Potential applications of laccase-mediator-systems for biorefinery purposes

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Vorwort

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Aachen, im März 2016 Simon Roth

Success is peace of mind which is a direct result of self-satisfaction in knowing you did your best to become the best you are capable of becoming.

John Robert “The Wizard of Westwood“ Wooden
(1910 – 2010, basketball legend)

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Kurzfassung

Zeitgemäße Konzepte für Bioraffinerie-Prozesse streben die Nutzung lignocellulotischer Biomasse als Rohstoff zur Gewinnung von Plattformchemikalien und neuartiger Treibstoffe an. Bei Lignocellulose handelt es sich um einen festen Verbund aus verschiedenen Makromolekülen, die von dem phenolischen Polymer Lignin zusammengehalten werden. Lignin verleiht Lignocellulose ihre Festigkeit und Stabilität und erschwert dadurch den Zugang zu deren einzelnen Bestandteilen, was die stoffliche Umwandlung in wertschöpfende Chemikalien erschwert. Weiterhin stellt Lignin die mit Abstand größte natürliche Quelle für phenolische Verbindungen dar. Allerdings fehlt es heutzutage noch an effizienten Prozessen, um Phenolverbindungen aus Lignin zu gewinnen, weshalb der Ligninanteil hölzerner Biomasse vielfach ungenutzt bleibt. Für die wirtschaftliche Realisierung lignocellulose-basierter Bioraffinerieprozesse ist eine wertschöpfende Verwertung von Lignin jedoch Voraussetzung.

In der Natur finden sich saprophytische Organismen, die Lignin biokatalytisch zersetzen können. Weißfäulepilze sind in der Lage, Lignin selektiv abzubauen. Dazu sezernieren sie einen Enzymcocktail, in dem Laccasen eine zentrale Rolle spielen, was diese zu vielversprechenden Enzymen für die biokatalytische Umsetzung von Lignin in Bioraffinerieprozessen macht.

Ziel dieser Arbeit war es, mögliche Prozessanwendungen für Laccasen bzw. Laccase-Mediator-Systeme (LMS) im Kontext moderner Bioraffinerien zu identifizieren und zu validieren.

Für die weitere Verwertung in der Bioraffinerie muss Lignocellulose zunächst vorbehandelt und desintegriert werden. Bereits dieser erste Prozessschritt ist extrem energieaufwendig und

beeinflusst die Effizienz der nachfolgenden enzymatischen Hydrolyse der Cellulose. Da Cellulasen einen wesentlichen Kostenfaktor für Bioraffinerieprozesse darstellen, ist eine hohe Kompatibilität zwischen Vorbehandlung und enzymatischer Hydrolyse wichtig für die Wirtschaftlichkeit des Prozesses.

Im Rahmen dieser Arbeit wurde der Einsatz von Laccase-Mediator-Systemen für die Vorbehandlung von Biomasse erprobt. Es konnte gezeigt werden, dass die enzymatische Vorbehandlung von unbehandeltem Buchenholz mit LMS einen positiven Einfluss auf die nachfolgende enzymatische Hydrolyse der Cellulose hat. Dieser Effekt war jedoch nur zu beobachten, wenn die LMS-Vorbehandlung mit einer nachfolgenden Gefriertrocknung kombiniert wurde. So konnte eine Steigerung der Glucose-Ausbeuten um den Faktor 1,3 erzielt werden. Es ist davon auszugehen, dass die Gefriertrocknung der Lignocellulose zu einer Auflockerung der Struktur der Biomasse und damit zu einer größeren Oberfläche führt, auf der mehr Lignin zugänglich wird, welches Cellulasen unproduktiv bindet. Den Effekt der Oberflächenvergrößerung und der damit verbundenen gesteigerten Exposition von Lignin beobachtet man in deutlich stärkerem Ausmaß auch bei den meisten physikochemischen Vorbehandlungsmethoden. Es wird angenommen, dass eine LMS-Behandlung die unproduktive Bindung der Cellulasen durch oxidative Modifikationen des Lignins reduziert. So konnte im Rahmen von Adsorptionsstudien mit dem Fluoreszenzprotein mCherry gezeigt werden, dass eine LMS-Behandlung von rohem Buchenholz die unspezifische Proteinadsorption an die Oberfläche des Materials signifikant reduziert.

Der positive Effekt auf die Zuckerausbeute der enzymatischen Hydrolyse konnte nur für Buchen-, nicht aber für Fichtenholz beobachtet werden, vermutlich aufgrund unterschiedlicher chemischer Eigenschaften des Lignins. Eine Kombination von Vorbehandlung mit LMS und der ionischen Flüssigkeit [EMIM]Ac wurde getestet, jedoch konnte kein Synergismus zwischen beiden Vorbehandlungsmethoden festgestellt werden, entweder aufgrund der weitgehenden Delignifizierung durch die ionische Flüssigkeit oder durch chemische Modifikationen des Lignins während der Behandlung.

Insgesamt stellte sich die LMS-Vorbehandlung als eine vielversprechende Möglichkeit heraus, um die Kompatibilität von klassischen Vorbehandlungsverfahren und der

enzymatischen Hydrolyse zu steigern und die Lignocellulose für diesen Prozessschritt zu konditionieren.

Während der experimentellen Studien zur enzymatischen Vorbehandlung von Biomasse wurde deutlich, dass es an effizienten Nachweismethoden zur quantitativen Bestimmung der tatsächlichen Zugänglichkeit der Cellulose für Cellulasen in lignocellulotischen Substraten fehlt. Daher wurden fluoreszierende Proteinsonden mit einem cellulose-bindenden Motiv (CBM) aus *C. fimi* entwickelt und getestet. Dabei stellte sich heraus, dass die unspezifische Bindung dieser Proteinsonden an die Lignocellulose die CBM-vermittelte, cellulose-spezifische Affinität überdeckt und den Einsatz dieser Sonden auf ligninhaltigen Substraten zur Quantifizierung der Cellulosezugänglichkeit ausschließt.

Das Kernziel dieser Arbeit war die Entwicklung und Validierung LMS-katalysierter Prozesse für die enzymatische Depolymerisation von Lignin. Dafür galt es zunächst, geeignete Reaktionsbedingungen zu identifizieren. Vier organische Lösemittel wurden als potentielle Additive getestet, um die Ligninlöslichkeit im Reaktionssystem zu erhöhen und wässrig-organische Zwei-Phasen-Systeme für eine eventuelle *in-situ* Extraktion von Reaktionsprodukten zu realisieren. Die beiden untersuchten kommerziellen Laccase-Präparate aus *P. ostreatus* und *T. versicolor* zeigten eine vergleichsweise hohe Lösemitteltoleranz. Das Ausmaß der Inaktivierung von Laccase durch das Lösemittel war in absteigender Reihenfolge 1-Butanol > 2-MTHF > n-Heptan > 1-Hexanol, wobei die Inaktivierung durch n-Heptan nur im Zwei-Phasen-System aufgrund von Grenzflächeninaktivierung zu beobachten war. Die beste Kompatibilität zwischen Laccase und Lösemittel wurde für 1-Hexanol gefunden, weshalb es für Depolymerisationsversuche mit Lignin im Zwei-Phasen-System eingesetzt wurde.

Weiterhin wurde der Einfluss von zwei synthetischen Mediatoren auf die Laccase-Stabilität untersucht. Im Vergleich zu der unerwartet starken inaktivierenden Wirkung der Mediatoren 1-Hydroxybenzotriazol (HBT) und Violursäure (VA) war der Einfluss der zuvor getesteten organischen Lösemittel vernachlässigbar. Die Stabilität von Laccase war in Anwesenheit von VA geringer als in Experimenten mit HBT, weshalb letzteres für weitere Versuches eingesetzt wurde. In Reaktionen mit Laccase und HBT wurde ein nicht weiter charakterisiertes bzw. identifiziertes Nebenprodukt gefunden, das nicht mehr als Mediator der Laccase-Reaktion

fungieren konnte, sodass von einem Verlust des Mediators über die Reaktionszeit ausgegangen werden muss. Insgesamt wurde der Mediator als die kritischste Komponente in LMS-basierten Reaktionssystemen identifiziert.

Die LMS-katalysierte Depolymerisation von Organosolv-Lignin wurde mit Laccase aus *T. versicolor* und HBT als Mediator sowohl im wässrigen Ein-Phasen- als auch im wässrig-organischen Zwei-Phasen-System getestet. Die Lignin-Proben wurden mit Gelpermeationschromatographie (GPC) analysiert. Unter keiner der getesteten Reaktionsbedingungen konnte eine Depolymerisation des Lignins nachgewiesen werden. Vielmehr wurden in Reaktionsansätzen mit Laccase bzw. LMS Hinweise auf Polymerisation des Lignins gefunden, die sich in einer Verschiebung der GPC-Chromatogramme hin zu kleineren Elutionsvolumina darstellten.

Die angestrebte Depolymerisation von Lignin mit Hilfe von LMS konnte nicht gezeigt werden. Dennoch leiten sich aus den Ergebnissen dieser Arbeit wertvolle Erkenntnisse hinsichtlich der Grenzen und Möglichkeiten für den Einsatz von LMS in Bioraffinerieprozessen ab: Die hohe Stabilität von Laccase, auch unter Einfluss von Lösemitteln, macht sie zu einem vielversprechenden Enzym für die biokatalytische Realisierung oxidativer Prozesse in der Bioraffinerie. So stellte sich die oxidative Konditionierung mild vorbehandelter Biomasse mit LMS zur Steigerung der Effizienz der enzymatischen Hydrolyse als potenzielles Einsatzgebiet in der Bioraffinerie heraus. Um die unerwünschte Rückpolymerisation von Lignin und seinen Spaltprodukten zu unterdrücken und somit LMS-katalysierte Lignindepolymerisation zu ermöglichen, wurde die Integration von LMS in Multienzymsysteme als vielversprechender Ansatz identifiziert, der zukünftig experimentell adressiert werden sollte.

Abstract

Modern biorefinery concepts focus on lignocellulosic biomass as a feedstock for the production of next generation biofuels and platform chemicals. Lignocellulose is a recalcitrant composite consisting of several tightly packed components which are stuck together by the phenolic polymer lignin hampering the access to the carbohydrate compounds of biomass. Furthermore, lignin represents the most important natural resource of phenolic compounds but its efficient conversion into value-added products is still challenging why lignin remains largely unutilized today.

Certain saprophytic organisms are able to degrade lignin by the use of an enzymatic cocktail. Laccases have been found to play a central role during lignin degradation and are therefore of major interest as biocatalysts for biomass processing. Within this work potential applications of laccases and laccase-mediator-systems (LMS) for biorefinery purposes were investigated and evaluated regarding their practical implementation into biorefinery process routes.

The initial pretreatment of biomass in biorefinery processes is energy intensive and impacts the efficiency of cellulases during the enzymatic hydrolysis of cellulose. Since cellulases are a major cost factor in those processes, best possible compatibility between biomass pretreatment and enzymatic cellulose hydrolysis has to be achieved.

It was demonstrated that LMS can assist the pretreatment of biomass and promote the subsequent enzymatic cellulose hydrolysis by oxidative modification of the surface lignin. The pretreatment of raw beech wood with LMS only had a marginal influence on the subsequent cellulose hydrolysis but when LMS pretreatment was combined with a freeze-drying step the obtained glucose yields were increased by up to 1.3 fold.

It can be assumed that freeze-drying shares with classical physico-chemical pretreatment techniques like e.g. steam explosion an increasing effect on the lignin-displaying surface of the lignocellulosic material which unproductively binds cellulases. LMS are supposed to reduce unspecific protein adsorption onto the substrate surface by oxidation of the surface lignin and thereby, enhance the efficiency of cellulases. Adsorption studies using the fluorescent protein mCherry confirmed that LMS pretreatment of raw beech wood decreases the amount of unspecifically bound protein.

The enhancing effect of LMS pretreatment on enzymatic cellulose hydrolysis was only found for beech but not for spruce, most likely due to different chemical properties of the lignin. Besides, LMS pretreatment was tentatively combined with disintegration of the lignocellulose in the ionic liquid (IL) [EMIM]Ac, but no synergism between both treatment methods was found, either due to extensive delignification by the IL or IL-induced changes in the structure of the residual lignin.

The results of this work suggest LMS pretreatment subsequent to physico-chemical pretreatment techniques as promising method to condition biomass for an enhanced performance of expensive cellulases.

The investigations regarding biomass pretreatment revealed a demand for an assay technique to quantify the actual accessibility of lignocellulosic substrates for cellulases and to characterize how pretreatment methods influence this parameter. Therefore, fluorescent protein probes consisting of a cellulose binding module (CBM) from *Cellulomonas fimi* and the fluorescent protein mCherry were cloned, expressed and tested for biomass characterization. It turned out that unspecific binding onto the lignocellulose predominates the binding behavior of the probes and masks the CBM-specific adsorption to cellulose.

The core objective of this work was the design and development of a LMS-catalyzed process for lignin depolymerization. Therefore, potential process conditions were tested to identify an optimal reaction environment for LMS. To enhance lignin solubility and realize compartmentalization of the reaction system and thereby, *in-situ* extraction of potential reaction products, organic solvents were tested as process additives. The investigated commercial laccase preparations from *P. ostreatus* and *T. versicolor* showed high tolerance against the applied organic solvents. The inactivating effect on laccase was in descending

order 1-butanol > 2-MTHF > n-heptane > 1-hexanol, why the latter was used for depolymerization experiments with LMS and organosolv lignin in a two-phase-system. The inactivating effect of n-heptane was only observed in a two-phase system and originated from inactivation at the interphase.

The impact of the two synthetic mediator compounds 1-hydroxybenzotriazole (HBT) and violuric acid (VA) on laccase stability was investigated and found to exceed that of the tested organic solvents by one order of magnitude. The inactivation by VA was faster than by HBT why depolymerization experiments were performed with the latter. During experiments with laccase and HBT an unidentified, unreactive side product of HBT was detected indicating a loss of mediator over time due to undesired side reactions. Considering the afore mentioned drawbacks the mediator was identified as the most crucial component of LMS reaction systems.

The application of LMS for lignin depolymerization was studied with a reaction system containing laccase from *T. versicolor*, the mediator HBT, and organosolv lignin as the substrate. Lignin samples were analyzed with gel permeation chromatography (GPC). Under none of the tested reaction conditions lignin depolymerization was observed. In fact, when laccase or LMS was present in the reaction system, GPC chromatograms were shifted towards smaller elution volumes indicating polymerization of the lignin. Even if LMS caused lignin bond cleavage its polymerizing activity predominates in the reaction system. Therefore, continuative research is necessary to suppress undesired coupling reactions and shift LMS reactions towards lignin depolymerization. The integration of LMS in multi-enzyme-systems was identified as promising approach to address this issue in future.

Contents

Nomenclature

Abbreviations

[EMIM][Ac]	1-ethyl-3-methylimidazolium acetate
1-PS	one-phase system
2-MTHF	2-methyltetrahydrofuran
2-PS	two-phase system
5-HMF	5-hydroxymethylfurfural
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
APE	alkaline peroxide extraction
AS	acetosyringone
BCA	bicinchoninic acid
BET	Brunauer-Emmett-Teller nitrogen adsorption
BSA	bovine serum albumin
CAC	cellulose accessibility to cellulases
CBM	cellulose binding module
CBM4	family-4 carbohydrate-binding-module
CBQ	cellobiose:quinone oxidoreductases
CMC	carboxymethylcellulose
CV	column volume
DC	detergent compatible protein assay
DMP	dimethoxyphenol
EC	enzyme commision number
EG	endoglucanase
ESCA	electron spectroscopy for chemical analysis
ET	electron transfer
EtOH	ethanol
FITC	fluoresceinisothiocyanate
FPLC	fast protein liquid chromatography
GFP	green fluorescent protein

GOX	glucose oxidase
GPC	gel permeation chromatography
GSH	glutathione
GSSG	glutathione disulfide
HAA	3-hydroxyanthranilic acid
HAT	hydrogen atom transfer
HBT	1-hydroxybenzotriazole
HPAEC-PAD	high pressure anionic exchange chromatography coupled with a pulsed amperometric detector
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence
IL	ionic liquid
IMAC	immobilized metal ion affinity chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
LMC	lignin model compound
LMS	laccase-mediator-system
MOPS	3-morpholinopropane-1-sulfonic acid
MTP	microtiter plate
NaAc	sodium acetate
NADH / NAD^+	nicotinamide adenine dinucleotide
NHA	N-hydroxy-N-phenylacetamide
P.o.	*Pleurotus ostreatus*
PASC	phosphoric acid swollen cellulose
PEG	polyethylene glycol
RAC	regenerated amorphous cellulose
SA	syringaldehyde
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size exclusion
T.v.	*Trametes versicolor*
TEMPO	(2,2,6,6-Tetramethyl-piperidin-1-yl)oxyl
TMFB	Cluster of Excellence "Tailor-made Fuels from Biomass"
VA	violuric acid

Symbols

[S]	substrate concentration	[mM]
A_{420nm}	absorption at 420 nm	[-]
A_{max}	total capacity of the adsorbant	[mol/g]
A_{max}	maximum measured enzyme activity	[U/mg]
A_{spec}	specific enzyme activity	[U/mg]
A_t	calculated enzyme activity	[U/mg]
c_{ABTS}	concentration of oxidized ABTS	[M]
$c_{enzyme,well}$	enzyme concentration in reaction mixture	[mg/L]
d	path length of light	[cm]
df	dilution factor	[-]
f	proportion factor	[-]
k_1	inactivation constant 1	[1/h]
k_2	inactivation constant 2	[1/h]
K_M	Michaelis-Menten constant	[mM]
K_P	equilibrium constant of adsorption	[L/mol]
$P_{ads,\ eq}$	adsorbed protein at equilibrium	[mol/$g_{substrate}$]
P_{free}	concentration of unbound protein in solution	[mol/L]
t	time	[h]
v	reaction rate	[U/mg]
v_0	initial reaction rate	[µmol/(min L)][U/L]
v_{max}	maximal reaction rate	[U/mg]
$\Delta A/t$	change in absorption per time	[1/min]
ε_{420}	extinction coefficient at 420 nm	[L/(µmol cm)]
λ_{em}	emission wavelength	[nm]
λ_{ex}	excitation wavelength	[nm]

List of Figures

List of Tables

Chapter 1

Introduction and theoretical background

Facing the foreseeable depletion of fossil energy sources we are entering a new era of an economy which is more and more shifting the production of fuels and chemicals from a petrochemical basis to renewable resources via biorefining (Kamm and Kamm, 2004). Biorefinery concepts of the first generation mainly focused on easily accessible and convertible biomass such as starch- or sugar-containing crops and thereby came into conflict with food production which is based on limited resources like cultivable land (Graham-Rowe, 2011).

Lignocellulose as feedstock for biorefineries offers a good prospect to escape from the dilemma of biofuel versus food production. The abundance of lignocellulose as a resource can only be compared to that of water (Leonowicz et al., 2001) and besides its occurrence as the actual wooden plant material it is also available in form of nowadays unutilized agricultural and municipal residues (Doherty et al., 2011; Fava et al., 2015; Perlack et al., 2005). However, due to the composite structure of lignocellulose, its initial break-up is energy-intensive and requires a high technical effort (Himmel et al., 2007; Wyman et al., 2005; Yang and Wyman, 2008; Zhang, 2011). Therefore, suitable concepts for biomass pretreatment and conditioning as well as for the efficient utilization of all components are essential to push biorefinery processes towards energetic and economic feasibility.

In nature the decomposition and recycling of lignocellulosic biomass is an integral part of the circle of life, where saprophytic organisms decompose and metabolize lignocellulose like

"natural biorefineries" (Fischer et al., 2013; Ke et al., 2012). The transfer of these natural mechanisms to technical processes is a promising strategy to develop innovative biorefinery concepts. While the enzymatic depolymerization of cellulose has already been realized in technical scale, the utilization of lignin is still challenging but essential to realize economically feasible biorefineries. The branched polyphenolic lignin acts as the "glue" between the different biomass components (Chandra et al., 2007; Zakzeski et al., 2009). Thus, the depolymerization of lignin is not only necessary to gain access to its valuable phenolic subunits but also to disintegrate the biomass during the initial pretreatment. Certain fungi have been found to depolymerize lignin by the use of an enzymatic cocktail in which laccases play a central role.

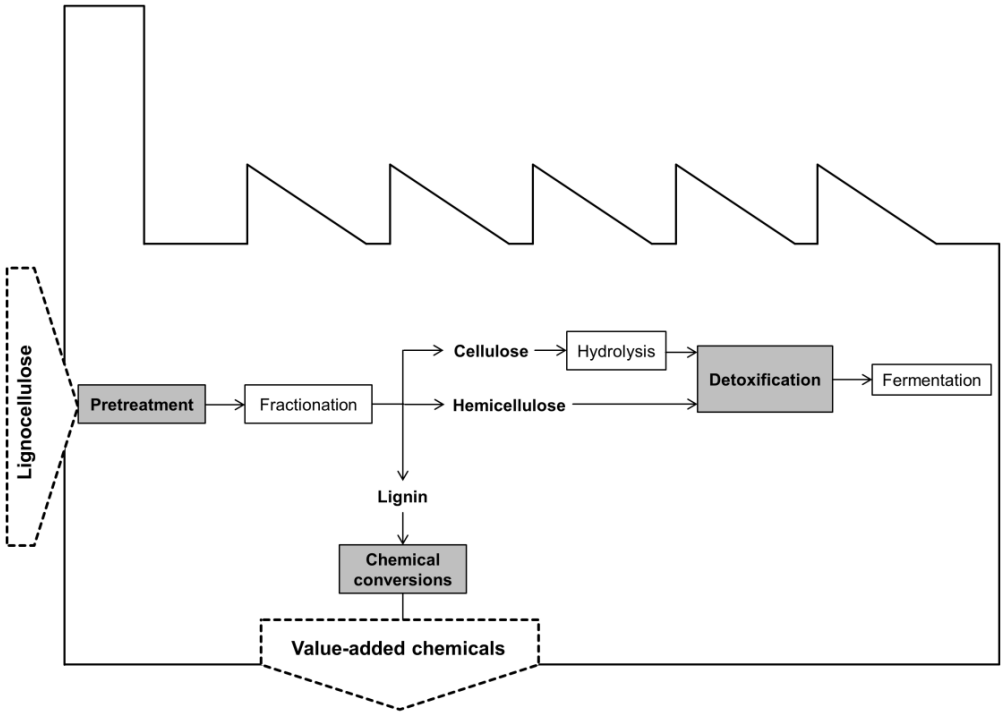

Figure 1.1: Potential fields of application for laccases in biorefinery processes (highlighted in grey) are (1) biomass pretreatment, (2) lignin conversion and (3) hydrolyzate detoxification (Roth and Spiess, 2015).

With regard to their natural function in wood-decaying species, laccases are promising biocatalysts for the realization of several process steps in biorefinery concepts. The potential fields of application for laccases in this context are (1) the pretreatment of the biomass, (2)

lignin depolymerization and modification and (3) the detoxification of biomass hydrolyzates (Figure 1.1) from which the first two are experimentally addressed in this work.

1.1 Lignin

Since laccase-mediator-systems in all biorefinery applications discussed in this work act on lignin and laccase is involved in both lignin biosynthesis and degradation a brief overview of lignin and its structure is provided in the following. The term lignin originates from the Latin expression "lignum" for wood, representing the essential role of this phenolic polymer within the lignocellulosic composite. In the cell walls of wooden plants lignin glues the carbohydrate polymers cellulose and hemicellulose, thereby providing recalcitrance and strength to the lignocellulose matrix (Argyropoulos and Menachem, 1997). Lignin is the subject of intensive research since decades and among the manifold reviews especially the one of Zakzeski et al. deserves particular mention (Argyropoulos and Menachem, 1997; Boerjan et al., 2003; Chakar and Ragauskas, 2004b; Lewis and Yamamoto, 1990; Vanholme et al., 2010; Zakzeski et al., 2009).

Lignin makes up 15 - 35 weight percent of wooden biomass and can account for even 40% of its energy content (Demirbaş, 2001; Perlack et al., 2005; Zakzeski et al., 2009). The lignin content of softwood (25-35 wt%) is typically higher than that of hardwood (18-25 wt%) (Azadi et al., 2013). Monocotyledonous plants like grasses and grains have comparatively low lignin contents of 15-19 wt% (Azadi et al., 2013; Demirbaş, 2001). Lignin is a branched, heterogeneous and amorphous polymer basically formed from three hydroxycinnamyl alcohol monomers which are also referred to as monolignols (Figure 1.2). These monolignols *p*-coumaryl, coniferyl and sinapyl alcohol differ in their degree of methoxylation (Boerjan et al., 2003). During lignin biosynthesis the monolignols are coupled by radical polymerization and form the phenylpropanoid lignin subunits *p*-hydroxyphenyl (H, derived from p-coumaryl alcohol), guaiacyl (G, derived from coniferyl alcohol) and syringyl (S, derived from sinapyl alcohol). The subunits are linked by several types of bonds (Figure 1.2, Figure 1.3) from which the β-O-4 bond has the highest abundance (45-60 bonds per 100 C9-units), followed by the 5-5'- (3-24 bonds per 100 C9-units) and the β-5-bond (3-12 bonds per 100 C9-units) (Azadi et al., 2013; Zakzeski et al., 2009). The composition of lignin varies among taxa and

species and even between different parts of the same plant (Boerjan et al., 2003; Vanholme et al., 2010).

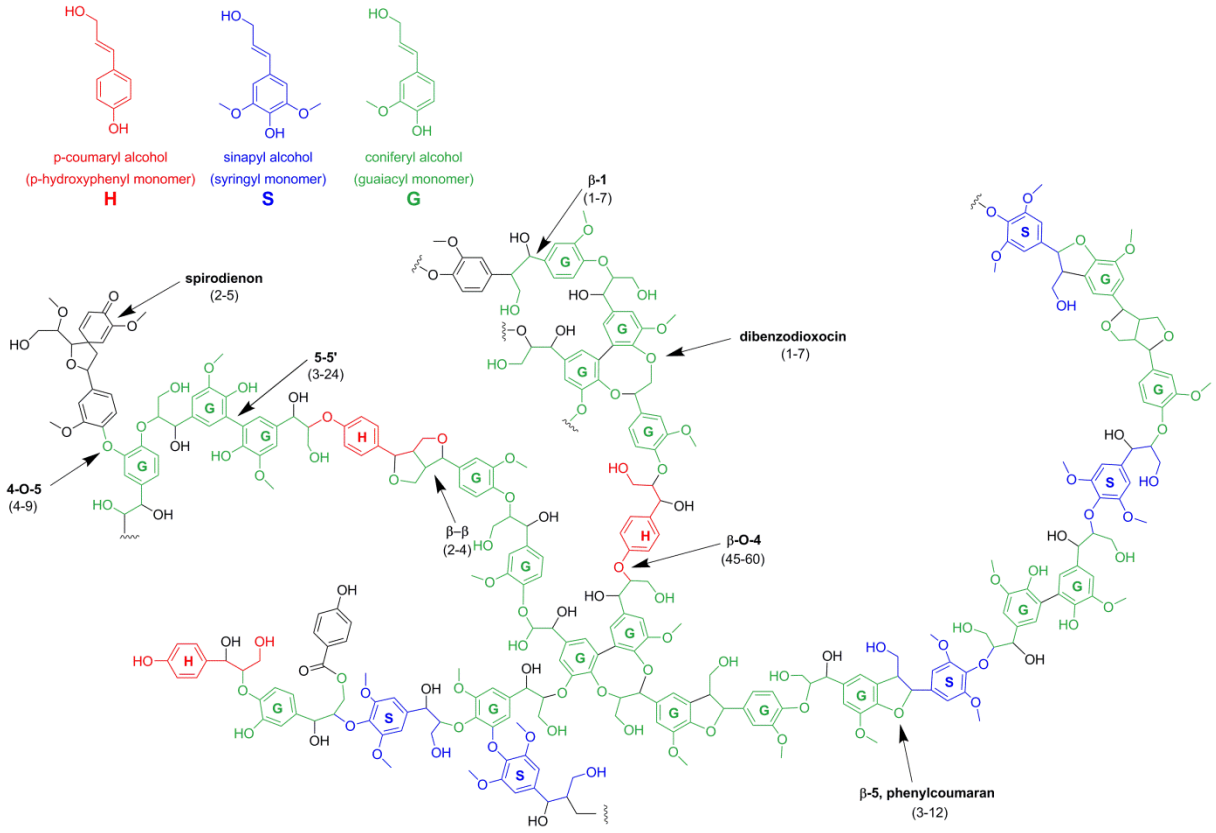

Figure 1.2: Schematic illustration of a representative lignin structure. According to Vanholme et al., 2010 and Zakzeski et al., 2009 and in Roth and Spiess, 2015. Numbers in brackets indicate the average abundance of the particular bond type per 100 C_9-units (Azadi et al., 2013; Zakzeski et al., 2009).

1.2 Laccases

Laccase has already been described in 1883 by Yoshida as the coagulant of the sap of the Japanese lacquer tree *Rhus vernicifera* (Yoshida, 1883). The resulting lacquer was historically used as coating for wooden commodities and was designating for the enzyme laccase. As one of the oldest known enzymes laccase has been intensively researched and exhaustively reviewed. Relevant key reviews cover most aspects concerning laccases and their applications (Table A.1, appendix). Therefore, this chapter gives only a brief overview about the most important characteristics of laccases.

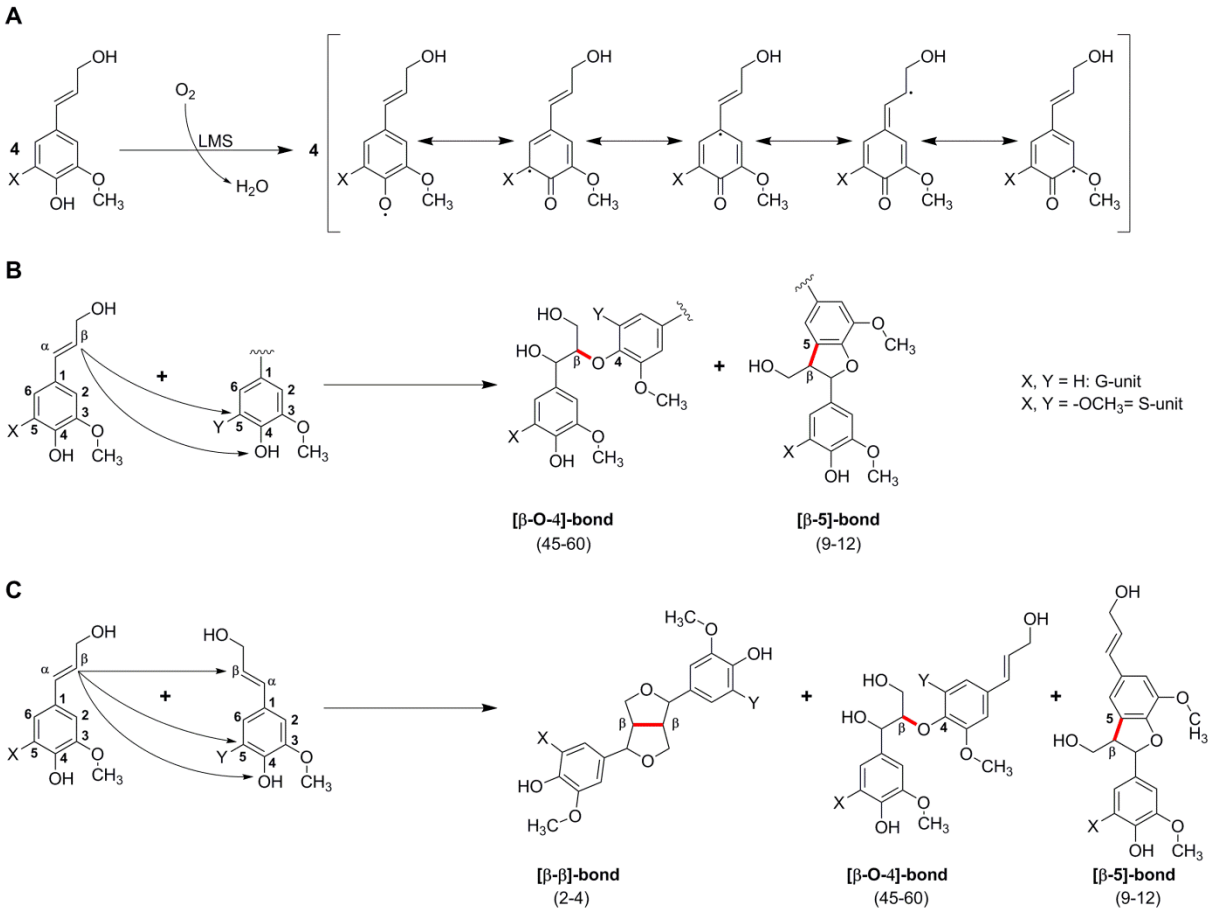

Figure 1.3: Laccase-induced oxidative coupling in lignin biosynthesis forming the typical lignin bonds (Roth and Spiess, 2015). A: Oxidative deprotonation of monomeric phenols by LMS. B: Coupling of monolignols to phenolic end groups of lignin and (C) with each other. X and Y can be -H or –OCH_3. Formation of β-O-4 bonds occurs under water incorporation. Illustration according to van Parijs et al., 2010 and Vanholme et al., 2010. Numbers in brackets indicate the average abundance of the particular bond type per 100 C_9-units (Azadi et al., 2013; Zakzeski et al., 2009).

Laccases are ubiquitary enzymes and are found in plants, fungi, bacteria and insects (Dwivedi et al., 2011; Giardina et al., 2010). In plants laccases are involved in the biosynthesis of lignin by inducing radical polymerization of the monolignolic building blocks to the branched lignin network resulting in various bond types (Figure 1.3) (Argyropoulos and Menachem, 1997; Boerjan et al., 2003; Vanholme et al., 2010). In wood decaying fungi laccases play a key role in the opposed process of lignin degradation. In particular white-rot fungi cause a selective delignification of wood, resulting in so called “white rot”, named after the remaining whitish cellulose fibers. White-rot fungi are the most important producers of laccases and secrete an

enzymatic cocktail to degrade lignin (Dashtban et al., 2010), which contains besides laccases also several heme peroxidases like lignin peroxidase, manganese peroxidase and versatile peroxidase and auxiliary enzymes that provide reduction equivalents for the peroxidases such as hydrogen peroxide. In contrast to heme peroxidases laccases only require air oxygen as electron accepting co-substrate making them promising biocatalysts for reactions in technical scale.

Laccases (EC 1.10.3.2, p-diphenol:dioxygen oxidoreductase) belong to the group of "blue-copper proteins" named by their blue color arising by absorption at 610 nm from one of the four copper ions located in their active site. The copper ions are classified into three types according to their spectroscopic properties. As common for all multicopper oxidases, substrate oxidation takes place at the "blue" T1 copper ion from where the abstracted electron is transferred to the trinuclear copper cluster T2/T3, where oxygen is finally reduced to water (Solomon et al., 2008). Since laccase performs a one-electron-oxidation of its substrates, but four electrons have to be transferred during the oxygen reduction, electrons must be stored by the enzyme in a "battery-like" manner (Thurston, 1994). It is believed that electron storage is realized by oxygen intermediates temporarily bound to the T2/T3 copper cluster (Lee et al., 2002; Shleev et al., 2006b; Yaropolov et al., 1994). The T1 copper is the primary electron acceptor during substrate oxidation and thereby determines the redox potential of the laccase, which has to be higher than the redox potential of the substrates which the laccase can oxidize directly (Morozova et al., 2007a). Hence, the scope of oxidizible substrates rises with the redox potential of the laccase, which renders laccases with high redox potential interesting as biocatalysts for technical applications. The redox potential of laccases ranges from 420 – 790 mV vs. NHE (Giardina et al., 2010; Morozova et al., 2007a). Plant laccases have comparatively low redox potentials, whereas the highest values of 730 – 790 mV are reported for fungal laccases (Morozova et al., 2007a). Nearly all laccases with technical relevance origin from wood-decaying basidiomycetes, mainly out of the group of white-rot fungi. Certain mediator molecules can even extend the redox potential of laccase-catalyzed reactions (see chapter 1.3 below).

The so-called "yellow laccases" lack the typical absorption maximum at 610 nm and have been found to oxidize also non-phenolic substrates in the absence of a mediator (Leontievsky et al., 1997). Based on the high similarity in structural and catalytic properties, Leontievsky et

al. proposed that yellow laccases evolved from blue laccases by incorporation of a small lignin-derived phenolic compound acting as intrinsic mediator (Leontievsky et al., 1999; Pozdnyakova et al., 2004). Due to their mediator independence they are of high interest for technical applications, but the current knowledge on these enzymes does not exceed basic characterization.

Fungal laccases have an average molecular weight of 66 kDa (Baldrian, 2006). Their pH optima and pI values lie in the acidic range (pH 2-5) (Baldrian, 2006) and are thereby conflicting with lignin solubilization working best at high pH values. Laccases are quite thermo-tolerant, with reported temperature optima for fungal laccases from 25°C up to 80°C (Baldrian, 2006; Morozova et al., 2007a). Laccases are glycoproteins, with the carbohydrate content of fungal laccases being typically lower (10–25%) than that of plant laccases (22-45%) (Dwivedi et al., 2011). The glycosylation is often considered causing high stability of laccases against inactivating conditions such as solvents, radicals or temperature. A high tolerance of laccases against solvents as reported for ethanol (Maijala et al., 2012; Rodakiewicz-Nowak et al., 1999; Rodakiewicz-Nowak and Jarosz-Wilkołazka, 2007), dioxane (Barreca et al., 2003; Cantarella et al., 2003a; d'Acunzo et al., 2004), acetone (d'Acunzo et al., 2004; Fiţigău et al., 2013; Rodakiewicz-Nowak and Jarosz-Wilkołazka, 2007), isopropanol (d'Acunzo et al., 2004), DMSO (Rodakiewicz-Nowak and Jarosz-Wilkołazka, 2007) or ionic liquids (Harwardt et al., 2014; Liu et al., 2013; Rehmann et al., 2012) is advantageous for their use in technical processes, especially for lignin conversion, where added solvents increase the lignin solubility in aqueous media at moderate pH values.

1.3 Mediators in laccase-mediator-systems (LMS)

Laccases are able to oxidize phenolic compounds, but their comparatively low redox potential prevents them from oxidizing the non-phenolic structures which represent the main fraction of the lignin polymer. Certain mediator molecules can act as electron shuttle between the substrate and the enzyme (Figure 1.4). Mediators modulate the redox potential of the reaction system and thereby, expand the scope of laccase-oxidizable structures also to non-phenolic structures. Furthermore, as small, diffusible molecules they extend the laccase-induced oxidation to lignin and lignocellulose, which are sterically not accessible for laccase itself (Crestini and Argyropoulos, 1998). Since mediators are essential for many biorefinery

applications of laccases, especially for the lignin modification, laccase mediators are discussed in more detail below.

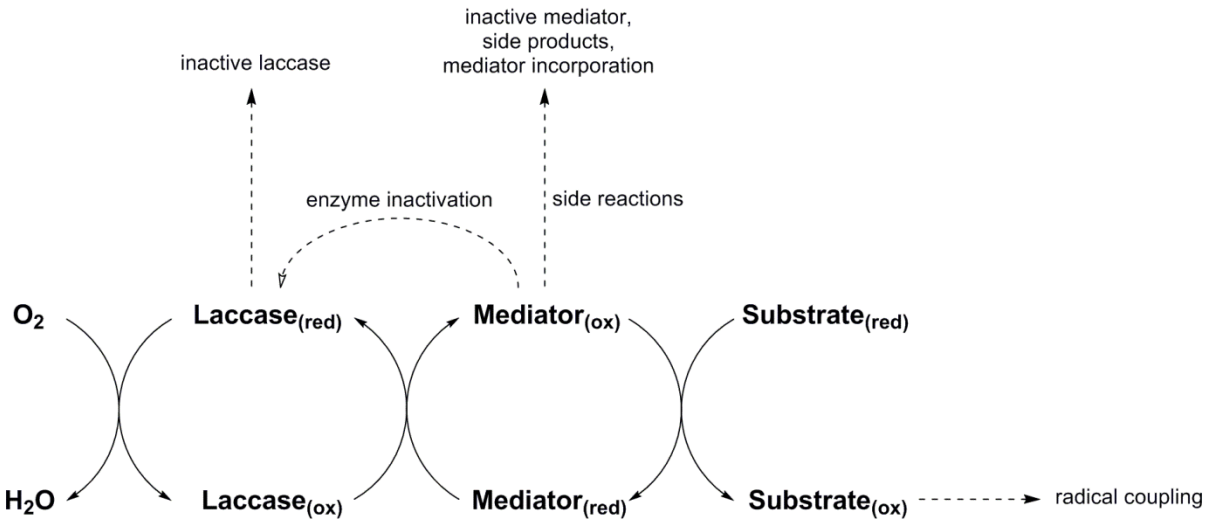

Figure 1.4: Laccase-mediator-system (LMS) and potential undesired side effects (Roth and Spiess, 2015).

The first substance identified as laccase mediator was 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS (Figure 1.5). In 1990 Bourbonnais et al. showed for the first time that suitable mediators can enable laccases to oxidize non-phenolic compounds and cleave C_α-C_β bonds (Bourbonnais and Paice, 1990). Henceforth, the so called laccase-mediator-systems (LMS) were intensively studied. The potential of LMS to realize enzymatically catalyzed lignin degradation brought laccases in the focus of pulp and paper industry. Further substances were found to perform as laccase mediators; especially compounds with N-hydroxy functionality were discovered to exhibit high redox potentials and a strong reactivity with lignin. The most prominent one is 1-hydroxybenzotriazole (HBT) followed by violuric acid (VLA), N-hydroxyphthalimide (HPI) and TEMPO (Figure 1.5).

Three reaction mechanisms for the oxidation of non-phenolic substrates by the mediators have been identified and serve to group the mediators, namely (I) electron transfer (ET), (II) radical hydrogen atom transfer (HAT) or (III) ionic mechanism (Shiraishi et al., 2013) (Figure 1.5). While ABTS follows the ET route (Bourbonnais et al., 1998; Bourbonnais and Paice, 1990), N-hydroxy compounds like HBT, VLA or HPI perform radical hydrogen atom transfer (Fabbrini et al., 2002a, 2002b; Galli and Gentili, 2004; Xu et al., 2000). The efficiency of

substrate oxidation by electron transfer strongly depends on the redox potential of the substrate as shown for ABTS (Baiocco et al., 2003; Fabbrini et al., 2002a), in contrast to the HAT route, which therefore entails a much broader scope of oxidizable substrates. For the stable N-oxyl radical TEMPO and its derivatives (e.g. PROXYL) an ionic mechanism is proposed (Astolfi et al., 2005; Fabbrini et al., 2002a; Galli and Gentili, 2004), where laccase oxidizes the stable oxyl-radical to the oxoammonium ion (Fabbrini et al., 2001) which then abstracts the α-proton from the substrate (Galli et al., 2004). The recycling of the resulting hydroxylamine is not fully understood yet, but Arends et al. supposed a laccase-catalyzed re-oxidation as more likely than oxidation by oxygen or the oxoammonium ion (Arends et al., 2006).

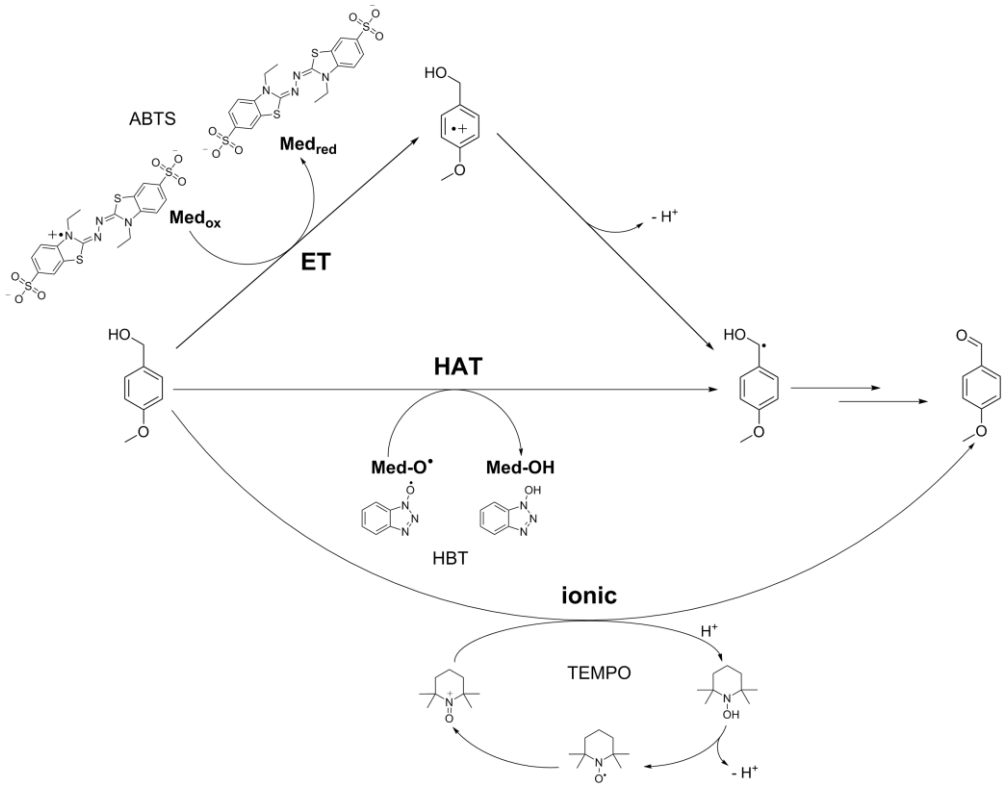

Figure 1.5: Reaction mechanisms of different types of laccase mediators. ET: electron transfer mechanism representative for ABTS; HAT: hydrogen atom transfer using HBT as example for N-hydroxyl mediators; ionic mechanism of aminoxyl radical mediators like TEMPO. Illustration assembled from Astolfi et al., 2005; Baiocco et al., 2003; Canas and Camarero, 2010; D'Acunzo et al., 2006; Galli and Gentili, 2004 and in Roth and Spiess, 2015.

All mediators mentioned until now belong to the group of synthetic mediators which is not exhaustively covered, but contains many more substances (Astolfi et al., 2005; Shin et al., 2006). The use of synthetic mediators implicates several drawbacks: Their prize impairs the economic feasibility of LMS applications in industrial scale. Issues like ecotoxicology as well as disposal have to be considered. Most notably, however, mediators are known to provoke several undesired side effects (Figure 1.4): Due to their radical state after enzymatic oxidation most mediators have an inactivating effect on enzymes in general and on laccase in the LMS in particular (Rehmann et al., 2013; Soares et al., 2001) most likely due to reactions with aromatic amino acids (Bendl et al., 2008). Formation of side products after laccase oxidation detracts mediators from the LMS and impedes the redox cycle between laccase and substrate. For example, after oxidation by laccase, HBT can form unreactive side products not further serving as laccase mediator (Bourbonnais et al., 1997; Li et al., 1998; Xu et al., 2000). Also covalent binding of mediators to the substrate can eliminate them from the reaction system (Matsumura et al., 1986; Rittstieg et al., 2002). The discussed effects overlap during LMS reactions and create a complex reaction network (Figure 1.4), whose description and understanding is essential for further optimization of LMS reaction systems.

Palonen et al. approached the inactivating effect of mediators on enzymes by gradually activating an unreactive acetylated derivate of the mediator *N*-hydroxy-*N*-phenylacetamide (NHA) via an *in-situ* lipase-catalyzed deacetylation (Palonen and Viikari, 2004). This "slow-release-mediator" reduced enzyme inactivation, but since the mediator activation is irreversible, the positive effect might be limited to a certain time frame. Preventing undesired side effects caused by oxidized mediators in general remains a challenge which has to be addressed in the future.

The realization of LMS-catalyzed processes in industrial scale essentially requires solutions to overcome obstructive factors like costs, ecotoxicity and disposal of mediators. Biomass-derived, i.e. so called natural, mediators are promising alternatives to the synthetic mediators like ABTS or HBT. E.g. the fungal metabolite 3-hydroxyanthranilic acid (3-HAA) was found to act as laccase mediator in a study with lignin model compounds and synthetic lignin (Eggert et al., 1996). However, 3-HAA was used in high excess (15:1 mediator:substrate), and later studies report a rapid dimerization of this compound (Cantarella et al., 2003a), rendering a technical application of 3-HAA doubtful. More interesting are mediator molecules derived

directly from lignin, which would allow for an intrinsic mediator functionality of the substrate itself (Canas and Camarero, 2010). Already in 1989 syringaldehyde was shown to promote the oxidation of a non-phenolic lignin model compound via a phenoxy radical of syringaldehyde which was formed by *Trametes versicolor* laccase (Kawai et al., 1989). In a later study D'Acunzo et al. proved on the example of Phenol Red that phenolic compounds can act as laccase mediators and proposed the underlying mechanism (D'Acunzo and Galli, 2003): Phenolic mediators are monoelectronically oxidized by laccase to the corresponding phenoxy radical which then causes the rate-limiting HAT oxidation at the non-phenolic substrate (Figure 1.6) (Calcaterra et al., 2008). Besides acting as laccase mediator, phenoxy radicals also can undergo oligomerizing coupling reactions (D'Acunzo et al., 2006). The stability of the phenoxy radical seems to be crucial for its efficiency as mediator. Resonance-stabilized phenoxy radicals are supposed to have longer half-life times counteracting self-coupling and promoting HAT oxidation (Calcaterra et al., 2008; D'Acunzo et al., 2006; D'Acunzo and Galli, 2003).

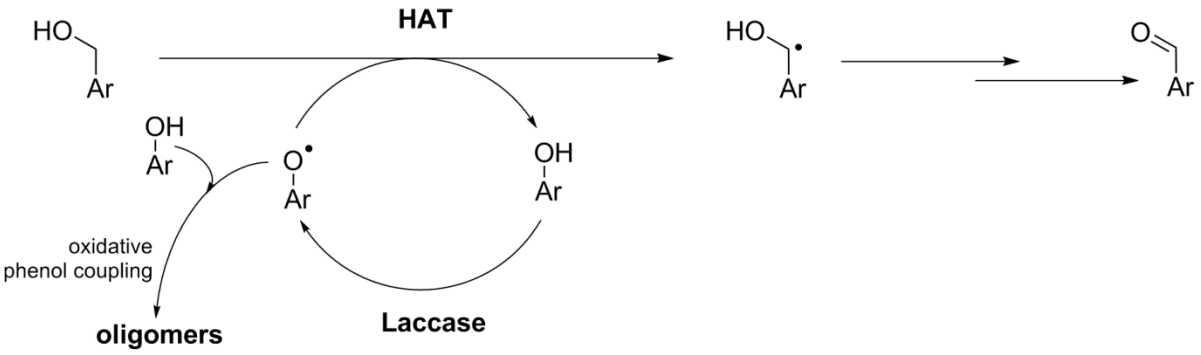

Figure 1.6: Reaction mechanism of phenolic compounds acting as natural laccase mediators. Reaction scheme according to Calcaterra et al., 2008; D'Acunzo et al., 2006 and in Roth and Spiess, 2015.

Acetosyringone is an example for a natural phenolic compound forming resonance stabilized phenoxy radicals after laccase oxidation (Martorana et al., 2013). It is an efficient mediator in LMS-catalyzed decolorization of various recalcitrant dyes (Camarero et al., 2005) as well as in depolymerization of lignosulfonates (Cho et al., 2004). Besides acetosyringone, many other natural phenolics have been identified as laccase mediators, mainly syringyl-type compounds (Camarero et al., 2005; Du et al., 2013; Nousiainen et al., 2009; Rico et al., 2014). However,

several studies report grafting of those mediators onto lignin surfaces (Du et al., 2013; Fillat et al., 2012; Moilanen et al., 2014; Vila et al., 2011).

1.4 Enzymatic biomass pretreatment with LMS

The entangled, recalcitrant architecture of lignocellulose poses the key challenge of utilizing wooden biomass in biorefineries. The initial step of all biorefinery process routes is the disintegration and fractionation of the lignocellulosic composite for individual treatment of the different biopolymers. There exist manifold techniques to break-up lignocellulose (Kumar et al., 2009; Menon and Rao, 2012) but most are very energy-intensive and involve harsh or even toxic chemicals (Zhu and Pan, 2010), thereby negatively impacting the overall energy balance and significantly contributing to the production cost (Viell et al., 2013; Wyman et al., 2005; Zhang, 2011). Hence, innovations in the field of biomass pretreatment are essential to shift biorefineries towards economic feasibility. Besides the optimization of classical methods like e.g. organosolv pulping (Schmiedl et al., 2012), the development of new technologies for biomass disintegration is fostered by current research.

Biological pretreatment methods are promising nature-inspired alternatives to the established physico-chemical methods and might at least supplement them to lower the energy and chemical demand. As mentioned above, white-rot basidiomycete fungi disintegrate lignocellulose via delignification in order to get access to its nutritive compounds, resulting in an eased access to the carbohydrates. Among the secreted lignolytic enzyme cocktail, laccases are supposed to support biomass disintegration by oxidative modifications of lignin (Gutiérrez et al., 2012; Rico et al., 2014).

The first technical processing of lignocellulose using lignolytic enzymes came up long before the idea of biorefineries: The delignification and bleaching of the lignocellulosic pulp for paper making are energy-intensive steps requiring harmful chemicals, forming an early stimulus for the exploration of lignolytic enzymes in the pulp and paper industry. LMS have been found to assist the delignification of pulp, and their application was developed to pilot scale (Call and Mücke, 1997). Even if pulp delignification and biomass pretreatment share several aspects, their goals are different: In paper mills the delignification success is measured by the brightness of the paper and the required amount of chemicals and energy to bleach the

pulp. The purity of the cellulose fibers is essential whereas the properties and utilization of the other biomass components like lignin is of no relevance. This most traditional field of LMS-catalyzed pulp delignification has been intensively reviewed (Table A.1, appendix) (Call and Mücke, 1997; Virk et al., 2012; Widsten and Kandelbauer, 2008) and is not further addressed in this work.

Biomass pretreatment, however, is benchmarked in terms of sugar yields obtained during saccharification as well as fermentability of the hydrolyzates. Furthermore, the separability of the individual biomass components in pure streams for further processing is an important criterion. During the last two decades LMS were studied for enzymatic biomass pretreatment following basically two types of approaches (Table A.2): (1) LMS pretreatment subsequent to a classical physico-chemical pretreatment (e.g. steam explosion) of the lignocellulose and (2) LMS pretreatment of raw or pretreated lignocellulose followed by a physico-chemical treatment method like alkaline extraction.

1.4.1 LMS pretreatment should precede enzymatic hydrolysis

In one of the first studies Palonen et al. applied laccase from *Trametes hirsuta* in combination with the mediator NHA to steam pretreated softwood, increasing the sugar yields during enzymatic hydrolysis by up to 21% (Palonen and Viikari, 2004). Even a treatment with laccase alone enhanced the sugar release by around 13%. A LMS pretreatment simultaneous to enzymatic hydrolysis with cellulases resulted in slightly lower sugar yields due to the inactivating effect of oxidized mediators on the cellulases. These results were confirmed for steam exploded wheat straw incubated with cellulases, a laccase from *Sclerotium sp.*, and with and without the mediator ABTS: Laccase alone enhanced the sugar release during enzymatic hydrolysis, whereas the sugar yields were decreased compared to the untreated reference in the presence of laccase and mediator (Qiu and Chen, 2012). Although the earlier mentioned slow release mediator (NHA-Ac in combination with lipase) minimized the inactivation of the cellulases by the oxidized mediators (Palonen and Viikari, 2004), the effort is considered too high for technical realization, meaning that LMS pretreatment and enzymatic hydrolysis should be performed as separate process steps to avoid inactivating effects of the LMS.

1.4.2 LMS pretreatment modifies the biomass surface functionalities.

The LMS induced increase of hydrolysis yield was attributed to modifications in the surface functionalities of lignocellulose, particularly, an increase of carboxylic groups detected by ESCA (electron spectroscopy for chemical analysis) (Palonen and Viikari, 2004). The resulting decrease in hydrophobicity might lower irreversible, unproductive binding of cellulases to lignin. Furthermore, the carboxylic groups increase the negative surface charge potentially repulsing cellulases, which are also negatively charged at pH 4-5 due to their low pI values (Palonen and Viikari, 2004). The increase of carboxylic groups in lignin after LMS treatment was also described in the context of pulp biobleaching (Chakar and Ragauskas, 2004a).

Further lignin modifications were found, namely an increased ratio of acidic to aldehyde functionalities of the lignin monolignols which were attributed to LMS-induced C_α-C_β cleavage (Chen et al., 2012). In contrast, 2D-NMR (heteronuclear single quantum coherence, HSQC) analysis of lignin extracted from steam exploded eucalyptus wood which was pretreated by LMS revealed more secondary hydroxyl groups, less methoxyl groups, but no decrease in β-O-4 structures which would hint at lignin depolymerization (Martin-Sampedro et al., 2011). And finally, oxidation of the aromatic rings in lignin after LMS treatment of steam exploded wheat straw were observed using FT-IR spectroscopy (Qiu and Chen, 2012).

Phenolic hydroxyl groups were found important for the inhibition of cellulases by lignin, not only via unspecific protein adsorption, but presumably also via interaction of phenolic groups with the substrate binding site of the cellulases (Pan, 2008). Chemical blocking of these groups by hydroxypropylation significantly decreased the inhibitory effect of several tested lignins (Pan, 2008). In summary, LMS pretreatment alters the lignin functionality by oxidation to carboxylic, and phenolic hydroxyl groups, thereby improving the interaction with cellulases.

1.4.3 Biomass, mediator, and cellulase influence unspecific protein adsorption to LMS treated biomass

Moilanen et al. compared the sugar yields of steam pretreated biomasses after treatment with laccases from *Cerrena unicolor* and *T. hirsuta* without any mediator on the subsequent enzymatic hydrolysis: While the sugar yield was enhanced by 12% for spruce, the opposite was found for giant reed with sugar release lowered by 17% (Moilanen et al., 2011). The different sugar yields were attributed to the chemical composition of the lignin of both plant species, which after laccase treatment resulted in lower protein adsorption to lignin from steam pretreated spruce than to lignin from steam pretreated giant reed. Since lignin origin and extraction method strongly influence unspecific protein adsorption and thereby, cellulase inhibition (Nakagame et al., 2010), unspecific interactions between laccase treated lignin and cellulases were deduced as decisive for cellulase hydrolysis yields (Moilanen et al., 2011, 2014; Palonen and Viikari, 2004). The laccase treatment also increased the carbohydrate content of the residual lignin of steam pretreated giant reed obtained after extensive enzymatic hydrolysis compared to the untreated control from 18% to 24%, whereas that of the residual lignin of steam pretreated spruce remained unchanged (Moilanen et al., 2011). An increased carbohydrate content of lignin after laccase treatment was mentioned earlier to impede cellulose hydrolysis (Nakagame et al., 2010).

In their most recent study Moilanen et al. showed that the adsorption of the cellulases Cel5A (endoglucanase) and Cel7A (cellobiohydrolyse) on lignin isolated from steam pretreated spruce was significantly lowered after LMS treatment, whereas the adsorption of the β-glucosidase Cel3A from Novozym 188 was not affected (Moilanen et al., 2014). Different mediators (ABTS, AS, HBT, TEMPO) were compared and the strongest impact was observed for ABTS, with unspecific binding of Cel5A and Cel7A on isolated lignin reduced by the factor 1.75 and 3.5, respectively, and degree of hydrolysis of pretreated spruce improved by 54%. The mediator HBT had almost no effect, most likely because it is slowly oxidized by *T. hirsuta* laccase. The mediator AS seemed to polymerize on lignin by laccase treatment, indicated by a decrease of phenolic compounds in the sample supernatants, resulting in decreased cellulase adsorption to the now syringyl-grafted softwood (guaiacyl) lignin. Interestingly, TEMPO as ionic oxidation mediator caused oxidation primarily of reducing ends of cellulose at the C6-position to 6-aldehydo-D-glucose (Moilanen et al., 2014). An

oxidative attack of the TEMPO-oxoammonium ion on cellulose was reported earlier by Galli and Gentili (Galli and Gentili, 2004) and is the subject of several patents (Besemer and De, 1995; Jetten et al., 2000; Johanna et al., 1999). Oxidized groups in cellulose are supposed to hamper the performance of cellulases, especially of cellobiohydrolases and β-glucosidases, preventing full conversion of cellulose to glucose.

1.4.4 LMS treatment following and preceding physicochemical biomass pretreatment

The disintegration of biomass by physico-chemical pretreatment methods always exposes lignin on the biomass surface. Lignin is known to adsorb proteins and thereby is the most inhibiting factor during enzymatic hydrolysis of pretreated lignocellulose (Berlin et al., 2006; Chernoglazov et al., 1988; Converse et al., 1990). Especially, pretreatment by steam explosion was shown to promote unproductive binding of enzymes to lignin (Palonen et al., 2004; Rahikainen et al., 2013). The main reason for the positive effect on enzymatic cellulose hydrolysis of a LMS treatment following a physico-chemical pretreatment can be assumed to be the oxidation and functional modification of exposed lignin, whereas the often presumed disintegration or delignification by LMS treatment is of minor relevance. This becomes particularly obvious by the fact, that a mediator is not necessary to achieve enhanced cellulase performance by laccase treatment (Moilanen et al., 2011, 2014; Palonen and Viikari, 2004). In no present study mere LMS treatment of physico-chemically pretreated lignocellulose could be clearly demonstrated to result in removal or cleavage of lignin, but LMS treatment can only be considered as conditioning of lignin to enhance the efficiency of the subsequent enzymatic hydrolysis by reducing unspecific binding of enzymes.

In contrast, LMS pretreatment followed by alkaline peroxide extraction (APE) significantly enhances lignin removal and sugar recovery (Gutiérrez et al., 2012; Heap et al., 2014; Rico et al., 2014), e.g. the lignin content of eucalyptus wood and elephant grass decreased by up to 50% after a sequence of LMS and APE treatment in comparison to a slight decrease for APE without previous LMS pretreatment (Rico et al., 2014). The most important structural changes to lignin induced by LMS treatment were identified as C_α-oxidation of syringyl units using 2D-NMR analysis as Gutiérrez et al., 2012; Rico et al., 2014 (Figure 1.7 A). The synergism between LMS treatment and APE is mostly attributed to LMS-induced oxidation of lignin,

promoting its alkaline cleavage (Balakshin et al., 2001; Barreca et al., 2003; Fillat and Roncero, 2009; Heap et al., 2014): After LMS has oxidized the α-hydroxyl group to the corresponding α-ketone by hydrogen atom abstraction the C_α-C_β bond of the phenacyl-aryl ether is cleaved under alkaline conditions and in presence of hydrogen peroxide according to the mechanism published by Gierer et al. (Gierer et al., 1977; Heap et al., 2014) (Figure 1.7 B). This is in line with the natural lignin degradation which is also initiated by side chain oxidation and was referred to as "enzymatic combustion" by Kirk (Kirk and Farrell, 1987; Rico et al., 2014).

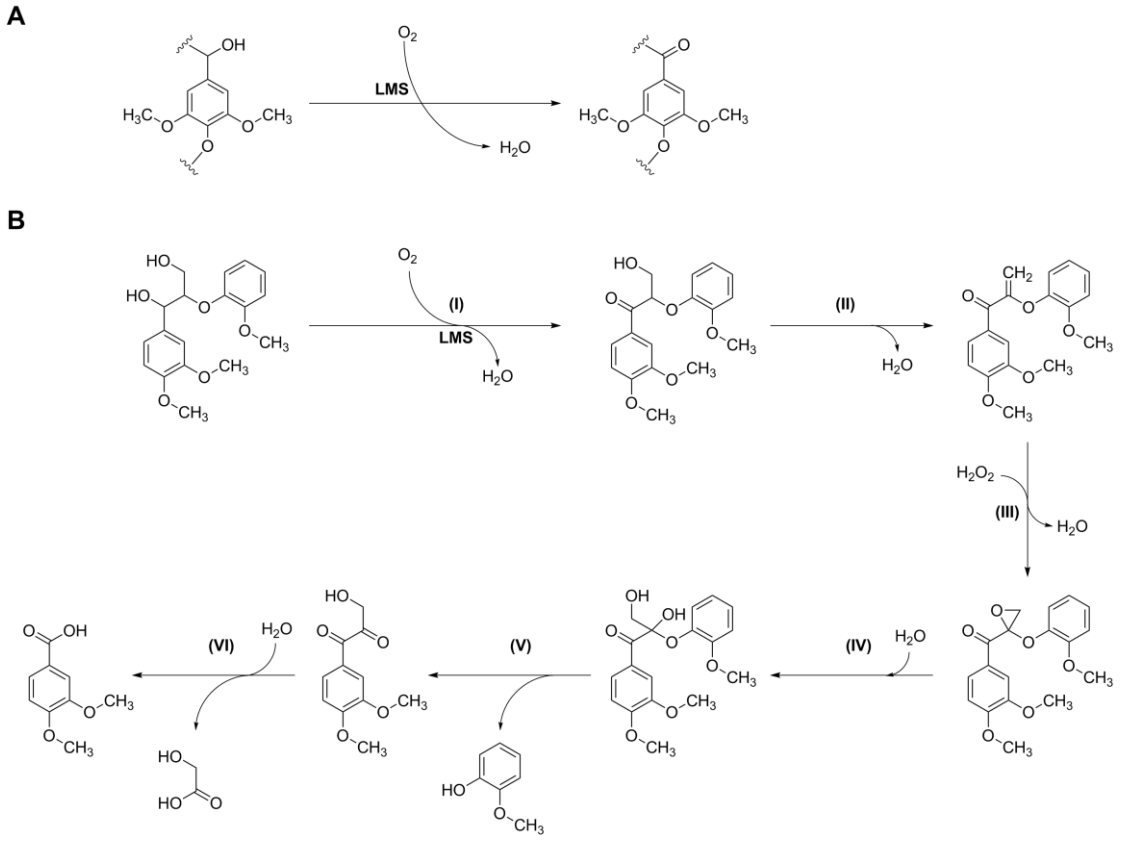

Figure 1.7: LMS treatment promotes alkaline cleavage of C_α-C_β bonds. A: Rico et al. found C_α-oxidation of syringyl units as the most important structural changes induced by LMS treatment (Rico et al., 2014). B: Reaction mechanism of lignin cleavage during alkaline peroxide extraction (APE) as proposed by Gierer et al. (Gierer et al., 1977). Reaction scheme according to Heap et al., 2014 and in Roth and Spiess, 2015. I: LMS-catalyzed oxidation, II: dehydration, III: epoxidation, IV: alkaline opening of epoxide, V: cleveage of hemiketal, VI: cleveage of 1,2-diketone (formely C_α-C_β bond).

Even if lignin cleavage by alkyl-aryl ether breakdown might occur to a certain extent during LMS pretreatment (Du et al., 2013), the dominating effect in all approaches is the oxidation of lignin reducing unspecific binding of cellulases or promoting chemical cleavage. Modern biorefinery concepts include steps for biomass disintegration and fractionation into the single biopolymers. The cellulose fraction, however, will contain impurities of residual or decomposed lignin. Therefore, laccase or LMS pretreatment promises to enhance the cellulase performance during subsequent enzymatic hydrolysis and thereby, limit their otherwise significant cost contribution to biofuel production.

1.5 Lignin depolymerization and modification by LMS

The economic feasibility of biorefinery concepts depends on the exploitation of the complete lignocellulosic biomass. While cellulose and hemicellulose are well accessible by current process routes, the generation of value from lignin is yet a challenge. Lignin represents the most abundant natural feedstock for phenolic and aromatic compounds which are currently only obtained from petrochemical resources. Hence, strategies for a targeted, selective breakdown of lignin are essential to tap the full potential of lignocellulosic biomass.

Biocatalysis is a promising approach to decompose the lignin polymer in a directed manner while preserving the natural phenolic phenylpropane subunits. Enzyme-catalyzed processes are state-of-the-art for the hydrolysis of the cellulose fraction of biomass even in production scale. In analogy to cellulose hydrolysis, enzymatic lignin depolymerization has been intensively researched during the last decades to complement biorefinery concepts.

1.5.1 How laccases arose as potential lignin degrading enzymes

When laccases were identified as part of the extracellular enzyme system of wood-decaying basidiomycete fungi (Fårhraeus, 1952; Law, 1950), their actual biological function and particularly the question whether laccases are involved in lignin polymerization or depolymerization was disputed controversially: Researchers split into two groups (Cho et al., 2004; Leonowicz et al., 1985) of followers (e.g. Ander and Eriksson, 1976; Eggert et al., 1997) and opponents (e.g. Haars and Hüttermann, 1980) of the hypothesis that laccase can cause lignin depolymerization. First clues of an active role of laccases in lignin degradation were inferred from experiments using whole cultures or unpurified culture supernatants (Ander and Eriksson, 1976; Eggert et al., 1997; Haars and Hüttermann, 1980; Kirk et al., 1968; Vliet, 1954), but a clear evidence that laccase alone can depolymerize lignin was not provided (Gierer and Opara, 1973). Kirk summarized the reasons, why the ability to degrade lignin was so often attributed to phenol oxidases like laccase: (I) Lignin degrading white-rot fungi produce extracellular phenol oxidases which can easily be detected by simple assays in contrast to the lignin non-degrading brown-rot fungi, (II) the polyphenolic structure of lignin makes it per se a substrate for phenol oxidizing enzymes and (III) the doctrine that lignin

degradation is an oxidative process perfectly fits to the kind of reaction catalyzed by phenol oxidases (Kirk, 1971). However, *in-vivo* experiments do not allow for drawing reliable conclusions regarding the capability of laccases for lignin depolymerization, but experiments under precisely controlled conditions with purified enzymes and defined substrates are mandatory.

1.5.2 Studies on lignin model compounds

A generally accepted way to test the catalytic properties of lignolytic enzymes are experiments with lignin model compounds (LMC) representing typical bonds or substructures in lignin. The β-O-4 ether linkage (Figure A.1, appendix) is the most prevalent bond within the lignin polymer with a ratio of 45 – 60% (Azadi et al., 2013; Zakzeski et al., 2009) and therefore, the primary target for a directed lignin degradation and the most studied bond in LMC-based investigations.

These β-arylether-dimer LMC are consistently subject to oxidization of the α-hydroxyl group to the α-carbonyl derivative by laccase (Figure A.1), but a suitable mediator is essential to overcome the redox barrier of this reaction (Barreca et al., 2003; Bourbonnais and Paice, 1990; Fabbrini et al., 2002a; Heap et al., 2014; Li et al., 1999; Srebotnik and Hammel, 2000). Besides this oxidation of adlerol to the adlerone form, several authors also report the LMS-catalyzed cleavage of the β-O-4-ether bond (Figure A.1). Moreover, even aromatic ring cleavage is possible with LMS (Kawai et al., 1999b). The careful comparison of the applied β-arylether-dimers and the obtained degradation products reveals that especially the substitution of the phenolic rings influences the cleavability of the β-O-4-ether-bond and alters the spectrum of reaction products. Noteworthy, LMC possessing a phenolic hydroxyl group lead to coupling reactions whether a mediator was present or not (Heap et al., 2014; Leontievsky et al., 1999; Rittstieg et al., 2002, 2003). LMS-catalyzed cleavage of the β-O-4 bond was exclusively demonstrated with LMC lacking phenolic hydroxyl groups ignoring the fact that this functionality is of widespread occurrence in lignin (0.2 phenolic hydroxyl groups per C_9 unit in softwood lignin; Chakar and Ragauskas, 2004b). Furthermore, 5-5 and β-1 linkages have been addressed with correspondent LMC as potential targets for LMS (Figure A.2 and A.3, appendix).

All obtained results report the successful cleavage of LMC containing the most relevant bonds present in lignin and thereby, suggest that LMS are able to degrade lignin. However, the focus lies often on a qualitative identification of degradation products to elucidate the reaction pathways, but not on the quantitative evaluation that shows that in most of the studies less than 20% of the starting material is converted into degradation products (Castro et al., 2003; Kawai et al., 1999a; Srebotnik and Hammel, 2000). Furthermore, each LMC only represents a detail of the heterogeneous lignin polymer. Therefore, it is doubtful whether those findings can be directly transferred to reaction systems containing LMS together with genuine lignin (Azarpira et al., 2013; Xu et al., 2014). Hence, the conclusions drawn from experiments with LMC as substrates have to be confirmed with real substrates.

1.5.3 Studies on lignin substrates

Lignosulfonates were the first substrates used for testing the ability of laccases for lignin degradation (Table A.3, appendix) (Haars and Hüttermann, 1980; Leonowicz et al., 1985). They are abundant byproducts from pulp and paper processes using sulfite pulping. Lignosulfonates have a high molecular weight which is considered to be representative for genuine lignin, and they have a good water solubility that promotes application under aqueous conditions.

Early on, lignosulfonate was incubated with a culture of the laccase secreting white-rot fungus *Fomes annosus* with a strong laccase inhibitor (Haars and Hüttermann, 1980). Instead of the expected reduction in lignosulfonate degradation, the inhibitor prevented the repolymerization of resulting low molecular weight compounds, which were not detectable when laccase activity was present (Haars and Hüttermann, 1980). Therefore, laccase seemed not to be essential for lignin degradation but to possess a strong polymerizing activity. Upon incubation of isolated laccase from *T. versicolor,* a low molecular weight lignosulfonate (1 kDa) was polymerized, whereas a high molecular weight fraction (97 kDa) with laccase resulted in new product fractions with decreased molecular weight (Leonowicz et al., 1985). Thus, Leonowicz et al. could confirm, that laccases possess both a polymerizing as well as a depolymerizing activity depending on the molecular weight of the substrate (Leonowicz et al., 1985), which appears logical as the ratio of potential cleavage and coupling sites correlates with the degree

of polymerisation. Indeed, the polymerization reactivity of *T. hirsuta* laccase was higher for lignins with lower molecular weight and a higher content of small phenolics (West et al., 2014). Summarizing, polymerization dominates over depolymerization and therefore, laccase-catalyzed depolymerization can never yield considerable amounts of low molecular weight degradation products.

The studies above did not explicitly include mediators, even if lignin derived low molecular compounds could fulfill the role of mediators. Mediators have always been proposed to shift the activity of laccase towards lignin degradation. When incubating kraft lignin with a purified laccase from *T. versicolor*, the lignin molecular weight strongly increased over time, whereas there was no significant change in presence of the mediator ABTS (Bourbonnais et al., 1995). When ABTS was later added to the reaction mixture, the polymerized lignin was depolymerized to the molecular weight of the starting material (Bourbonnais et al., 1995). Also the natural mediators acetovanillone and acetosyringone enhanced the depolymerization of fractionated lignosulfonates by a fungal laccase from *C. unicolor* in comparison to *C. unicolor* laccase alone (Cho et al., 2004). Bourbonnais et al.'s explanation that the mediator coupled covalently to the phenolic groups of lignin, blocking potential polymerization sites (Bourbonnais et al., 1995), was later confirmed for ABTS (Matsumura et al., 1986; Rittstieg et al., 2002). Reactions incorporating ABTS supposedly increase lignin water solubility and molecular weight, which might explain the often reported delignifying effect of this LMS in pulping processes (Bourbonnais et al., 1995). Therefore, the fate of the mediator during LMS reactions should always be addressed analytically and considered for the interpretation of the results.

1.5.4 Polymerization versus depolymerization

The mentioned consecutive polymerization and depolymerization was confirmed by Potthast et al. on an artificial system containing phenolic monomers which were initially polymerized by LMS to oligomeric products and reversely degraded afterwards (Potthast et al., 1999), whereas others found an initial occurrence of low molecular weight intermediates e.g. from a β-O-4 LMC with laccase and a subsequent polymerization even in presence of a mediator,

indicating repolymerization of monomeric degradation products (Leontievsky et al., 1999; Rittstieg et al., 2002, 2003).

In their review Leonowicz et al. concluded that the depolymerizing activity of laccases is of low efficiency and requires a rapid removal of reactive radical or quinone species to avoid coupling reactions and thereby, allow for lignin degradation (Leonowicz et al., 2001). Almost fifteen years later these findings are still valid: Even if several publications indicate the possibility to degrade lignin by use of LMS (Bourbonnais et al., 1995; Cho et al., 2004; Hernández Fernaud et al., 2006; Leonowicz et al., 1985; Shleev et al., 2006a), the large majority of studies describe polymerizing effects (Table A.3), regardless of whether mediators are present or not. To date no study on LMS exists which reliably reports a significant lignin degradation yielding considerable amounts of degradation products. This statement is in line with the review by Munk et al. published while this work was prepared (Munk et al., 2015). Many publications report the laccase-catalyzed polymerization of phenol and a wide range of its derivatives (Aktas and Tanyolaç, 2003; Ikeda et al., 1996a, 1996b; Mita et al., 2003; Potthast et al., 1999; Rittstieg et al., 2002, 2003; Sun et al., 2013). Since phenolic groups are widespread in lignin (Chakar and Ragauskas, 2004b) and its degradation products (Azadi et al., 2013; Xu et al., 2014), the structural properties of lignin itself intrinsically promote polymerization and thereby, counteract oxidative degradation. With respect to the role of laccase in lignin biosynthesis (Figure 1.3), it is not surprising that oxidative coupling reactions occur in presence of phenolic substrates. The dualism of conflicting polymerizing and depolymerizing activities is the widely accepted doctrine and at the same time the key challenge for realizing lignin degradation by LMS (Cho et al., 2004; Hernández Fernaud et al., 2006; Leonowicz et al., 1985; Majumdar et al., 2014; Youn et al., 1995).

1.5.5 Counteracting undesired repolymerization

Compartmentalization is a promising strategy to minimize undesired repolymerization of degradation products. In a proof-of-concept for membrane-based compartmentalization of a LMS reaction system, a dialysis membrane module (cellulose membrane, 10 kDa cutoff) separated ^{14}C-labeled artificial lignin and a fungal laccase from *Pycnoporus cinnabarinus*,

while the natural mediator 3-hydroxyanthranilic acid (3-HAA) was activated by laccase outside of the membrane module, and then after membrane diffusion degraded the synthetic lignin inside to degradation products, again detected outside via radioactivity measurements (Eggert et al., 1996). Potential drawbacks to membrane-based compartmentalization are the similar molecular weight of mediators and monomeric degradation products as well as loss of mediators during product separation and repolymerization caused by activated mediators at the membrane outside.

An alternative compartmentalization can be realized by multiphase reaction systems. Laccases are well compatible with a wide range of organic solvents that could solubilize lignin and allow for different partitioning of substrate and degradation products in a biphasic system. LMS reactions have been realized in biphasic systems combining aqueous buffers with organic solvents, but not yet for lignin processing (Arca-Ramos et al., 2012, 2014; Mustafa et al., 2005). Ionic liquids are interesting alternative solvents since they are able to dissolve and extract lignin (Viell et al., 2013), even if only to a low extent of 0.1 wt% in water-immiscible [BMIM][PF6] (Lee et al., 2009), and to stabilize laccases (Harwardt et al., 2014; Rehmann et al., 2012). Some water-immiscible ionic liquids in combination with suitable surfactants can form micro-emulsions preserving laccase activity and stability (Xue et al., 2011; Zhou et al., 2008). The partitioning of the mediator molecules like ABTS or TEMPO into the non-aqueous phase protects the enzyme from the inactivating effect of the oxidized mediator (Rehmann et al., 2013). The efficient compartmentalization using water-immiscible ionic liquids depends on the partitioning coefficients of all reactants, especially lignin, mediators and expected degradation products, but prize, eco-toxicity, compatibility with downstream processing and recycling have to be considered.

Also enzymes can be employed to counteract undesired repolymerization. The laccase-catalyzed oxidation of lignin leads to the formation of transitory quinoid intermediates or quinone degradation products which are known to undergo further polymerization. The elimination of these quinones would prevent repolymerization (Leonowicz et al., 2001). Glucose oxidase was found to accept quinones as electron-receiving co-substrates (Alberti and Klibanov, 1982) and combined with laccase forms a quinone-reducing redox cycle (Green, 1977) (Figure 1.8), supporting the laccase-catalyzed depolymerization of lignosulfonates (Szklarz and Leonowicz, 1986) and Björkman lignin (Leonowicz et al., 1999).

A similar mechanism was found for veratryl alcohol oxidase, requiring veratryl alcohol as electron donor (Marzullo et al., 1995). Westermark and Eriksson showed for the first time the reduction of *ortho* and *para* quinones by cellobiose:quinone oxidoreductases (CBQ) under simultaneous oxidation of cellobiose to cellobionic acid and proposed a cooperative interaction between CBQ and laccase during lignin degradation (Westermark and Eriksson, 1974). CBQ indeed decreased the polymerization of kraft lignin initiated by lignin peroxidase (Ander et al., 1990). Direct evidence that CBQ can avoid repolymerization also for LMS is yet lacking, but in the light of the current knowledge about CBQ (Eriksson et al., 1993; Henriksson et al., 2000), it appears worth testing those enzymes together with LMS for lignin depolymerization.

In nature fungi secrete a cocktail of enzymes that synergistically depolymerize lignin. Therefore, combining laccase with auxiliary enzymes to a multi-enzyme-system might help to avoid repolymerization and should be considered for future process development. All auxiliary oxidases discussed here accept lignocellulose-derived molecules (e.g. glucose, cellobiose, veratryl alcohol) as reducing agents, which is important for their economic integration into biorefinery processes.

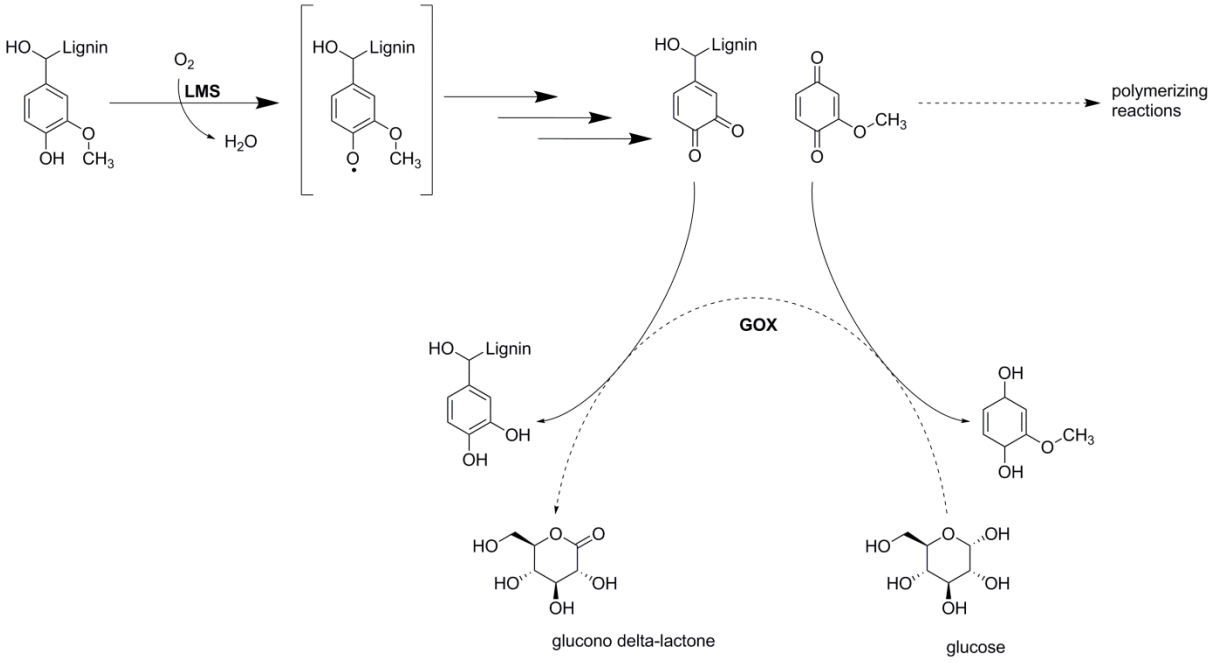

Figure 1.8: Reaction scheme for the reductive removal of quinones by glucose oxidase. According to Green, 1977 and in Roth and Spiess, 2015.

1.5.6 Laccase in a multi enzyme system for lignin bond cleavage

Picart et al. recently qualified β-etherase as a potential partner for laccase (Picart et al., 2014). β-etherases belong to the superfamily of glutathione transferases and selectively cleave β-O-4 bonds with a carbonyl group at the C_α-position (Figure 1.9). This C_α-carbonyl could be provided by LMS from the C_α-hydroxyl groups in lignin β-O-4 bonds. Unfortunately, fungal laccases are most active under acidic conditions, whereas β-etherases show highest activities in the alkaline range. To meet the pH optima of both enzymes, the two enzyme reactions should be performed stepwise. The pH shift between both reactions would inactivate laccase and thus prevent LMS-induced repolymerization and inactivation of β-etherase by radical laccase mediators.

The supply and recycling of oxidized glutathione (GSSG) as essential co-substrate of β-etherases was demonstrated by a NADH-dependent glutathione reductase from *Allochromatium vinosum* which was coupled to a NAD^+-reducing C_α-lignin-dehydrogenase closing the regeneration cycle of both co-factors (Reiter et al., 2013). In case of LMS for biorefinery applications, a suitable enzyme for NADH regeneration would have to be implemented into the multi-enzyme system.

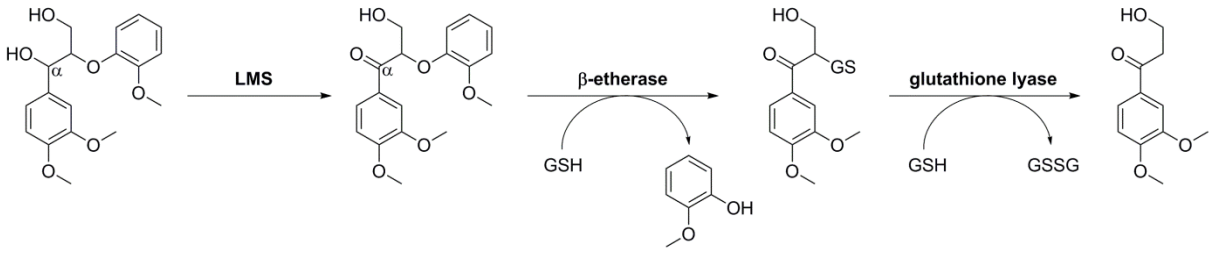

Figure 1.9: Potential synergism between LMS and a β-etherase in cleaving β-O-4 lignin bonds. Pre-oxidation of the C_α-hydroxyl group to the corresponding carbonyl by LMS followed by the stereoselective cleavage of the β-O-4 bond by the β-etherase. GSH: glutathione, GSSG: glutathione disulfide. Reaction scheme according to Picart et al., 2014.

1.6 Detoxification of biomass hydrolyzates

For efficient utilization of biomass, pretreatment methods disintegrate and foster further fractionation of the biomass (Kumar et al., 2009; Zhu and Pan, 2010), mostly involving harsh conditions and chemicals that lead to partial degradation of the biomass components. In particular lignin releases small phenolic fragments which can impede the following cellulose hydrolysis or fermentation, e.g. vanillin, vanillic acid, syringaldehyde, 4-hydroxybenzoic acid, acetovanillone or coniferyl alcohol (Chandel et al., 2012; Klinke et al., 2004).

Lignin-derived phenolics inhibit or even deactivate cellulases (Kim et al., 2011; Tejirian and Xu, 2011; Ximenes et al., 2010, 2011), especially at high solids loadings (Kim et al., 2011). The underlying mechanism is not fully understood but it is assumed that phenolic compounds complex with proteins and adsorb onto the surface of cellulose hampering its hydrolysis (Tejirian and Xu, 2011). Furthermore, phenolics in the fermentation stage partition into the cell membrane of the fermentation organisms and thereby, affect the integrity (Heipieper et al., 1994; Mussatto and Roberto, 2004; Palmqvist and Hahn-Hägerdal, 2000b). Generally, the removal of pretreatment-derived inhibitory phenolics from biomass hydrolyzates improves the yield of fermentative ethanol production in second-generation lignocellulose biorefineries (Table A.4, appendix).

Enzymatic methods for the removal of phenolics offer several advantages over physico-chemical methods like liming, alkaline or sulfite treatment, precipitation, extraction, steam stripping, or ion exchange (Olsson and Hahn-Hägerdal, 1996; Palmqvist and Hahn-Hägerdal, 2000a): The technical effort is relatively low since detoxifying enzymes only have to be added to the process stream, being compatible with cellulose hydrolysis or fermentation and waste water treatment. Laccases removing phenolic inhibitors from biomass hydrolyzates exploit their *in-vivo* function of eliminating toxic phenolic metabolites from the fungal lignin decomposition (Ander and Eriksson, 1976; Gierer et al., 1977; Haars and Hüttermann, 1980). The laccase-catalyzed detoxification is based on the radical coupling of small phenolic compounds to oligomeric products (Jönsson and Palmqvist, 1998; Jurado et al., 2009), which cannot partition into the cell membrane. The toxicity of phenolics therefore, inversely correlates with their molecular weight (Chandel et al., 2012; Mussatto and Roberto, 2004). Furthermore, oligomerization facilitates precipitation and removal from the process streams.

The elimination of phenols by laccases was found to be more efficient than physico-chemical approaches, such as liming, alkaline or sulfite treatment and comparable to anion exchange (Chandel et al., 2007; Larsson and Reimann, 1999; Martín and Galbe, 2002)

Laccases, however, do not affect carbohydrate-derived inhibitors like furfural or 5-hydroxymethylfurfural (5-HMF), which may be one reason why laccase detoxification alone does not necessarily lead to improved fermentation yields (Chandel et al., 2007). For fermentations with organisms sensitive to those compounds, activated charcoal sorbents or ion exchange resins unspecifically adsorb all classes of inhibitors, but at higher technical and material effort and lower specific removal of phenolics (Chandel et al., 2007; Larsson and Reimann, 1999). The combination of a laccase-catalyzed phenol removal with subsequent adsorption of carbohydrate-derived inhibitors promises a longer lifetime for the adsorbents.

The laccase-catalyzed detoxification of phenolics needs to be properly integrated with the adjacent process steps of the biorefinery. Laccase treatment can decrease sugar recovery in a subsequent enzymatic hydrolysis finally leading to lower ethanol yields (Jurado et al., 2009; Moreno et al., 2013b; Moreno and Ibarra, 2012). Laccase forms phenolic polymerization products in hydrolyzate slurries (Heap et al., 2014; Jurado et al., 2009), which may precipitate, and adsorb onto the cellulose fibers, thereby reducing the cellulase accessibility, or unproductively adsorb the cellulases as already shown for different types of lignins (Guo et al., 2014; Rahikainen et al., 2013). In case of high solid loadings, where high concentrations of phenolics hamper the enzymatic cellulose hydrolysis, laccase detoxification might therefore, not to be the method of choice but should be replaced by ion exchange or activated char coal adsorption. In a contradicting study, laccase treatment before enzymatic hydrolysis of solid-containing samples enhanced sugar yield (Lee et al., 2012), potentially due to similarities with LMS pretreatment, where the modified biomass surface decreases unspecific cellulase adsorption. Therefore, LMS catalyzed detoxification should be clearly defined as the removal of soluble monomeric phenolic contaminants, and be performed after enzymatic hydrolysis to avoid deposition of the polymeric products onto the cellulose fibers.

For an economically feasible detoxification, reuse of enzymes was demonstrated using laccase immobilized on Sepabeads® carriers that converted toxic phenolics from an organosolv hydrolyzate to polymerization products precipitating onto the immobilisates,

which could be removed by washing with 45% ethanol solutions (Ludwig et al., 2013). The laccase immobilizates were highly stable during this process, retaining around half their initial activity after five recycling and two washing steps (Ludwig et al., 2013). Immobilization would not only allow for recycling of the biocatalysts, but also for a continuous operation, for example in a packed-bed reactor.

While laccase-catalyzed detoxification of biomass hydrolyzates has been investigated for fermentative ethanol production with yeasts, no such studies exist for third generation biorefineries, which employ organisms such as *Aspergillus terreus* or *Ustilago maydis* to produce new platform chemicals like itaconic acid (Jäger and Büchs, 2012; Klement and Büchs, 2012). The demand of these organisms for hydrolyzate detoxification should be analyzed in future.

The need for laccase-catalyzed detoxification of phenolics from hydrolyzates becomes very obvious, considering the essential recycling of all aqueous streams like fermentation broth, or hydrolysis buffers in economically feasible processes: Accumulation of phenolics over time would increase the inhibitory effects on enzymes and fermentation organisms (Palmqvist et al., 1996) and increase the effort for treatment for waste water streams. Therefore, phenolics removal after biomass pretreatment should be integral part of the conceptual design of biorefinery processes.

1.7 Project objectives

This work was performed as part of the cluster of excellence "Tailor-made fuels from biomass". The TMFB cluster follows a holistic research approach for the conversion of lignocellulosic biomass into next generation biofuels. Therefore, the whole process chain from biomass disintegration, fractionation and molecular conversion to identification and combustion of promising fuel candidates is covered by interdisciplinary research projects. While in the first funding phase of TMFB (11/2007 – 10/2012) the research projects especially focused upon conversion and utilization of the carbohydrate fraction, in the second funding phase (11/2012 – 10/2017) special regard is paid to lignin to accomplish complete exploitation of lignocellulosic feedstocks. In the TMFB cluster lignin conversion is addressed by both chemo- and biocatalytic approaches. Following the second type of approach the core

objective of this project was the conceptual design of LMS-based reaction systems for the enzymatic processing of lignin and their reasonable implementation into a biorefinery process.

The biorefinery concept of the TMFB cluster favors an initial fractionation of the biomass by organosolv-like processes (Viell et al., 2013; vom Stein et al., 2011). The resulting lignin stream will contain appreciable amounts of the extraction solvent and it has to be expected that residual lignin in the solid cellulose fraction will influence the subsequent enzymatic hydrolysis. These circumstances defined the scope of investigations conducted within this research project:

An approach for LMS-catalyzed biomass conditioning was investigated (chapter 2). The aim was to develop an enzymatic pretreatment method to enhance the compatibility of physico-chemical pretreatment techniques like e.g. organosolv pulping and the subsequent enzymatic hydrolysis. The LMS pretreatment was supposed to promote the efficiency of cellulases by oxidative modifications of the surface lignin decreasing unproductive binding of the enzymes.

It is commonly accepted that biomass pretreatment influences the accessibility of cellulose for hydrolytic enzymes, but a quantitative description of this effect is still difficult. Therefore, CBM-based fluorescent protein probes were tested as a tool for the characterization of lignocellulosic biomass and the impact of pretreatment techniques on cellulose accessibility (chapter 3). This sub-project was performed in cooperation with Dirk Heesel from the chair for Molecular Biotechnology (Prof. Fischer, Dr. Ulrich Commandeur), who provided the *E coli* strains expressing the tested protein probes. The goal of this sub-project was to provide the TMFB cluster with a new assay technique for benchmarking lignocellulosic substrates concerning their actual accessibility for cellulases to assist the optimization of pretreatment methods with regard to this parameter.

The development of a LMS-catalyzed process for lignin modification and depolymerization was the core subject of this study (chapter 4). The conceptual design of such a process requires a detailed understanding of the reaction mechanisms and interactions of all components of the LMS. Furthermore, suitable reaction conditions compromising between the requirements of the LMS and the adjacent process steps in the biorefinery have to be identified. Therefore, the influence of typical mediator compounds and relevant solvents on

laccase activity and stability was studied. A simplified reaction system using a LMC as substrate was established to investigate the reaction of LMS with typical lignin structures. The applied β-O-4-type LMC was synthesized and provided by Jakob Mottweiler (IOC, Prof. Bolm) and used as model substrate by most of the TMFB research projects addressing lignin conversion.

The reaction of LMS with organosolv lignin was tested under different conditions. The lignin samples were analyzed with aqueous GPC. The method for GPC analysis of lignin was established and optimized in cooperation with Serafin Stiefel (AVT.CVT, Prof. Wessling) and Thomas Schmidt (DWI, Leibniz Institute for Interactive Materials) and shared with all TMFB projects dealing with lignin.

Chapter 2

Pretreatment of wooden biomass with a laccase-mediator-system – enzymatic biomass conditioning for more efficient cellulose hydrolysis

2.1 Introduction

One potential field of application for LMS in biorefinery processes is the pretreatment or conditioning of biomass in order to improve the compatibility between physico-chemical pretreatment and the subsequent enzymatic hydrolysis. Since hydrolytic enzymes are one of the major cost factors in biorefineries (Brijwani et al., 2010; Klein-Marcuschamer et al., 2012), the conditioning of the raw lignocellulose during pretreatment must aim at a most efficient performance of the cellulases to keep their required amount as low as possible. Especially lignin impairs the efficiency of cellulases since it restricts the access to cellulose and binds proteins unproductively (Berlin et al., 2006; Chernoglazov et al., 1988; Converse et al., 1990; Rahikainen et al., 2011). Enzymatic pretreatment is a promising way to render established process steps more efficient in order to decrease the demand of energy and chemicals.

The enzymatic pretreatment of biomass with LMS generally splits up into two approaches (chapter 1.4), namely (1) LMS treatment subsequent to a physico-chemical pretreatment (e.g. steam explosion) of the biomass to reduce unproductive adsorption of cellulases (Moilanen et al., 2011, 2014; Palonen and Viikari, 2004; Qiu and Chen, 2012) and (2) LMS treatment

followed by an alkaline extraction step (Gutiérrez et al., 2012; Heap et al., 2014; Rico et al., 2014).

This study (Kress, 2013; designed and supervised by Simon Roth) shares most aspects with the first type of approach for LMS pretreatment aiming at a reduction of unproductive cellulase binding by oxidative lignin modifications, but also addresses the interplay of LMS and physico-chemical pretreatment by subsequent freeze-drying or incubation in the ionic liquid [EMIM][Ac]. To our knowledge, this is the first study investigating the effect of a LMS pretreatment on raw wood and in combination with freeze-drying and IL pretreatment.

2.2 Materials and Methods

2.2.1 Chemicals

All chemicals were of analytical-reagent grade or higher and purchased from Sigma Aldrich (Hamburg, Germany) or Carl Roth (Karlsruhe, Germany), if not stated otherwise. Carboxymethyl cellulose 4M is a product of Megazyme International (Wicklow, Ireland). The ionic liquid 1-ethyl-3-methylimidazolium acetate (EMIM Ac) was kindly provided by BASF (Ludwigshafen, Germany).

2.2.2 Lignocellulosic substrates

Wood chips from beech (“Räuchergold” KL 1/4, JRS – J. Rettenmaier & Söhne GmbH + Co. KG, Rosenberg, Germany) and spruce (“Räuchergold” KL 9, JRS – J. Rettenmaier & Söhne GmbH + Co. KG, Rosenberg, Germany) were milled in a bench scale shredder (Waring Laboratory Blender, Waring, Torrington, USA) and sieved to defined particle sizes using a sieve shaker (AS 200 digit, Retsch, Haan, Germany).

2.2.3 Enzymes

A powdery commercial preparation of laccase from *Trametes versicolor* (51639, Sigma-Aldrich, St. Louis, USA) was used unpurified. The commercial cellulase mixture from *Trichoderma reesei* Celluclast® 1.5 L ATCC26621 (Novozyme, Bagsværd, Denmark) utilized for the hydrolysis of lignocellulosic samples was desalted and rebuffered according to the protocol published by Jäger et al. (Jäger et al., 2010) using column chromatography with an Äkta FPLC system and a HiPrep 26/10 desalting column (GE Healthcare, Buckinghamshire, UK). Prior to FPLC purification, Celluclast® was diluted (1:1 v/v) in 0.1 M sodium acetate buffer (pH 4.5). The same buffer was used as running buffer for FPLC.

2.2.4 Enzyme activity assays

Laccase activities were quantified using the ABTS assay adopted from Harwardt et al. and Bourbonnais et al. (Bourbonnais et al., 1995; Harwardt et al., 2014). The assay mixture contained 0.5 mM ABTS, 0.1 M sodium acetate buffer (pH 4.5) and 30 mg/L of commercial laccase preparation. The reaction was started by adding the diluted laccase sample (0.1 g/L powder in sodium acetate buffer). The oxidation of ABTS was measured spectrophotometrically at 420 nm for 2 min at 30 °C in a Synergy Mx microplate reader (Biotek, Winooski, VT, USA). The enzyme activity was calculated from the initial reaction rate determined from the linear slope of the progress curves. 1 Unit was defined as the amount of enzyme oxidizing 1 µmol of ABTS in 1 min at 30 °C and pH 4.5.

For the quantification of cellulase activities the hydrolysis rate of carboxymethylcellulose (CMC) was measured. The CMC substrate solution was prepared by suspending 10 g/L CMC in 0.1 M sodium acetate buffer (pH 4.5) under continuous stirring over 48 h at 90 °C to achieve maximum dissolution. The slightly turbid, homogeneous substrate solution was aliquoted and stored at -20 °C until use. To determine the cellulase activity, assay mixtures consisting of 5.4 mL CMC solution and 0.6 mL enzyme sample were incubated at 40 °C and sampled at 10 min intervals over 2 h. Samples were inactivated for 10 min at 90 °C, solids were spun down and the glucose concentration in the sample supernatants was determined by HPLC. The cellulase activity was calculated from the linear increase of glucose concentration

over time. 1 Unit was defined as the amount of enzyme releasing 1 µmol glucose per minute at 40 °C and pH 4.5.

To calculate specific enzyme activities, the applied protein amount has to be related to the measured enzyme activity. Protein concentrations were determined by the bicinchoninic acid assay (Smith et al., 1985) using the BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA) and bovine serum albumin as a standard according to the manufacturers' protocol. The absorbance at 562 nm was measured with a Synergy Mx microtiter plate reader (Biotek, Winooski, VT, USA).

2.2.5 Pretreatment of lignocellulose

The procedure for pretreatment of the lignocellulosic material prior to enzymatic hydrolysis includes different methods whose sequence was altered for different experimental approaches (Figure 2.1). All experiments were performed according to the protocol of Kress as described in Kress, 2013.

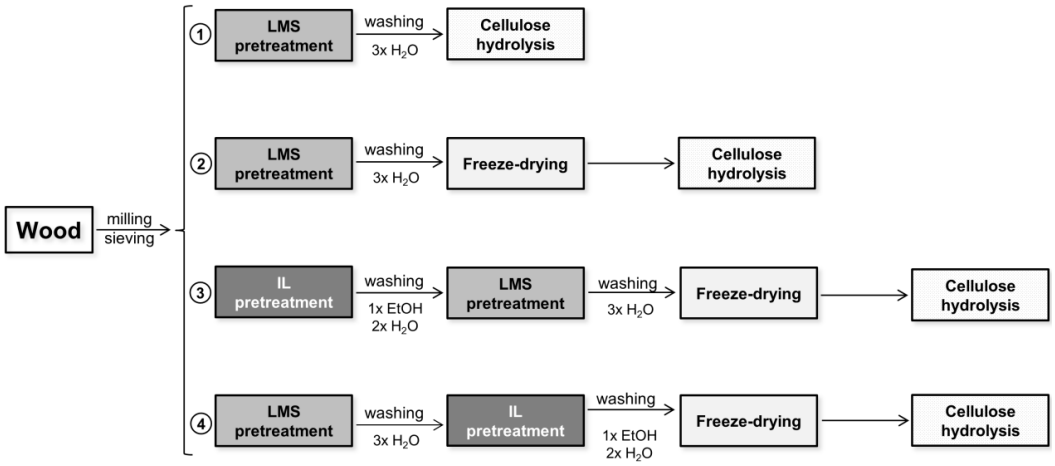

Figure 2.1: Overview of the experimental approaches for LMS pretreatment of lignocellulose.

The enzymatic LMS pretreatment of lignocellulose was conducted in shake flasks, using 20 mL 0.1 M sodium acetate buffer (pH 4.5), 5 % (m_{DW}/V) lignocellulose, 60 $U_{ABTS}/g_{lignocellulose}$ laccase, and 5 mM mediator if not stated otherwise. A blank experiment only containing lignocellulose in buffer was conducted as reference. Bovine serum albumin

(BSA) was used as non-catalytic protein reference in corresponding concentrations. The shake flasks for LMS pretreatment were sealed with cotton plugs and incubated at 40 °C and 200 rpm for 48 – 72 h. The lignocellulose was then separated from the reaction broth using a vacuum driven Büchner funnel and washed three times with de-ionized water.

Freeze-drying of lignocellulosic samples was realized using a laboratory freeze-dryer VaCo 2 (Zirbus technology GmbH, Bad Grund, Germany). Before freeze-drying, samples were flash-freezed in liquid nitrogen.

For pretreatment with ionic liquid (IL), 5 % (m_{DW}/V) of the powdery lignocellulose were mixed with [EMIM][Ac] and incubated at 100 °C for 2 h. The solids were separated from the ionic liquid, washed once with ethanol (70 % v/v) and twice with de-ionized water. For solid-liquid-separations during this procedure a vacuum driven Büchner funnel was used.

2.2.6 Enzymatic hydrolysis of lignocellulose

The enzymatic hydrolysis was performed with 1 % (m_{DW}/V) lignocellulose and 10 % ($m/m_{lignocellulose}$) purified Celluclast® in 0.1 M sodium acetate buffer (pH 4.5) at 40 °C. Samples were taken over time and analyzed via HPLC as described above for the CMC assay. Each experiment was conducted with three biological replicates. Immediately after starting the reaction by adding the cellulase preparation a first sample was taken, defined as blank and the determined glucose concentration was subtracted from all further sample values.

2.2.7 HPLC analysis of sugar concentrations

Glucose concentrations were determined via HPLC (Dionex Ultimate 3000, Thermo Scientific, Waltham, USA) with an organic acid resin column (CS-Chromatography, Langerwehe, Germany), 5 mM H_2SO_4 as mobile phase and an refractive index detector (RI-101, Shodex, Munich, Germany). The flow rate of the mobile phase was 0.8 mL/min and the oven temperature was 60 °C. The standard retention time for glucose was 6.5 min.

2.3 Results and discussion

2.3.1 Evaluation of suitable mediators for LMS pretreatment

The effect of LMS pretreatment on raw beech wood was addressed by incubating the lignocellulose with laccase alone and in combination with different mediators. While ABTS and HBT are known as classical synthetic molecules mediating the laccase reaction (Bourbonnais et al., 1998) syringaldehyde (SA) belongs to the group of natural mediators with a lignin-related syringyl structure (Camarero et al., 2005; Canas and Camarero, 2010). To evaluate the impact of laccase and the different mediators on the cellulose accessibility within the lignocellulosic composite, an enzymatic saccharification subsequent to the LMS pretreatment was conducted as an analytical benchmark (Figure 2.2). Therefore, saturating enzyme loadings (10% $m/m_{lignocellulose}$) beyond the economically feasible range were used to avoid limitations in the applied cellulase activities even when substrates of different particle size were compared.

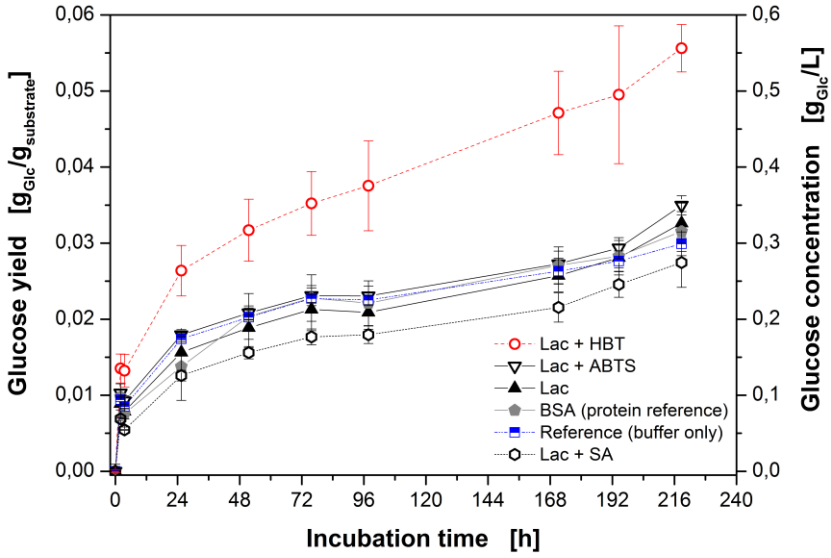

Figure 2.2: Influence of mediator choice on glucose release during a subsequent cellulose hydrolysis. Beech wood (1 % m_{DW}/V, particle size < 0.6 mm), 5 mM mediator, freeze-drying after LMS pretreatment.

All progress curves show the typical non-linear shape with a saturation-like behavior originating from overlaying effects like product inhibition, enzyme inactivation and substrate

heterogeneity (Desai and Converse, 1997; Luterbacher et al., 2013; Zhang et al., 1999). Since the degree of disintegration as well as the portion of hydrolysable cellulose in raw or LMS pretreated wood is significantly lower than in pretreated and partially delignified substrates, all experiments conducted with raw wood resulted in minor mass-related sugar yields (maximum 0.056 $g_{glucose}/g_{lignocellulose}$). For the experiment depicted in Figure 2.2, wood chippings were only roughly sized (< 0.6 mm) after milling, leading to increased standard deviations due to a higher substrate heterogeneity. The recurring rise in glucose release rate after 168 h or 192 h, respectively, was only observed for the experiment with the largest particle size (< 0.6 mm, Figure 2.2) and most likely results from experimental artefacts like time-depending swelling of the biomass or loss of volume by evaporation.

Pretreatment by laccase together with ABTS did not influence the cellulose hydrolysis. A pretreatment with laccase and syringaldehyde reproducibly leads to lower sugar yields, probably due to polymerization reactions on the biomass surface (Fillat et al., 2012; Liu et al., 2009; Rittstieg et al., 2002). In contrast to other studies where an enhancing effect of laccase was observed without a mediator (Moilanen et al., 2011, 2014), in our experiments laccase alone slightly decreased the obtained sugar yields, potentially also because of polymerization effects. However, these differences might result from the structural and chemical changes induced by the SO_2 and steam pretreatment before LMS treatment as conducted in (Moilanen et al., 2011, 2014). Furthermore, a covalent coupling of laccase itself on the biomass surface by a Michael addition of amino groups of laccase to phenyl groups of the surface lignin (Witayakran and Ragauskas, 2009) can impair substrate accessibility and hamper cellulase performance. BSA had no effect on the cellulose hydrolysis excluding effects by the LMS pretreatment that were only due to the presence of protein.

Only the combination of laccase and HBT had an enhancing effect on cellulose hydrolysis represented by significantly higher glucose yields and an increased initial hydrolysis rate (Figure 2.2). This result is consistent with HBT being one of the strongest mediators for laccase reactions (Bourbonnais et al., 1997; Cantarella et al., 2003b) and several studies reporting HBT as suitable mediator for oxidative biomass conditioning with LMS (Chen et al., 2012; Gutiérrez et al., 2012; Heap et al., 2014; Lee et al., 2012).

In a different study HBT was the least effective under the tested mediators because it is oxidized very slowly by the applied laccase from *Trametes hirsute* (Moilanen et al., 2014).

The underlying mechanism of the enhancing effect of LMS pretreatment on enzymatic cellulose hydrolysis is not fully understood yet, but all published results indicate a functional modification of lignin on the substrate surface as the main reason for a better cellulase performance. Carboxylic groups on the surface of steam-pretreated soft wood increased after LMS treatment, presumably decreasing the unspecific adsorption of cellulases as a consequence of the reduced hydrophobicity and a more negative charge of the material surface (Palonen and Viikari, 2004). By means of a raise in the content of acid soluble lignin after LMS treatment, the correlation between LMS-catalyzed lignin oxidation and a reduced unproductive binding of cellulases could be clearly demonstrated (Moilanen et al., 2014). In summary, the prevention of unproductive binding of cellulases by oxidative modification of lignin is the accepted hypothesis to explain the enhancing effect of LMS pretreatment subsequent to a physico-chemical pretreatment on the enzymatic cellulose hydrolysis. The experimental conditions of our study might even foster this effect: The mass-related accessible surface of untreated wood is very low compared to pretreated substrates. In addition, we used high cellulase loadings potentially saturating the unspecific binding sites on the substrate surface, thereby, hampering substrate access for the free cellulases.

2.3.2 Influence of the mediator HBT on LMS impact and cellulose hydrolysis

After identifying HBT as suitable mediator for LMS pretreatment of lignocellulose (Figure 2.2), it was of particular interest to further characterize the increased glucose release and to distinguish a reactive or adsorptive effect of HBT from an enzymatically induced reaction.

The incubation of beech wood with two concentrations of HBT (5 and 10 mM) without laccase reproducibly led to slightly increased sugar yields during the subsequent hydrolysis (Figure 2.3, open blue symbols). This could either be due to a reactive modification of the lignocellulosic surface by HBT or due to HBT adsorption. A non-covalent adsorption of HBT to the biomass surface could change hydrophobicity and polarity and thereby, reduce the unproductive binding of the cellulases. Furthermore, the blocking of cellulase binding sites by

adsorbed HBT might be an explanation. The concentration dependency of the enhancing effect of HBT on the enzymatic hydrolysis matches with both hypotheses of reactive surface modification and HBT adsorption. However, an adsorptive effect would require a very strong interaction between HBT and the lignocellulose since it had to endure the washing step following the LMS pretreatment (cp. Figure 2.1). Further investigations of this phenomenon are advisable, especially regarding the fate of the mediator.

As expected, the combination of laccase and HBT further increased the obtained sugar yields compared to experiments without enzyme (Figure 2.3, closed symbols). It is widely accepted that LMS oxidizes the surface lignin and thereby, reduces unproductive adsorption of cellulases. Nevertheless, the glucose release profile found for the experiment with HBT and laccase most likely results from a combination of LMS-catalysed lignin oxidation and the effect of HBT in absence of laccase.

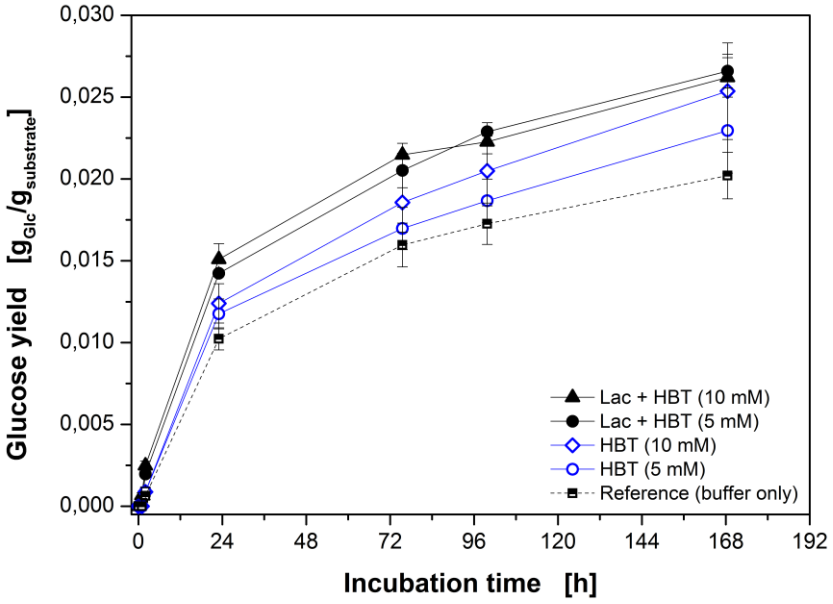

Figure 2.3: Influence of mediator HBT on the impact of LMS pretreatment and the subsequent glucose release during cellulose hydrolysis. Beech wood (1 % m_{DW}/V, particle size 200-250 µm), freeze-drying after LMS pretreatment.

2.3.3 Influence of particle size on the impact of LMS pretreatment

The inverse correlation of particle size and specific surface area leads to higher cellulose hydrolysis rate and yield for smaller substrate particles. The same effect might be true for the impact of the LMS pretreatment, since more target molecules may be accessible for LMS-induced surface modifications. Therefore, the influence of the particle size was addressed performing pretreatment experiments with milled beech wood sized with sieves of two different mesh sizes (< 100 µm / 200 – 250 µm). In all runs, LMS pretreated material was compared with a reference incubated in pure buffer (Figure 2.4).

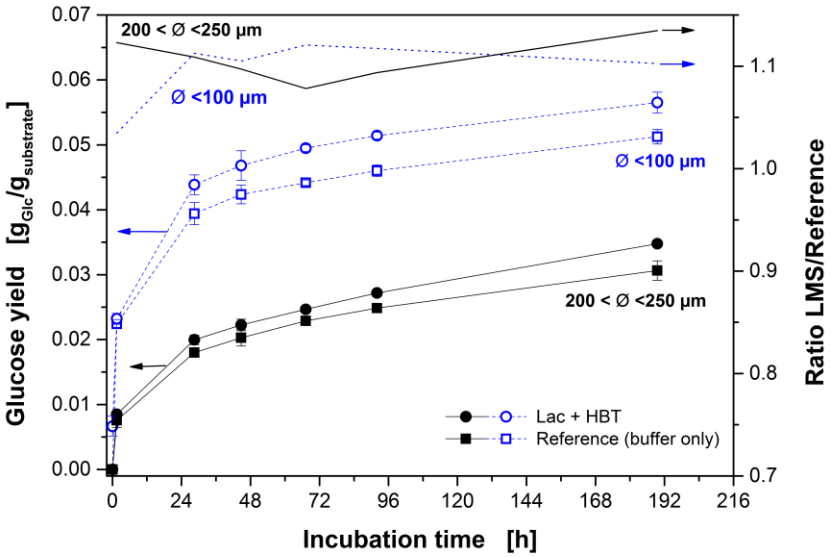

Figure 2.4: Influence of particle size on the impact of LMS pretreatment and the subsequent glucose release during cellulose hydrolysis. Beech wood (1 % m_{DW}/V, particle size < 100 µm / 200-250 µm), 5 mM HBT, freeze-drying after LMS pretreatment. Ratio of hydrolysis yields with and without LMS pretreatment reflects improvement factor of LMS pretreatment.

As expected, smaller particle sizes led to higher hydrolysis rates and yields both with and without LMS pretreatment (Figure 2.4). To evaluate the impact of the LMS pretreatment irrespectively of the two tested particle sizes, the ratio of the glucose yield of samples with LMS pretreatment to reference samples without LMS pretreatment was calculated. These values represent the factor by which LMS pretreatment enhanced the hydrolysis yields and amounted to ~1.1 for both particle sizes, excluding correlation between particle size and

impact of the LMS pretreatment. Probably, the efficiency of the applied LMS is high enough to oxidize all potential reaction sites on the substrate surface.

2.3.4 LMS pretreatment does not affect the hydrolysis of spruce

The positive results obtained with the LMS pretreatment of beech, representing a hard wood species, motivated comparative experiments with softwood from spruce (Figure 2.5). The positive effect of the LMS pretreatment of beech could not be found for spruce, most likely due to differences in structure and composition of these wood sources, especially, in the chemical properties of lignin. The higher content of lignin and resins in spruce might also impede the disintegrating effect of the freeze-drying step.

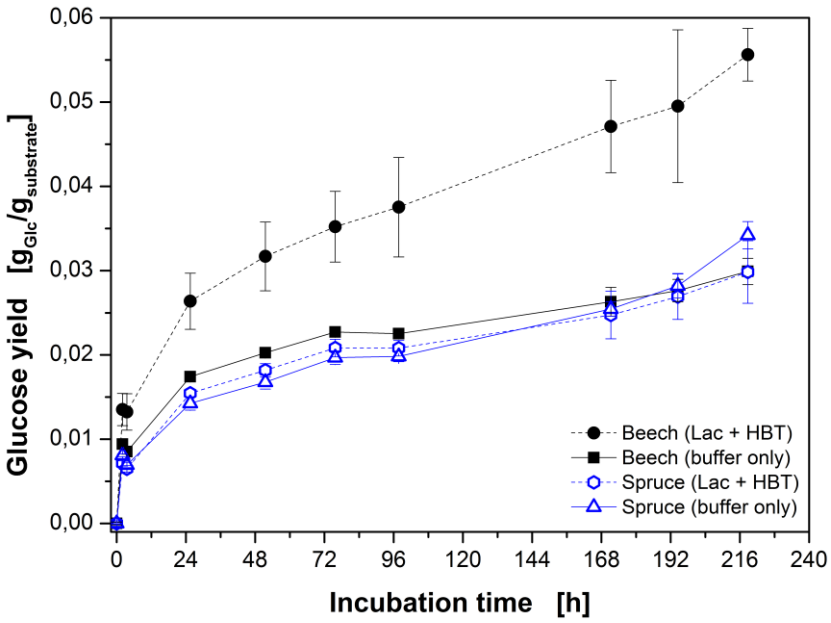

Figure 2.5: Influence of LMS pretreatment on beech in comparison to spruce. Beech / spruce wood (1 % m_{DW}/V, particle size < 0.6 mm), 5 mM HBT, freeze-drying after LMS pretreatment.

A positive effect of a laccase treatment on the enzymatic hydrolysis of SO_2 and steam-pretreated spruce was found (Moilanen et al., 2011). However, the opposite was shown for SO_2 and steam-pretreated giant reed which could be explained by a different chemical structure and composition of the lignin of both plant species (Moilanen et al., 2011). Since the physico-chemical pretreatment with SO_2 and steam most likely changes the structural and

chemical properties of the lignin these results are not directly comparable with ours. As typical for soft wood, spruce lignin mainly consist of guaiacyl subunits and lacks syringyl subunits which are characteristically found in hard wood lignins (Moilanen et al., 2011; Rencoret et al., 2009). Since the C_α-hydroxyl groups of syringyl subunits are a main target for LMS-catalyzed lignin oxidation (Rico et al., 2014), this might explain why in our study LMS pretreatment had no effect on the enzymatic cellulose hydrolysis of spruce.

2.3.5 Influence of a freeze-drying step on LMS impact and cellulose hydrolysis

Our standard protocol applied for the LMS pretreatment of lignocellulose included freeze-drying of the samples to ease storage and transfer to the subsequent hydrolysis step. The influence of the freeze-drying step was addressed with a series of experiments, comparing runs with and without freeze-drying based on the same dry weight amount of lignocellulose. Figure 2.6 reveals that freeze-drying of the biomass prior to the enzymatic hydrolysis significantly increased the initial hydrolysis rates (2.6 – 3.8x) as well as the obtained sugar yields (1.9 – 2.2x). The positive effect of the LMS pretreatment was clearly enhanced by subsequently freeze-drying the samples (from 1.11x to 1.31x increase of glucose yields).

The freezing of the wet wood samples most likely leads to an expansion and loosening of the fiber composite by an increase in volume of the contained pore water which then evaporates during vacuum drying. The resulting extension of the specific surface area of the material makes the cellulose more accessible for cellulases and enhances hydrolysis rate and yield. Therefore, freeze-drying shares the effect of surface and cellulose accessibility increase with pretreatment methods like e.g. steam explosion. While cellulose is packed into structurally organized fiber bundles, lignin acts as the cement in the lignocellulosic composite and fills the space between the cellulosic fibers. It can be assumed that after breakup of the lignocellulosic composite due to freeze-drying, lignin still adheres to the outside of the intact cellulose bundles so that more lignin per surface is exposed and can hamper the cellulases by unproductive binding.

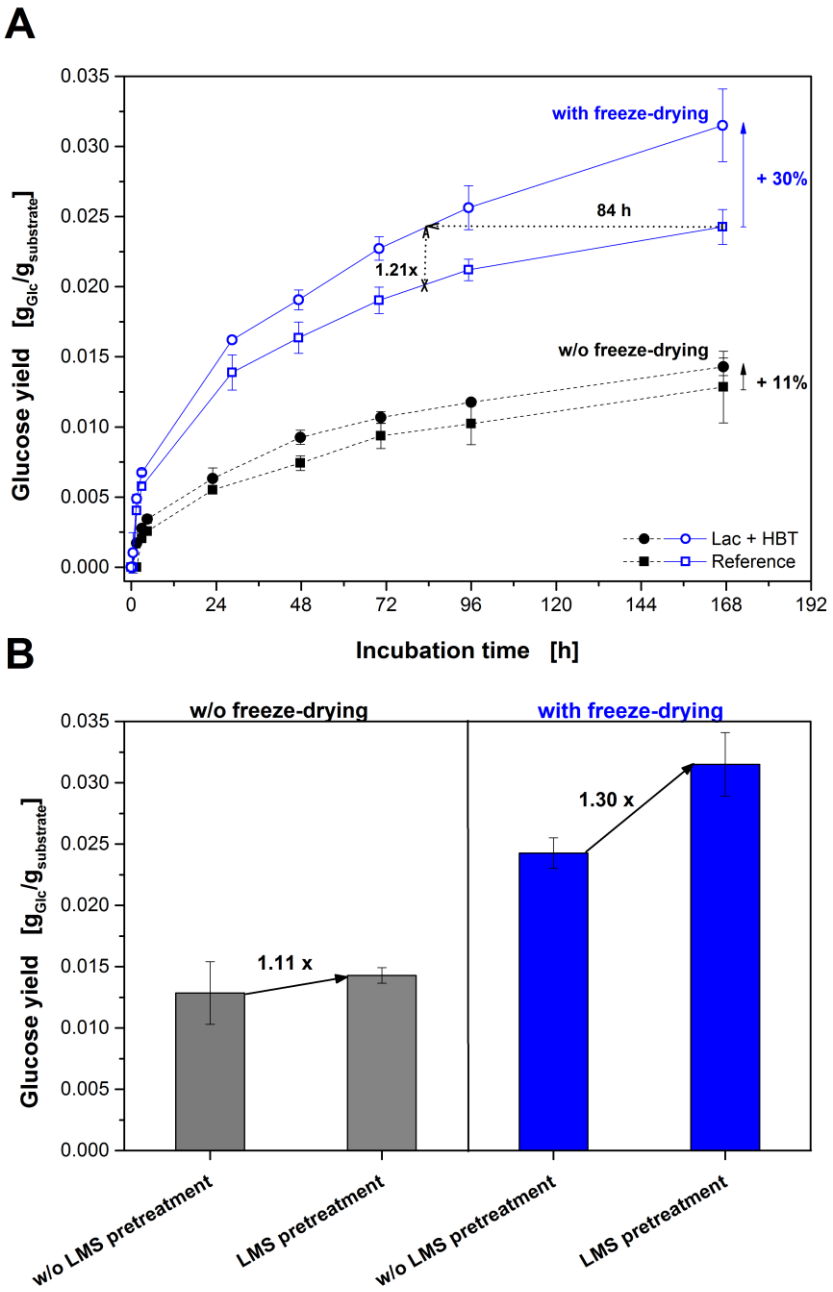

Figure 2.6: Influence of sample freeze-drying on impact of LMS pretreatment and cellulose hydrolysis. Beech wood (1 % m_{DW}/V, particle size 200-250 µm), 5 mM HBT, with and w/o freeze-drying after LMS pretreatment. A: Time-resolved glucose release during enzymatic hydrolysis. Numbers and arrows indicate acceleration of cellulose hydrolysis and increase of glucose yields due to LMS pretreatment. B: Comparison of obtained glucose yields after 168 h.

This hypothesis would explain why the impact of the LMS pretreatment is more intense after freeze-drying. Also the fact that laccase alone has no or even a negative influence (Figure **2.2**)

on cellulose hydrolysis, supports this theory: The physical freeze-drying treatment is performed after LMS treatment. When LMS is applied, the lignin is still packed in the lignocellulosic composite. The small mediators penetrate deeper into the material and can thereby reach lignin structures which are sterically not accessible for laccase itself and are later exposed on the material surface due to the effect of freeze-drying.

Besides increasing the final glucose yields, the LMS pretreatment also accelerates the enzymatic cellulose hydrolysis significantly (cf. arrows in Figure 2.6 A): The final glucose yield of the reference experiment (0.024 $g_{glc}/g_{substrate}$) obtained within 168 h was already reached after 84 h when the lignocellulose was LMS-pretreated. This 50 % process time reduction nicely demonstrates the synergism between LMS pretreatment and enzymatic cellulose hydrolysis and qualifies it as an interesting method for biomass conditioning subsequent to a physical pretreatment method. While freeze-drying represents a typical lab scale procedure, scalable mechanical pretreatment steps using e.g. cutting mill or screw press (Yan and Miazek, 2014) could benefit from a subsequent LMS treatment even in industrial scale. The synergism between steam pretreatment and subsequent LMS pretreatment has already been demonstrated by the group around Viikari (Moilanen et al., 2011, 2014; Palonen and Viikari, 2004).

2.3.6 Combination of LMS and ionic liquid pretreatment

The disintegration or dissolution of polysaccharides and lignin from lignocellulose with ionic liquids (IL) is a pretreatment method for biorefinery processes (Viell and Marquardt, 2011; Zavrel et al., 2009). Even if IL pretreatment can remove the major part of lignin, it must be assumed that residual lignin will remain on the cellulose fibres and influence the enzymatic hydrolysis.

Here, we intended to address the two questions whether (1) LMS pretreatment of raw beech wood before the IL pretreatment can assist lignin dissolution by oxidative lignin modifications and whether (2) LMS pretreatment subsequent to the IL pretreatment can improve the enzymatic cellulose hydrolysis by modifying the residual lignin and thereby, reducing the unproductive binding of cellulases.

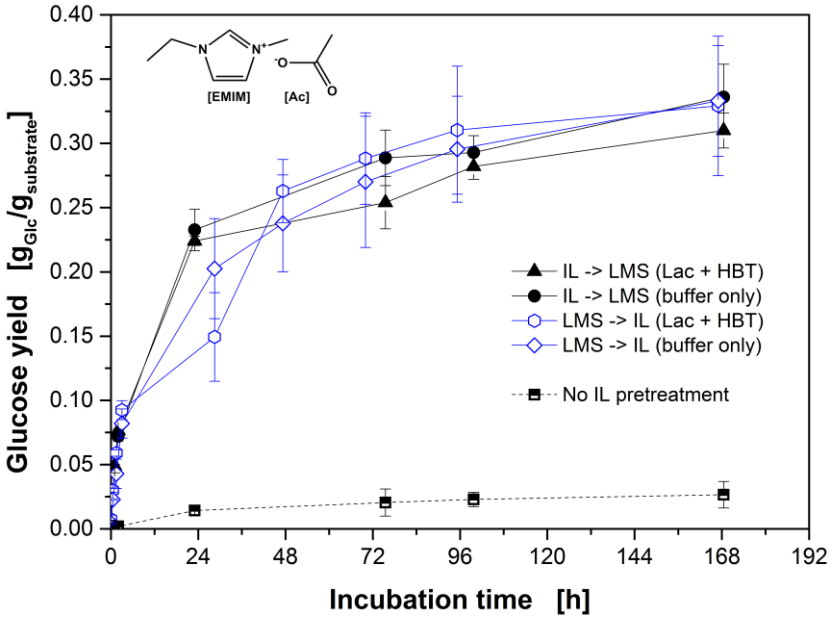

Figure 2.7: Combination of LMS pretreatment with IL pretreatment. Beech wood (1 % m_{DW}/V, particle size 200-250 µm); pretreatment with [EMIM][Ac]: 5 % ($m_{lignocellulose}/V_{IL}$), 5 mM HBT, freeze-drying after LMS pretreatment.

The extensive impact of the IL pretreatment on wooden lignocellulose and the subsequent cellulose hydrolysis becomes obvious by the huge difference between the results of the reference and the IL-pretreated samples (Figure 2.7). Due to the strong disintegrating and delignifying effect of the [EMIM][Ac] pretreatment, the cellulose hydrolysis rate and yield are one order of magnitude higher. For benchmarking the results it should also be considered that the relative portion of digestible cellulose of the IL-pretreated material is strongly increased due to the removal of lignin and hemicellulose.

No significant influence of the LMS pretreatment, either before or after the IL pretreatment, could be found. Either the extent of delignification by [EMIM][Ac] is high enough to reduce the residual lignin below inhibiting amounts, or the IL treatment influences the structure and chemical functionality of the residual lignin in a way that unproductive binding of cellulases is prevented. A synergism between LMS pretreatment and a subsequent IL treatment as demonstrated for LMS pretreatment combined with subsequent alkaline extraction (Heap et al., 2014) was not found. This result also indicates that in contrast to pretreatment methods like e.g. steam explosion, an unproductive binding of cellulases to residual lignin on the material surface is not an issue for IL-pretreated lignocellulose. This potential advantage

should be confirmed by further adsorption studies (Moilanen et al., 2014) and by experiments at lower, non-saturating cellulase loadings. If indeed no unproductive protein binding occurs after IL pretreatment, the decreased cellulase requirements might compensate for the disadvantages attributed to ionic liquids such as ecotoxicity, recycling issues or costs.

2.4 Conclusions and outlook

We have investigated the capability and feasibility of LMS pretreatment for biomass conditioning to improve efficiency of enzymatic cellulose hydrolysis (Kress, 2013). While LMS pretreatment of raw wood was inspired by the natural example of white-rot fungi, freeze-drying and IL pretreatment address the potential synergism between LMS pretreatment and further disintegrating pretreatment techniques.

The impact of LMS pretreatment on the hydrolysis of raw wooden biomass was quite low. Only in combination with a freeze-drying step a significant influence on rate and yield of the subsequent cellulose hydrolysis was observed. It can be assumed that freeze-drying leads to a disintegration of the biomass and, hence, an increased surface area displaying lignin, but the particular influence on the biomass structure should be further investigated. The effect of freeze-drying is most likely similar to physico-chemical pretreatment-methods like e.g. steam explosion, but to a much lower extent.

It was shown by several studies that the enhancing impact of LMS treatment subsequent to physico-chemical methods originates from oxidative modifications of the residual surface lignin, represented by an increase in the content of acid soluble lignin after LMS treatment (Moilanen et al., 2014), and a resulting decrease in the unproductive binding of the cellulases (Moilanen et al., 2011, 2014; Palonen et al., 2004; Palonen and Viikari, 2004). We suggest that our experimental findings share this mechanism. Further adsorption experiments should however be conducted to confirm this mechanism and to clarify the effect of HBT in absence of laccase. Also, structure-elucidating methods like HSQC would help with a deeper understanding of the underlying mechanisms. Our experiments with spruce wood demonstrate that the effect of LMS pretreatment on lignocellulose strongly depends on the type of biomass or the particular lignin characteristics, respectively. A synergism between LMS and IL

pretreatment was not found, probably because of the extensive delignification or structural lignin modifications caused by the IL treatment. In the light of previous studies our findings affirm that in combination with disintegrating pretreatment steps LMS treatment is a promising method for biomass conditioning in order to enhance the efficiency of enzymatic cellulose hydrolysis and to reduce the required amount of expensive cellulases.

Chapter 3

Application of fluorescent protein probes for biomass characterization regarding cellulose accessibility

3.1 Theoretical background

The efficiency of enzymatic cellulose hydrolysis strongly depends on the accessibility of the cellulose for glucanases (Arantes and Saddler, 2011), which is termed as "cellulose accessibility to cellulases" (CAC) and expressed as mass-related accessible surface ($m^2/g_{substrate}$) (Gao et al., 2014b; Hong et al., 2007; Wang et al., 2012; Zhu et al., 2009). The initial biomass pretreatment in biorefinery processes aims at disintegration of the lignocellulosic composite to increase the CAC and thereby cellulase efficiency. There exist diverse analytical methods to determine structural properties of biomass or cellulose, respectively, but they do not necessarily represent the actual CAC. Especially when samples are measured under conditions deferring from those of enzymatic hydrolysis, the results are not directly transferable. This is particularly true, when analytical techniques require dried cellulose samples (like e.g. x-ray diffraction or nitrogen adsorption) or involve non-aqueous solvents (Zhang and Lynd, 2004).

Besides biomass integrity also the cellulose structure determines hydrolyzability. While many cellulases are unable to hydrolyze crystalline regions, cellulases perform best on amorphous cellulose. The efficiency of enzymatic cellulose hydrolysis is often correlated with the crystallinity of the cellulosic substrates (Hall et al., 2010), which is usually determined via x-ray diffraction and quantified in terms of the crystallinity indexes (CrI) (Zhang and Lynd,

2004). Although CrI values and hydrolysis rates correlate to a certain extent (Bansal et al., 2010), these tendencies do not allow reliable predictions regarding the hydrolyzability of genuine substrates (Zhang and Lynd, 2004; Zhang et al., 2006) nor a mechanistic understanding how pretreatment methods affect cellulose hydrolysis. Even if crystallinity of cellulose influences hydrolyzability, its overall accessibility predominantly determines cellulase efficiency (Arantes and Saddler, 2011; Fierobe et al., 2002; Zhang and Lynd, 2004).

The enzymatic hydrolysis of insoluble cellulosic substrates occurs at the substrate surface why material properties determining the specific accessible surface area like particle size, porosity or pore size and volume strongly influence cellulase efficiency and thereby, hydrolysis rates. Sorption-based methods like e.g. Brunauer-Emmett-Teller (BET) nitrogen adsorption (Strømme et al., 2003) or Simons' stain methods (Chandra et al., 2008) are typical analytical techniques to characterize and compare the surface accessibility of different lignocellulosic substrates (Zhang and Lynd, 2004), but they also do not reflect the actual substrate accessibility for cellulases, since the probe molecules are usually magnitudes smaller compared to cellulases.

Protein adsorption studies using genuine cellulases promise to give a realistic picture of the cellulose accessibility, but are facing the problem of simultaneous cellulose hydrolysis altering the material properties during the assay (Hong et al., 2007; Tanaka et al., 1986). Furthermore, common protein determination methods, especially photometric assays, are error-prone and possess comparatively low sensitivity. Also rates of glucose release during enzymatic hydrolysis are often used to benchmark hydrolyzability of substrates and to describe the impact of pretreatment techniques, but they reflect the sum of all effects influencing cellulase performance and do not provide a segregated impression of the accessibility of cellulose for the glucanases.

Therefore, it was of particular interest to develop and establish an analytical method covering all structural aspects of substrate access for cellulases and allowing a defined determination of the actual CAC. Fluorophore-tagged carbohydrate-binding-modules (CBM) are suggested to characterize the cellulose accessibility and to determine the CAC of cellulosic substrates (Hildén et al., 2003; Hong et al., 2007; Jäger, 2012). CBM effect the specific binding of cellulases to cellulosic substrates. Therefore, isolated CBM can represent the affinity of the

whole enzyme to the substrate while lacking the assay-disturbing hydrolytic activity. One potential approach to use CBM as an analytical tool for substrate characterization is chemical labeling of these cellulose-binding proteins to generate cellulose-specific fluorescent protein probes which can be used for adsorption experiments (Hildén et al., 2003; Jäger, 2012). The chemical labeling of CBM with synthetic dye molecules can involve several drawbacks like yielding inhomogeneous populations of proteins labeled with different numbers of dye molecules per protein or changed binding properties due to the presence of dye molecules on the protein surface or even in the cellulose binding site (Moran-Mirabal et al., 2009). Fluorescein isothiocyanate (FITC)-labeled CBM have successfully been used for qualitative binding studies on lignocellulosic substrates (Hildén et al., 2003; Jäger, 2012), but the labelled CBM cannot represent the actual size of cellulases. Furthermore, the preparation of chemically labeled CBM is labor-intensive, since it requires reactive dye-coupling and subsequent removal of unbound fluorophore molecules.

An alternative approach is the application of fusion-proteins consisting of a CBM linked to a fluorescent protein such as e.g. GFP (Gao et al., 2014b; Hong et al., 2007; Kawakubo et al., 2010; Wang et al., 2012; Zhu et al., 2009). Table 3.1 displays approaches of using such fusion-proteins for characterization of cellulosic substrates. As an advantage over chemically labeled CBM, those fluorescent protein probes carry exactly one fluorophore per CBM and do not require additional preparation steps after they have been expressed and purified. Furthermore, the molecular weight of these fusion-proteins is more similar to genuine cellulases. A first fusion-protein-based system for quantitative determination of the CAC consisted of the type-A (surface-binding) CBM3 from the cipA gene of *Clostridium thermocellum* and the fluorescent protein GFP (Hong et al., 2007). This system was extended by using two fusion-proteins differing in type of CBM and fluorescent protein. The two probes had diverse cellulose binding properties and the different fluorescent proteins (GFP, mCherry) made them analytically distinguishable (Gao et al., 2014b). While CBM3 from *C. thermocellum* binds both amorphous and crystalline regions in cellulose, the type-B (chain-binding) CBM17 from the cellulase 5A of *Clostridium cellulovorans* selectively binds only on amorphous cellulose strands. By comparing the binding behavior of the two fluorescent protein probes CBM3-GFP and CBM17-mCherry on different materials structural characteristics of the tested substrates and the resulting accessibility for cellulases could be elucidated (Gao et al., 2014b).

Table 3.1: Studies using fluorescent, CBM-based fusion-proteins for the characterization of cellulosic substrates.

Substrate	CBM	CBM binding	Fluorescent protein	A_{max} [µmol/g]	Source
Avicel / cellulose CF1 / filter paper / BMCC / RAC[a]	CBM3 (*C. thermocellum*)	amorphous/ crystalline[+]	GFP	0.338 ± 0.014 (Avicel) 0.644 ± 0.021 (CF1) 1.39 ± 0.045 (filter paper) 4.76 ± 0.21 (BMCC) 5.97 ± 0.31 (PASC)	Hong et al., 2007
Avicel / RAC[a] / corn stover[d]	CBM3[*] (*C. thermocellum*)	amorphous/ crystalline[+]	GFP	7.83 ± 0.13 (RAC) 0.32 ± 0.01 (Avicel) 2.05 ± 0.15 (COSLIF[1] corn stover) 1.09 ± 0.08 (DA[2] corn stover)	Zhu et al., 2009
japanese cedar wood[d]	CBM3 (*C. josui*) CBM28 (*C. josui*)	crystalline (*C.j.* CBM3); amorphous (*C.j.* CBM28)	CFP[3]	163[e] 286[e]	Kawakubo et al., 2010
Hornified[4] substrates from lodge pine	CBM3[*] (*C. thermocellum*)	amorphous/ crystalline[+]	GFP	n.s.	Wang et al., 2012
Avicel / RAC[a]	CBM3[*] (*C. thermocellum*) CBM17 (*C. cellulovorans*)	amorphous/ crystalline (CBM3);[+] amorphous (CBM17)	GFP (CBM3); mCherry (CBM17)	8.64 ± 0.15[b] 11.28 ± 0.26[c]	Gao et al., 2014b
α-cellulose	CBM4 *(C. fimi)*	amorphous	mCherry	n.d.	This study

[a] regenerated amorphous cellulose prepared from Avicel according to (Percival Zhang, et al. 2006)
[b]CBM3-GFP on PASC
[c]CBM17-mCherry on PASC
[d]different pretreatments
[e]organosolv pulp from japanese cedar wood
[*]according to (Hong et al., 2007)
[+](Gao et al., 2014b)
[1]cellulose solvent and organic solvent lignocellulose fractionation (treatment with phosphoric acid / acetone)
[2]dilute sulfuric acid pretreated
[3]cyan fluorescent protein
[4]dried pulp, technical term from pulp and paper context
n.s.: not specified
n.d.: not determined

The goal of the study presented in this chapter was quite similar to (Gao et al., 2014b). Two fluorescent protein probes, one containing an amorphous-binding CBM and one containing a crystalline-binding CBM, labeled with two different fluorescent proteins, should be used to characterize lignocellulosic substrates regarding cellulose accessibility and crystallinity. A mixture of both probes should be incubated with the substrate. While the total amount of adsorbed probes should reflect the overall cellulose accessibility of the substrate, the ratio between both probes measured in terms of relative fluorescence intensities should give information about the crystallinity of the substrate (Figure 3.1).

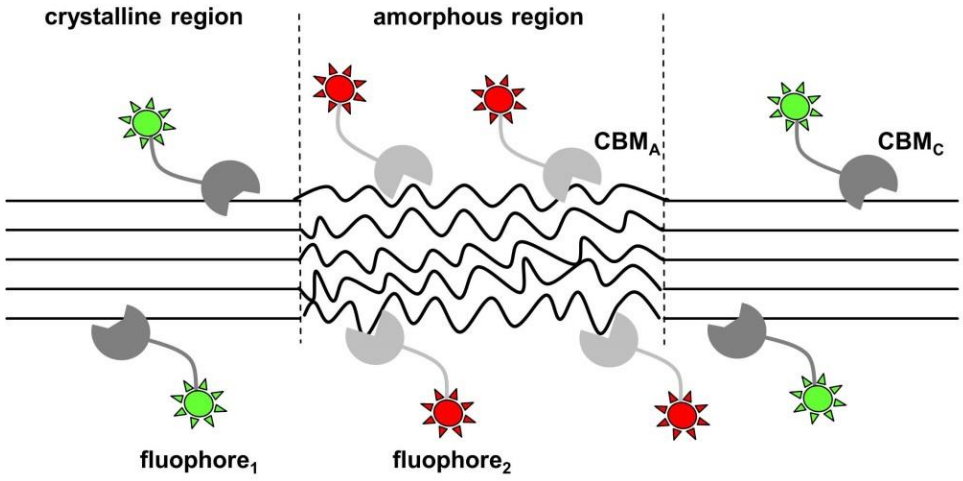

Figure 3.1: Concept for the characterization of cellulosic substrates by use of CBM-based fluorescent protein probes. CBM_A: amorphous-binding CBM; CBM_C: crystalline-binding CBM; $fluorophore_{1/2}$: fluorescent proteins with distinguishable fluorescent properties.

A particular intent of these fluorescent protein probes was the investigation of the enhancing influence of LMS pretreatment on the enzymatic hydrolysis of wooden biomass. For a first feasibility study and to validate the concept of CBM-based fluorescent protein probes for biomass characterization, a fusion-protein consisting of the first N-terminal, family-4 carbohydrate binding module (CBM_{N1}, below referred to as CBM4) of the *Cellulomonas fimi* CenC 1,4-β-endoglucanase (Johnson et al., 1996; Tomme et al., 1996b) and the fluorescent protein mCherry (Campbell et al., 2002; Gross et al., 2000; Shaner et al., 2004) was designed and recombinantly produced. The CenC 1,4-β-endoglucanase of *C. fimi* possesses two CBMs at its N-terminus. In contrast to the majority of CBM having a planar architecture, they are

cleft-shaped leading to a high specificity for amorphous, single-stranded cellulose (Brun et al., 2000; Tomme et al., 1996a). This feature makes this CBM a promising candidate to realize protein probes reflecting cellulose accessibility in general as well as the degree of crystallinity.

3.2 Materials and Methods

3.2.1 Chemicals

All chemicals were of analytical-reagent grade or higher and purchased from Sigma Aldrich (St. Louis, USA) or Carl Roth (Karlsruhe, Germany) if not stated otherwise. Bovine serum albumin (BSA) was used in form of a calibration solution (2 g/L, ThermoFisher Scientific, Rockford, USA).

3.2.2 Cellulosic substrates

α-Cellulose was purchased from Sigma-Aldrich (St. Louis, USA). Detailed physical properties of this substrate are given in (Jäger, 2012). The beech wood and its mechanical preparation was identical to that described in chapter 2.2.2 ("Räuchergold" KL 1/4, JRS - J. Rettenmaier & Söhne GmbH + Co. KG, Rosenberg, Germany). The particle size used here was < 100 µm. LMS pretreatment of the beech wood was conducted according to chapter 2.2.5 and as described in (Kress, 2013). Briefly, 5 % (m_{DW}/V) lignocellulose were incubated with 60 $U_{ABTS}/g_{lignocellulose}$ laccase and 5 mM HBT in 0.1 M sodium acetate buffer (pH 4.5) at 40 °C for 72 h. Afterwards, the beech wood was washed and freeze-dried.

3.2.3 Strains

The two *E. coli* strains expressing the fluorescent protein probes mCherry and CBM4-mCherry were kindly provided by the institute for Molecular Biotechnology at RWTH Aachen University (Rußkamp, 2013) and are briefly described in the following. *E. coli* SHuffle (genotype: F´ lac, pro, lacIQ / Δ(ara-leu)7697 araD139 fhuA2 lacZ::T7 gene1 Δ(phoA)PvuII phoR ahpC* galE (or U) galK λatt::pNEB3-r1-cDsbC (SpecR, lacIq) ΔtrxB rpsL150(StrR) Δgor Δ(malF)3), a derivate of *E.coli* BL21, was used as expression host. The expression vector was based on the pET22b(+) plasmid and encodes for mCherry or the fusion-protein of mCherry and CBM4, respectively. The construct for the fusion-protein CBM4-mCherry is shown in Figure 3.2. The family-4 carbohydrate-binding-module (CBM4) originates from the CenC-gene of *Cellulomonas fimi* ATCC 484 (Figure 3.2 C) (Kormos et al., 2000; Tomme et al., 1996b).

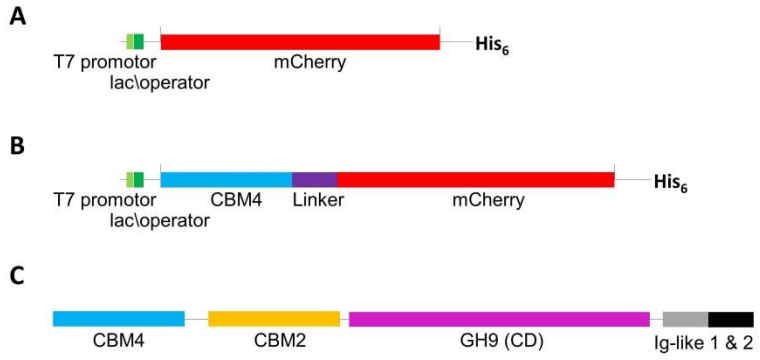

Figure 3.2: Used constructs for the fluorescent protein probes (A: single mCherry, B: CBM4-mCherry) and CenC gene of *Cellulomonas fimi* ATCC 484 (C). Illustration not in scale with the original length of the base sequence.

3.2.4 Cultivation and expression

Cultivations for expression of the fluorescent protein probes were performed at the chair for Molecular Biotechnology. The cultivation conditions were adapted and optimized from (Kormos et al., 2000). LB-medium (10 g/L bacto-tryptone, 5 g/L yeast extract, 10 g/L NaCl, 100 µg/mL ampicillin) was used for all cultivations. The cultures were grown at 37°C and 180 rpm in 100 mL Erlenmeyer shake flasks containing 20 mL LB-medium, which was

inoculated from overnight grown pre-cultures at an initial OD_{600} of 0.01 – 0.03. After OD_{600} reached 0.5, the protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the temperature was reduced to 20°C. Cultivations were run over night (~18 h) and cells were harvested by centrifugation (4°C, 18000 g, 10 min) the next day. If the pellets were not used immediately, they were stored at -20°C.

3.2.5 Initial strategy for cell disruption and protein purification

At the beginning of this study, factors influencing the purification of the fluorescent protein probes were investigated to design an optimal purification strategy. Initially, purifications were conducted in small bench scale using gravity-flow columns for IMAC and SEC according to the following protocol:

In 25 mL of 20 mM Tris lysis buffer (1 M NaCl, 5 % (v/v) glycerol, 10 mM imidazole, 0.05 % (v/v) Triton X-100, pH 6.5) one tablet of protease inhibitor was dissolved under stirring for 10 min at 4°C. 25 mL of 1x BugBusterTM (Novagen, USA) were added, and an appropriate amount of cell pellet was resuspended in the solution. A spatula tip of DNAseI (Sigma Aldrich) was added, and the suspension was incubated for 15 minutes. Then, cells debris was spun down (4°C, 15000 g, 10 minutes), and the clear supernatant was given on a 10 mL Ni-NTA agarose gravity-flow column (QIAGEN, Germany) for IMAC purification. Therefore, the column was equilibrated with two column volumes (CV) of 250 mM imidazole in water and followed by 3 CV of lysis buffer, before the lysate was applied. After sample application the column was washed with 9 CV of washing buffer (50 mM Tris, 0.5 M NaCl, 20 mM imidazole, pH 6.5). For protein elution 10 mM MOPS buffer (pH 6.5) containing 10 % (v/v) glycerol, 250 mM imidazole and 0.2 % (v/v) Triton X-100 was used. The elution volume depended on the amount of bound protein. All flow-through fractions were collected and the content of target protein was determined by fluorescent measurement (cp. 3.2.9).

Fractions rich in target protein were further purified with SEC. FPLC-SEC was conducted using an Äkta pure 25 device (GE Healthcare Life Sciences) containing a HiPrep 16/60 or HiPrep 26/60 column. 15 mM citrate buffer pH 5.2 containing 10 % (v/v) glycerol, 30 or 150 mM NaCl and optionally 0.2 % (v/v) Triton X-100 was used as running buffer. The

columns were equilibrated before purification by purging with 5 CV of running buffer. The flow rate was adjusted to 0.5 mL/min. The purification protocol was further developed and optimized during this study resulting in the procedure described in section 3.2.6 (see below).

3.2.6 Optimized strategy for cell disruption and protein purification

All adsorption experiments were conducted with protein probes purified according to the following optimized protocol.

Cell pellets are disrupted using ultra sonication (Hielscher Ultrasound Technology, UP 200S). Therefore, cell pellets from 500 mL cultivation broth were resuspended in 7 mL of the buffer used for the subsequent purification by immobilized metal ion affinity chromatography (IMAC). 20 µg DNAseI, 0.5 % (v/v) Triton X-100, and 0.5 mM phenylmethylsulfonylfluoride (PMSF) were added. Sonication was performed in 10 cycles of 15 seconds each with a break of 15 seconds in between. After sonication the cell broth was centrifuged (4°C, 18000 g, 10 minutes) and the supernatant was directly applied to IMAC purification.

IMAC was performed via FPLC on an Äkta pure 25 device (GE Healthcare Life Sciences) containing a Ni-NTA Superflow 5 mL cartridge (QIAGEN). 25 mM MES buffer, pH 7.0, containing 10 % (v/v) glycerol, 1 M NaCl, and 10 mM (loading buffer A) or 200 mM (elution buffer B) imidazole was used as running buffer. All buffer solutions were filtered before use (pore size < 0.22 µm). The flow rate was adjusted to 1.0 mL/min. For column equilibration the system was flushed with 3 CV (CV=5 mL) of buffer A, until the UV signal became constant. After sample loading (0.5 – 3 CV) the column was washed with 5 CV of buffer A until reaching a stable UV signal. For elution a gradient of buffer A and B (5 – 100 % B over 50 min) was run, and the protein containing eluent was collected in 1 mL fractions. Fractions containing target protein were pooled and further concentrated using VivaSpin ultrafiltration spin tubes (Sartorius Stedim Biotech GmbH). Afterwards, the concentrated solution was purified by size exclusion chromatography with the same Äkta device. Depending on the sample volumes two different columns were used. For large sample volumes the HiPrep 26/60 Sephacryl S-100 High Resolution column (GE Healthcare Life Sciences) and for smaller

sample volumes the Superdex^{TM} 75, 10/300 GL column (GE Healthcare Life Sciences) were applied. The running buffer was 50 mM citrate buffer, pH 5, containing 1 M NaCl and 10 % (v/v) glycerol. The column was purged (0.4 – 1.5 mL/min) with 1 - 2 CV to reach equilibrium. After sample loading (0.4 mL/min) elution was performed with at least 1 CV at a flow rate of 0.4 – 1.5 mL/min. After pooling the fractions of the target protein they were concentrated with VivaSpin ultrafiltration spin tubes. The resulting solution was used as stock solution for protein determination and recording of adsorption isotherms.

3.2.7 SDS gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the purity of protein probe preparations. The gels consisted of a 5 % (v/v) polyacrylamide stacking gel and a 12 % (v/v) polyacrylamide separating gel. For sample preparation, 10 µL of loading buffer (NuPAGE® LDS Sample Buffer 4x) were added to 40 µL sample. The samples were heated to 70°C for 10 min or to 90°C for 2 - 5 min. 2 - 15 µL of each sample were loaded into the gel pockets. The running buffer contained 30.3 g/L Tris buffer, 144 g/L glycerol and 1 % (w/v) SDS. SDS-PAGE was run for 35 min at 100 - 150 V and the gel was stained overnight in a solution of 0.25 % (w/v) Coomassie Brilliant Blue G-250, 50 % (v/v) methanol and 10 % (v/v) acetic acid. Destaining was conducted in deionized water with 5 % (v/v) methanol and 7.5 % (v/v) acetic acid, until protein bands were clearly visible.

3.2.8 Determination of protein concentration

The concentrations of the protein probe stock solutions were determined using the detergent compatible (DC) protein assay kit (Bio-Rad, Hercules, CA, USA). Measured protein concentrations were correlated with the fluorescence of the protein probe solutions (cp. Figure 3.5 B). A calibration curve with BSA standards of concentrations from 125 µg/mL to 2000 µg/mL in citrate buffer was used. 10 µL of sample or standard were pipetted into a microtiter plate well and mixed with 25 µL of DC reagent A and 200 µL of DC reagent B. The assay was incubated for 15 min at room temperature, and absorbance at 750 nm was

measured with a Synergy Mx microtiter plate photometer (Biotek, Winooski, VT, USA). Absorption values were converted into protein concentrations by use of the BSA calibration curve.

3.2.9 Recording of adsorption isotherms

The adsorption of the fluorescent protein probes on different cellulosic substrates was studied with adsorption isotherm experiments. Basically, the same amount of substrate was incubated with different concentrations of the protein probes, and after reaching adsorption equilibrium free protein in the sample supernatant was measured by means of fluorescence. Ten dilutions with concentrations from 6 – 100 µg/mL in 50 mM citrate buffer (pH 5, 1 M NaCl) were prepared from the fluorescent protein probe stock solutions. 5 mg of substrate was weighed in 1.5 mL Eppendorf tubes and mixed with 300 µL of the particular dilution. Samples were incubated at 4°C for 2 h on a roller shaker. After incubation the solids were spun down (5 min, 13000 g, 4°C), and 100 µL of supernatants were transferred to a MTP. Fluorescence (λ_{ex}: 550 nm / λ_{em}: 610 nm) was measured with a Synergy Mx microtiter plate reader at 30°C and a gain factor of 100. Protein concentrations were calculated from the fluorescence values using the correlation determined with the DC assay. Dilutions were also incubated without substrate and measured as reference representing the whole amount of applied protein. The difference of the reference value and the value measured in the sample supernatants accounts for the protein amount bound to the substrate. The calculated values for protein bound to the substrate (µmol/$g_{substrat}$) were plotted against free protein in solution (µmol/L) and fitted using the Langmuir adsorption model according to equation (3.1).

$$P_{ads,eq} = \frac{A_{max} \cdot K_P \cdot P_{free}}{1 + K_p \cdot P_{free}} \tag{3.1}$$

$P_{ads,\,eq}$:	adsorbed protein at equilibrium	[mol/$g_{substrate}$]
A_{max}:	total capacity of the adsorbant	[mol/g]
P_{free}:	concentration of unbound protein in solution	[mol/L]
K_P:	equilibrium constant	[L/mol]

3.2.10 Influence of foreign protein and detergent on unspecific adsorption of mCherry

The influence of foreign protein, potentially blocking sites for unspecific adsorption, was investigated using bovine serum albumin (BSA) as blocking protein and mCherry as fluorescent protein probe. Furthermore, the influence of the detergent Tween-20 was tested. Therefore, the substrate was mixed and pre-incubated with BSA or Tween-20 before isotherm experiments. 5 mg of substrate (beech wood) was mixed with 150 µL of a solution containing either 0.18 g/L BSA, 0.36 g/L BSA or 5 g/L Tween 20. Afterwards, 150 µL of the protein probe solution was added and incubation and sample handling were conducted as described in 3.2.9.

3.3 Results and Discussion

3.3.1 Optimization of purification of fluorescent protein probes

The application of fluorescent protein probes as an analytical tool for biomass characterization requires an efficient concept for the production of the fusion proteins since comparatively high protein amounts are required to record adsorption isotherms. For this purpose the purification strategy was examined in detail.

After expression in *E .coli* and cell disruption the fusion proteins were purified over their His-tag by immobilized metal affinity chromatography (IMAC) and subsequent size exclusion chromatography for removal of imidazole and buffer exchange. First, bench scale gravity-flow columns were used for IMAC. SEC was realized with a FPLC device and monitored by UV detection (Figure 3.3). The chromatogram of the FPLC-SEC purification showed three major protein peaks (Figure 3.3 A). Fractions of each peak were collected and analyzed with SDS-PAGE (Figure 3.3 B). In the fractions of the first protein peak two double bands at around 43 kDa and 25 kDa were identified. According to its size the first double band can be attributed to the full length fusion protein (43 kDa) consisting of the CBM and mCherry, whereas the second double band at around 25 kDa most likely derived from mCherry alone

(27 kDa) as a degradation product of the fusion protein. The double band at ~25 kDa can also be found together with two sharper bands in the fraction of the second FPLC peak. The molecular weight of the sharp upper band in this fraction roughly matches with that of mCherry (27 kDa) suggesting that the second peak found in the FPLC chromatogram represents single mCherry as a cleavage product of the fusion protein. The third FPLC peak supposably derived from another unidentified degradation product of the fusion protein.

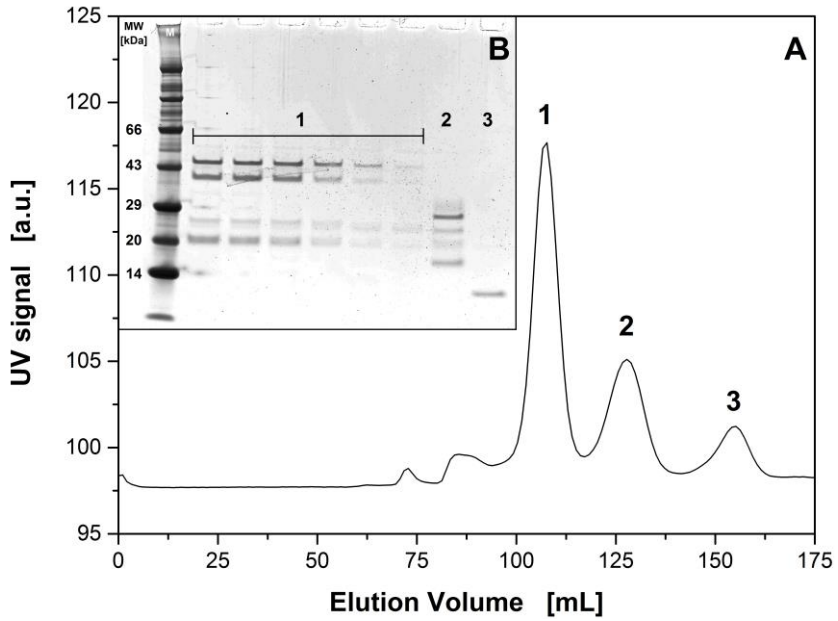

Figure 3.3: Purification of CBM4-mCherry using the initial purification strategy. A: Chromatogram (UV detection) of SEC purification. B: SDS-PAGE of obtained protein fractions. Numbers indicate the individual fractions in the chromatogram and SDS gel.

The occurrence of double or multiple bands with similar molecular weight presumably results from partial degradation or refolding of mCherry during sample preparation for SDS-PAGE. The same phenomenon has been observed for dsRed, which is the natural fluorescent protein mCherry originates from, and two fragment bands with a difference in their molecular weight of around 7 kDa were found as the result of incomplete denaturation (Gross et al., 2000). It is noteworthy that bands representing the full-length fusion protein and mCherry alone are found together in the fractions of the first FPLC peak indicating either cleavage of the fusion protein within the linker region during sample preparation for SDS-PAGE or dimerization of mCherry with the mCherry part of the fusion protein during SEC. The occurrence of a

separate peak for mCherry in the FPLC chromatogram reveals that the fusion protein is prone to fragmentation. Since the His-tag is attached to the mCherry part of the fusion protein, mCherry fragments are retained during IMAC purification whereas CBM fractions elute from the column.

The purification protocol was adapted to address the potential dimerization of mCherry and the incomplete protein denaturation during sample preparation for SDS-PAGE. The concentration of sodium chloride in the SEC running buffer was increased from 30 to 150 mM, and 0.2 % (v/v) Triton X-100 was added to suppress undesired protein interactions like dimerization. The temperature for sample preparation before SDS-PAGE was increased from 70°C to 90°C and the incubation time was reduced from 10 to 2-5 minutes. Figure 3.4 shows the results of the modified protocol.

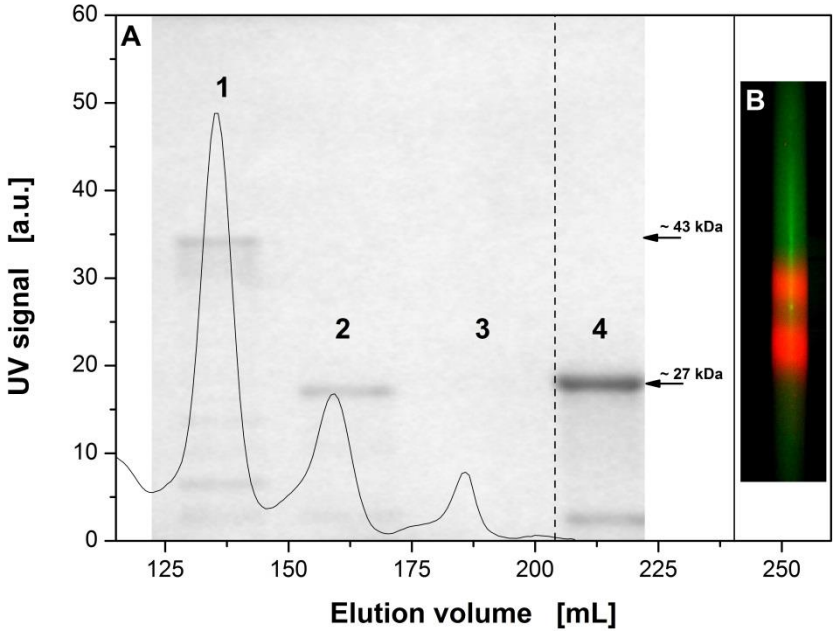

Figure 3.4: Purification of CBM4-mCherry using the optimized purification strategy. A: Chromatogram (UV detection) of SEC purification. Background: Bands in SDS-PAGE for each of the obtained protein fractions (1-3); molecular weights were derived from standard marker (not shown, q.v. Figure 3.3 B). 4: mCherry reference. B: Photography of SEC purification column under green light with red light filter.

The occurrence of mCherry-derived double bands could be avoided due to the adapted conditions for sample preparation. The fraction of the first FPLC peak consisted purely of the

full length fusion protein. No band for mCherry was found in this fraction. It is unclear, if this is a result of suppressed dimerization during SEC purification or if the shorter but higher heating during sample preparation prevents cleavage of the protein probe. The relative height and distance of the three FPLC peaks among each other is almost the same for both purification protocols casting dimerization of single mCherry with the fusion-protein during SEC into doubt. Dimerization would increase the apparent molecular weight and change the relative distance between the first and the second peak. Besides UV detection and SDS-PAGE the appearance of two fluorescent protein fractions during SEC purification could also be visualized by illuminating the SEC column with green light with a wavelength spectrum in the range of mCherry excitation (587 nm) and photographing through a red filter (Figure **3.4** B).

The results of the purification study show that it is essential to perform a suitable purification to obtain a pure preparation of the protein probe and to remove single mCherry. The presence of single mCherry indicates that the fusion protein is prone to fragmentation which is a drawback for its use as an analytical tool. The purification protocol was further developed and optimized at the chair for Molecular Biotechnology to enhance yields and accelerate FPLC purifications. All further results were conducted with protein probes produced with the optimized protocol described in chapter 3.2.6.

3.3.2 Characterization of mCherry as fluorescent reporter

The fluorescent mCherry part of the protein probe was initially characterized regarding the use in adsorption experiments. The measured wavelength spectra for excitation and emission (Figure 3.5 A) exactly match with literature values (Shaner et al., 2005, 2004). For the applied CBM4-mCherry protein probe a linear correlation between protein concentration and fluorescence intensity could be shown enabling a fast and simple quantification of the protein probe (Figure 3.5 B). Since factors like type and concentration of buffer, pH, impurities as well as age of the preparation can influence the correlation factor for fluorescence intensity and protein concentration, calibration measurements have to be performed together with each adsorption experiment.

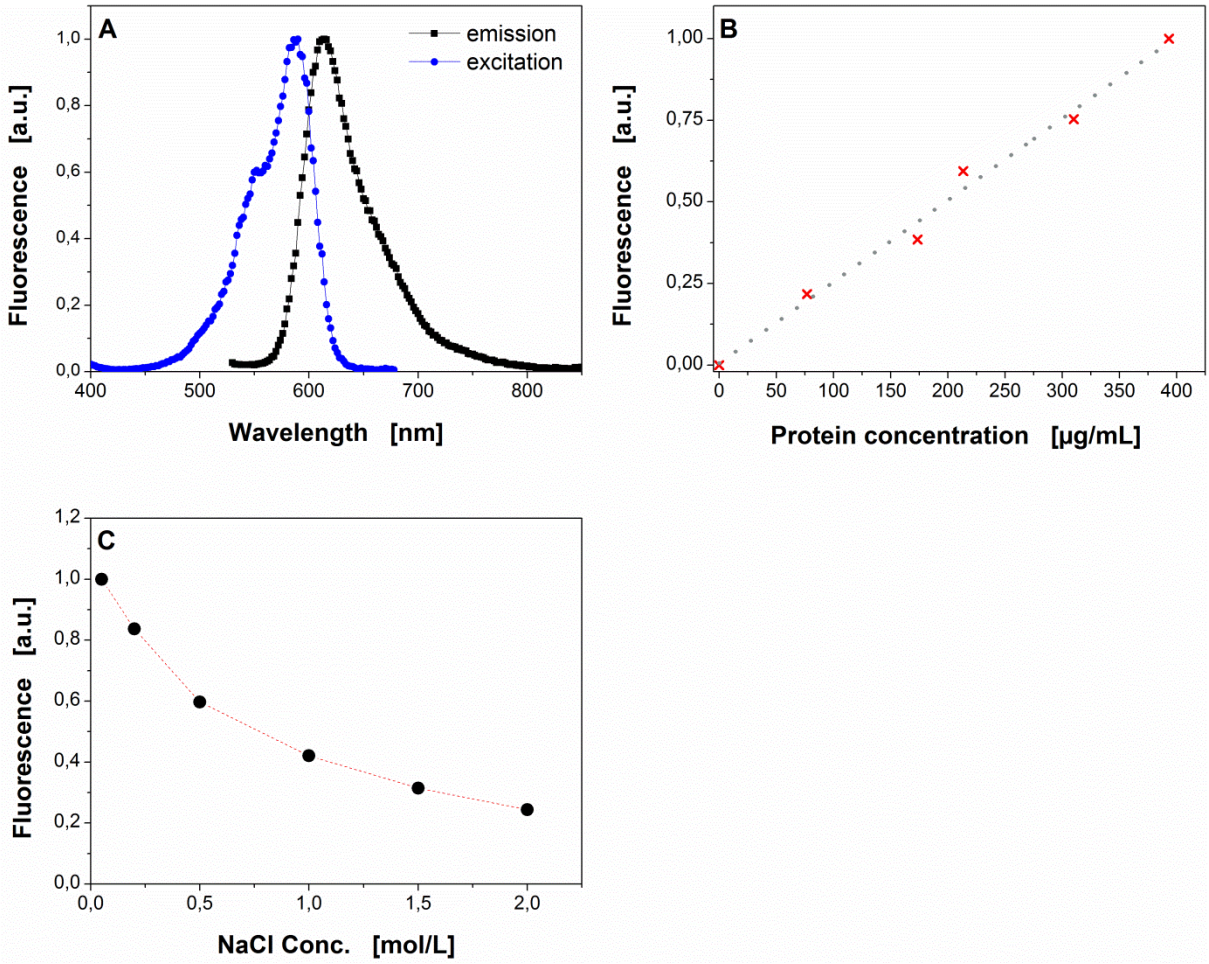

Figure 3.5: Properties of mCherry as fluorescent reporter protein. A: Excitation and emission spectrum of mCherry. B: Calibration curve for correlation of protein amount and fluorescence of CBM4-mCherry; protein concentration was determined by DC assay. C: Influence of the NaCl concentration on the fluorescence intensity of mCherry.

One factor with major impact is the concentration of NaCl, which reduces the fluorescence intensity of mCherry (Figure 3.5 C). Even if some β-barrel-type fluorescence proteins like GFP or YFP show a high sensitivity to halides (Arosio et al., 2010; Wachter and Remington, 1999), this is unlikely for mCherry since its natural origin dsRed was shown to fluoresce independently of the chloride concentration (Arosio et al., 2010). Therefore, the ionic strength seems to be the influencing factor. This fact is of particular relevance, since high salt concentrations are sometimes applied to suppress unspecific protein adsorption of fluorescent protein probes. For example, cellulose was characterized with fluorescent CBM fusion proteins at NaCl concentration of 2 M (Gao et al., 2014b). Compared to a salt-free system the

fluorescence of mCherry is reduced by 80% when 2 M NaCl are present leading to a strong loss of sensitivity in the fluorescence measurements (Figure 3.5 C).

3.3.3 Adsorption of CBM4-mCherry on α-cellulose

The CBM4-mCherry protein probe was tested on α-cellulose as substrate. The adsorption of the fluorescent protein probe was compared to that of mCherry alone to distinguish CBM-mediated specific cellulose binding from unspecific protein adsorption. α-Cellulose was chosen as substrate because of its structure: With a crystallinity index of 0.64 (Jäger, 2012) it possesses both crystalline and amorphous cellulose regions (Zhang et al., 2006) making it a suitable test substrate for this study (Figure 3.1). Furthermore, with a residual content of hemicellulose (22 % xylan; Jäger, 2012) and a comparatively high degree of polymerization (DP_W= 2140 – 2420; Jäger, 2012) α-cellulose shares more structural aspects with native lignocellulose than Avicel, Sigmacell, and other model substrates like PASC (phosphoric acid swollen cellulose), Whatman paper, or CMC (Zhang et al., 2006).

The adsorption experiments show a high scatter due to the small sample volume of 300 µL and the solid content. Ignoring outliers (crossed circles in Figure 3.6) the adsorption of CBM4-mCherry on α-cellulose can approximately be fitted using the Langmuir adsorption model, but the data only allow a rough estimation of magnitudes, why no distinct values for the model parameters A_{max} (total adsorption capacity) and K_P (equilibrium constant) are given here. The adsorption of mCherry on α-cellulose shows an even higher scatter and cannot reasonably be fitted. Compared to CBM4-mCherry the values have the same order of magnitude. Again, considering the high scatter of the data it can be assumed that only a slight unspecific protein adsorption was observed here. The maximum amount of bound protein (A_{max}) can be assessed at around 0.02 – 0.03 µmol/$g_{substrate}$.

Compared to reported A_{max} values (Table 3.1; Gao et al., 2014b; Hong et al., 2007) these values are one order of magnitude lower. A fluorescent protein probe consisting of the CBM3 from *Clostridium thermocellum* and GFP as fluorophore had an A_{max} value of 0.338 ± 0.014 µmol/g (Hong et al., 2007) or 0.32 ± 0.01 µmol/g (Zhu et al., 2009), respectively, for adsorption on Avicel. The crystallinity index of Avicel is similar (0.5 – 0.6, Zhang and Lynd,

2004) or even higher (0.82 for Avicel PH101, Jäger, 2012) compared to that of α-cellulose (0.64; Jäger, 2012) and the microscopic structure as well as the actual CAC of the substrates differ so that direct comparability of the A_{max} values is not given. Furthermore, the binding specificity of the CBM3 from *C. thermocellum* is much lower since it accepts both crystalline and amorphous cellulose. However, whole cellulases purified from the *T. reesei* preparation Celluclast™ also adsorbed to α-cellulose with A_{max} values of 0.155 ± 0.003 µmol/g (CBH I) and 0.212 ± 0.010 µmol/g (EG I), respectively, (Jäger, 2012) confirming the magnitude of the values by Gao and Hong (Gao et al., 2014b; Hong et al., 2007) for Avicel using the fluorescent protein probes.

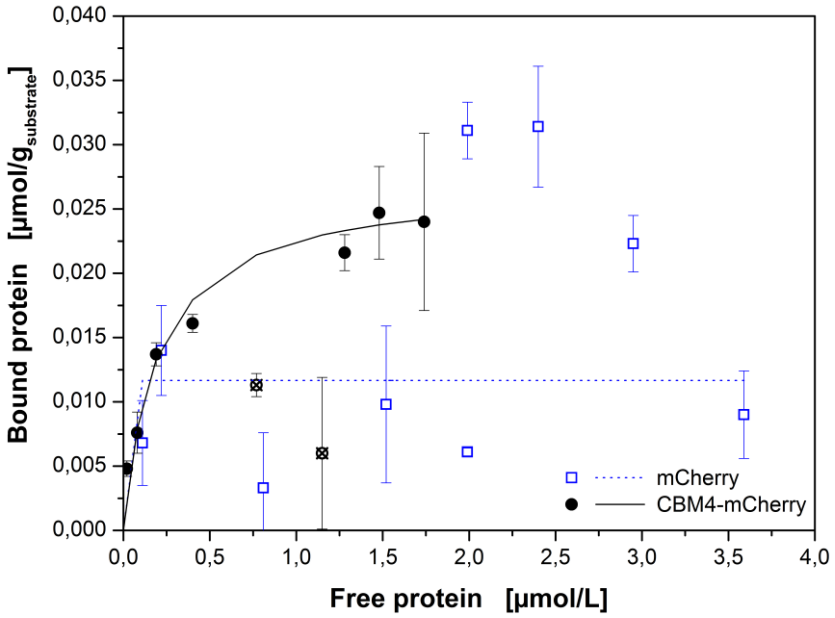

Figure 3.6: Adsorption of mCherry-CBM4 and single mCherry on α-cellulose. (1 M NaCl, 50 mM citrate buffer, pH 5). Fit according to the Langmuir adsorption model. Crossed circles were treated as outliers and excluded from the fit.

In summary, a specific binding of the CBM4-mCherry protein probe, standing out from a basal, unspecific adsorption of single mCherry, was not found, but the A_{max} values determined in this study indicate only a marginal unspecific adsorption. The reasons for the inadequate binding of the tested fluorescent protein probe remain unclear. Besides binding properties of the CBM4 from *C. fimi*, also the folding of the protein probe might influence its binding behavior. Since the fluorophore mCherry and the CBM4 are connected over a single-chain

peptide linker, the final protein fold could sterically hinder the binding of the CBM on cellulose.

3.3.4 Unspecific adsorption of mCherry on cellulosic substrates

In order to use fluorescent protein probes for characterizing the accessibility of lignocellulosic substrates for cellulases, specific, CBM-mediated cellulose binding has to be clearly discriminated from unspecific protein adsorption. It is well known that proteins unspecifically adsorb on lignocellulose mainly due to interactions with lignin (Converse et al., 1990; Gao et al., 2014a; Nakagame et al., 2010; Palonen et al., 2004; Rahikainen et al., 2011, 2013). If unspecific protein adsorption exceeds the effect of specific cellulose binding by the CBM, fluorescent protein probes become pointless for the characterization of cellulose accessibility. To get an impression of the magnitude of unspecific protein binding on different (ligno-) cellulosic substrates, adsorption studies using single mCherry were conducted (Figure 3.7).

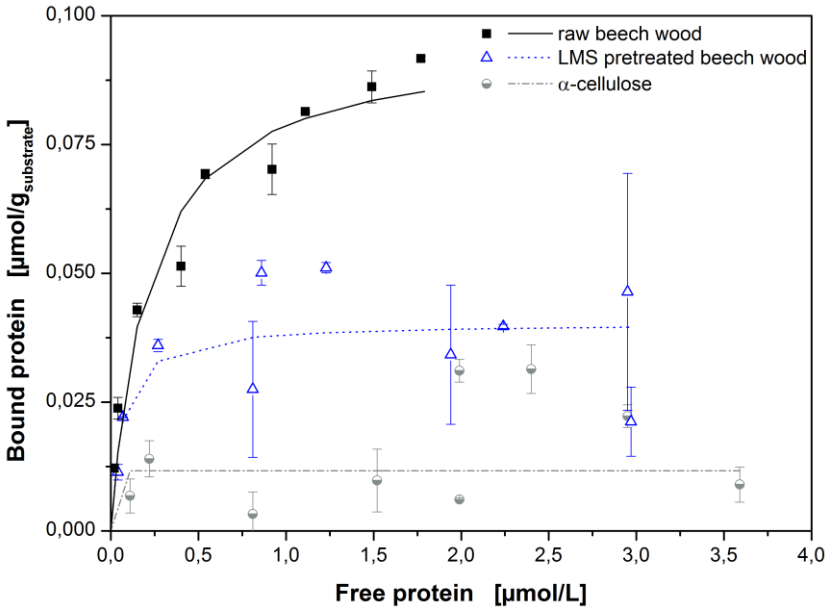

Figure 3.7: Adsorption of mCherry on several cellulosic substrates. (1 M NaCl, 50 mM citrate buffer, pH 5). Fit according to the Langmuir adsorption model.

Beech wood as representative for wooden lignocellulose was tested as sorbent for mCherry. The maximum amount of mCherry adsorbed to beech wood was calculated from the Langmuir adsorption model to A_{max}= 0.1 $\mu mol_{mCherry}/g_{beech\ wood}$, which is equivalent to 2.7 $mg_{mCherry}/g_{beech}$. Although a direct comparison of unspecific protein adsorption on raw wooden biomass is difficult due to variable substrate properties like e.g. particle size, an adsorption capacity of up to 180 mg/g for the adsorption of BSA on sulfuric acid pretreated straw gives a rough impression of the magnitudes (Yang and Wyman, 2006). This number also illustrates that unspecific protein adsorption has an even higher importance for substrates pretreated with lignin exposing techniques.

The adsorption capacity of beech wood which was previously pretreated with the laccase-mediator-system (LMS) is more than halved compared to the raw material (Figure 3.7). Due to the high scatter of the values and the low accuracy of the Langmuir fit (dashed line in Figure 3.7) no distinct value for A_{max} is given. This reduced unspecific protein adsorption after LMS pretreatment was also found as increased efficiency of enzymatic hydrolysis after LMS pretreatment due to a decreased unproductive cellulose binding (e.g. Moilanen et al., 2014). Relative to both lignocellulosic substrates the adsorption of mCherry to α-cellulose can be considered as negligible and in the range of error.

To verify unspecific protein adsorption as the mechanism behind the adsorption of mCherry on lignocellulose, beech wood was pre-incubated with BSA to block potential protein binding sites on its surface (Wang et al., 2012; Yang and Wyman, 2006). Two final concentrations of BSA were tested: One equaled the applied mCherry concentration (90 mg/L), and the other was twice as high (180 mg/L). Furthermore, a pre-incubation with Tween-20 was tested, since surfactants suppress unspecific protein adsorption on lignocellulose (Eriksson et al., 2002; Zheng et al., 2008).

After pre-incubation with BSA and Tween, respectively, a saturating amount of mCherry (Figure 3.8, red point in insert) was added. Pre-incubation with BSA indeed reduced the adsorption of mCherry on raw beech wood to nearly a half independent of the tested BSA concentrations (Figure 3.8). Apparently, already 90 mg/L BSA are saturating since the double amount of BSA (180 mg/L) did not further decrease the binding of mCherry on the substrate. Probably, due to a higher adsorption affinity to the lignocellulose surface, mCherry might

displace BSA from adsorption sites to certain extent. The addition of high concentrations (5 g/L) of BSA as suggested to suppress the distorting effect of unspecific protein binding, when CBM-based protein probes are used for the characterization of cellulosic materials (Wang et al., 2012), appears hardly advantageous since also an excess of BSA cannot totally avoid unspecific protein adsorption. Furthermore, adsorbed BSA might even hinder CBM binding on the substrate surface or interact with the protein probe.

As expected, the presence of 2.5 mg/L Tween-20 effectively prevented unspecific adsorption of mCherry on beech wood, but as a surfactant it might also influence the specific cellulose binding of the CBM, why it is not reasonable to combine it with the fluorescent protein probes.

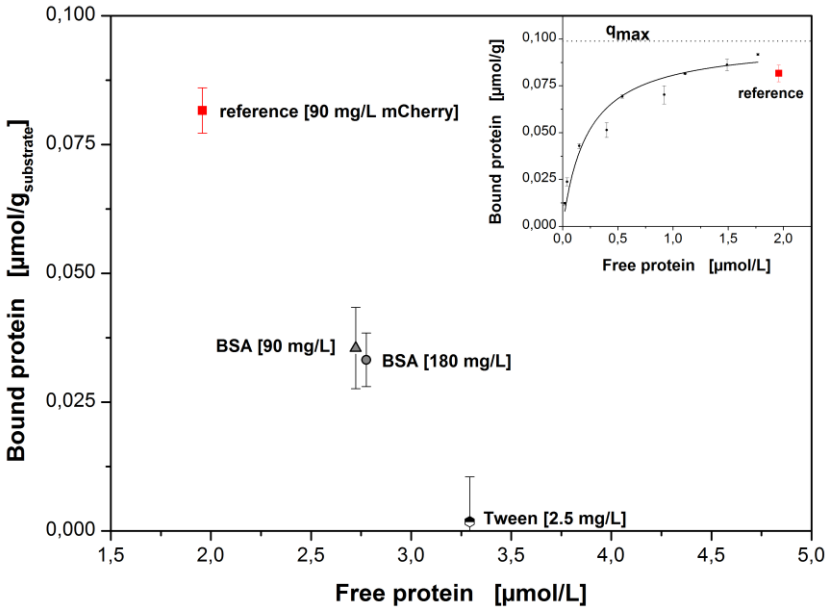

Figure 3.8: Influence of pre-incubation with BSA or Tween-20 on the unspecific adsorption of mCherry on beech wood (90 µg/mL mCherry, 1 M NaCl, 50 mM citrate buffer, pH 5). Insert: adsorption isotherm of mCherry on raw beech wood. The used protein concentration of 90 µg/mL is marked in red as reference.

3.4 Conclusions and Outlook

In order to quantify the actual accessibility of lignocellulosic substrates for cellulases, the binding of a fluorescent protein probe consisting of CBM4 from *C. fimi* coupled to mCherry was validated. The outcome of this study revealed that biomass characterization with CBM-based fluorescent protein probes involves several challenges and drawbacks:

The adsorption studies require a sufficient amount of pure protein probe but, the yields obtained during production of the probes were unsatisfactorily low and suggested further necessary optimization of expression and purification of the protein probes. Furthermore, we found hints that the probes are prone to cleavage within the linker peptide during purification. Cleavage products of the probe complicate purification and influence the adsorption experiments. Potentially, an adaption of the amino acid sequence might stabilize the linker peptide between CBM and fluorescence reporter and avoid cleavage.

CBM4 from *C. fimi* was tested as the cellulose-binding part of the protein probe. Against expectations, no significant binding on α-cellulose was found. It remains unclear, if the insufficient binding derived from α-cellulose structure, which was not recognized by CBM4 or from an inappropriate folding conformation of the protein probe, which could hamper the CBM binding on cellulose. However, a protein probe for the quantification of cellulose in biomass should bind to α-cellulose, since α-cellulose mostly resembles the cellulose in native lignocellulosic substrates. Alternative CBMs could be tested to figure out if the lacking affinity to α-cellulose was due to the choice of the CBM.

That CBM-based fluorescent protein probes are promising tools to investigate cellulose accessibility was concluded only from studies with purified cellulosic substrates lacking the lignin part which is mainly responsible for unspecific protein adsorption (Gao et al., 2014b; Hong et al., 2007; Zhu et al., 2009). However, adsorption studies with mCherry alone indicated that unspecific protein adsorption strongly contributes to the binding of the protein probes onto lignocellulose and most likely masks the cellulose-specific binding of the CBM. A reasonable application of CBM-based protein probes for the characterization of lignocellulosic substrates regarding their cellulose accessibility essentially requires minimization of the unspecific protein adsorption.

Since it was demonstrated that mCherry unspecifically adsorbs onto lignocellulose, the exchange of the large (27 kDa) β-barrel-shaped mCherry against alternative fluorescence reporters such as the smaller (16-19 kDa) light-oxygen-voltage-sensing (LOV) domain-based flavin-mononucleotide-binding fluorescent proteins (Fbfp, Drepper et al., 2010, 2007) might reduce the unspecific adsorption of the protein probe. Also chemical labeling of the CBM is thinkable, but a defined ratio of CBM and fluorophore has to be guaranteed and, the labeling must not disturb the binding of the CBM to cellulose.

However, single mCherry, representing an easily detectable protein, turned out as a promising tool to characterize lignocellulose regarding unspecific protein adsorption. The unproductive binding of proteins to lignocellulosic materials inversely correlates with the efficiency of the enzymatic cellulose hydrolysis and can be influenced by pretreatment. Since hydrolytic enzymes are a major cost factor in biorefinery processes, it makes sense to select and develop pretreatment methods regarding their compatibility with the enzymatic hydrolysis. Besides cellulose accessibility this means the lowest possible unspecific adsorption of cellulases on the substrate. As demonstrated here, adsorption studies with fluorescent proteins like mCherry represent a feasible tool to characterize biomass as well as the influence of pretreatment methods on unspecific protein adsorption.

Chapter 4

Conceptual design and feasibility study for LMS-based lignin depolymerization in biorefineries

4.1 Introduction

The ability of laccase-mediator-systems to depolymerize lignin is discussed controversially (see chapter 1.5.4., Leonowicz et al., 2001; Munk et al., 2015). Laccase-catalyzed lignin cleavage has been claimed, but reliable experimental evidence is still missing (Bourbonnais et al., 1995; Call and Mücke, 1997; Cho et al., 2004; Giardina et al., 2010; Leonowicz et al., 1985; Shleev et al., 2006a; Thurston, 1994). Processing of lignin by LMS in biorefineries requires a suitable reaction environment which compromises between the stability of laccase and solubilization of the barely water-soluble lignin. Furthermore, lignin degradation products as well as lignin itself are prone to repolymerization under oxidizing conditions.

Therefore, a rapid removal of degradation products or a spatial separation of LMS and reactants, respectively, is essential to avoid undesired radical re-coupling (Leonowicz et al., 2001). Organic solvents are promising process additives to solubilize lignin. Aqueous-organic two-phase systems potentially allow for *in-situ* extraction of reaction products and compartmentalization of the reaction system. The advantages of organic solvents for lignin processing are countered by their inactivating effect on enzymes. Besides, the laccase-catalyzed oxidation or even cleavage of lignin essentially depends on the presence of suitable redox mediators, which are also known to inactivate laccase due to their radical character after enzymatic oxidation.

The aim of this study (Höfler, 2014; designed and supervised by Simon Roth) was to validate the concept of LMS-catalyzed lignin depolymerization as well as to identify and validate potential strategies that address e.g. inactivation of laccase by the mediator, lignin solubilization, and undesired repolymerisation. Three commercial preparations of fungal laccases were characterized regarding purity, specific activity, and basic kinetic parameters. Beyond, the influence of different organic solvents with potential as solubilizer for lignin on laccase activity and stability was investigated, and two typical synthetic mediator compounds (HBT, VA) were tested concerning their inactivating effect on laccase. A reaction system based on a lignin model compound (LMC) was established to gain insights into the reactions between laccase, mediator and substrate. LMC oxidation was monitored using high performance ion exchange chromatography coupled to pulsed amperometric detection (HPAEC-PAD). The applicability of LMS for the processing of genuine lignin was evaluated using organosolv lignin as the substrate. The experiments were conducted under pure aqueous conditions and in aqueous-organic two-phase systems. Lignin samples were analyzed by use of gel permeation chromatography (GPC).

4.2 Materials and Methods

4.2.1 Chemicals

All chemicals were of analytical-reagent grade or higher and purchased from Sigma Aldrich (St. Louis, USA) or Carl Roth (Karlsruhe, Germany), if not stated otherwise. The lignin model compound was synthesized and kindly provided by Jakob Mottweiler from the Institute for Organic Chemistry (IOC, Prof. Bolm) at RWTH University. Organosolv lignin was obtained from the Fraunhofer Institute for Chemical Technology ICT in Pfinztal and extracted as described in (Schmiedl et al., 2012).

4.2.2 Enzymes

Laccase preparations of different biological origin were purchased from Sigma Aldrich (St. Louis, USA). The product specifications are given in Table 4.1:

Table 4.1: Commercial laccase preparations.

Laccase origin	Art.-Nr.	Batch#	Appearance	Supplier
Agaricus bisporus	40452-100MG	BCBK6143V	powder	Sigma Aldrich
Pleurotus ostreatus	75117-1G	BCBH4411V	powder	Sigma Aldrich
Trametes versicolor	51639-1G	BCBF7247V	powder	Sigma Aldrich

4.2.3 Determination of the protein content of laccase preparations

The mass-related protein content in the laccase preparations was determined with three different colorimetric assays. The assays were conducted as prescribed in the manufacturer's manual, if not stated otherwise.

The bicinchoninic acid protein (BCA) assay was conducted by use of the Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Absorption measurements were performed at 562 nm and 30 °C. The DC assay (Bio-Rad, Hercules, CA, USA) was performed as described in chapter 3.2.8. Absorption values were determined at 750 nm and room temperature. The Coomassie (Bradford) protein assay was applied according to the manual instructions for MTPs with a sample volume of 5 µL. Samples were incubated for 10 min at room temperature and measured at 595nm and 30°C.

1 g/L stock solutions of the laccase preparations were prepared. The concentration of the stock solution of laccase from *A. bisporus* was 0.5 g/L due to limited solubility. The stock solutions were diluted 1:1, 1:2.5, 1:6.25, 1:10 and 1:25 for measurement. BSA standards in a concentration range of 25 µg/mL to 2000 µg/mL were used for assay calibration.

Photometric absorption measurements were performed using a *Synergy MX* microtiter plate photometer (Biotek, Winooski, VT, USA). The protein concentration was derived from the slope of the regression curve over all dilutions.

4.2.4 ABTS-based activity assay for determination of laccase activity and kinetic parameters according to Michaelis and Menten

A colorimetric assay based on the laccase-catalyzed oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was used to quantify laccase activity. The ABTS assay was always conducted at 30°C in 96-well MTPs containing 200 µL 0.1 M NaAc buffer (pH 4.5) resulting in a path length of 0.618 cm. The reaction was started by the addition of laccase to the reaction mixture: 60 µL of laccase sample were mixed with 140 µL ABTS solution (0.715 mM) directly within the MTP well, leading to a final ABTS concentration of 0.5 mM. All solutions were preheated to 30°C. The initial linear increase in adsorption at 420 nm was monitored spectrophotometrically using a *Synergy MX* microtiter plate photometer (Biotek, Winooski, VT, USA). The specific laccase activity was calculated according to equation (4.1) using the molar extinction coefficient of oxidized ABTS (radical cation $ABTS^{\bullet+}$) of ε_{420}= 0.036 L/(µmol cm) (Collins et al., 1998; Harwardt et al., 2014; Johannes and Majcherczyk, 2000).

$$A_{spec} = \frac{v_0}{c_{enzyme,well}} = \frac{\frac{\Delta A}{t}}{\varepsilon_{420} \cdot d \cdot c_{enzyme,well}} \tag{4.1}$$

A_{spec}:	specific enzyme activity	[U/mg]
$\Delta A/t$:	change in absorption per time	[1/min]
$c_{enzyme,well}$:	enzyme concentration in reaction mixture	[mg/L]
d:	path length of light	[cm]
ε_{420}:	extinction coefficient of ABTS at 420 nm	[L/(µmol cm)]
v_0:	initial reaction rate	[µmol/(min L)][U/L]

To determine kinetic parameters according to Michaelis and Menten, initial reaction rates were measured at initial ABTS concentrations in a range of 0.015 - 0.93 mM. Appropriate ABTS dilutions were prepared from a 5 mM stock solution in 0.1 M NaAc buffer (pH 4.5). Laccase preparations were diluted from 1 g/L stock solutions in NaAc buffer to adjust suitable volumetric activities for an accurate measurement of the initial increase in absorption. The final concentrations of laccase in the assay were 30 mg/L (preparation from *A. bisporus*) or 3 mg/L (preparation from *P. ostreatus* / *T. versicolor*), respectively, due to their different specific activities. Initial reaction rates were plotted over applied ABTS concentrations, and the kinetic parameters were obtained by fitting the Michaelis-Menten equation (4.2) to these data.

$$v = \frac{v_{max} \cdot [S]}{K_M + [S]} \tag{4.2}$$

K_M:	Michaelis-Menten constant	[mM]
[S]:	substrate concentration	[mM]
v:	reaction rate	[U/mg]
v_{max}:	maximal reaction rate	[U/mg]

4.2.5 Determination of laccase stability in presence of organic solvents and mediator compounds

The stability of laccase from *P. ostreatus* and *T. versicolor* in solvent-saturated buffer as well as in aqueous-organic two-phase-systems was investigated. The tested solvents were 1-butanol, 2-methyltetrahydrofuran (2-MTHF), n-heptane and 1-hexanol. The solvent-saturated buffers were produced by mixing an excess of organic solvent with 0.1 M NaAc buffer (pH 4.5) resulting in a two-phase-system. The solvent-saturated buffer was separated from the organic phase using a separatory funnel. 1 g/L of laccase preparation was dissolved in 10 mL of each solvent saturated buffer and incubated under stirring at room temperature. As reference, 1 g/L of each laccase preparation was incubated in pure 0.1 M NaAc buffer (pH 4.5) and compared to the solvent-saturated buffers. Over time, each 100 µL samples were taken and diluted 1:10 with 900 µL 0.1 M NaAc buffer (pH 4.5) to minimize the influence of the solvent on the ABTS assay. 60 µL diluted sample was applied for the ABTS assay

resulting in a final concentration of 30 mg/L laccase preparation. The ABTS assay was conducted as described above (4.2.4).

For stability tests in aqueous-organic two-phase-systems 10 mL of solvent-saturated buffers containing 1 g/L laccase preparation were prepared as described above and mixed with the same amount of the particular organic solvent. The mixtures were incubated in 50 mL centrifuge tubes at 25 °C and 700 rpm to maximize the aqueous-organic interface. The two-phase-systems were compared to samples with solvent-saturated buffer as reference to observe the influence of inactivation at the interface. Aqueous phase samples were handled and measured as described above for the experiments with solvent-saturated buffers. The ratio of aqueous and organic phase was kept constant by removal or addition (in case of evaporation) of the organic solvent.

The influence of concentration of the synthetic mediator compounds 1-hydroxybenzotriazol (HBT) and violuric acid (VA) on laccase stability was tested. Therefore, 1 g/L of the laccase preparations from *P. ostreatus* and *T. versicolor* were incubated with 5 mM and 10 mM of each mediator in 10 mL 0.1 M NaAc buffer (pH 4.5) in 15 mL centrifuge tubes at 25°C and 300 rpm. Experiments without mediator were conducted as reference. The incubation mixtures were sampled over time and residual laccase activity was determined using the ABTS assay as described above. The sample volume was 100 µL for the trial with HBT and 10 µL for the trial with VA. The samples were diluted 1:10 (HBT) or 1:100 (VA), respectively, to minimize the influence of the oxidized mediator on the ABTS assay. The dilution factor for the samples containing VA was chosen higher due to the stronger reactivity of VA. The resulting laccase concentrations in the ABTS assay were 30 mg/L (HBT trial) or 3 mg/L (VA trials).

4.2.6 Modelling of laccase inactivation

The inactivation of laccase in the presence of solvents or mediators was described by a phenomenological model (Aymard and Belarbi, 2000) representing a biexponential decay (Eq. 4.3). The model assumes the influence of two inactivating effects. The proportion of their contribution to enzyme inactivation is described by the factor f. The half-lives calculated from

the model can be used to quantify and compare laccase inactivation under different conditions.

$$A_t = f \cdot A_{max} \cdot e^{-k_1 \cdot t} + (1 - f) \cdot A_{max} \cdot e^{-k_2 \cdot t} \quad (4.3)$$

A_t:	Calculated enzyme activity	[U/mg]
A_{max}:	Maximal measured enzyme activity	[U/mg]
f:	Proportion factor	[-]
k_1:	Inactivation constant 1	[1/h]
k_2:	Inactivation constant 2	[1/h]
t:	Time	[h]

4.2.7 Oxidation of a lignin model compound with LMS

The compound 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol was used as model substrate to study the reaction of LMS with β-O-4-type lignin structures. It is referred to as lignin model compound (LMC) in the following. If not stated otherwise, the reactions conditions were the following: A stock solution with 400 mg/L LMC was prepared and diluted by mixing with the other components of the reaction system resulting in a final concentration of 200 mg/L LMC which equals to 0.6 mM (MW_{LMC} = 334.37 g/mol). VA and HBT were tested as mediators. The applied standard concentration of 5 mM was diluted from 12.5 mM stock solutions in the reaction mixture. The reaction was started by adding laccase preparation from *T. versicolor* from a 10x stock solution (0.1 g/L) leading to a final concentration of 10 mg/L. All solutions were prepared in 0.1 M NaAc buffer (pH 4.5). The reaction mixture was incubated at 30°C and sampled over time.

When samples were taken manually laccase was heat inactivated for 10 min at 95°C. When an automated sampling was realized by use of the Dionex ICS-5000+ AS-AP autosampler (Thermo Fisher Scientific, USA), enzyme inactivation was achieved by an immediate mixing of the sample with the eluent (100 mM NaOH, pH > 12). The samples were analyzed regarding their residual concentration of reduced LMC using HPAEC-PAD. Before analysis, samples were filtered through a polyethersulfone (PES) filter with a pore size of 0.2 µm.

4.2.8 Analysis of LMC using HPAEC-PAD

The concentration of the reduced diol-form of the LMC was measured with high HPAEC using a Dionex ICS-5000$^+$ device (Thermo Fisher Scientific, USA) coupled to a PAD (ICS-5000 ED, Thermo Fisher Scientific, USA). The detector was equipped with a gold electrode and an Ag/AgCl reference electrode. The analytical column was a CarboPac PA100 (4 x 250 mm, Thermo Fisher Scientific, USA). The column was tempered to 30°C and the flow rate was adjusted to 0.5 mL/min. The injection volume was 10 µL. For sample elution a gradient blended from four different eluent solutions and varying over elution time was applied. The composition of the eluent blend is given in Table 4.2. Under these conditions the retention time of the LMC was 5.8 ± 0.2 min.

Table 4.2: Eluent blend for concentration gradient for HPAEC analysis.

Eluents:				
A:	100 mM NaOH			
B:	500 mM NaAc with 100 mM NaOH			
C:	200 mM NaOH			
D:	ultrapure water			
Gradient composition:				
Time after injection [min]	**Percentage of eluent [%]**			
	A	**B**	**C**	**D**
equilibration (15 min)	50	-	-	50
0.0	50	-	-	50
7.0	50	-	-	50
10.0	65	10	-	25
12.0	65	10	-	25
14.0	40	35	25	-
22.0	40	35	25	-
26.0	55	10	10	25
28.0	50	-	-	50

4.2.9 Incubation of lignin with a laccase-mediator-system in aqueous environment

The influence of a LMS consisting of laccase from *T. versicolor* and the mediator HBT on organosolv lignin in aqueous environment was investigated. The experiments were performed in 50 mL centrifuge tubes at 30°C and 900 rpm for 48 h. 1 g/L laccase preparation together with 1.0, 2.5 or 5 mM HBT were dissolved in 10 mL of 0.1 M MOPS buffer (pH 7). Experiments without LMS or mediator, respectively, were conducted as reference. 0.1 g (final concentration 10 g/L) of organosolv lignin was suspended in the LMS containing buffer. After incubation for 48 h solid lignin was spun down for 30 min at 4000 rpm and 4°C. The pellets were washed three times with 20 mL 0.1 M NaAc buffer (pH 4.5) for 5 min at 900 rpm, spun down as described above and separated from the supernatant by decantation. Washing was performed to remove potential residues of the mediator or its side products. The solid lignin samples were analyzed with GPC.

4.2.10 Incubation of lignin with a laccase-mediator-system in an aqueous-organic two-phase-system

0.1 g organosolv lignin was dissolved in 10 mL 1-hexanol (final concentration 5 g/L) and mixed with 10 mL 0.1 M MOPS buffer (pH 7) containing 1 g/L laccase preparation from *T. versicolor* and 1 mM HBT. Experiments without LMS were conducted as reference. The reaction mixture was incubated in 50 mL centrifuge tubes for 48 h at 30°C and 700 rpm. Afterwards, the samples were centrifuged for 5 min at 4000 rpm and 4°C and phases were separated. The organic phase was fully vaporized to solid state by use of a rotary evaporator. The aqueous phase as well as the crud layer (layer of solids at the interphase) were first lyophilized, and residual 1-hexanol was removed by evaporation afterwards. 10 mg of the obtained solids were applied for GPC analysis.

4.2.11 Lignin analysis with gel permeation chromatography

Lignin fractions were analyzed using gel permeation chromatography (GPC) to detect shifts within the molecular weight distributions of the samples.

5 mg of solid lignin samples were dissolved in 1 mL of eluent resulting in a final sample concentration of 5 g/L. Samples were filtered before injection through a PVDF filter with 0.22 µm pore size to exclude any solids.

GPC measurements were performed with help of Dr. Thomas Schmidt at the DWI - Leibniz Institute for Interactive Materials. GPC analysis was conducted by use of a EcoSEC semi-micro device (PSS - Polymer Standard Services, USA) equipped with a PSS Suprema Pre Column connected in series to three PSS Suprema 5 µm 100Å columns (PSS - Polymer Standard Services, USA). Chromatograms were recorded by absorption measurement at 280 nm with an Agilent 1200 VWD Variable Wavelength UV-Detector. A 20 mM solution of Na_2HPO_4 (pH 12) containing 0.01 % (m/m) NaN_3 and 0.5 g/L PEG 6000 was used as eluent. PEG 6000 was added to avoid agglomeration of lignin molecules. The flow rate was adjusted to 1 mL/min, and the injection volume was 100 µL. Measurements were performed at 40°C.

The UV-signals were normalized to one and plotted over the elution volume. Chromatograms were interpreted qualitatively by comparing elution volumes and curve shapes (Mattinen et al., 2008).

4.3 Results and Discussion

4.3.1 Characterization of commercial laccase preparations

Three commercial laccase preparations were characterized regarding their suitability for lignin processing. Initially, basic parameters of the preparations were determined (Table 4.3). The mass-related protein content of the powdery preparations gave first information about the purity of the product and was used to calculate specific enzyme activities.

The protein content was measured by use of three different colorimetric assays. The BCA assay is a simple and sensitive standard method for protein determination, but prone to errors due to possible sugar impurities or protein glycosylation (Brown et al., 1989). Since fungal laccases are glycoproteins with an extent of glycosylation of 10 – 25 wt % (Baldrian, 2006;

Dwivedi et al., 2011; Giardina et al., 2010) the protein content was double-checked with two other assays. Like the BCA assay, the DC assay is based on the Biuret reaction (Brown et al., 1989), but involves a different reagent forming the detectable chromophore which makes it generally insensitive to a broad range of impurities like e.g. sugars or detergents (Peterson, 1979). The Bradford assay uses the binding of the dye Coomassie Brilliant Blue G-250 to basic amino acids, especially arginine (Bradford, 1976).

BCA and DC assay resulted in comparable values. This was unexpected since the BCA assay was assumed to overestimate the protein content due to the glycosylation of laccase. The protein contents determined with the Bradford assay differed from the results of BCA and DC assay and had a much higher error of up to 38 %, why they were not further evaluated. Referring to the results of BCA and DC assay, the preparations of *P. ostreatus* and *T. versicolor* have a comparable protein content of 16 - 18 wt % which is significantly higher than that of the *A. bisporus* preparation (6 - 11 wt %). The non-protein content of the powdery preparations most likely originates from residues of the fermentation medium or stabilizers. Since the BCA assay was the standard procedure for protein quantification from the beginning of this project and its reliability was confirmed by the DC assay, its results were used to calculate specific laccase activities.

Table 4.3: Basic parameters of the characterized laccase preparations.

Laccase origin	Protein content [wt %]			v_{max}* [U/mg]	K_M* [mM]
	BCA assay	DC assay	Bradford assay		
Pleurotus ostreatus	16.5 ± 1.6	18.0 ± 0.1	14.2 ± 4.6	36.6 ± 0.5	0.0482 ± 0.0025
Trametes versicolor	17.1 ± 2.3	17.5 ± 1.2	24.7 ± 7.7	35.2 ± 0.8	0.0508 ± 0.0041
Agaricus bisporus	6.9 ± 3.5	10.8 ± 1.0	21.2 ± 8.2	11.4 ± 0.8	0.3680 ± 0.0602

*determined with ABTS as substrate and referred to the protein content determined with BCA assay

Table 4.4: Typical parameters of fungal laccases.

Laccase origin	K_M (ABTS) [mM]	pH	Temp. Optimum [°C]	Source
T. versicolor	0.0128	3.0	50	(Dwivedi et al., 2011)
	0.0373	4.0	n.s.	(Höfer and Schlosser, 1999)
	0.0508	4.5	30	this study
P. ostreatus	0.0390[a]	5.5	n.s.	(Garzillo et al., 2001)
	0.4700[b]	3.5	n.s.	(Garzillo et al., 2001)
	0.2800	3.0	25	(Palmeiri et al., 1993)
	0.0482	4.5	30	this study
Median for fungal laccases[#]	0.0390[*]	-	-	(Baldrian, 2006)

a: POXC
b: POXA1b
[*]n=36, Q_{25}=18, Q_{75}=100, min=0.004, max=0.77
[#]calculated and presented in the review by Baldrian, 2006

The laccase preparations were also analyzed regarding their kinetic parameters according to Michaelis and Menten (Michaelis and Menten, 1913). Michaelis-Menten plots were recorded using ABTS as the substrate (Figure 4.1). While the K_M values represent substrate affinity, the v_{max} correspond to the specific laccase activity under substrate saturation. The laccase preparations from *P. ostreatus* and *T. versicolor* behave almost identically. The determined K_M values are similar to those found in literature (Table 4.4). In case of *P. ostreatus* two laccase-like enzymes are reported from which POXC has a K_M value in the same order of magnitude as found in this study (Garzillo et al., 2001). Compared to the laccase preparations from *P. ostreatus* and *T. versicolor* the *A. bisporus* preparation had a significantly lower specific activity and substrate affinity. The weak performance, also in terms of protein content, was the reason to exclude the laccase preparation from *A. bisporus* from further investigations.

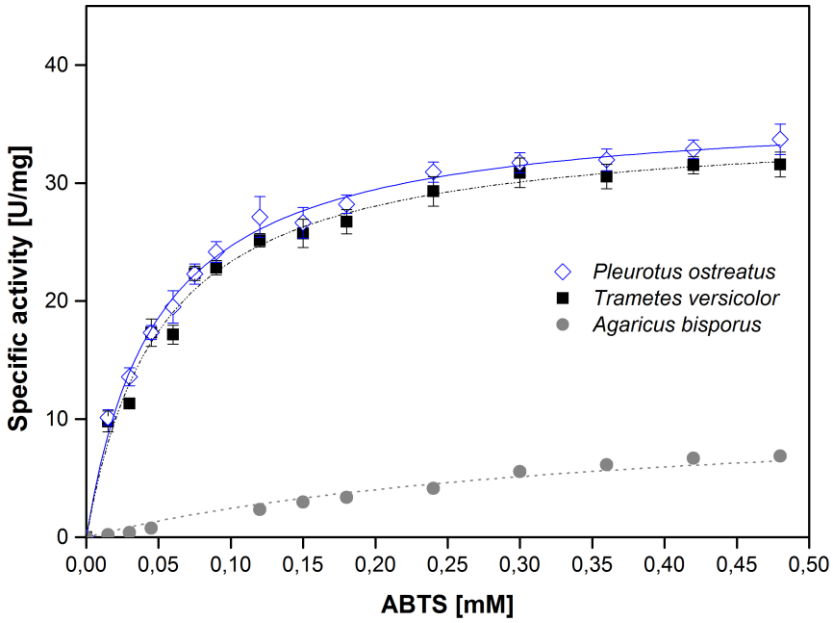

Figure 4.1: Laccase activity as a function of substrate concentration (Michaelis-Menten plot). Data points were fitted to the Michaelis and Menten equation.

4.3.2 Stability of laccase in presence of selected organic solvents

Many current concepts for third generation biorefineries include process steps for biomass fractionation or lignin extraction, respectively, based on organic solvents (Zhang, 2011; Zhao et al., 2009). Lignins extracted by organosolv-like processes are usually barely water-soluble (Schmiedl et al., 2012). The addition of organic solvents to the aqueous medium, in which the enzymatic reaction takes place, is a promising strategy to increase lignin solubility and thereby, its availability for the enzymatic reactions. Fungal laccases have been reported to exhibit comparatively high stability in various organic solvents (see chapter 1.2).

Four organic solvents (Table 4.5) were tested as aqueous one-phase- and aqueous-organic two-phase-systems regarding their impact on laccase activity and stability. The one-phase-systems represent the molecular influence of the solvent on the enzyme, whereas the experiments with two-phase-systems also address inactivation at the interface. The homogeneous systems with solvent-saturated buffer were compared to solvent-free buffer, and the two-phase-systems were compared to solvent-saturated buffer to distinguish interface effects (Figure 4.2). The inactivation behavior of laccase in presence of organic solvents, both

in one- and two-phase-systems, was fitted by a biexponential model (eq. (4.3) for the phenomenological description of enzyme inactivation irrespective of the underlying mechanism (Aymard and Belarbi, 2000). The solvent influences are discussed solvent by solvent below.

Table 4.5: Properties of organic solvents tested regarding compatibility with laccase.

Solvent	Water solubility [% (m/m_{water})]	LogP	Lignin solubility*
NaAc buffer (0.1 M, pH 4.5)	-	-	-
2-MTHF	14	0.82	++
1-Butanol	8	0.88	+
1-Hexanol	0.6	1.94	+
n-Heptane	$2*10^{-4}$	4.47	-

*simple solubility test with organosolv lignin by visual evaluation (cp. Figure 4.10). Rough classification in not soluble (-), slightly soluble (+), soluble (++)

The organocat process for biomass fractionation which was developed within the TMFB uses 2-methyltetrahydrofuran (2-MTHF) as organic phase for lignin extraction (vom Stein et al., 2011). 2-MTHF has a high water solubility of 140 g/L and a comparatively high polarity represented by a LogP value of 0.82, promising increased lignin solubility in a 2-MTHF-saturated aqueous phase (Table 4.5). Even though 2-MTHF led to a strong initial decrease of laccase activity (Figure 4.2 A), resulting in 74 – 85 % lower half-lives (Table 4.6), residual activities equal to the solvent-free reference approach from approximately 260 h, demonstrating good long-term stability in presence of 2-MTHF. No inactivating interface effects were found in the two-phase-system (Figure 4.2 E). These results suggest the possibility to feed the lignin-containing 2-MTHF-phase of the organocat process directly into the process step for LMS-catalyzed lignin processing.

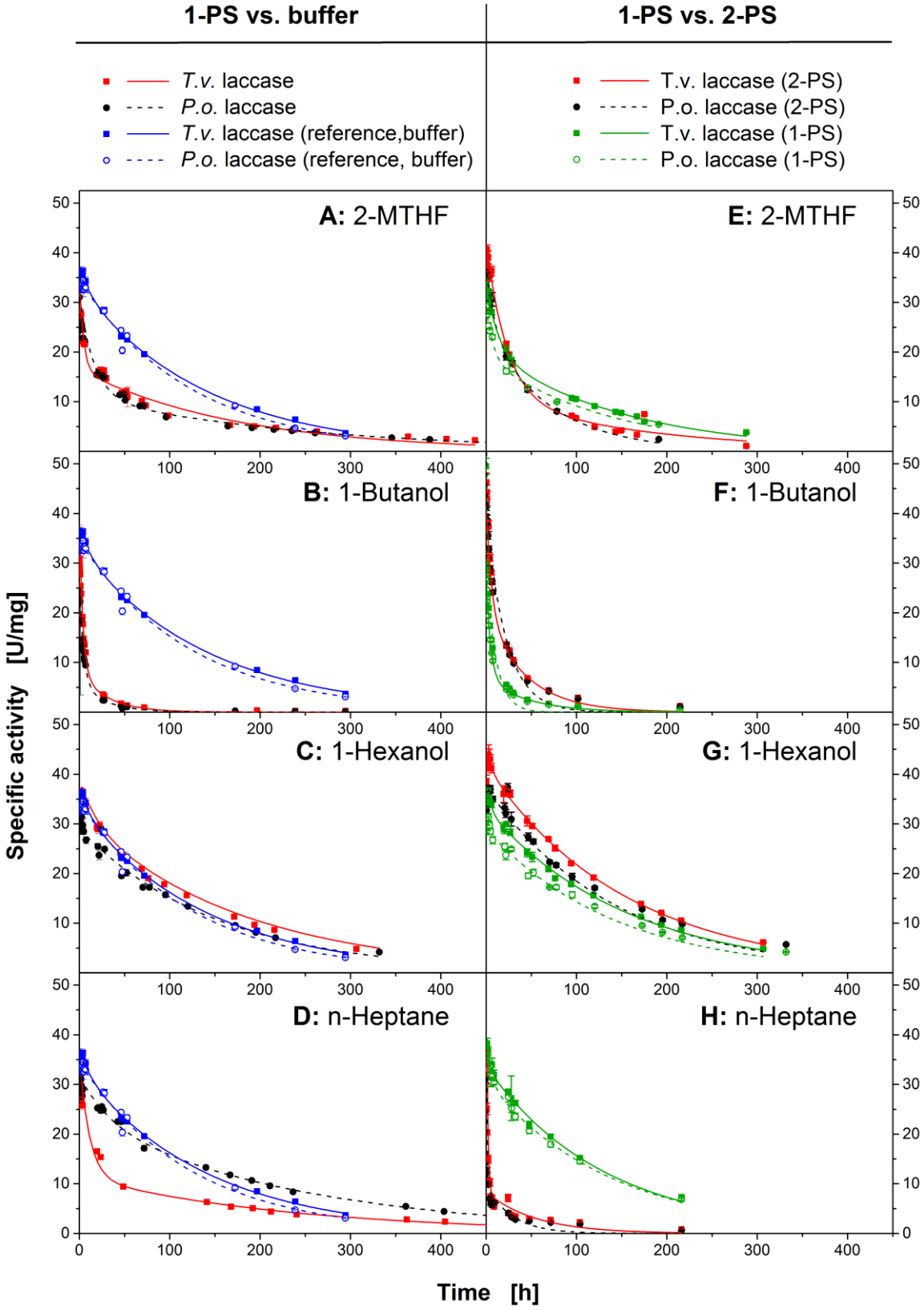

Figure 4.2: Stability of laccase in the presence of different organic solvents in aqueous one- and aqueous-organic two-phase systems. Laccase activity was determined by ABTS assay. Data points were fitted to a biexponential model (eq. 4.3, Aymard and Belarbi, 2000).

1-Butanol had the highest inactivating effect among the tested solvents (Figure 4.2 B, F). Half-lives were decreased by ~95 % excluding 1-butanol as solvent for LMS-catalyzed lignin processing. Stability of laccase in presence of 1-hexanol was comparable to the solvent-free system (Figure 4.2 C), which is most likely due to the low water solubility of 6 g/L. The experiments with the two-phase-system did not reveal any negative effect of the interface (Figure 4.2 G). The opposite was true for n-heptane. Due to its negligible water solubility, no inactivating effect was found in the one-phase-system with laccase from *P. ostreatus* (Figure 4.2 D). The decreased laccase stability in the experiment with the laccase from *T. versicolor* was probably caused by an experimental error where insufficient phase separation after preparation of the solvent-saturated buffer resulted in an undesired second phase leading to enzyme inactivation at the interface. This interpretation is in-line with the results of the experiment with a two-phase-system, where a strong interface inactivation due to the highly hydrophobic properties of n-heptane, promoting protein unfolding, was observed (Figure 4.2 H).

Table 4.6: Kinetic parameters and half-life times derived from fitting laccase activities in presence of organic solvents (Figure 4.2) to a biexponential model (eq. 4.3, Aymard and Belarbi, 2000).

	1-PS							
	f [-]		**k_1 [h^{-1}]**		**k_2 [h^{-1}]**		**$t_{1/2}$ [h]**	
	T.v.	***P.o.***	***T.v.***	***P.o.***	***T.v.***	***P.o.***	***T.v.***	***P.o.***
NaAc buffer (reference)	0.899	0.972	0.007	0.008	0.083	31.300	82.60	81.50
2-MTHF	0.499	0.357	0.006	0.004	0.214	0.062	12.40	21.30
1-Butanol	0.224	0.228	0.033	0.033	0.265	0.211	3.58	4.44
1-Hexanol	0.840	0.910	0.006	0.007	0.057	0.921	87.40	91.70
n-Heptane	0.347	0.262	0.004	0.031	0.089	0.004	14.90	100.30
	2-PS							
	f [-]		**k_1 [h^{-1}]**		**k_2 [h^{-1}]**		**$t_{1/2}$ [h]**	
	T.v.	***P.o.***	***T.v.***	***P.o.***	***T.v.***	***P.o.***	***T.v.***	***P.o.***
2-MTHF	0.264	0.675	0.006	0.014	0.044	0.098	23.00	25.60
1-Butanol	0.628	0.491	0.045	0.023	1.820	0.171	5.09	9.31
1-Hexanol	0.930	0.991	0.006	0.007	0.197	11.600	96.30	104.40
n-Heptane	0.229	0.298	0.018	0.035	0.761	3.390	1.35	0.36

No significant differences in solvent compatibility were found between both laccases from *T. versicolor* and *P. ostreatus*. The compatibility results based on laccase activity and stability in one- and two-phase systems together with a rough determination of lignin solubility (cp. Table 3.1) suggest 2-MTHF and 1-hexanol as promising solvents for LMS-catalyzed lignin processing.

4.3.3 Influence of typical mediator compounds on laccase stability

Suitable redox mediators are essential for the laccase-catalyzed oxidation of non-phenolic lignin structures, but common synthetic mediators are known to inactivate laccase due to their high reactivity as a radical after enzymatic oxidation (Camarero et al., 2007; Fillat et al., 2010; Li et al., 1999; Rehmann et al., 2013). Due to their high redox potentials of E_0=1.08 V and E_0=0.916 V (Fabbrini et al., 2002a), respectively, HBT and VA belong to the most reactive mediators and are therefore, commonly used in LMS. The influence of HBT and VA was investigated by a stability study to quantify the inactivating effect.

As expected, both mediators caused a rapid inactivation of laccase, independent of the tested concentrations and the origin of laccase (Table 4.7, Figure 4.3). Compared to experiments with 5 mM mediator, inactivation was only slightly faster in presence of 10 mM mediator, suggesting these concentrations being close to saturation regarding laccase inactivation. VA was found to inactivate laccase much faster than HBT despite its lower redox potential. Potentially, the lower redox potential of VA allows a higher turnover rate causing a faster inactivation of the enzyme. Indeed, for laccases of different origins higher k_{cat} values for VA compared to that of HBT have been reported (Li et al., 1999), supporting the above hypothesis that laccase inactivation correlates with mediator turnover rates. For example, for a laccase from *Trametes villosa* the k_{cat} value for VA (260 ± 20 min^{-1}) was three times higher than that of HBT (84 ± 6 min^{-1}).

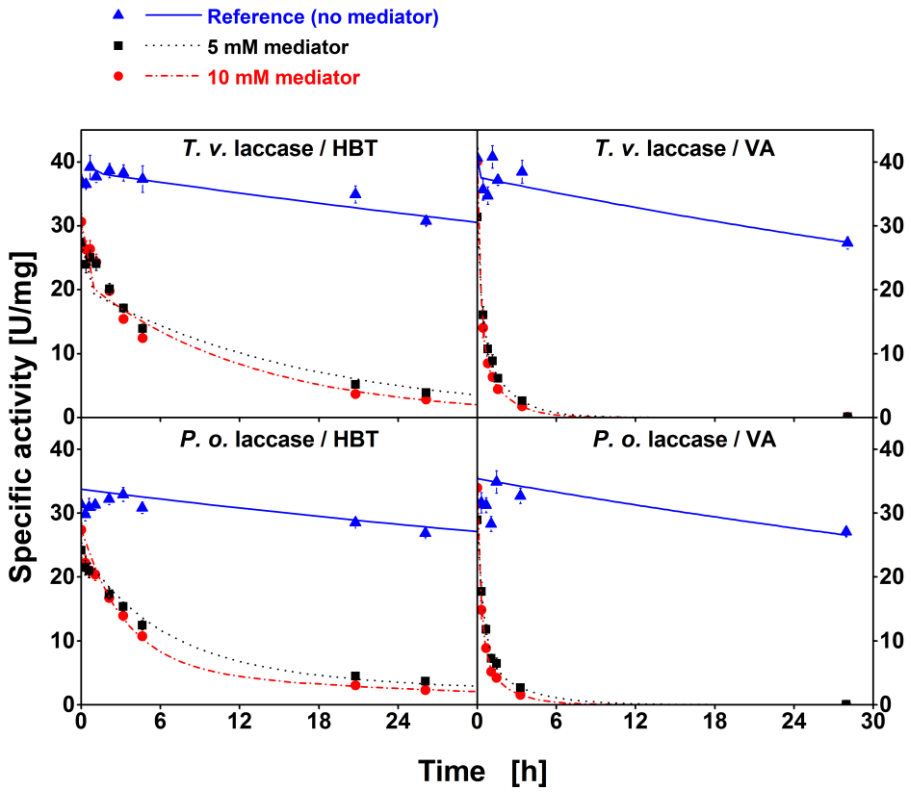

Figure 4.3: Influence of the mediator compounds HBT and VA on laccase stability. Laccase activity was determined by ABTS assay. Data points were fitted according to the biexponential model (eq. 4.3, Aymard and Belarbi, 2000).

Compared to the inactivation due to organic solvents (Table 4.6), the effect of the mediator compounds HBT and VA is magnitudes higher. A reaction of radical mediators with aromatic amino acids like tryptophane or tyrosine is commonly assumed as the mechanism behind mediator-induced laccase inactivation (Aracri et al., 2009; Arca-Ramos et al., 2012; Li et al., 1999; Soares et al., 2001). Especially mediator compounds with N-hydroxy functionality like HBT or VA have been found to possess a strong inactivating effect on enzymes whereas natural phenolic mediators are much less harmful but limit the scope of oxidizible substrates due to their lower redox potentials (Aracri et al., 2009). Newly identified high-redox-potential mediator compounds might cause less extensive laccase inactivation. Replacement of aromatic amino acids on the enzyme surface by protein engineering might prevent mediator-induced laccase inactivation (Li et al., 1999). Beyond, medium engineering can reduce the undesired inactivation effect of mediators, e.g. by surfactants protecting laccase from mediator-induced inactivation (Ji et al., 2009). Alternatively, water-immiscible ionic liquids

formed two-phase systems, in which the mediator partitioned into the IL phase and was thereby, separated from the laccase resulting in reduced inactivation (Rehmann et al., 2013).

Table 4.7: Tested mediator compounds and half-lives of laccase in presence of the mediator.

Mediator	Redox potential E_0 [V]*	Conc. [mM]	Half-life $t_{1/2}$ [h]	
			T.v.	***P.o.***
HBT	1.08	5	6.82	5.7
		10	4.38	3.09
VA	0.916	5	0.47	0.48
		10	0.27	0.28
NaAc buffer			82.6	81.5

*(Fabbrini et al., 2002a)

4.3.4 Investigation of an artificial, LMC- based reaction systems for studying LMS reaction networks

LMC contain defined lignin sub-structures (cp. chapter 1.5.2) and are ideally suited to investigate the reaction network of LMS. The compound 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol (Figure 4.4, 1) represents the typical β-O-4 bond accounting for 45-60 % of the inter-subunit linkages in lignin (Azadi et al., 2013; Zakzeski et al., 2009). In combination with suitable redox mediators laccase is able to oxidize the adlerol form of the LMC (Figure 4.4, 1) to the corresponding adlerone (Figure 4.4, 2; Bourbonnais et al., 1997; Eggert et al., 1996; Li et al., 1999; Majumdar et al., 2014). The reduced adlerol form can be detected via HPAEC-PAD analysis so that the decrease of the educt concentration was followed over time.

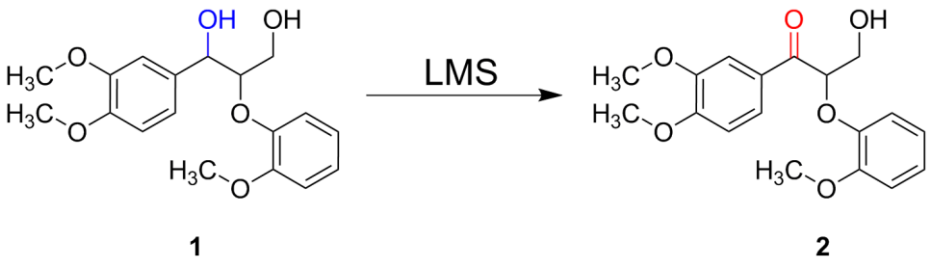

Figure 4.4: Lignin model compound 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol (1) and its LMS-catalysed oxidation to the corresponding adlerone (2).

Three artificial mediators (ABTS, HBT, VA) were tested for LMS-catalyzed oxidation of the LMC and compared regarding reaction rate and conversion (Figure 4.5). When VA was used as mediator, fastest and nearly quantitative conversion of the LMC was observed, whereas the reaction system containing HBT as mediator showed a slower and incomplete conversion. Concerning the redox potentials of both mediators and their impact on laccase stability (Table 4.7) these results are unexpected. VA inactivates laccase ($t_{1/2}$ = 0.47 min) much faster than HBT ($t_{1/2}$ = 6.82 min), and its redox potential (E_0 = 0.916 V) is lower than that of HBT (E_0 = 1.08 V). The lower redox potential of VA might allow for a faster oxidation yielding higher concentrations of oxidized mediator in the system before laccase is inactivated. Oxidized mediator accumulates and continuously oxidizes the LMC, why the reaction lasts much longer than laccase was active in the presence of VA in the stability investigations (~ 6 h, Figure 4.3). The results also indicate that the reaction between mediator and substrate is the rate-limiting step in the reaction system. Also for a laccase from *T. villosa* and the same LMC, higher reaction rates were obtained with VA than with HBT-containing LMS, but in both cases the total conversion was limited to 70% (Li et al., 1999), probably due to the higher concentrations of LMC (1 mM) and mediator (10 mM) in the reaction system.

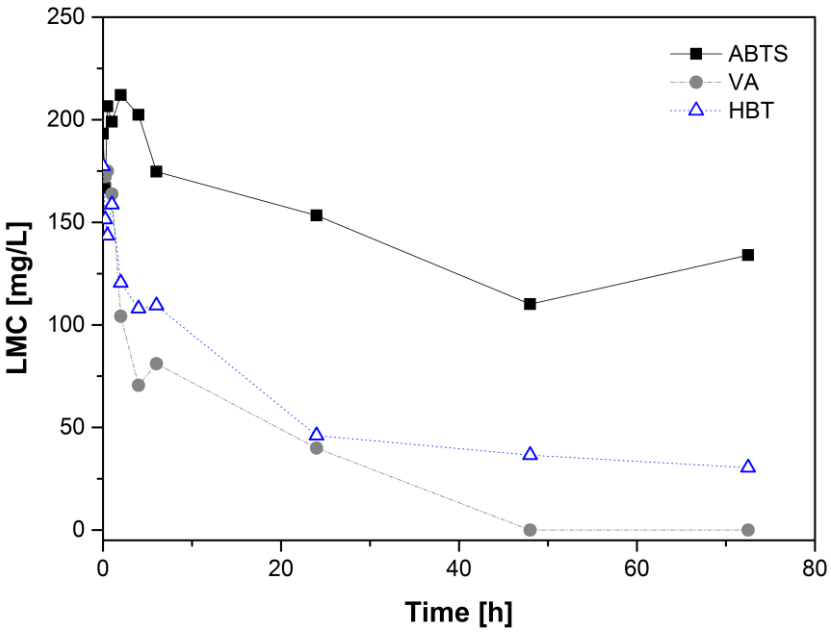

Figure 4.5: Oxidation of LMC by laccase from *T. versicolor* in presence of 5 mM artificial mediators (ABTS, HBT, VA) in NaAc buffer (0.1 M, pH 4.5). LMC concentrations were analyzed by HPAEC-PAD.

Slowest rate and lowest conversion were observed when ABTS was used as mediator. The oxidation of ABTS can yield two different species: The radical cation $ABTS^{\bullet+}$ and the dication $ABTS^{++}$ (Bourbonnais et al., 1998). Even if differing values for the redox potential of both species are reported, the more stable radical cation $ABTS^{\bullet+}$ has the lowest redox potential, which is below that of laccase (Bourbonnais et al., 1998; Fabbrini et al., 2002a). $ABTS^{\bullet+}$ alone was shown being unable to oxidize veratryl alcohol, but laccase allowed for oxidation of veratryl alcohol most likely by conversion of $ABTS^{\bullet+}$ into the more reactive dication (Bourbonnais et al., 1998; Bourbonnais and Paice, 1990). Therefore, laccase inactivation would terminate the reaction. Furthermore, ABTS is known to react via the electron transfer (ET) route (Bourbonnais et al., 1998; Bourbonnais and Paice, 1990), where oxidation strongly depends on the redox potential of the substrate and therefore, is comparatively low for non-phenolic structures like the applied LMC (Baiocco et al., 2003; Fabbrini et al., 2002a). For a deeper understanding, how the mediator influences the reaction system and its conversion efficiency, it would be essential to analyze and monitor the fate of the mediator over reaction time, which requires suitable analytics detecting the different oxidation stages of the mediator as well as potential side products.

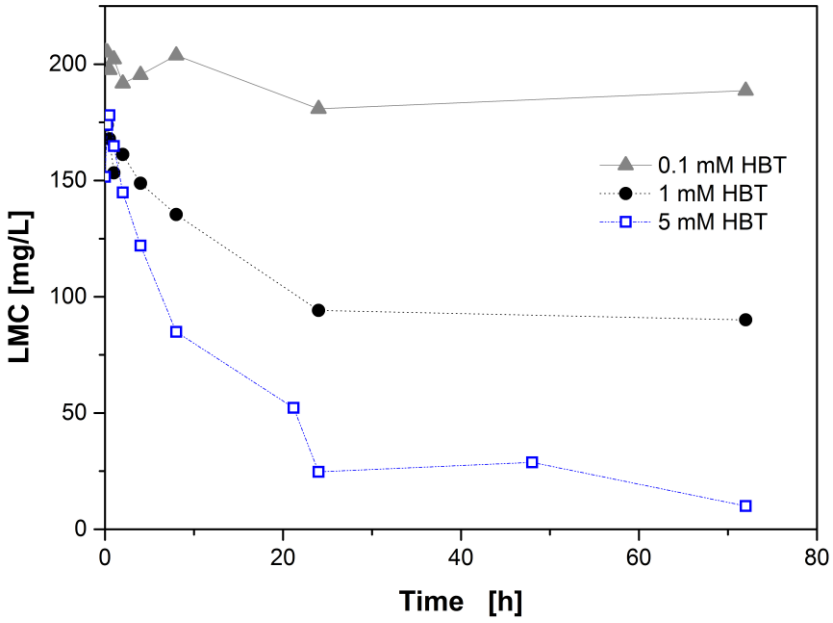

Figure 4.6: Influence of the concentration of the mediator HBT on rate and conversion of LMC oxidation by laccase from *T. versicolor* in NaAc buffer (0.1 M, pH 4.5). LMC concentrations were analyzed by HPAEC-PAD.

HBT is one of the most common mediators which showed much better compatibility with laccase in stability investigations than VA. Therefore, it was applied for further experiments with LMS regarding the role of the mediator during LMC conversion. The influence of the concentration of HBT on the oxidation of the LMC was investigated (Figure 4.6). The mediator is essential to oxidize this non-phenolic LMC (Heap et al., 2014), and no conversion was observed without HBT (data not shown, Heap et al., 2014). When the concentration of HBT was increased from 0.1 mM to 5 mM, also rate and conversion were enhanced, but in both cases full conversion of the LMC was not achieved. With regard to the molar ratio of mediator (0.1 mM / 5 mM / 10 mM) and LMC (0.6 mM) of 0.17 / 8.3 / 16.7, respectively, the incomplete conversion is unexpected, especially, when it is assumed that the enzymatic oxidation of the mediator is not rate-limiting and oxidized mediator can accumulate in the reaction system and convert LMC even if laccase is already inactivated.

Also the influence of the concentration of laccase from *T. versicolor* in the reaction system was investigated regarding rate and conversion (Figure 4.7). 5 mg/L and 10 mg/L laccase preparation led to almost the same results whereas rate and conversion were significantly lower, when only 1 mg/L laccase preparation were applied. 5 mg/L laccase preparation seem

to be saturating regarding the rate of LMC oxidation. This is surprising since mediator-induced laccase inactivation was assumed to be a major reason for the termination of the reaction and the resulting incomplete conversion of the LMC. Therefore, the loss of enzyme activity over reaction time cannot be the only reason, why the reaction stops, before all LMC is oxidized.

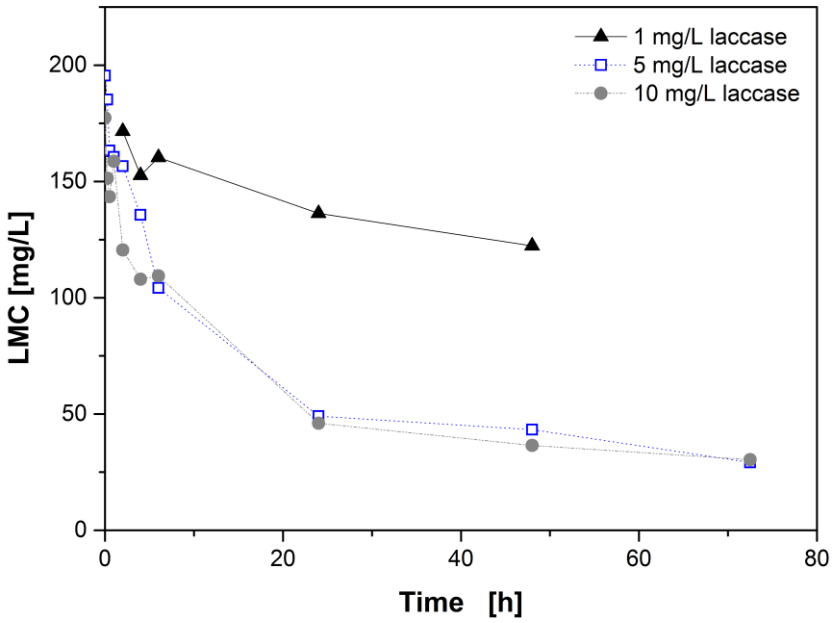

Figure 4.7: Influence of the concentration of laccase from *T. versicolor* on the oxidation of 0.6 mM LMC in NaAc buffer (0.1 M, pH 4.5) with 5 mM HBT. LMC concentrations were analyzed by HPAEC-PAD.

To gain a comprehensive picture of the reaction system, the reaction was automatically sampled with high frequency using the HPLC autosampler (Figure 4.8). Additionally, the residual enzyme activity was determined manually via ABTS assay. The LMC is oxidized and its concentration decreases over time (Figure 4.8). Simultaneously, laccase is inactivated by the oxidized mediator, but even after termination of the LMC conversion, a certain amount of residual laccase activity is found in the reaction system. Therefore, enzyme inactivation is not the reason for incomplete conversion of LMC. Besides the decrease of LMC concentration, also the formation of an unidentified, mediator-derived compound was detected with HPAEC-PAD. The signal for the unidentified compound rises over reaction time and saturates after approximately 12 – 15 h. The compound was attributed to an unreactive side product of HBT since its signal also occurred, when only laccase and HBT and no LMC were in the reaction

system (data not shown). When LMC was given to a solution of abreacted HBT no conversion was observed (data not shown). It is reported in literature, that HBT can react to certain unreactive side products like e.g. benzotriazole (BT) (Bourbonnais et al., 1997; Li et al., 1998; Xu et al., 2000). BT was excluded as the found side product on the basis of a comparative reference measurement of a BT standard. Therefore, structure elucidating analysis techniques are necessary to identify the side product of HBT. However, the mediator HBT is detracted from the reaction system by an undesired side reaction. Since residual laccase activity is found in the reaction system even after termination of the reaction and the concentration of the unidentified side products saturates approximately when the LMC conversion stops, it has to be assumed, that the loss of reactive mediator over time is the major reason for the incomplete conversion of the LMC.

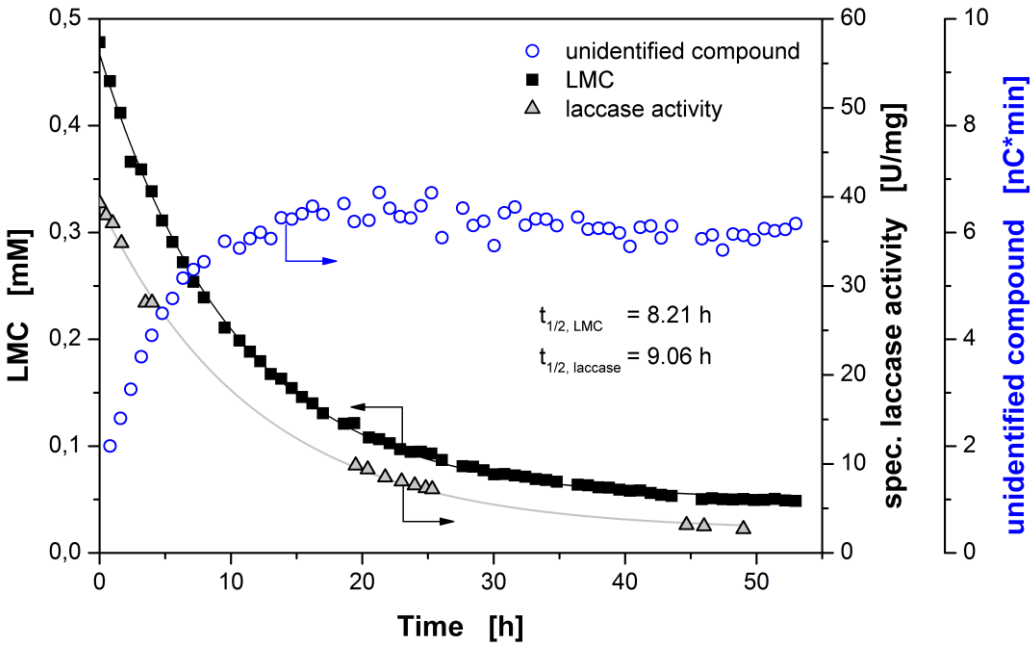

Figure 4.8: Reaction of LMS (laccase from *T. versicolor*, 5 mM HBT) with LMC in NaAc buffer (0.1 M, pH 4.5). Concentrations of LMC and an unidentified mediator side product were followed over time by an automated HPAEC-PAD method. Laccase activity was determined manually via ABTS assay. Decrease in LMC concentration and laccase activity was fitted to a first order decay. The half-lives are given in the diagram.

The results presented in this sub-chapter reveal that the LMS-catalyzed oxidation of a LMC representing a typical lignin structure involves a complex reaction network (cp. Figure 1.4). A detailed understanding of the mechanisms and interactions of the different occurring reactions

is essential for process design and requires further experiments addressing the influence of parameters like concentration of enzyme, mediator and substrate as well as the fate of all compounds during the reaction.

4.3.5 Conversion of organosolv lignin with LMS

Based on the natural example of delignifying white-rot fungi laccases are commonly supposed to contribute to lignin depolymerization (Giardina et al., 2010; Thurston, 1994), although the ability of LMS to depolymerize lignin has been discussed controversially over decades (Leonowicz et al., 2001; Munk et al., 2015). Especially in paper pulping, the ability for lignin degradation was attributed to laccase in combination with certain redox mediators, but opposed repolymerization activity was also reported (Bourbonnais et al., 1995; Call and Mücke, 1997; Cho et al., 2004; Leonowicz et al., 1985; Shleev et al., 2006a).

Here, the question of lignin depolymerization was experimentally addressed by investigating the influence of a LMS consisting of laccase from *T. versicolor* and the mediator HBT on organosolv lignin. The experiments were performed in an aqueous buffer system as well as in an aqueous-organic two-phase system. The influence of LMS on the molecular weight distribution of organosolv lignin was analyzed via GPC.

For the experiments in aqueous environment organosolv lignin was suspended in MOPS buffer with a pH of 7. Although this pH value differs from the acidic pH optimum of laccase from *T. versicolor* (Baldrian, 2006; Han et al., 2005), it compromises between activity and stability of laccase and lignin solubility. Since the standard ABTS assay does not work at elevated pH values due to precipitation of reaction products, and all available colorimetric assays react sensitively to pH shifts, the determination of absolute and specific activities for laccase at pH 7 was not possible. Therefore, relative activities were monitored over time, from which half-lives could be derived. At pH 7 the half-life of laccase from *T. versicolor* was determined to be 75 – 95 h (data not shown) and thereby, identical to pH 4.5 (Table 4.6). Compared to the inactivating effect of HBT (cp. Table 4.7) the influence of the higher pH value on laccase stability can be considered as negligible.

The laccase-catalyzed oxidation of compounds involves the transfer of electrons from the substrate to molecular oxygen, which is then reduced to water. Since the terminal reduction of oxygen requires protons, it has to be assumed that neutral or even alkaline pH values hamper the reaction and reduce its rate, but laccase was proved to be active at pH 7 by a qualitative colorimetric assay using dimethoxyphenol (DMP) as substrate (data not shown).

To analyze the impact of LMS on organosolv lignin, the insoluble lignin fraction was separated, dried and then applied for GPC analysis using an aqueous eluent. Since standards allowing for a calibration on absolute molecular weights are not available for lignin (Mattinen et al., 2008), the normalized detector signals are plotted over elution volumes, and chromatograms are interpreted qualitatively. Thus, relative changes in the molecular weight distribution of a sample can be compared, but no information regarding absolute concentrations or actual molecular weights is derived.

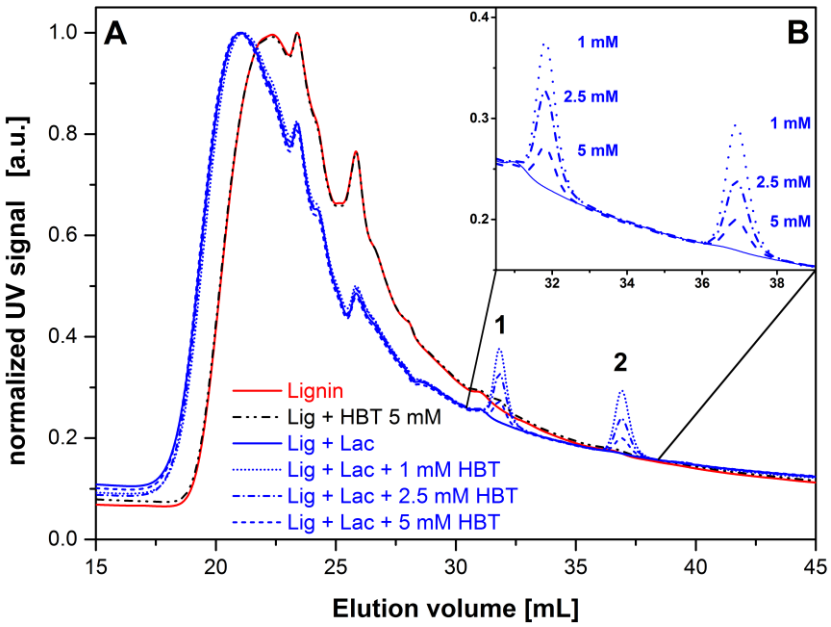

Figure 4.9: Influence of laccase from *T. versicolor* with varying concentrations of the mediator HBT on organosolv lignin. Samples were analyzed by GPC. B: Close-up of HBT-related peaks. Numbers at peak maxima indicate the applied concentration of HBT in the particular experiment.

The results of the treatment of organosolv lignin with LMS in MOPS buffer are shown in Figure 4.9. Untreated lignin alone (red curve) or in combination with the mediator HBT (black curve) were incubated as reference and were compared to experiments also containing

laccase (blue solid curve) or the LMS with varying concentrations of HBT (blue dashed curves). Laccase shifted chromatograms towards smaller elution volumes, whether mediator was present or not, indicating an increase in the molecular weight of the lignin. These findings are in line with a polymerizing effect of laccase on lignin due to radical coupling reactions (Mattinen et al., 2008; Pas et al., 2011; West et al., 2014). Even if cleavage reactions might have occurred to certain extent, the polymerizing activity of laccase predominates in the reaction system.

When laccase and mediator were combined, two additional sharp peaks (1, 2 in Figure 4.9 A) occurred in the chromatograms, whose heights inversely correlate with the mediator concentration (Figure 4.9 B). These peaks were not found when the incubation trials solely contained HBT without laccase (black curve). Therefore, these peaks can be attributed to the presence of oxidized forms of HBT. Also the defined peak shape indicates individual substances behind. The appearance of two additional peaks as a consequence of the presence of HBT together with laccase is also a hint at the formation of mediator side products during the LMS reaction as already discussed earlier (cp. 4.3.4). It was unexpected to find the water-soluble mediator or its side products in the solid lignin fraction, especially since samples were washed three times before GPC analysis. Benzotriazol, known as a side product of oxidized HBT (Li et al., 1999; Xu et al., 2000), can be incorporated into the lignin polymer as a result of LMS treatment (Ibarra et al., 2007). Presumably, side reactions of HBT led to insoluble products precipitating together with the solid lignin fraction, potentially also due to coupling of HBT with low molecular weight lignin compounds. The inverse correlation of peak height and mediator concentration is remarkable, but might originate from faster side reactions at higher mediator concentrations detracting HBT from the reaction system.

These findings are of major relevance for the design of LMS-based processes for lignin conversion, since side reactions of the mediator would withdraw an essential component from the reaction system over time and thereby, reduce its efficiency. Furthermore, undesired incorporating coupling reactions of the mediator with lignin could lead to impurities in potential products. Further structure-elucidating investigations are advisable to identify the reaction products behind the additional GPC peaks and explain the fate of the mediator in LMS reaction systems.

To validate if spatial separation of high and low molecular lignin fractions or potential reaction products due to their different partitioning between aqueous and organic phase could help to avoid undesired (re-)polymerization reactions, the conversion of organosolv lignin with the LMS was also tested in an aqueous-organic two-phase-system consisting of MOPS buffer (pH 7) and 1-hexanol. 1-Hexanol was chosen as organic solvent since its inactivating effect on laccase was insignificant (cp. 4.2.5). Despite its low solubility in water leading to a fast and almost complete phase separation, 1-hexanol is comparably polar (logP = 1.94) and provides acceptable lignin solubility. In a two-phase system the organosolv lignin mainly dissolves in the upper organic phase (Figure 4.10). At high lignin loadings exceeding its solubility in the system a crud layer of lignin solids was formed at the interphase after phase separation (not shown).

Figure 4.10 Lignin (0.5 g/L) dissolved in an aqueous-organic two-phase system consisting of NaAc buffer (0.1 M, pH 4.5) and 1-hexanol. At non-saturating concentrations (< 1 g/L) no crud layer at the interphase was formed.

Figure 4.11 shows the results for the 48 h incubation of organosolv lignin with LMS in the two-phase system consisting of 1-hexanol and MOPS buffer. Samples were taken from each phase, dried, and the remaining solvent was removed by evaporation. The solid samples were then analyzed by aqueous GPC. The samples of the incubation with LMS were compared to lignin, which was incubated in the two-phase system without LMS (black curves in Figure 4.11).

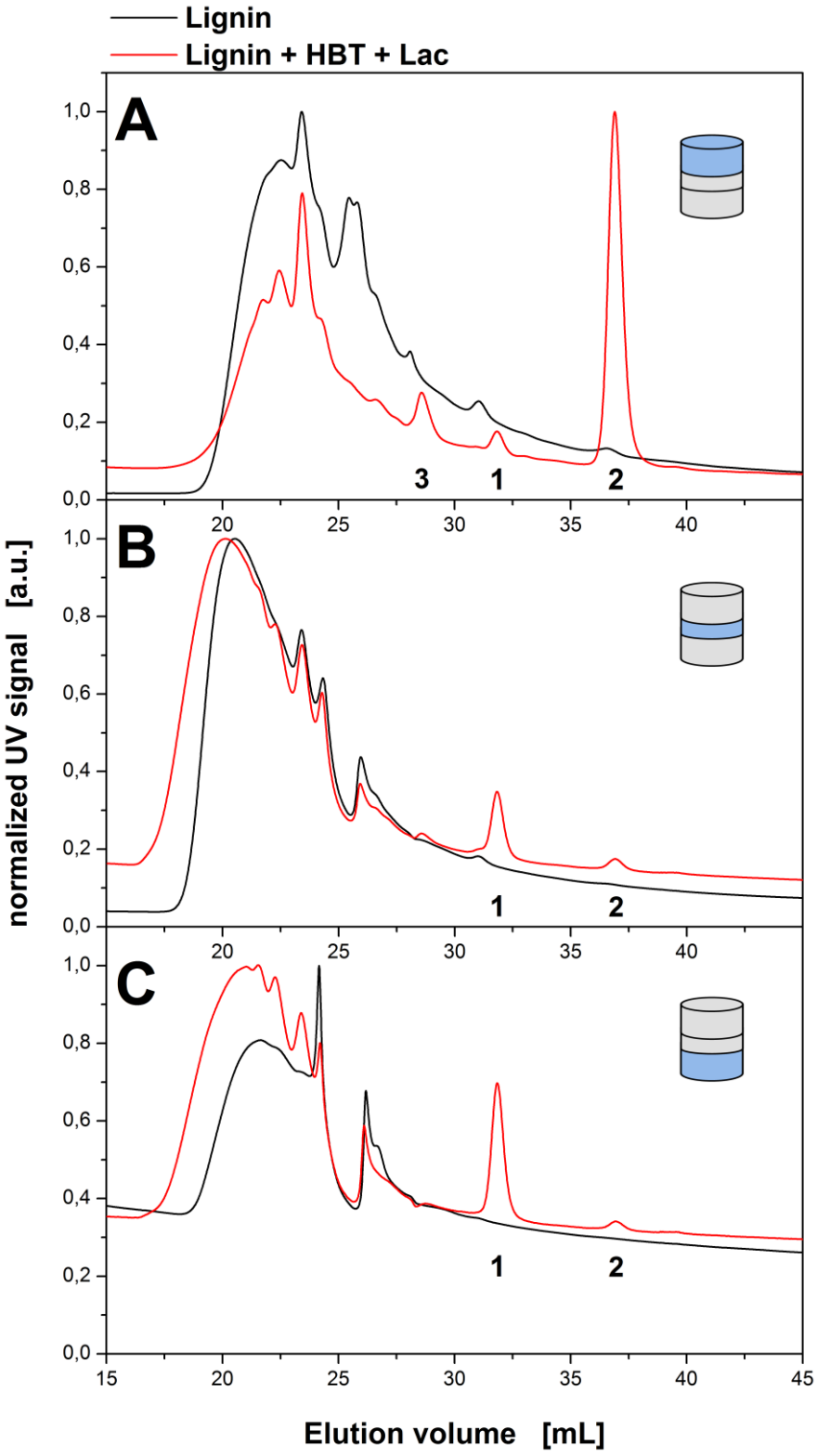

Figure 4.11: Reaction of LMS (laccase from *T. versicolor*, 5 mM HBT) with organosolv lignin in an aqueous-organic two-phase system consisting of NaAc buffer (0.1 M, pH 4.5) and 1-hexanol. Samples were taken from all three fractions and analyzed by GPC. A: upper organic phase; B: crud layer at interphase; C: lower aqueous phase.

The two-phase system obviously led to lignin fractionation indicated by differing molecular weight distributions between the samples from the different phases. As already observed for the pure aqueous system, the presence of LMS shifts the chromatograms towards smaller elution volumes representing higher molecular weights and suggesting LMS-induced polymerization of the material. While this effect is clearly visible for samples from the lower aqueous phase (Figure 4.11 C) and the interphase (Figure 4.11 B), it is much less apparent for samples from the organic phase (Figure 4.11 A). Aqueous phase and interphase basically represent the solid, insoluble lignin fraction, whereas the organic phase only contains dissolved lignin. It can be assumed that LMS-induced oxidation and polymerization alters lignin solubility and potentially withdraws lignin from the organic phase by precipitation, explaining more distinct shifts in the elution volume for samples from aqueous phase and interphase.

As mentioned above changes in concentration are not reflected by the normalized GPC chromatograms. The two peaks previously discussed for the aqueous system and attributed to the oxidized mediator or its side products, respectively, are also found in the two-phase system (1, 2 in Figure 4.11). The compound represented by the second peak at around 37 mL elution volume seems to accumulate in the organic phase. While the absolute height of the peaks does not provide any information about the concentration of the compounds and depends on the concentration of all other substances detected, the ratio of the first and the second peak gives an impression of the partitioning. In the chromatograms for aqueous phase and interphase the first peak is one order of magnitude higher than the second one, whereas it is vice versa for the organic phase.

Furthermore, a third defined peak is found in the chromatogram of the organic phase potentially also originating from a side product of HBT (3 in Figure 4.11 A). This peak does not appear in the chromatograms of the aqueous and interphase probably due to the low concentration of the related compound. Since it occurs at lower elution volumes than the two other mediator-related peaks (1, 2), it possesses a higher molecular weight potentially resulting from coupling of a mediator derived product with lignin compounds.

The experiment with LMS in an aqueous-organic two-phase system reveals that organosolv lignin is indeed fractionated and partitions between both phases, but undesired LMS-induced

polymerization could not be avoided. Rather, as already found and discussed for the pure aqueous system, the LMS shifts chromatograms towards smaller elution volumes indicating an increase in the molecular weights of the compounds as a result of polymerizing effects. GPC analysis just gives a rough glimpse, how LMS influences the molecular weight distribution of lignin. A combination of this analytical method with structure elucidating techniques like e.g. HSQC-NMR would be necessary to understand the reaction mechanisms behind polymerization, and how LMS alters lignin functionalities and thereby, solubility in aqueous and organic environment. Furthermore, the structural identification of the mediator side products should be addressed, particularly with regard to potential coupling products of mediator and lignin compounds.

4.4 Conclusions and Outlook

This chapter studied the feasibility of a process for LMS-catalyzed lignin depolymerization (Höfler, 2014; designed and supervised by Simon Roth).

Three commercial laccase preparations were initially characterized and compared regarding product purity and basic kinetic parameters, leading to the choice of sufficiently active preparations from *P. ostreatus* and *T. versicolor*, whereas the preparation from *A. bisporus* was excluded from further experiments due to its low purity and activity.

Organic solvents were considered as additives for lignin processing either by increasing lignin solubility or by compartmentalization of the reaction system as aqueous-organic two-phase systems. The influence of four organic solvents on laccase stability was tested in solvent-saturated aqueous one-phase systems as well as in aqueous organic two-phase-systems. 1-Butanol had the strongest inactivating effect on laccase, both in the one- and two-phase systems. Even though 2-MTHF caused a fast initial inactivation of laccase it provided long term stability of laccase comparable with the pure buffer system. As a potential fuel candidate of the TMFB process and the solvent used for organocat fractionation of the biomass, it is a promising candidate for lignin processing. The effect of n-heptane on laccase stability in the aqueous one-phase system was negligible because of its very low water solubility, but

significant in the two-phase system due to laccase inactivation at the interphase. The best compatibility with laccase stability was found for 1-hexanol, why it was chosen as solvent for further experiments regarding LMS-catalyzed lignin depolymerization.

The mediator was identified as the most crucial component of the reaction system: The influence of the two synthetic mediators HBT and VA on laccase stability was investigated. The inactivating effect of the oxidized, radical mediators exceeded that of all tested organic solvents by one order of magnitude. Despite its lower redox potential, VA caused a faster inactivation of laccase than HBT, probably due to a higher reaction rate. Therefore, HBT was selected as the mediator for the depolymerization experiments with lignin. Besides the inactivating effect on laccase, HBT was also found to undergo side reactions after enzymatic oxidation. The removal of reactive mediator by transformation into a non-reactive side product most likely limited the LMC oxidation. Furthermore, during the depolymerization studies with LMS and lignin we found hints that HBT reacts with lignin compounds. Peaks occurring in the GPC chromatograms of the solid lignin fraction were attributed to the presence of oxidized HBT indicating a coupling of the mediator with lignin compounds and/or the formation of precipitating products.

Hence, a reasonable application of LMS for biorefinery purposes in larger scale essentially requires solutions for mediator-induced laccase inactivation as well as undesired mediator side reactions. Since the reaction mechanism of LMS generally involves radical intermediates of the mediator compound it is more promising to address the reaction sites of mediator-induced laccase inactivation than to screen for mediators with higher LMS-compatibility. Genetic engineering of laccase could reduce the number of aromatic amino acid side chains which are prone to reactions with radical mediators, but the enzyme structure providing catalytic activity has to be preserved. Also adaption of the reaction conditions could help to minimize laccase inactivation by the mediator. Compartmentalization of the reaction system can decrease the inactivating effect of mediators, when enzyme and mediator partition into different phases, as shown for water-immiscible ionic liquids (Rehmann et al., 2013). Furthermore, the realization of an economically feasible process based on LMS requires cheap, preferably biomass-derived mediators to substitute the expensive artificial mediators like HBT, VA or ABTS.

The influence of LMS on organosolv lignin was tested under different conditions. The molecular weight distribution of the lignin samples was analyzed with GPC. In all experiments where LMS was present a shift towards smaller elution volumes was found during GPC analysis indicating polymerization of the lignin. Under none of the tested conditions LMS-catalyzed depolymerization of lignin was observed. Also compartmentalization of the reaction system by use of an aqueous-organic two-phase system could not avoid polymerization. According to several publications, laccase exhibits a strong polymerizing activity countering potential cleavage of lignin. A rapid removal of degradation products appears as the most reasonable strategy to shift LMS-catalyzed oxidation of lignin towards depolymerization. Besides physical removal techniques like extraction or membrane separation, a fast conversion of degradation products into compounds which cannot react further with LMS is a promising approach to avoid repolymerization. This could be realized by combining laccase with additional enzymes like certain oxidases (e.g. glucose oxidase) which can reduce the quinonic cleavage products.

Chapter 5

Summary, conclusions and outlook

Lignocellulose is a promising feedstock to replace oil as the key resource of today's economy, but its efficient and complete utilization is still challenging. Especially the lignin part of wooden biomass hinders complete exploitation of lignocellulose, since reasonable strategies for the conversion of lignin into value-added products are still lacking. Furthermore, lignin and lignin-derived compounds can impede process steps for the conversion of the carbohydrate fraction. Hence, new concepts for the processing of lignin have to be developed to close the "lignin gap" in biorefinery processes.

Mimicking natural reaction mechanisms, biocatalytic processes are promising approaches to address the challenge of a sustainable, economically feasible lignin conversion: In contrast to chemo-catalytic processes, enzyme reactions avoid harsh, energy-intensive reaction conditions as well as toxic or expensive chemicals and catalysts. Furthermore, lignolytic enzymes are supposed to catalyze very specific reactions leading to a defined spectrum of products and preserving the valuable phenolic sub-structures of lignin.

White-rot fungi are commonly known as lignin degrading organisms and have been intensively researched over the last decades to benefit from their strategies for lignin depolymerization. Laccases were found to play a central role in the enzymatic cocktail, which white-rot fungi secrete for lignin decomposition. Thus, laccases or laccase-mediator-systems respectively, arose as promising biocatalysts for lignin modification and depolymerization in biorefinery processes.

The objective of this work was the concept evaluation of LMS-based processes for lignin processing in a biorefinery. The LMS-catalyzed depolymerization of lignin was focused as the main subject, but also the application of LMS for biomass pretreatment was investigated.

Following the natural example of wood delignification by white-rot fungi the impact of a LMS pretreatment on raw beech wood was tested, but the influence on the subsequent enzymatic hydrolysis was negligible. However, when the LMS pretreatment was followed by a freeze-drying step, the glucose yield obtained during enzymatic hydrolysis was significantly increased by up to 1.3 fold. The freeze-drying of the raw beech wood most likely leads to expansion and loosening of the lignocellulosic composite resulting in an increased lignin displaying surface which can unproductively bind cellulases. A LMS-induced oxidative modification of the surface lignin, reducing the unproductive binding of cellulases, is suggested as the mechanism behind the enhancing effect of this LMS pretreatment. The enhancing influence of LMS on enzymatic hydrolysis was only found for beech but not for spruce, most likely due to differences in composition and functionalities of the lignin. No synergism was observed for LMS pretreatment in combination with pretreatment with the ionic liquid [EMIM]Ac, either due to extensive delignification by the IL pretreatment or chemical lignin modifications avoiding the unproductive binding of cellulases.

The positive results obtained with LMS pretreatment in combination with freeze-drying representing a physico-mechanical pretreatment suggest laccase in sequence with classical, mechanical pretreatment techniques worthy to be considered as additional process step for biomass conditioning to eliminate negative influences of physico-chemical biomass pretreatment on the efficiency of expensive cellulases.

The investigations regarding the enzymatic biomass pretreatment triggered a demand for an assay that quantifies the accessibility of lignocellulosic substrates for cellulases to analyze the influence of different pretreatment methods on this parameter. A fluorescent protein probe consisting of the CBM4 from *C. fimi* as cellulose-recognizing element and mCherry as fluorescent reporter was tested as an analytical tool to measure cellulose accessibility.

The adsorption of the CBM-containing protein probe was compared to that of mCherry alone. It turned out, that unspecific protein adsorption strongly contributes to the binding of the probe to lignocellulosic materials and masks its CBM-specific affinity. In order to

characterize the accessibility of lignocellulose via a CBM-based assay unspecific binding of the probe has to be prevented to isolate the CBM-mediated adsorption to cellulosic structures. The β-barrel-shaped mCherry could be exchanged by alternative fluorescence proteins like the LOV-domain-derived flavin-based fluorescent protein (FbFP) (Drepper et al., 2007) to reduce the unspecific binding of the probe. Also chemical labeling of the CBM with synthetic fluorophores like fluorescein is thinkable, but a defined ratio of CBM and fluorophore has to be guaranteed, and the labeling must not disturb the binding of the CBM to cellulose.

As a spin-off of this study, mCherry was found as a simple reporter to quantify unspecific protein adsorption to lignocellulosic substrates and to analyze, how pretreatment methods influence this parameter. E.g., adsorption studies with single mCherry revealed that LMS pretreatment of raw beech wood reduces unspecific adsorption of proteins onto the material surface. These findings support the theory that the enhancing effect of LMS pretreatment on enzymatic cellulose hydrolysis derives from a reduced unproductive binding of the cellulases. Adsorption studies with single mCherry or related fluorescence proteins like GFP can help to characterize and optimize pretreatment methods regarding their impact on unspecific protein adsorption onto the biomass.

The core objective of this work was the design and evaluation of a LMS-catalyzed process for lignin depolymerization. Therefore, the influence of process conditions and components of the reaction system on laccase was evaluated to define a suitable reaction environment.

Since lignin is barely water-soluble, several organic solvents were tested as process additives. The applied commercial laccase preparations from *P. ostreatus* and *T. versicolor* showed high stability in the presence of the four tested organic solvents. Best compatibility was found for 1-hexanol. 2-MTHF caused a strong initial decrease in laccase activity, but the long term stability was acceptable. Fastest inactivation of laccase was observed in the presence of 1-butanol. n-Heptane led to a significant loss of laccase activity due to inactivation at the interface, when it was tested in a two-phase system.

Thus, the application of laccases in solvent containing reaction systems is feasible and advantageous for process routes, where lignin is extracted by organosolv-like methods. The high solvent tolerance of the tested laccases would allow feeding the organic, lignin-

containing extraction phase directly into a LMS reaction step, enabling a simple integration into the process route of a biorefinery and avoiding the handling of solid lignin.

Also the impact of the synthetic mediator compounds HBT and VA on laccase stability was investigated. The inactivating effect of both substances was one order of magnitude higher than that of the tested organic solvents. Inactivation of laccase by VA was faster than in presence of HBT, why the latter was used for further experiments regarding lignin depolymerization. When HBT was used as laccase mediator for LMC conversion, unreactive side products were found, indicating a loss of mediator in the reaction system over time. Furthermore, during experiments with organosolv lignin, HBT apparently reacted with lignin compounds forming insoluble coupling products.

The mediator was identified as the most crucial component of LMS. Further research is necessary to address mediator-related challenges like enzyme inactivation and undesired side reactions. Laccase engineering might help to enhance compatibility of enzyme and mediator. The exchange of aromatic amino acids, which are prone to react with the radical form of the mediator, could avoid mediator-induced inactivation, but might also influence laccase activity and stability by conformational changes in the protein folding. Furthermore, processes based on artificial mediators like HBT or VA are far away from economic viability, why new cheap, natural and preferably biomass-derived mediators have to be identified to make the application of LMS for biorefinery purposes economically feasible.

Torres-Duarte et al. named the following three properties a good mediator should have (Torres-Duarte et al., 2009):

1. Good laccase substrate in terms of high turnover number in oxidation.
2. Formation of stable diffusible radicals.
3. Radical with high redox potential.

Summing up the results of this study the following points are added to be considered for the future development of economically feasible processes involving LMS:

- The mediator should not be prone to undesired side effects like enzyme inactivation, formation of catalytically inactive mediator compounds, or coupling to the substrate or its degradation products.
- The mediator should not promote polymerization of the substrate or its degradation products.
- The mediator should react in a stable, loss-free redox cycle between laccase and substrate such that the required amount of mediator is considerably lower than the stoichiometric amount of oxidized substrate.
- The mediator should be low priced and eco-friendly. Biomass- or even lignin-derived compounds are highly desired.

Beyond, strategies to separate the mediator from potential lignin degradation products have to be identified. Since mediator compounds and typical lignin-derived products are very similar in their molecular weight, processes separating by size, like e.g. membrane-based techniques, are unsuitable. A promising approach is two-phase extraction. Rehmann et al. showed, that typical mediators preferably partition into a second phase of water-immiscible ionic liquids which also reduces the inactivation of laccase by the mediator (Rehmann et al., 2013). The applicability of such a process depends on the partitioning of the target products between both phases. Furthermore, the utilization of ionic liquids in biorefinery processes is discussed controversially due to related drawbacks like prize, ecotoxicity and recycling of ionic liquids.

The application of LMS for lignin depolymerization was investigated with reaction systems consisting of laccase from *T. versicolor*, the mediator HBT and organosolv lignin as the substrate both in one- and two-phase systems. The lignin samples were analyzed with GPC. Under none of the tested conditions, depolymerization was observed. On the contrary, in all experiments where laccase or LMS was present, polymerization of the lignin occurred, indicated by a shift of the GPC chromatograms towards lower elution volumes.

Even if LMS can catalyze lignin bond cleavage, its polymerizing activity predominates in the reaction system. The rapid removal of reactive products like radicals or quinones is essential to prevent repolymerization of the lignin and enable LMS-catalyzed lignin depolymerization (Leonowicz et al., 2001). The compartmentalization of the reaction system by a second water-immiscible phase or a membrane is a potential approach to remove reactive intermediates but it is doubtful, if those separation techniques are fast and selective enough to push the LMS reaction towards lignin depolymerization.

The combination of LMS with additional auxiliary enzymes is a promising strategy to eliminate reactive species and avoid undesired coupling reactions. Certain oxidases like glucose oxidase (Alberti and Klibanov, 1982; Green, 1977), veratryl alcohol oxidase (Marzullo et al., 1995) or cellobiose:quinone oxidoreductases (Ander et al., 1990; Westermark and Eriksson, 1974) were found being able to reduce quinones and thereby, to prevent their recoupling in a LMS reaction system. All afore mentioned oxidases accept biomass-related compounds like e.g. glucose, veratryl alcohol or cellobiose as reducing agents allowing their integration into biorefinery processes.

The combination of LMS with other enzymes to multi-enzyme-systems is quite close to the natural example of white-rot fungi, which secrete a cocktail of enzymes to decompose lignin. β-Etherases are promising partners for laccase, since they are able to cleave β-O-4 bonds, but require a carbonyl group at the C_α-position (Picart et al., 2014). A previous oxidation of lignin with LMS can convert C_α-hydroxyl groups into the corresponding carbonyls allowing subsequent cleavage of β-O-4 bonds by a β-etherases. Since β-etherases require glutathione as co-substrate, a technical application of this enzyme depends on efficient strategies for supply and recycling of glutathione.

Considering the results of this work it becomes obvious that the application of LMS for lignin depolymerization has certain potential but, also holds some essential challenges, which have to be addressed by future investigations. The following points sum up the outcome of this project and shall serve as essential guideline for the conceptual design of processes using LMS for lignin degradation:

- **Repolymerization** is the most striking drawback of LMS-based processes, which can be avoided by rapid separation of degradation products as well as elimination of reactive species like radicals or quinones.
- **Mediators** are essential to LMS, but inactivate enzymes. Mediators tend to be lost due to side reactions. Oxidized mediators can initiate polymerization. Mediators cannot be easily separated from potential degradation products. Mediators should have a low prize and no eco-toxicity. Therefore, natural lignin-derived mediators are promising alternatives to synthetic mediators.
- **Properties of lignin** like molecular weight, functional groups, solubility in water or solvents influence its reactivity in LMS. Results obtained with lignin model compounds are often not transferrable to genuine lignin. The lignin extraction strongly influences the lignin properties and should be aligned to the requirements of the LMS reaction system. Process development should be performed using authentic substrates.
- **Suitable analytical methods** are essential to monitor the effect of LMS on lignin. GPC analysis should be combined preferably with structure elucidating methods like e.g. 2D-NMR.
- The **reaction environment** should promote laccase activity and stability, but also provide sufficient lignin solubility.
- All solvents, additives and mediators of the LMS reaction system should fit to the "**green intentions**" of biorefineries and be recyclable with moderate effort for economically feasible processes.

In final conclusion, laccases have certain potential to support biorefinery processes and shift them towards higher energy efficiency and economic feasibility, but continuing research is necessary to overcome existing limitations of laccases as technical catalysts.

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Appendix

Table A.1: Recommendable reviews to enter the topic of laccases with focus on applications in the context of biorefineries (Roth and Spiess, 2015).

Reference	Focus / Topics	Comments / Features
(Baldrian, 2006)	structural and catalytic properties of fungal laccases	extensive tables with structural and catalytic values for more than 100 fungal laccases
(Call and Mücke, 1997)	pulp delignification using LMS	key review, extensive overview about LMS with focus on pulp delignification
(Canas and Camarero, 2010)	natural mediators	good overview about the topic of natural mediators; ET / HAT oxidation mechanism
(Dwivedi et al., 2011)	structural comparison of laccases from different sources, relation between structural and catalytic properties	extensive table with K_M values for different laccases and substrates
(Giardina et al., 2010)	structural features and catalytic mechanisms of laccases	good overview on the structure of the active site of laccases and connection between structure and catalytic mechanism
(Kudanga and Le Roes-Hill, 2014)	applications of LMS in biofuel production, focus on ethanol and biogas production	Recent overview also addressing LMS pretreatment and detoxification
(Leonowicz et al., 2001)	lignin degradation, multi-enzyme-systems	key review with focus on role of laccase in lignin degradation
(Morozova et al., 2007a)	structural and catalytic properties of blue laccases	table with T1 redox potentials for more than 20 laccases of different origins; table with M_W, carbohydrate, pI, $T_{optimum}$, $t_{1/2}$ for more than 50 laccases of different origin; rough overview about potential applications in biotechnology
(Morozova et al., 2007b)	mediators, biotechnological applications, patents	overview about a variety of artificial mediators and their reaction pathways.

(Munk et al., 2015)	laccase-catalysed lignin cleavage	the question if “laccases can catalyze bond cleavage in lignin?” is addressed in detail and commented by the authors
(Riva, 2006)	laccases and its applications in organic synthesis	condensed overview, often cited standard review
(Thurston, 1994)	general survey of laccases	discussion on natural function of laccase ("To delignify or not to delignify")
(Virk et al., 2012)	applications of laccases in pulp and paper industry	current overview including waste water treatment; table of LMS used for pulp delignification studies including the obtained effects in terms of kappa numbers and brightness
(Widsten and Kandelbauer, 2008)	application of LMS in context of forest products	classical pulp and paper topics like biobleaching and wast water treatment; LMS-assisted biomass conditioning for production of wood-derived products like composite fiber boards

Table A.2: LMS pretreatment approaches in current literature (Roth and Spiess, 2015). Chronological order; ABTS: 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid, DNS: 3,5-dinitrosalicylic acid, GC: gas chromatography, HBT: 1-hydroxybenzotriazol, NHA: N-hydroxy-N-phenylacetamide, NHA-Ac: N-acetoxy-N-phenylacetamide, SSF: simultanious saccharification and fermentation

Raw material	Pre-pretreatment of lignocellulose before laccase-pretreatment	Laccase (origin/product)	Mediator	Treatment of substrate after laccase pretreatment	Enzyme preparation for hydrolysis	Order pretreatment / hydrolysis	Sugar analysis	Improvement factor for saccharification after LMS pretreatment	Reference
Soft wood	Steam pretreatment	*Trametes hirsuta*	2 - 20 mM NHA / NHA-Ac (lipase activation)	washing, laccase inactivation with 0.2% NaN_3	Celluclast 1.5 L (*T. reesei*) / Novozym 188 (*A. niger*)	stepwise / simultaneous	DNS / HPLC	1.21 (stepwise); 1.14 (simultaneous)	(Palonen and Viikari, 2004)
Eucalyptus globulus	Steam explosion	Novozyme 51003	5 mM HBT	-	-	only pretreatment, no hydrolysis	-	-	(Martín-Sampedro et al., 2011, a&b)
Spruce, giant reed	SO_2 impregnation / steam pretreatment	*Cerrena unicolor*, *Trametes hirsuta* (reference)	none	laccase denaturation by boiling (10 min)	Celluclast 1.5L (*T. reesei*)	stepwise in same broth	DNS	1.12 (spruce); 0.83 (giant reed)	(Moilanen et al., 2011)
Bambusa bambos	Air dried, milled to 0.2 mm particles	*Pleurotus sp.*	none	washing, air drying at 60°C	*Trichoderma reesei* Rut C30	stepwise	DNS	-	(Kuila et al., 2011)
Corn stover	Ensilage pretreatment with Multifect A40 (cellulase/hemicellulase mixture, Genencor)	*Trametes versicolor*	1.48 mM HBT	washing	Spezyme CP (commercial prep.) / Novozyme 188 (*A. niger*)	stepwise	glucose analyser YSI	1.37	(Chen et al., 2012)
Elephant grass (*P. purpureum*) Eucalyptus (*E. globulus*)	Air-dried, cutting mill, ball mill	*Trametes villosa* (Novozymes)	0.37 mM HBT	peroxide-reinforced alkaline extraction	Celluclast 1.5 L (*T. reesei*) / Novozym 188 (*A. niger*)	stepwise	DNS, HPLC, SSF-fermentation	1.12 (elephant grass); 1.61 (eucalypt)	(Gutiérrez et al., 2012)

Rice straw *(Oryza sativa L.)*	Milling, sulfuric acid, steam explosion pretreatment	*Yarrowia lipolytica*	1 mM HBT	-	Celluclast 1.5 L (*T. reesei)*	stepwise	DNS	1.73	(Lee et al., 2012)
Wheat straw	Steam explosion	*Sclerotium sp.*	none / 0.75 mM ABTS	laccase denaturation by boiling (5 min)	commercial cellulase preparation	stepwise / simultaneous	HPLC	1.25 (stepwise); 1.28 (simultanious)	(Qiu and Chen, 2012)
Eucalyptus globulus	Cutting mill, ball mill	*Myceliophthora thermophila*	4.71 - 14.14 mM methyl syringate	peroxide-reinforced alkaline extraction	Celluclast 1.5 L (*T. reesei*) / Novozym 188 (A. niger)	stepwise	GC (alditol acetates)	1.27	(Rico et al., 2014)
Wheat straw	Acid pretreatment	*Trametes versicolor*	8.2 - 24.5 mM HBT	alkaline peroxide extractions	GC-220 (Genencor)	stepwise	HPLC	1.35	(Heap et al., 2014)
Spruce	SO_2 impregnation / steam pretreatment	*Trametes hirsuta*	0.5, 1, 3, and 10 mM ABTS, HBT, TEMPO, acetosyringone (AS)	laccase denaturation by boiling (10 min)	Celluclast 1.5 L (*T. reesei*) / Novozym 188 (*A. niger*)	stepwise	HPAEC-PAD	1.19 (no mediator); 1.22 (HBT); 1.36 (TEMPO); 1.49 (AS); 1.54 (ABTS)	(Moilanen et al., 2014)

Table A.3: Approaches of laccase-catalyzed lignin modification (Roth and Spiess, 2015). Chronological order.

Laccase	Substrate class	Substrate	Substrate origin	Substrate MW [kDa]	Mediator	pH	Temp. [°C]	Main effect	Reference
Fomes annosus	Ligninsulfonate	Lignosulfonate (commercial, Roth GmbH), fractionated by GPC	n.s.	435	none	n.s.	25	Polymerisation	(Haars and Hüttermann, 1980)
Trametes versicolor	Lignosulfonate	Lignosulfonate (Peritan Na, fractionated by prep. GPC)	spruce	97	none	4.5	20	Polymerisation / Depolymerisation	(Leonowicz et al., 1985)
Trametes versicolor	Lignin	Kraft Lignin (14C-labled)	aspen	3.5	ABTS	5.0	n/a	Polymerisation / Depolymerisation	(Bourbonnais et al., 1995)
Phanerochaete chrysosporium	artificial Lignin	synthetic guaiacyl lignin (DHP, 14C-labeled)	coniferyl alcohol	> 9.1	3-HAA	4.0	RT	Depolymerisation	(Eggert et al., 1996)
Coriolus versicolor	artificial Lignin	synthetic guaiacyl lignin (14C-labeled)	coniferyl alcohol polymerised by horse radish peroxidase	> 4	HBT	4.0	RT	Depolymerisation	(Kawai et al., 1999a)
Trametes villosa	artificial Lignin	synthetic guaiacyl lignin (14C-labeled, phenolic and non-phenolic)	coniferyl alcohol polymerised by horse radish peroxidase	8.1 (phenolic) 7.6 (non-phenolic)	HBT	4.0	30	Polymerisation / Depolymerisation	(Srebotnik and Hammel, 2000)
Cerrena unicolor	Lignosulfonate	Lignosulfonate	spruce	97	acetovanillon, acetosyringone	5.0	27	Depolymerisation	(Cho et al., 2004)

Trametes hirsuta, Trametes ochracea	Lignin	Hydrolytic, organosolv, Kraft, and alkali lignins, Soluble sulphate pine lignin	n.s.	~ 28	ABTS, HBT	5.0	n/a	Polymerisation / Depolymerisation	(Shleev et al., 2006a)
Trametes villosa, Myceliophthora thermophila, Trametes hirsuta, Bacillus subtilis	Ligninsulfonate	Calcium lignosulfonate (industrial, Borregaard, Norway)	n.s.	28.4	ABTS, HBT	n.s.	30	Polymerisation	(Nugroho Prasetyo et al., 2010)
Trametes versicolor (soluble / immobilized)	Lignin	acidolysis lignin	milled wheat straw	16.9	HBT	6	30	Polymerisation / Depolymerisation	(Crestini et al., 2011)
Thielavia arenaria, *Trametes hirsuta*, *Melanocarpus albomyces*	Lignin	organosolv lignin (OS), steam-explosion lignin (SE); lignins fractionated by sequential org. solvent extraction	Alcell (OS), birch (OS), pine (SE), eucalypt (SE)	7.2 (Alcell, OS), 12.2 (birch, OS), 5.8 (pine, SE), 8.3 (eucalypt, SE)	none	5.0 / 6.0	30	Polymerisation	(Pas et al., 2011)
Streptomyces coelicolor	Lignin	ethanosolv lignin	*Miscanthus x giganteus*	0.2 - 1	none	n.s.	37	Polymerisation	(Majumdar et al., 2014)
Trametes hirsuta	Lignin / Ligninsulfonate	Kraft lignin (KL), ligninsulfonate (LS), steam explosion lignin (SE), enzymatic saccharification lignin (ES)	n.s., eucalypt / pine (SE) *Pinus radiata* (ES)	5.3 (KL), 26.4 (LS) 2.8 (SE pine), 3.2 (SE eucalypt), 4.0 (ES)	none	5.5	n/a	Polymerisation	(West et al., 2014)

Table A.4: Approaches to laccase-catalyzed detoxification of biomass hydrolyzates (Roth and Spiess, 2015). Chronological order; AH: acid hydrolysis, EH: enzymatic hydrolysis, EtOH: ethanol, F: fermentation, LD: laccase detoxification, P: pretreatment, SSCF: simultaneous saccharification and co-fermentation, STY: space-time-yield

Raw material	Pretreatment method	Laccase origin	Process sequence	Further processing of hydrolyzate	Effect of laccase detoxification	Reference
Willow	steam / SO_2	*Trametes versicolor*	P-LD-F	fermentation with *Saccharomyces cerevisiae* for ethanol production	significant reduction of monoaromatic phenolic compounds (detected by GC-MS), shift towards higher MW in GPC analysis, EtOH conc. (g/L): +46% EtOH yield (g/g): -4% EtOH STY (g/L/h): +72%	(Jönsson and Palmqvist, 1998)
Spruce	(dilute acid)	*Trametes versicolor*	AH-LD-F	fermentation with *Saccharomyces cerevisiae* for ethanol production	reduction of total phenolics by 80%, EtOH conc. (g/L): +46% EtOH yield (g/g): 5.5x EtOH STY (g/L/h): 17x	(Larsson and Reimann, 1999)
Sugarcane bagasse	steam explosion	*Trametes versicolor*	P-EH-LD-F	fermentation with *Saccharomyces cerevisiae* for ethanol production	removal of 80% of phenolics, EtOH conc. (g/L): +38.3% EtOH yield (g/g): +50% EtOH STY (g/L/h): +84.6%	(Martín and Galbe, 2002)
Sugarcane bagasse	(acidic hydrolysis)	*Cyathus stercoreus*	AH-LD-F	fermentation with *Candida shehatae* for ethanol production	reduction of total phenolics by 77.5%, EtOH conc. (g/L): +88% EtOH yield (g/g): +93% EtOH STY (g/L/h): +87.5%	(Chandel et al., 2007)

Wheat straw	steam explosion	*Coriolopsis rigida* / *Trametes villosa*	P-LD-EH-F / P-EH-LD-F	fermentation with *Saccharomyces cerevisiae* for ethanol production	reduction of total phenolics by 70 - 75%, LD before EH lowers sugar yields by ~ 3 -8%, shift towards higher MW in GPC analysis of supernatants, 2 - 2.7x EtOH conc. after LD	(Jurado et al., 2009)
Rice straw	mild acid/steam explosion	*Coltricia perennis* / *Novozym 51003*	P-LD-EH	none	removal of up to 76% of phenolics, up to 48% higher sugar yields after EH	(Kalyani et al., 2012)
Rice straw	mild acid / steam explosion	*Yarrowia lipolytica*	P-LD-EH	none	removal of 52% (w/o mediator) / 75% (with 1 mM HBT) of phenolics, increase of sugar yields after EH by 30.3% (w/o mediator) / 73.2% (with 1 mM HBT),	(Lee et al., 2012)
Wheat straw	mild acid / steam explosion	*Pycnoporus cinnabarinus* / *Trametes villosa*	P-LD-EH-F / P-EH-LD-F	fermentation with *Kluyveromyces marxianus* for ethanol production	removal of up to 94% of phenolics higher removal rates, when LD before EH, lower glucose yields when LD before EH, EtOH conc. (g/L): 13.6x (high solid loading) / 1.05x (low solid loadings) EtOH yield (g/g): 1.06x (high solid loading) / 11.6x (low solid loadings) EtOH STY (g/L/h): 2.81x (high solid loading) / 11.5x (low solid loadings)	(Moreno and Ibarra, 2012)
Wheat straw	liquid hot water	*Trametes versicolor*	P-LD	none	nearly all phenolics removed after 24 h. Classification of phenolics into three groups depending on efficiency of LD.	(Kolb et al., 2012)

Wheat straw	organosolv	*Trametes versicolor* (immobilized laccase)	P-LD-F	fermentation of the aqueous xylan-rich fraction obtained by organosolv extraction with *Pichia stipitis* for ethanol production	removal of 82% of phenolics, EtOH yield (g/g): +2% EtOH STY (g/L/h): +30.2%	(Ludwig et al., 2013)
Wheat straw	steam explosion	*Pycnoporus cinnabarinus*	P-LD-EH / P-LD-SSCF	fermentation with xylose-consuming *Saccharomyces cerevisiae* for ethanol production	removal of 50 - 80% of phenolics, reduced sugar yields in EH after LD, lower productivity and EtOH yields after LD in SSF. Increased cell viability after LD in SSCF.	(Moreno et al., 2013b)
Wheat straw	steam explosion	*Pycnoporus cinnabarinus*	P-LD-SSCF / P-(LD+EH)-SSCF	fermentation with *Kluyveromyces marxianus* or *Saccharomyces cerevisiae* for ethanol production	increased cell viability after LD in SSCF, shorter lag phases, no influence of LD on ethanol yields (*S. cerevisiae*), significantly improved fermentation performance (*K. marxianus*)	(Moreno et al., 2013a)

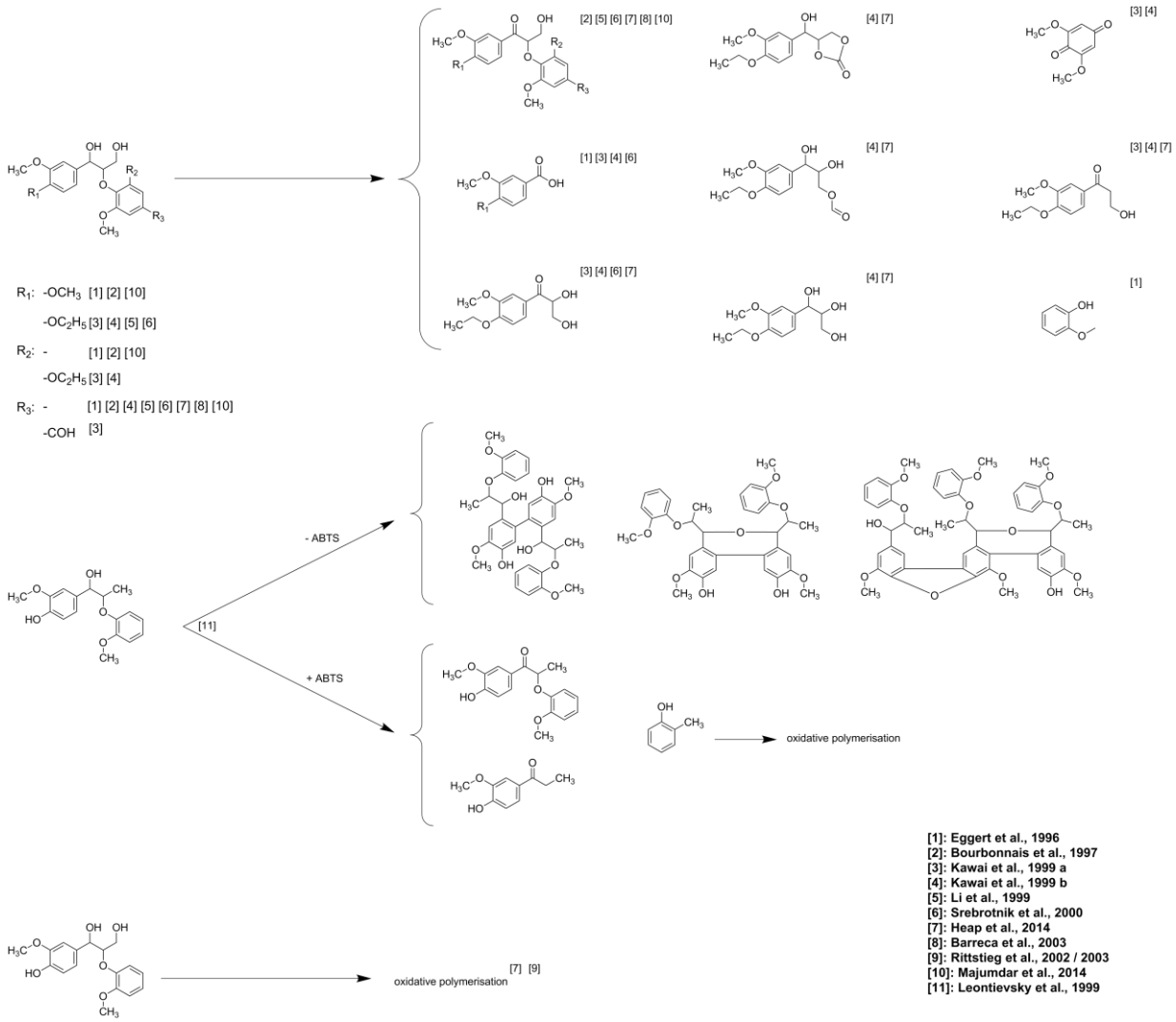

Figure A.1: Overview about studies using LMC representing a β-O-4 bond as a substrate for LMS and obtained reaction products (Roth and Spiess, 2015).

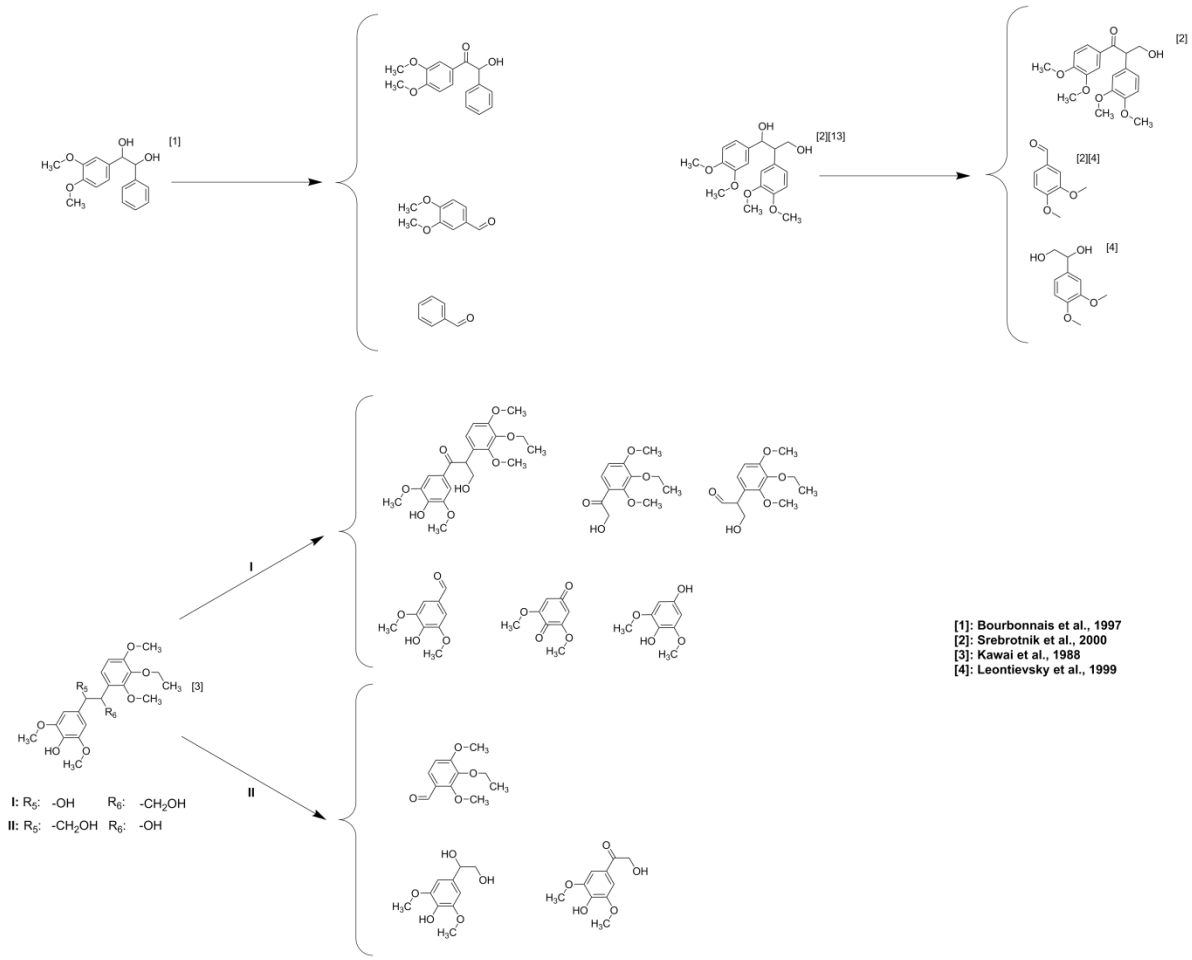

Figure A.2: Overview about studies using LMC representing a β-1 bond as a substrate for LMS and obtained reaction products (Roth and Spiess, 2015).

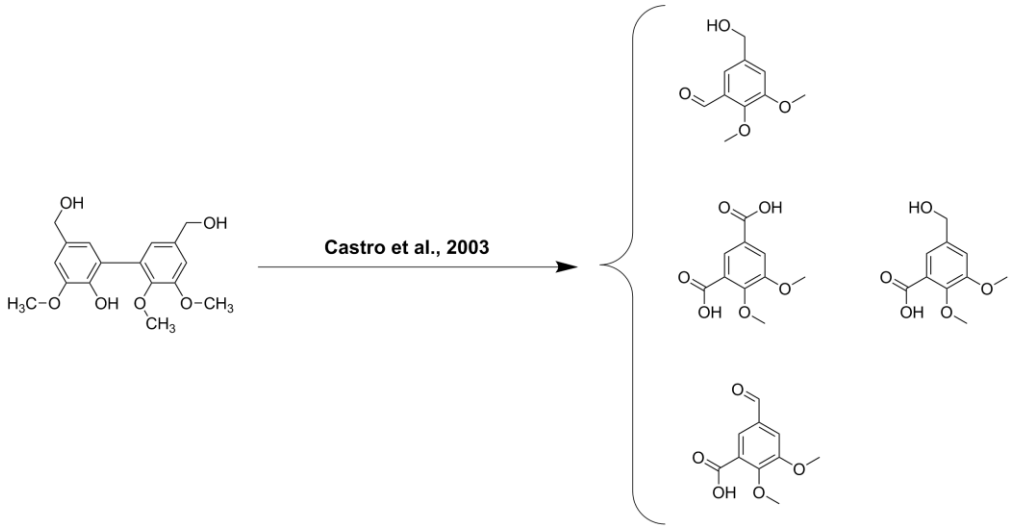

Figure A.3: LMS catalysed cleavage of a 5-5 bond in a LMC as demonstrated by Castro et al. (Castro et al., 2003) and in (Roth and Spiess, 2015).