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Enzymatic elimination of toxicants

Master Thesis

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Abstract

Air pollution is a global health problem of increasing importance. Several methods to reduce different air toxicants are known. This research focuses on the synthesis of an enzyme-based filter system which is able to filter air pollutants especially from combustion processes. Many typical toxicants were tested for their ability to be eliminated by laccases by direct oxidation, in the presence of mediators or by coprecipitation in the presence of other phenolics. Enzymatic elimination of toxicants was mechanistically investigated using spectrophotometry, HPLC/MS, GC/MS, and oxygen consumption. For in situ removal of the air pollutants, the enzyme was successfully immobilized onto a solid support maintaining enzymatic activity.

Kurzfassung

Luftverschmutzung ist ein weltweites Gesundheitsproblem von zunehmender Wichtigkeit. Verschiedene Methoden wurden bereits untersucht um einzelne, unterschiedliche Giftstoffe aus der Luft zu reduzieren. Diese Arbeit beschäftigt sich mit der Synthese eines Enzym-basierten Filtersystems, welches fähig sein soll Luftschadstoffe, vor allem von Verbrennungsprozessen, zu filtern. Einige typische Giftstoffe wurden auf der Fähigkeit von Laccasen abgebaut zu werden getestet, sei es durch direkte Oxidation, in Gegenwart von Mediatoren oder durch Copräzipitation. Diese Abbau-Prozesse konnten erfolgreich mit unterschiedlichen analytischen Methoden mechanistisch erforscht werden, u.a. Spektrophotometrie, HPLC/MS, GC/MS und Sauerstoffverbrauch. Um die Luftschadstoffe in situ entfernen zu können, wurde das Enzym erfolgreich auf einem Festkörper unter Beibehaltung der enzymatischen Aktivität immobilisiert.

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1. Introduction

1.1. Environmental pollutants from combustion processes

1.1.1. Introduction

Air pollutants are a complex mixture of different compounds existing in gaseous (e.g. NO_x, CO, O_3, SO_2), liquid and solid phases (particulate matter)[1]. Airborne particulate matter (PM) is a heterogeneous mixture of solid and liquid particles suspended in air. These particles may vary heavily in size and chemical structure. The sources are both natural and man-made and include amongst others emissions from motor vehicles, road dust, industrial combustion, agriculture, wood burning. Many chemical compounds of particle matter were identified such as nitrates, sulfates, organic compounds (e.g. polycyclic aromatic hydrocarbons), biological compounds and metals[2].

Because of the complex composition of particulate matter the analysis and regulation is based on the particle sizes; PM10, for example, includes particulate matter with a median diameter of smaller than 10 µm. Over time the standards started considering smaller particles with diameters smaller than 2.5 µm (PM2.5) because of their ability to reach the small airways of human body. PM2.5 consists mostly from combustion processes, whereas the particles with diameters between 2.5 and 10 µm are predominantly from natural sources[2].

The main source of air pollution in the modern urban and industrial world is the combustion of fossil fuels (e.g. automobiles, industry). In developing countries cooking and the burning of wood are other important sources of air pollution[2]. Table 1 shows a shortened list of relevant pollutants produced during the smoking of tobacco. This list is an example of the diversity of air pollutants and is an example for the complexity of air pollution. The last column indicates the phase of the individual contaminant, either vapor or particulate phase.

Table 1. Examples of phase conditions of some combustion products using cigarette smoke as model[3]

Major classes of compounds produced during smoking	Constituents	Smoke phase
Major products	Tar	P
	Nicotine	P
	CO	V
Inorganic gases	NH_3	V
	HCN	V-P
	NO_x	V
Tobacco-specific nitrosamines	N'-Nitrosonornicotine (NNN)	P
	N'-Nitrosoanabasine (NAB)	P
	N'-Nitrosoanatabine (NAT)	P
	4-(Methylnitrosamino)-1-(3-pyridyl)-1 butanone	P
Phenols	Phenol, o-cresol, m-cresol, p-cresol	V-P
	Catechol, hydroquinone	P
	Resorcinol	P
Carbonyls	Formaldehyde	V-P
	Acetaldehyde, acetone	V-P
	Propionaldehyde, methyl ethyl ketone, acrolein	V-P
	Butyraldehyde, crotonaldehyde	V-P
Polynuclear aromatic hydrocarbons (PAHs)	Benzo[α]pyrene	P
Aromatic amines	1-aminonaphthalene, 2-aminonaphthalene	P
	3-aminobiphenyl, 4-aminobiphenyl	P
Semi-volatiles	Pyridine, styrene	P
	Quinoline	P
Vapour phase	1,3-Butadiene, benzene, toluene, acrylonitrile	V-P
	Isoprene	V-P
Metals	Pb, Cr, Ni, Hg, As, Cd, Se	P

*P: particulate; *V: vapor

1.1.2. Polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic compounds containing two or more aromatic rings[4]. They are produced by incomplete burning of coal, oil, gas, wood and other organic substances such as tobacco. This chemical class consists of more than 100 different substances. Usually PAHs are found as a complex mixture and not as single compounds[5]. Some important PAHs are listed in Fig. 1.

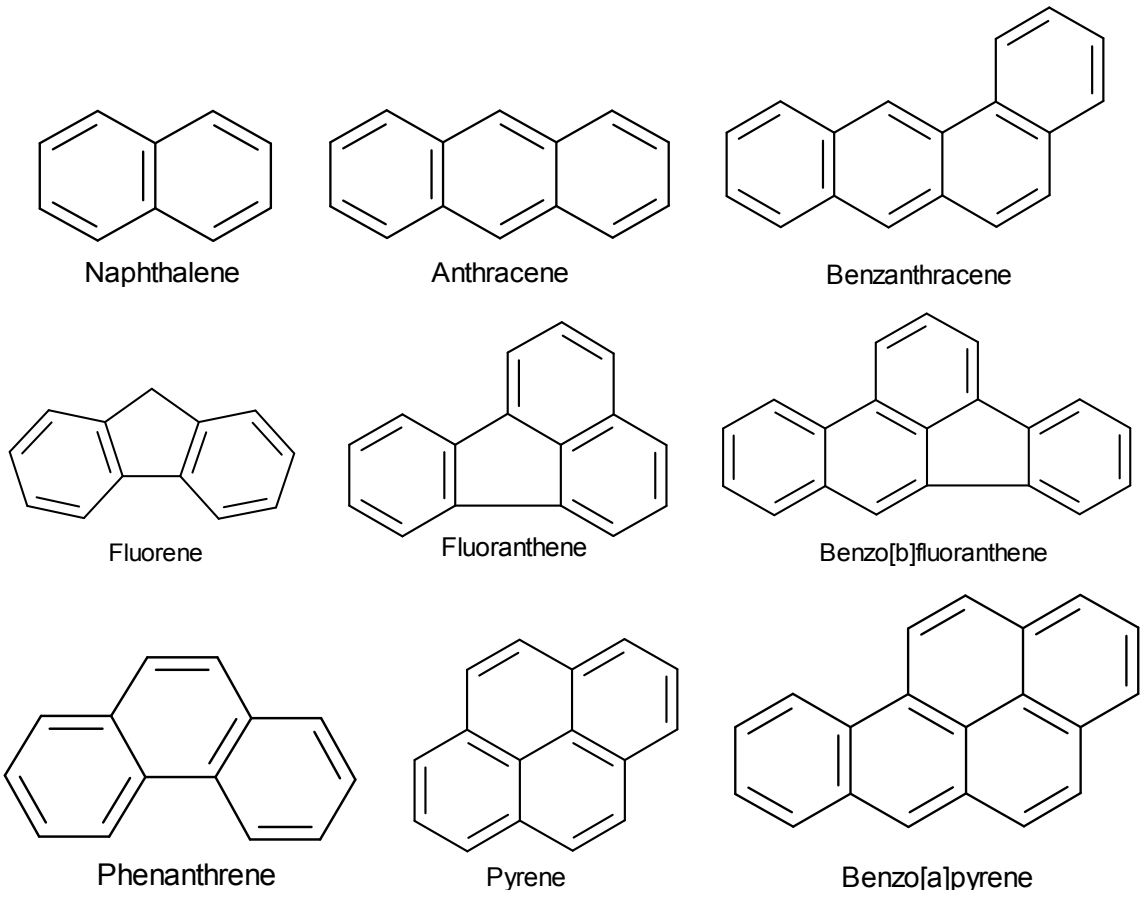

Fig. 1. Selection of important PAHs

At room temperature PAHs are solids. They have commonly high melting- and boiling-points and their solubility in water is very low. PAHs are soluble in many organic solvents and are lipophilic[4].

Sources of human and environmental exposure

PAHs occur in the environment most of the time in air from wood burning, volcanoes and from the exhaust of automobiles and trucks. They also can enter soil at hazardous waste sites and water from industrial plants. The environmental movement of the individual PAHs depends on different properties, such as solubility in water and how easily they evaporate into the air. PAHs tend to stick on solid particles in air, water and soils. They break down in the air in some days to weeks due to sunlight and other chemicals; in soil and water it might take weeks to months[5].

Humans are exposed to PAHs indoor and outdoor. Outside sources are cigarette smoke, exhausts from vehicles, coal, wood burning and industrial waste incineration. The main indoor sources are as well tobacco smoke and smoke from wood fires but also some food might be contaminated[5]. Contaminated food is a result of direct

contact with PAHs containing soil or air, or processing, baking or frying where PAHs are produced. High temperature cooking of food increases the amount in the food. The levels of individual PAHs tend to be higher in winter than in summer due to additional residential heating during the cold season[4].

PAHs can enter the body via inhalation, nutritional uptake and direct contact with the skin. They tend to be stored in fat parts of the body. PAHs are transformed in the body into different substances. Animal studies showed that they are not stored for a long time in the body but leave it within a few days[5].

Effects on human health

PAHs, such as benzo[α]pyrene or benzo[β]fluoranthene, caused tumors in laboratory animals, which were in contact with them through air, food or by constant direct contract with the skin. Studies of humans showed that longtime exposure might results in developing cancer[5].

Other studies in animals showed harmful effects on skin and body fluids after short- and longtime exposure. Studies on pregnant mice fed with benzo[α]pyrene showed difficulties in reproducing and caused birth defects. Similar effects are also possible for humans, but there is no data available to confirm this assumption[5]. This lack of data is mainly due to the fact that humans are normally not exposed to individual PAHs but to a complex mixture[4].

1.1.3. Nitrogen oxides

Nitrogen oxides are substances consisting out of nitrogen and oxygen. Several molecules belong to this group such as nitric oxide (NO), nitrogen dioxide (NO_2), nitrogen trioxide, nitrogen tetroxide (N_2O_4) and dinitrogen pentoxide (N_2O_5)[1]. NO_x is a collective name for all the nitrogen and oxygen containing substances in the air[1] or just the sum of NO and NO_2[6], depending on the source of the information. The main focus of toxicological research of nitrogen oxides lies on NO_2. This is because of several reasons:

- NO_2 is a regulated air pollutants with available standards
- NO from exhaust and power plants is largely converted to NO_2
- NO_2 plays an important role in the formation process of ozone (O_3)[1]

Sources of human and environmental exposure

Nitrogen oxides are formed by combustion processes in motor vehicles and industrial processes and are exposed to the air mainly in the form of NO and NO_2[6]. The exposure is related to the amount of traffic, which is shown by peaks in the NO_x concentrations in the morning and late afternoon when it is rush-hour traffic[1]. In atmospheric processes they are part of complex chemical reactions. NO is oxidized to NO_2 and other products. The oxidized nitrogen compounds have a negative impact on human health and the environment and play an important role in the formation of tropospheric ozone[6].

The exposure to humans varies greatly. It depends on time of day, season, whether it is in urban or rural area, indoor or outdoor. Indoor concentrations depend heavily on ventilation and places of possible combustion[6].

Effects on human health

As mentioned before NO_2 is the most studied molecule of the nitrogen oxides. In animal studies NO_2 leads to effects on host defense against infectious pulmonary diseases and lung problems. The respiratory tract is a first barrier against inhaled gaseous agents. NO_2 exposure can result in malfunctions of this line of defense. Tests on lab animals showed an increased mortality with increased NO_2 concentration or duration of exposure when inducing additional infections[6].

As humans are exposed to NO_2 all the time, longtime studies on animals are very important and showed a variety of structural alterations in the pulmonary system. Mutagenic effects were only observed in bacterial cells but none in mammalian cell cultures[6].

1.1.4. Aldehydes

Aldehydes are organic compounds containing a carbonyl group and are commonly present in both indoor and outdoor environments. Formaldehyde and acetaldehyde

play an important role to the air quality because of the presence in greater quantities but also due to the different effects on human health[7,8].

1.1.4.1. Formaldehyde

Formaldehyde is a colorless, flammable gas at room temperature. It has a pungent, irritating odor and may cause burning to the eyes, nose and lungs at higher concentrations. Formaldehyde reacts with many other chemicals, and breaks down into methanol and carbon monoxide at very high temperatures[7].

Sources of human and environmental exposure

Formaldehyde is present in the environment because of natural processes and from man-made sources, although combustion is the largest source. A formation in large quantities occurs in the troposphere by the oxidation of volatile hydrocarbons. Smaller natural sources are the decomposition of plant residues and the transformation of various chemicals emitted by foliage[7,9]. Formaldehyde is naturally produced in very small amounts in human bodies as part of metabolism and causes no harm[7].

The main sources of formaldehyde in human households are building materials, new furniture out of plywood, resins containing formaldehyde, as well as tobacco smoke and combustion processes in general[9]. It is also used as a preservative in some foods, such as some types of Italian cheeses, dried foods and fish[7].

Formaldehyde is released to the air from many other home products as well and is exposed to the humans via breathing while using these products. Small amounts of formaldehyde are released by some paper products, such as grocery bags and paper towels. Because these products contain formaldehyde, the skin might also be a point of exposure by touching or coming in direct contact with them[7].

The age of the building and the different seasons are factors which influences the levels of the formaldehyde emission, so are temperature, humidity, ventilation rate, product usage, presence of combustion sources and the smoking habits of occupants[8,9].

The amount of formaldehyde in mobile homes is usually higher than it is in normal households because of their lower air turnover[7]. Employees in hospitals and

scientific facilities might be exposed to higher amounts of formaldehyde as it has to be used as disinfectant or preservative[9].

The levels of formaldehyde found outdoors are generally significantly lower than those inside the households[8]. Outdoor formaldehyde pollution is largely caused by human activities including car exhaust fumes, above all engines without catalytic converters and those using oxygenated gasoline, and industrial plant emissions[9].

Most of the formaldehyde exposure in the environment is through the air. Formaldehyde is dissolved easily in water, but it does not last a long time in water and therefore is normally not found in drinking water supplies. Most formaldehyde in the air breaks down during the day. The breakdown products of formaldehyde in air include formic acid and carbon monoxide. Formaldehyde does not seem to build up in plants and animals, and although formaldehyde is found in some food, the quantity found is not high[7].

Effects on human health

Direct contact between formaldehyde and tissues leads to irritations. The effect of formaldehyde on people depends on the sensitivity of the individual person. The most common symptoms include irritation of the eyes, nose, and throat, along with increased tearing[7,9]. Moreover, formaldehyde is able to induce respiratory sensitization, inducing allergic asthma, as well as skin sensitization. It is one of the agents commonly responsible for contact dermatitis[10].

Drinking large amounts of formaldehyde can cause severe pain, vomiting, coma, and possible death. At direct contact with a strong solution of formaldehyde the skin can become irritated[7].

Several studies of laboratory rats where the test animals were exposed to high amounts of formaldehyde in air, resulted in rats developing nose cancer[7]. Some studies on humans exposed statistically significant exposure–response relationships for peak and cumulative exposure of formaldehyde and the increased number of nasopharyngeal cancer[10].

1.1.4.2. Acetaldehyde

Acetaldehyde is a colorless, volatile liquid with a pungent suffocating odor. It has a boiling point of 20.4 °C. Acetaldehyde is highly flammable and a reactive compound that is miscible in water and in the most common solvents[11].

Sources of human and environmental exposure

Acetaldehyde is naturally produced by combustion and photo-oxidation of hydrocarbons commonly found in the atmosphere[12]. It is suggested that the removal of acetaldehyde in the atmosphere is predominantly achieved via radical formation and photolysis[11]. It is also produced in surface water by photochemical processes[12].

As acetaldehyde is an important industrial chemical, it might be released into the air or in wastewater during production and use. At low levels it occurs in drinking-water, surface water, rainwater, effluents and engine exhaust[12].

Acetaldehyde is an intermediate product in the metabolism of humans and higher plants and is a product of alcohol fermentation. The metabolism of alcohol is the main source of exposure to acetaldehyde for the normal population[11]. Because of the human metabolism acetaldehyde occurs in trace quantities in human blood. It also can be found in small amounts in all alcoholic beverages, such as beer, wine and spirits and in plant juices and essential oils and roasted coffee[12].

The second most significant source of exposure is cigarette smoke. Other sources of exposure are degradation of hydrocarbons, sewage, and solid biological wastes, as well as the open burning and incineration of gas, fuel oil, and coal[11].

Effects on human health

Acetaldehyde is mildly irritating to the eyes and the upper respiratory tract by exposure of a short period according to limited studies on humans. Based on indirect evidence, acetaldehyde is presumably a toxic metabolite playing a role in the induction of alcohol-associated liver damage and developmental effects. On the other hand, the acute toxicity by inhalation or oral ingestion tested on animals is low[11].

The data available on longtime effects of acetaldehyde following an oral uptake are limited. Studies on rats showed increased hyper-keratosis of forestomach. Studies

on inhalation based on experiments with rats and hamsters identified the upper respiratory tract as the target issue in the body. In these studies increased numbers of different nasal and cell carcinomas were observed in the test animals[11]. Intratracheal instillation in hamsters did not cause an increased number of tumors of the respiratory tracts. But inhalation of acetaldehyde has an impact on the increased number of respiratory-tract tumors produced by the intratracheal instillation of benzo[α]pyrene[12].

The data available to conclude potential risks of acetaldehyde on reproductive, neurological and immunological aspects of organisms is inadequate and limited. Due to the short half-lives of acetaldehyde in air and water and its biodegradability, the effect of acetaldehyde on the environment is expected to be low[11].

A study of chemical workers with high exposure to different aldehydes showed an increased frequency of bronchial and oral cavity tumors. The risk of getting several diseases like oesophageal, oral and pharyngeal tumors and cancer is associated with high metabolic levels of acetaldehyde after heavy alcohol intake, according to the genetic polymorphism of the enzymes which are involved in the metabolism of alcohol to acetaldehyde and in the following metabolism of acetaldehyde itself. Although the studies had limitations in design and size, a consistent increased risk of alcohol-related cancers was observed among test persons with genetic polymorphism of the enzymes of the acetaldehyde metabolism compared to persons with other genetic polymorphisms. This is caused by the higher internal doses of acetaldehyde after heavy alcohol consumption[12].

Acetaldehyde is metabolized to acetic acid. It causes gene mutations in bacteria and mammalian cells as well as sister chromatid exchanges and micronuclei in the cultured mammalian cells. In vivo, acetaldehyde leads to DNA damage in cultured mammalian cells and in mice[12].

The evaluation of the International Agency for Research on Cancer (IARC) concluded that there is sufficient evidence for the carcinogenicity of acetaldehyde concerning experimental animals. Due to the lack of available data there is inadequate evidence concerning humans, but acetaldehyde is classified as possibly carcinogenic to humans[12].

1.1.4.3. Acrolein

Acrolein is a clear or yellow, volatile and highly flammable liquid with a burnt, pungent odor[13,14]. It is highly soluble in water and in solvents like ethanol and diethylether. Due to the conjugation of a carbonyl group with a vinyl group it has high reactivity. Acrolein is involved in many different types of reactions such as Diels-Alder condensations, di- and polymerization, oxidation, reduction and some more. Without inhibitor an exothermic polymerization occurs enhanced by light and air. In the presence of acids or strong bases this polymerization takes place even with an inhibitor[14].

Sources of human and environmental exposure

Acrolein is found in outdoor air because of the breakdown of many pollutants. Incomplete combustion and thermal degradation of tobacco, wood and plants produce acrolein. Other combustion processes like the burning of gasoline in automobile engines cause as well its formation. Acrolein might also be found near hazardous wastes sites in which it is not stored properly. Here there is a possible exposure via air, drinking water or direct contact with contaminated soil[13].

The human body is able to produce small amounts of acrolein as breakdown products of amino acids and fatty molecules. When overheating fats, acrolein is formed as well. Therefore it is also be found in foods such as fried foods or roasted coffee[13].

Acrolein present in the air as vapor reacts into other chemicals within days. In water it dissolves easily and parts of it change into vapor and enter the air. The remaining acrolein is transformed into other chemicals which are broken down or removed. Acrolein present in soil might be quickly evaporated, washed out with water or inactivated by binding to other materials in soil[13].

The principal use of acrolein is being an intermediate of the production of other chemicals. In direct use it is applied as biocide to recirculating process water systems and irrigation canals to control algae, slime and plants[13,14].

Effects on human health

Acrolein may cause toxic effects after oral, inhalation or dermal exposures. It is irritating the mucous membranes and, at high concentration, leads to irritation of the skin. Due to acrolein uptake a change in body and organ weight and in serum biochemistry have been observed in animals, as well as skeletal malformations. These might be secondary effects caused by irritations of the respiratory or the gastrointestinal tract[13].

Acrolein, as vapor or liquid, can cause eye irritation by direct contact. Depending on the concentration it leads to stinging, increased blinking and lacrimation. At lower levels of exposure the human eye seems to adapt to the irritation as a study reports no increase in discomfort after a certain timeframe at a constant level of acrolein vapor exposure. Dogs and monkeys appear to be more sensitive to acrolein than rodents, as they showed lacrimation and blinking of the eyes, whereas there was no ocular irritation observed with guinea pigs and rats. The relation between exposure level and structural damage of the eye is unknown due to inadequate data[13].

Acrolein may also affect the entire respiratory tract, the nasal irritation being the most sensitive effect. The type and level of the effect depends on the exposure level; from reduced breathing rates to pulmonary edema and death. Compared to the ocular effects, the respiratory effects appear to be similar across species and the duration of the exposure[13].

Oral exposure leads primarily to an irritation of the gastrointestinal mucosa, although human data is not available. The effects in animals are similar and depending on the exposure level across the tested species for acute and intermediate exposures. During chronic exposures an adaptation to the irritation might take place. Increasing the levels leads to vomiting, ulceration and edema of the stomach mucosa[13].

There is a weak mutagenic potential in acrolein by having negative impact on the DNA repair mechanisms as shown in in vitro studies[13]. In vivo and in vitro studies showed the inhibition potential of acrolein in the synthesis of nucleic acids[14]. The data proving carcinogenicity of acrolein is insufficient. Therefore the Department of Health and Human Services (DHHS) and the International Agency for Research on Cancer (IARC) did not classify it as carcinogenic in humans[13].

1.1.5. Carbon monoxide (CO)

Carbon monoxide is a colorless, odorless, tasteless and highly poisonous gas. It is a product of incomplete combustion of carbon containing fuels and is found in outdoor and indoor air[15,16]. It is produced naturally and by humans for example from the exhaust of automobiles or wood burning stoves. Industrially carbon monoxide is used in the synthesizing process of different compounds such as acetic acid or polycarbonates[15]. With increasing levels of exposure it leads to severe effects on human health, even to death. An important role in these effects plays the formation of carboxyhaemoglobin, which interferes with oxygen transport in the blood[16].

Sources of human and environmental exposure

Carbon monoxide is a trace part of the troposphere and is produced by both nature and human activities. Plants are able to produce and metabolize carbon monoxide, so it is part of the natural environment. There is no data available indicating that carbon monoxide has any negative effects on plants or microorganisms. But there is a harmful effect on human health, depending on the levels[16].

Humans are exposed to carbon monoxide during their typical daily activities. The levels vary depending on the place and time which often correlate with the traffic level[15]. It appears in different microenvironments: while travelling with motorized vehicles, cooking or heating with domestic gas or wood, while working at the job as well as in burnt tobacco products (active smoking and second-hand smoke). The biggest impact for human health are the concentrations of carbon monoxide indoor and in motor exhaust[16].

The physiological norm for carboxyhaemoglobin for non-smokers is estimated to be approximately 0.3-0.7 %. The general population is exposed to a small to medium amount of carbon monoxide leading to 1-2 % carboxyhaemoglobin. Some workplaces or even homes exceed 10 %. Regular smokers are able to increase their carboxyhaemoglobin concentration to 3-8 % because of the inhaled smoke[16].

Indoor concentrations depend on outdoor concentrations, sources of carbon monoxide inside and ventilation. The highest amounts are found in enclosed parking garages, service stations and restaurants. The working place is an important factor in the daily carbon monoxide uptake. Every occupation related with vehicle exhaust,

such as auto mechanics, bus, truck or taxi drivers have an increased risk of exposure[16].

Effects on human health

Carbon monoxide influences the metabolism of cells via hypoxic and non-hypoxic modes of action. It is able to bind to heme and alter function and metabolism of heme proteins. The formation of carboxyhaemoglobin reduces the oxygen transport in the blood. It is also able to reduce the oxygen storage in muscle cells. The most vulnerable tissues for hypoxic injuries are therefore those having the highest need of oxygen, such as brain and heart. The non-hypoxic mechanisms involve the binding of carbon monoxide to other heme proteins and therefore interfere with the physiological regulatory systems[15].

Human clinical studies and animal studies showed a relation between adverse cardiovascular effects and carbon monoxide exposure leading to a certain carboxyhaemoglobin level, enhancing problems of patients who already had coronary diseases. Different animal studies over a wide range of exposure conditions showed that carbon monoxide exposure leads to effects such as cardiac hypertrophy, cardiac arrhythmias and possibly atherosclerosis[15].

Carbon monoxide poisoning is one of the leading causes of death caused by poisoning in the United States of America. The extent of problems from acute exposure depends on the concentration and duration and the health of the individual person. As carbon monoxide affects nearly all tissues, the symptoms may vary, including headache, vomiting, dizziness and blurred vision. As the symptoms of a mild carbon monoxide poisoning are similar to flu-like viral diseases, there might be misdiagnosis. Symptoms of more severe poisoning include cardiac arrhythmias, cardiac arrest, hypotension or respiratory arrest. During pregnancy carbon monoxide might lead to fetal death. The severity of the symptoms doesn’t always correlate with the actual level of poisoning because it depends as well on other factors like health status of the individual, length of the exposure, time between the exposure and the measurement of the carboxyhaemoglobin and supplemental oxygen treatment of the patient[15].

1.2. Oxidative stress and its consequences on human health

1.2.1. Introduction

Combustion processes generate a complex mixture of aerosol and gases containing reactive species, toxic compounds, metals etc. These compounds have been identified as the causes of more than 50 oxidative stress related diseases including cancer, cardiovascular diseases, diabetes, atherosclerosis and many others[17,18].

Oxidative stress is a consequence of an increase in oxidants in the body; a condition which overcomes the body's ability to remove these reactive species. This imbalance leads to the damage of biomolecules. This might be accomplished by an excess of oxidants or by a decrease of the available antioxidants. Atoms having a single unpaired electron have increased reactivity and are referred as free radicals. These free radicals react with neighboring molecules and depending on the number of reactions they can cause severe cellular damage[19].

Fig. 2 describes this balance which is on the side of the free radicals and therefore on the side of the oxidative species. This excess on free radicals leads to oxidation and damage of several important biomolecules, such as lipids, proteins or nucleic acids, in the human body leading to various diseases and problems.

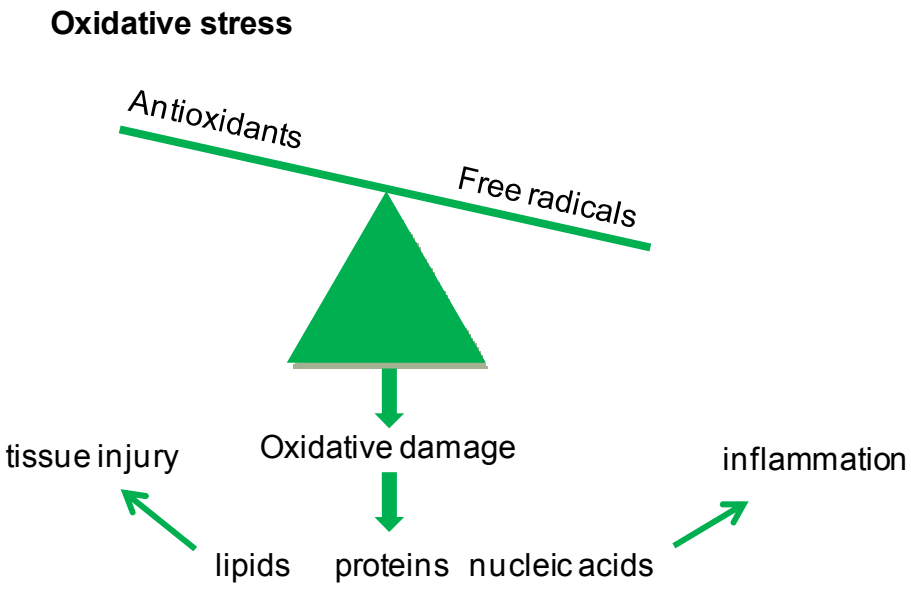

Fig. 2. Overview of oxidative stress, its oxidant-antioxidant balance and its consequences[19]

Free radicals affect the human body from endogenous (cellular metabolism) and exogenous (metals, air pollution, solvents, pesticides, etc.) sources. For protection biological systems developed a complex antioxidant system against reactive species. This protection system includes an enzymatic system (e.g. glutathione peroxidase, catalase, superoxide dismutase) and a non-enzymatic system (e.g. glutathione, ascorbic acid, α-tocopherol, β-carotene)[20,21]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are major concern on health. They both play a dual role by being beneficial for the body, as part of the normal cellular metabolism, and as well being harmful[22]. To obtain better conditions for human health there are two possibilities: on the one hand increasing the level of antioxidants and enhancing the protection system, and on the other hand decreasing the level of free radicals and their sources.

1.2.2. Oxidative stress associated diseases

Oxidative stress is the reason for the start of many different diseases. It plays a role in cardiovascular and neurological disorders, diabetes, pulmonary diseases or aging[23,24,25,26]. The lung is one of the primary targets of oxidative damage caused by air combustion pollution. However the surface of the lung is protected by a thin layer of fluid with antioxidants to prevent oxidative stress. Healthy persons have an antioxidant enriched lung lining fluid, but asthmatic patients for example, who are more sensitive to air pollution, have lower levels of this protection fluid[19].

1.3. Strategies for pollution control and the prevention of health related problems

In order to prevent the human body acquiring oxidative stress related diseases, different strategies have been developed including

a. Methods for monitoring oxidant and antioxidant levels. One example developed in our lab is the use of tetramethoxy azobismethylene quinone (TMAMQ) in an assay to measure antioxidant capacity. This assay uses the fact the TMAMQ is reduced by a wide variety of structurally different antioxidants and is therefore able to determine the level of the antioxidant capacity[27,28,29]. By using methods like this it is possible to monitor the total antioxidant capacity of a system.

b. Strategies for removal/sequestrating combustion toxic products
c. Methods for detecting the presence of toxic products from combustion processes

1.3.1. Pollution control strategies

In general there are two main control strategies for pollution, “dilution and dispersal” and on the other hand “concentration and containment” “Dilution and dispersal” means that the pollutants are attenuated by spreading out the molecules. This results in a reduction of the concentration in a specific point and therefore reducing the risk for health related problems of humans. The second main strategy, “concentration and containment”, tries to accomplish the exact opposite. Instead of spreading the pollutants over a large area, the goal is to concentrate and capture them in a specific place. The best method has to be chosen depending on the individual problem which has to be solved; there is no “best” method in general[30].

The strategies for the removal/sequestration of air pollutants can also be classified into physical methods (adsorption, etc), chemical methods (chemical oxidation etc.) and biological methods.

1.3.2. Absorption

Absorption is a diffusional mass-transfer operation where a soluble component of the gas phase is removed in a liquid by dissolving it. The driving force here is the difference in the concentration of the individual substance between gas and liquid phase. Therefore by having a higher concentration in the liquid phase than in the gas phase, desorption might occur. The liquid phase mainly consists of water but it is possible to use low volatility organic liquid as well[31].

The two phases (gaseous and liquid) have to be in direct contact in order to have an exchange. It is possible to run these streams in the same direction (co-current flow) or in opposite direction (countercurrent flow). A combination of both running methods may improve the removal of the contaminants[31].

The solvent might be recovered by distillation or by stripping the absorbed material from the solvent (desorption). Absorption is efficient by having high concentrations of

the pollutants and works especially well with inorganic acid gasses. Some absorption processes include the removal of hydrochloric or hydrofluoric acid vapor in water or chlorine gas removal of an alkali solution. This method might be working most effectively when combining it with other methods such as adsorption or incineration[31].

1.3.3. Adsorption

Adsorption is as like absorption a mass-transfer process. In this case solids are brought in contact with an adsorbing material. The individual particulate matter must have the ability to concentrate or adsorb on the surface of this adsorbing material to obtain a positive effect. There are two phenomena which occur during adsorption: Physical adsorption at low-temperatures, which is similar to condensation, and chemisorption at high temperatures. There are different adsorbents which are used; typically activated carbon, silica gel and alumina[31].

Adsorption is applied in the recovery of components, valuable solvents from air streams and odor removal. The applications vary from solvent reclamation in dry cleaning to the recovery of ethyl acetate and toluene from cellophane drying operations. The limitation of the method depends above all on the properties of the adsorbents as well as on the properties of the adsorbate. If the concentration is too high exothermic reactions might occur and even an explosion might follow. The molecular mass of adsorbate plays as well an important role as low mass components are highly volatile and therefore less likely to be adsorbed and high mass components are strongly adsorbed but are difficult to remove from the adsorbent during the recovering process. Problems for this method might be moisture, as water might block the adsorbent for the adsorbate, and the temperature of the gas because of possible exothermic reactions[31].

1.3.4. Thermal oxidation

Volatile organic compounds (VOCs) are easily combustible and therefore appropriate for combustion processes. During these processes the organic material is oxidized to CO_2 and water. There are three types of combustion processes which are used for emission control[31]:

- Thermal oxidation – flares
- Thermal oxidation and incineration
- Catalytic oxidation

In the process of flaring VOCs are burned in either an open or an enclosed flame. It is used to control a variety of flammable VOC streams and is able to tolerant differences in VOC concentrations and flow rate. Flares are ideal for infrequent, large volumes of concentrated hydrocarbon emissions[31].

A thermal oxidizer, or incinerator, burns VOCs in gas streams in an enclosed chamber with one or more burners. The incoming gas can be burnt with additional natural gas to ensure high oxidation temperatures. By proper control of the combustion process high destruction efficiency is possible. The most important parameters for this method are: time, temperature and turbulence. The installation of this method is costly due to equipment, fuel and process-control[31]. This system works efficiently with high concentrated pollutants; diluted gas streams are too energy intensive and therefore too costly[30]. They are also a major source of environmental pollution therefore considered environmentally unfriendly[31].

Catalytic oxidation uses as well thermal oxidation to destroy hydrocarbon in contaminated streams, but using lower temperature and with the help of a catalyst. Therefore due the reduction of fuel needed, the operating costs are reduced as well. Another advantage is the reduction of NO_x and CO because this system enhances the oxidation of these molecules. In order to save additional fuel costs, a heat exchanger might be used for heat recovery. The ideal working conditions for this system include low concentrations in the stream, because by using higher concentrations overheating and deactivating the catalyst is possible. Materials such as platinum or palladium, cobalt oxide or copper/manganese oxide are used as catalysts. The ideal working temperature depends heavily on the catalyst and its properties. This method might be limited because of various contaminants in the gas stream which inhibit the catalyst[31].

1.3.5. Condensation

Condensation of vapor might occur as a film of the material on a wall of the condenser tube or as several drops forming various points on the surface. The film-

type condensation is more common und more often used. The lack of information on the dropwise condensation supports this fact. The film covers the surface uniformly, whereas the dropwise method is not that evenly distributed. The individual drops grow on the surface and coalescent with other drops. At a certain point gravitation leads to running off the surface[31].

Using high concentrations of pollutants, condensation systems are used as preliminary pollution control followed by other already described methods. During the condensation a phase change occurs from gas to liquid which can be enforced by increasing pressure and maintaining the temperature, or by lowering the temperature while maintaining the current pressure. Depending on the boiling points of the individual compounds and therefore the volatility, the temperature has to be decreased in order to obtain a successful condensation.

1.3.6. Biofiltration

Biofilters consist of a container with a filter medium in it. This medium is out of organic material such as peat and bark chips. The contaminated gas is forced through the filter[30]. Here biotransformations occur as well as adsorption, absorption to remove the pollutants from the gaseous stream[31]. The microorganisms present on this filter degrade the pollutants and thereby providing a detoxifying step. The medium has to offer perfect conditions for the microbial growth to obtain the ideal activity[30]. The organic matrix provides the necessary salts and trace elements; the pollutants are the food source[31]. For maintaining this condition the filters have to be watered but an excess of water is to be avoided[30]. Overloading the filter by providing more pollutants than might be adsorbed and oxidized leads to a decrease of removal because the contaminants can pass through the filter[31].

Biofilters work efficiently and economically for the removal of different pollutants such as volatile organic carbons (VOCs) and odor, even in low concentration. Beside this fact, the low costs of installation and operation, the longevity of the biofilter itself and the environmental safety of the operation are advantages to other control technologies[31].

There are many factors which have an impact on the rates of removal of the biofilter, such as the composition of the media, pH, moisture level, temperature, the length of

time in which the gas is in the biofilter and also the properties of the individual compounds, which are planned to be removed. The rates of degradation of the different pollutants depend on their chemical structure. Table 2 shows a list of gases classified according to their degradability. This table shows that for example alcohols and organic acids are more easily degraded than polyaromatic hydrocarbons.

Table 2. Gases classified according to their degradability in biofilters[31]

Rapidly degradable	Rapidly reactive	Slowly degradable	Very slowly degradable
Alcohols	H_2S	Hydrocarbons	Halogenated hydrocarbons
Aldehydes	NO_x (not N_2O)	Phenols	Polyaromatic hydrocarbons
Ketones	SO_2	Methylene chloride	CS_2
Ethers	HCl		
Esters	NH_3		
Organic acids	PH_3		
Amines	SiH_4		
Thiols	HF		
Other molecules with O, N or S functional groups			

Biofilters are not limited to gaseous molecules alone; they are also able to remove particulate matter and liquids from the gas streams. Nevertheless these molecules are able to plug the biofilter. The field of application for biofilters is large including above all chemical and pharmaceutical industry, wastewater facilities and food processing as a technology for odor control[31].

Biotrickling filter

Biotrickling filters are an intermediate technology between biofilters and bioscrubbers. In the vessel there is again filter medium, but it is an inert material, for example clinker or slag. As the compactibility of this material is not that good, the space between the single particles increases and therefore the surface area as well. The microbes form a biofilm on the surface of the medium particles. The contaminated air is forced through the filter, while water circulates through it, trickling down from the top. This counter flow between rising gas and falling water improves the efficiency of the method. The process is monitored by taking water samples

within the vessel. Process control might be achieved by additions to the circulating water, as needed[30].

Bioscrubber

Bioscrubbers are included in this same group of biofiltration system, although it is not truly a biological treatment system. But nevertheless it is an efficient method of removing odor components by dissolving them. It is as well suitable for hydrophilic compounds such as acetone or methanol. The contaminated gas passes through a fine water spray in the first bioreactor. The pollutant is absorbed into the water which is collected at the bottom of this bioreactor. This contaminated solution is then transferred to a second bioreactor, where the actual biodegradation occurs[30].

Biofilters compared to other pollution methods

In comparison to other methods biofilters have the advantage of being able to treat gas streams in an economically manner. Other technologies have disadvantages such as high fuel use, high maintenance requirements or high capital costs. An additional disadvantage of other technologies is the fact that pollutants are just transferred from the gaseous stream to the wash water. Biofilters are able to perform a biotransformation of the contaminants to less toxic forms[31].

1.3.7. Emerging technologies: Enzymes on biofilters or as detoxifying agents

Using whole cells for the bioremediation process can be quite difficult because of the special requirements of the living organisms on the polluted site. The most important factors here are growth and activity of the microorganisms, which can be stimulated by adding nutrients and oxygen. Microbial growth and activity also depends on the pH, temperature and moisture of the site. Most of the microorganisms only grow optimally in a small range of conditions, which makes optimal growth even harder to obtain[32].

Enzymes are the biological catalysts which facilitate the conversion from substrates to products which is achieved by the microorganisms. Enzymes are divided into six classes, namely oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases (synthetases). The major enzyme classes involved in the degradation/detoxification of wastes are hydrolases and oxidoreductases.

There are several advantages for the use of enzymes over traditional technologies and even microbial remediation:

- overcoming inhibitors of microbes
- use in extreme conditions when microbial activity is limited
- effective at low pollutant concentrations
- active in the presence of microbial predators or antagonists[33]
- no nutrient supply needed
- and others

Microbial hydrolytic enzymes

Extracellular enzyme activity is an important step in the degradation and utilization of organic polymers since only small molecules can pass through cell pores. Hydrolytic enzymes are able to disrupt major chemical bonds in the toxic molecules which results in the reduction of their toxicity. This mechanism is used for the biodegradation of oil spill, organophosphates and carbamate insecticides. Complex polymers are broken into smaller soluble molecules, which then are able to enter cells for further metabolism. Hydrolases have their advantages in the availability, lack of cofactor stereoselectivity and the tolerance of additional solvents, if they are miscible with water. Extracelluar hydrolytic enzymes relevant for pollution control include amylases, proteases, lipases, DNases, pullulanases, pectinases, cutinases, cellulases, glycosidases etc.[34,35].

Microbial oxidoreductases

Oxidoreductases mediate the detoxification of toxic organic compounds through oxidative coupling. These enzymes are able to cleave chemical bonds and to assist the electron transfer from a reduced substrate to another chemical compound. The oxidation-reduction reactions lead to oxidized contaminants, which are harmless. Oxidoreductases include several different enzymes such as oxygenases, laccases or peroxidases[34].

Plant families of Fabaceae, Gramineae, and Solanaceae release oxidoreductases which take part in the oxidative degradation of chlorinated solvents, explosives, and petroleum hydrocarbons[36]. Oxygenases participate in the metabolism of organic

compounds e.g. chlorinated aliphatic, halogenated organic compounds (herbicides, insecticides, fungicides, etc.)[34].

Oxidoreductases are also used to remove oxidized soluble metals including radioactive metals by transferring electrons from organic compounds to the metals leading to their precipitation[34,37]. Bacteria, such as *Escherichia coli, Bacillus cereus* and fungi (*Aspergillus niger*) accumulate Cd^{2+} and reduce Cr^{6+} to Cr^{3+} and *Enterobacter cloacae* precipitates Cr^{3+} as a metal hydroxide[38]. Hg^{2+} and several of its organic compounds (methyl mercury, ethyl mercuric phosphate) are transformed into volatile Hg^0, which is less toxic[39]. Among the oxidoreductases laccase has proved to be versatile, being able to mediate the removal of toxicants from different environments and also structurally different pollutants.

1.4. Laccase

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is a blue multi-copper oxidase, which is able to catalyze the oxidation of various phenolic compounds; at the same time oxygen is reduced to water (Fig. 3).

Due to the fact that oxygen is the only co-factor needed and it is usually present in the environment, laccases are often used. The versatility, broad substrate specificity, high stability and the mild reaction conditions are additional advantages for the work with laccases.

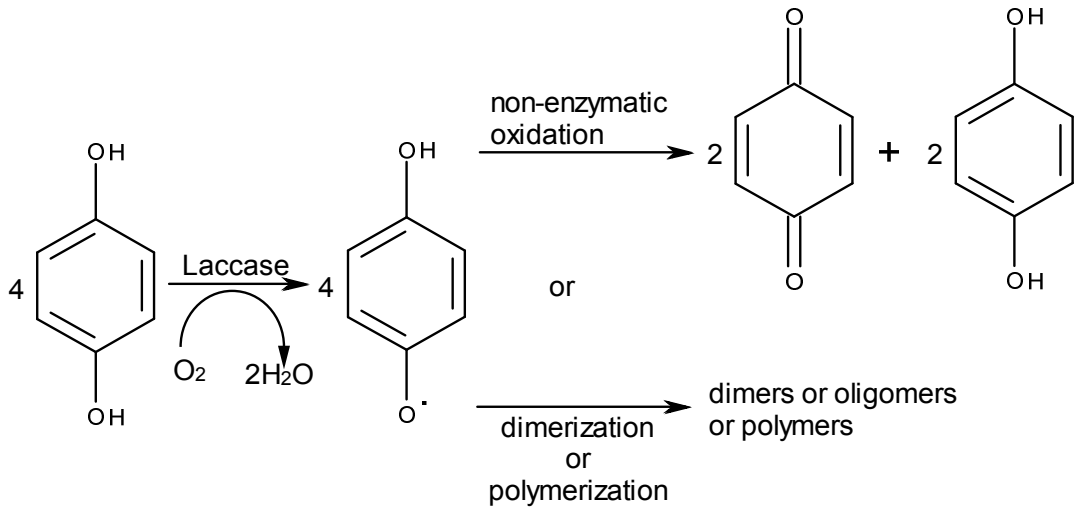

Fig. 3. Example of the oxidation of hydroquinone by laccase[40]

1.4.1. Distribution

Laccase was discovered by Yoshida in 1883 in the Japanese lacquer tree *Rhus vernicifera*[41]. Besides the plant laccases there are reports about laccases in bacteria[42,43] and insects[44] as well. But most predominantly laccases have been found and isolated from fungi. In comparison to the plant laccases which participate in the radical-based mechanisms of lignin polymer formation[45,46], the fungal laccases probably have more different ways of usage including morphogenesis, fungal plant-pathogenesis, stress defense and lignin degradation[47].

1.4.2. Structure of Laccase

Fungal laccases are glycoproteins which often occur as isoenzymes that oligomerize to form multimeric complexes. The molecular mass of the monomer ranges from about 50 to 100 kDa[48]. For the catalytic activity four copper atoms per active protein unit are needed. As shown in Fig. 4there are three types of copper atoms: one type 1 (T_1) copper (Cu_1) and a three-nuclear cluster consisting of one type 2 (T_2) copper (Cu_4) and two type 3 (T_3) coppers (Cu_2 + Cu_3). The type 1 copper is responsible for the typical blue color of the laccase due to the covalent copper-cysteine bond which has a strong absorption at 600 nm[40].

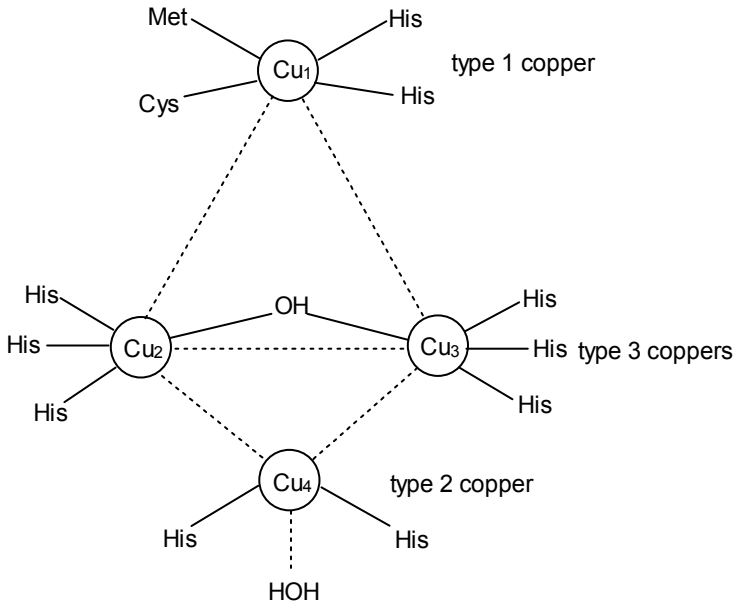

Fig. 4. Active site of laccase CotA from *Bacillus subtilis*[40]

1.4.3. Reaction mechanism & properties

The catalytic properties of laccase have been attributed to the following three major steps[49]:

I) Type 1 copper is reduced by accepting electrons from the reducing substrate.

II) Electrons are transferred from T_1 to the trinuclearT_2/T_3 cluster.

III) Molecular oxygen is activated and reduced to water at the trinuclearT_2/T_3cluster.

Fig. 5 shows the catalytic mechanism of laccase involving a four-electron reduction of the dioxygen molecule to water at the enzyme copper sites[40].

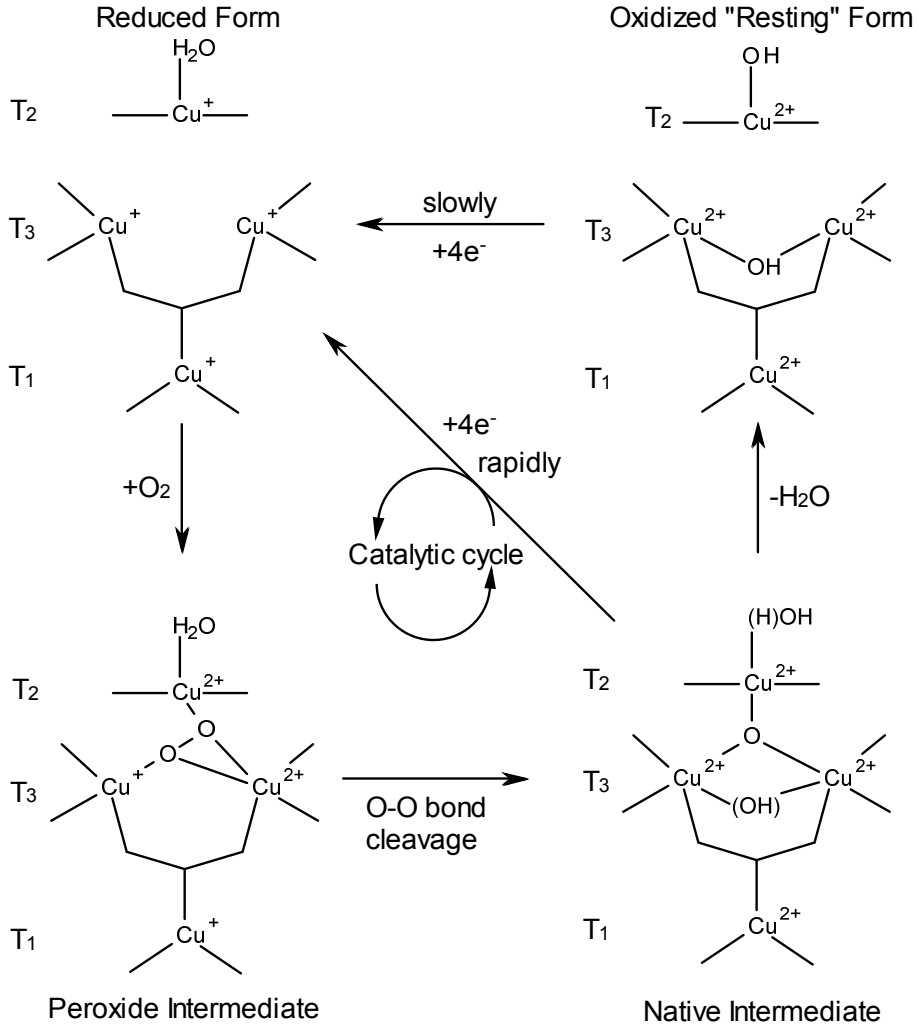

Fig. 5. Mechanism of the four-electron reduction of a dioxygen molecule to water at the laccase copper sites[40]

The dioxygen molecule interacts with the completely reduced trinuclear cluster to produce the peroxide intermediate which contains the dioxygen anion[50]. One oxygen atom of the dioxygen anion is bound with the T_2 andT_3 copper ions and the other oxygen atom is coordinated with another copper ion of T_3. Then, the peroxide O-O bond is ruptured to produce a native intermediate which is the fully oxidized form

with the three copper centers in the trinuclear site mutually bridged by the product of full O_2 reduction[51].

The native intermediate provides a relatively stable structure that serves as the thermodynamic driving force for the $4e^-$ process of O_2 reduction, and also, provides efficient electron transfer (ET) pathways from the T_1site to the other copper centers in the trinuclear cluster. This efficient ET pathway leads to the fast reduction of the fully oxidized trinuclear cluster in the native intermediate to generate the fully reduced site in the reduced form for further turnover with O_2. The native intermediate can slowly convert to a completely oxidized form called “resting” laccase, which has the T_2 copper isolated from the coupled binuclear T_3centers. The native intermediate only rearranges to the resting form in the absence of reductant[52].

Laccase can catalyze the oxidation of a variety of compounds, including ortho- and para-benzenediols, polyphenols, aminophenols, polyamines, lignin, aryldiamines, and a number of inorganic ions. To accomplish this, laccase abstracts an electron from a substrate to produce a free radical, and reduces oxygen to water. Fig. 3 illustrates the simplified laccase-catalyzed oxidation with hydroquinone.

Thus the application of laccases in detoxification of pollutants is achieved through:

a. Oxidative degradation
b. Generation of reactive species which then react among themselves or with other molecules leading to products with high molecular weight due to polymerization
c. Generation of reactive species which attack other chemical compounds within their vicinity leading to hybrid less toxic compounds

Fungal laccases typically have pH optima in the range from 3,5 to 5,0 when the substrates are organic donors of hydrogen atoms, and the pH-dependence curve is bell-shaped[53,54,55]. The optimum pH for phenolics compounds can actually increase at higher pH values but this is complicated by the stability of the enzyme. This pH effect results from the balance between the ionization potential of the phenolic substrate and the inhibition of the T_2/T_3 copper site by the binding of OH^- ion[56].

The optimal temperature for laccase-catalyzed oxidations typically ranges from 50 to 70 °C, which doesn’t differ from other extracellular ligninolytic enzymes[57]. However,

there are a few fungal laccases with an optima oxidative profile below 35 °C such as the laccase from *G. lucidum* with its highest activity observed at 25 °C[58].

There is a wide spectrum of compounds which have been described to inhibit laccase. These inhibitors include small anions such as azide, cyanide, fluoride and hydroxide. These ions bind with the trinuclear cluster and therefore prevent the electron transfer from the type 1 copper onto the T_2/T_3 site inhibiting the enzymatic activity[56,59,60,61]. Metals (Hg, Cd, Co, Mn) are as well able to inhibit laccase activity[62].

1.4.4. Laccase-Mediator system

As shown in Fig. 6 laccase is oxidizing a substrate while reducing oxygen to water. In reactions where the substrate to be oxidized has a higher redox potential than laccase or the substrate is too large to enter the enzyme active site, the presence of a so-called chemical mediator may used to facilitate the reaction.

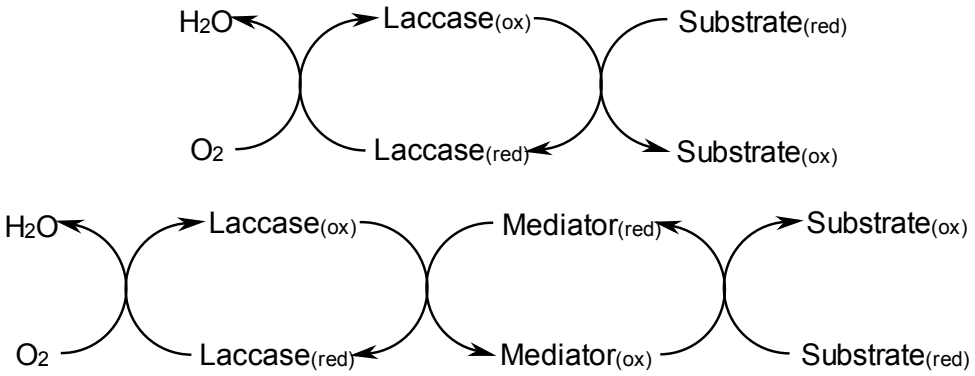

Fig. 6. Schematic representation of the laccase-mediator redox cycle[40]

The mediator first reacts with the laccase to form a reactive oxidized intermediate which then oxidizes the target substrate.

The mechanism of the mediator-substrate oxidation is suggested to occur via electron transfer (ET), radical hydrogen-atom transfer (HAT), or ionic oxidation routes, depending on the structure of the oxidized mediator.[63]

1.4.5. Immobilization

In order to achieve a successful removal of PAHs, phenolics and other environmental pollutants a filter is used. Cellulose acetate was chosen as the filter material for the work on this thesis.

Immobilization onto solid carriers is perhaps the most used strategy to improve operational stability of biocatalysts and in this case it is expected to enhance in situ removal of toxicants. An overview of the possibilities of immobilizing enzymes is shown in Fig. 7.

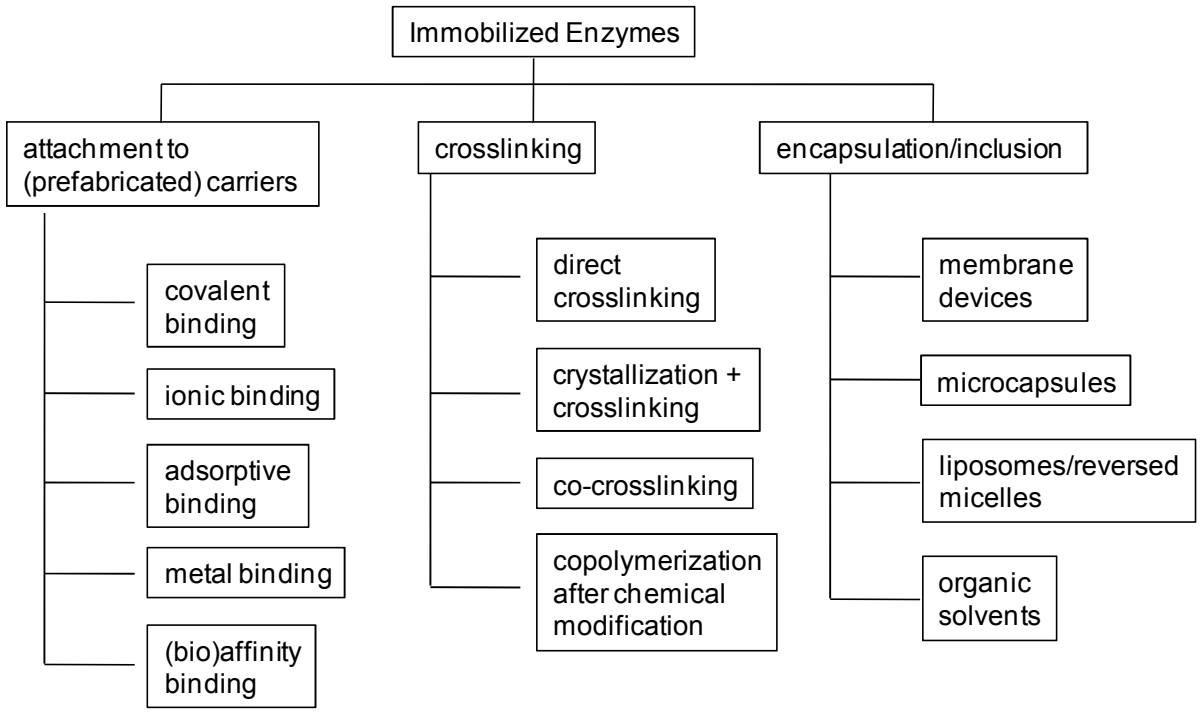

Fig. 7. Different possibilities for the immobilization of enzymes[64]

These strategies allow enzymes to be easily reused multiple times for the same reaction, increase enzyme stability and have been reviewed recently[65,66,67]. Examples of bifunctional crosslinkers, include glutaraldehyde and ethylene glycol bis-(succinimidyl succinate).

For environmental use covalent binding appears to be the most promising strategy. Adsorption, for example, might be easier and cheaper in production but there is the possibility of losing enzyme because the binding isn't as strong as a covalent bond. The following list gives an overview of some promising possibilities and methods for this immobilization process:

1. $NaIO_4$ activation and coupling with and without spacer arm
    a. Direct coupling after activation with $NaIO_4$ (Fig. 8+Fig. 9)
    b. Hexamethylenediamine used as a spacer arm after activation with $NaIO_4$ (Fig. 11)

2. Amino functionalization
    a. using silanes (Fig. 12)
    b. using phenolic amines
    c. using RB5-dye (Fig. 13)

The first strategy involves a direct covalent bond between the enzyme and the cellulose acetate filter. Through the usage of periodate the glucose molecules are oxidized, opened and aldehyde groups are produced. (Fig. 8+Fig. 9) The acetyl-groups of the cellulose-acetate might be a problem, due to sterical and reactivity reasons. To increase the reactivity of the filter material and improve the binding of the enzyme a partial hydrolysis might be necessary (Fig. 10)[68].

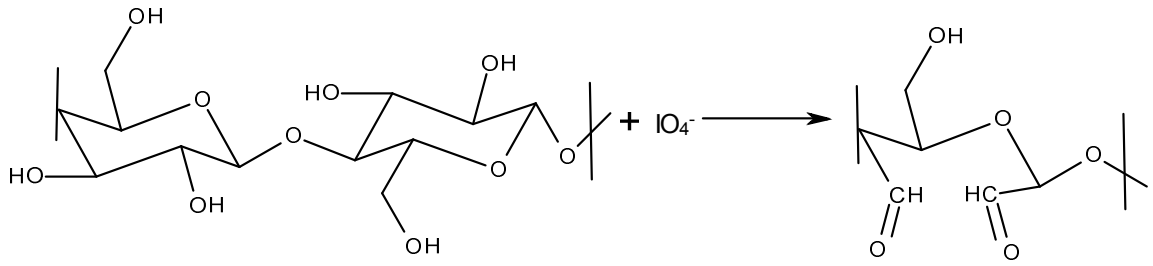

Fig. 8. Cellulose oxidation with periodate[68]

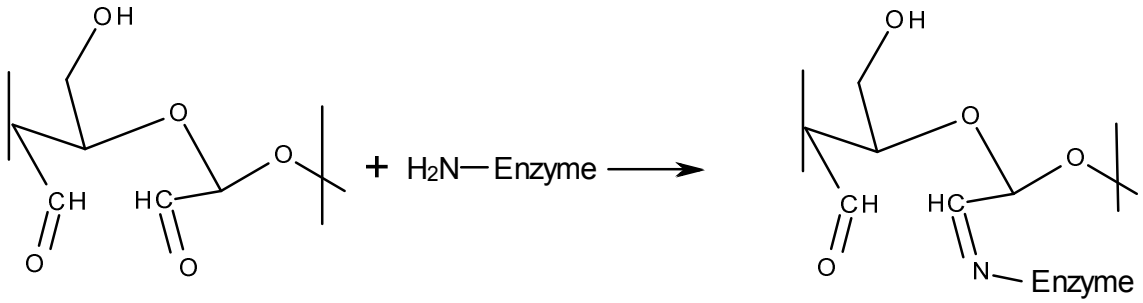

Fig. 9. Covalent binding of the lipase to the activated cellulose[68]

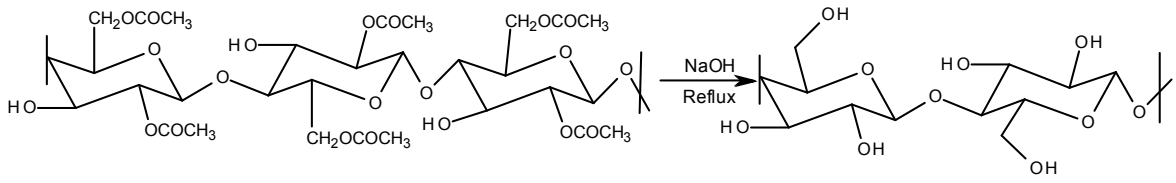

Fig. 10. Alkaline reduction of the acetyl groups[68]

If the resulting imine-bond between the enzyme and the filters is not stable enough, a reduction of the imine to an amine provides a better stability[68]. Additionally there is

a possibility to insert a spacer arm between the enzyme and the filter like hexamethylenediamine (Fig. 11)[68,69].

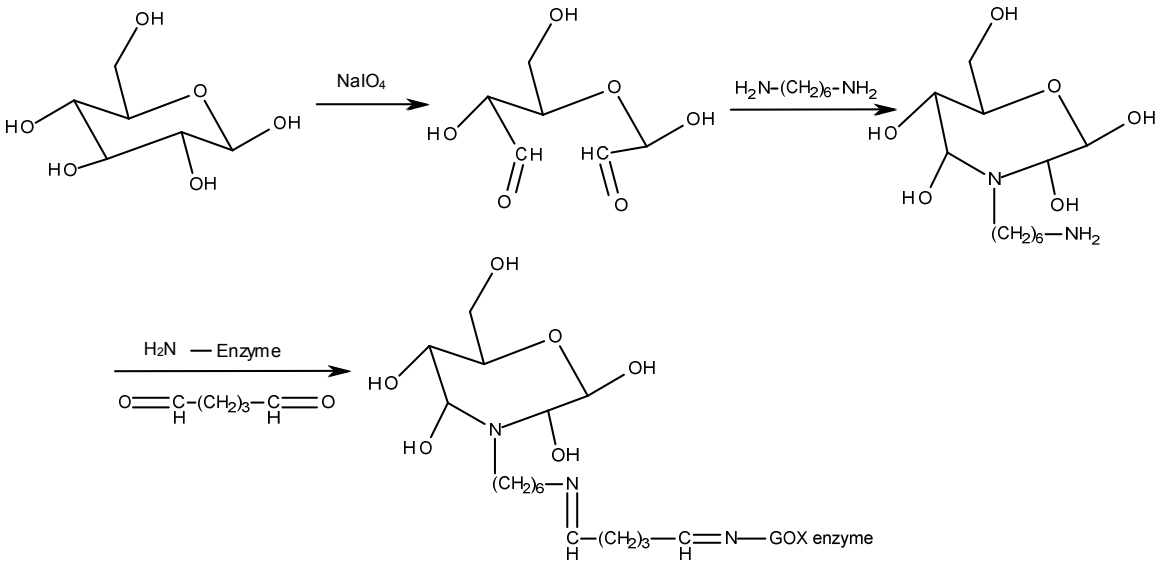

Fig. 11. Enzyme immobilization process on a cellulose/cellulose acetate membrane using $NaIO_4$ and hexamethylenediamine[69]

An additional possibility is an amino functionalization of the cellulose acetate. The first method introducing amino groups is the use of silanes. By using these introduced amino-groups and the amino-groups of the enzyme, glutaraldehyde, for example, could manage to crosslink the filter and an enzyme (Fig. 12)[70].

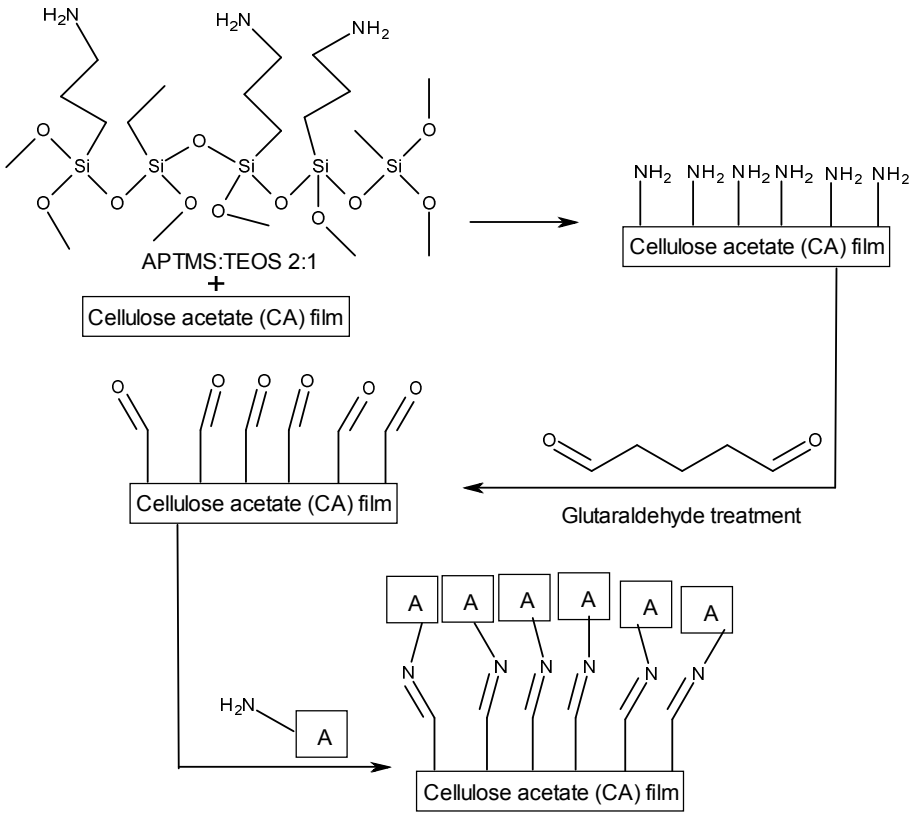

Fig. 12. Schematic representation of functionalization and protein immobilization on the cellulose acetate surface[70]

A second approach for amino functionalization is the use of phenolic amines. By using the laccase to oxidize the phenolic part, the molecule is activated and a coupling to the cellulose acetate is induced. This kind of reaction is already used for enzymatic grafting to lignocelluloses material[71]. The coupling takes place preferably at a hydroxyl-group of the filter, for that reason a partial hydrolysis is needed probably (Fig. 10). The amine-group of the coupled molecule is then able to couple with the enzyme.

A third strategy for amino functionalization might be the use of a RB5-dye and a following reduction with sodium hydrosulphite (Fig. 13)[72].

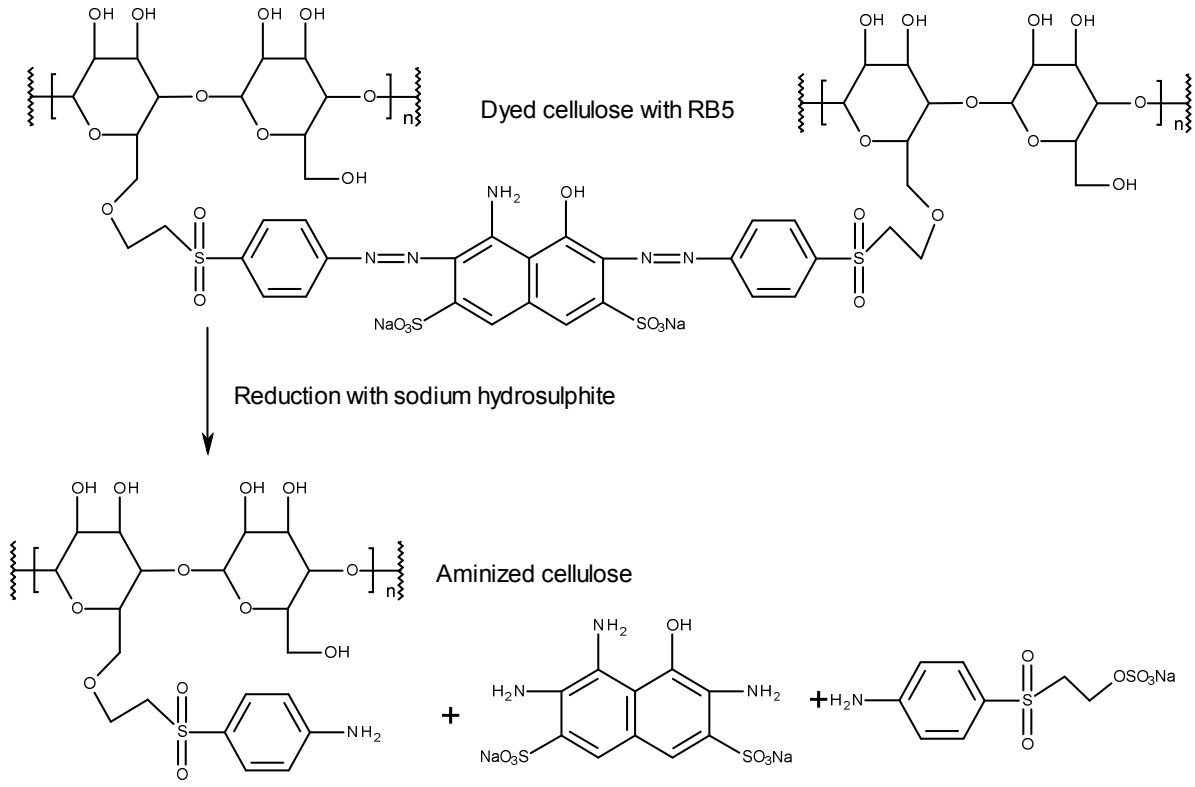

Fig. 13. Reduction of RB5-dyed cellulose with sodium hydrosulphite to get an aminized cellulose[72]

1.5. Aim of the thesis

This research aims to synthesize a biofilter system which is able to filter air pollutants especially from combustion processes. The objectives of this thesis in particular are:

a) Screening laccases for their ability to degrade or remove pollutants in order to prevent air pollution
b) Immobilizing laccases onto solid supports for in situ removal of air pollutants in filters
c) Evaluating the removal of air pollutants by the synthesized filter

2. Material & Methods

2.1. Enzyme activity

Laccase activity was measured according to a method by Liu et al.[73] using an 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Sigma-Aldrich, Steinheim, Germany) activity assay. Two different laccases from different producers were used; one laccase from *Trametes villosa* (*Tv*) heterologously expressed in *A. oryzae* (a donation from Novozyme A/S, Denmark), another laccase from *Trametes hirsuta* (*Th*) secreted by this organism. The *Trametes hirsuta* laccase was produced and purified as described by Almansa et al[74].

The enzyme of *Tv* was diluted 1:50000 and the laccase of *Th* was diluted 1:5000 with 50 mM succinate buffer (pH 4.5). 650 µL of this diluted enzymatic solution were put into a plastic cuvette and 200 µL of a 10 mM ABTS solution in ddH_2O were added to start the reaction.

The absorbance change of ABTS, due to its oxidation, was followed by a time scan using a Hitachi U2001 UV-VIS-Spectrophotometer at 420 nm for 1 minute. The enzyme activity was expressed in units, calculated using the following equation. Each enzyme was measured 5 times.

Equation 1. Formula for the calculation of the enzymatic activity

$$v_0 = \frac{\Delta abs}{\Delta t} = \frac{\Delta abs}{min} \times \frac{Vtot}{Vsample \times \varepsilon \times d} \times f$$

v_0: volumetric enzyme activity [U/mL]
$\Delta abs/\Delta t$: change of absorbance per defined time
ε: molar extinction coefficient of ABTS at 420 nm and pH 4.5
ε=36.0 [mL/(µmol x cm)][73]
V_{tot}: total volume (850 µL)
V_{sample}: sample volume (650 µL)
d: path length (1 cm)
f: dilution factor, (50000 (*Tv*)/5000 (*Th*))

2.2. Purification of the laccase

A number of purification steps were carried out including ammonium sulfate precipitation followed by other chromatographic steps. In preliminary tests 2 mL of

the crude enzyme of *T. hirsuta* were mixed with different amounts of ammonium sulfate ranging from 40% to 80% (w/v). The samples were stirred using a thermomixer (Thermomixer Comfort Eppendorf AG, Hamburg, Germany) and were cooled with ice. In the sample with 75% salt concentration the entire protein precipitated. Therefore this concentration was used for the precipitation of 200 mL of the crude enzyme which was stirred over night in a cooling room.

Further chromatographic separations techniques used a Fast Flow pressure Liquid chromatography instrument ÄKTA purifier system 900 (Amersham pharmacia biotech, Uppsala, Sweden). The most important settings are listed in Table 3. A hydrophobic interaction chromatography (HIC) column was chosen taking advantage of the salt introduced during ammonium sulphate precipitation step. This approach promotes the binding of laccase through hydrophobic interaction on the stationary phase. The crude enzyme of *T. hirsuta* was loaded onto the column using 10 mM succinate buffer pH 4.5 supplemented with 1 M ammonium sulphate as running buffer. This led to the immobilization of laccase onto the stationary phase. The salt blocks the hydrophilic pockets on the enzyme thereby promoting hydrophobic interaction between hydrophobic pockets on laccase and the hydrophobic groups on the stationary phase. To desorb the laccase, the salt concentration was lowered (by introducing 10 mM succinate buffer without salt) which weakens the hydrophobic interactions forces leading to elution of laccase. The concentration was changed in steps of 20 % after one column volume.

Table 3. Most important settings for the laccase purification using the ÄKTA purifier system

Column	HIC, self-packed with butyl sepharose 4 fast flow (Amersham Biosciences, Uppsala, Sweden)
Column volume (CV)	40 mL
Flow rate	2,2-2,5 mL/min (reduced during the run because of too high pressure)
Loading volume	500 µL
Fraction size	5 mL
Solvents	A= 10 mM succinate buffer pH 4.5 with 1 M ammonium sulfate; B= 10 mM succinate buffer
Gradient	2 CV 0% B, 1 CV 30% B, 1 CV 60% B, 1 CV 100% B

2.3. Investigating the ability of laccase to oxidize toxicants

The investigation of the oxidation potential of laccase to oxidize toxicants was carried out using two approaches:

1. Direct oxidation: individual pure toxicants were incubated with laccase in order to find out which are laccase substrates
2. Copolymerization: If no direct oxidation occurred, these non-laccase substrates were incubated with laccase and laccase substrates which were acting as mediator to oxidize these non-laccase substrates

2.4. Monitoring the removal of toxicants

Several complimentary analytical techniques were used to monitor the removal of the different toxicants: UV-VIS-Spectrophotometry, oxygen electrode, HPLC-MS, GC-MS and different toxicant specific assays. All potential laccase substrates (phenolics, PAHs, aromatic amines) were purchased from Sigma-Aldrich (Steinheim, Germany).

2.4.1. Spectrophotometer

Spectroscopy as the technically simplest method in wavelength scan mode was used to monitor a possible direct oxidation of toxicants. Measurements were carried out using a Hitachi U2001 UV-VIS-Spectrophotometer in the range of 900 nm to 200 nm in order to monitor the changes in chromatographic spectrums.

Three measurements for each molecule were executed using different conditions at different times. The first scan was made using 0.25 mM of the possible laccase substrate in 50 mM ammonium acetate buffer pH 4.5. If the resulted absorption was too high a dilution of the sample was made.

The composition for the reaction of the second scan is listed in Table 4. For comparing reasons the dilution of the first scan was applied on this reaction as well to have the same conditions.

The third spectrophotometric measurement was made of the laccase reaction after 15 minutes incubation time to see a possible shift of the wavelengths over time.

Table 4. Laccase reaction for the substrate scan using spectrophotometry

Amount	Substance
0.25 mM	possible laccase substrate
0.95 U	crude laccase *T. villosa*
50 mM	ammonium acetate buffer pH 4.5

2.4.2. Oxygen electrode (Clark-Cell)

As described in the introduction laccases reduce oxygen to water and at the same time a substrate is oxidized. (Fig. 6) Due to this fact oxygen consumption measurements can be used to follow the reaction.

The oxygen electrode (Rank brother Ltd. Dual digital model 20 (Cambridge, UK)) 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 silver ring reference electrode (anode) and the central platinum disc working electrode (cathode) where O_2 is reduced. (Fig. 14) 3 M potassium chloride solution is used to reach conduction between those two electrodes and a gas permeable, ionimpermeable membrane separates the sensing electrodes from the test system[75].

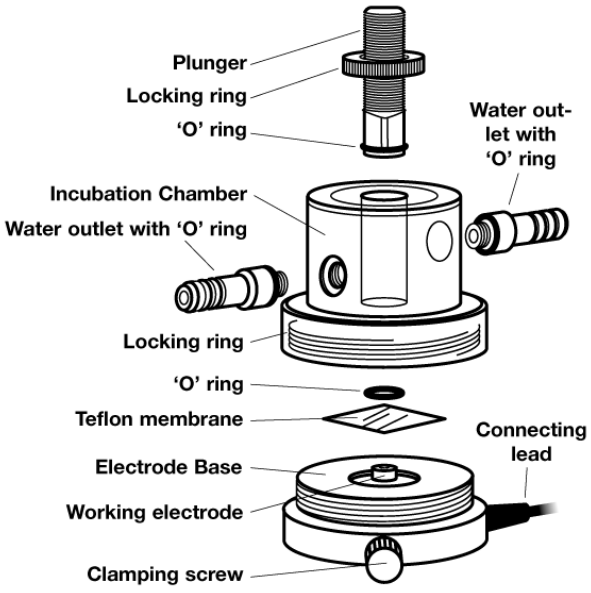

Fig. 14. Setup of the oxygen electrode device[75]

The Clark electrode - Principle of operation

The platinum cathode is polarized at -0.6 V 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 reaction at the silver anode generates silver chloride.

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

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

This electrochemical process adds up to the following equation:

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

The oxygen partial pressure in the tested solution or gas phase is proportional to the resulting current between the two electrodes[75].

50 mM ammonium acetate buffer (pH 4.5) was put into a stirred incubation chamber. 0.83 mM hydroquinone (HQ), methylhydroquinone (MHQ), trimethylhydroquinone (TMHQ), 4-methylcatechol (4MCat), 2-naphthylamine (2NA), or quinoline was added to test for possible laccase substrates. Afterwards 0.8 U of the laccase *T. villosa* were added and the oxygen concentration followed via the Clark cell.

2.4.3. HPLC and HPLC/MS

High performance liquid chromatography (HPLC) on its own and coupled with mass spectrometry (MS) was used to proof a reaction from a non-laccase-substrate caused by laccase by using a mediator which is oxidized by the enzyme.

On Table 5 the starting conditions of the coupling reactions with benzo[α]pyrene are shown. Due to the low concentration of our stock solution available the laccase-substrate amount had to be reduced in comparison to the other reactions to assure a 1:1 mixture between laccase-substrate and non-laccase-substrate. Table 6 shows the conditions for the other non-laccase-substrates. As substrates hydroquinone, methylhydroquinone, trimethylhydroquinone, 4-methylcatechol and 2-naphthylamine were used due to some preliminary tests using UV-VIS spectrophotometry and the

Clark cell. Tested non laccase substrates were 3-aminobiphenyl, 4-aminophenyl, benzo[α]pyrene and 1,2-benzanthracene.

Table 5. Coupling reaction with benzo[α]pyrene and laccase substrates

Amount	Substance
0.5 mM	benzo[α]pyrene
0.5 mM	laccase-substrate
0.95 U	laccase *T. villosa*
50%/50%	50 mM ammonium acetate buffer pH 4.5/acetone

Table 6. Coupling reaction with laccase-substrates and non-laccase-substrates

Amount	Substance
2.5 mM	laccase-substrate
2.5 mM	non-laccase-substrate
0.95 U	laccase *T. villosa*
50%/50%	50 mM ammonium acetate buffer pH 4.5/acetone

The reactions measured using the HPLC and the HPLC/MS respectively were incubated for 48 hours at 30 °C and 600 rpm in an eppendorf Thermomixer comfort. In order to partially purify the reaction solutions because of the high sensitivity of the HPLC/MS system two extraction methods were applied using acetone and ethyl acetate respectively.

Sample purification for HPLC analysis using acetone:
100 µL reaction solution and 900 µL of ice-cold acetone were mixed. After 15 min incubation on ice they were centrifuged for 15 min at 14000 rpm and 0 °C in a Hettich Mikro 200R centrifuge.

Sample purification for HPLC analysis using ethyl acetate:
100 µL reaction solution and 900 µL of ice-cold ethyl acetate were mixed. After some minutes of waiting equilibrium is reached and two separate phases are clearly visible.

In both cases 600 µL of the upper part were transferred into the HPLC vials.
The HPLC analysis was performed using a system from Dionex (Dionex Softron Gmbh, Munich, Germany) equipped with a P580 pump, an ASI 100 Automated Sample Injector and a photodiode array detector (PDA-100). The separation of the

molecules was achieved by a C-18 column (X-Terra-MS, C18, 150 mm x 4.6 mm, Water, USA).

For the use of the HPLC-system two methods were established; one method for the analysis of samples with hydroquinone and methylated hydroquinone (Table 7 + Fig. 15) and another method for the rest of the samples (Table 8 + Fig. 16).

Table 7. HPLC method for the analysis of hydroquinone samples

Flow	1 mL/min
Solvents	A = ddH_2O, B = ACN, C = 0,1 % formic acid in ddH_2O
UV	254/218/295/374 nm
Oven	30 °C
Column	C-18 X-Terra
Duration of run	60 min

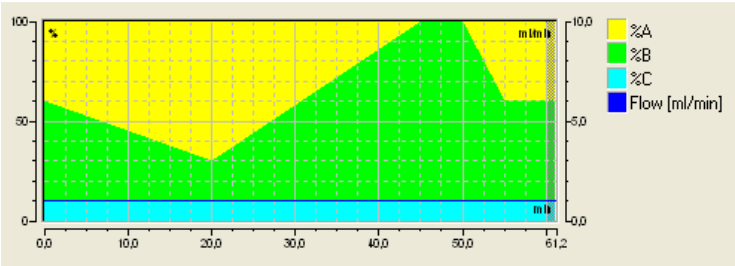

Fig. 15. Solvent gradient of the HPLC method for the analysis of hydroquinone samples

Table 8. HPLC method for the analysis of phenolic samples excluding the hydroquinone samples

Flow	1 mL/min
Solvents	A = ddH_2O, B = ACN, C = 0,1 % formic acid in ddH_2O
UV	254/218/273/374 nm
Oven	30 °C
Column	C-18 X-Terra
Duration of run	60 min

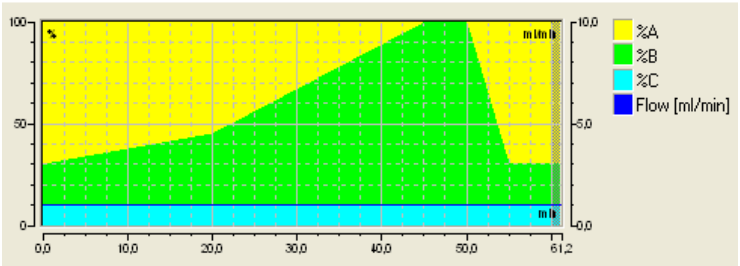

Fig. 16. Solvent gradient of the HPLC method for the analysis of phenolic samples excluding the hydroquinone samples

In order to get some additional proof of the success of the coupling reactions HPLC/MS was used to determine the masses of the coupling products. The MS spectra were acquired with an Agilent Ion Trap SL (Palo Alto, CA, USA) equipped with an electrospray ionization system. The HPLC methods were the same as described above and the MS conditions adjusted as in Table 9.

Table 9. Most important settings for the MS analysis of the coupling products

Scan range	100 – 600 m/z
Capillary	3500 V
Nebulizer	70.0 psi
Dry gas	12.0 L/min
Polarity	Positive
Max. accumulation time	300 ms

2.4.4. HS-SPME-GC/MS

The GC/MS system was used to monitor the removal of some more volatile compounds like acetaldehyde (Aa) and acrolein (Ac). (Fig. 17)

Acetaldehyde
Formula Weight : 44,052(2)

Acrolein
Formula Weight : 56,063(2)

Fig. 17. Acetaldehyde and acrolein; fragmentation products for MS-SIM analysis marked in red

As their boiling points are relatively low (20 °C and 52 °C respectively) headspace-solid phase micro extraction (HS-SPME) was used.

SPME is a microextraction technique; that means that the amount of extraction solvent is very small compared to the sample volume. Using this technique the analytes are not fully transferred to the extracting phase, but equilibrium is reached between the sample matrix and the extracting phase. For better usage in practice, the extracting phase is permanently attached to rods out of various materials. The extracting phase might be out of different materials, but mostly it is a polymeric organic phase cross-linked to the rod[76].

In the headspace mode (Fig. 18b), the vapor above the bulk matrix is sampled. Therefore the analytes have to be relatively volatile to be transported to the coated

rod. One big advantage of the headspace mode is the protection of the fiber because neither the solvent nor large molecules are able to harm the coating because there is no direct contact[76].

The choice of sampling mode plays a significant role on extraction kinetics. By sampling the headspace, the extraction of the analytes from the matrix is indirectly, as shown in Fig. 18b. Because of the fact, that more volatile components concentrate in the headspace, they are extracted faster than less volatile components[76].

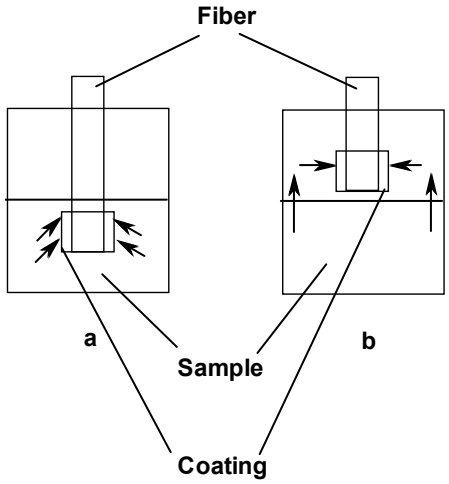

Fig. 18. Modes of SPME operation: (a) direct extraction, (b) headspace SPME[76]

Table 10 shows the starting conditions for the coupling reactions of acetaldehyde and acrolein (Fluka, Buchs, Switzerland). The reactions were incubated for 24 hours at 30 °C and 600 rpm in an eppendorf Thermomixer comfort, cooled down on ice for 30 min and then transferred into the GC vials.

Table 10. Reaction conditions for the reduction of acrolein and acetaldehyde

Mediator + Laccase	**Mediator**	**Laccase**	**Without treatment**
10 mM acetaldehyde/acrolein	10 mM Aa/Ac	25 mM Aa/Ac	25 mM Aa/Ac
10 mM mediator (HQ/MHQ/TMHQ)	10 mM mediator		
0.95 U laccase *T.villosa*		0.95 U lac	
in 50 mM citrate buffer pH 4.5	in buffer	in buffer	in buffer

The GC analysis was performed using an Agilent Technologies 7890A GC System with a HP-5MS (30m x 0.25 mm x 0.25 µm) column. For the sampling process a

Supelco SPME Fiber Assembly (1 cm, 23 GA, Auto; 60 µm; CW (PEG)) and a CTC Analytics Combi PAL autosampler was used. For the determination of the molecules the Agilent Technologies 5975C VL-MSD with Triple-Axis-Detector was used as described in Table 11. A gradient until 300 °C was chosen to assure that all molecules are leaving the column and none of them stays stuck there. The extraction time was chosen due to some preliminary tests where 300 s created the best results.

Table 11. HS-SPME-GC settings for the analysis of acetaldehyde and acrolein

Split	1:20
Inlet	240 °C
Oven Gradient	80 °C for 5 min
	Increase of 20 °C/min until 300 °C
	300 °C for 1 min
Preincupation time	300 s
Incubation temperature	50 °C
Preincupation agitator speed	500 rpm
Extraction time	300 s
Desorption time	300 s
GC runtime	1140 s

In order to get an overview of all possible coupling products, a wide area of masses was chosen (27/29 – 550 amu). Smaller masses than 27 were left out to avoid peaks of the solvent (water) and other small contaminants which might produce unwanted peaks.

As the signals in the MS scan mode were not satisfying, single ion monitoring (SIM) was used to get more accurate results concerning the investigated molecules. In this case single ions are chosen for the specific molecule of interest in comparison to the scan mode where a larger area of masses is scanned. For the analysis of acetaldehyde and acrolein the molecule peaks and the most common fragmentation products were chosen. (Fig. 17, fragmentation products marked in red) The MS settings are summarized in Table 12.

Table 12. MS settings for the analysis of acetaldehyde and acrolein

	Acetaldehyde	**Acrolein**
Scan parameter	29-550 amu	27-550 amu
SIM	29/44	27/56

2.4.5. Formaldehyde (Nash-reaction)

A sensitive possibility to measure the amount of formaldehyde is the use of a method published for the first time by Nash in 1953[77]. Within this method the Hantzsch reaction (Fig. 19) is used to form diacetyldihydrolutidine out of formaldehyde and acetylacetone in the presence of excess ammonium salt[78]. The reaction product diacetyldihydrolutidine can be easily measured using a spectrophotometer at the absorption maximum of 413 nm.

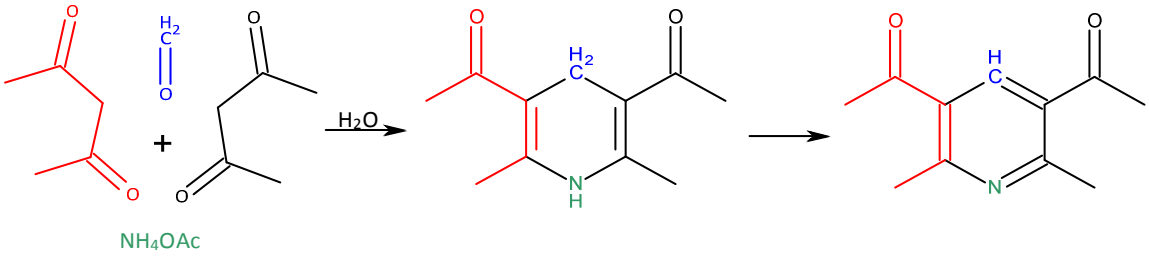

Fig. 19. Hantzsch reaction for the analysis of formaldehyde

The Nash reagent was prepared by dissolving 15 g of ammonium acetate, 300 µL of acetic acid, and 200 µL of acetylacetone (2,4-pentanedione) (Sigma-Aldrich, Steinheim, Germany) in water to make 100 mL of the reagent solution.

In order to calculate the amount of the reduction of formaldehyde (Carl Roth Gmbh, Karlsruhe, Germany) caused by the laccase a calibration curve was made.

Table 13 lists the different variations of reactions to find out the influence of the laccase and the laccase-mediator-system on the removal of formaldehyde.

Table 13. Reactions for the analysis of the formaldehyde reduction

Mediator + Laccase	**Mediator**	**Laccase**	**Without treatment**
2.68 mM formaldehyde (FA)	2.68 mM FA	2.68 mM FA	2.68 mM FA
2.5 mM mediator (HQ/MHQ/TMHQ/Cat/4MCat/2NA)	2.5 mM mediator		
0.95 U laccase *T.villosa*		0.95 U lac	
in 50 mM ammonium acetate buffer pH 4.5	in buffer	in buffer	in buffer

The samples were incubated for 24 hours at 25 °C and 600 rpm at an eppendorf Thermomixer comfort. After the incubation time they were put on ice for 30 min.

To perform the Hantzsch reaction, 20 µL of the incubated formaldehyde solution was added to 480 µL of 50 mM ammonium acetate buffer pH 4.5 and 500 µL of the Nash reagent. This mixture was incubated for 10 min at 60 °C in a water bath. Afterwards the samples were cooled at room temperature for 30 min.

The samples were measured using a Hitachi U2001 UV-VIS-Spectrophotometer at 413 nm.

2.4.6. Nitric oxide

For the analysis of nitric oxide an in situ method for generating the gas was chosen since nitric oxide is difficult to handle. The nitric oxide was generated in vitro using sodium nitroprusside (SNP). At around pH 7.4, nitric oxide is generated from an aqueous SNP solution and interacts with oxygen to produce nitrite ions, which are quantified by the Griess reaction (Fig. 20).

The reaction mixture contained 10 mM SNP (Merck, Darmstadt, Germany), phosphate buffered saline (pH 7.45), 10 mM laccase-mediator (hydroquinone, catechol, 4-methylcatechol) and laccase from *T. hirsuta*. After incubation for 150 min at 25°C, sulfanilamide (0.33% in 20% phosphoric acid) (Merck, Darmstadt, Germany) was added and the solution was incubated for 5 min. Naphthylethylenediaminedihydrochloride (NED) (0.1% w/v) (Merck, Darmstadt, Germany) was then added and the mixture was again further incubated for 30 min at 25°C for the development of the color[79].

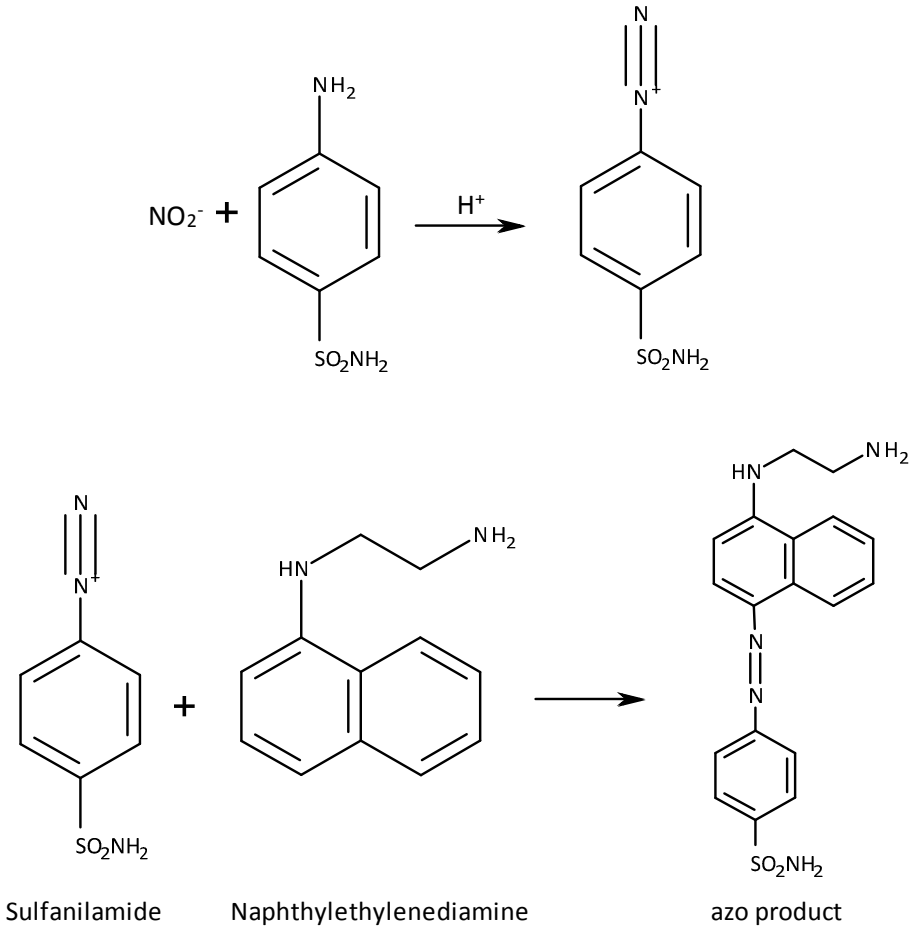

Fig. 20. Griess reaction to analyze nitrite ions

The pink chromophore generated during diazotization of nitrite ions with sulfanilamide and subsequent coupling with NED was measured spectrophotometrically at 540 nm against a blank sample. All tests were replicated four times. To quantify the results a calibration curve was made by using different concentrations of sodium nitrite.

2.5. Immobilization of laccase on solid supports

As already stated in the introduction immobilization of the enzyme is necessary for multiple uses of the enzyme and to improve the operational stability. To ensure that the enzyme stays on the filter and does not get into the environment, a covalent bond is necessary to permanently link the enzyme with the filter material. Cellulose acetate is not very reactive because of the acetyl groups, therefore it is necessary to partially remove them. An adjacent activation step provides better reactivity for the filter material. The use of cross-linkers between filter and enzyme further improves

the stability and the final activity of the enzyme. All chemicals used for the immobilization process were purchased from Sigma-Aldrich (Steinheim, Germany).

2.5.1. Deacetylation

In order to achieve an increased reactivity of the biofilter material and improve the binding of the enzyme a partial hydrolysis was necessary as described in Fig. 10.

Therefore 100 mL of a 1% (w/v) cellulose acetate solution in different concentrations of NaOH was used and boiled for 10 min under reflux. By using such a short amount of time it is assumed that the hydrolysis is not complete; by varying the concentrations of the NaOH the degree of the deacetylation might be regulated. For these experiments 10mM, 50mM and 100mM NaOH solution in water was used. After the hydrolysis the samples were washed several times with water to get rid of the rest of the NaOH. For the drying step of the filter material the Labconco Freeze dry system freezone 4.5 Liter Benchtop Model 77500 (Vienna, Austria) was used with a Labconco model 117 vacuum pump at about -50 °C.

Monitoring the deacetylation

Monitoring the changes between cellulose acetate and the deacetylated cellulose acetate (=cellulose) was carried out using Fourier transform infrared (FTIR) spectroscopy.

Because of the powder form of the samples attenuated total reflectance (ATR) was used. ATR is a technique with a wide range application methods and sample materials, such as liquids, pastes and powder[80]. For this technique no sample pretreatment is necessary.

This method is based on the phenomenon of total internal reflection (Fig. 21). When a beam from the IR source enters the crystal, this radiation will undergo a total internal reflection when the angle of incidence is greater than a certain critical angle. Due to the wave nature of radiation, the beam penetrates a bit beyond the reflecting surface and when the sample material, which is close to the reflecting surface, absorbs radiation, the beam loses energy at the wavelength where the material absorbs[81].

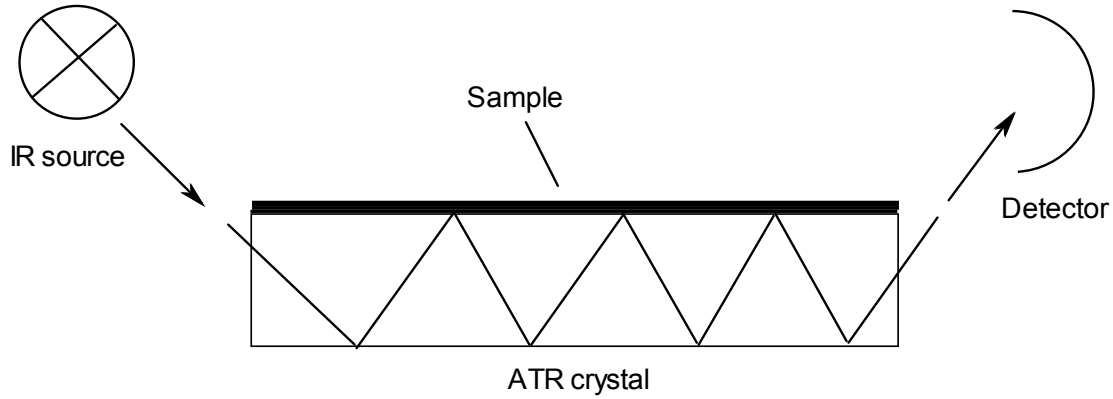

Fig. 21. Schematic of an attenuated total reflectance cell[80]

For the analysis of the solid cellulose acetate samples and the deacetylated variants a Perkin Elmer Spectrum 100 FT-IR Spectrometer was used using a scan range from 4000 to 650 $cm^{-1}$and a resolution of 4.00 cm^{-1}. For the ATR cell a Diamond/ZnSe crystal was used. The most important settings are summarized in Table 14.

Table 14. Important settings for the FTIR spectroscopy measurement of the deacetylation of cellulose acetate

Scan range	4000 – 650 cm^{-1}
Universal ATR Crystals	Diamond/ZnSe
Resolution	4.00 cm^{-1}

2.5.2. Periodate activation of cellulose

As described in the introduction the glucose rings of the cellulose acetate are opened by periodate (Fig. 8) to allow the coupling of a crosslinker onto the filter material. The glucose moieties are oxidized to aldehyde groups.

For the periodate reaction of the filter material 40 mg cellulose acetate and deacetylated cellulose acetate respectively were used and 1 mL of 0.5 M sodium periodate solution was added and incubated for 90 min in the dark[82]. After the incubation time the samples were washed thoroughly with ddH_2O several times in order to get rid of the excess of periodate.

2.5.3. The use of cross-linkers for enhancing immobilization of enzyme

Fig. 22 shows an overview of the cross-linking process.1 mL of an 1 % (v/v) 1,6-hexamethyldiamine (HMD) solution was added to the washed periodate

activated filter material from 2.5.2. This was incubated for 18 hours at 25 °C and 700 rpm in an eppendorf Thermomixer comfort. After this incubation time it was thoroughly washed several times with ddH_2O. For activating reasons and as a possibility of another cross-linker 1 mL of a 5 % solution of glutaraldehyde was added to the samples and incubated for 1 hour at 25 °C and 700 rpm. After removing the supernatant 1 mL of the laccase *T. hirsuta* with about 4 mg of dicyclocarbodiimide was added and incubated for 24 hours at 30 °C and 700 rpm in a Thermomixer. Afterwards the supernatant was removed and 1 mL of 0.5 M $NaBH_4$ was added for 10 min to reduce the imine bond between crosslinker and enzyme to an amine bond. A thorough washing step followed with 50 mM succinate buffer pH 4.5 to ensure the best conditions for the laccase.

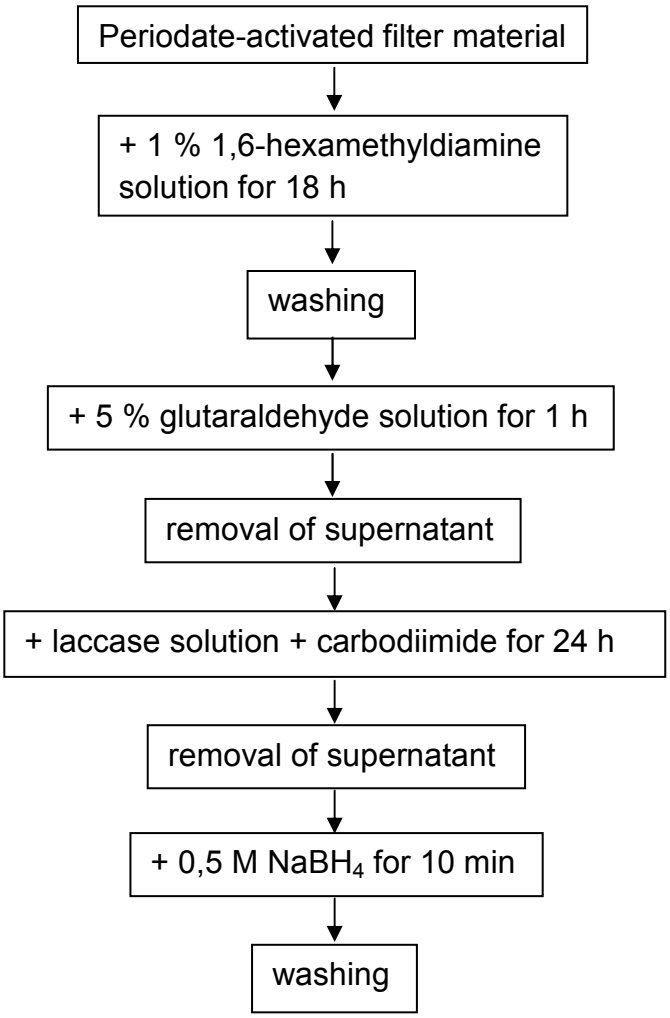

Fig. 22. Overview of the cross-linking process

For the analysis of the cross-linking process an ABTS assay to measure the laccase activity was used as the final goal is activity of laccase on the filter material. 1 mL of 7 mM ABTS in 25 mM Na-succinate buffer pH 4.5 was added to the samples and

incubated for 10 min at 35 °C and 750 rpm in an eppendorf Thermomixer comfort. As the centrifugation process and the mixing before the spectrophotometrically measurement take some time, the total reaction time was 12 min.

3. Results & Discussion

3.1. Enzyme activity

The laccase preparation from *Trametes villosa* had a higher volumetric activity with ABTS than the laccase from *Trametes hirsuta* (Table 15). The laccase from *T. villosa* is produced by a genetic modified microorganism, whereas the laccase from *T. hirsuta* is secreted by this organism.

Table 15. Determined laccase activities of the crude enzymes

Enzyme	Activity
Laccase *T. villosa*	475,86 U/mL
Laccase *T. hirsuta*	25,53 U/mL

3.2. Purification of the laccase

The laccase from *Trametes hirsuta* from our enzyme stocks were used during these studies. The enzyme particularly for immobilization studies was first partially purified in order to reduce the amount of contaminants. Ammonium sulfate precipitation was employed to reduce the volume from 200 mL to 30 mL and also resulted in the removal of 80 % of contaminating proteins.

Fast high pressure liquid chromatography using a hydrophobic interaction column resulted in further removal of over 90 % contaminants which can be clearly seen in Fig. 23; the first peak indicating the contaminants, the second peak predominantly the laccase. Starting with a high concentration of salt because of the ammonium precipitation the salt concentration was reduced step by step by introducing 10 mM succinate buffer without salt into the system. (Fig. 23, indicated by the greenish line). Peak 2 was identified as laccase by using ABTS. A fast change in color indicated the activity of the enzyme.

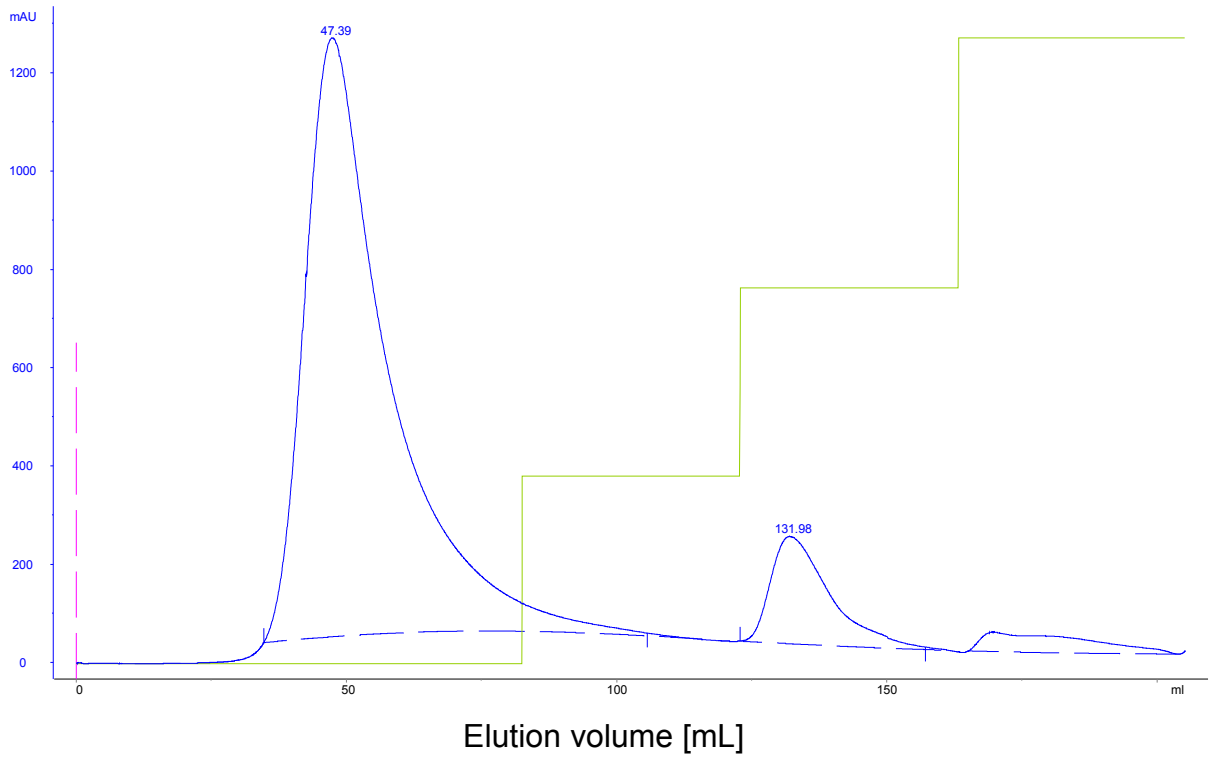

Fig. 23. Chromatogram of the purification of laccase using HIC column

3.3. Investigating the ability of laccase to oxidize toxicants

Emissions from combustion processes contain a large variety on different toxicants. It is possible to group these into groups according to their chemical structure. These groups include above all phenols, carbonyls, PAHs, inorganic gases and aromatic amines.

Laccase was therefore chosen since it could potentially detoxify a variety of toxicants through direct oxidative degradation, generation of reactive species which in turn react with each other or other molecules in the surrounding leading to polymerization, transformation, sequestration and ultimately detoxification.

3.3.1. Laccase oxidation of simple phenolics

Spectrophotometric measurements showed that laccase is able to oxidize simple phenolics. Fig. 24 shows the wavelength scans of hydroquinone, methylhydroquinone, trimethylhydroquinone and 4-methylcatechol. The dark green line indicates the phenols with buffer and without any enzyme. By adding enzyme to the system a clearly visible change of the absorption maximum was noticeable. This shift of the wavelength is caused by the oxidation of the phenols by the enzyme.

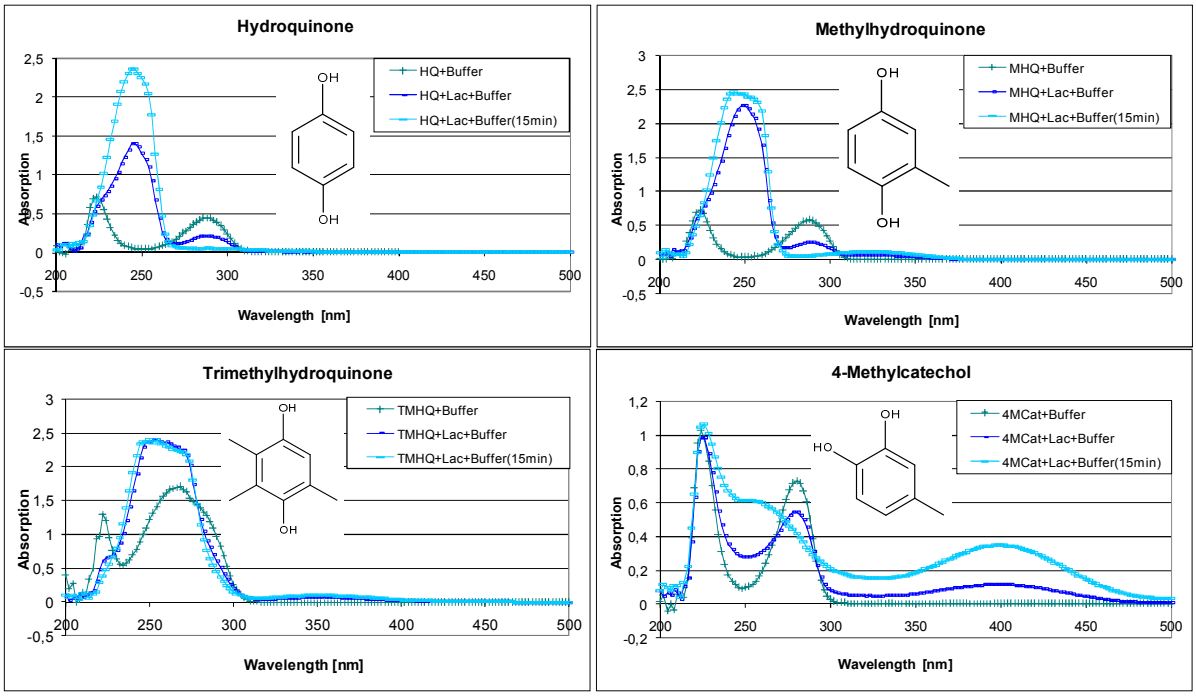

Fig. 24. Wavelength scans of some simple phenolics before and after oxidation with laccase

The changes in the wavelength scan were quite visible on the spectrum indicating oxidation of some tested compounds. Fig. 25 shows the development of the color over a time frame of 15 min. In this case catechol was oxidized which leads to a color change and a precipitation. Oxidation starts immediately and the first change is already visible within a few seconds.

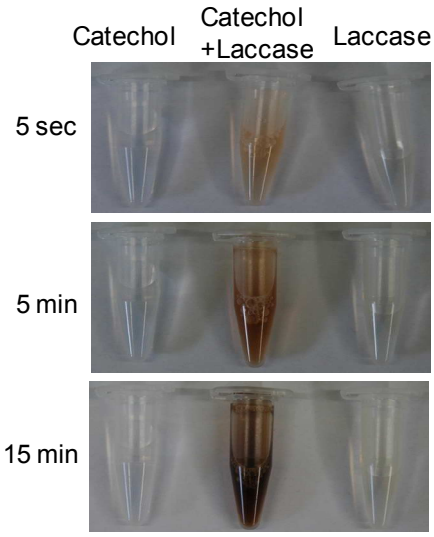

Fig. 25. Development of the color during oxidation of catechol with laccase

The ability of laccase to oxidize the phenolics was also investigated by monitoring oxygen consumption using the Clark cell. As already mentioned before adding laccase to the system will oxygen cause to be consumed by the laccase if oxidation

occurs. Fig. 26 shows the measurements of four simple phenolics, methylhydroquinone, hydroquinone, trimethylhydroquinone and 4-methylcatechol. These results confirm the results from the spectrophotometric measurements. As the slopes of the curves indicate the reactions occur very fast for these molecules.

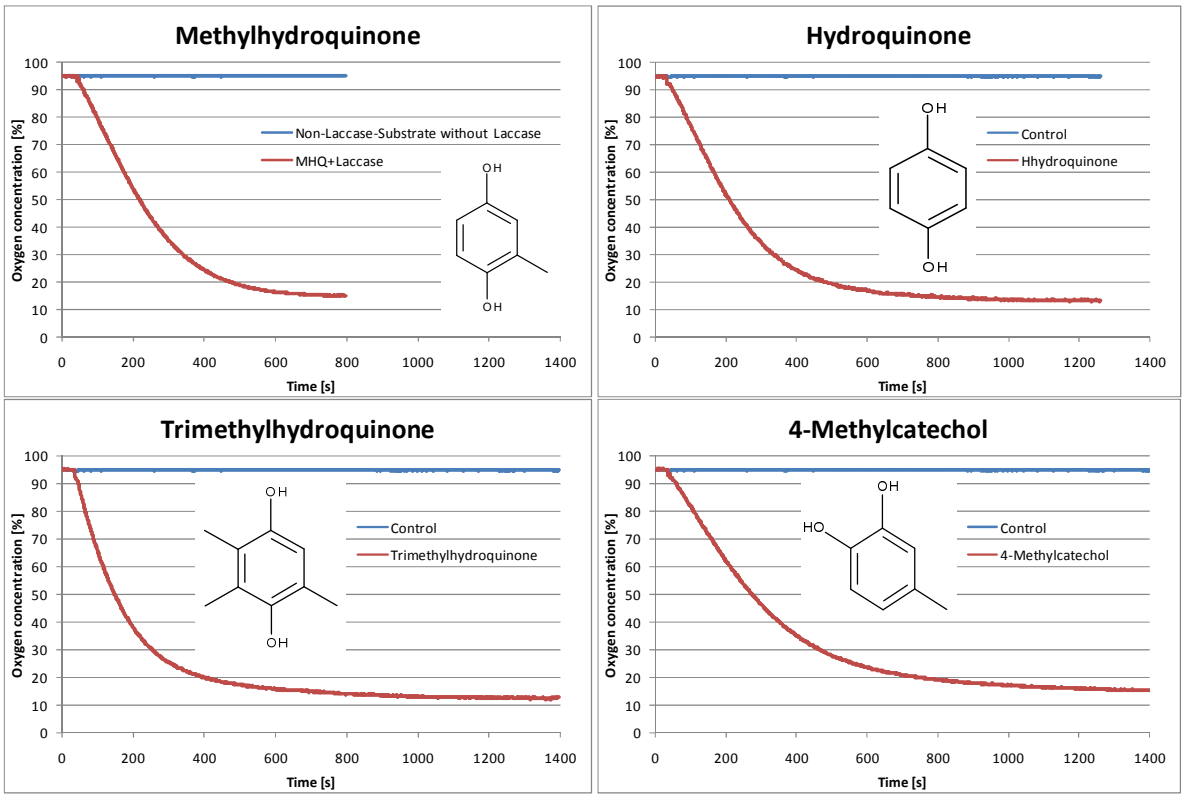

Fig. 26. Clark cell measurements of oxidation of simple phenolics with laccase (red) in comparison to a control

A disadvantage of this Clark cell system is the consumption of oxygen by the electrode. This problem is solved by continuously stirring the incubation chamber and therefore providing enough oxygen and ensuring 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 a regulation of the temperature in the system is important[75].

This data corresponds with the results of the literature. These simple diphenols belong to the main substrates for almost all laccases[47,83]. Spectrophotometric measurement as well as the O_2-consumption methods are standard methods to monitor a change in oxidation[59].

3.3.2. Laccase oxidation of nitrogen-containing molecules

For the scanning process of the laccase substrates not only small phenolics were tested, but nitrogen-containing molecules and PAHs as well. Fig. 27 shows the wavelength scans of 2-naphthylamine, 3-aminobiphenyl and quinoline. These three molecules do not show a significant shift of the wavelength because of the enzymatic treatment. The comparison of Fig. 28 and Fig. 27 show that 2-naphthylamine shows a significant change because of the laccase for the eye but on the wavelength scan no shift can be seen. At about 255 and 305 nm there is a small increase visible 15 minutes after the start of the laccase treatment, but the wavelengths of the absorption maxima stay the same.

3-aminobiphenyl was assumed as a possible laccase substrate as there were oxidation processes found with aromatic amines such as aniline, which was already used as a mediator in coprecipitation reactions[84], and even o-tolidine (3,3'-dimethylbenzidine) was used in a spectrophotometric assay with laccase[85]. The wavelength scan in Fig. 27 nevertheless does not show any change in the wavelength of the absorption maximum.

Quinoline was not expected to be a laccase substrate at all as the molecule structure is unlikely to give any access point for the enzyme. By catalyzing an oxidation reaction laccase abstracts an electron from the substrate to produce a radical which is highly reactive. The two aromatic rings without any functional group seem to be too stable for an electron to be abstracted. Typical laccase substrates are simple phenols or anilines with primary hydroxyl- and amino-groups which can be easily attacked.

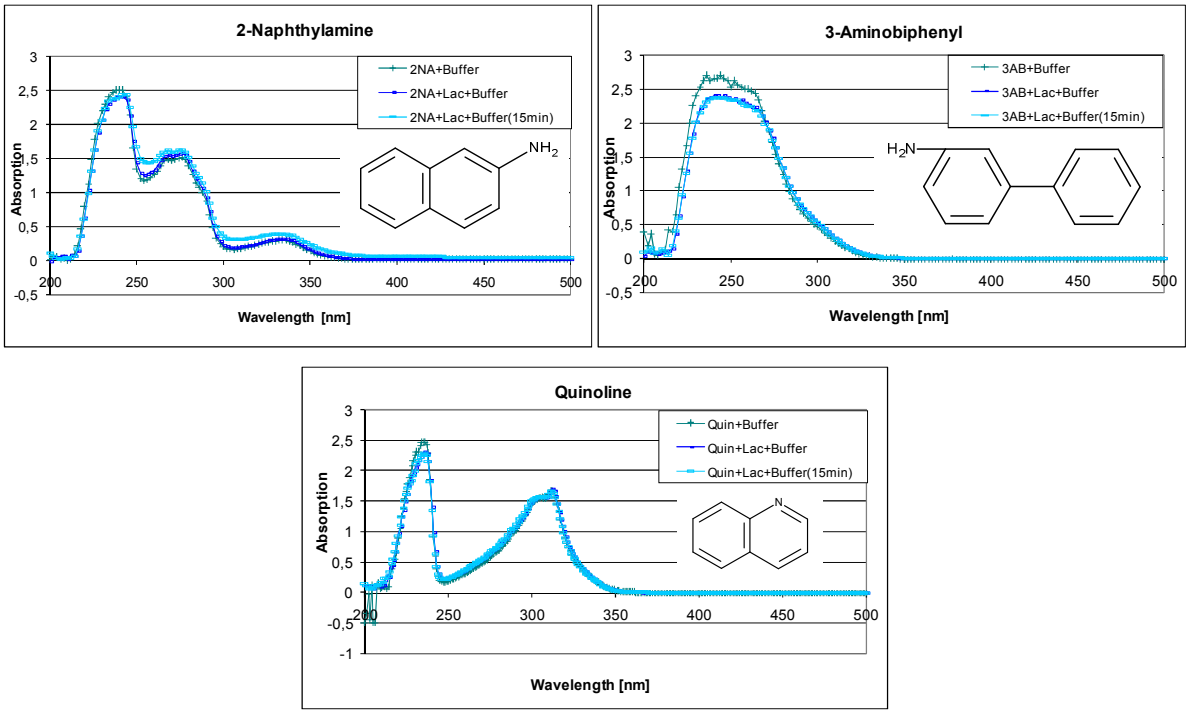

Fig. 27. Wavelength scans of some nitrogen containing molecules before and after oxidation by laccase

Fig. 29 shows the Clark cell measurement of 2-naphthylamine. This molecule did not show any change of the wavelength caused by laccase at all (Fig. 27). Nevertheless in Fig. 28 a change caused by laccase is clearly visible.

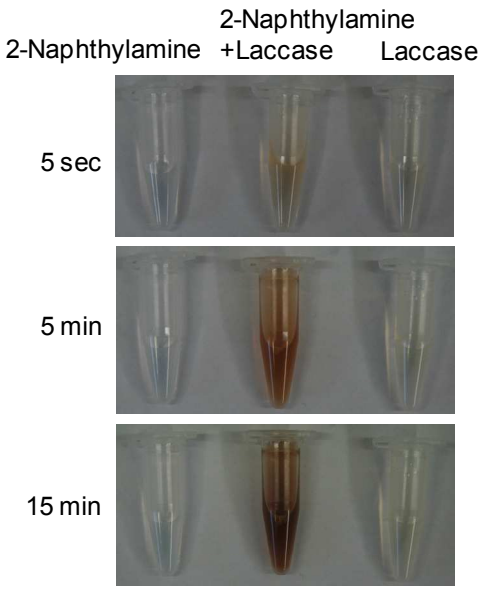

Fig. 28. Development of the color of 2-naphthylamine caused by the oxidation with laccase

The oxygen electrode confirms the expectation of the visual test. Oxygen is consumed after adding the laccase indicating an oxidation reaction. In comparison to the simple phenolics in Fig. 26, the slope of 2-naphthylamine is not so steep which

indicates a slower reaction rate. These facts agree with the literature where 1-naphthylamine was found to be laccase substrate for several laccases[86].

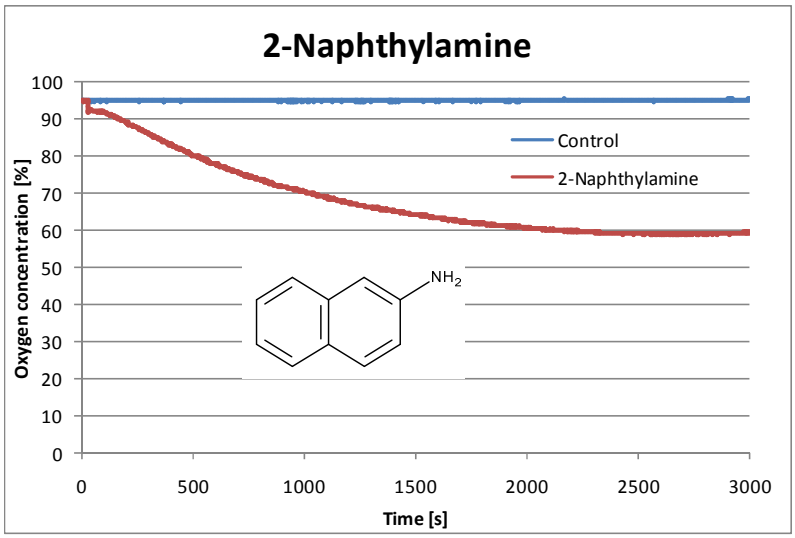

Fig. 29. Clark cell measurement of oxidation of 2-naphthylamine with laccase

The oxygen electrode measurement confirmed that quinoline is no laccase substrate (Fig. 30). There is no significant change of the oxygen concentration compared to the control.

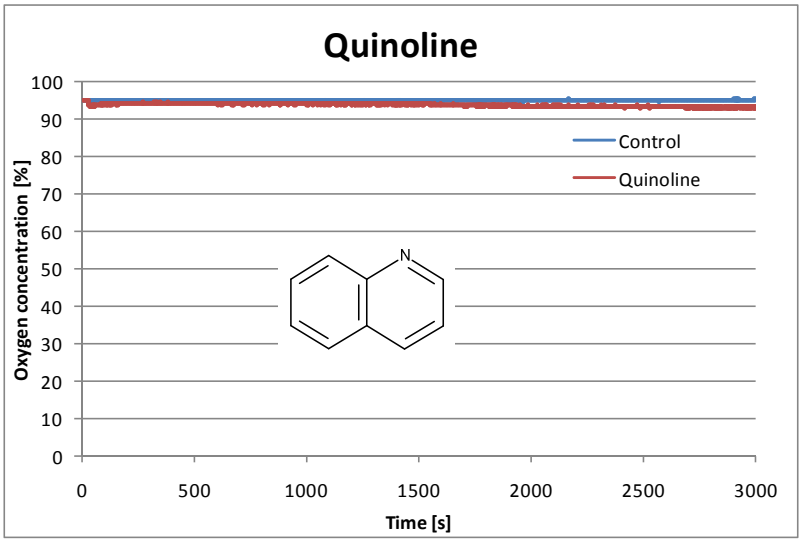

Fig. 30. Clark cell measurement of oxidation of quinoline with laccase

3.3.3. Laccase oxidation of PAHs

1,2-benzanthracene is the representative for the polycyclic aromatic hydrocarbons (PAHs). As expected there is no shift of the wavelengths seen in Fig. 31. This is because of the structure of the PAHs. Laccase is able to oxidize simple phenolics

and in cases amino functionalities. These polycyclic molecules with no functional groups are therefore no possible substrates for the oxidation by laccase and not useful as a mediator to oxidize other molecules.

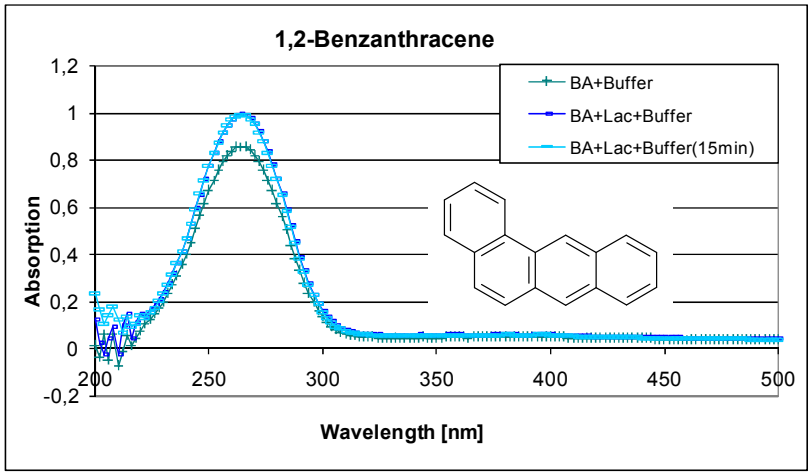

Fig. 31. Wavelength scan of 1,2-benzanthracene

This data is confirmed by other working groups using no direct oxidation of PAHs by laccase but the indirect pathway using laccase substrates as mediators[84,87,88,89].

3.4. Coprecipitation as removal strategy for non-laccase toxicants produced during combustion

Although laccase was able to attack a number of toxicants from different classes of toxicants, it was not able to oxidize all. The ability of laccase to generate reactive species which are able to react none specifically with any molecule in their surrounding was adopted. Those compounds like hydroquinone, catechol, which showed to be laccase substrates in the initial screening phase, were incubated with non-laccase substrates.

3.4.1. HPLC and HPLC/MS

HPLC and UV-VIS detector showed several new formed peaks after the treatment with the laccase and a mediator to proof a possible coupling reaction even with a non-laccase substrate. With the addition of MS as a detector distinct coupling products could be identified as an additional proof of the coupling process.

Fig. 32 shows the chromatogram of the coupling reaction between 3-aminobiphenyl and methylhydroquinone. Several peaks could be identified as indicated in the

picture by the arrows. In addition to the 1:1 coupling products of the starting substances, a larger molecule could be identified providing another proof of the possible polymerization and precipitation of the environmental pollutants.

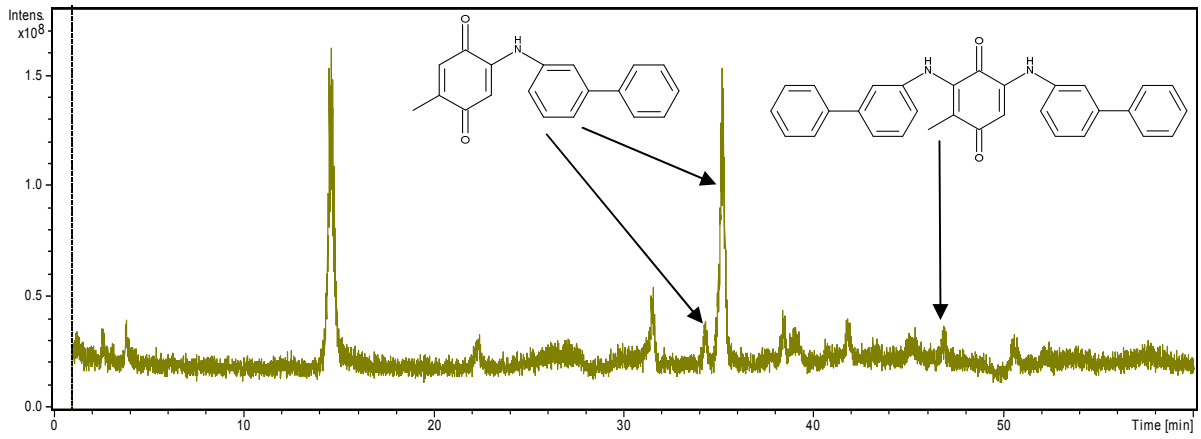

Fig. 32. Chromatogram of the products of the coupling reaction between 3-aminobiphenyl and methylhydroquinone

Fig. 33 and Fig. 34 show the MS spectra of the 1:1 coupling products between 3-aminobiphenyl and methylhydroquinone. Methylhydroquinone, reacting as the mediator, is oxidized in a first step from the hydroquinone to the quinone. While this reaction occurs 3-aminobiphenyl is attracted by the reactive species and is able to couple to the laccase substrate.

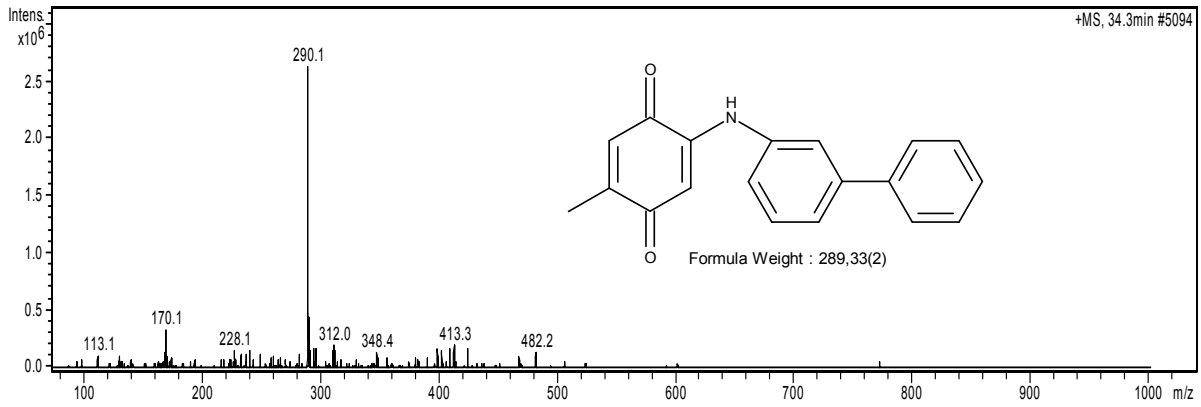

Fig. 33. MS spectrum of the coupling product between 3-aminobiphenyl and methylhydroquinone (34.3 min)

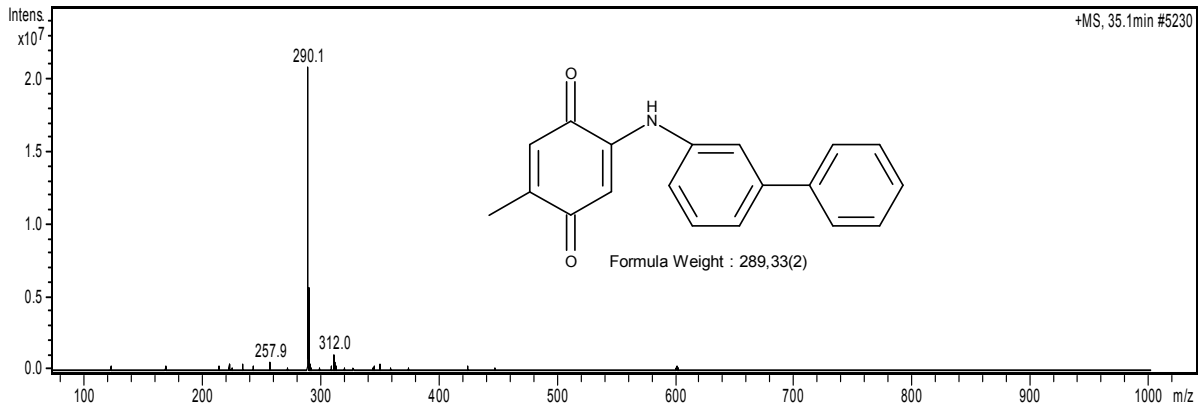

Fig. 34. MS spectrum of the coupling product between 3-aminobiphenyl and methylhydroquinone (35.1 min)

Fig. 35 shows the coupling product of two molecules of 3-aminobiphenyl and one molecule of methylhydroquinone. The laccase mediator is oxidized here as well in a first step before the coupling reaction occurs.

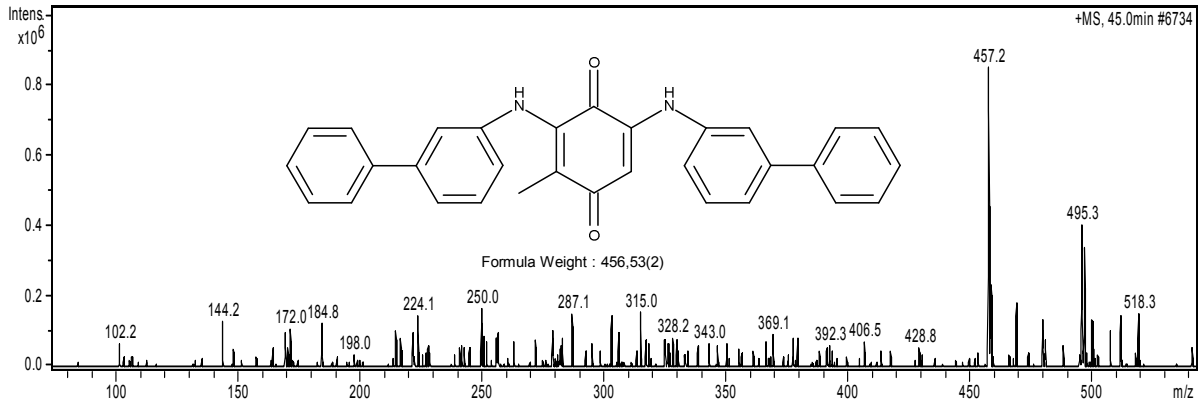

Fig. 35. MS spectrum of the coupling product between 3-aminobiphenyl and methylhydroquinone (45.0 min)

The position of the amino-group of the aminobiphenyl has no influence on the coupling possibilities. Neither is there an observed influence of the positions of the hydroxyl-groups of the simple phenolics which are used as mediator. For another analysis 4-aminobiphenyl and 4-methylcatechol were used for the coupling process. In contrast to methylhydroquinone which has two hydroxyl-groups in para-position, the hydroxyl-groups of 4-methylcatechol are in ortho-position. Fig. 36 shows the chromatogram of the coupling reaction between the above mentioned molecules. As the peaks were relatively small compared to the noise and above all compared to the big peak in the beginning, the analytical software allows a specific search for certain masses.

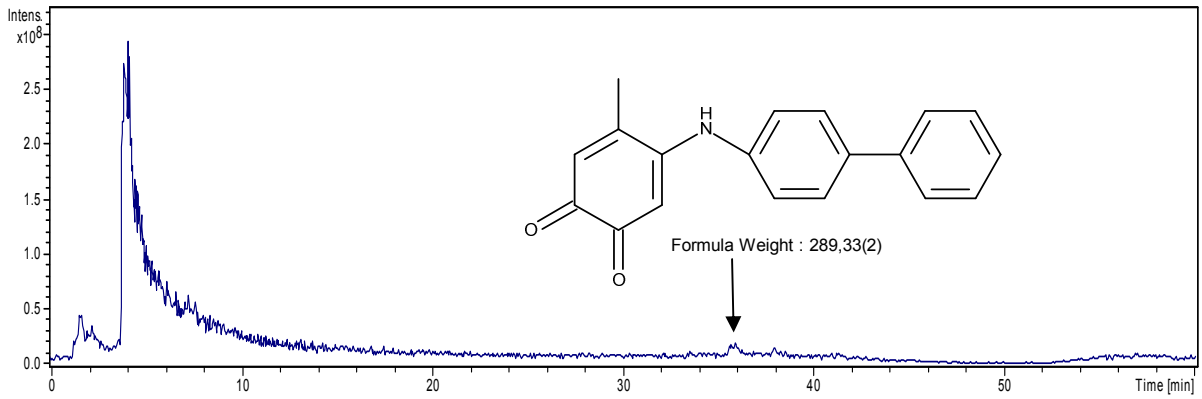

Fig. 36. Chromatogram of the coupling reaction between 4-aminobiphenyl and 4-methylcatechol

Fig. 37 shows the chromatogram of the coupling reaction but only considering the values where the mass of 290 is found. The mass of 290 was chosen because of the fact that the molecule mass of the suggested molecule is 289 and the analysis was made in positive mode which means that the molecule might be protonated. Using this analyzing tool the identification of this coupling product was simplified.

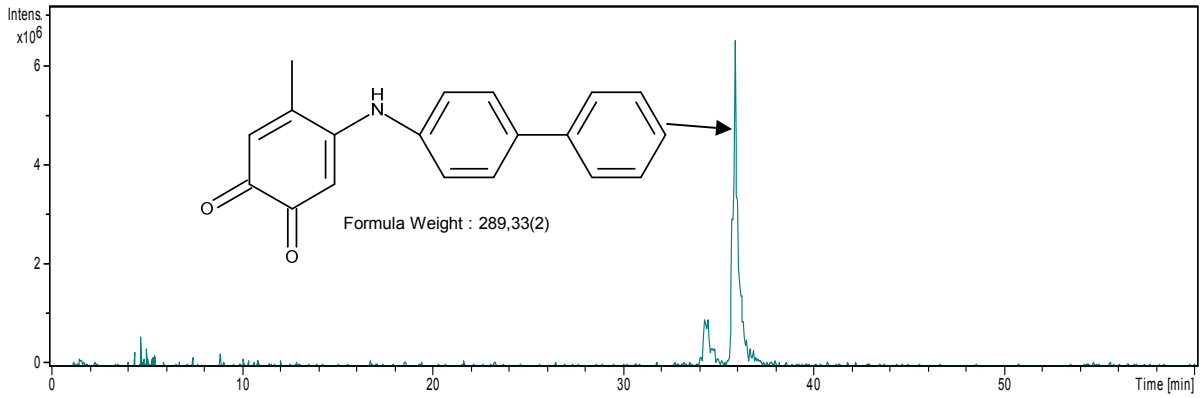

Fig. 37. Chromatogram of the coupling reaction between 4-aminobiphenyl and 4-methylcatechol scanning only for the mass of 290

Fig. 38 shows the MS spectrum of the peak marked in Fig. 37 at 29.6 min. The mediator is oxidized by the laccase, easily visible because of the oxo-functionalities, and therefore reactive enough to couple via the amino-group of the other molecule.

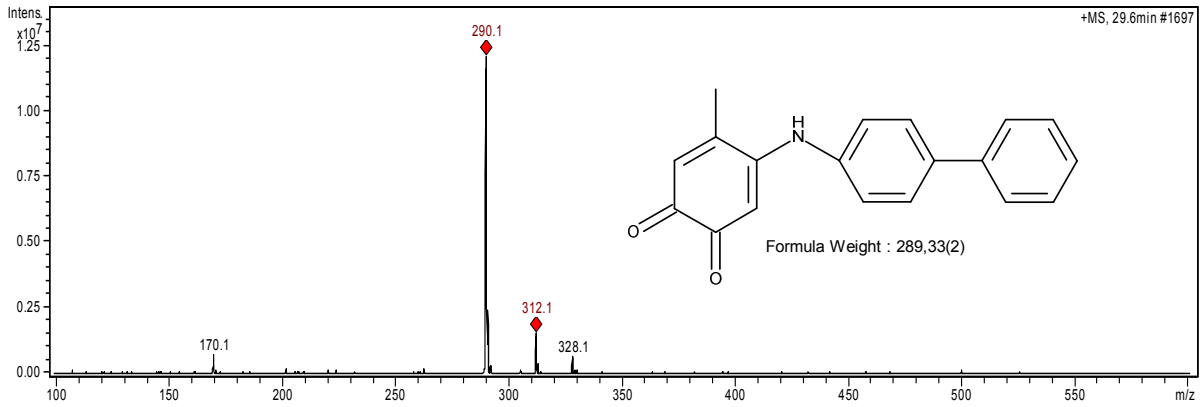

Fig. 38. MS spectrum of the coupling product between 4-aminobiphenyl and 4-methylcatechol (29.6 min)

The literature confirms these results having coupled hydroquinone and its derivatives to primary amines resulting in monoaminated or diaminated quinones[90].

Instead of just simple phenolics, amines might be used as a mediator as well. In this case 2-naphthylamine was used to couple with the polyaromatic hydrocarbon 1,2-benzanthracene. Fig. 39 shows the chromatogram of the coupling reaction between these reactants. Several of the reaction products could be identified.

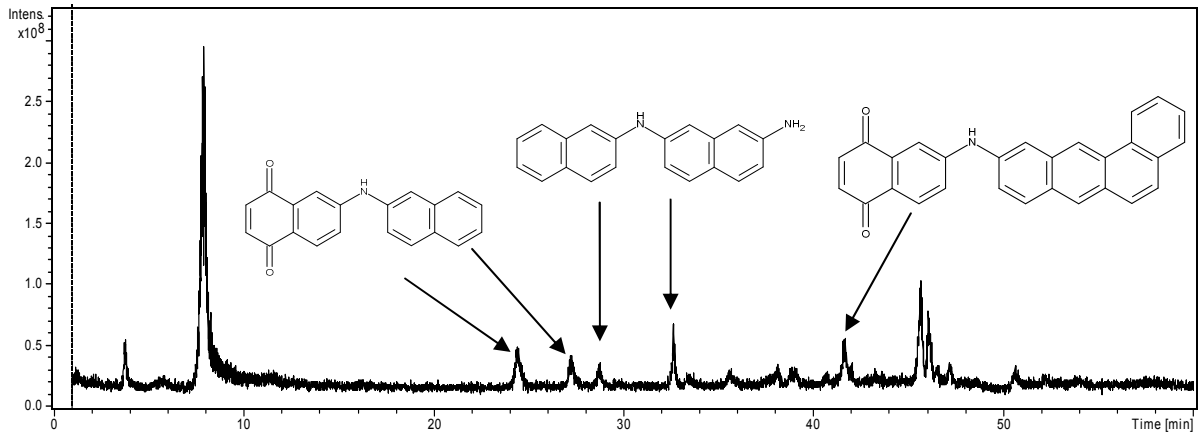

Fig. 39. Chromatogram of the coupling reaction between 2-naphthylamine and benzanthracene

The first two peaks which were identified are caused by a dimer of 2-naphthylamine. By producing reactive species not only the wanted coupling reaction takes place but also the formation of dimers and oligomers of the mediator was expected. During the formation process of this first dimer one molecule of 2-naphthylamine is oxidized and two oxo-functionalities are introduced. This reactive molecule then allows the coupling between the two amino groups of the molecules by abstracting NH_2. In this case just one molecule interacts as the mediator by being oxidized and therefore reactive enough to enhance to coupling process. Fig. 40 shows one of the MS spectra found for this kind of dimer of 2-naphthylamine.

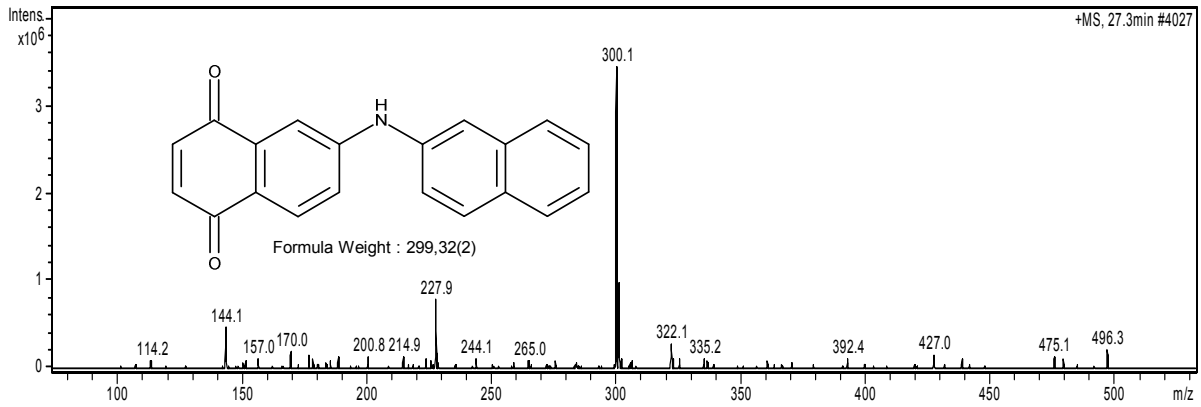

Fig. 40. MS spectrum of one possible dimer of 2-naphthylamine formed after activation by laccase (27.3 min)

The third and fourth peaks which were identified in the chromatogram of Fig. 39 represent another form of a dimer which might be built in a laccase coupling reaction. Fig. 41 shows the MS spectrum and the molecular structure of the expected molecule. In this reaction the amino-group of one molecule attacks the aromatic ring of a second molecule and enables the coupling.

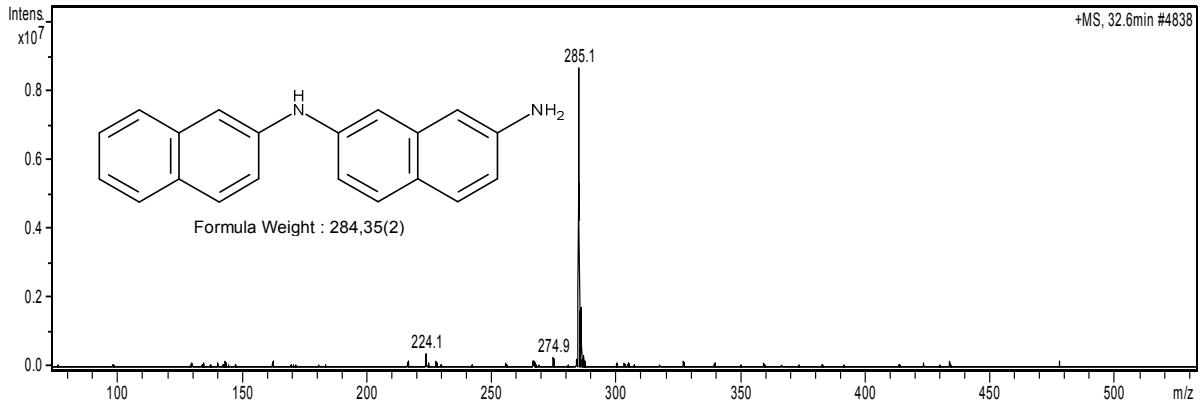

Fig. 41. MS spectrum of another possible dimer of 2-naphthylamine formed after activation by laccase (32.6 min)

The last identified peak of the chromatogram (Fig. 39) indicates one of the coupling products obtained through the laccase induced reaction. Fig. 42 shows the MS spectrum of this newly formed molecule. 2-naphthylamine is again fully oxidized and two oxo-functionalities are introduced. The oxidized molecule is now reactive enough to attack the rarely reacting polyaromatic backbone of 1,2-benzanthracene.

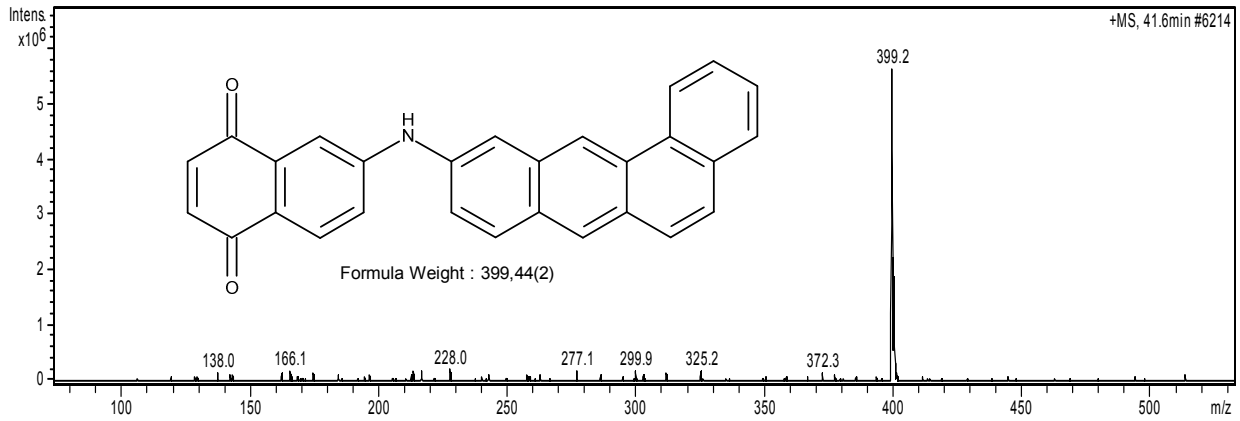

Fig. 42. MS spectrum of the coupling product between 2-naphthylamine and benzanthracene upon laccase oxidation (41.6 min)

The cross-coupling of PAHs induced by laccase and a mediator is found several times in the literature. Different mediators were already used; synthetic mediators such as ABTS or HBT or natural mediators such as simple phenols and anilines. All results agree in the fact that the addition of a mediator tremendously increases the degradation of the PAH by the laccase[84,87,89,91].

3.4.2. HS-SPME-GC/MS

As mentioned in the methods section the scan mode of the MS did not produce any results which can be evaluated. The signal to noise ratio was not good enough to be able to significantly distinguish between a wanted peak and the ground noise. Another problem was the solvent peak (Fig. 43). As acetaldehyde is a relatively small molecule and soluble in water; its peak and the solvent's peak were coming at similar times. By improving the method, it was able to remove the solvent peak.

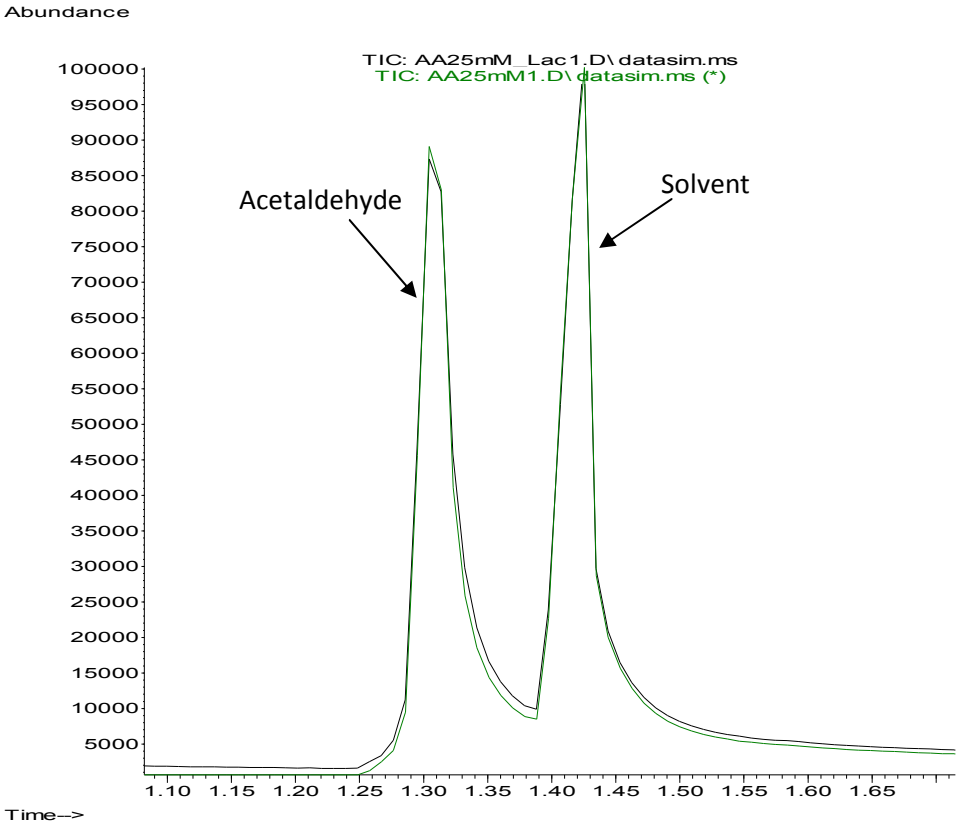

Fig. 43. GC/MS analysis of acetaldehyde (green) and acetaldehyde with laccase (black)

Acetaldehyde (Fig. 43) as well as acrolein (Fig. 44) is not oxidized by laccase directly. There is no change in concentration of these molecules after incubating with laccase. Therefore the only possible strategy for their removal with laccase is through co-polymerization of laccase oxidized substrates.

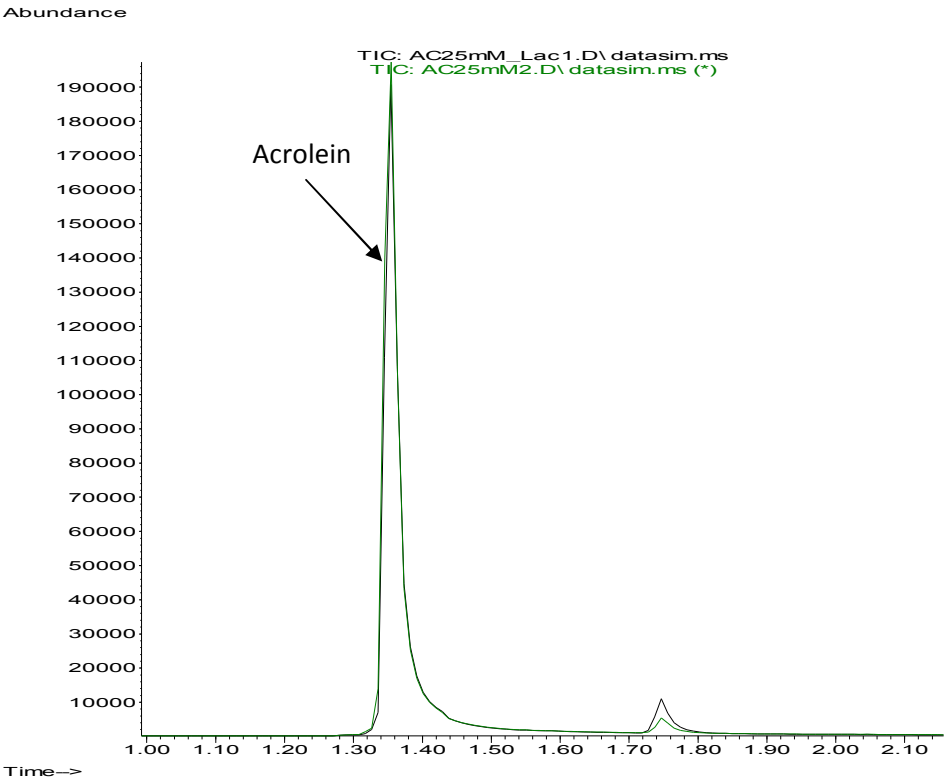

Fig. 44. GC/MS analysis in SIM mode: acrolein (green), acrolein with laccase (black)

Fig. 45 shows the influence of a mediator on the removal of acetaldehyde by laccase. The first peak, indicating the concentration of acetaldehyde, the sample with a mediator and laccase (green) is considerably lower than the sample with mediator but without enzyme (black). Additionally there is a new peak formed at the enzyme-mediator-sample, identified as acetic acid. Therefore laccase is able to oxidize acetaldehyde after adding a mediator which is hydroquinone in this case.

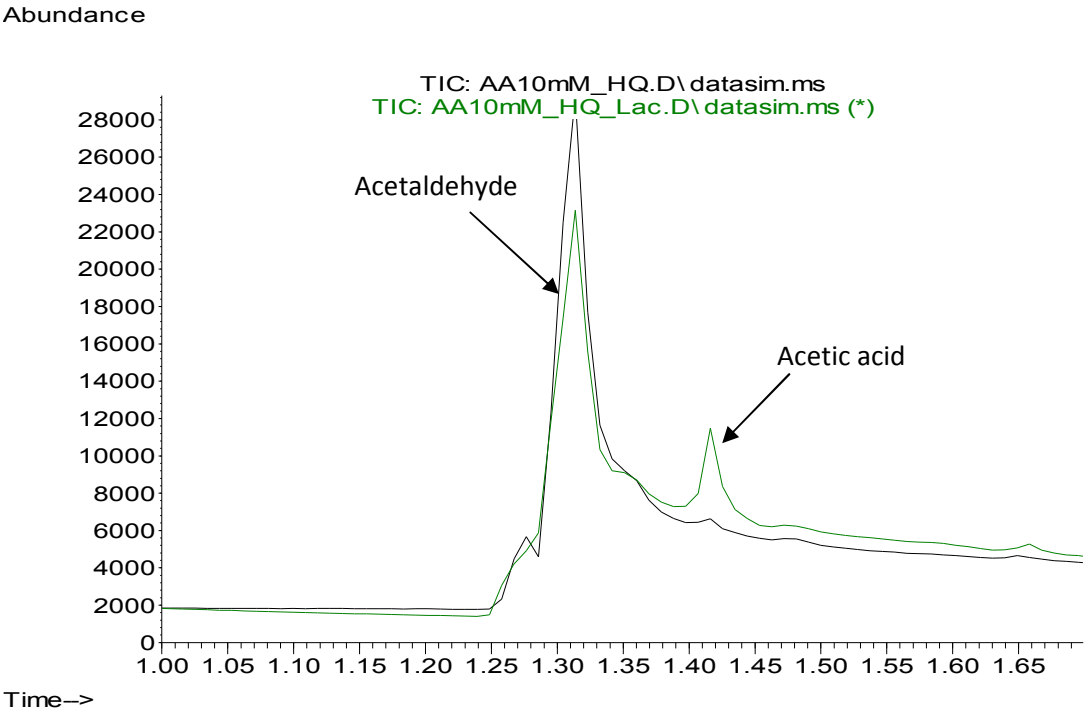

Fig. 45. GC/MS analysis in SIM-mode: acetaldehyde + HQ (black), acetaldehyde + HQ + laccase (green)

Acrolein showed similar results. The peak of acrolein had a significant decrease in the laccase-mediator sample compared to the sample without enzyme. On the other hand, no oxidation product could be found and identified.

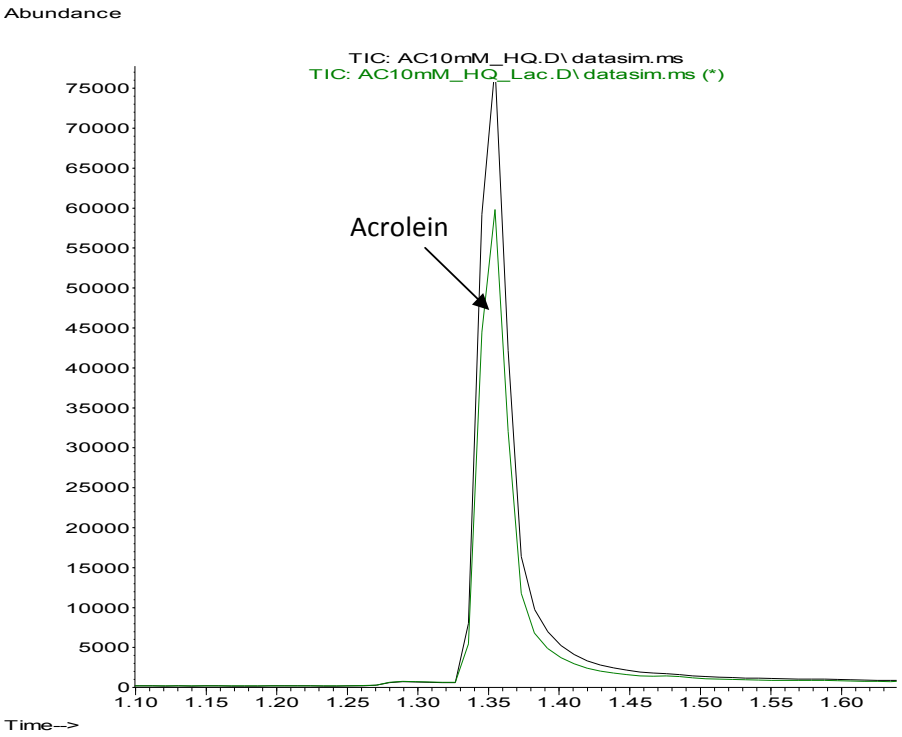

Fig. 46. GC/MS analysis in SIM-mode: acrolein + HQ (black), acrolein + HQ + laccase (green)

3.4.3. Formaldehyde (Nash-reaction)

Due to the chemical structure of formaldehyde, it was not expected to be directly oxidized by laccase. Therefore a co-removal of formaldehyde using phenolic laccase substrates was the reason for this analysis.

Table 16 shows the summarized results of the Nash-reaction after incubating the samples with the phenolics. In all samples there was a slight reduction of the formaldehyde concentration even without enzyme after incubating with the mediator. It would appear that formaldehyde reacts with the different phenolics even in the absence of laccase. 2-Naphthylamine produced very interesting results as the decrease of formaldehyde caused by this molecule was 79 % of the beginning concentration.

For most of the samples the adding of laccase caused a considerably larger decrease of the formaldehyde samples. The exceptions are trimethylhydroquinone and 2-naphthylamine. These two molecules appear to have their best formaldehyde removal potential without laccase.

That means by adding these mediators to the filter system environmental pollutants might be oxidized and co-polymerized with and without the presence of laccase.

Table 16. Co-removal of formaldehyde in the presence of laccase substrates

Sample/Mediator	Formaldehyde concentration without laccase [µM]	Std. dev.	Loss in %	Formaldehyde concentration with laccase [µM]	Std. Dev.	Loss in %
without Mediator	60,67	0,46	-	59,89	0,83	1,29
Hydroquinone	59,27	0,20	2,32	55,93	0,47	7,81
Methyl-hydroquinone	59,43	0,91	2,05	54,26	0,58	10,56
Trimethyl-hydroquinone	54,40	2,00	10,35	56,29	1,77	7,22
Catechol	59,46	1,02	1,45	54,20	3,38	13,90
Methylcatechol	59,79	1,22	1,99	52,24	0,68	10,67
2-Naphthylamine	12,68	0,54	79,11	35,17	1,82	42,03

3.4.4. Nitric oxide

As shown in Fig. 47, qualitative analysis shows that nitrite ions were highest in the control as compared to the samples incubated with the different phenolic substrates. This is good evidence that these different phenolics, when activated by laccase, lead to the co-removal of nitric oxide which is not a laccase substrate.

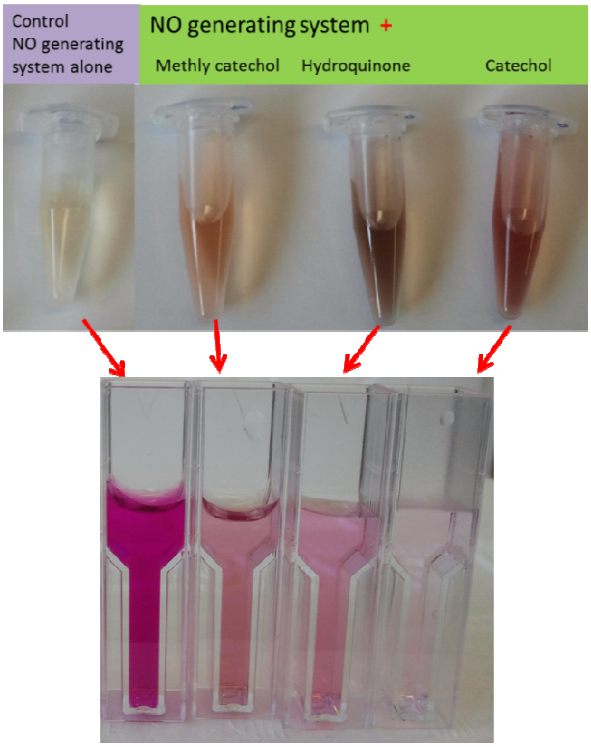

Fig. 47. Visualization of the quenching/sequestration of nitric oxide during the co-incubation of nitric oxide generating system with the laccase oxidized phenolics

Quantitative analysis showed that all the laccase oxidized phenolics were able to mediate the removal of over 92 % 7.8 mM nitric oxide generated in situ (Table 17). catechol was the highest achieving 93.72 %. This means that when laccase activates these molecules they will actively remove nitric oxide as well.

Table 17. % removal of nitric oxide in the presence of different laccase oxidized toxicants

Sample	mM Nitric oxide	% quenching/ sequestration
10 mM SNP (control)	7.80	0
10 mM SNP + 10 mM hydroquinone + laccase	0.63	92
10 mM SNP + 10 mM catechol + laccase	0.54	93.72
10 mM SNP + 10 mM 4-methylcatechol + laccase	0.67	92.58

3.5. Immobilization of laccase on solid support

Since the combustion products are released as a mixture containing gases, aerosols, vapor, for the successful continual removal of toxicants a laccase loaded filter was decided to be the best removal strategy. It was therefore decided to use cellulose acetate as the solid support since it is largely used as a filter. Unfortunately cellulose acetate is none reactive due to acetyl groups which therefore demands prior activation procedures.

The removal of acetyl groups was achieved by hydrolysis using NaOH. These acetyl groups were the main point of the analysis of the deacetylation because they are the difference between cellulose acetate (Fig. 48) and the deacetylated cellulose acetate (=cellulose) (Fig. 48). These acetyl groups can be detected using FTIR spectroscopy.

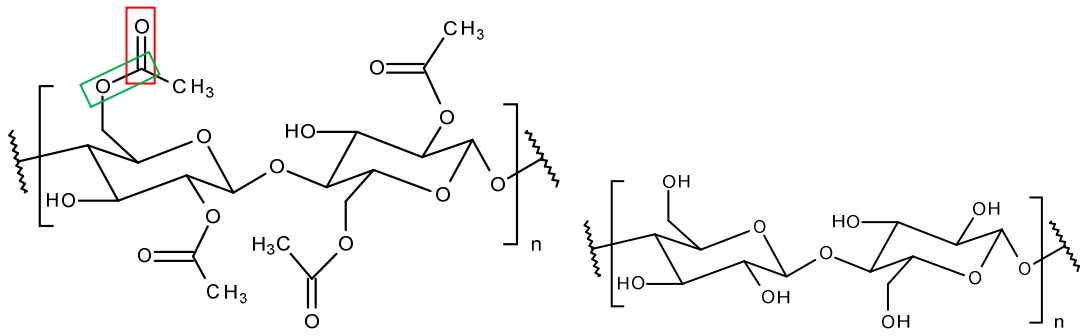

Fig. 48. Structures of cellulose acetate and cellulose, C=O bond marked in red, C-O bond marked in green

Fig. 49 shows the FTIR-spectrum with the two most important peaks for the determination of acetyl-groups in a molecule. The first peak at a wavenumber of about 1737 cm^{-1} represents the C=O stretching vibrations of the acetate group (Fig. 48, marked in red). This goes in line with several publications finding this peak at wavenumbers between 1735 and 1765 cm^{-1} [92,93,94]. The black line indicates the cellulose acetate used as the starting point for the modifications. According to the producing company the degree of acetylation is about 40%. The blue line indicates cellulose which has no acetyl groups which is indicated that there is no peak at about 1737 cm^{-1}. The red and the green lines show the samples which were treated with 50 mM and 100 mM NaOH. Due to the height of the peaks of the different samples the degree of acetylation is reduced by using a higher concentration of NaOH for the deacetylation process.

The second peak marked in Fig. 49 is at the wavenumber of 1216 cm^{-1}. This peak represents the C-O stretching vibration of the ester bond (Fig. 48, marked in green). Toprak et al. found this peak at about 1235 cm^{-1}, but as their C=O stretching vibrations were found as well at a bit higher numbers, they appear to be the same[92]. Similar to the first peak the cellulose spectrum (blue) doesn't show any peak at all. The other lines indicate the different degrees of acetylation.

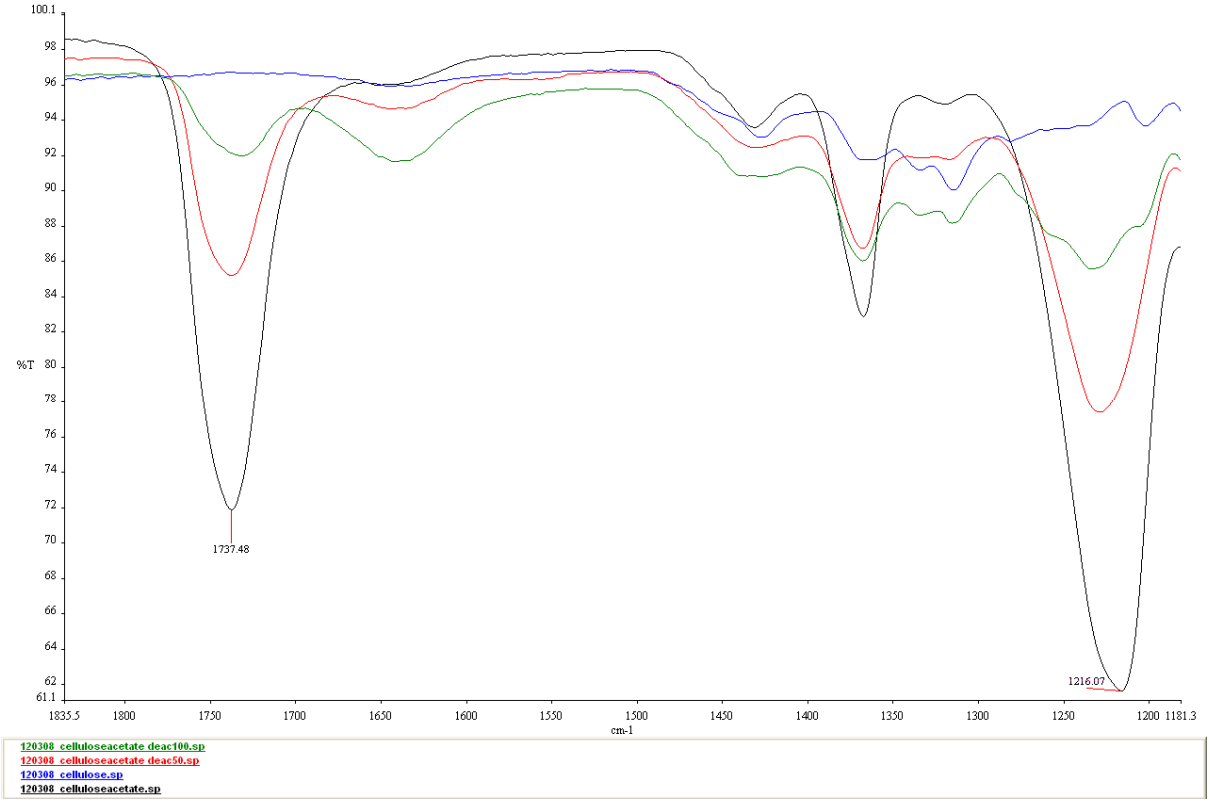

Fig. 49. IR-spectrum of the two most important peaks (1737 cm^{-1} and 1216 cm^{-1}) of cellulose (blue), cellulose acetate (black), cellulose acetate treated with 50 mM NaOH (red) and cellulose acetate treated with 100 mM NaOH (green).

3.5.1. Use of cross-linkers to immobilize laccase

In order to improve the stability and reactivity of the enzyme glutaraldehyde and hexamethylenediamine (HMD) were introduced as cross-linkers and their influence on the activity of the enzyme was investigated.

Fig. 50 shows the influence of the deacetylation on the possibility to bind the crosslinker HMD on the cellulose acetate. All samples were treated the same way with a periodate oxidation and the addition of HMD. Sample No. 4 is cellulose acetate. The samples No. 1-3 are partial deacetylated cellulose acetate samples;

No. 1 having the highest and No. 3 the lowest degree of deacetylation. This shows that the deacetylation has an influence on the coupling of the cross-linkers.

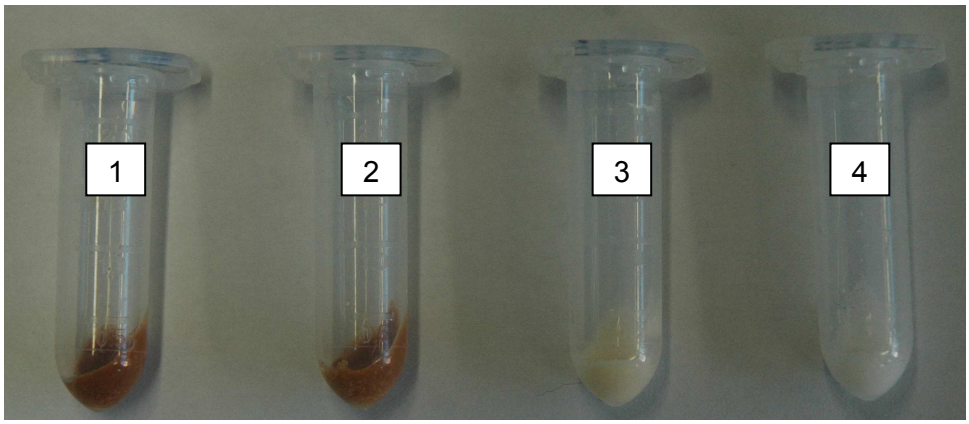

Fig. 50. Influence of the deacetylation on the binding of the crosslinker HMD. All samples were treated with periodate and the crosslinker. No. 4 shows cellulose acetate, No. 1-3 partially deacetylated cellulose acetate, No. 1 having the highest degree of deacetylation.

Enzyme activity tests

The activities of immobilized enzymes are summarized in Table 18.

Table 18. Enzymatic activity of the modified cellulose acetate filter after immobilization

Sample	Activity (U/g Filter)
Cellulose acetate (Adsorption)	0,40
Partial deacetylated cellulose acetate (Adsorption)	1,10
Partial deacetylated cellulose acetate + HMD	1,00
Partial deacetylated cellulose acetate + glutaraldehyde	1,91
Cellulose acetate + HMD + glutaraldehyde	1,31
Partial deacetylated cellulose acetate + HMD + glutaraldehyde	2,26

Cellulose acetate with a higher degree of deacetylation (Fig. 50 samples No. 1+2) led to problems with the ABTS assay. Because of that only sample No. 3 (Fig. 50) could be used for the measurement of the activity. During the incubation time the green color of the oxidized ABTS faded away for the higher deacetylated samples. The immobilization process might have worked well and the enzymatic activity might

be higher than the measured samples, but due to the assay conditions it was not possible to quantify the laccase activity for these samples. Regarding this problem the results for the lowest deacetylated cellulose acetate sample might be higher than measured.

The results in Table 18 show clearly the positive impact of the treatment of the filter material on the enzymatic activity. Adsorption as immobilization method has lower activity than covalent bound enzyme because it can't be washed away easily. Partial deacetylation of the cellulose acetate improves the adsorption process by raising the activity level.

The chemical treatment using crosslinkers achieved the highest measured activity. HMD used as the only crosslinker did not result in better activity than the adsorption approach. Glutaraldehyde has obviously great importance on the positive results, achieving higher values than HMD. The best results nevertheless are obtained by a combination of the two crosslinkers.

Glutaraldehyde is able to react with several functional groups of amino acids and therefore of enzymes. Above all the free amino groups are the main target of the reaction[95]. Therefore by providing amino groups on the filter material by functionalization with HMD, glutaraldehyde can react very well with both the functionalized filter and the enzyme resulting in a covalent connection.

Of big importance as well is the fact of the activation of the cellulose acetate filters which was done to all the samples in Table 18 prior to the treatment with the crosslinkers. This activation achieved by sodium periodate opened up the glucose rings which enables the attack of the crosslinkers or the enzyme respectively. There are other methods described in the literature to obtain such an activation of cellulose acetate such as the use of Ce(IV), but according to Sternberg et al.[96] this was the method resulting in the highest final activity.

4. Conclusion

Air pollution is a global health problem of increasing importance. Pollutants are a complex mixture of different compounds existing in gaseous, liquid and solid phases. They are harmful to animals and humans and are responsible for numerous diseases. Several methods to reduce different air toxicants are known. The aim of this research was to develop an enzyme-filter system to eliminate air pollutants especially from combustion processes.

Many typical toxicants from combustion processes were tested for their ability to be eliminated by laccases by direct oxidation, in the presence of mediators or by coprecipitation in the presence of other phenolics. Enzymatic elimination of toxicants was mechanistically investigated using spectrophotometry, HPLC/MS, GC, oxygen consumption, and different molecule-specific assays.

Direct oxidation occurred as suspected with simple phenolics as they are known laccase substrates. In nitrogen-containing molecules oxidation depended on the position of the nitrogen. Amino groups such as in 2-naphthylamine are possible points for enzymatic attack. On the other hand molecules such as quinoline with the nitrogen included in the aromatic ring provide no possibility to be oxidized by laccase. Due to their stable structure PAHs are as well not affected by direct oxidation by the laccase.

In the presence of simple phenolics, oxidation of molecules, which were not oxidized by laccase directly, could be achieved. This group of molecules includes different volatile compounds such as acetaldehyde, acrolein, formaldehyde and nitric oxide.

Coupling reactions induced by the laccase-mediator-system were also observed. Simple phenolics were coupled to aminobiphenyl which showed no direct oxidation with laccase. 2-naphthylamine was able to attack and couple to benzanthracene, a PAH which is hardly reactive.

For in situ removal of the air pollutants, the enzyme was successfully immobilized onto a solid support, cellulose acetate, maintaining enzymatic activity. In order to increase the reactivity of the support it was hydrolyzed by NaOH. By varying the concentration of NaOH the degree of deacetylation changes which was detected by

ATR-FTIR spectroscopy. Deacetylation improved the final activity of the enzyme on the solid support. A further increase of the activity was possible through the introduction of crosslinkers. By using HMD amino-groups were introduced to the cellulose acetate. By adding glutaraldehyde which is able to react with amino groups and therefore with the amino functionalized cellulose acetate and the enzyme, it is possible to improve the final enzymatic activity on the filter material.

Nevertheless there is room for improvement and possibilities for additional investigations. The list of analyzed toxicants could be expanded to get an overview of all the possibilities of this method. Additionally different methods for the immobilization process could be investigated to ensure the best possible method. In this case there are several options to change the crosslinkers, obtain an amino functionalization differently and finally optimize the best procedures.

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6. Abbreviations

2NA	2-Naphthylamine
3AB	3-Aminobiphenyl
4AB	4-Aminobiphenyl
4MCat	4-Methylcatechol
Aa	Acetaldehyde
ABTS	2,2-azinodi-3-ethylbenzothiazoline-6-sulphuric acid
Ac	Acrolein
ACN	Acetonitrile
ATR	attenuated total reflectance
BαP	Benzo[α]pyrene
CA	Cellulose acetate
Cat	Catechol
ddH_2O	double distilled water
FA	Formaldehyde
FTIR	Fourier transform infrared
GC	Gas chromatography
h	hour(s)
HIC	hydrophobic interaction chromatography
HPLC	High Performance Liquid Chromatography
HQ	Hydroquinone
HS	Head-space
Lac	Laccase
MHQ	Methylhydroquinone
min	minute(s)
MS	Mass spectrometry
SIM	Single Ion Monitoring
SPME	Solid phase micro extraction
TMHQ	Trimethylhydroquinone

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8. Declaration

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.

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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.

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