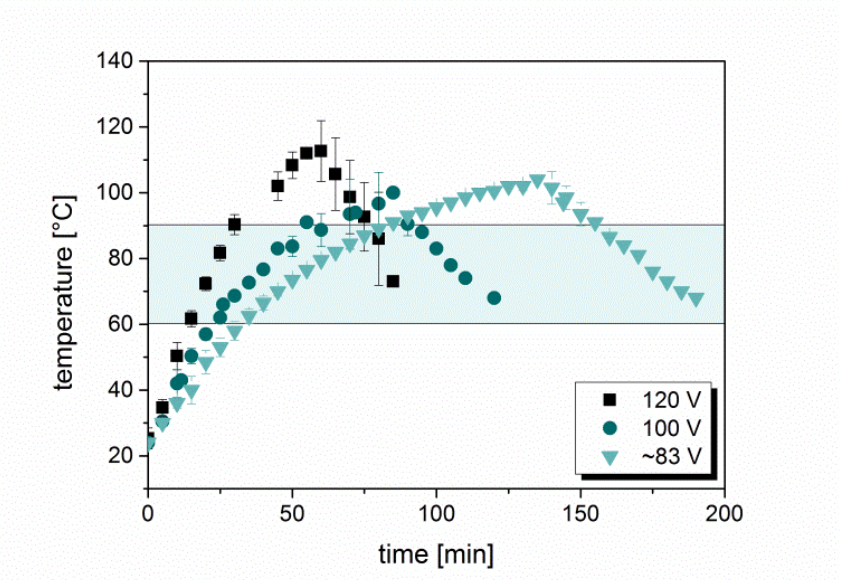


Figure SI-1. Temperature-current dependency measured over the time.

Viscosity-temperature dependency determined with a rheometer

For the viscosity measurements a rheometer MCR 302 (Anton Paar, Austria) as described in chapter 4.1.2. Due to limitations of the method and the rheometer the values for the viscosities 10 °C above the melting point were estimated by extrapolation of the measured values.

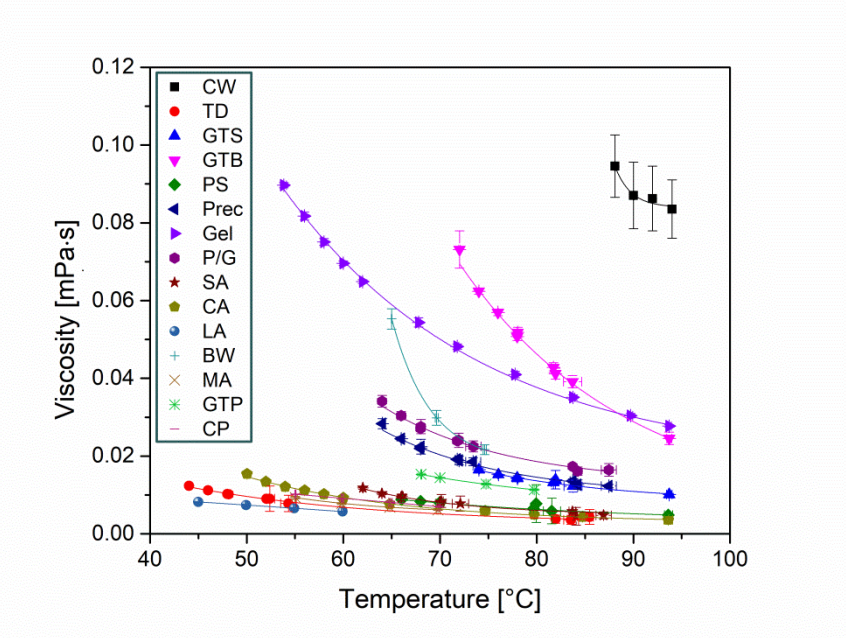
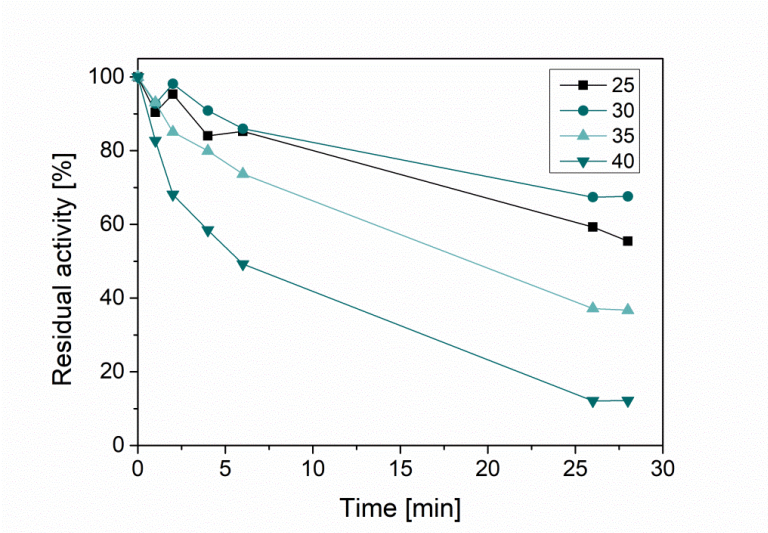


Figure SI-2. Rheological measurements of different carriers. CW – Carnauba wax, TD - Te

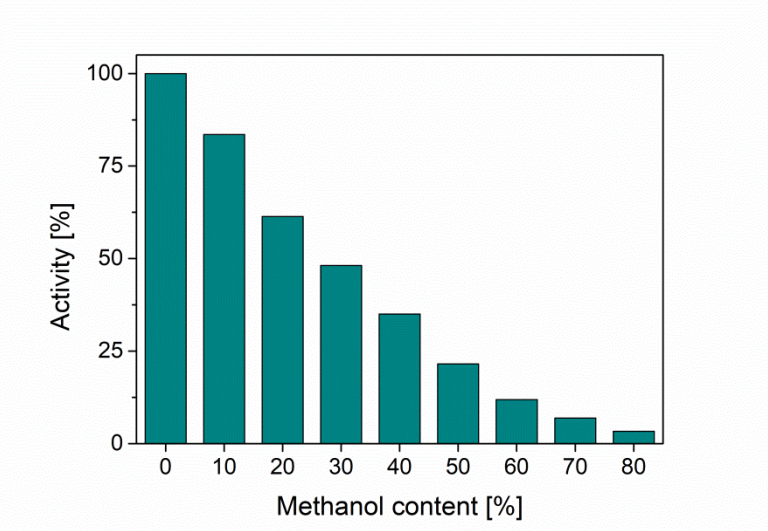
Characterization of the used enzymes

The enzyme characterization profiles were measured over the time with the corresponding standard activity assay of the enzymes described in chapter 4.1.1. Therefore 2 mL of enzyme solution were incubated at the given conditions.

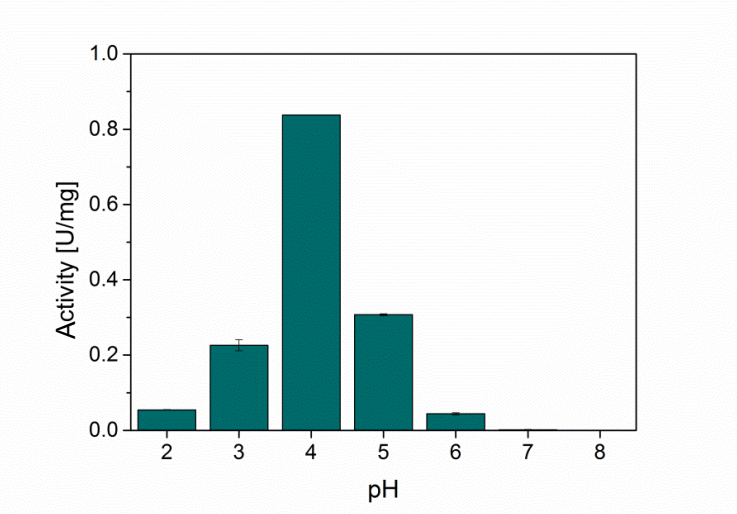
Laccase from Trametes versicolor



SI-3. Temperature profile of the laccase from *TvL*. Cond.: Citrate phosphate buffer pH 4, c_{ABTS} = 5 mM, λ = 420 nm.

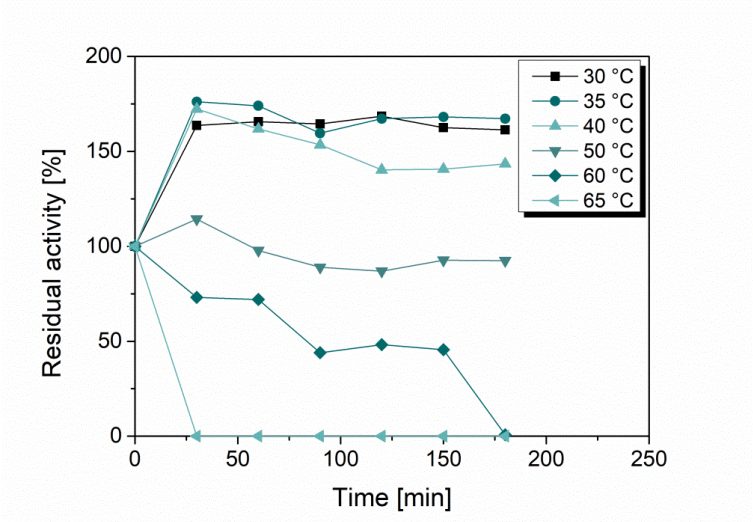


SI-4. Methanol stability of the laccase from *TvL*. Cond.: 30 °C, citrate phosphate buffer pH 4, c_{ABTS} = 5 mM, λ = 420 nm.



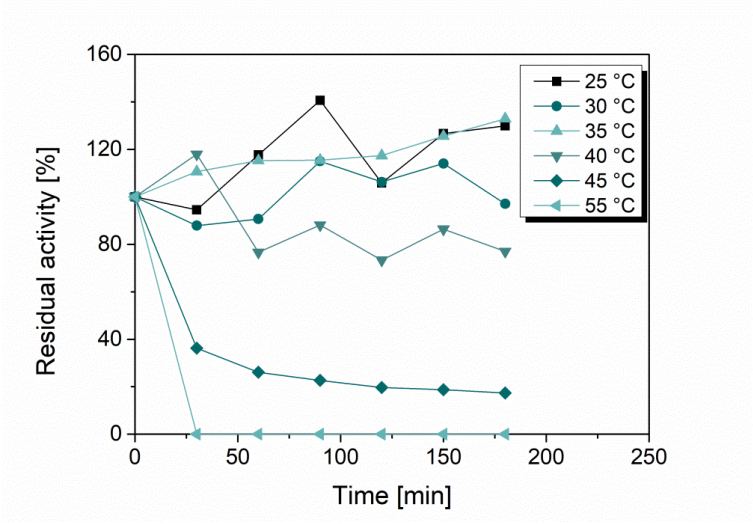
SI-5. Profile of the pH of the laccase from *TvL*. Cond.: 30 °C, citrate phosphate buffer, c_{ABTS} = 5 mM, λ = 420 nm.

Laccase from Myceliophthora thermophile



SI-6. Temperature profile of the laccase from *MtL*. Cond.: Phosphate buffer pH 6, 0.1 M, c_{ABTS} = 5 mM, λ = 420 nm.

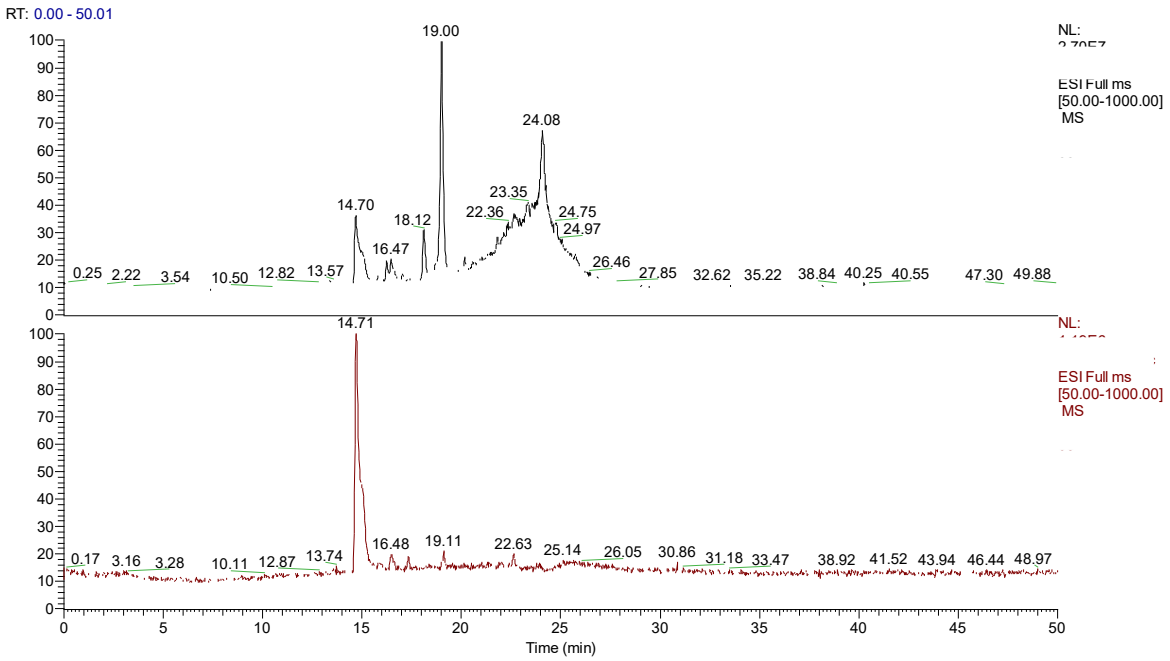
Pig liver esterase



SI-7. Temperature profile of the pig liver esterase. Cond.: Phosphate buffer pH 7.5, $c_{p\text{-}NPA}$ = 10 mM in DMSO, λ = 401 nm.

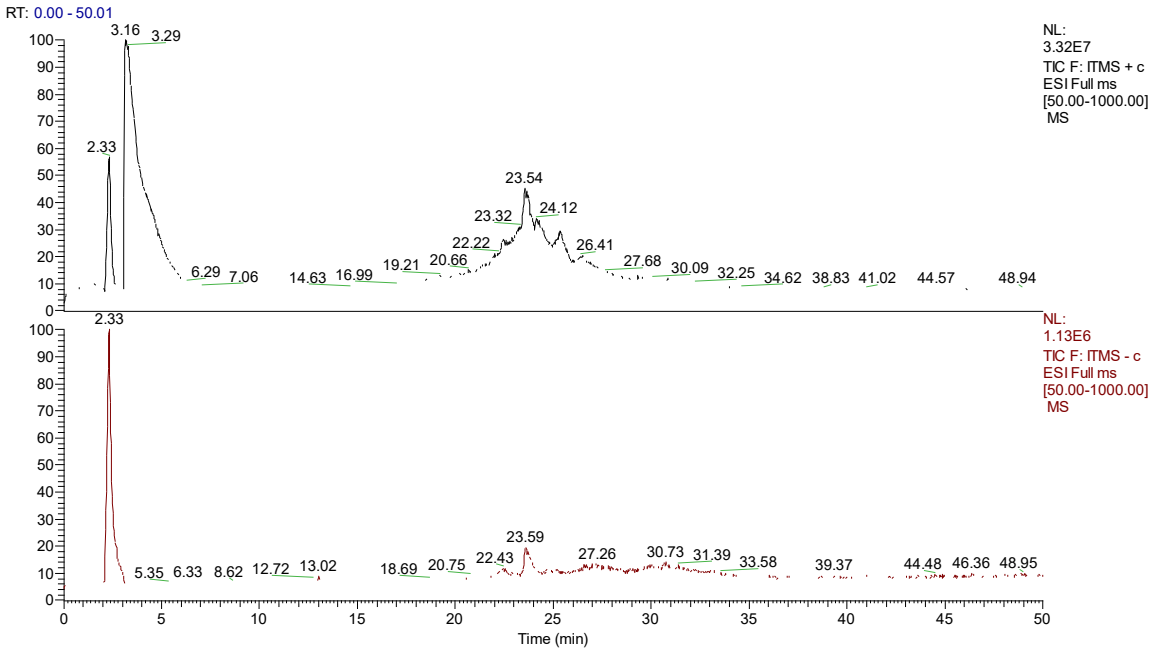
LC/MS data

4-Methylcatechol



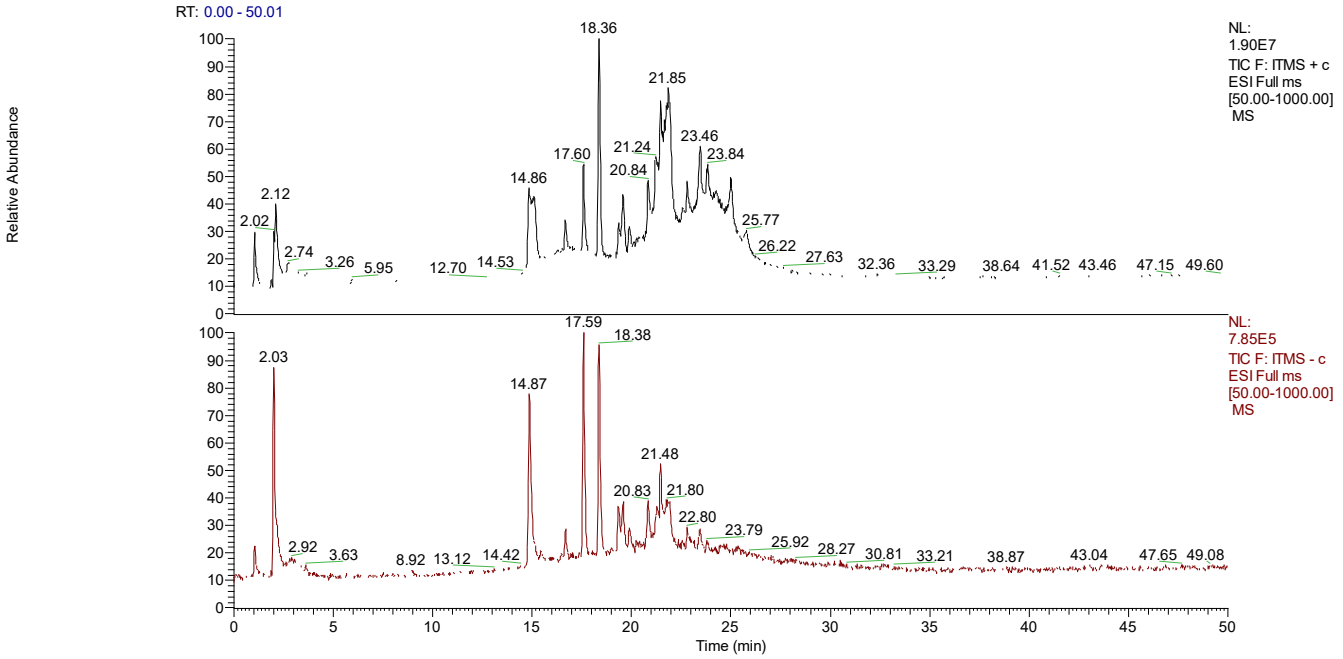
SI-8. TIC chromatogram of the 4-methylcatechol (c = 5 mM) in sodium acetic acid buffer pH 5 in the positive and negative mode.

Cyclohexylamine



SI-9. TIC chromatogram of cyclohexylamine (c = 5 mM) in sodium acetic acid buffer pH 5 in the positive and negative mode.

Laccase initiated reaction of 4-MC without amine



SI-10. TIC chromatogram of a laccase-initiated oxidation of 4-MC without amine in citrate buffer in the positive and negative mode. Cond.: Sodium acetic acid buffer pH 4, V = 20 mL, $c_{4\text{-MC}}$ = 5 mM, $A_{laccase}$ = 5 U/mL.

CURRICULUM VITAE

PERSONAL DATES

Name:	Claudia Engelmann
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Address:	Compeweg 6 21073 Hamburg
Phone:	+49-162-9866643

CAREER SUMMARY

Since April 2017 — **Research Associate**
Institute of Technical Biocatalysis, Technical University Hamburg, Germany

- Investigation of enzyme cascades with *in situ* product removal
- HPLC and GC maintenance
- Analytical training of employees
- Supervision of practical courses, trainees, master and PhD students

Sept. 2013 – Feb. 2017 — **Research Associate**
Industrial Chemistry, University of Rostock, Germany

- Enzyme immobilization and characterization of different enzymes
- Laccase catalyzed C-C and C-N coupling reactions
- Analytical investigation *via* HPLC, GC, NMR and *in situ* IR
- Supervision of practical courses, bachelor and master theses

Jan. 2016 – July 2016 — **Research Stay at the Research Group of Prof. NADIA PASSERINI**
Università di Bologna, Italy

- Enzyme immobilization with a spray congealing apparatus
- Characterization of immobilized enzymes

Sept. 2015 — **Research Stay at the Research Group of Prof. KARL-HEINZ VAN PÉE**
Technical University, Dresden, Germany

- Enzyme expression

April 2012 – Nov. 2012 — **Scientific Assistant at the Research Group of Prof. UDO KRAGL**
Technical Chemistry, University of Rostock, Germany

- Downstream processing of fermentation broths
- Enzyme immobilization
- Redox-biotransformations

ACADEMIC SUMMARY

Sept. 2013 – Feb. 2017	**PhD thesis Prof. UDO KRAGL** Industrial Chemistry, University of Rostock, Germany „Improving biocatalytic reactions – Immobilization *via* spray congealing & promising coupling reactions “
Oct. 2008 – Aug. 2013	**Degree in Chemistry** Industrial Chemistry, University of Rostock ***Master thesis:*** „*In situ* IR-Spektroskopie zur Reaktionsverfolgung ausgewählter enzymkatalysierter Reaktionen“ ***Bachelor thesis****:* „Methodenentwicklung zur Extraktion aus wässrigen Medien in Mikromischern“

FURTHER EDUCATION

Sept. 2016	NaWuReT-Summerschool: „Beiträge der Reaktionstechnik zur Bewältigung von Ressourcenknappheit“, Bayreuth, Germany.
July 2016	„Project Management for Young Scientists”, University of Rostock, Germany.
Sept. 2015	Seminar for spray drying (Büchi), Berlin, Germany.
Jan. – Mai 2015	„Grundlagen der Betriebswirtschaftslehre”, University of Rostock, Germany
Sept. 2013	PhD Workshop „Catalysis for Sustainable Synthesis” (CaSuS), Rostock, Germany.
Feb. 2013	Seminar: „Experimental Design and Analysis with STAVEX, Rostock, Germany.

FURTHER QUALIFICATIONS

IT skills	*Microsoft Office:*	Word, Excel, PowerPoint, Visio
	Graphic programs:	Corel Draw, PhotoShop
	Further programs:	Origin, Endnote, ChemDraw, Matlab, iC IR, TopSpin, Eurochrom, Varian Star, Clarity, Xcalibur, OpenLab, ChemStation
Analytical skills	UV/Vis-Spectroscopy GC, GC/MS, HPLC, LC/MS, IC FT-IR, NMR, DSC	
Language skills	Deutsch	(native)
	English	(fluent)
	Italian	(conversational)
	French	(basic knowledge)