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New enzymatic methods for the drying of alkyd resins

MASTERARBEIT

zur Erlangung des akademischen Grades einer Diplom-Ingenieurin

des Masterstudiums Biotechnologie

erreicht an der

TECHNISCHEN UNIVERSITÄT GRAZ

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Institut für Umweltbiotechnologie

2011

Abstract

The work carried out in this master thesis was embedded in the development of a completely new technology that might revolutionize the paint and coating industry. The basic idea was to cross-link alkyd resins via unsaturated fatty acids side chains with the aid of a laccase mediator system (LMS). This new technology will replace the currently used toxic heavy metal based siccatives.

A mediator screening was performed by means of oxygen consumption measurements. Gel permeation chromatography confirmed a molecular weight increase of LMS treated alkyd resins. Additionally the LMS catalyzed curing process of the model resin was followed over time via FTIR spectroscopy and Raman spectroscopy. The obtained data revealed that the peak assigned to double bonds significantly decreased and around the same time OH-containing groups were formed. Both the positive control (cobalt siccative treated alkyd resin) and the LMS treated samples showed these effects.

According to drying time recorder results a good drying performance of the resin was seen when using the LMS. The system still needs further optimization while the high potential for industrial applications was clearly demonstrated.

Kurzfassung

Die vorliegende Diplomarbeit und die damit verbunden Experimente sind Teil der Entwicklung einer neuen Technologie die die Farb- und Lackindustrie revolutionieren könnte. Die grundlegende Idee ist, Alkydharze über die ungesättigten Fettsäureseitenketten mit Hilfe eines Laccase-Mediatorsystems (LMS) querzuvernetzen und somit bisher verwendete toxische Sikkative, die Schwermetalle enthalten, zu ersetzten.

Mittel Sauerstoffverbrauchsmessungen wurde nach geeigneten Mediatoren gescreent. Die Zunahme des Molekulargewichtes der Harze nach LMS-Behandlung konnte anhand von Gel-Permeations-Chromatographie gezeigt werden. Zusätzlich wurde der LMS-katalysierte Aushärtungsprozess mittels FTIR und Raman Spektroskopie über die Zeit verfolgt. Die erhaltenen Daten zeigten eine deutliche Verkleinerung eines Peaks dem das Vorhandensein von Doppelbindungen zugeordnet werden kann. Etwa zeitgleich bilden sich OH- enthaltende Gruppen. Sowohl in der Positivkontrolle (die mit einem cobalthältigen Trockenmittel behandelt wurde), als auch in den Proben die das Laccase-Mediator System enthielten, konnten diese Effekte gemessen werden.

Mittels Trocknungszeitrekorders wurde für LMS-quervernetzte Harze ein zufriedenstellendes Trockenverhalten gemessen. Das Verfahren muss zwar noch weiter optimiert werden vielversprechende Resultate demonstrieren das hohes Potential dieser Technologie in Hinblick auf eine industrielle Anwendung.

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1 Introduction

New government regulations (i.e. the "EU's regulation on the Registration, Evaluation, Authorization and Restriction of Chemicals" = REACH) dictate that cobalt containing coatings need to be registered, classified and labeled [1]. Coating industry is actively looking for a replacement since when the new regulation is introduced it would be a competitive advantage to have a product without such labeling. There are several approaches at which industry is looking at to overcome the regulatory issue. Siccatives containing other metals (e.g. manganese or iron) are one example for a possible cobalt surrogate. Nevertheless most of those driers contain heavy metals that sooner or later might be banned from products as well.

The present work is part of a project of the Austrian Center of Industrial Biotechnology (ACIB), accomplished at the Institute of Environmental Biotechnology of the Graz University of Technology together with the industrial partner Cytec. Cytec is one of the world market leaders concerning coatings and the center involved is Graz. The project aims to develop a method to enzymatically cross-link waterborne alkyd resins in order to find an environmentally friendlier alternative for the currently used cobalt containing siccatives.

An enzyme catalyzed drying process would definitely be a breakthrough for the industry. The main idea of the present work is to use a laccase mediator system with the capability of cross-linking unsaturated fatty acids as they exist in alkyd resins. Since laccase needs a certain water activity waterborne alkyd resins were tested. Beside the mentioned advantage of replacing a carcinogenic substance by a harmless one, compared to other metal containing siccatives, laccase has the extra plus that it is completely biodegradable and may be produced in a sustainable way from renewable materials.

The basic steps of the project were to identify a laccase mediator combination that is able to harden the model alkyd resin. The whole process should be improved and the final goal is to develop the system further to get an optimized and applicable method for the drying of waterborne alkyd resins. To reach this target a fundamental understanding of the whole reaction will be necessary.

The following chapter will give some basic information about paints and coatings. Additionally a deeper insight into the chemical equations and mechanisms will allow better understanding of the reaction. Furthermore an overview about laccases and LMS will be given. Finally possible laccase mediator reaction mechanisms are discussed.

1.1 Coatings and paints

Coatings and paints are mostly liquids, paste, or rarer powder products which are applied to surfaces by various methods in layers of given thickness. These layers form then adherent films on the surface of the material to be coated [2].

Coatings and paints can be found everywhere in our everyday live as they are applied to all kind of surfaces. To give some examples were coatings are used walls, wood, furniture, cars, cans and houses should be mentioned. Less evidently they are also utilized on the wires of motors, discs or inside television sets. They do not only protect the materials from environmental impacts like chemical invasion, UV radiation or mechanical stress but in many cases also have a decorative function [3].

1.1.1 General compounds of coatings / paints

Coatings are mixtures of many chemical compounds that can be classified in four big groups: binders, volatile compounds, pigments and extenders and additives. A binder is an (organic) polymer that binds all the substances present in a coating and forms a film that adheres to the surface that needs to be coated. Binder polymers and their precursors are frequently named resins. The focus of this study lies on alkyd resins that are described in the following chapters. Further examples of important resin types in terms of production volume are acrylics, polyvinyl acetates, epoxies and amino resins [3].

Volatile compounds are responsible for the consistence of the coating. They are the fluid compounds that make an application of the resin possible and evaporate during and after the application. Until the 1945 the majority of the used volatile compounds were low molecular weight organic solvents. Since there is the need to reduce volatile organic carbon (VOC) emissions there was on the one hand the development towards higher solids coatings that contain a higher concentration of solids. On the other hand the use of water as a solvent in so called waterborne coatings got more important. In contrast to a solvent-borne alkyd resin were the resin is dissolved in an organic solvent, in waterborne coatings the alkyd resin is emulsified in water [3,4].

Other important ingredients of a coating are pigments and extenders that are powders of natural or synthetic materials. Pigments are dispersed in the paint and do not only produce the color of the paint and hides the substrate surface but also enhance the strength of the film. Moreover pigments and extenders can be a protection against corrosion and modify application properties [4,5].

The last important group of ingredients are additives. There are various types of additives with most diverse function but generally it can be said that additives are compounds that are added to the formulation in low contents to modify some characteristics of the coating. The name of the additive typically reflects the function of this additive in the formulation. Leveling agents for example help to form a smooth and homogeneous surface. Antifoaming agents inhibit foaming during production and application of the paint. A typical concentration of additives lies between 0.01 to 1%. The most important additives might be catalysts that lower activation energy of the reaction and therefore enhance the velocity of the cross-linking reaction (so called siccatives). Other additives are stabilizers, anti-skinning additives, flow modifiers, and many more [2,6].

1.2 Drying oils and oil paints - an overview

Oil paints are the oldest form of modern coatings and the used binders are natural occurring vegetable oils as for instance soybean or linseed oil. Both of them count to the drying oils [4]. A drying oil is defined as fatty oil that forms a coating by reactions with atmospheric oxygen if exposed to air. This autooxidation takes place in the presence of fatty acids that contain unsaturations with more than two double bonds per molecule. These oils that react with oxygen may be unmodified or modified. In the first case we are talking about drying oils in the narrow sense. Linseed oil counts among those. Oils that are only able to form films in a modified form are called semidrying oils [7]. In general it can be said that a higher amount of unsaturated C-C bonds lead to a better drying ability.

During the experiments of this master thesis the main focus was set on linseed oil since the model alkyd resin also contained linseed oil. In the following figure a typical triglyceride that can be found in linseed oil is shown.

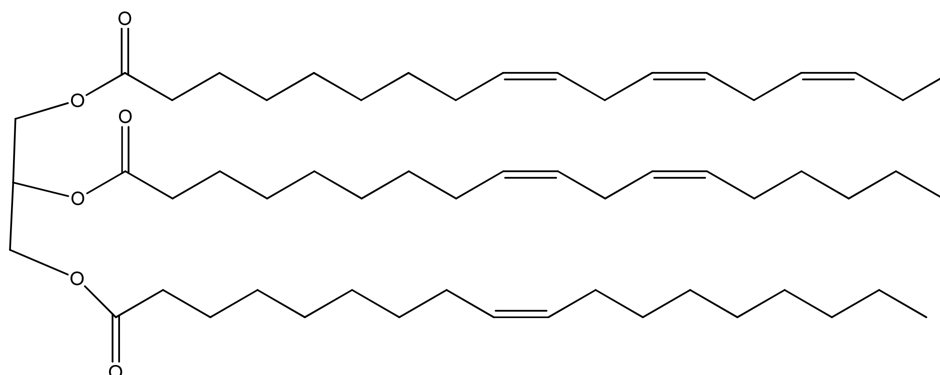


Figure 1: Typical triglyceride found in linseed oil. The triester (top down) consists of α-linolenic acid, linoleic acid and oleic acid.

Linseed oil is obtained from the seed of flax (*Linum usitatissimum*). This plant has already been cultivated by humans since the Stone Age. The seeds of *Linum usitatissimum* have an oil content of 32 to 43% and the main fatty acids are linolenic acid and linoleic acid [7]. Both fatty acids are illustrated in Figure 2: Linoleic acidFigure 2.

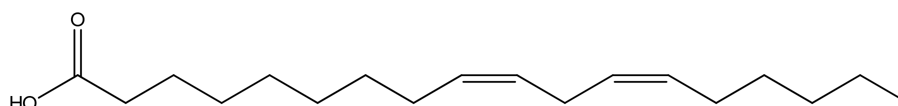


Figure 2: Linoleic acid

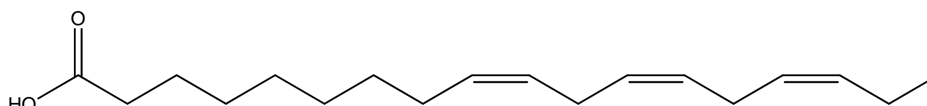


Figure 3: α-Linolenic acid

In the coating industry linseed oil is above all used as a feedstock for coatings like varnishes, alkyds and many others as well as for the production of linoleum and for glazier's putty. Films formed from linseed oil have good properties in respect of stability against water and other agents. One disadvantage is that they are not very light-stable and therefore tend to yellow [7].

1.3 Alkyd resins

Alkyd resins are utilized for the decoration and protection in nearly all sectors including wood protection, automotive coating, finishes of metal, steel and decorative paints. They are also important as corrosion protection coatings [8].

In contrast to oil paints, alkyd paints do contain synthetic resins that are called alkyd resins. The term alkyd resin originates from the polyhydric *ALkohols* and polybasic *aCIDs* (CID was modified to KYD). Consequently in a chemical sense alkyds and polyesters can be regarded as synonyms [4].

Basically alkyd paints are prepared by heating up a poly alcohol (for example glycerol), a polybasic acid (e.g. phthalic acid anhydride) and a fatty acid or triglyceride. During these esterification reactions ester bonds are formed between the alcohol and the phthalic acid anhydride as well as the alcohol and the fatty acid. Only in the second case water is formed.

The following figures give a deeper insight into the reaction mechanism. In Figure 4 a chemical equation of the reaction between an alcohol and the phthalic anhydride is shown. As can be seen no water is formed during this reaction. In the majority of alkyd resins phthalic anhydride is present. During the reaction the anhydride ring opens at approximately 160°C and a phthalate monoester is formed. The remaining acid group starts to react at circa 180°C [4,9].

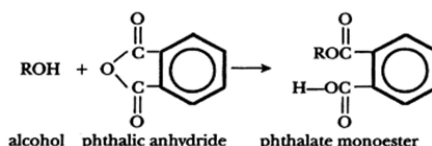


Figure 4: During the reaction of alcohol and phthalic anhydride a phthalate monoester but no water is formed [9]**.**

The reaction between glycerol and monobasic fatty acids and the reaction between monoglyceride and phthalic anhydrate are outlined in the subsequent Figure 5. Monoglyceride is formed when glycerol is converted with a fatty acid. In this instance water is formed as a byproduct. If monoglyceride then reacts with phthalic anhydride an alkyd resin is formed.

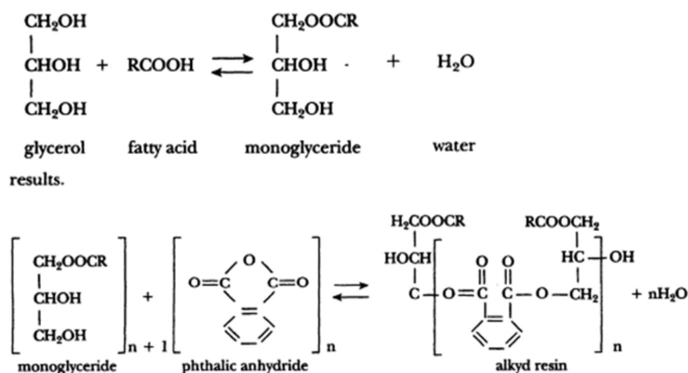


Figure 5: The conversion of glycerol and a fatty acid leads to the formation of monoglyceride. If monoglyceride reacts in a subsequent reaction with phthalic anhydride an alkyd resin is formed. In both cases water is generated as a byproduct [9]**.**

A simplified structure of the obtained polyester with a linoleic acid fatty chain is shown in Figure 6. Nevertheless this structure is only a simplification of the real alkyd resin that contains a variety of molecules with different shape, molecular weight or composition [9].

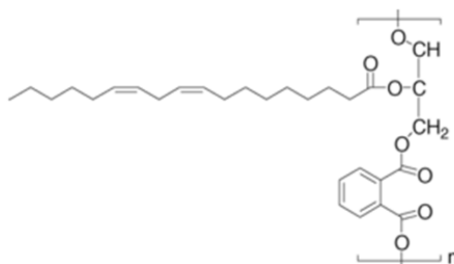


Figure 6: Schematic illustration of an alkyd resin with a linoleic acid side chain [4]**.**

Due to the fact that the amount of ingredients can be modified and the huge diversity of possible components that exist, alkyd resins are available with a multifarious number of characteristics and for various applications. Commonly alkyd resins are classified by means of oil type and oil length [5]. The oil content is referred to as "oil length". An oil content ≤ 40% is defined as short oil and 41 – 60% oil content as medium oil. Long oils contain 61 – 70% oil and very long oil alkyds are those containing 71 – 85% oil [8].

1.3.1 Drying mechanism with Cobalt

As mentioned previously, the aim of this project is to find an enzyme based alternative able to replace heavy metal siccatives in particular cobalt based siccatives. For this reason knowledge about the cobalt catalyzed drying process is of importance. As noted before additives that catalyze the drying reaction have to be put in the formulation. Oxidative drying systems have in common that they are formulated with siccatives. These siccatives are oil-soluble metal salts of organic acids e.g. ethylhexanoic acid and naphthenic acid. The metal occurs in different oxidation states and is able to undergo redox reactions. The most active metal in siccatives is Co, but also Fe, Mn, Ce, Pb and Zr are utilized. These metal siccatives are typically added in concentration of 0.005 to 0.2 wt%. [7]

Generally the siccative accelerates two steps of the drying process. In the first place the methylene bonds are activated and hydroperoxides are formed. Thereafter, radicals are formed from the hydroperoxides in a redox reaction and the metal catalyst is re-oxidized. The whole cobalt catalyzed drying process can be summarized as followed:

1. Formation of a hydroperoxide
2. Decomposition of the hydroperoxide with Co^{2+}
3. Combination with another unsaturated side chain
4. Generation of a carbon-based radical
5. Further polymerization

The cross-linking reaction steps are schematically described in the following Figure 7.

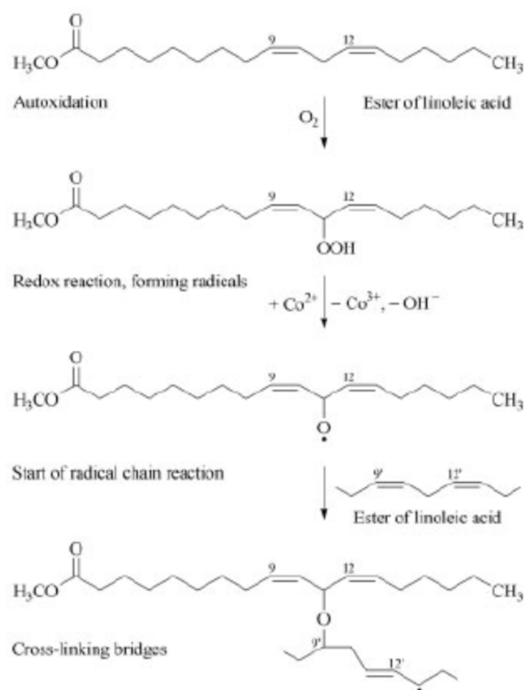


Figure 7: Schematic illustration of the proposed reaction mechanism [7]**.**

Oyman proposed a detailed mechanism of the cobalt catalyzed drying process. Besides the reaction steps mentioned in Figure 8, cobalt also seems to deactivate natural antioxidants [5].

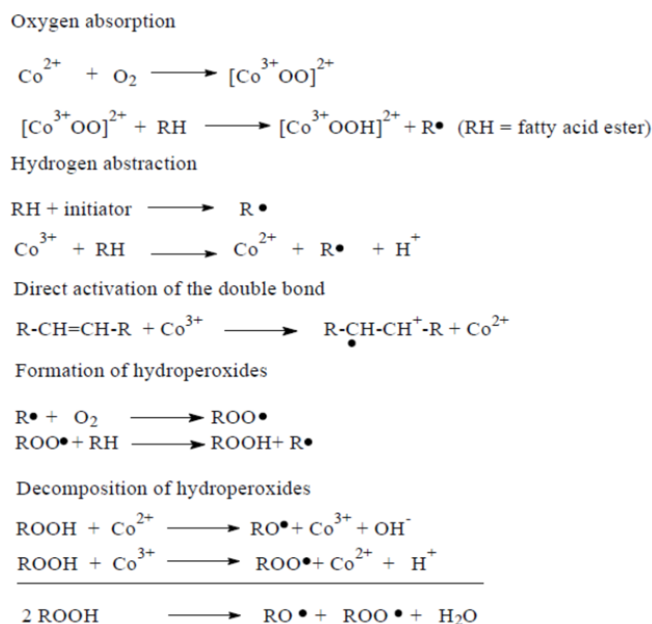


Figure 8: Reactions that are most likely involved in the cobalt catalyzed drying reaction of alkyd resins [5].

In order to prevent the too fast cross-linking of the surface layer leading to insufficient oxygen diffusion and wrinkles, lead was added in earlier times. Cobalt supports the formation of hydroperoxides especially on the topmost layer of the film and lead promotes the polymerization and an evenly formed film. For the simple reason that lead is now defined as dangerous, it is replaced by zirconium that has the same effect [7]. Unfortunately also Co is suspected to be cancer-causing.

1.4 Undesired effects during the drying process of alkyd paints

During the curing process some reactions might occur that are unfavorable and therefore undesirable. With respect to the present work some points require specific attention and are shortly described below.

One example for an unwanted reaction is skin formation that occurs very likely on air-drying alkyd paints. Especially as soon as the can has been opened the uppermost layer of the coating is at risk to harden and form a film. Anti - skinning additives as for example phenol derivatives or oximes restrain skin formation [8].

Another important point is the yellowing effect. Yellowing of dried oil films is defined as the discoloration over time. Yellowing is a general problem of alkyd paintings and is provoked by over-oxidation of the resin [4]. Alkyd resins with a high content of unsaturated oils have the tendency to yellow under certain conditions. Especially linseed oil is not sufficiently light stable and tends to yellow. Responsible for the yellowing are colored polyenes or quinone structures that are formed starting from unsaturated hydroperoxides. Ketones are present as intermediates. As a consequence it is generally recommended to replace linseed oil by products that are less likely to discolorate in coatings of white and other light colors. Additionally it has to be considered that also siccatives that contain manganese or cobalt can cause yellowing in films due to complexes that are formed [7].

Both reactions are directly connected to the drying process and play a major role for the quality of a coating. Of course these are no major topics of the present work but they should be kept in mind as they may become important during a subsequent stage of the project.

1.5 Laccases

Laccase (EC 1.10.3.2) belong to the blue copper family of phenoloxidases that is produced by white-rot fungi, different bacteria and plants [10]. Laccases catalyze the oxidation of substituted phenols and other aromatic compounds. During this reaction radicals are formed and molecular oxygen is reduced to water (see Figure 9) [11]. An impressive fact is that in contrast to other proteins laccases have high resistances against organic solvents like methanol, ethanol, acetone and acetonitrile [10].

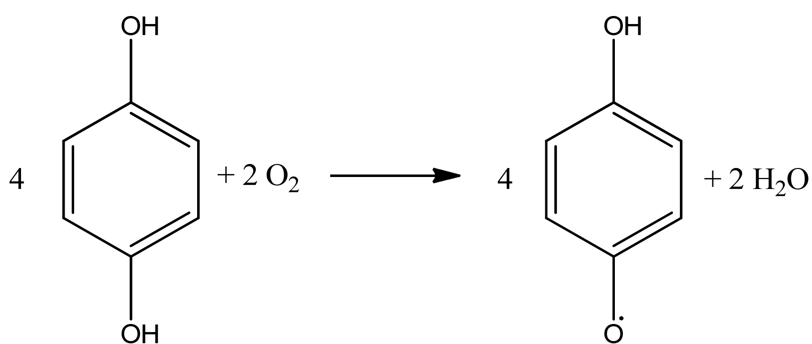


Figure 9: Reaction catalyzed by laccase. In the presence of oxygen four benzendiol molecules are converted to four benzosemiquinone molecules and water.

Laccases secreted by white-rot fungi are glycoproteins that consist of one mononuclear domain containing one type-1 copper and a trinuclear domain with one type-2 and two type-3 copper centers. The type-1 copper center has a strong absorbance near 600 nm and as a consequence the blue color is provoked. Type-1 copper is also reported to be the primary electron acceptor from the substrate. Following this, electrons are delivered to the trinuclear site were the molecular oxygen binding site is located. Here the reduction of dioxygen to water takes place. Within the trinuclear site the type-3 copper centers consist of a pair of copper atoms that is bridged by a hydroxide molecule. The third copper atom present in the trinuclear domain (type-2 copper) has no ligand that links it to the type-3 copper centers [12,13]. In Figure 10 the schematic constitution of trinuclear copper site of ascorbate oxidase is illustrated which is similar to the active side of laccase.

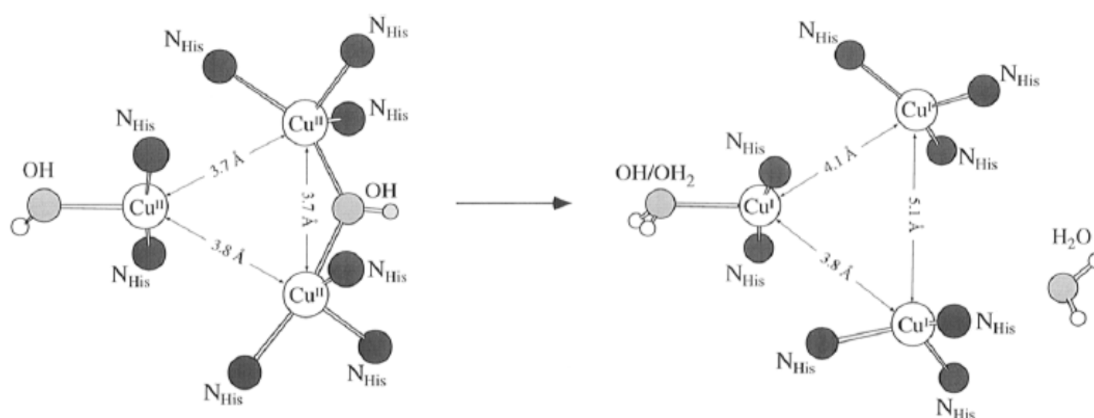


Figure 10: Structure of the active-site of a trinuclear copper cluster in ascorbate oxidase. On the left-hand side the oxidized form is shown and the reduction is illustrated [13]**.**

In this study we mainly focused on a laccase that was purified from the basidiomycete white-rot fungus *Trametes hirsuta* (Figure 11) that causes wood decay. This laccase was reported to have a pH optimum of 3.0, an isoelectric point of pH 7 and a molecular weight of 62 kDa. The optimum temperature was 55°C [14].

Figure 11: White-rot fungus *Trametes hirsuta* [15]**.**

The very essential property of laccase is its low specificity for substrates when compared to other enzyme classes such as hydrolases. Already for a long time laccase has been used in biotechnologies due to the broad spectrum of possible substrates. Some examples are the transformation of aromatic compounds as for instance dyes or the application in bioremediation processes like wastewater treatment [10]. Laccase catalyzes not only the oxidation of polyphenol analogues, but also oxidizes aniline derivates, phenol analogues, L-ascorbic acid and others. Nevertheless laccase is not able to oxidize conjugated nonpolar groups and unconjugated polar groups that have a higher redox potential than the mentioned substrates [16].

In nature, laccases play an important role in the synthesis of lignin and chitin in the case of higher plants and insects. In contrast to that fungal laccases are involved in the degradation of lignin and humic acids. Apparently, bacterial laccases are responsible for the resistance of spores and pathogenesis and pigmentation. Especially for lignolytic basidiomycetes, laccases and peroxidases are of immense importance as they impart the ability to degrade lignin in decayed wood. Additionally they are able to detoxify aromatic compounds. Summarizing it can be said, that laccases are widely distributed in the soil and in the whole nature and catalyze a huge number of reactions [17,18].

1.6 Mediators

To open the range to an even wider spectrum of substrates, so called “mediators” can be used to extend the enzymatic activity. A mediator is a compound of low molecular weight that can be oxidized by the laccase and acts then as an electron shuttle between the laccase and the substrate. As soon as this mediator is oxidized by the enzyme it is able to diffuse away from the active site and oxidizes a substrate that cannot interact directly with the laccase for any reason, for instance because of its size or redox

potential. In addition to that, the oxidized mediator can start an oxidation mechanism that otherwise is not accessible to the enzyme. In such a way the number of potential laccase substrates can be raised a lot [19].

Figure 12 illustrates how oxygen is reduced to water in order to oxidize the substrate. The reaction takes place in two steps. In a first step, the mediator is oxidized by the enzyme and in a second step the oxidized mediator is reduced and regenerated and the target molecule is oxidized [20].

Figure 12: The role of a mediator during the substrate oxidation [21]**.**

For the present study the fact, that oxygen is consumed during the catalyzed reaction, was utilized to follow the reaction via oxygen consumption measurement. For these experiments two methods to determine oxygen concentration in solution were available - the oxygen electrode (also Clark cell) and a fiber-optic oxygen micro sensor. Both systems are shortly described in the materials and methods section.

1.6.1 Laccase mediator systems

Laccase mediator systems have already been used for various industrial applications such as pulp delignification, oxidation of organic pollutants and even in the development of biosensors and biofuel cells. There exist many different inorganic and organic molecules that are reported to be effective mediators as for instance phenol and thiol aromatic derivates and N-hydroxy compounds [20].

But which requirements need to be fulfilled so that a molecule is an excellent mediator? First of all the mediator should be a good laccase substrate. Secondly its oxidized and reduced forms should be stable. Another important point is that the enzyme reaction should not be inhibited by the mediator and the mediator should not take part in the reaction [11]. Considering the thermodynamic aspect, an ideal electron donor has a reduction potential that lies between the one of the type-1 copper of the laccase and that of oxygen. By means of voltammetric monitoring of the laccase mediated reaction it is possible

to identify mediators that have stable electrochemical states and show a reversible electrodic behavior [20]. Two synthetic chemicals that accomplish these objectives are 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO) and 2,2’-azino-bis(3-ethyl)benzothiazoline-6-sulfonic acid (ABTS). Many of the compounds that are described as mediators in the literature have intermediates of low stability that easily fall out of the catalytic cycle. For that reason they cannot be defined as mediators in the classical sense [11].

Some different mechanisms of oxidation of non phenolic substrates by laccase mediator systems that can be found in the literature [17,19] are:

1) An ionic mechanism as in the case of TEMPO where N-O$^{\cdot}$species are present.

2) A radical H-abstraction also called HAT (hydrogen atom transfer) route. >N-OH species as for instance 1-hydroxybenzotriazole (HBT), violuric acid (VLA) or N-hydroxyphthalimide (HPI) follow this mechanism.

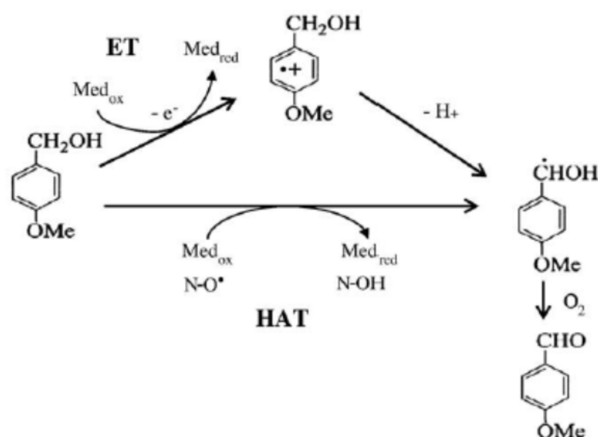


Figure 13: Oxidation of lignin model compound (p-anisilic alcohol) by a laccase mediator. Two different oxidation mechanisms are proposed: electron transfer (ET) and hydrogen atom transfer (HAT) [17]**.**

3) Electron transfer (ET): A redox mechanism which exists with molecules that can easily get oxidized by the laccase as for example ABTS. In Figure 14 the oxidized states of ABTS are displayed.

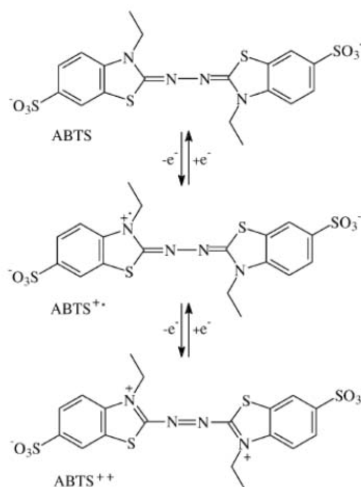


Figure 14: ABTS and its oxidized states [19]**.**

All in all it can be recorded that the industrial application of synthetic mediators is limited because of their relatively high costs. Some of them also show toxicity. Thus it is not surprising that the interest in cheaper not toxic natural occurring mediators is high and many groups carry on research in order to find natural mediators [17,18,22,23]. The comparison of free radicals released during the oxidation of HBT or ABTS leads to the assumption that radicals that are produced during the oxidation of typical laccase substrates are as well able to mediate laccase reactions [11,24]. Some compounds that are mentioned in the literature [17,19] as proved mediator compounds are listed in Figure 15 and Figure 16.

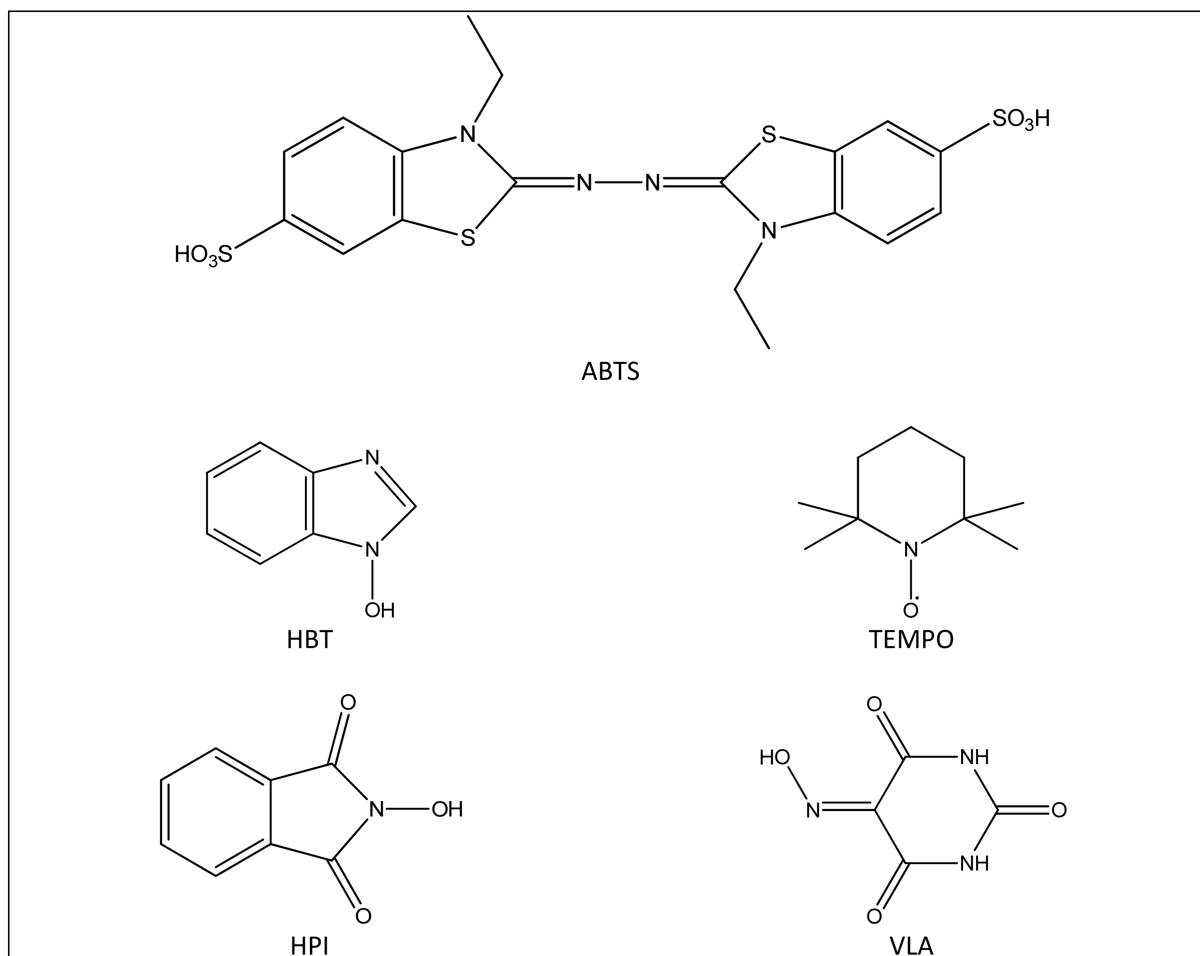


Figure 15: List of synthetic laccase mediators.

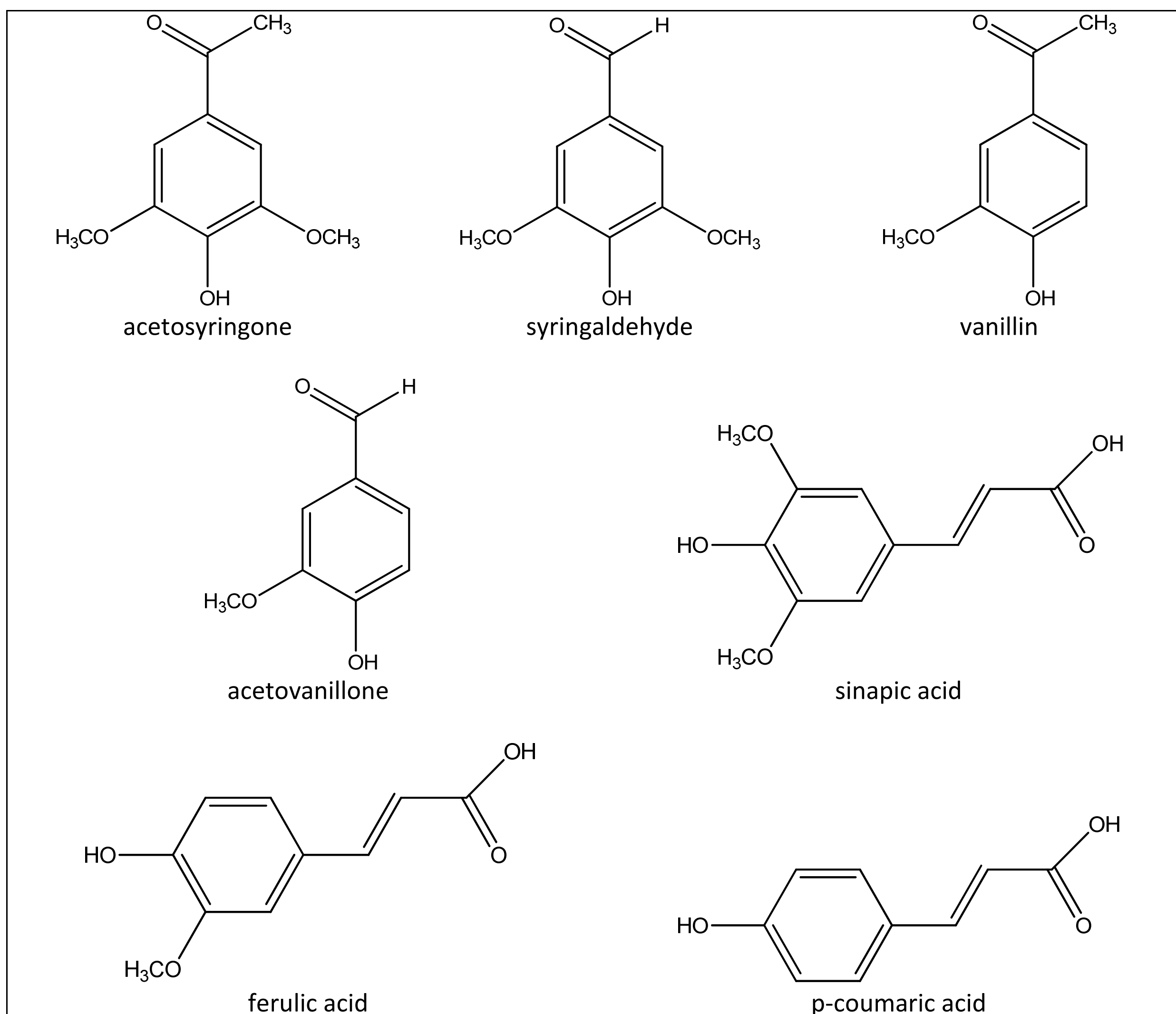


Figure 16: Examples of natural laccase mediators.

1.6.2 Mechanism of a LMS catalyzed conversion of unsaturated lipids/fatty acids

Several research groups [16,22,25-27] already have shown that unsaturated triglycerols and fatty acids can be oxidized by laccase mediator systems. Good results have been achieved by using ABTS or HBT as mediators.

Research from Camarero et al. [18] suggest the assumption that phenoxyl radicals as generated in the laccase catalysis e.g. from p-hydroxycinnamic acid follow the HAT oxidation mechanism which is similar to the one described for HBT nitroxyl radicals.

In Figure 17 a suggested reaction mechanism for the peroxidation of unsaturated lipids is presented.

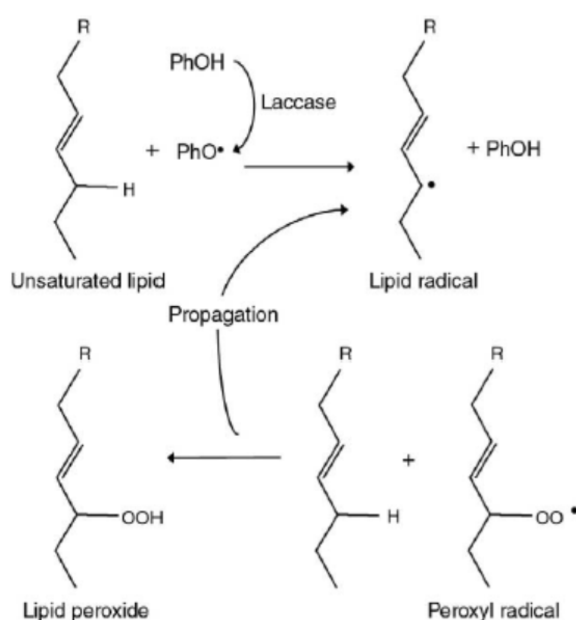


Figure 17: Mechanism of peroxidation of unsaturated lipids by phenoxyl radicals that are generated by laccase during oxidation of natural phenolic mediators [17]**.**

Zhang et al. [26] also proposed a reaction scheme similar to that in which the radicals are produced via hydrogen abstraction and a lipid radical is formed. The double bonds rearrange and the consequence is the formation of a conjugated diene. This compound is easily attacked by molecular oxygen which results in the generation of peroxy radicals that can then undergo chain reactions. A schematic illustration of the process is shown in Figure 18.

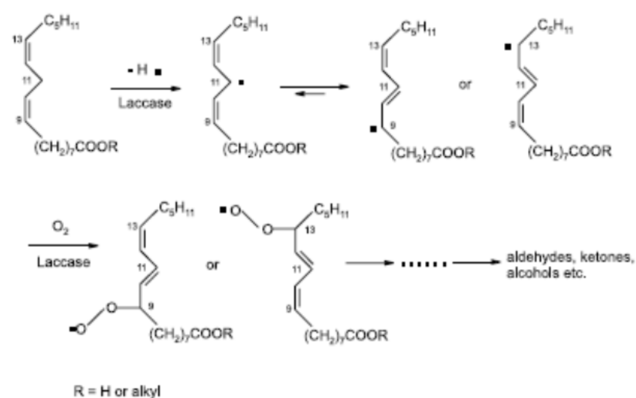


Figure 18: Mechanism of the formation of peroxy radicals [26]**.**

1.7 Aim of this work

The aim of this project is to cross-link unsaturated groups in waterborne alkyd resins by using laccase / mediator systems. Different alkyd resins containing linseed oil fatty acids provided by Cytec Graz were used as model substrates. The expected advantage of this method is the elimination of heavy metal siccatives (e.g. Co- or Pb- containing substances that are known to be toxic / carcinogenic) from the formulation.

2 Materials and Methods

2.1 Characterization of Laccases

Three laccases from *Trametes hirsuta* (*Th*L), *Trametes villosa* (*Tv*L) and *Myceliophthora thermophila* laccase (*Mt*L) with the potential ability to catalyze the cross-linking reaction, were analyzed. pH optimum, kinetics, activity and protein concentration were determined in order to select the most appropriate laccase for the following tests including cross-linking reaction experiments, oxygen consumption measurements, FTIR and Raman spectroscopy etc. Laccase from *Trametes hirsuta* was produced at the institute [14]. *Trametes villosa* laccase and *Myceliophthora thermophila* laccase were purchased from Novozymes, Denmark.

2.1.1 Laccase activity

Laccase activity was measured according to Liu et al. [28]adapting an ABTS activity method. *Th*L and *Tv*L were diluted 1:10000 with 50 mM succinate buffer (pH 4.5) and *Mt*L was diluted 1:10000 with 100 mM potassium phosphate buffer (pH 7). 650 µL of buffer diluted enzyme was put into a cuvette and the reaction was started by adding 200 µL of ABTS solution (10 mM in ddH$_2$O). It is essential to temper the reaction mixture precisely as the reaction rate strongly depends on the temperature. The assay was carried out at 27°C (standard conditions). As Figure 19 shows the absorption maximum of oxidized ABTS was identified as 414 nm in advance.

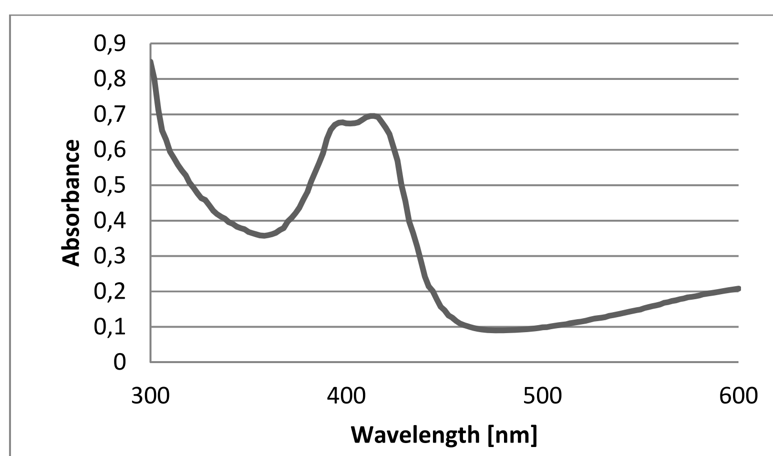


Figure 19: Wavelength scan of ABTS that was oxidized by *Th*L.

The ABTS absorbance change, due to oxidation of the ABTS, was measured at 420 nm for 1 minute with a photometer. Enzyme activity was expressed in units. One unit (U) is defined as the amount of enzyme that catalyzes the conversion of one micro mole of substrate per minute (U = µmol/min) [28]. One unit is equal to 16.67 nkat.

For each sample a threefold determination was accomplished. The volumetric activity was calculated by the following equation:

Equation 1

$$v0 = \frac{\Delta\, abs}{\Delta\, t} = \frac{\Delta abs}{min} * \frac{Vtot}{Vsample * \varepsilon * d} * f$$

v0:	volumetric enzyme activity [U/mL]
$\Delta abs/\Delta t$:	change of absorbance per minute [abs/min]
ε_{ABTS}:	molar extinction coefficient of ABTS at 420 nm and pH 7 ε=43.2 [mL/(µmol*cm)] [29]
ε_{ABTS}:	molar extinction coefficient of ABTS at 420 nm and pH 4.5 ε=36.0 [mL/(µmol*cm)] [28]
V_{tot}:	total volume [µL], (850 µL)
V_{sample}:	sample volume [µL], (650 µL)
d:	path length [cm], (1 cm)
f:	dilution factor (10000)

2.1.2 Kinetic parameters

Kinetic parameters were detected by performing a slightly modified version of the laccase activity assay described in chapter 2.1.1. 650µL of buffer diluted enzyme (50 mM succinate buffer pH 4.5) were transferred to a cuvette and the reaction was then started by adding 200 µL of 20, 10, 5, 1, 0.75, 0.5, 0.25, 0.1, 0.05 and 0.01 mM ABTS solution respectively. The absorbance change of ABTS at 420 nm was determined at 27°C and recorded with the photometer and the volumetric enzyme activity was calculated as described above (see equation 1).

2.1.3 pH-Optimum

In order to determine the effective pH range of each laccase an activity assay with pH dependent profile was measured.

100 mM sodium acetate buffer at pH 3.07, pH 3.32, pH 3.59, pH 4, pH 4.5, pH 5 and pH 5.4; potassium phosphate buffer pH 6, pH 7 and pH 8 and 100 mM tris buffer pH 9 was used to dilute the laccase of interest. 650 µL of the laccase sample were pipetted into a cuvette and the reaction was started by adding 200 µL of ABTS solution (10 mM in ddH_2O). Activity was determined at standard conditions.

2.1.4 Protein concentration

Total protein concentration of the selected laccases was measured with a kit based on the Bradford protein method [30]. A calibration curve was prepared to define the protein concentration in µg/mL using bovine serum albumin (BSA). 10 µL of the BSA standard solution and the diluted samples respectively were pipetted into the wells of a 96 well microtiter plate. After addition of 200 µL Bradford reagent the reaction mixture was incubated for 15 minutes at room temperature. Absorption measurements were performed with a plate reader at 595 nm.

2.1.5 SDS-PAGE analysis of the tested laccases

In order to determine the size of the enzymes sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. All laccase samples were diluted with ddH_2O, whereby *Mt*L was diluted 1:50, 1:70 and 1:100, *Tv*L 1:10, 1:30 and 1:50 and *Th*L was diluted 1:5, 1:10 and 1:30. As a standard “Broad Range SDS-PAGE Molecular Weight Standards” from Bio-Rad were used. 10 µL of the dilutions and the standard solution were then mixed with 10 µL sample buffer respectively and denaturized for 5 minutes at 95°C. 10 µL were loaded onto the gel and separation was done via electrophoresis at a charge of 100 V for approximately 90 minutes. Staining was implemented by shaking the gel for 20 minutes in 0.25 % (w/v) Coomassie Blue solution at room temperature. After removal of the Coomassie Blue solution the gel was incubated in destaining solution until the bands could be seen properly.

Table 1: Composition of buffers, gels and solutions needed for SDS-PAGE

	Reagent	Volume [mL] (unless otherwise stated)
10 % Separating gel	30 % Acryl amide bisacrylamide	3.35
	1.5 M Tris-HCl pH 8.8	2.5
	ddH_2O	4
	10 % SDS	0.1
	10 % APS	0.05
	TEMED	0.007
10 % Stacking gel	30 % Acryl amide bisacrylamide	1.3
	0.5 M Tris-HCl pH 6.8	2.5
	ddH_2O	6.1
	10 % SDS	0.1
	10 % APS	0.05
	TEMED	0.007
Sample buffer	0.5 M Tris-HCl pH 6.8	2
	ddH_2O	1.2
	10 % SDS	3.2
	Glycerol	1.6
	0.05 % Bromphenol blue	2
Running buffer (5x concentrated)	Tris	15 g
	Glycerol	72 g
	SDS	5 g
	ddH_2O	1000 ml
Staining solution	0.25 % (w/v) Coomassie Blue	
Destaining solution	dH_2O	700
	Acetic acid	150
	Methanol	150

2.1.6 Native gel electrophoresis

Additionally to the SDS gel electrophoresis a native gel electrophoresis was done according to Zhang et al. [26], Nagai et al. [31] and Srinivasan et al. [32] with some modifications to detect bands that show laccase activity. The native gel was prepared as described in Table 1 with the following modifications. Instead of 10 % SDS, ddH_2O was used and also the sample and running buffer did not contain SDS. Laccase solutions were diluted with ddH_2O as follows: ThL 1:5, 1:10 and 1:20; MtL 1:50, 1:100, and 1:150; TvL 1:10, 1:20, 1:50 and 1:100. 20 µL of the dilutions were then mixed with 20 µL sample buffer respectively. 15 µL of each sample were applied to the gel and separated by electrophoresis at a charge of 100 volt for about 90 minutes. Two gels were processed at the same time in order to compare two dying methods. One gel was stained with Coomassie Brilliant Blue R-250 solution. This dying method

shows all protein bands. Additionally, ABTS staining was performed to detect bands with laccase activity. For that reason the second gel was stained by soaking it in a 50 mM succinate buffer solution (pH 4.5) with 5 mM ABTS for approximately 7 minutes. Like that it was possible to detect laccase activity in the protein bands by the color change of ABTS.

2.1.7 Analysis via Fast protein liquid chromatography (FPLC) - ÄKTA

The three laccases were analyzed via Fast protein liquid chromatography (FPLC). The enzyme solutions were diluted with ddH_2O to a final concentration of 300 µg/mL ascertained by the above mentioned Bradford protein assay. Samples were centrifuged for 10 minutes at 10000 rpm and room temperature to precipitate particles from the solution. Separation of the samples was achieved with a size exclusion column (SEC, Superdex 75 10/300 GL). 200 µL of the sample were applied. Filtrated and degassed potassium phosphate buffer (50 mM potassium phosphate buffer pH 7 containing 100 mM sodium chloride) was used as eluent at a flow rate of 0.7 mL/min. Detection of the protein was realized with UV detection at 280 nm.

2.2 Screening for potent laccase mediators

As described previously laccases reduce oxygen to water and at the same time a substrate (e.g. a mediator) is oxidized. This fact can be utilized to follow the reaction via oxygen consumption measurement. A faster decrease of oxygen concentration would indicate a faster reaction. An important point to keep in mind is that we are dealing with a two-step reaction. In a first step the mediator is oxidized. In a second step the oxidized mediator transfers electrons to the desired substrate [21] being subsequently reoxidized by the laccase. In order to correctly follow the reaction via O_2 consumption it is important to wait until the tested mediator is completely oxidized and a stable oxygen concentration is reached before adding the substrate. This principle is visualized in Figure 20.

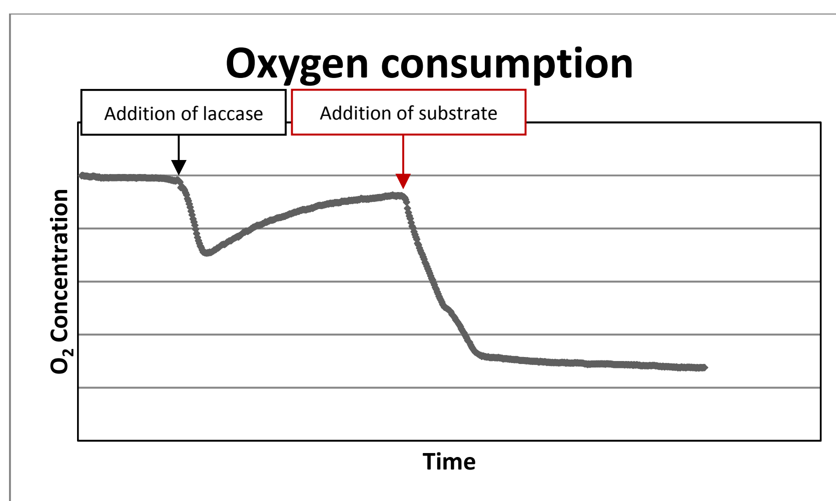


Figure 20: Decrease of oxygen concentration after the addition of laccase and long alkyd resin in the presence of a mediator.

After addition of the laccase (black arrow) the mediator is oxidized, oxygen is consumed and therefore the oxygen concentration in the solution decreases. Since the present experimental set-up is an open system the oxygen concentration increases again as soon as the entire amount of mediator is oxidized. The red arrow indicates the addition of the substrate after stabilization of oxygen concentration. The subsequent measured oxygen decrease depends on how effective the LMS reacts with the substrate. In this way, the amount of oxygen needed to oxidize the substrate could be evaluated.

For our studies two methods to determine oxygen concentration in solution were used, an electrochemical system (Clark electrode) and a fiber-optic oxygen micro sensor. Both systems are shortly described below.

2.2.1 Methods for oxygen concentration determination

2.2.1.1 Oxygen electrode (Clark cell)

The oxygen electrode is one of the most commonly used tools to measure oxygen partial pressure in solution or in the gas phase. The system consists of two electrodes, the central platinum disc working electrode (cathode) where O_2 is reduced and the silver ring reference electrode (anode). 3 M potassium chloride solution is used to reach conduction between those two electrodes and a gas permeable, ion-impermeable membrane separates the sensing electrodes from the test system [33].

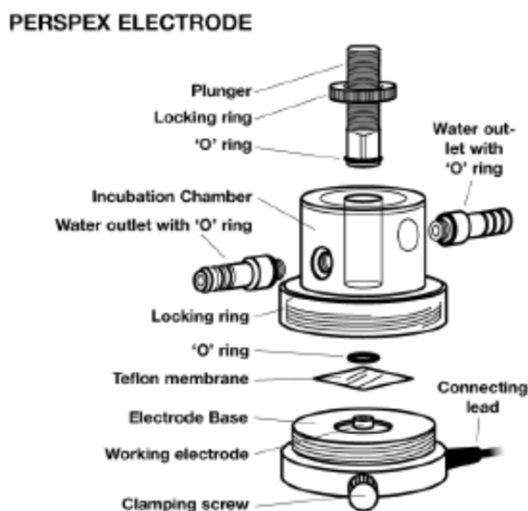
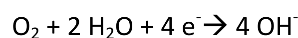


Figure 21: Setup of the Clark electrode device [33]**.**

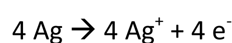
The Clark electrode - Principle of operation

The platinum cathode is polarized at -0.6V with respect to the silver anode and every oxygen molecule that diffuses from the test medium through the membrane to the cathode is reduced to water.



$$O_2 + 2\ H_2O + 4\ e^- \rightarrow 4\ OH^-$$

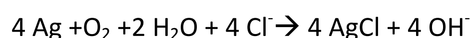
The corresponding oxidation occurs at the silver anode where silver chloride is generated.



$$4\ Ag \rightarrow 4\ Ag^+ + 4\ e^-$$

$$4\ Ag^+ + 4\ Cl^- \rightarrow 4\ AgCl$$

The overall electrochemical process can therefore be summarized as followed:



$$4\ Ag + O_2 + 2\ H_2O + 4\ Cl^- \rightarrow 4\ AgCl + 4\ OH^-$$

The oxygen partial pressure in the tested solution or gas phase is proportional to the resulting current between the two electrodes.

One disadvantage of this system is the consumption of oxygen by the electrode. Nevertheless this fact does not influence the measurements if the test medium in the incubation chamber is stirred continuously. Like this it can be guaranteed that the partial pressure of oxygen in the test solution and

the membrane-medium interface is the same. Furthermore the oxygen electrode is temperature sensitive and therefore it is advantageous to temper the system [33].

2.2.1.2 *Fiber-optic oxygen micro sensor (optrode)*

Fiber-optic oxygen micro sensors are so called optrodes and are the optical analog to electrodes. In these sensors a change in optical properties is caused by interaction of the analyte with the optrode. Changes in the fluorescence properties of oxygen-quenchable fluorophores or color changes based on pH-dependent acid-base indicators are typical. For the measurements a needle-type oxygen micro sensor based on dynamic fluorescence quenching was used [34,35].

Fiber-optic oxygen micro sensor - Principle of operation

The sensor consists of a thin silica fiber whose tip is coated with an oxygen-sensitive layer and a black silicone film.

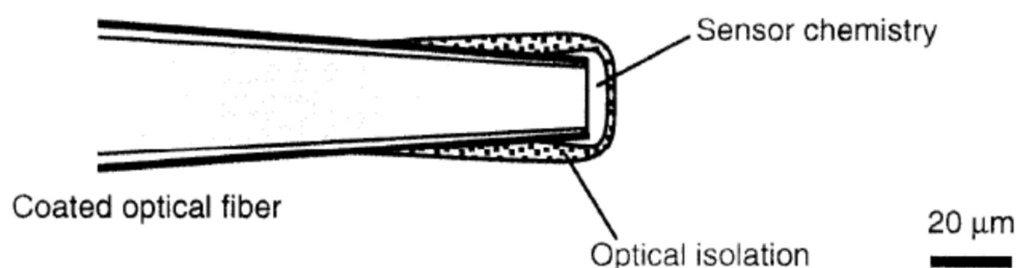


Figure 22: Design of an oxygen microoptrode [34]**.**

Blue LED excites the immobilized indicator at the sensor tip to emit light. If an oxygen molecule is present a non-radiative energy transfer takes place and the excess energy is transferred to the oxygen molecule. Thereby fluorescence is quenched or decreased and the degree of quenching corresponds to the oxygen concentration in the matrix which is in a dynamic equilibrium with oxygen in the sample (Figure 23). The black coat acts as an optical isolation that makes the signal independent of the optical

properties of the analyte medium such as coloration, turbidity-reflectivity or refractive index and avoids interferences from ambient light and background fluorescence [34].

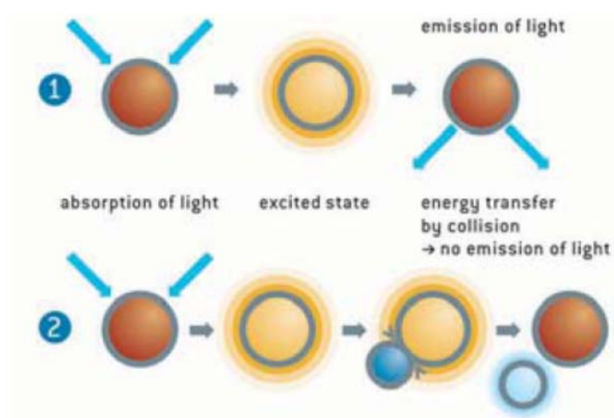


Figure 23: Principle of oxygen caused fluorescence quenching [35].

In comparison to the Clark cell a microoptrode does not consume oxygen. Furthermore the signal of the oxygen sensor is based on a thermodynamic equilibrium and is independent from diffusion. Some other advantages of the system are that high concentrations of CO_2 and H_2S do not affect the signal. The response time of the oxygen microoptrode depends on the thickness of the sensing layer and the black isolation coat [34]. One big disadvantage of the system is the fragility of the silica fiber which makes an extremely careful handling necessary (Figure 24).

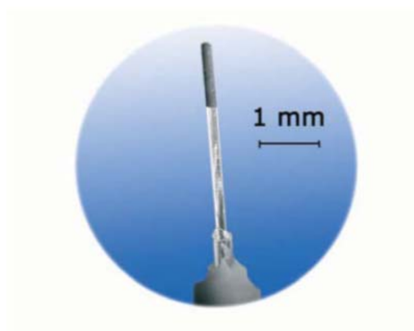


Figure 24: Needle type oxygen micro sensor [35].

In summary it can be said that both systems show advantages for specific applications and no significant differences in the obtained results could be seen.

2.2.1.3 Oxygen consumption measurement

Oxygen consumption measurements were performed on both the Rank Brothers Ltd Oxygen Electrode and the PreSens needle type Oxygen micro sensor.

Oxygen consumption measurement using alkyd resin as substrate

220 µL of 50 mM succinate buffer (pH 4.5) were put into the stirred incubation chamber, 20 µL of a 10 mM ABTS solution and 20 µL *Trametes hirsuta* laccase solution were pipetted into the stirred reaction vessel. After the recovering of O_2 concentration (see Figure 20) 250 µL of alkyd resin UE HSW 323/23c were added. From now on UE HSW 323/23c will be called "long (alkyd) resin" since this alkyd resin contains 65% oil.

Screening for potential mediators

920 µL of 50 mM succinate buffer (pH 4.5) were put into a stirred beaker, 40 µL of *Trametes hirsuta* laccase solution were added. 40 µL 10 mM ABTS in dH_2O, 40 µL 10 mM HBT in dH_2O, 40 µL 10 mM syringaldazine in DMF, 40 µL 50 mM TEMPO in ethanol or 20 µL 10 mM syringic acid in dH_2O solution were added to reach the final concentration mentioned in Figure 41. After the recovering of the O_2 concentration, 40 µL long alkyd resin were added.

Testing different substrates

920 µL of 50 mM succinate buffer pH 4.5 were put into a stirred beaker, 40 µL *Trametes hirsuta* laccase solution and 40 µL 10 mM ABTS in dH_2O were added. After the recovering of O_2 concentration 40 µL fatty acid neutralized, 40 µL long alkyd resin and 40 µL linseed oil respectively were added. For the "linseed oil emulsified-sample" 50 µL of *Trametes hirsuta* laccase solution and 50 µL 10 mM ABTS in dH_2O were mixed and then 1500 µL linseed oil emulsified were added. Linseed oil emulsified consisted of 9% emulsifier, 56% linseed oil and 35% water.

2.3 Measurement of the molecular weight increase- Gel permeation chromatography

Gel permeation chromatography (GPC) was used to detect and quantify molecular weight increase in the resin after laccase/mediator treatment.

For each sample 3 g of long alkyd resin diluted with 50 mM succinate buffer pH 4.5 1:2 respectively were put into a 15 mL glass beaker. 100 µL *Trametes hirsuta* laccase (141 U/mL) and 100 µL of one of the following mediator solutions were then added respectively: 10 mM ABTS in dH_20, 10 mM HBT in dH_20, 50 mM 2,2,6,6-*Tetramethylpiperidine*-1-oxyl (TEMPO) in ethanol, 10 mM syringaldazine in dimethylformamide (DMF), 10 mM syringic acid in dH_20. Table 2 shows the entire list of samples for two experimental setups. The bulk samples were stirred for 6 or 18 hours at room temperature and analyzed at Cytec Graz.

For the 1st experiment5 ml THF were used to dissolve 3.0 g sample. 100 µL of this solution were then diluted in 10 mL THF + Sulphur (0.02 %) and filtrated with MILLIPORE GLASS FIBRE PREFILTERS + MILLIPORE FLUOROPORE™ MEMBRANE FILTERS (0.45 µm).

The samples of the 2nd experiment were prepares as followed: 25-50 mg sample were diluted in 10 mL THF + TFAA + Sulphur (0.02 %) and filtrated with MILLIPORE GLASS FIBRE PREFILTERS + MILLIPORE FLUOROPORE™ MEMBRANE FILTERS (0.45 µm) (glass syringe and steel filter housing) or with WHATMAN® syringe filter (0.45 µm PTFE) (PP syringe and PP filter housing).

The samples of both experiments were analyzed by size-exclusion chromatography (SEC) using the following conditions:

- *Instrumentation:* Agilent 1100 Isocratic Pump G 1310A and Agilent 1100 Auto-sampler G 1313 A
- *Concentration detector:* Agilent RID 1200 G 1362A
- *Columns:* 2 x Polymer Labs PL Mixed C, 5µm, 7.8mm x 300 mm
- *Calibration: polystyrene calibration:* SET E 2010 Feb 16 Sauer, Fit Polynomial 4
- *Mobile phase:* THF + 5g/L TFAA
- *Flow rate:* 1.0 mL/min

- *Standard:* polystyrenes 7500000, 2560000, 841700, 280500, 143400, 63350, 31420, 9920, 2930, 580
- *Injection volume:* 100µL
- *Sample concentration:* approximately 1.5g/L
- *Sample solvent:* THF + Sulphur (0.02%)

Data were collected and analyzed using Polymer Standards Service WINGPC Unity software.

Table 2: Prepared samples for the two GPC experiments.

		Long alkyd resin [g]	Laccase	Mediator
1st Experiment	Stirred for 6 hours	3		
		3		100 µL ABTS (10 mM in H_2O)
		3	100 µL *Th*L	100 µL ABTS (10 mM in H_2O)
		3	100 µL *Th*L	---
2nd Experiment	Stirred for 18 hours	3g alkyd resin / succinate buffer 1:2	100 µL *ThL*	---
		3g alkyd resin / succinate buffer 1:2	100 µL *ThL*	100 µL ABTS (10 mM in H_2O)
		3g alkyd resin / succinate buffer 1:2	100 µL *ThL*	100 µL HBT (10 mM in H_2O)
		3g alkyd resin / succinate buffer 1:2	100 µL *ThL*	100 µL TEMPO (50 mM in Ethanol)
		3g alkyd resin / succinate buffer 1:2	100 µL *ThL*	100 µL Syringaldazine (10 mM in DMF)
		3g alkyd resin / succinate buffer 1:2	100 µL *ThL*	100 µL Syringic Acid (10 mM in H_2O)
		3g	---	Cobalt 200 ppm

2.4 Optimization of the long alkyd resin

The formulation of the tested long alkyd resin contains about 20 different compounds. Three of them namely acticide (a biocide), tin(II)ethylhexanoate and triphenylphosphite were identified to be potential laccase activity inhibiting substances. Acticide consists of the two compounds 5-chloro-2-methyl-1,2-thiazol-3-one and 2-methyl-1,2-thiazol-3-one. The structures of these compounds are can be found in Figure 25.

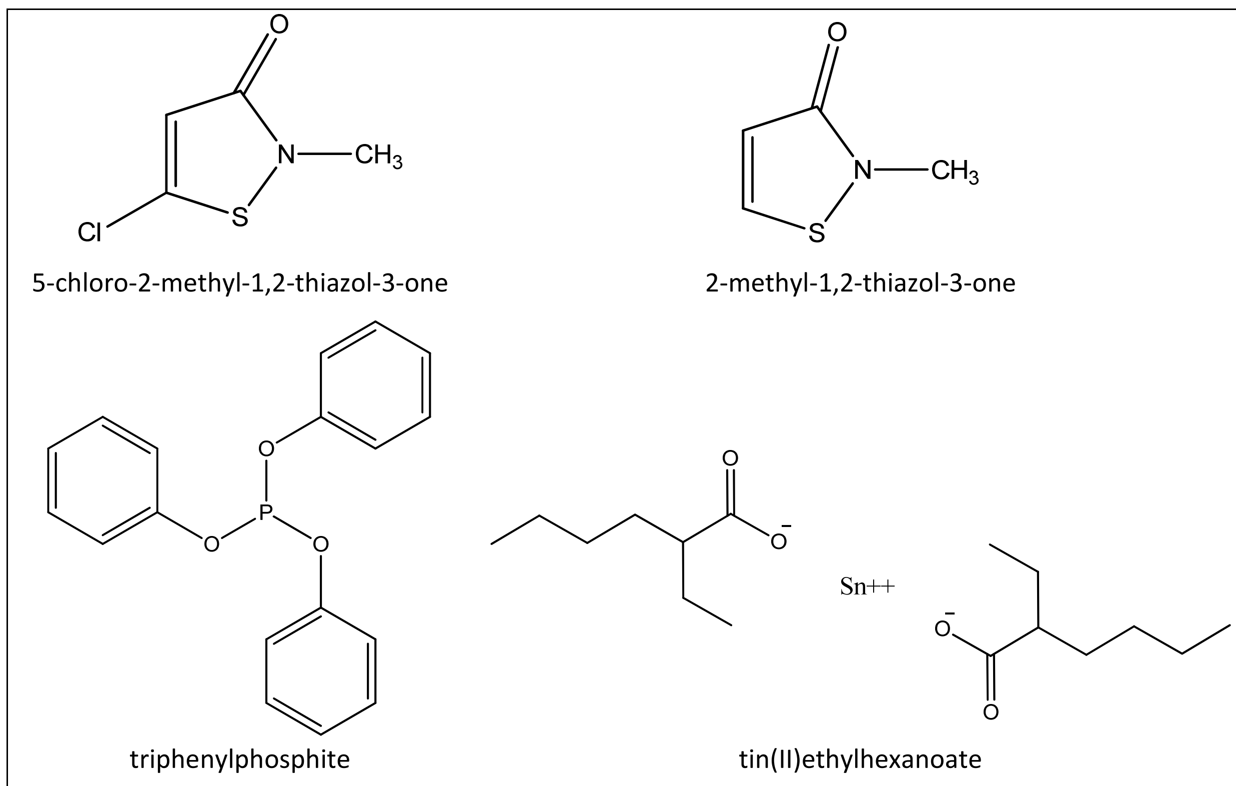


Figure 25: Structural formula of the potential laccase inhibitors5-chloro-2-methyl-1,2-thiazol-3-one, 2-methyl-1,2-thiazol-3-one, tin(II)ethylhexanoate and triphenyl phosphite.

To verify this assumption a laccase inhibition test was performed. In order mimic the conditions that happen in the alkyd resin the concentrations of the three compounds in the test were chosen as close to the concentration in the formulation as possible. Due to difficulties during the sample preparation it was not possible to reach the exact concentration of each compound. The needed amounts are very low and since the compounds are very viscous it was not possible to weight out exactly the required quantity.

2.4.1 Laccase inhibition tests

In a first test *Trametes hirsuta* laccase was diluted 1:10000 in 50 mM succinate buffer (pH 4.5) and acticide (A), triphenylphosphite (TPP) or tin(II)ethylhexanoate (THE) were diluted with this solution until the final concentrations shown in Table 3were reached. Enzyme activity was determined at different times by following ABTS oxidation spectrophotometrically as described in chapter 2.1.1. Enzyme activity was calculated with equation 1 and expressed in units per milliliter (U/mL). Incubation of the samples was done at 125 rpm at a temperature of 25°C and 37°C respectively.

Table 3: Overview of the prepared samples of the laccase inhibition tests.

	Incubation at 25°C		Incubation at 37°C	
Sample name	**g of the pure compound**	**mL *Th*L solution**	**g of the pure compound**	**mL *Th*L solution**
Acticide (0.0778%)	0.01035	13.3	0.01347	17.31
Tin(II)hexanoate (0.0048%)	0.00194	40.4	0.00154	32.08
Triphenylphosphite (0.0228%)	0.00346	15.8	0.01048	45.96
Control	---	30.0	---	30.0

In order to see if a combination of the three compounds shows different behavior regarding to laccase inhibition an additional test with a mixture of all three compounds was performed. A 1:5000.25 dilution of *Trametes hirsuta* Laccase in 50 mM succinate buffer (pH 4.5) was prepared and added to the amount of acticide, tin(II)hexanoate and triphenyl phosphite mentioned in Table 4. Enzyme activity was determined at different times by following ABTS oxidation spectrophotometrically as clarified in chapter 2.1.1. Samples were incubated at 125 rpm and a temperature of 25°C.

Table 4: Sample preparation for the additional laccase inhibition test. * Original concentration in long alkyd resin.

Sample name	added substance	[g]	Enzyme solution [ml]	Concentration [%]	Concentration [%] *
all 3 compounds	**A**	0.03242	41.67	0.0777	0.0778
	TEH	0.00321		0.0077	0.0048
	TPP	0.01157		0.0278	0.0228
Acticide	**A**	0.03148	40.46	0.0777	0.0778
Tin(II)ethylhexanoate	**TEH**	0.00275	35.71	0.0077	0.0048
Triphenyl phosphite	**TPP**	0.00992	35.68	0.0278	0.0228

2.5 Testing the drying performance

2.5.1 Tack free time analysis

As a first step drying performance was tested by tack free time analysis. The tack free time is defined as the time that is necessary to get a tack free surface where no stickiness can be observed under

moderate pressure [36,37]. In our case a surface was defined as tack free in case a dry finger did not leave behind any imprints and the film was not sticky anymore.

During the sample preparation an aqueous 20 mM HBT solution was prepared. 10 g of long alkyd resin were mixed together with 500 or 1000µL 20 mM HBT solution. 500 µL undiluted, 1:10, 1:100 and 1:1000 with 50 mM succinate buffer pH 4.5 diluted *Th*L solution was added respectively and the formulations were mixed properly with a spatula. Pure long alkyd resin was used as a standard. The samples were then applied on glass plates with a film thickness of 30 µm with a film applicator and incubated in an air draught free cupboard at room temperature. Every day the film was tested as mentioned above.

2.5.2 Drying time recorder measurements

Tack free time analysis is a fast and good first testing method but it is not automatable and the comparability between the results obtained by different operators is doubtful as the result of the investigation of the surface with the fingertip is a subjective value. For that reason drying performance was also studied by means of drying time recorder. The drying time recorder system consists of a glass strip on which the film is applied and a machine that moves a needle along this surface. As soon as the coating that needs to be tested is applied to the glass stripe the needle of the drying time recorder is placed on the strip and moves with a defined velocity along the coated glass plate. Since the time of application is known it is possible to draw a conclusion about the time that is needed to reach specific drying stages. Typical stages of drying that occur in films are shown in Figure 26.

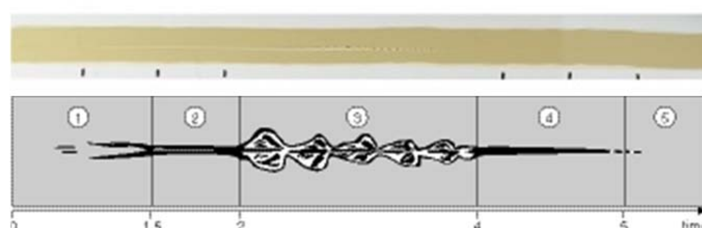


Figure 26: Stages of drying are 1) Leveling, 2) Basic trace, 3) Ripped film, 4) Surface trace, 5) Dry [38]

Vialkyd AY 6120 / 60% (pH 7) (from now on called medium alkyd resin) has a lower concentration of linseed oil (only 60%) in comparison to the model resin “long alkyd” which contains 65% linseed oil. The project partner Cytec Graz was also interested in the drying behavior of this medium alkyd resin. The

tested resins medium alkyd (pH 7) and long alkyd (pH 4) were mixed with *Th*L, 10 mM aqueous ABTS and 10 mM aqueous HBT solution respectively so that the concentrations mentioned in Table 5 were reached. The samples were then applied to elongated glass plates in a layer thickness of 76 µm and put into the drying time recorder. Measurements were performed at room temperature.

Table 5: Samples prepared for drying time recorder measurements. * dry weight.

	Control A	Cobalt A1	ABTS A2	ABTS A3	HBT A4	HBT A5
Medium alkyd resin (pH 7) [g]	25	25	25	25	25	25
Cobalt 12% emulsion		2000 ppm				
Laccase [U/g resin]*			9.4	9.4	9.4	9.4
ABTS 10 mM [mL]			1.25	0.75		
HBT 10 mM [mL]					1.25	0.75

	Control B	Cobalt B1	ABTS B2	ABTS B3	HBT B4	HBT B5
Long alkyd resin (pH 4) [g]	25	25	25	25	25	25
Cobalt 12% emulsion		2000 ppm				
Laccase [U/g resin]*			9.4	9.4	9.4	9.4
ABTS 10 mM [mL]			1.25	0.75		
HBT 10 mM [mL]					1.25	0.75

Since we found out that some components of the long alkyd resin are laccase activity inhibitors a new model alkyd resin (long alkyd resin (inhibitor-free)) without acticide, tin(II)ethylhexanoate and triphenyl phosphite was provided by Cytec Graz. To see if a progress in drying time could be achieved a new drying performance test was done. HBT treated samples showed better surface characteristics. Therefore there was a focus on the mediator HBT.

The pH of long alkyd resin (inhibitor-free) was adjusted to pH 4.5 using triethylamine. 23 g of the resin were put in a sarstedt flask and cobalt or 20 mM aqueous HBT solution and *Th*L were added until the concentration mentioned in Table 6 were reached. The samples were mixed properly with a spatula and the drying time was determined by means of drying time recorder as described above.

Samples were prepared at ACIB Graz and the drying time recorder tests were performed at Cytec Graz.

Table 6: Sample preparation for the drying test with long alkyd resin (inhibitor-free).* dry weight

	Control C	Cobalt C1	HBT C2	HBT C3	*Th*L C4
Long alkyd resin (inhibitor-free) (pH 4.5) [g]	23	23	23	23	23
Cobalt 12% emulsion		200 ppm			
***Trametes hirsuta* Laccase**			4 U / g resin*	4 U / g resin*	4 U / g resin*
HBT 20 mM [mL]			1.25	2	

2.6 Process monitoring via Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) shows a high potential to detect ongoing reactions due to the possibility to measure changes of the spectrum of a substance over time. FTIR spectroscopy is a method to monitor the drying reaction on a molecular level [4,39,40].

2.6.1 FTIR spectroscopy of LMS treated long alkyd resin (inhibitor free)

Long alkyd resin (inhibitor-free) was adjusted to pH 4.5 with triethylamine. For each sample 23 g of the resin were put in a sarstedt flask and 2 mL 20 mM aqueous HBT solution, 2 mL 20 mM aqueous ABTS solution were inserted respectively and then 1.25 mL *Th*L solution (51 U/mL) were added. As a positive control long alkyd resin (inhibitor-free)) with 200 ppm Cobalt was prepared. In addition to that pure long alkyd resin (inhibitor-free) and 23 g long alkyd resin (inhibitor-free) mixed with 1.25 mL *Th*L solution (51 U/mL) were set up as negative controls. All samples were mixed properly with a spatula and then applied to a CaF_2 crystal window with a film thickness of 30 µm. Measurements were performed over time on a FTIR Spectrometer Spectrum 100 from Perkin Elmer (10 scans per spectrum with a resolution of 4.00 cm^{-1}).

2.6.2 FTIR spectroscopy of Linseed oil

Linseed oil is one of the main compounds of the tested alkyd resins and it is the substance that should cross-link via the unsaturated fatty acid side chains. Therefore the behavior of linseed oil is of fundamental relevance and an extra test was carried out with linseed oil as model substrate.

Linseed oil was emulsified by shaking 0.5 g linseed oil, 10 mL 50 mM succinate buffer pH 4.5 and 0.05 g Tween 20 for 30 minutes on a vortex. Emulsion with and without cobalt siccative was applied to a CaF_2 crystal window and measurements were performed on a FTIR spectrometer as described above.

2.7 Process monitoring via Raman spectroscopy

Another very useful technique to follow oxidation reactions of model compounds is Raman spectroscopy. In comparison to FT-IR spectroscopy, Raman spectroscopy has advantages as double bonds show stronger Raman absorptions [39].

The hardening process of both long alkyd resin (inhibitor-free) and linseed oil were monitored via Raman spectroscopy. One drop of the samples of chapter 2.6.1 and 2.6.2 were applied to an aluminum foil rapped microscope slide. Since the intensity of the observed signals with a film thickness of 30 µm was very low this method was used to qualitatively asses the reaction.

2.8 Film properties

To get a better understanding of the processes involved in the enzymatic drying of alkyd resins specially regarding oxygen transfer across the film, enzyme diffusion and drying of the surface that take place in the film some additional analysis were done. Microscopic observations of the surface were made in order to learn more about these mentioned mechanisms.

2.8.1 Microscopic observations of the dried surface

For this purpose a drying test was performed with different concentrations of *Th*L and HBT. 10 g long alkyd resin were mixed with aqueous HBT solution and *Th*L solution as mentioned in Table 7. *Th*L was diluted with 50 mM succinate buffer pH 4.5 and in each case 500 µL were taken in order to get samples with a constant volume. Samples were applied to glass plates with the film applicator and the thickness of the wet films was kept at 30 µm. Samples were incubated in the dark at room temperature. Drying performance (tack free time) was checked every day. Additionally surface structure was documented by photos and microscope analysis where 10x magnification and TL-DIC contrast method were used.

Table 7: Samples for the microscopic observations of the dried surface.

Sample name	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Co 200 ppm	Blank
Long Alkyd resin [g]	10	10	10	10	10	10	10
20 mM HBT [µL]	500	500	500	500	1000	---	---
500 µL of *Th*L (51 U/mL), dilution	undiluted	1:10	1:100	1:1000	undiluted	---	---

2.8.2 Oxygen concentration in the film

A possible limiting factor of the whole reaction might be the limited oxygen diffusion. As noted in the introduction the oxidation reaction needs oxygen and therefore a restricted oxygen concentration in the film might lead to slower drying of the alkyd resin.

In order to measure the oxygen consumption in the film the alkyd resin was knife coated on an optical oxygen sensor film (film thickness 60 µm). The used sensor film was obtained as followed: 1 mg of platinum(II)-meso-Tetra(pentafluorophenyl)porphin (PtTFPP) and 100 mg of polystyrene (MW 250000) were dissolved in $CHCl_3$ and knife coated on a PET support to give, after solvent evaporation, a phosphorescent sensor film with a thickness of 20 µm. Phase shifts and subsequently oxygen concentration were measured with a phase-fluorimeter with the corresponding 2 mm optical fibre purchased from PreSens. The modulation frequency was set to be 4920 Hz.

After coating the resin with a film thickness of 60 µm on top of the sensor film oxygen concentration was measured every 30 sec at a single spot. A scheme of the test set-up is illustrated in Figure 27.

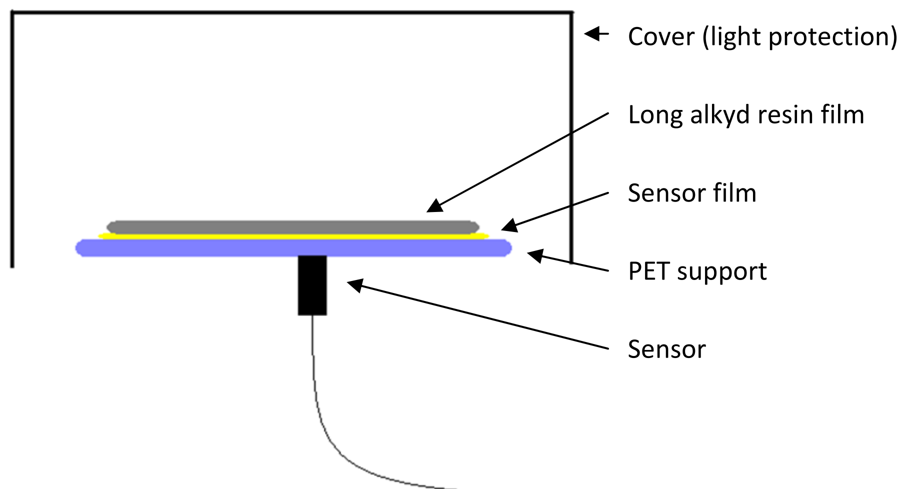


Figure 27: Set-up for the oxygen concentration measurement in films.

The measurement of the oxygen concentration in the alkyd resin film was performed at the Institute of Analytical Chemistry and Food Chemistry.

2.8.3 Fluorescence labeling of the Laccase

The aim of this experiment was to analyze the distribution of the laccase in the film and reveal if there are potential reaction limitations due to insufficient laccase diffusion or heterogeneous enzyme distribution. The idea of this experiment is to label the enzyme and study the spreading of the laccase via confocal laser scanning microscope (CLSM).

For this reason Rhodamine B solution with 0.0084 g Rhodamine B and 300 µL 50 mM potassium phosphate buffer pH 7 and NHS (N-Hydroxysuccinimide) solution with 0.2518 g NHS and 400 µL 50 mM potassium phosphate buffer pH 7 were prepared. 0.0172 g EDAC (N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide), 20 µLNHS solution and 250 µL Rhodamine B solution were incubated for 30 min at 25°C and mixed at the speed of 500 rpm in order to activate the fluorescent dye. Subsequently, 1 mL of *Th*L stock solution was added and incubation was proceeded for another 3 hours. As the fluorescent dye is UV sensitive Rhodamine B containing solutions were kept in the dark and the reaction vessel was rapped with aluminum foil.

Purification of the Rhodamine B labeled laccase was performed by means of FPLC. Therefore the sample was centrifuged for 10 minutes at 10000 rpm and room temperature to precipitate particles from the solution and then diluted 1:10 with 50 mM Succinate buffer pH 4.5. Separation of the samples was achieved with a size exclusion column (SEC, Superdex 75 10/300 GL). 200µL of the sample were applied on the column..Filtrated and degassed succinate buffer (50 mM, pH 4.5) was used as eluant at a flow rate of 1.0 mL/min and fractions of 0.5 mL were collected. Detection was realized at two different wavelengths - 280 nm (protein) and 552 nm (Rhodamine B). Prior wavelength scan identified the absorption maximum of Rhodamine B to be 552 nm.

One part of the obtained *Th*L - Rhodamine B solution was concentrated using a Vivaspin from Sartorius Stedim biotech with a molecular cut off of 30000 Da in order to enrich the remaining activity. 1 g long alkyd resin was mixed with 87 µL 20 mM HBT solution and 1 U of Rhodamine B-labeled *Th*L was added. The sample was applied to a microscope slide with a film thickness of 30 µm and measurements were performed at the confocal laser scanning microscope. The excitation wavelength was set to 532 nm and detection was done at 625 nm.

3 Results

3.1 Characteristics of the tested laccases

The three following laccases from *Trametes hirsuta* (*Th*L), *Trametes villosa* (*Tv*L) and *Myceliophthora thermophila* (*Mt*L) were characterize in regard to the pH optimum, kinetics, activity and protein concentration in order to find the most suitable laccase for the subsequent experiments.

3.1.1 Laccase activity

Laccase activities of the stock solutions of the three enzymes are summarized in Table 8. As the enzymes were stored in the fridge for a longer period and therefore a loss of activity is expected the activity was measured before experiments and always mentioned in the materials and methods chapter.

Table 8: Determined laccase activities.

Enzyme	Activity [U/mL]
Trametes hirsuta **laccase**	141.0 ± 2.2
Trametes villosa **laccase**	829.7 ± 8.5
Myceliophthora thermophila **laccase**	228.6 ± 0.7

3.1.2 Kinetic parameters

The software Origin (Version 7.5) was used to plot the measured activities and the kinetic parameters K_m and v_{max} were determined by solving the Michaelis Menten equation 2 and are shown in Figure 28, Figure 29 and Figure 30.

Equation 2

$$vo = \frac{vmax * [S]}{Km + [S]}$$

v_0: current reaction rate or volumetric enzyme activity [U/mL]
v_{max}: maximum reaction rate [U/mL]
[S]: substrate concentration [mM]
K_m: Michaelis constant [mM]

Table 9: Kinetic parameters K_m and v_{max} of *Th*L, *Tv*L and *Mt*L.

Enzyme	K_m [mM]	v_{max} [U/mL]
Trametes hirsuta **laccase**	0.025 ± 0.002	164.3 ± 3.7
Trametes villosa **laccase**	0.024 ± 0.004	776.6 ± 29.3
Myceliophthora thermophila **laccase**	0.287 ± 0.010	258.1 ± 2.6

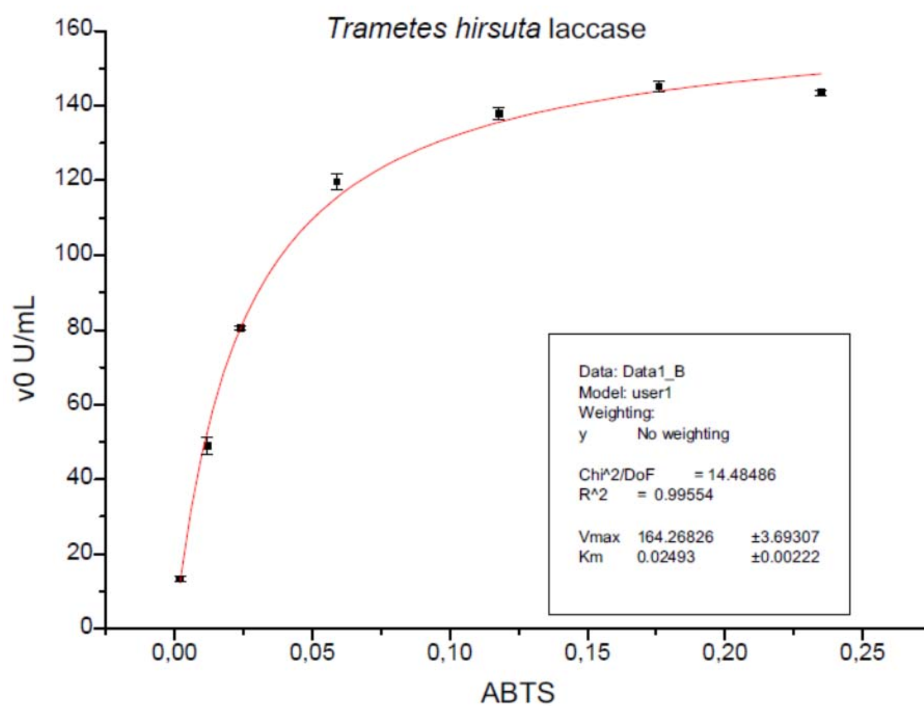


Figure 28: Determination of the kinetic parameters of *Th*L.

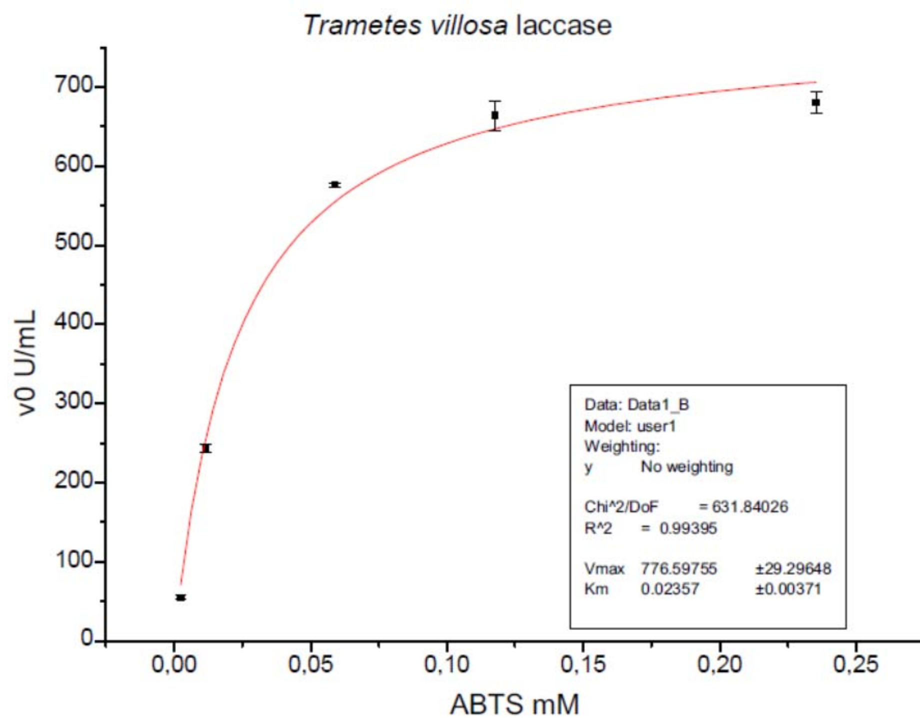


Figure 29: Determination of the kinetic parameters of *Tv*L.

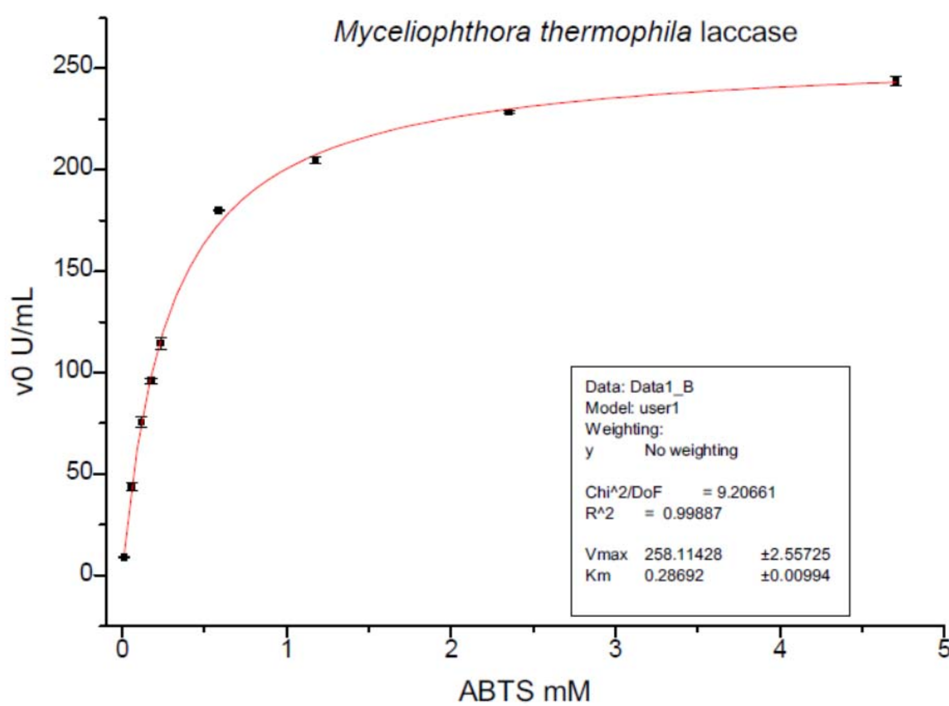


Figure 30: Determination of the kinetic parameters of *Mt*L.

The Michaelis constant K_m is defined as the substrate concentration at which the reaction catalyzed by an enzyme reaches the half of its maximum velocity. Under specific conditions of temperature, pH, ionic strength, etc. the Michaelis constant is a characteristic parameter for an enzyme and one substrate. A low K_m value means that the affinity of the enzyme to the tested substrate is high and vice versa. This means that the higher the Michaelis constant is the more substrate is needed to reach the maximum conversion rate. Therefore K_m and v_{max} are parameters that make a comparison between more or less suitable substrates/enzymes possible [41].The three tested enzymes have quite different v_{max} and K_m values (see Table 9). *Tv*L and *Th*L show almost the same K_m value for the tested substrate ABTS but v_{max} of *Tv*L is much higher than the one of *Th*L. This means that the conversion of ABTS by *Tv*L happens faster than by *Th*L or *Mt*L. Compared to the other two laccases *Mt*L has the highest K_m value and as a consequence the lowest affinity to ABTS.

3.1.3 pH optimum

pH optimum measurements with ABTS as substrate revealed that all three tested laccases have a different pH optimum and pH range in which the enzyme is active. The optimum pH range is an important factor because the pH of different alkyd resin varies a lot and although it can be adjusted to a certain pH there is always the possibility that pH changes occur during long time storage. Therefore a laccase with a wider pH range is definitely an advantage. The three tested laccases cover already a broad pH spectrum from lower than pH 3 up to at least pH 8.

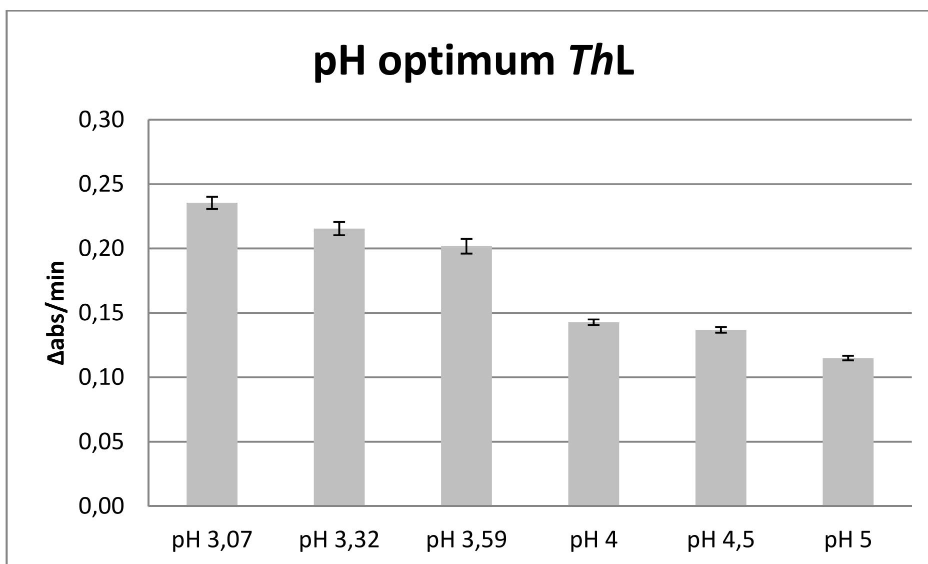


Figure 31: pH range of *Th*L.

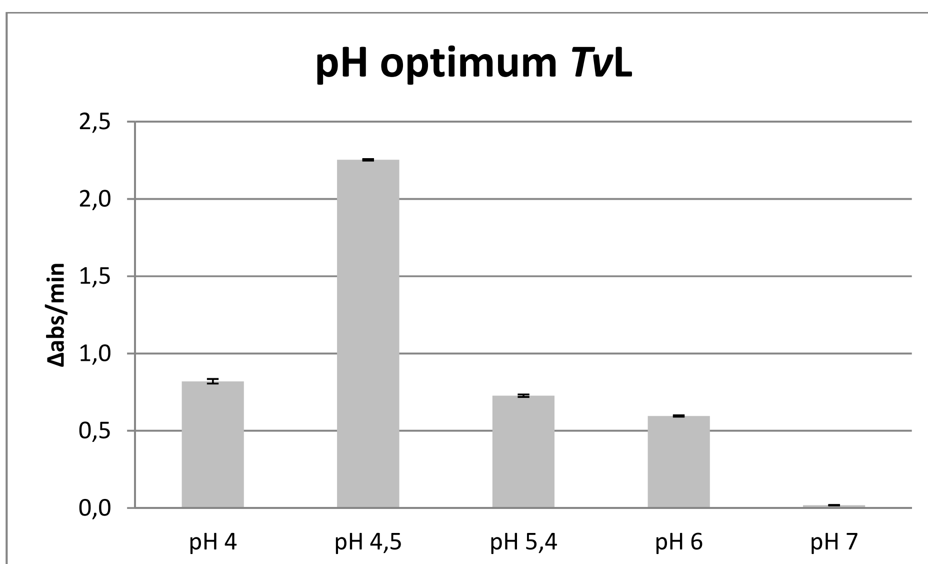


Figure 32: pH range of *Tv*L.

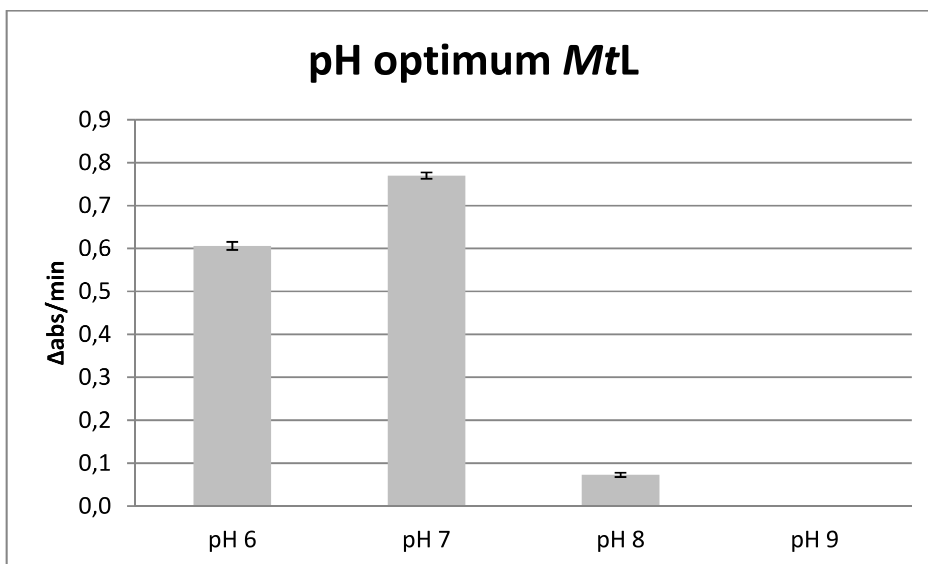


Figure 33: pH range of *Mt*L.

3.1.4 Protein content of the laccase solutions

To determine the protein concentration of the three laccase solutions a calibration was done with BSA in a concentration from 10 to 500 µg/mL. The protein concentration was calculated by using the equation mentioned in Figure 34.

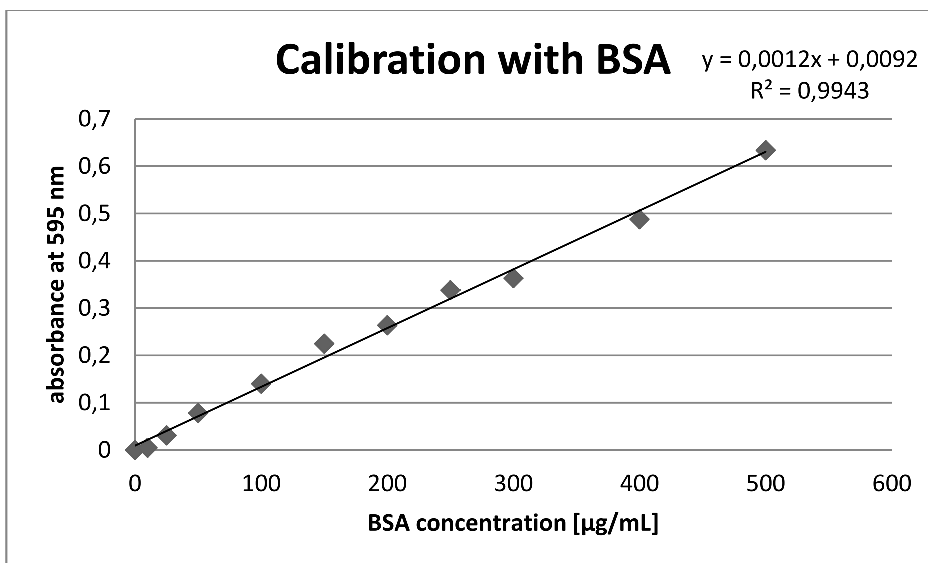


Figure 34: Calibration curve for determination of protein concentration of the stock solutions of *Th*L, *Tv*L and *Mt*L.

Total protein content of the laccase solutions was calculated by using equation 3, which correlates the absorbance at a wavelength of 595 nm with the protein concentration (µg protein per mL solution).

Equation 3

$$protein\ concentration\ \left[\frac{\mu g}{mL}\right] = \frac{abs[595\ nm] - 0.009}{0.001}$$

Table 10: Identified protein concentrations of the laccase stock solutions.

Enzyme	Protein concentration [µg/mL]
Trametes hirsuta **laccase**	3280 ± 380
Trametes villosa **laccase**	12550 ± 150
Myceliophthora thermophila **laccase**	18570 ± 130

*Th*L has the lowest protein content, which explains the lower activity. As the *Th*L was produced at the institute and the obtained protein solution from the batch was not concentrated, this result was expected.

3.1.5 SDS-PAGE analysis

SDS-PAGE analysis was performed to determine the size and purity of the protein solutions. During the denaturation step the enzymes gets linearized and SDS binds to the amino acids so that the protein has a uniform charge. As a result, the conformation of the protein does not have an effect to the movement of the protein in the electric field and the velocity of the sample in the electric field depends in the first place on the size. Figure 35shows the stained SDS-PAGE gel with the three tested laccases.

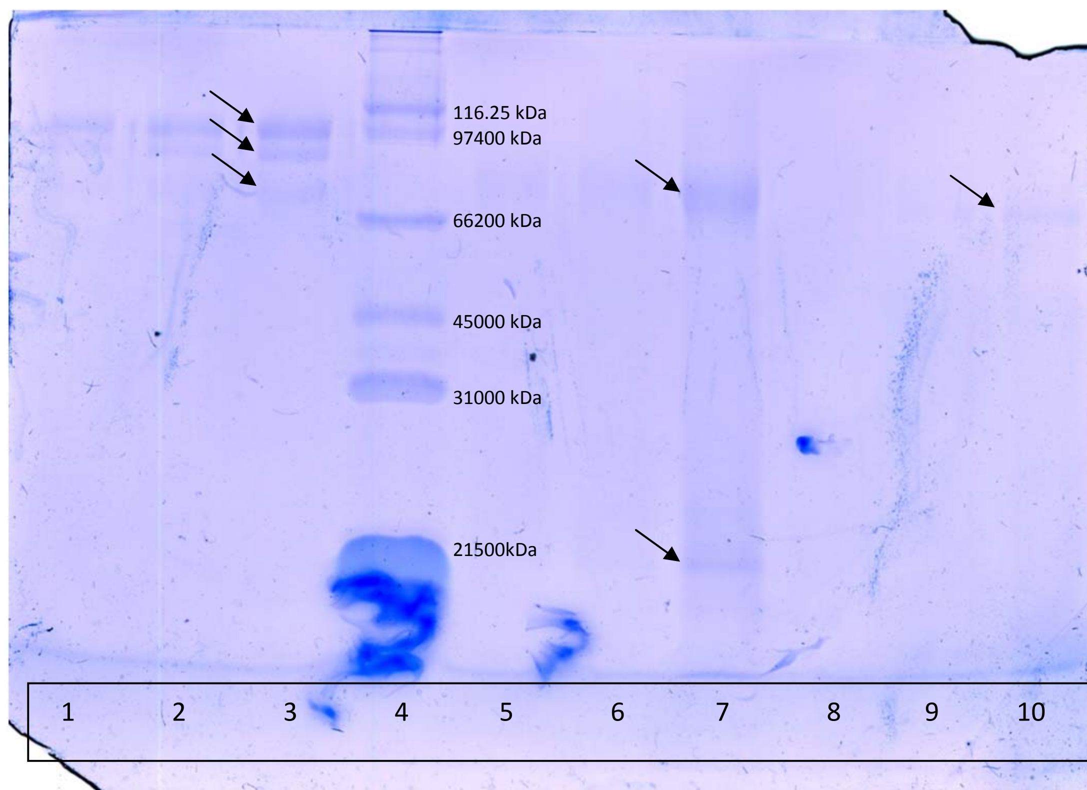


Figure 35: SDS-PAGE gel stained with Coomassie Blue solution. 1) Myceliophthora *thermophila* Laccase 1:100, 2) Myceliophthora *thermophila* Laccase 1:70, 3) Myceliophthora *thermophila* Laccase 1:50, 4) Standard, 5) Trametes *villosa* Laccase 1:50, 6) Trametes *villosa* Laccase 1:30, 7) Trametes *villosa* Laccase 1:10, 8) Trametes *hirsuta* Laccase 1:30, 9) Trametes *hirsuta* Laccase 1:10, 10) Trametes *hirsuta* Laccase 1:5.

Myceliophthora thermophila laccase shows two bands at about 97 kDa and 90 kDa and a weaker band at approximately 75 kDa. In the *Trametes villosa* laccase sample a smear along the whole lane can be observed. Additionally two bands can be seen at roughly 70 and 21 kDa. A similar situation was found in the case of *Trametes hirsuta* laccase where a smear along the lane was found and only one very weak band exists at about 66 kDa. The smear in the case of *Th*L and *Tv*L indicates that the laccase solutions are not purified and/or impurities are still in the sample. Another factor are proteases that might be in the solution in low concentrations and bit by bit cut the laccases into smaller pieces. This explains smear above all in lower molecular weight regions of the gel. Smear of higher molecular weight might be caused by protein aggregates that are formed during the storage of the solutions. One indication for aggregated proteins is the fact that especially the *Th*L solution formed pellets during centrifugation, that contains besides impurities possibly denaturized and aggregated protein.

3.1.6 Native Gel Electrophoresis (PAGE)

In contrast to SDS-PAGE proteins are not denatured in native gel electrophoresis. This means that the movement of the samples on the gel depends on the mass to charge ration and the conformation of the protein. Therefore is it obvious that the band patterns of one sample look differently in SDS and native gels. The aim of this experiment was to detect bands that show laccase activity and to see if there is also activity in areas with smear.

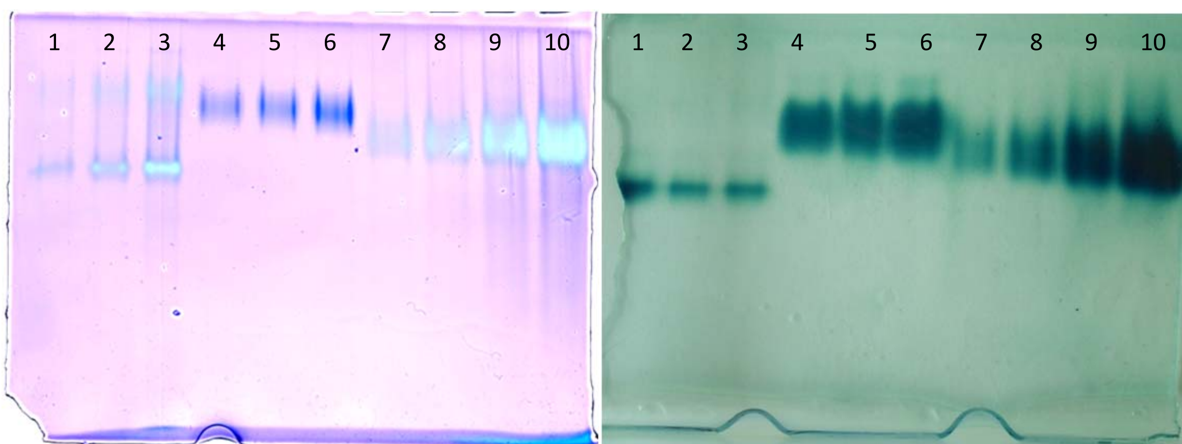


Figure 36: Native PAGE gels. The gel on the left side was stained with Coomassie Brilliant Blue R-250 and the gel on the right side was stained with ABTS solution. 1) Trametes *hirsuta* Laccase 1:20, 2) Trametes *hirsuta* Laccase 1:10, 3) Trametes *hirsuta* Laccase 1:5, 4) Myceliophthora *thermophila* Laccase 1:150, 5) Myceliophthora *thermophila* Laccase 1:100, 6) Myceliophthora *thermophila* Laccase 1:50, 7) Trametes *villosa* Laccase 1:100, 8) Trametes *villosa* Laccase 1:50, 9) Trametes *villosa* Laccase 1:20, 10) Trametes *villosa* Laccase 1:10.

As can be seen in Figure 36the gel on the left hand side was stained with Coomassie Brilliant Blue R-250 so that the entire protein on the gel was identified. The gel on the right hand side was put into an ABTS solution so that only those bands that contain active laccase turned green due to oxidation of the ABTS. *Th*L (line 1 to 3) exhibits a long smear with two prominent bands but only one of the two shows laccase activity. *Mt*L has only one band with laccase activity and *Tv*L sample displays a long smear and one major band with laccase activity.

3.1.7 Analysis via Fast protein liquid chromatography (FPLC) - ÄKTA

The measurements of the three samples were performed with the aid of fast protein liquid chromatography and confirmed the results from the gel electrophoresis. Especially *Th*L and *Tv*L have a high content of impurities which can be seen on the chromatogram as a very broad peak eluting from 7 to around 20 mL. The chromatogram of *Mt*L is very interesting since to peaks one eluting after 9.76 mL and one after 16.96 mL can be seen. Just like native gel electrophoresis, FPLC is a method were the enzyme stays in its active (native form). Therefore it is not clear why *Mt*L showed only one band at the native electrophoresis gel but two peaks after FPLC.

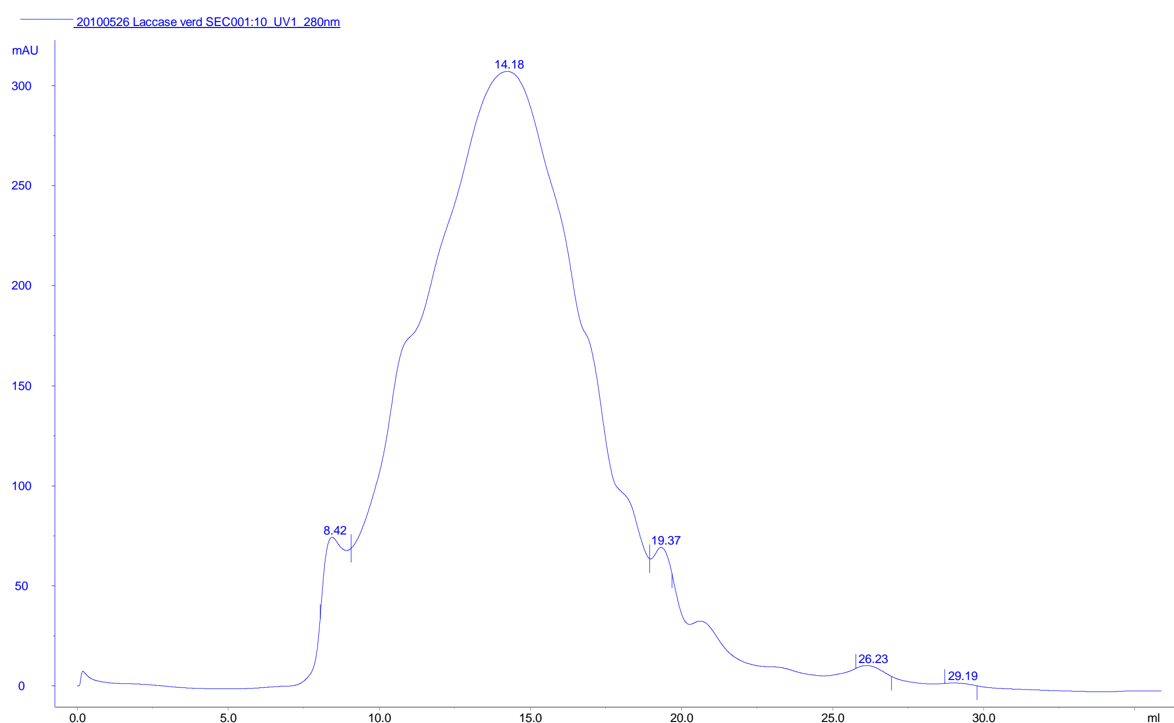


Figure 37: Chromatogram of *Th*L.

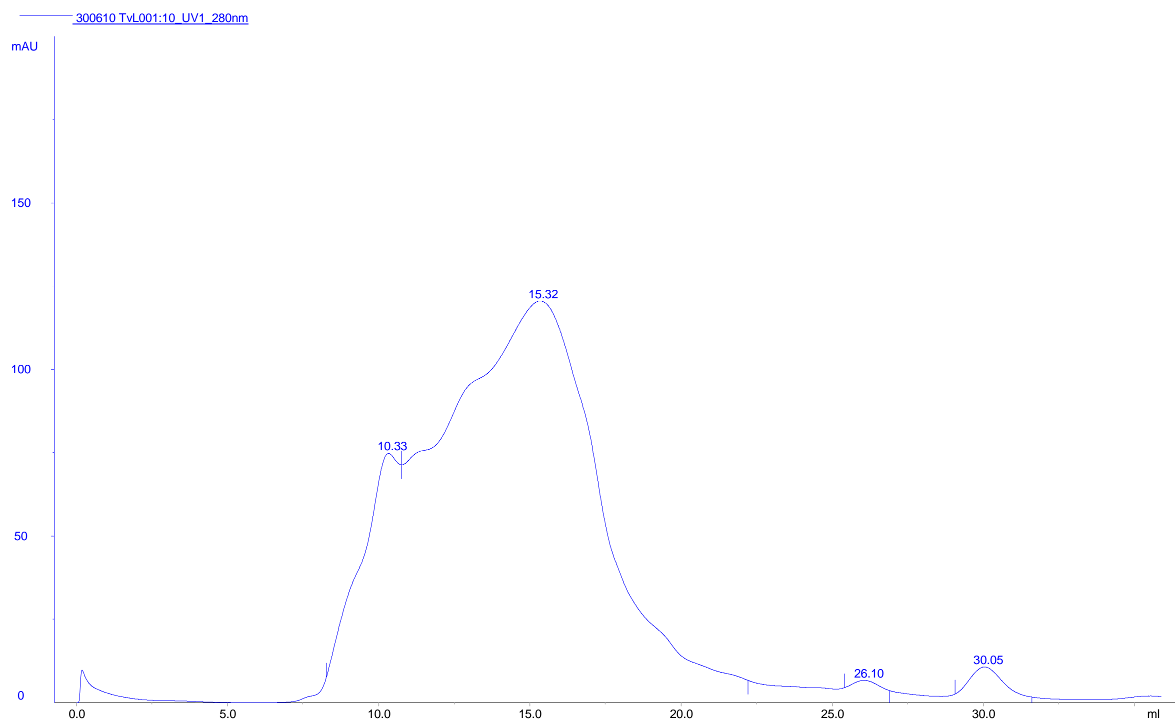


Figure 38: Chromatogram of *Tv*L.

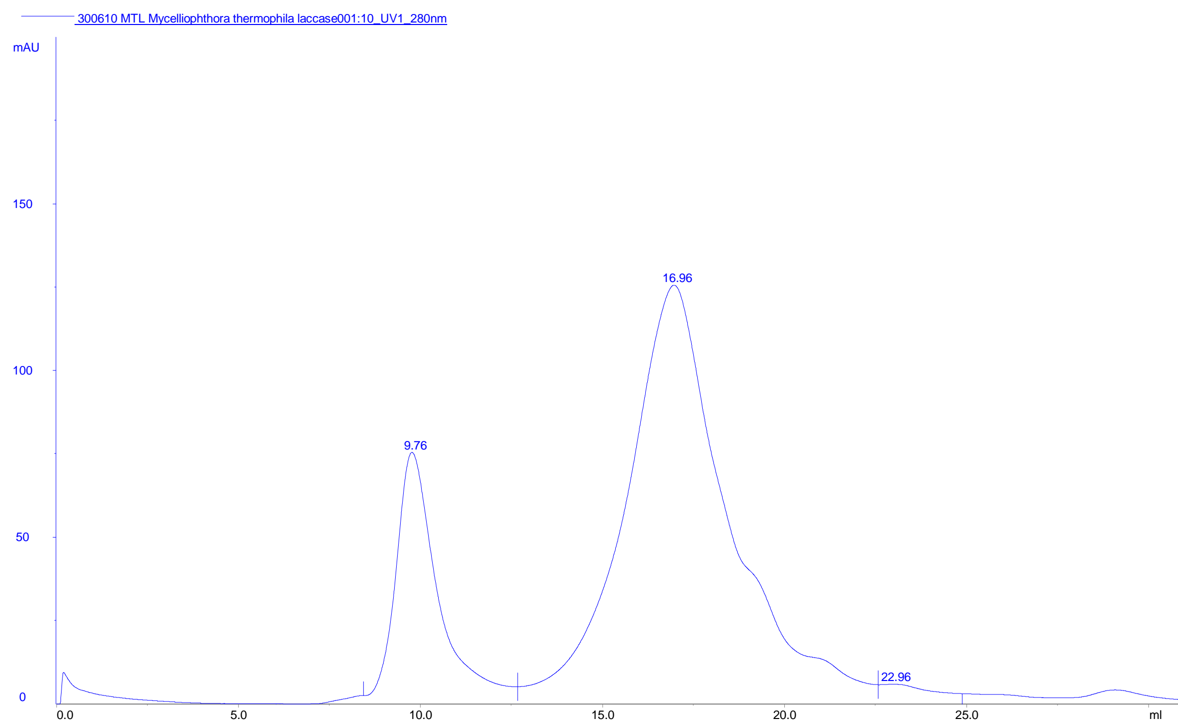


Figure 39: Chromatogram of *Mt*L.

3.1.8 Results of enzyme characterization

After analysis of all tested parameters and characteristics of the three enzymes, *Trametes hirsuta* laccase was chosen for further experiments because this laccase shows high enzyme activity especially in a lower pH range as it is required for the model resin and high stability also during rough conditions like higher temperatures or long time storage [14]. The other two enzymes might be used for future experiments especially if an enzyme with a pH range around 7 is needed *Myceliophthora thermophila* laccase might be the enzyme of choice. Hollmann et al. [42] investigated *Mt*L and observed more than 75% of the maximal activity in the range of pH 5 to pH 7.5.

3.2 Measurement of the oxygen consumption - mediator screening

A typical oxygen consumption curve obtained with the oxygen electrode is shown in Figure 40.

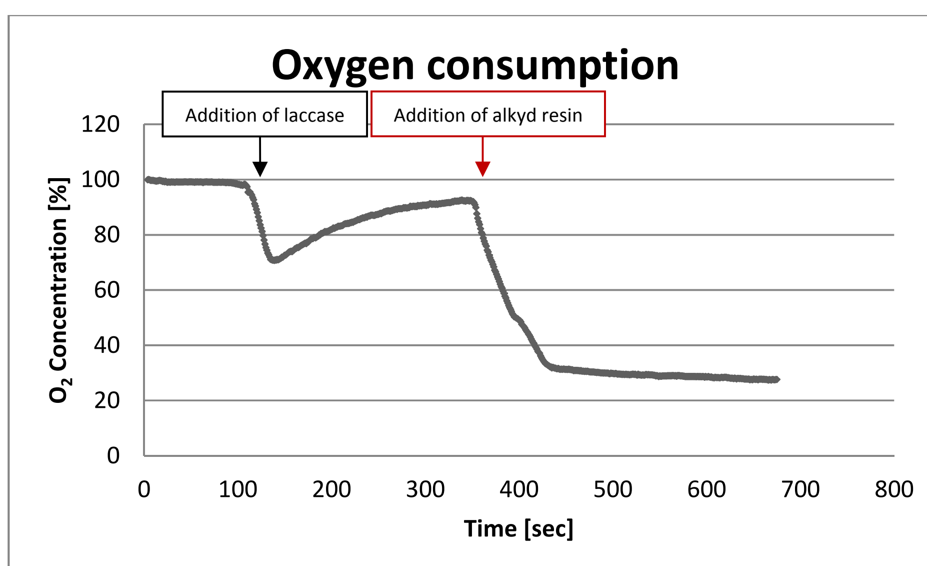


Figure 40: Typical oxygen consumption curve after the addition of *Th*L and long alkyd resin.

It can be clearly seen that as soon as the *Trametes hirsuta* laccase is added to the buffer-ABTS solution (black arrow) the enzyme starts to oxidize the ABTS molecules and the first O_2 decrease can be seen. After the complete oxidation of ABTS the oxygen concentration in the sample recovers since the experimental set-up is an open system. The red arrow indicates the addition of 250 µL long alkyd resin. Again a significant decrease in oxygen concentration is measured.

Similar results were obtained with the fiber-optic oxygen microsensor. Both methods were used to screen for the ability of potential mediators to oxidize the buffer diluted alkyd resin (Figure 41) and to measure oxygen consumption of different substrates (Figure 42).

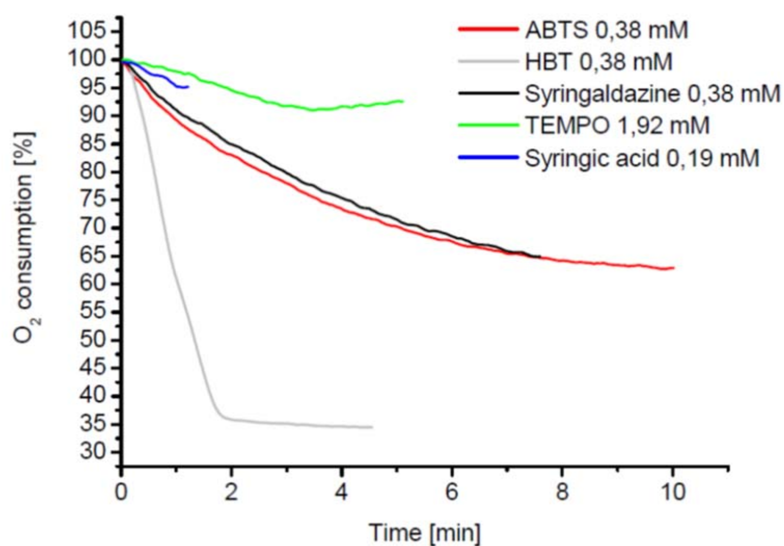


Figure 41: Oxygen consumption during the screening for appropriate laccase mediators.

Figure 41 shows oxygen consumption after addition of different potential mediators to the buffer diluted alkyd resin. Only the second decrease of oxygen concentration after addition of alkyd resin is given. HBT seems to have the highest ability to oxidize alkyd resin but also ABTS and syringaldazine show a promising decrease in oxygen concentration.

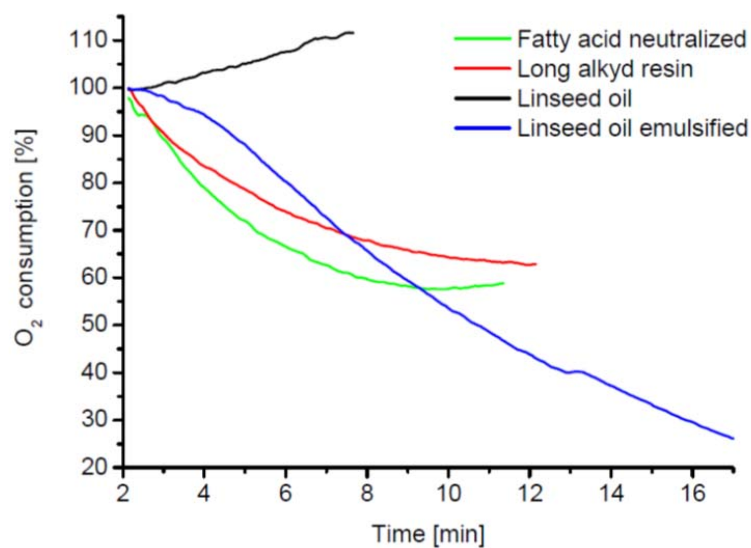


Figure 42: Oxygen consumption of different substrates after laccase / ABTS treatment.

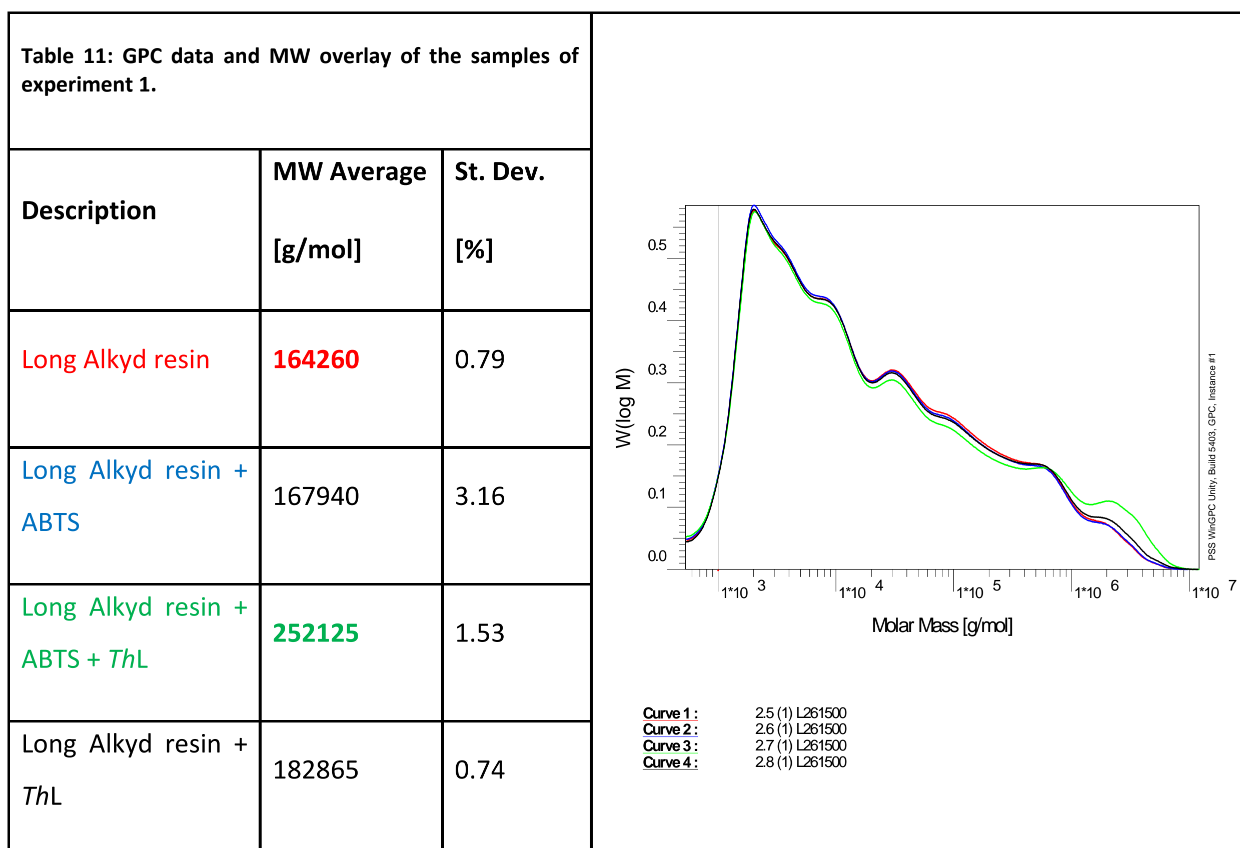
In Figure 42 again only the second decrease of oxygen concentration is given. It is clearly shown that oxygen is consumed only if the substrate is dissolved in water like in the case of neutralized linseed oil fatty acids (fatty acid neutralized, green) or in presence of a surfactant like in the cases of the long alkyd resin which contains an emulsifier in the formula or linseed oil emulsified which is a sample that contained 9% emulsifier, 56% linseed oil and 35% water.

3.3 Gel permeation chromatography– molecular weight increase

In order to quantify the molecular weight (MW) increase of mediator/enzyme treated alkyd resin gel permeation chromatography (GPC) measurement was performed.

Table 11: GPC data and MW overlay of the samples of experiment 1.

Description	**MW Average [g/mol]**	**St. Dev. [%]**
Long Alkyd resin	**164260**	0.79
Long Alkyd resin + ABTS	167940	3.16
Long Alkyd resin + ABTS + *Th*L	**252125**	1.53
Long Alkyd resin + *Th*L	182865	0.74



GPC was used to characterize the molecular weight increase of the enzyme/mediator treated samples.

A clear increase in the molecular weight was measured with GPC in sample "Long Alkyd resin + ABTS + *Th*L" from **164260** g/mol to **252125** g/mol compared with the blank which proves the effective enzymatic cross-linking of alkyd resins with a mediator/laccase system.

Results of Experiment 2 where long alkyd resin was treated with *Th*L and different laccase mediators are given in Table 12. Prior GPC analysis, samples were filtered in order to remove high molecular weight

compounds that could block the column. Therefore in addition to the increase of MW the formation of precipitates in the analyzed samples was considered to be indicative of a possible cross-linking reaction.

Table 12: Results from the GPC measurements and noticed formation of precipitates. Long alkyd resin was used as a blank and tested with different *Th*L/mediator combinations.

Drying system	Formation of precipitates	Mw compared to blank
Blank	normal	---
***Th*L/ABTS**	**strong**	**higher MW**
***Th*L/HBT**	**strong**	**lowest MW**
***Th*L/TEMPO**	strong	highest MW
***Th*L/Syringaldazine**	normal	lower MW
***Th*L/Syringic acid**	normal	lower MW
Cobalt (200 ppm)	**very strong**	**No change of MW**

It can be observed that all presented samples show different unusual behavior during the sample preparation step in particular increased filtering resistance that indicates formation of precipitates. GPC exhibited a significant increase of molecular weight for the tested ABTS and TEMPO samples indicating a certain degree of cross-linking. Nevertheless the positive control (cobalt 200 ppm) did not show a change in MW. Most likely a possible loss of high mass portions during the filtering step cannot be excluded and there is an uncertainty whether the real MW distribution of the samples can be detected, when the samples are filtered. An alternative method for molecular weight increase measurement would be needed. Possible alternative methods for MW detection would be MALDI-TOF or Zetasizer measurement. Nevertheless if a higher MW is detected there is an increase of MW, but the problem arises since there is no MW change and it is known to cross-link like in the case of cobalt treated long alkyd resin.

3.4 Optimization of the alkyd resin – Laccase inhibition tests

For the better comprehensibility two types of charts were chosen: In Figure 43 and Figure 45 the overall activity of all tested solutions is plotted and Figure 44 and Figure 46 show a bar graph with the remaining activity that is charted in relation to the control. The data from the first test show clearly that all three compounds have an inhibiting effect on laccase activity. Especially acticide lowers the activity of the enzyme over time significantly. During the incubation at 37°C the loss of activity of the control and the samples was generally higher than the one of the samples incubated at 25°C.

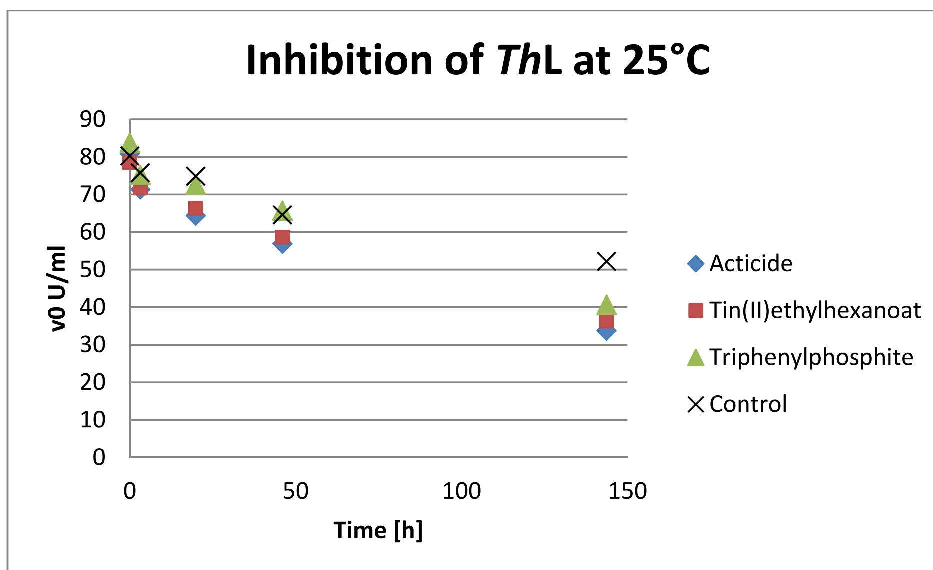


Figure 43: Overall activity of the tested solutions that have been incubated at 25°C.

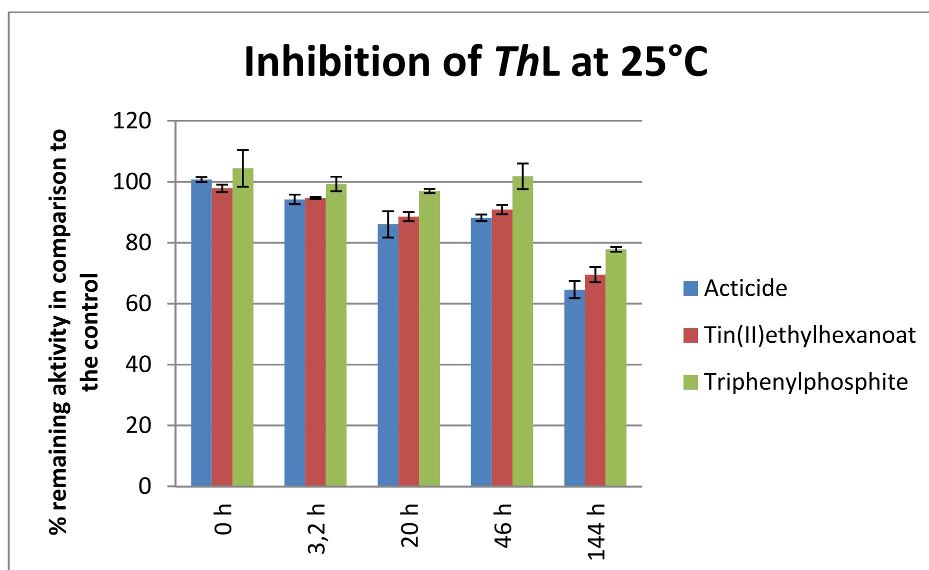


Figure 44: Bar graph with the remaining activity after incubation at 25°C that is charted in relation to the control.

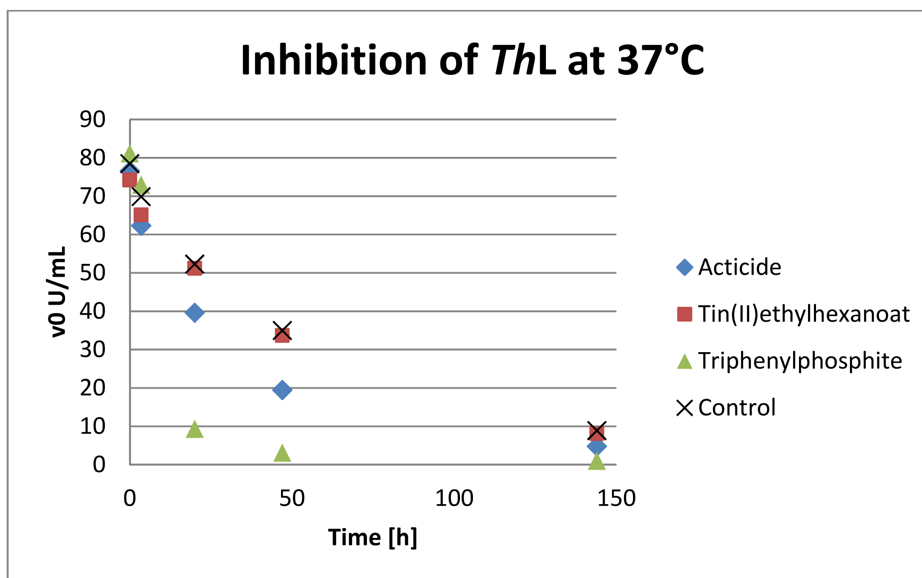


Figure 45: Overall activity of the tested solutions that have been incubated at 37°C.

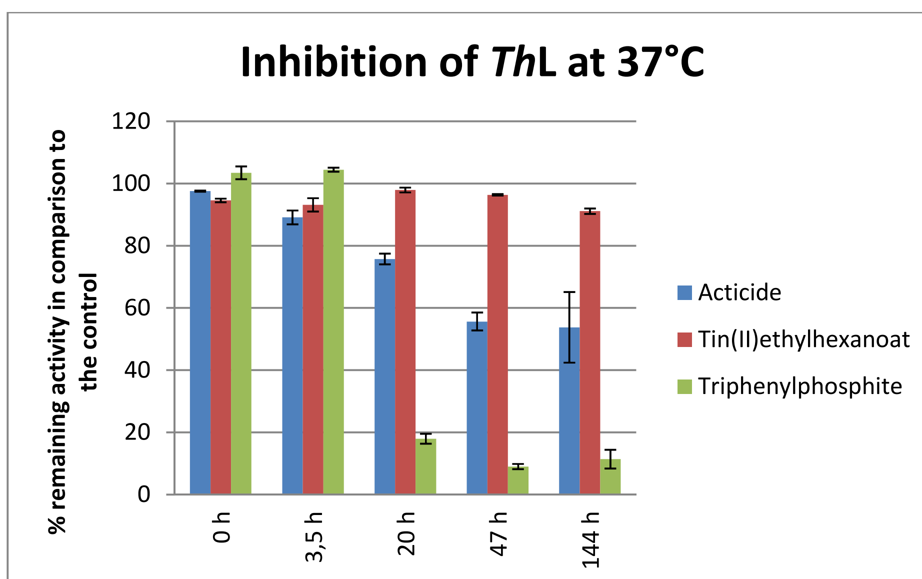


Figure 46: Bar graph with the remaining activity after incubation at 37°C that is charted in relation to the control.

The second test was carried out to monitor if a combination of all three compounds (as they are found in the alkyd resin) exhibits different behavior concerning laccase inhibition as the single chemical solution.

Already the very first activity measurement revealed that a combination of acticide, tin(II)ethylhexanoate and triphenyl phosphite leads to a prominent loss of activity in comparison to both the control and the single component tests (see Figure 47).

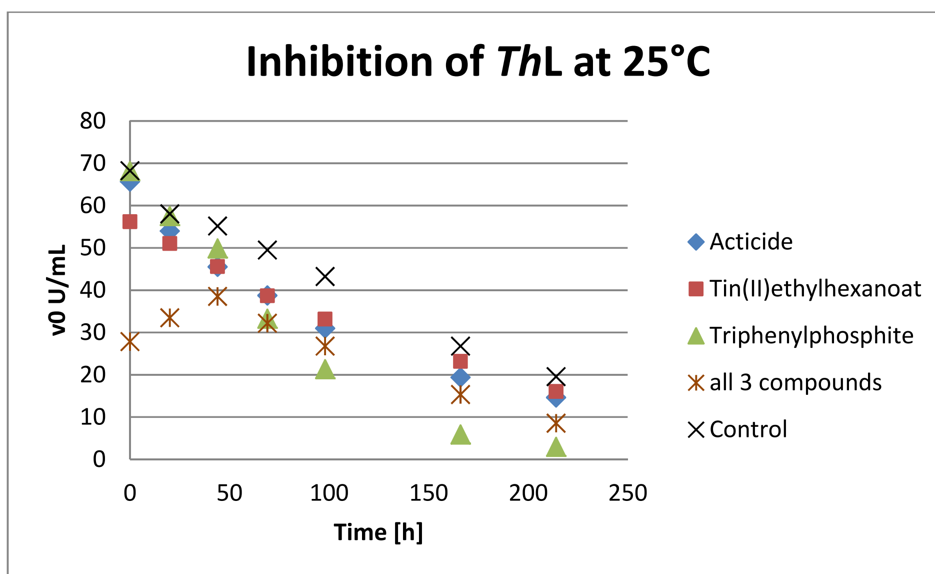


Figure 47: Overall laccase activity in the presence of all three compounds. Samples were incubated at 25°C.

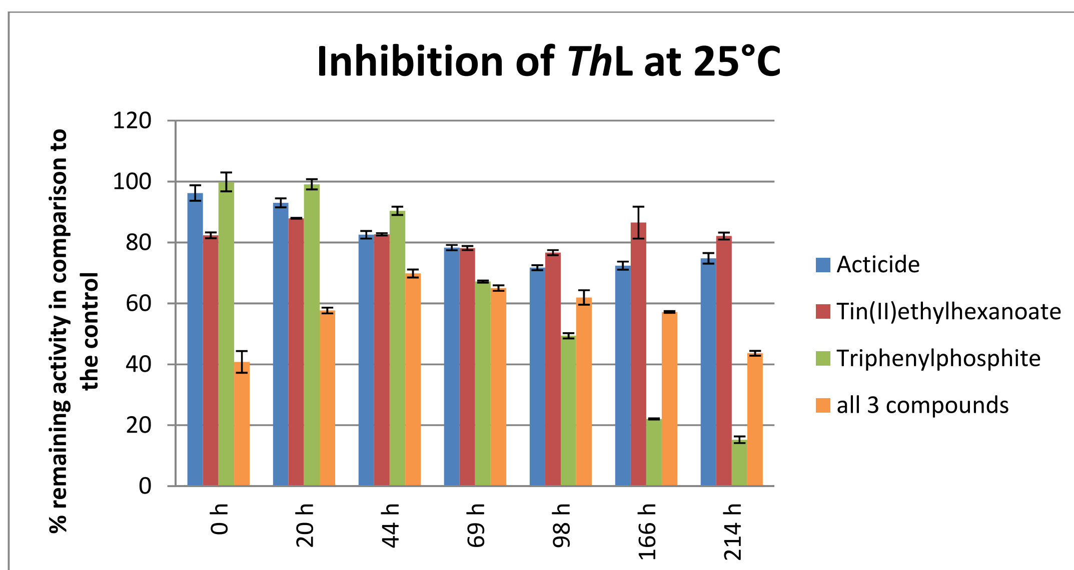


Figure 48: Laccase activity charted in relation to the control. Samples were incubated at 25°C.

An interesting point is that the activity of the “all 3 compounds” sample in contrary to expectations increased again during the first 44 hours and decreased once more until the final measurement. The reason for this behavior is not fully understood. One possible explanation is that the three chemicals interfere with each other in a way that parts of at least one compound precipitate. This would lead to a lower concentration in the solution and parts of the previously inhibited enzyme are active again. If this is the case the present inhibition can be called a reversible one. An indication for the mentioned theory is that after two days of incubation a yellowish precipitate was formed.

The activity tests showed that all three tested compounds provoke a significant decrease in laccase activity over time in comparison with the control. Consequently it was suggested to our partner Cytec Graz to produce a model resin that does not contain these three compounds to avoid adverse effects on drying performance due to enzyme inactivation during further experiments.

This important learning has to be considered when formulating alkyd resins containing laccases as drying agents. New laccase compatible biocides would need to be assessed in future formulas.

3.5 Drying performance

3.5.1 Tack free time analysis

Table 13: Results from the tack free time analysis. * adhesiveness after 7 days of incubation: -- very sticky, - sticky, ~ medium, + little sticky, ++ tack free.

Sample name	1	2	3	4	5	6	7	8	Blank
Sample description	10 g alkyd resin, 500 µL HBT 20 mM, 500 µL ThL	10 g alkyd resin, 500 µL HBT 20 mM, 500 µL ThL 1:10	10 g alkyd resin, 500 µL HBT 20 mM, 500 µL ThL 1:100	10 g alkyd resin, 500 µL HBT 20 mM, 500 µL ThL 1:1000	10g alkyd resin , 1000 µL HBT 20 mM, 500 µL ThL	10 g alkyd resin , 1000 µL HBT 20 mM, 500 µL ThL 1:10	10 g alkyd resin , 1000 µL HBT 20 mM, 500 µL ThL 1:100	10g alkyd resin , 1000 µL HBT 20 mM 500 µL ThL 1:1000	Only alkyd resin
Incubation time	**Observations and remarks**								
10 h	No changes observed								
1 day	No significant changes, very sticky								
2 days	very small rips appear, sticky	sticky	sticky	sticky	Rips bigger than those of sample 1 appear, sticky	sticky	sticky	sticky	very sticky
3 days	More rips appear, sticky	Samples 2-4 are stickier than samples 5-8			Rips all over the surface, bigger than those of sample 1, sticky	Stickier than sample 5	Stickier than sample 6	Stickier than sample 7	Very sticky
4 days	Even more rips appear, sticky	Some rips appear, stickier than 1	1 very small rip	sticky	Many big rips, sticky	sticky	1 rip, sticky	sticky	very sticky
7 days	Many fine rips	Some fine rips	1 rip	No rips	Many big rips	No rips	1 rip	No rips	No rips
adhesiveness*	-	-	~	-	+	~	-	-	--

The tack free time analysis revealed that HBT concentration as well as laccase concentration has an impact on the tack free time and therefore the velocity of the hardening process. After one week of incubation all tested samples were analyzed a last time to see if they are tack free or not. Apparently higher Laccase and mediator concentrations have a positive impact on the drying performance. One reason for that might be that a sufficient diffusion of the two compounds is possible in the film and therefore a higher concentration is needed to overcome this problem. Another important point is the laccase mediator distribution during sample preparation. As it is known from cobalt samples, sample preparation needs to be done at least 24 h before the application to ensure an optimal cobalt distribution in the resin and a following efficient hardening of the resin.

3.5.2 Drying time recorder measurements

Drying time recorder measurements revealed that no hardening of medium alkyd resin with the laccase mediator system is possible (see Table 14, samples A to A5). One reason for that result might be that the pH of medium alkyd resin (pH 7) was too high for *Th*L. The industrial partner Cytec Graz preferred to focus on long alkyd resin and therefore no further experiments were performed with this coating.

In contrast to that it was possible to cure long alkyd resin with the laccase mediator system (see Table 14, samples B – B5). Sample B2 treated with ThL and ABTS was tack free after 72 hours.

Table 14: Results from drying time recorder measurements. Long alkyd resin was applied with a film thickness of 76 µm wet film.

Sample name	leveling	basic trace	ripped film	surface trace	dry	Tack free
control A	---	---	---	---	> 24 h	---
Co / A 1	50 min	---	---	600 min	650 min	---
ABTS / A 2	---	---	---	---	> 24 h	---
ABTS / A 3	---	---	---	---	> 24 h	---
HBT / A 4	---	---	---	---	> 24 h	---
HBT / A 5	---	---	---	---	> 24 h	---
control B	---	---	---	---	---	---
Co / B 1	5 h	10 h	---	---	12 h	6 h
ABTS / B 2	---	---	---	---	---	72 h
ABTS / B 3	---	---	---	---	---	---
HBT / B 4	---	---	---	---	---	---
HBT / B 5	---	---	---	---	---	---

After the laccase inhibition test it was suggested to our industrial partner Cytec Graz to produce a new model resin (long alkyd resin Enzyme) without acticide, tin(II)ethylhexanoate and triphenyl phosphite. To see if a progress in drying time could be achieved a new drying performance test was done. HBT treated samples showed better surface characteristics. Therefore there was a focus on the mediator HBT.

As can be seen in Table 15the drying time of long alkyd resin was reduced from more than 3 days in the initial test to less than 43 h by changing the HBT concentration and the formulation of the long alkyd resin. In comparison to the blank it could significantly be shown that the *Th*L in combination with the mediator HBT is able to cure the alkyd resin.

Table 15: Results from the additional drying time recorder measurements. Long alkyd resin (inhibitor-free) was applied in a 76 µm wet film.

Sample name	Tack free time [h]	leveling [h]	Basic trace [h]	dry [h]
Control C	120	25	39	>48
Cobalt C1	6	5	10	12
HBT C2	72	19	46	>48
HBT C3	48	18	39	43
***Th*L C4**	100	21	48	>48

3.6 Process monitoring via Fourier transform infrared spectroscopy

A new FTIR spectroscopy method was implemented to characterize the drying reaction of long alkyd resin. The hardening process of a Cobalt siccative and *Th*L/HBT and *Th*L/ABTS treated alkyd resin was investigated over time via FTIR spectroscopy and compared to an untreated long alkyd resin and an only laccase treated alkyd resin. In the cases of Cobalt and the two different laccase/mediator systems a significant decrease of the peak at 3010 cm^{-1}, assigned to the double bonds (*cis* **H-C**=CH stretching vibration) and an increase of the band assigned to OH-containing compounds (3100-3600 cm^{-1}) was observed with the only difference that the Cobalt treated alkyd resin hardens much faster than the alkyd resin with the laccase/mediator systems. Table 16 gives summary of all observed peaks and an overview of the peak assignments.

Table 16: Peaks observed during FTIR measurements and their assigned chemical groups. Data adapted from [43]

Observed peaks (cm^{-1})	Assignment
965	C-H out-of plane bending, *trans* R-CH=CH-R'
1040	C-O stretching, saturated aliphatic primary alcohols
1072	C-O stretching, saturated aliphatic primary alcohols
1121	Weak peaks, overlay of more than one peak
1136	
1162	
1269	C-O stretching, carboxylic acid dimers
1350	Weak peaks, overlay of more than one peak
1379	
1416	
1464 (1426-1480)	
1487	
1580	
1599	
1738	C=O stretching, saturated aliphatic esters ketones and aldehydes, very strong!
2855	C-H symmetric stretching, CH_3 or C-H stretching. aldehydes
2927	C-H asymmetric stretching, CH_3-Ar, -CH_2-alkanes
2956	C-H asymmetric stretching, CH_3
3010	*cis* **H-C**=CH stretching vibration
3100-3600	Formation of OH containing species, (alcohols, acids, hydroperoxides), O-H stretching
Peak decreasing **Peak increasing**	

One point that is still not understood completely is that the laccase/mediator catalyzed reaction seems to have a long delay compared with Cobalt based system. The reason for this behavior needs to be identified in order to accelerate the drying reaction. A possible explanation is that there is another substrate in the formulation that is at first converted by the laccase and only if this substrate is consumed the reaction of interest can be catalyzed.

An interesting point is that also the Cobalt catalyzed drying reaction shows a significant lag phase of about 100 minutes. Only then the drying reaction seems to start leading to an immense increase of the 3100-3600 cm^{-1} and a decrease of the 3010 cm^{-1} peak (Figure 50). It is known that Cobalt needs some time to diffuse into the alkyd resin. According to the industrial partner Cytec Graz it is a standard practice to mix the cobalt siccative to the alkyd resin at least one day before application in order to

achieve an optimal drying performance. Possibly a similar procedure is beneficial as well for the enzyme mediator system.

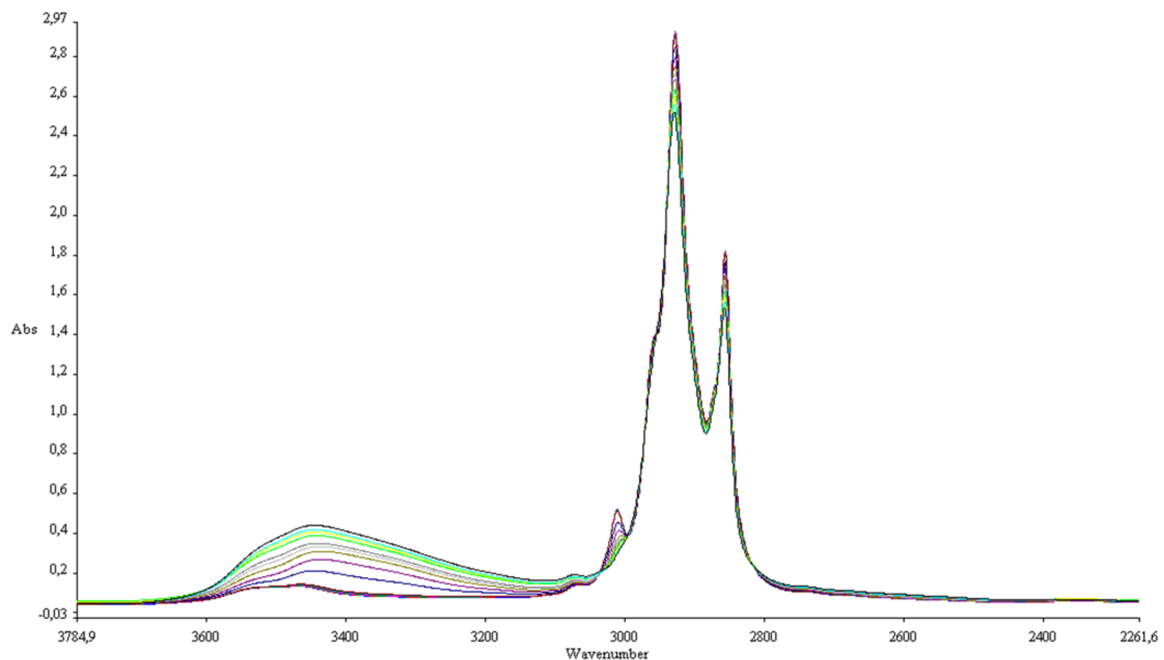


Figure 49: Full range of the time scan of 200 ppm cobalt treated long alkyd resin.

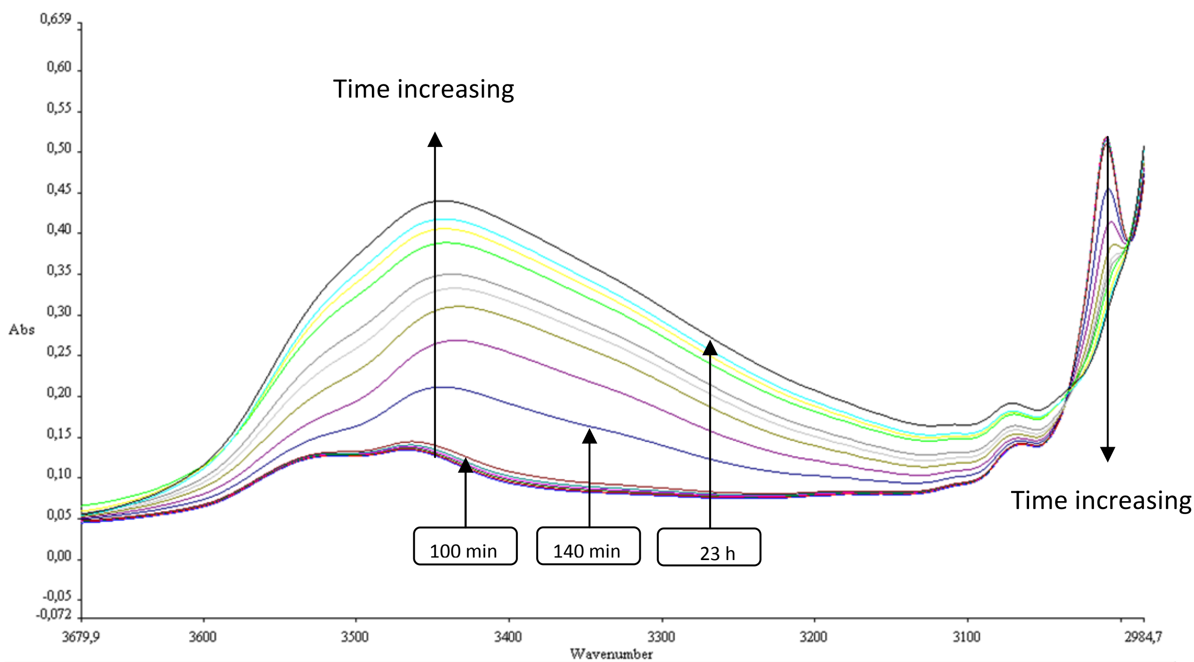


Figure 50: Time scan of the 200 ppm Cobalt treated long alkyd resin. Only the wavenumber range of interest is shown.

Nevertheless the consumption of double bonds can be correlated to the drying reaction since Cobalt treated samples show the same reaction principle as laccase/mediator treated resins. Furthermore untreated alkyd resin did not show a decrease of the double bond peak. The slight raise of the peak assigned to -OH containing compounds is definitely caused by autooxidation that is a common reaction affecting every type of (natural) oil with a certain degree of unsaturation. The achieved spectra from the FTIR measurements can be found in the appendix.

3.6.1 FTIR spectroscopy of Linseed oil

Long Alkyd resin (inhibitor-free) contains about 20 different compounds and therefore an obvious question is if the during FTIR measurements observed peaks are an overlay of more than one compound. Linseed oil is the main ingredient of the tested resin and in order to prove that the monitored double bond peak is caused by linseed oil, linseed oil was used as model substrate in this test.

It could be clearly demonstrated that linseed oil shows exactly the same behavior (increase of peak at 3100-3600 cm^{-1} and decrease of the peak at 3010 cm^{-1}) during the drying reaction as the tested resin and its fingerprint look almost the same as the one of long alkyd resin (see Figure 51 and Figure 52).

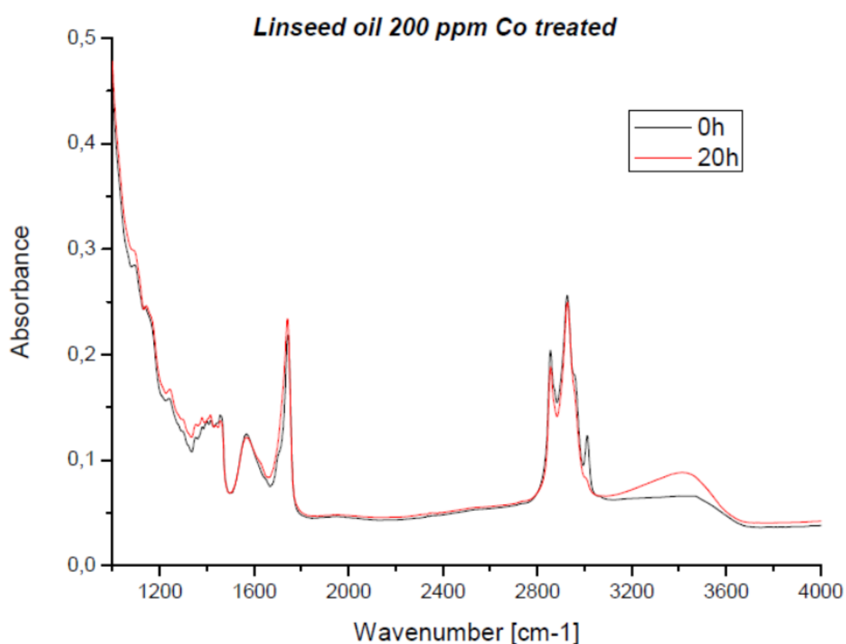


Figure 51: Full range of the spectrum obtained from 200 ppm cobalt treated linseed oil.

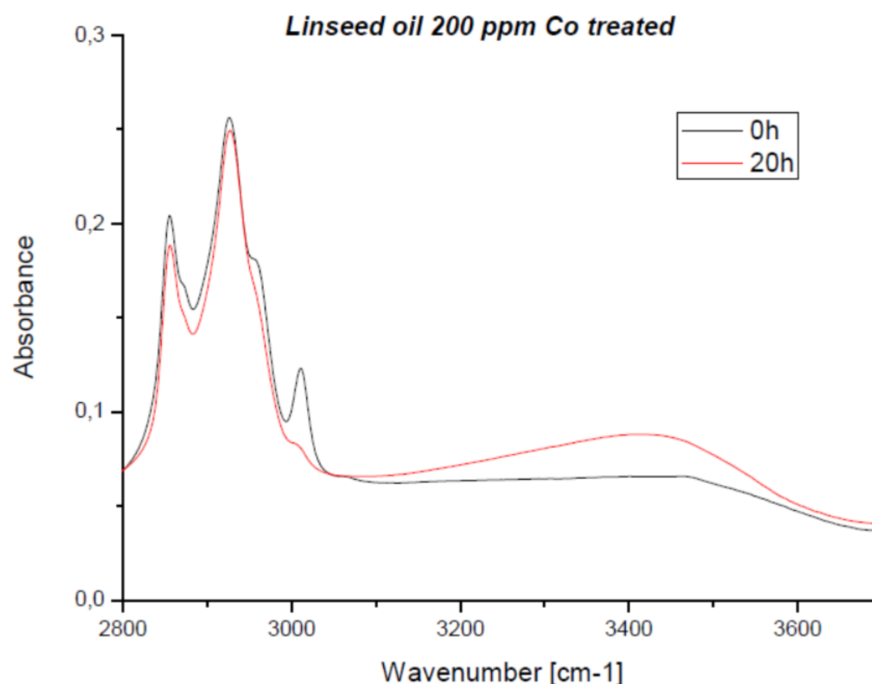


Figure 52: Spectrum obtained from 200 ppm cobalt treated linseed oil. Only the wavenumber range of interest is shown.

3.7 Process monitoring via Raman spectroscopy

As described in the previous chapter FTIR spectroscopy was used to detect the decrease of double bonds present in the sample. However, due to Oyman et. al. [39,40] double bonds show more intense Raman absorption if compared to the peaks measure with FTIR spectroscopy. Furthermore water does not interfere with the major peaks and that is a notable advantage as we are dealing with a waterborne resin. For these reasons high expectations were laid on Raman spectroscopy since those measurements worked very well for different applications were double bonds needed to be measured.

Unfortunately it turned out to be quite difficult to obtain acceptable results in the case of long alkyd resin and linseed oil because the signal-to-noise ratio was very low. Especially for the alkyd resin samples no clear results were achieved. Not even the cobalt treated sample showed significant changes over time.

Emulsified linseed oil that was dried with a cobalt siccative revealed that there are significant changes of prominent peaks. Despite of a low signal-to-noise ratio and a drift that may be caused by fluorescence of the sample the peak at 1655 cm^{-1} that is assigned to non-conjugated symmetric *cis*-C=C stretching and

the peak at 1265 cm^{-1}corresponding to non-conjugated *cis*-C=CH symmetric rock was clearly visible and a decrease in the intensity of both peaks over time was observed. At the same time a slight increase of 1634 cm^{-1} peak (symmetric conjugated C=C stretching (*cis-trans* and *trans-trans*) and/or terminal C=C stretching) was measured. This indicates the formation of conjugated double bonds [40].

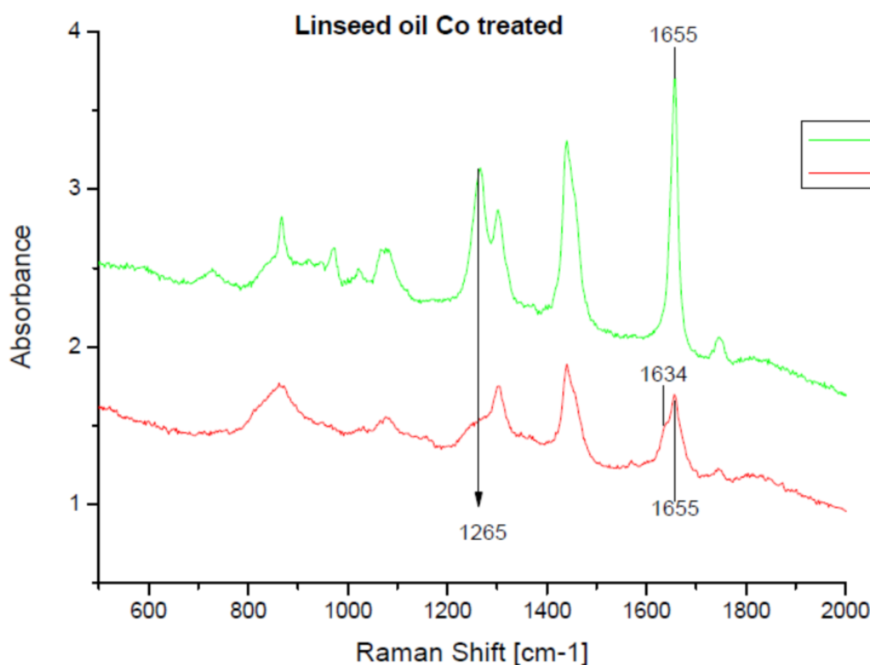
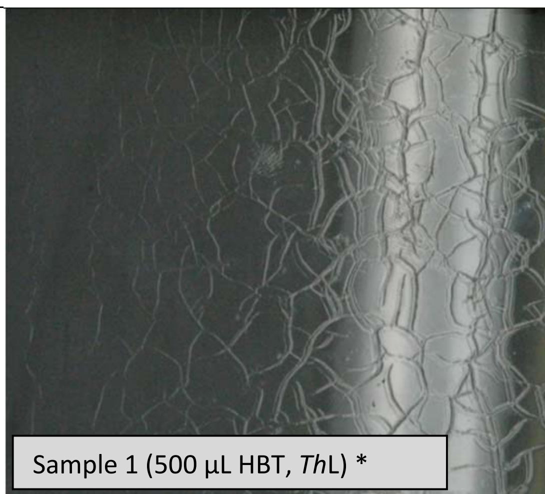


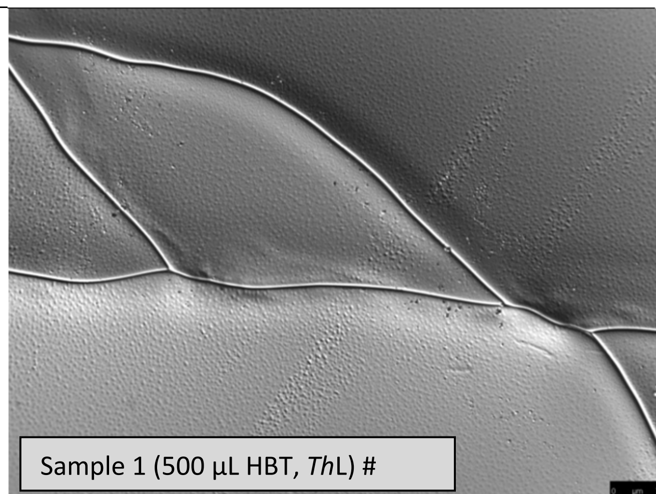
Figure 53: Measured Raman shift of cobalt treated linseed oil (Green: sample after 0 hours, red: sample after 20 hours.**) 1655 cm^{-1}: Symmetric *cis*-C=C stretching (non-conjugated), 1634 cm^{-1}: Symmetric conjugated C=C stretching (*cis-trans* and *trans-trans*) and/or terminal C=C stretching, 1265 cm^{-1}: Non-conjugated *cis*-C=CH symmetric rock.**

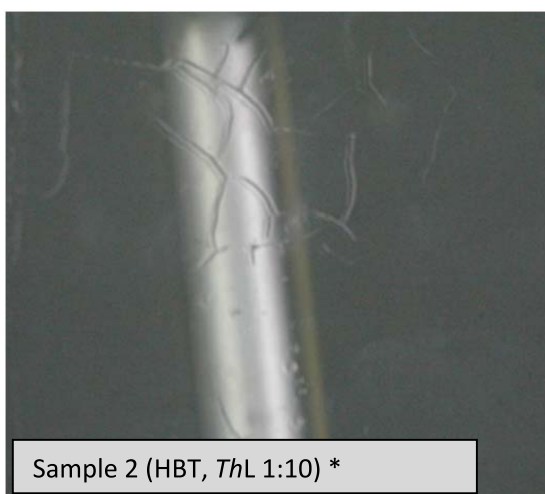
3.8 Film properties

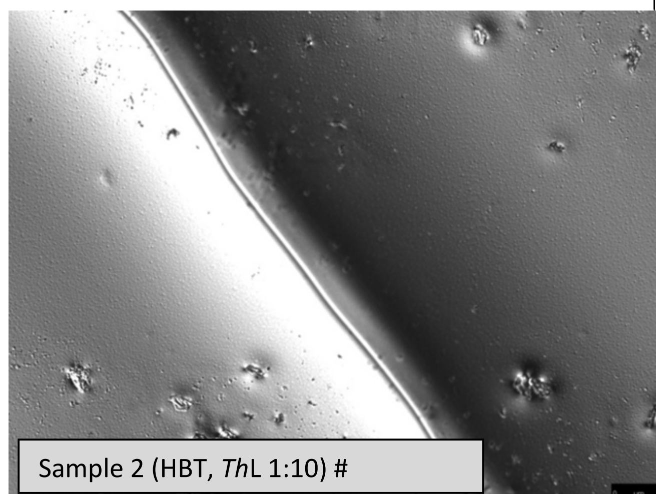
3.8.1 Surface analysis with the microscope

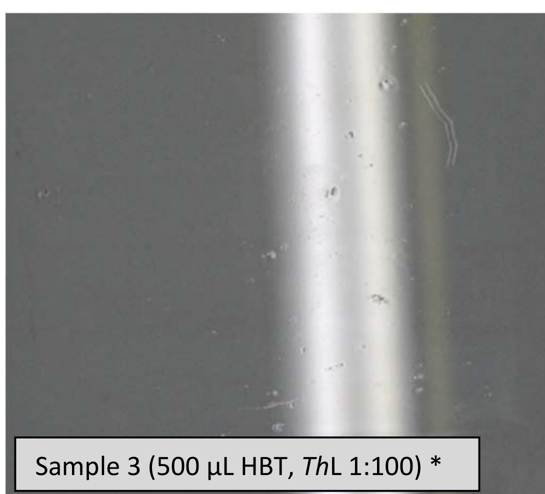
During earlier performed drying tests it was apparent that the surfaces of the films show different behavior depending on laccase and mediator concentration. Stronger or weaker developed ripped and wrinkled structures were found. For that reason a study of the surface was carried out to get a better overview about those surface characteristics. Film properties were recorded by means of conventional photography and additionally pictures were taken via microscope to get a closer look at the surfaces. Figure 54 shows a collection of the analyzed samples.

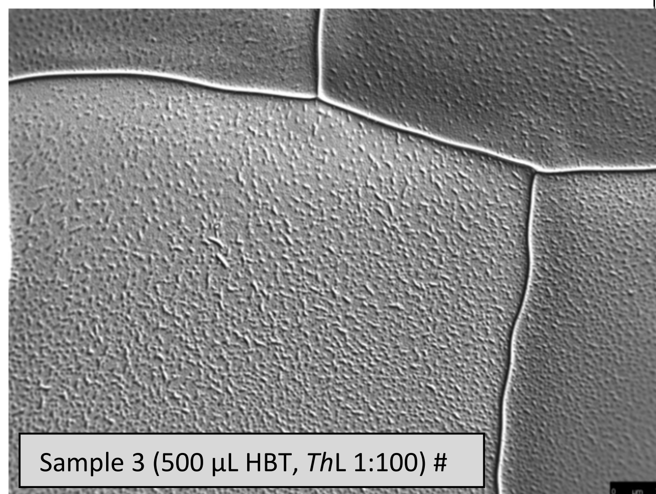

Sample 1 (500 µL HBT, *Th*L) *


Sample 1 (500 µL HBT, *Th*L) #


Sample 2 (HBT, *Th*L 1:10) *


Sample 2 (HBT, *Th*L 1:10) #


Sample 3 (500 µL HBT, *Th*L 1:100) *


Sample 3 (500 µL HBT, *Th*L 1:100) #

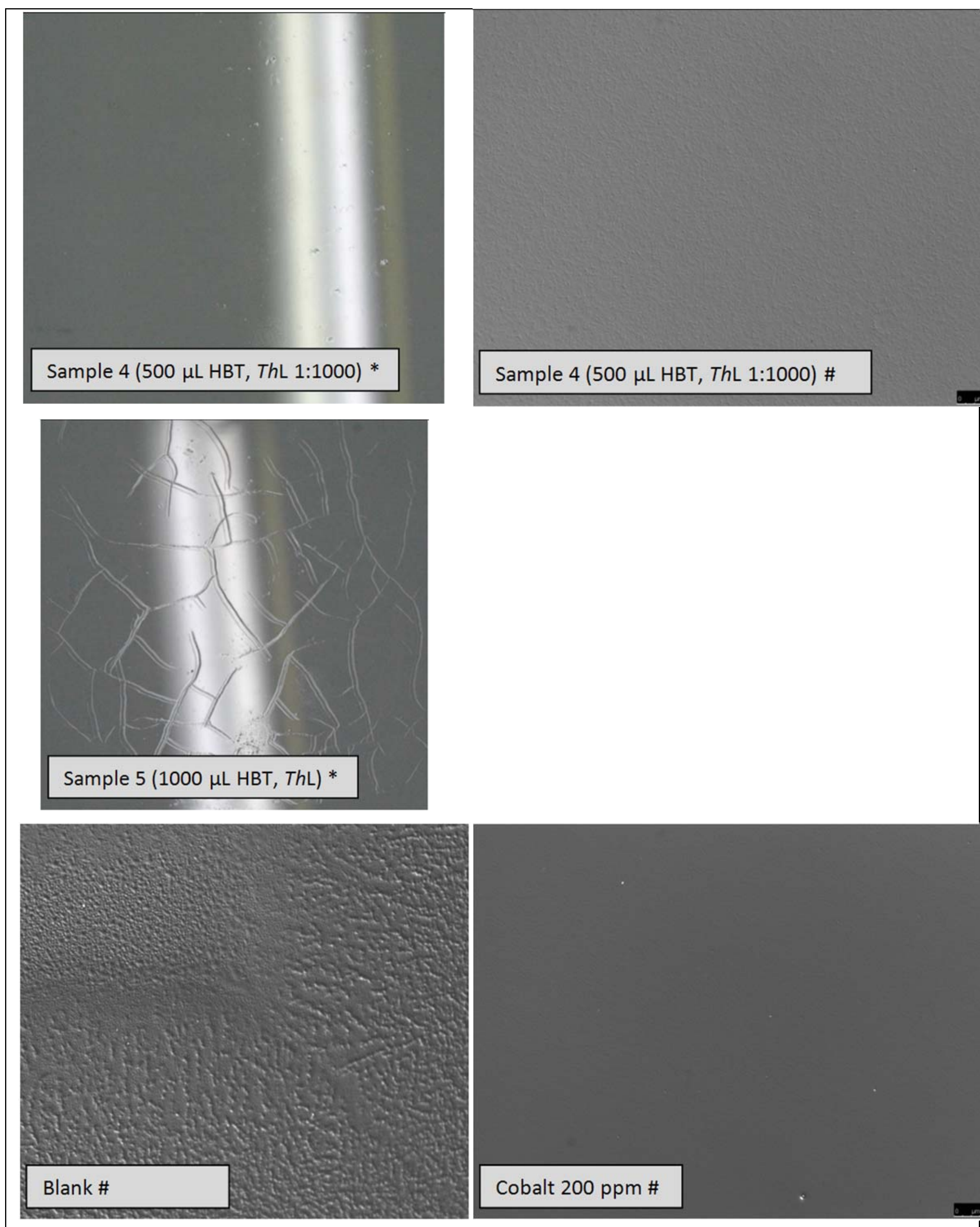


Figure 54: Conventional photos and pictures taken with the aid of CLSM of the *Th*L HBT treated long alkyd resin. * Conventional photo, #10 fold magnification.

On the left-hand side photos from the films surfaces can be seen and on the right side the corresponding picture with 10 fold magnification can be found. Comparing the structure of the blank and the positive control (200 ppm cobalt) it is obvious that the surface of the cobalt treated sample is much more even than all the laccase/mediator dried films and the blank. Looking at sample 1, 2, 3 and 4 it is impressing, that the different laccase concentration cause significantly different surface characteristics. Sample 4 with the lowest laccase concentration did not show any rips and the drying needed almost as long as the blank film. Sample 1 and 5 reveal that also different mediator concentrations have an impact on the surface structure.

The mentioned rips are most likely caused by the fact that during the drying of the resin the topmost layer of the film dries faster than the layers beneath. During this process tensions occur and rips appear.

In summary it can be concluded that it is quite difficult to set a clear trend based on the presented pictures as with the microscope only a small area can be examined at one time and many different parameter effect the drying reaction. It can be stated that a higher laccase concentration generates the formation of more rips and the alkyd resin gets faster tack free. A larger amount of HBT produces less rips and the film dries faster. The appearance of rips seems to be an indication of drying since both the sample with the lowest laccase concentration and the blank did not show these rips and did not dry during the fixed period of the experiment. Still the cobalt siccative treated resin did not exhibit a wrinkled or ripped surface.

3.8.2 Oxygen concentration in the film

As already mentioned before one very interesting question that needs to be answered is the one concerning oxygen diffusion in the film. Therefore oxygen concentration measurements in the film were performed to monitor the oxygen concentration during the drying reaction. In Figure 55 the oxygen content of a cobalt siccative treated sample over the course of the whole drying reaction is illustrated.

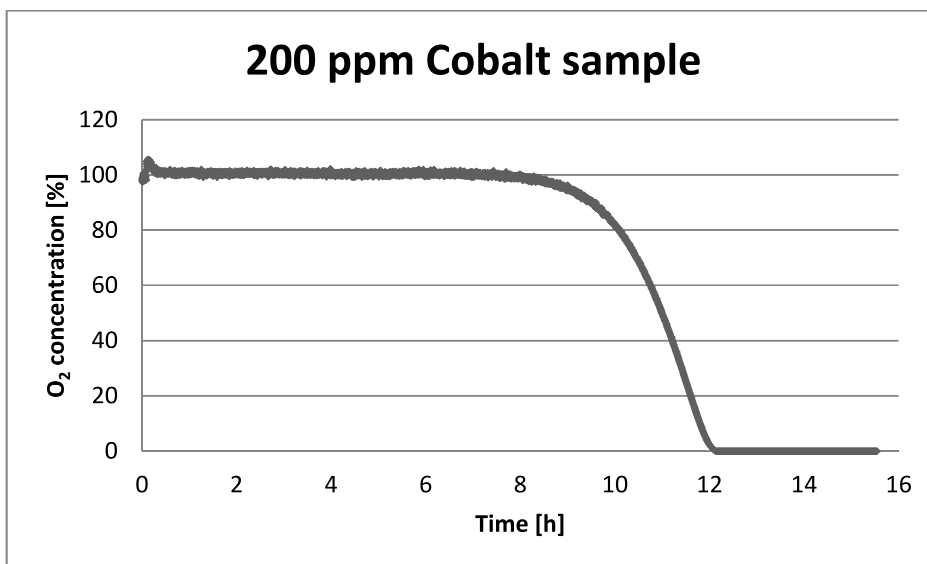


Figure 55: Oxygen consumption measured in the film of cobalt siccative treated long alkyd resin over the course of the whole drying reaction.

The measurements displayed that oxygen concentration is constant during the first 8 ½ hours until it decreases abruptly and reaches the concentration of 0 % oxygen 12 hours after application of the resin. This is a very interesting outcome because a continuous decrease of oxygen concentration during the hardening process was expected. Comparing these data with the results from the tack free time analysis and the drying recorder test it is remarkable that diffusion of oxygen through the cobalt film is still possible after 6 hours although the cobalt sample has a tack free time of 6 hours.

The measurement of the resin that was mixed with HBT and *Th*L (Figure 56) showed no considerable change in the oxygen concentration during the period of observation. Apparently the oxygen consumption during the first 30 hours after the application of the resin can be compensated by oxygen diffusion through the film. On the one hand this leads to the assumption that during the first 30 hours of the reaction the uppermost layer of the film is not yet dry and a sufficient oxygen transport is possible. This is a positive sign as it is important that the drying takes place throughout the whole film and not only on the surface. On the other hand the demand of oxygen in the sample might be that low that no difference of oxygen concentration can be measured. As the film of the tested HBT/*Th*L treated alkyd resin has a tack free time of more than 30 hours the test set-up was definitely not appropriate to follow the reaction.

Nevertheless the suggested technique for testing oxygen concentrations directly in the film could turn into an effective method to determine oxygen concentrations as soon as the laccase/mediator system for drying of alkyd resins is a little more optimized and therefore faster.

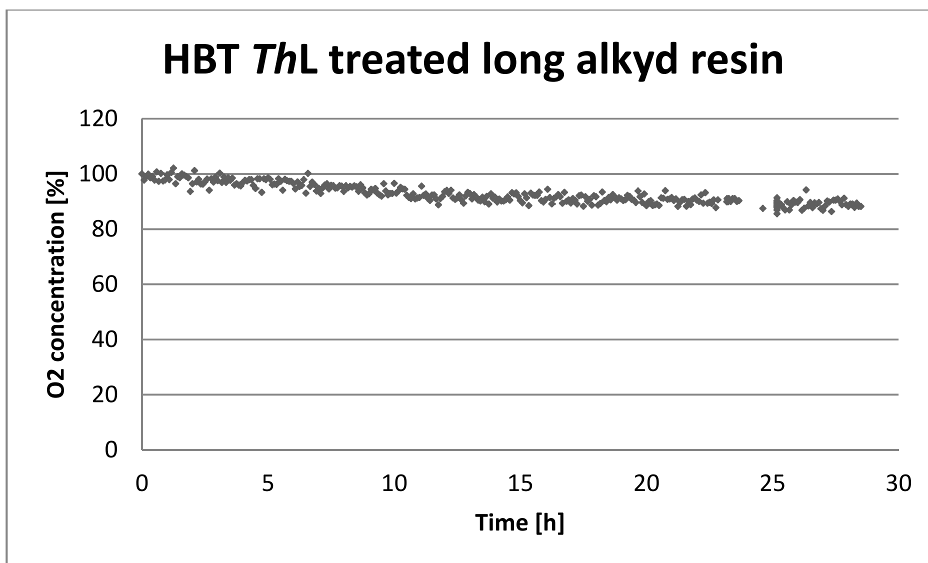


Figure 56: Oxygen consumption measured in the film of *Th*L HBT treated long alkyd resin.

3.8.3 Fluorescence labeling of the Laccase

As can be seen in Figure 57 we successfully coupled the fluorescent dye Rhodamine B to *Th*L. A large excess of Rhodamine B was added.

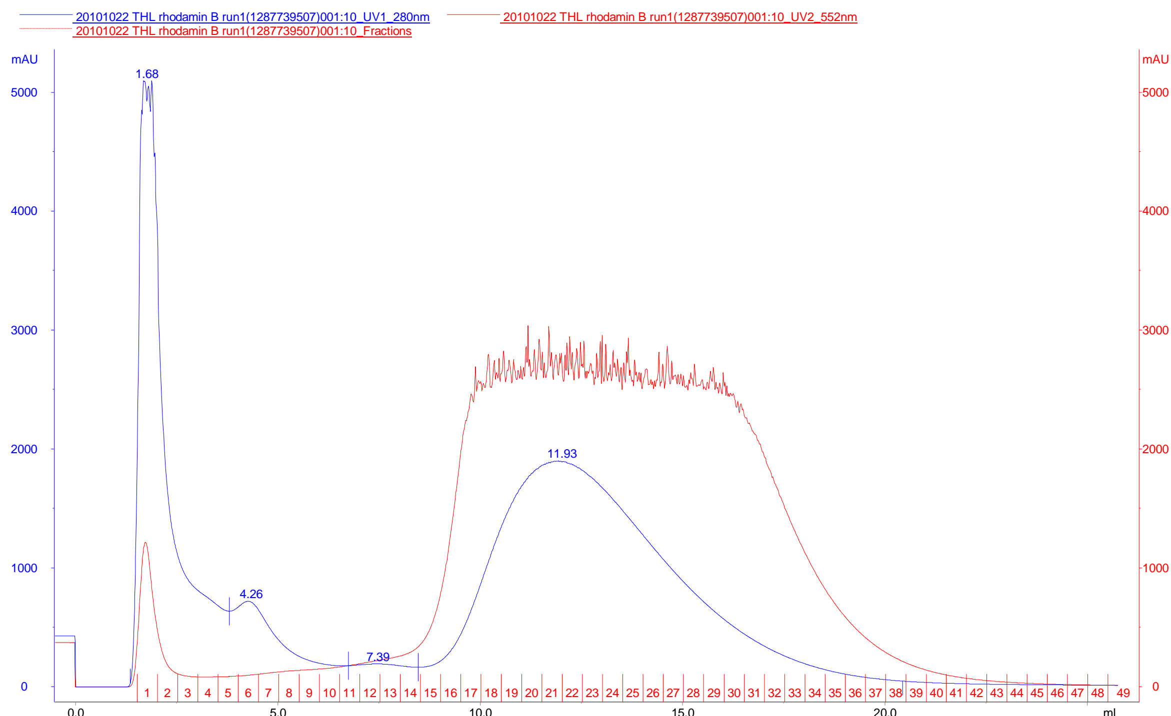


Figure 57: Separation of *Th*L, Rhodamine B coupled *Th*L and Rhodamine B by means of FPLC.

Uncoupled Rhodamine B eluted starting from 16 to approximately 31 mL of running buffer. Therefore in future experiments a lower concentration of the fluorescent dye is advised to be used. The fractions containing the coupled enzyme (in this case fraction 1 and 2 see Figure 57) were collected and stored in the dark at 4°C. An activity test with ABTS exhibited that the laccase was still active and so ready to use for drying tests.

Figure 58: Rhodamine B labeled *Th*L in the long alkyd resin film.

The fluorescent measurements (see Figure 58) on the microscope showed that a strong background noise is present and some brighter red dots are visible. Those red dots are generated by Rhodamine B labeled *Th*L molecules that most likely formed aggregates.

In Figure 59 a cross section of the alkyd resin film is presented.

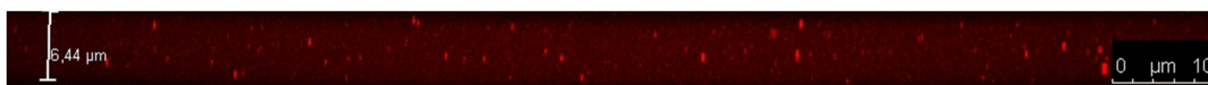


Figure 59: Cross section of Rhodamine B coupled *Th*L in a long alkyd resin film.

It can be clearly seen that also after 4 days of incubation the *Th*L aggregates are distributed all over the film and present also in lower layers. A clustering of laccase on the surface cannot be observed.

It was not possible to clarify what happens to the laccase directly after the application of the resin since the resin is milky in the first minutes and therefore measurements with the CLSM are impossible during this time. Maybe the laccase is able to diffuse / move around in the film as long as a certain concentration of water is present but it is also possible that the mobility of the enzyme is quite limited. In this case a good distribution of the enzyme in the alkyd resin before the application would be crucial. Good mixing is also important for cobalt siccative. However to answer these questions further experiments would be necessary.

4 Discussion

The project this master thesis is embedded in, aims to develop an enzymatic method to cross-link waterborne alkyd resins in order to find an environmentally friendlier alternative for the currently used cobalt containing siccatives. Laccases have been identified as potential enzymes to catalyze cross-linking.

Laccases have a very broad substrate spectrum but they are not able to convert non-phenolic substrates directly. However there exist natural and synthetic laccase substrates that have the capability to abstract electrons from non-phenolic substances [44-46]. These so called mediators are electron shuttles that broaden the substrate spectrum of laccases to an even wider range and make an enzymatic catalysis of the reaction of interest (cross-linking of unsaturated fatty acids) possible. Some research groups have already proven the oxidation of unsaturated groups of triglycerides and fatty acids using a laccase mediator system [16,22,25-27].

Three laccases, namely from *Trametes hirsuta*, *Myceliophthora thermophila* and from *Trametes villosa* were tested regarding their capability to catalyze the drying of alkyd resins. After characterization of the three enzymes, *Th*L was chosen for further experiments due to its good properties concerning pH-optimum. Almansa et al. [14] also proved that *Th*L has a broad temperature range and shows high stability during long time storage. Nevertheless *Mt*L and *Tv*L might play an important role if a different pH range is needed since the pH optimum of *Mt*L is at pH 7 and *Tv*L is most active at pH 4.5. The MW of the *Th*L and *Tv*L were determined to be about 66 kDa and 70 kDa respectively by SDS-PAGE. These results can also be confirmed if compared to date from the literature. The MW of *Th*L is reported to 62 kDa [14] and the one of *Tv*L is mentioned to be about 66 kDa [47]. In the case of *Mt*L three peaks at about 97 kDa, 90 kDa and 75 kDa were observed. Hollmann et al. reported that *Mt*L has a molecular weight of 85 kDa [42]. Knowledge about these enzymes characteristics was important in order to indentify the enzyme in GPC analysis of LMS-cross-linked resins.

To adapt a laccase for this application it might also be interesting to modify the laccase genetically and optimize the laccase for a specific resin for instance in terms of pH, water activity or resistance to compounds that are present in the resin. Previously, the solvent stability of a laccase has been dramatically improved by directed evolution [48]. Also the substrate specificity to the used mediator might be optimized as Koschorreck et al. [49,50] demonstrated successfully.

In this study, the screening for suitable mediators by means of oxygen consumption measurements was performed using both the Clark electrode and a fiber optic oxygen microsensor. Both methods showed comparable results. During oxygen concentration measurements the experimental setup was chosen in a way that it could be clearly differentiated between the two steps of the mediated reaction. In the first place oxygen is consumed during the oxidation of the mediator and in the second step the oxidized mediator transfers electrons to the substrate of interest being re-oxidized by the laccase [19]. Consequently, during this second step consumption of molecular oxygen continues.

The measurements revealed that HBT has the highest ability to oxidize long alkyd resin but also ABTS and syringaldazine showed a promising decrease in oxygen concentration. In contrast, with the mediators TEMPO and syringic acid no significant decrease in oxygen concentration was observed.

Oxidation of different resins and resin components with a combination of *Th*L and ABTS was investigated. Water solubility seemed to be an essential prerequisite for successful LMS oxidation as there was no oxygen consumption in case of poorly soluble or insoluble samples. For neutralized linseed oil fatty acids and the samples that contain a surfactant, like in the cases of the long alkyd resin or linseed oil emulsified, a clear decrease in the concentration of oxygen was measured. In contrast, it was not possible to oxidize linseed oil without being emulsified with the LMS. It is very likely that the exchange surface between the aqueous phase of the buffer system that contained the *Th*L and the mediator and the organic phase consisting of linseed oil was just too small. As a result of this small exchange surface an electron transfer from the mediator to the substrate was not possible in a large-scale. However if the substrate is present in an emulsified form the surface between the two phases is multiplied and therefore an oxygen consumption is measured.

The molecular weight increase of the laccase mediator catalyzed cross-linking reaction was quantified with the aid of gel permeation chromatography. The increase of long alkyd resin treated with ABTS a mediator and *Th*L was significantly improved to 252 kDa when compared to the control (164 kDa). Consequently an effective cross-linking of long alkyd resin with the laccase mediator system was successfully demonstrated.

Still, formation of precipitates removed prior to GPC analysis needs to be taken into account. High molecular weight compounds formed in some samples precipitated and were consequently removed by filtration prior to GPC analysis. The positive control (cobalt treated sample) showed for instance strong precipitation but no change of the molecular weight was determined in the remaining soluble fraction. In contrast, in the control sample no precipitates were formed. For this reason, formation of precipitates indicates a certain degree of cross-linking. Taking into account all those mentioned factors besides the cobalt siccative treated sample, the samples containing *Th*L in the combination with the mediators ABTS, HBT and TEMPO raised the molecular weight of long alkyd resin. To detect the absolute values of molecular weight an alternative analysis to GPC would be necessary. Possible techniques that might be suitable could be MALDI-TOF or Zetasizer measurements.

In the laccase activity inhibition test the three chemicals acticide, tin(II)ethylhexanoate and triphenyl phosphite were identified to inhibit laccase activity considerably. In different experimental setups (incubation temperature 25°C or 37°C, containing all three or only one substance per sample) laccase activity was monitored over time. The concentrations of the compounds were chosen with the same concentrations as they are present in the long alkyd resin. Generally a very high stability of *Th*L was observed at 25°C and even at 37°C incubation temperature. This is an important factor for subsequent applications in the coating industry. Nevertheless *Th*L activity decrease in the presence of acticide, tin(II)ethylhexanoate and triphenyl phosphite. The loss of activity of the samples incubated at 37°C was in general higher than the one of the samples incubated at 25°C. Especially samples containing acticide or all three compounds had a prominent loss of laccase activity over time compared to the control. As a result it was suggested to our industrial partner Cytec Graz to produce a model resin that does not contain any of these three compounds. Like this adverse effects on drying performance due to enzyme inactivation during further experiments will be eliminated.

Until now the mechanism of this laccase inhibition is not known and a literature research was done in order to get an idea what might happen during the laccase inhibition. Triphenyl phosphite was detected to be a neurotoxic esterase inhibitor [51,52]. Tanzi et al. [53] identified a significant cytotoxicity of tin(II)ethylhexanoate against Swiss 3T3 mouse fibroblasts and human endothelial cells. Acticide consists of the two compounds 5-chloro-2-methyl-1,2-thiazol-3-one and 2-methyl-1,2-thiazol-3-one. King et al. [54] showed that similar isothiazolinone-based enzyme inhibitors interact with cysteine residues of

monoacylglycerol lipase. Hence, a comparable inhibition mechanism might be possible in the case of laccase.

In the future this obtained knowledge needs to be considered when formulating alkyd resins containing laccases as drying agents. Additionally new laccase compatible biocides would need to be assessed in future formulas to prevent microbial growth.

The drying performance of the laccase mediator catalyzed reaction was measured by means of tack free time analysis and drying time recorder. Both analyses showed that both HBT concentration as well as laccase concentration have an impact on the tack free time and hence on the velocity of the hardening process. Apparently higher laccase and mediator concentrations influence the drying performance positively. One possible explanation is that the method - to bring the two compounds into the solution - is not yet optimal and a homogeneous distribution in the resin is not reached. In this case a higher laccase and mediator concentration would lead to a faster cross-linking reaction.

Cobalt cross-linked coatings are pre-incubated with the cobalt siccative for at least 24 h to ensure an optimal cobalt distribution in the resin and a following efficient hardening. This effect also needs to be considered in the case of the laccase mediator systems since laccase aggregates were detected with CLSM. Laccase was successfully coupled to the fluorescent dye Rhodamine B and this tagged laccase was utilized for measurement on the CLSM. A cross section of the sample indicated that the distribution of the laccase is not homogenous. It is most likely that the enzyme aggregates since the dots that can be seen are too big for a single *Th*L molecule. The average particle size of long alkyd resin is 600 nm. It is possible that the enzyme accumulates around these micelles present in the alkyd resin.

Drying time recorder measurements showed that it was not possible to cross-link medium alkyd resin with the laccase mediator system. One inhibiting factor was probably that the pH of medium alkyd resin (pH 7) was too high for *Th*L. Nevertheless the drying time of long alkyd resin was reduced from more than 3 days in the initial test to less than 43 h by changing the HBT concentration and the formulation of the long alkyd resin. (Long alkyd resin without acticide, tin(II)ethylhexanoate and triphenyl phosphite called long alkyd resin (inhibitor-free) was used.) In comparison to the blank it could significantly be shown that the *Th*L in combination with the mediator HBT is able to cure the alkyd resin.

An FTIR method was implemented to characterize the drying reaction of long alkyd resin. Similar measurements have already been done by Oyman et al. [39,40]. Cobalt siccative, *Th*L/HBT and *Th*L/ABTS treated alkyd resin was investigated over time via FTIR spectroscopy. Laccase only treated long alkyd resin and pure long alkyd resin served as negative controls.

The cobalt siccative treated sample and the two different laccase/mediator systems revealed asignificant decrease of the peak at 3010 cm^{-1}, assigned to the double bonds (*cis* **H-C**=CH stretching vibration) and an increase of the band assigned to OH-containing compounds (3100-3600 cm^{-1}) [43]. The only difference that was found between the benchmark (cobalt treated alkyd resin) and the laccase mediator catalyzed samples was that until now the cobalt siccative hardens the coating much faster. However the consumption of double bonds can be correlated to the drying reaction, as the sample with cobalt shows similar results.

Linseed oil is the main compound of long alkyd resin and is expected to cross-link via its unsaturations on the fatty acid side chains. Previously, Zhang et al have demonstrated that laccases are able to oxidize lipids and unsaturated fatty acid esters [26]. This is why linseed oil was used as model substrate in an additional drying experiment. The cobalt siccative treated emulsified linseed oil sample was monitored over time by means of FTIR spectroscopy. It was possible to prove that linseed oil shows exactly the same behavior (increase of peak at 3100-3600 cm^{-1} and decrease of the peak at 3010 cm^{-1}) during the drying and its fingerprint look almost the same as the one of long alkyd resin. Consequently, for future mediator screening a combination of oxygen consumption measurements and FTIR spectroscopy seems to be a good method to detect suitable laccase mediators.

Raman spectroscopy was performed to measure the change of double bonds present in the alkyd resin samples. Oyman et al. [39] reported that double bonds exhibit strong Raman absorption. Furthermore in contrast to FTIR spectroscopy Raman measurements are not disturbed by a water peak. The results of the Raman spectroscopy were difficult to interpret since the signal-to-noise ratio was very low. Especially samples of long alkyd resin regardless whether they were treated with cobalt siccative or the laccase mediator system did not show a significant peak change over time.

Only emulsified linseed oil that was dried with a cobalt siccative revealed clear changes of prominent peaks. An overtime decrease of the peak at 1655 cm^{-1} that is assigned to non-conjugated symmetric *cis*-C=C stretching and the peak at 1265 cm^{-1} corresponding to non-conjugated *cis*-C=CH symmetric rock

[39] was detected. Also a slight increase of the 1634 cm^{-1} peak (symmetric conjugated C=C stretching (*cis-trans* and *trans-trans*) and/or terminal C=C stretching) was noticed. This might indicates the formation of conjugated double bonds [40]. Nevertheless it has to be considered that the signal-to-noise ratio and a drift that may be caused by fluorescence of the sample overlaid the detected chromatogram and therefore it is difficult to make a clear statement about the ongoing reactions.

Oyman et al. [40] reported that oxidation of conjugated and non-conjugated double bonds takes place differently. Unsaturated fatty acids containing conjugated double bonds seem to produce a higher amount of cross-links than fatty acids with non-conjugated double bonds. This might be an important point that needs to be considered in future experiments. Besides linseed oil also other natural oils with a high content of unsaturations might be of interest.

Microscopic surface analysis showed that the surface of the cobalt treated sample is much more even than all the laccase/mediator dried films and the blank. It turned out that different laccase concentrations cause significantly different surface characteristics. Rips were observed in the case of a higher laccase concentration compared to the sample with the lowest laccase concentration and the blank. It could also be shown that the mediator concentration has an impact on the surface structure.

The mentioned rips result from the fact that the topmost layer of the film dries faster than the lower layers. On the one hand this is a good sign since this means that some kind of cross-linking takes place. On the other hand cobalt dried alkyd resin do not display these kinds of wrinkles. Usually so called anti skinning additives are mixed to the formulation to inhibit those kind of unwanted reactions [6]. Still it is not clear why the surface behavior of the cobalt catalyzed reaction is that different if compared to the laccase mediator catalyzed one. (The cobalt siccative treated long alkyd resin does not have a ripped surface.)

To sum up it can be stated that a higher laccase concentration leads to the formation of more rips and the alkyd resin gets faster tack free. A higher concentration of HBT produces less rips and the film also dries faster and seems to be more homogeneous. The development of rips seems to be an indication of an ongoing curing reaction since the surface of the negative control did not show rips and did not dry during the fixed period of the experiment.

The oxygen concentration of the film was measured by means of optical oxygen sensor. The experiment showed that the oxygen concentration of a cobalt treated film is constant for about 8 hours and then suddenly decreases until 0% oxygen is reached. This result raises questions about the permeability of the alkyd resin surface. (Prior drying recorder tested indicated that the drying time of cobalt treated alkyd resin lays at 6 hours). Obviously a diffusion of oxygen throughout the surface is possible after a sample is defined as tack free. Similar measurements were performed with HBT/*Th*L treated alkyd resin but no significant change in oxygen consumption was detected since the drying time of this sample is still too high for this method. The technique seems to have a high potential in the future when the laccase mediator system is a little more optimized and therefore faster.

Coming back to the used mediators some more points need to be mentioned. Synergic effects in laccase mediator systems using both HBT and ABTS as mediator at the same time have already been described during anthracene oxidation [55]. Also a combination of phenolic mediators has been tested but no improved effect was reported [17]. The mediation via HBT and ABTS follow different reaction mechanisms and therefore it is possible that only a combination of two or more mediators that follow different reaction routes is a useful method to enhance also the cross-linking reaction. Furthermore not only HBT and ABTS should be considered but also new mediators or mediator combinations should be tested. Additionally the reaction mechanism of the mediators has to be taken into account.

The reaction mechanism of the laccase mediator catalyzed cross-linking reaction is not yet revealed and further experiments have to be performed to get a better insight into the chemical action that takes place during the hardening of the alkyd paint. Until now it can only be stated that unsaturated fatty acids present in the coating are activated most likely by the formation of peroxides and peroxide radicals.

The generated peroxides and peroxide radicals then probably cross-link directly with the unsaturated double bonds of other fatty acids and form cross-linking bridges. Since the oxygen-oxygen bond in peroxides is labile it is also possible that the peroxides split into other reactive compounds such as radicals that are formed during homolytic cleavage. One way or the other at this stage of the reaction double bonds would be consumed in a radical chain reaction. This can be measured by means of FTIR spectroscopy because the peak at 3010 cm^{-1} assigned to *cis* **H-C**=CH stretching vibration would decrease. As a matter of course, other reactions might consume oxygen as well as the number of double bond

might decrease without the formation of cross-linking bridges. Especially autooxidation or unwanted reactions where peroxides fragment and volatile compounds are formed should here be mentioned [56].

As opposed to this mechanism the formation of epoxides (also called oxirane) would be particularly advantageous since Kudanga et al. [57] showed that oxiranes react with most different functional groups and therefore are suitable to react with lignocelluloses materials. Kudanga et al. coupled lignin model compounds to linoleic acid using oxiranes. Alkyd resins are amongst others applied to wooden surfaces therefore a possible cross-link directly with the wooden surface would lead to an additional benefit besides the replacement of toxic cobalt siccatives.

5 Conclucion

The objective of this research was to cross-link unsaturated groups in alkyd resins by using a laccase mediator system. The expected advantage of this method is the elimination of heavy metal siccatives (e.g. Co- or Pb- containing substances that are known to be toxic / carcinogenic) from the formulation.

Due to its good properties in respect of pH range, activity, stability and temperature range *Trametes hirsuta* laccase was chosen for detailed experiments. During a mediator screening potential laccase mediators for this purpose were identified including HBT and ABTS.

Enzymatic hardening of alkyd acids was clearly demonstrated by means of oxygen consumption measurements, GPC analysis, and drying recorder tests while FTIR spectroscopy and Raman spectroscopy revealed supporting mechanistic details.

Nevertheless there are still open questions and to get an overall image of the process further investigations are needed. In order to optimize the process it is necessary to completely understand the chemical process.

Future work should thus include amongst others a detailed analysis of oxygen diffusion and LMS distribution in drying films, an optimization of the reaction conditions and screening for high potential enzymes and mediators. Additionally, genetic engineering would offer a large potential for the adaptation of the laccase for a specific resin for instance in terms of pH, water activity or resistance to compounds that are present in the resin. Also the substrate specifity to the used mediator might be optimized.

In addition to this, it is well known, that the cobalt siccative needs to be premixed for at least 24 hours with the resin before the coating is applied on a surface. This ensures an optimal cobalt distribution in the resin and a following efficient hardening. This effect likewise seems to be important for laccases as seen with CLSM studies.

6 Declaration

STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

…………………………… ……………………………………………….

date (signature)

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt, und die den benutzten Quellen wörtlich und inhaltlich entnommene Stellen als solche kenntlich gemacht habe.

Graz, am …………………………… ……………………………………………….

(Unterschrift)

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8 Abbreviations

%	percent
°C	degree Celcius
ε	molar extinction coefficient
µL	microliter
µmol	micromol
Δabs/Δt	change of absorbance per minute
ABTS	2,2'-azino-bis(3-ethyl)benzothiazoline-6-sulfonic acid
APS	ammonium persulfate
BSA	bovine serum albumin
d	pathlength
DMF	dimethylformamide
dH_2O	destilled water
ddH_2O	double destilled water
e.g.	for example
EDAC	(N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide)
f	dilution factor
FPLC	fast protein liquid chromatography
FTIR	fourier transform infrared spectroscopy
GPC	gel permeation chromatography
HBT	1-hydroxybenzotriazole
HCl	hydrochloric acid
H_2O	water
Hz	Hertz
kDa	kilo Dalton
K_m	Michaelis constant
LMS	laccase mediator system
M	mole per liter
min	minute
mL	milliliter
mM	millimole per liter

*Mt*L	*Myceliophthora thermophila* laccase
MW	molecular weight
NHS	(N-Hydroxysuccinimide)
Nkat	nanokatal
nm	nanometer
PAGE	polyacrylamide gel electrophoresis
ppm	parts per million
rpm	revolutions per minute
[S]	substrate concentration
SDS	sodium dodecyl sulfate
sec	second
SEC	size exclusion column
TEMED	tetramethylethylenediamine
TEMPO	2,2,6,6-tetramethyl-1-piperidinyloxyl
*Th*L	*Trametes hirsuta* laccase
*Tv*L	*Trametesvillosa* laccase
U	Unit
U/mL	Units per milliliter
V	Volt
v0	current reaction rate or volumetric enzyme activity
v_{max}	maximum reaction rate
V_{sample}	sample volume
V_{tot}	total volume
(w/v)	weight per volume

9 Equipment

analytic balance	Sartorius 2004 MP
analytic balance	KERN PB
balance	DENVER INSTRUMENT S-4002
centrifuge	Eppendorf mini spin
centrifuge	Hettich EBA 3S
column	Size exclusion column (SEC), Superdex 75 10/300 GL
CLSM	Leica TCS SPE - Confocal Microscope, Leica microsystems
electrophoresis power supply	BIO-RAD POWER PAC 1000
fiber-optic oxygen meter	MICROX TX3, PreSens Precision Sensing GmbH
film applicator	Erichsen, Quadruple Film Applicator Model 360
FPLC	amersham pharmacia biotech, ÄKTA purifier, BOX-900, pH/C-900, UV-900, P-900
FT-IR Spectrometer	Perkin Elmer, Spectrum 100
magnetic stirrer	VARIOMAG Electonicrührer MULTIPOINT HP
phase-fluorimeter	Fibox, PreSens GmbH
pH-meter	METTLER TOLEDO Seven Easy
photometer	HITACHI U-2001 Spectrophotometer
pipette	SOCOREX ACURA 825
pipette	Biozym PreCision
pipette	Carl Roth GmbH
plate reader	TECAN infinite M200
scanner	HP Scanjet 4890
shaker	INFORS HT Multitron
thermomixer	eppendorf Thermomixer comfort
ultrasonic bath	Elma TRANSSONIC DIGITAL S
ultrasonic bath	BANDELIN SONOREX SUPER RK 102H
vortex	IKA VORTEX GENIUS 3
well plates	Greiner bio-one
oxygenmeter	RANK BROTHERS LTD. DUAL DIGITAL MODEL 20

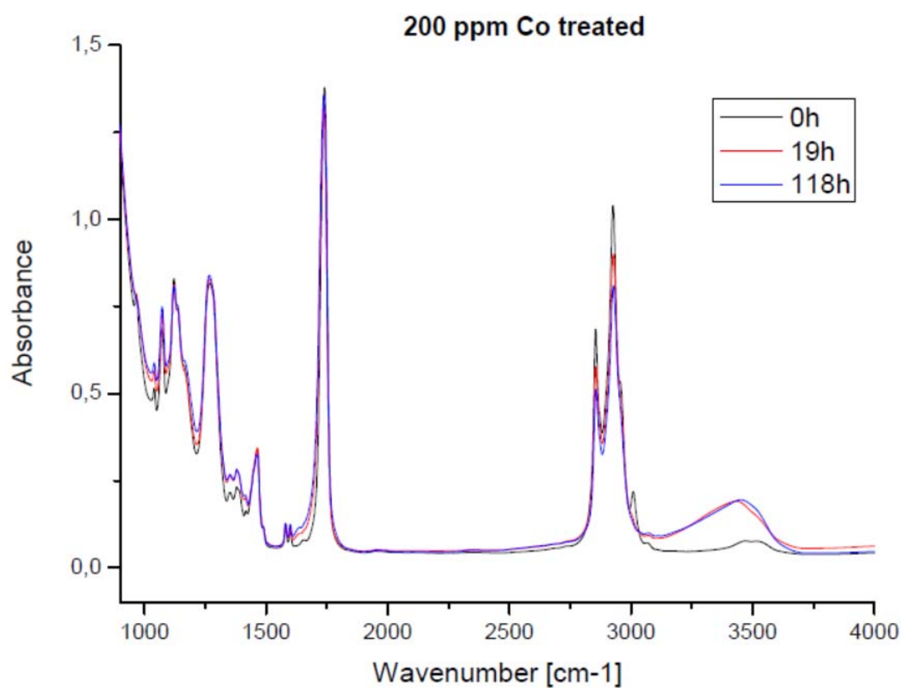
Raman Spectrometer	Perkin Elmer precisely, Raman Station 400
waterbath	B. BRAUN THERMOMIX 1420

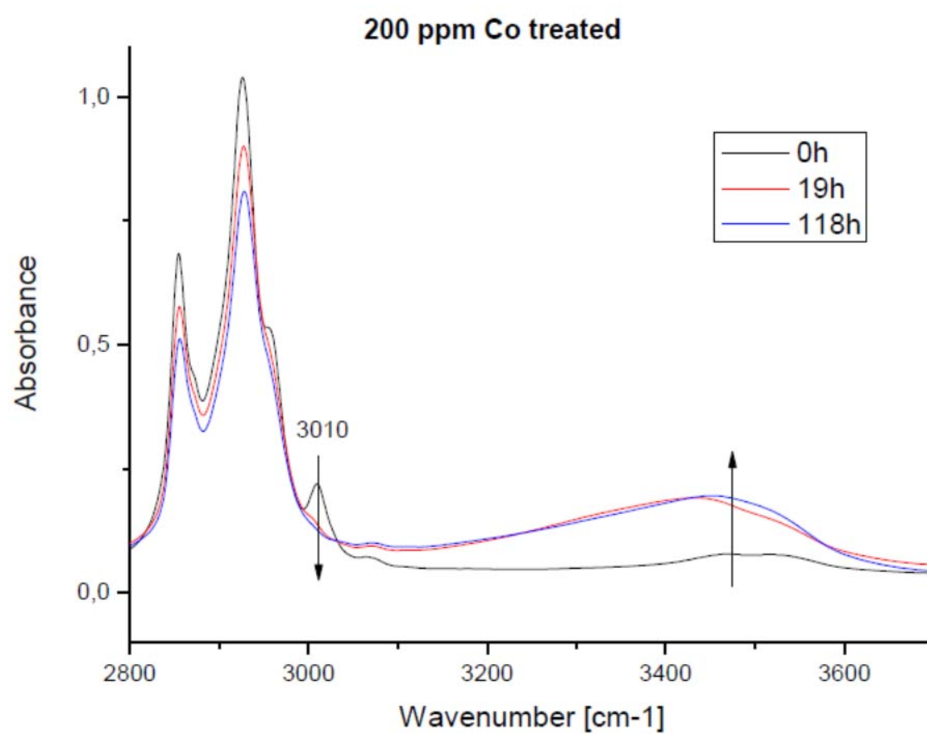
10 Chemicals

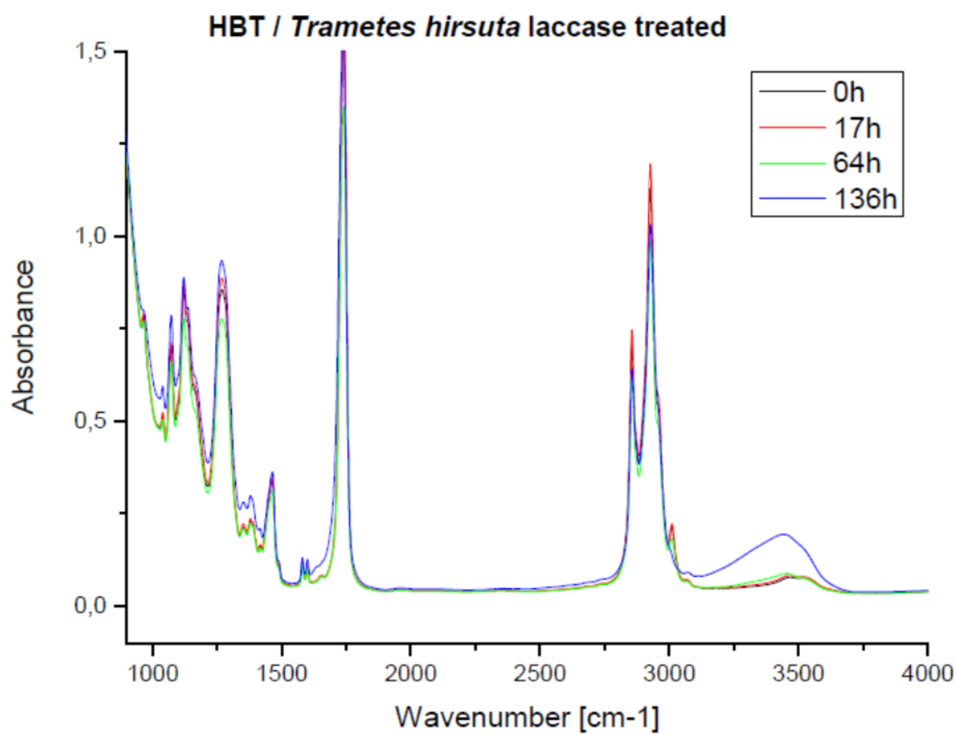
Acetic acid	Carl Roth GmbH
Acticide	Cytec
Albumin, bovine serum	Sigma
2,2‘-azinobis-(3-ethylbenzothiazoline)-6-sulphonate	Sigma
Bradford Reagent	Carl Roth GmbH
CaF2 Crystal window	Sigma
Cobalt oil drying agent	Cytec Graz
Coomassie Brilliant Blue R-250	BIO-RAD
Copper(II)sulfate-pentahydrate	Merck
Dimethylformamide	Merck
di-Potassium hydrogen phosphate anhydrous	Carl Roth GmbH
Folin	Sigma-Aldrich
Glucose	Sigma
Glucose oxidase	Sigma
1-Hydroxybenzotriazole hydrate	Aldrich
Iron(II)sulfate	Merck
Linseed oil	Cytec Graz
Manganese(II)sulfate	Merck
Myceliophthora thermophila Laccase	Novozymes
N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide	Aldrich
N-Hydroxysuccinimide	Fluka
Potassium phosphate monbasic	Sigma
Potassium sodium tartrate tetrahydrate	Fluka
Rhodamin B	Sigma
SDS-PAGE Molecular Weight Standards, Broad Range	Bio Rad
Sodium acetate	Carl Roth GmbH
Sodium carbonate anhydrous	Carl Roth GmbH
Sodium chloride	Carl Roth GmbH
Succinic Acid	Sigma Aldrich
Surfactant	Cytec Graz

Syringaldazine	Sigma
Syringic Acid	Sigma
2,2,6,6-Tetramethyl-1-piperidinyloxy, free radical	Aldrich
Tin(II)ethylhexanoate	Cytec
Trametes hirsuta Laccase	Institute of Environmental Biotechnology of the Graz University of Technology
Trametes villosa Laccase	Novozymes
Triethylamine	Cytec
Triphenyl phosphite	Cytec
Tris	Carl Roth GmbH
Tween 20	Sigma
UE HSW 323/23c / 65% (pH 4) = long alkyd resin	Cytec
Vialkyd AY 6120 / 60% (pH 7) = medium alkyd resin	Cytec

11 Appendix






HBT / *Trametes hirsuta* laccase treated
Absorbance
Wavenumber [cm-1]
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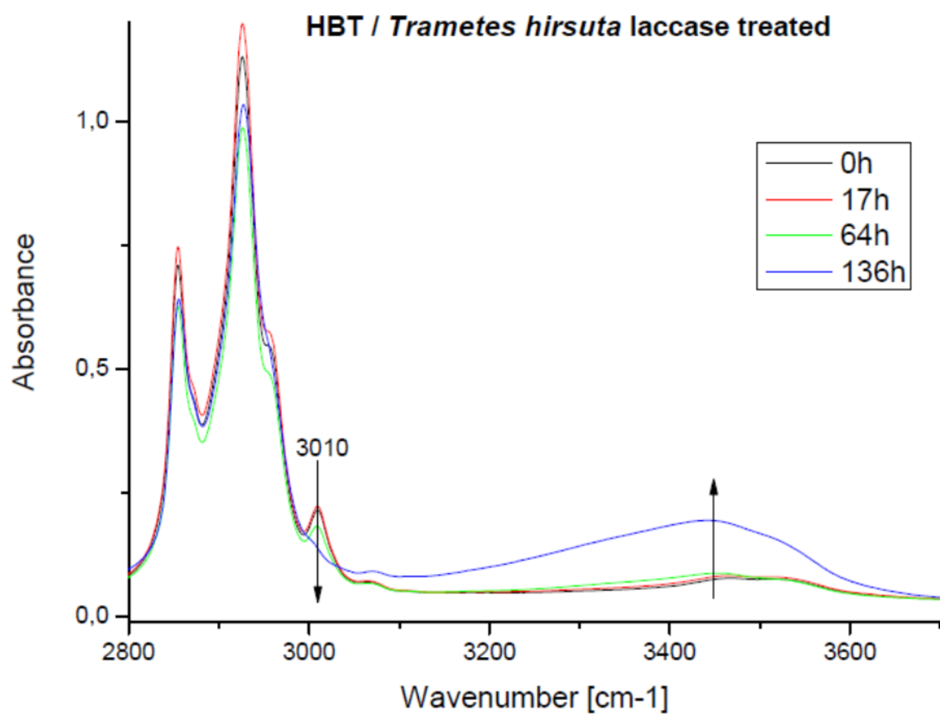
64h
136h
1,5
1,0
0,5
0,0
1000
1500
2000
2500
3000
3500
4000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Absorbance
Wavenumber [cm-1]
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3400
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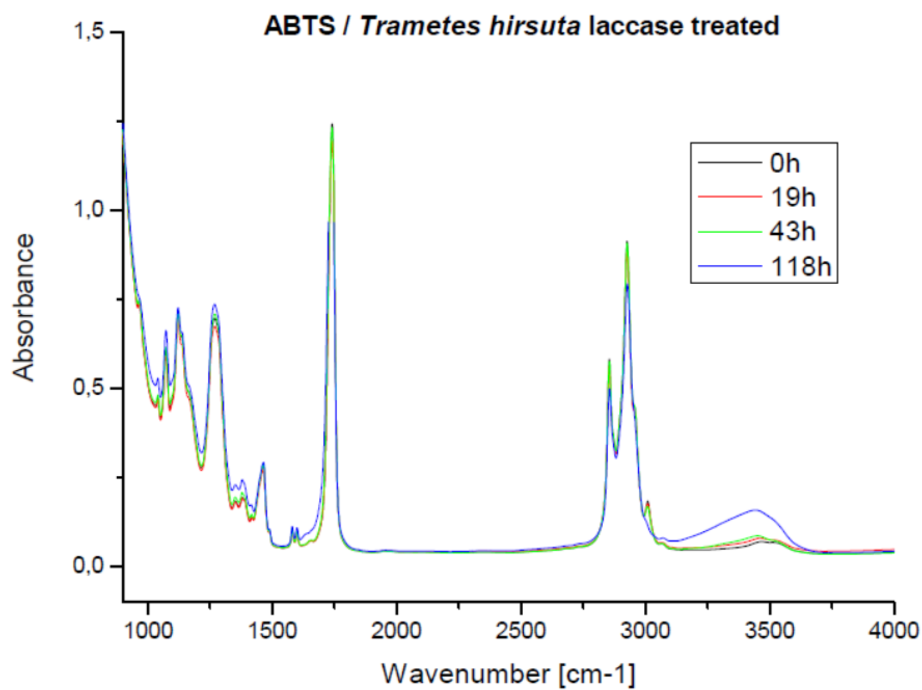
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43h
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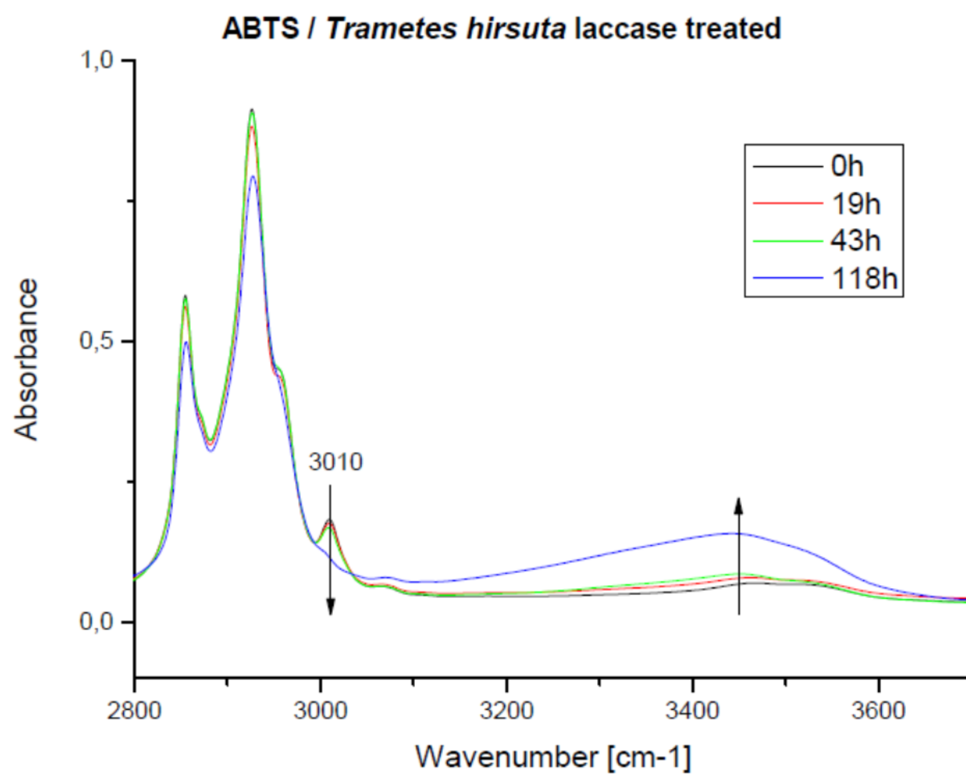
1000
1500
2000
2500
3000
3500
4000
Wavenumber [cm-1]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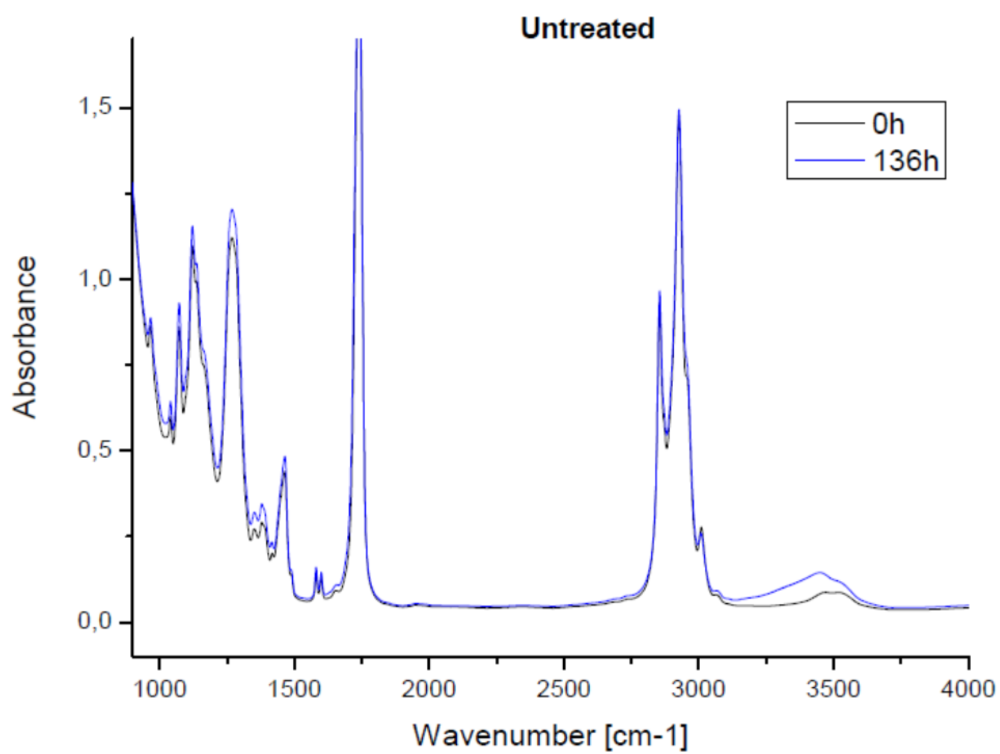
Wavenumber [cm-1]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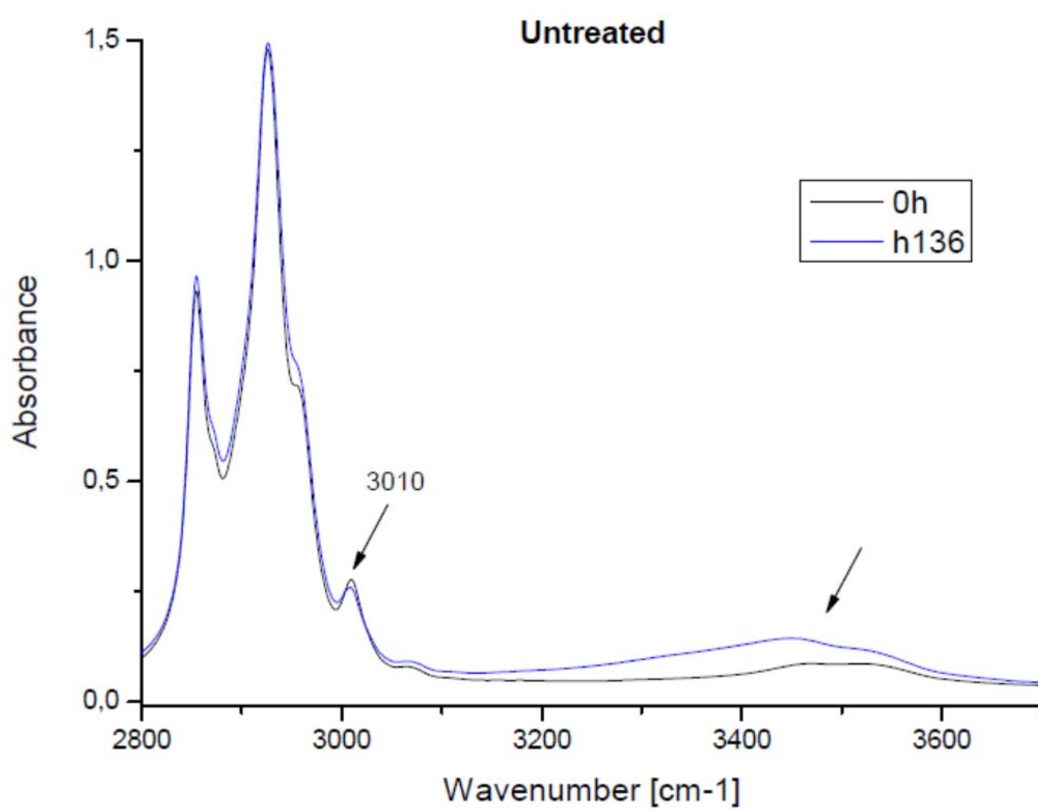
4000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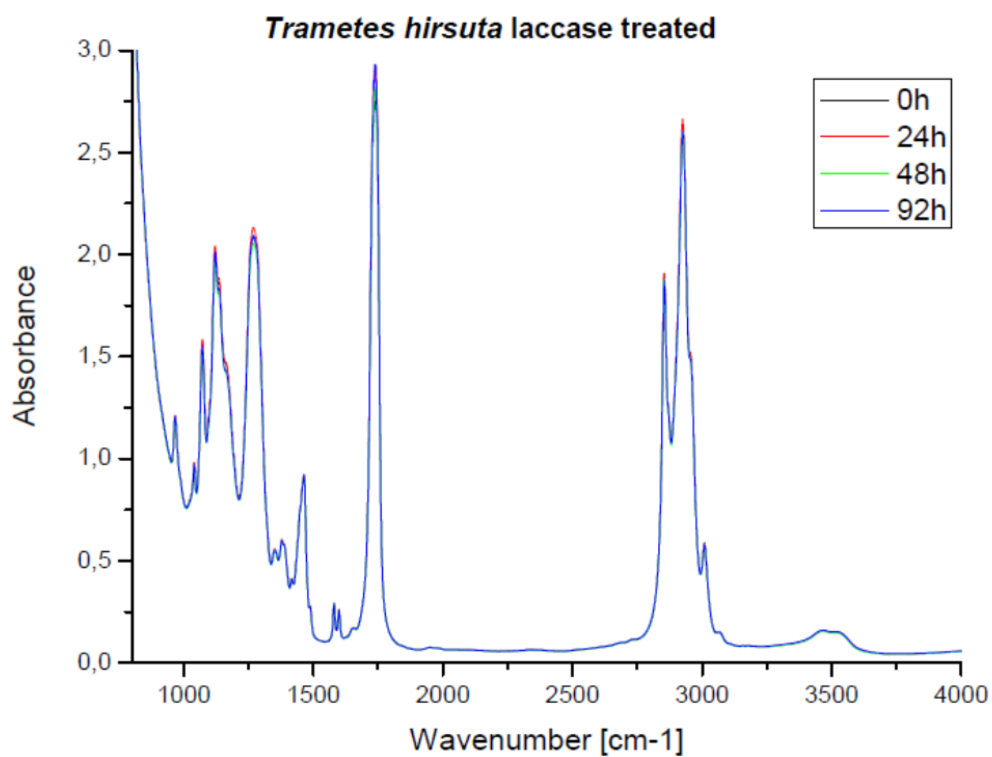
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1000
1500
2000
2500
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3500
4000
Wavenumber [cm-1]
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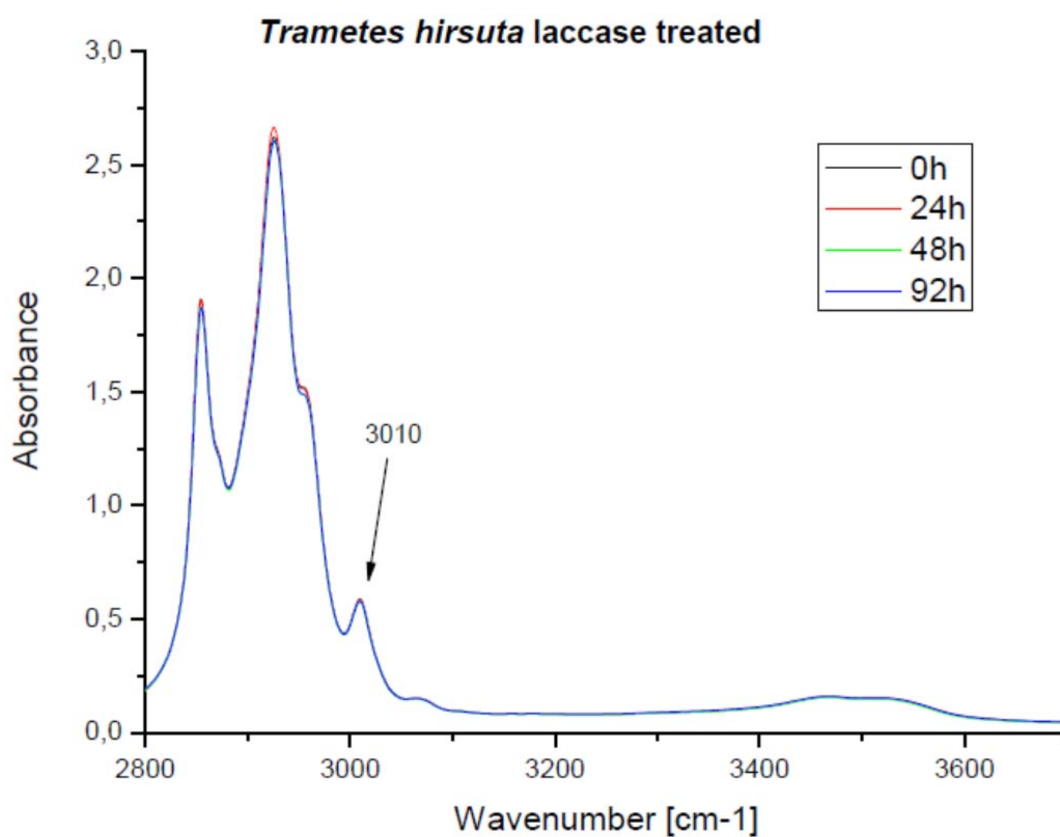
24h
48h
92h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Absorbance
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3200
3400
3600
Wavenumber [cm-1]
0h
24h
48h
92h
3010

12 Acknowledgement

The research underlying this master thesis was realized within the Austrian Centre of Industrial Biotechnology (ACIB) and work has been supported by the Federal Ministry of Economy, Family and Youth (BMWFJ), the Federal Ministry of Traffic, Innovation and Technology (bmvit), the Styrian Business Promotion Agency SFG, the Standortagentur Tirol and ZIT - Technology Agency of the City of Vienna through the COMET-Funding Program managed by the Austrian Research Promotion Agency FFG.

Many colleagues and friends contributed to the success of this master thesis.

First of all I would like to thank **Prof. Georg Gübitz**, who gave me the possibility to work on this project, for his guiding comments and ideas, for the good cooperation and for his support.

I would also like to thank especially **Katrin Greimel** and **Enrique Herrero Acero,** who supervised me during this project, for the numerous supportive and motivating discussions and the always helpful assistance. I really enjoyed working with you! ☺

Thank you to **Klaus Koren** for his support during oxygen concentration measurements.

Many thanks to the whole Gübitz group for the great working atmosphere (even if we had to share computers, tables and benches ;-)) In particular I would like to thank **Tamara Reiter**, **Angelika Schäfer**, **Konstantin Schneider** and **Doris Steifer** for their help during the experiments.

Above all I would like to thank my **parents** who always believe in me and supported me during all those years.

And finally I would like to thank my beloved family, friends and my boyfriend Alex for their patience, motivation, mental support and love.

THANK YOU!