Mechanistic Studies of Laccase/TEMPO Catalyzed Aerobic Oxidation of Alcohols

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Mechanistic Studies of Laccase/TEMPO Catalyzed Aerobic Oxidation of Alcohols

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Summary

The topic of this thesis is the aerobic alcohol oxidation by the Laccase/TEMPO system. Laccases are glycosylated oxidases which have four copper atoms in the active site, and which can catalyze the one-electron oxidation of substrate molecules, such as phenolic derivatives, concomitant with the four electron reduction of molecular oxygen to water. TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) molecule has a redox active nitroxide group and is employed as so-called mediator. The combination of laccase with TEMPO results in the selective oxidation of a wide range of non-electron rich alcohols. The integration of biocatalysis and organic synthesis is an important development in the fine chemical industry. Especially in the area of oxidation there is a stringent need to replace classic stoichiometric reagents with green catalytic alternatives. The introduction of catalytic systems in the field of oxidation can afford both economical and environmental benefits.

The laccase used in this study was the one from *Trametes versicolor*. Previous studies indicated that 15-30 mol % of TEMPO and high enzyme concentrations were required for the oxidation of benzylic alcohols. It was therefore important to study the reaction mechanism and limiting factors in a great depth. Based on this knowledge, the goal was to make the laccase/TEMPO system more economically viable for the oxidation of industrially relevant aliphatic alcohols.

The thesis consists of six chapters. In Chapter 1 an overview is given of laccases: Its biochemical characteristics, biological role in nature and application in organic synthesis and industry are discussed.

Chapter 2 describes the postulated mechanism for the laccase/TEMPO reaction. The function of laccase is to oxidize TEMPO to the corresponding oxoammonium ion, which is the actual oxidant in the system. Subsequently, oxoammonium oxidizes alcohol to aldehyde in a two-electron step. The resulting hydroxylamine can then be re-oxidized to TEMPO by laccase, or by disproportionation with another molecule of oxoammonium. Initial studies were carried out to determine the optimum reaction conditions (pH, temperature, concentrations of the reaction components) required to obtained sufficient conversions on a synthetic scale for a variety of aromatic alcohols. It turned that that 10 U/ml of laccase and 15 mM of TEMPO were required to oxidize 160 mM of substrate. Moreover it was investigated how sufficient aeration of the solution could be achieved. It was shown that TEMPO, next to 4-acetamido TEMPO is the most effective mediator for conversion of alcohols. Under these conditions the system displays a high selectivity for primary alcohols and only activated secondary alcohols such as 1-phenyl could be converted to the corresponding aldehyde.

In Chapter 3, the studies on the oxidation of linear aliphatic alcohols are described. In this case an optimum temperature of 35 ℃ was determined. Under these conditions, 65% of 1-octanol could be converted to aldehyde in 7 hours. The initial oxidation rates were determined for a wide range of alcohols (C5-C12). In this system aeration had to be slowed down in order to prevent further oxidation of the aldehyde to the acid. The investigation of aliphatic alcohols showed that the oxidation rate does not depend on the solubility; when dioxane was used one phase resulted, and we observed that the yield decreased

with increasing chain length of the substrate. This decrease in rate reflects the decrease in reactivity of the alcohols. Although solubility restraints are elevated, in general the yields are lower than observed previously under aqueous conditions. Apparently the presence of co-solvent lowers the activity of the enzyme. The activity profile of the enzyme during the reaction was investigated, and according to the standard assay no significant deactivation took place.

In Chapter 4 the optimized purification method for laccase from *Trametes versicolor* is presented. Purified blue enzyme was employed for spectroscopic kinetic studies of each separate step of the overall reaction. The optimized purification scheme based on two-step purification, first ion exchange, than size exclusion chromatography, can be applied for fungal laccase purification. The laccase from *Trametes versicolor* was purified with approximately 60% yield and a purification factor of 4. Stopped-flow studies unequivocally showed that TEMPO interacts directly with laccase. Rapid electron transfer from TEMPO to the Type 1 Cu took place and from there to the other copper sites resulting in a steady-state oxidation of the substrate. The complete oxidation of the reduced T1 Cu within ~ 100 µs after mixing with oxygen indicates a fast internal electron transfer (> 25.000/s) and rapid binding of oxygen (k_{on} > $5x10^7 M^{-1}s^{-1}$). These results indicated that neither internal electron transfer nor binding of oxygen are limiting the turnover rate of the enzyme.

Chapter 5 describes the kinetic studies and at line EPR experiments to establish rate constants for the individual steps in the reaction cycle of the laccase/TEMPO system. Rate constants were determined for the

two regeneration pathways for hydroxylamine: the oxidation by laccase and the recombination with the oxoammonium cation. In addition, alcohol oxidation rates by oxoammonium were determined at various pH values. Based on these rates, kinetic modeling was performed for the oxidation of various benzyl alcohol concentrations at 25 °C, using 10 U/ml of laccase and 15 mM of TEMPO. From these studies we conclude that only a fraction of the laccase appears to be active upon dispersion in the reaction mixture. We further found that besides by TEMPO the alcohol oxidation can be initiated by the hydroxylamine, but not by the oxoammonium ion, indicating that the system contains a (near) irreversible step.

Three sources of laccase, *Trametes versicolor, Trametes villosa, Agaricus bisporus,* were applied for cross-linked enzyme aggregate (CLEA) preparation and the results thereof are presented in Chapter 6. The optimum conditions for precipitation (best precipitant, pH, temperature) and cross-linking (concentration of cross-linker – glutaraldehyde, cross-linking time) steps were investigated. An increase in stability of laccase in its CLEA formulation was observed during the alcohol reaction. Moreover laccase-CLEA could be recycled 3 times significant without loss of activity. The reactions in buffer produced higher conversions of aliphatic alcohols using only 1 U/ml of laccase CLEA, in comparison with 10 U/ml of laccase that was required in case of soluble enzyme. These appointed results together with reusability data make the biocatalytic applications of laccase CLEAs very attractive.

Samenvatting

Het onderwerp van dit proefschrift is de oxidatie van alcoholen met luchtzuurstof, gekatalyseerd door het laccase/TEMPO systeem. Laccases zijn oxidases met suikerresiduen die vier koper atomen in het actieve centrum bevatten. Ze katalyseren de één-electron oxidatie van substraten, zoals fenolderivaten, gekoppeld aan de vier-electron reductie van moleculaire zuurstof naar water. TEMPO (2,2',4,4'-tetramethylpiperidinyl-1-oxyl) heeft een redox-actieve nitroxide groep en wordt ingezet als mediator. De combinatie van laccase met TEMPO resulteert in de selectieve oxidatie van een breed spectrum aan niet-electronrijke alcoholen. De integratie van biokatalyse en organische synthese is een belangrijke ontwikkeling in de fijnchemische industrie. Vooral op het gebied van oxidatie is er een drijfveer om klassieke stoichiometrische reagentie te vervangen door groene katalytische alternatieven. De introductie van katalytische systemen op het gebied van oxidatie kan zowel economische als milieuvoordelen opleveren.

De laccase in dit onderzoek is afkomstig van "*Trametes versicolor*". Eerdere studies hebben aangetoond dat 15-30 mol % TEMPO en hoge enzymconcentraties nodig zijn om de oxidatie van benzylalcoholen uit te voeren. Het was daarom belangrijk om het reactiemechanisme en de beperkende factoren grondig te bestuderen. Op basis van deze kennis was het doel om een laccase/TEMPO systeem te ontwerpen dat economisch aantrekkelijk is voor de oxidatie van industrieel relevantie alcoholen.

Het proefschrift bestaat uit zes hoofdstukken. In hoofdstuk 6 wordt een overzicht gegeven van laccases in het algemeen: De biochemische kenmerken van laccases, hun biologische rol in de natuur en hun mogelijke synthetische en industriële toepassingen worden besproken.

In hoofdstuk 2 wordt het voorgestelde mechanisme voor de laccase/TEMPO gekatalyseerde reactie besproken: De rol van laccase is om TEMPO te oxideren naar het oxoammonium ion, dat de werkelijke oxidant in het systeem is. Vervolgens oxideert oxoammonium het alcohol naar een aldehyde in een twee-electron stap. Het resulterende hydroxylamine kan dan worden gereoxideerd tot TEMPO òf door laccase, òf door disproportionering met oxoammonium. Initiële studies werden uitgevoerd om de optimale reactiecondities (pH, temperatuur, concentraties van de reactiecomponenten) te bepalen die nodig zijn om een reeks van aromatische alcoholen om te zetten op een synthetisch relevante schaal. Het bleek dat 10 units/ml van het laccase en 15 mM TEMPO nodig zijn om 160 mM substraat om te zetten. Er werd bekeken hoe voldoende beluchting van de oplossing kon worden bereikt. Ook werd bepaald dat uit de reeks van onderzochte TEMPO-derivaten, TEMPO, naast acetamido-TEMPO, de meest effectieve mediator is. Onder deze omstandigheden vertoont het systeem een hoge selectiviteit voor primaire alcoholen. Alleen geactiveerde secondaire alcoholen zoals 1-fenylalcoholen worden omgezet naar het overeenkomstige aldehyde.

In hoofdstuk 3 worden de studies naar de oxidatie van lineaire alifatische alcoholen beschreven. Bij een optimale temperatuur van 35 ℃ kon 65% 1-octanol worden omgezet naar aldehyde binnen 7 uur. De

initiële oxidatie snelheden voor een reeks van alcoholen (C5-C12) werden bepaald. In dit systeem werd de beluchting beperkt om de dooroxidatie van aldehyden naar carbonzuren te voorkomen. Deze studie laat zien dat de oxidatiesnelheid niet afhangt van de oplosbaarheid van het alcoholische substraat: Wanneer dioxaan werd gebruikt als co-oplosmiddel, resulterend in één fase, nam de opbrengst af met toenemende ketenlengte van het substraat. We concluderen dat deze afname gerelateerd is aan een afname in reactiviteit. Onder deze éénfase condities, zijn echter de opbrengsten lager dan onder waterige condities. Blijkbaar desactiveert de aanwezigheid van oplosmiddel het enzym. Deze desactivering van enzym werd gevolgd gedurende de reactie in een standard assay test. Echter met dit assay kon een afname van de enzymactiviteit tijdens de reactie niet worden waargenomen.

In hoofdstuk 4 wordt een geoptimaliseerde zuiveringsmethode voor laccase van *Trametes versicolor* beschreven. Deze zuiveringsprocedure bestaat uit twee stappen, ionuitwisseling wordt gevolgd door “size-exlusion”-chromatografie. Laccase werd gezuiverd met 60 % opbrengst en een zuiveringsfactor van 4. Gezuiverd blauw enzym werd gebruikt in spectroscopische kinetische studies van de enzymstappen in de reactiecyclus. “Stopped-flow” studies tonen onomstootbaar aan dat TEMPO direct met laccase reageert. Snelle electronenoverdracht van TEMPO naar Type 1 koper vindt plaats and wordt gevolgd door elecronenoverdracht naar andere kopercentra, resulterend in een “steady-state” oxidatie van het substraat. De volledige oxidatie van het gereduceerde T1 Cu binnen ~ 100 µs na het mengen met zuurstof, komt overeen met een snelle interne electronoverdracht (> 25.000/s) en snelle binding met zuurstof ($k_{on} > 5x10^7$ $M^{-1}s^{-1}$). Deze resultaten geven

aan dat noch interne electronoverdracht noch binding met zuurstof de turnoversnelheid van het enzym beperken.

Hoofdstuk 5 beschrijft de kinetische studies en on-line EPR experimenten die zijn uitgevoerd om de reactiesnelheidsconstanten te bepalen voor de individuele reactiestappen in de cyclus van het laccase/TEMPO systeem. Snelheidsconstanten werden bepaald voor de twee regeneratiepaden voor hydroxylamine: De oxidatie door laccase en de disproportionering met het oxoammonium cation. Daarnaast werd de snelheidsconstante voor de oxidatie van alcohol met oxoammonium bepaald bij verschillende pHs. Op basis hiervan werd een kinetische modellering uitgevoerd van de reactie zoals die plaatsvindt voor benzylalcohol oxidatie bij verschillende concentraties (25 °C, 10 U/ml laccase en 15 mM TEMPO). Uit deze studies kunnen we concluderen dat slechts een deel van het laccase aanwezig in het reactiemengsel actief blijkt te zijn gedurende de eerste twee uur. Verder werd gevonden dat de reactie naast TEMPO ook kan worden uitgevoerd met het hydroxylamine, maar niet met oxoammonium. Dit toont aan dat het systeem een (nagenoeg) irreversibele stap bevat.

De bereiding van “cross-linked” enzymaggregaten (CLEA) voor drie verschillende laccases, *Trametes versicolor, Trametes villosa,* en *Agaricus bisporus*, wordt beschreven in hoofdstuk 6. De optimale condities voor de bereiding (precipitant, pH, temperatuur) en de “cross-linker” (concentratie glutyraldehyde, tijd van cross-linker) werden onderzocht. Een toename in de stabiliteit van laccase enzym werd waargenomen, wanneer het als CLEA-laccase wordt gebruikt in de

TEMPO/laccase oxidatie van alcoholen. Bovendien kon laccase-CLEA driemaal worden gerecycled zonder dat significant verlies van de activiteit kon worden waargenomen. De reacties in buffer geven hoge omzettingen van alifatische alcoholen met slechts 1 U/ml laccase-CLEA (in vergelijking met 10 U/ml benodigd voor natief laccase). Deze resultaten, samen met de potentie tot recycling, maken laccase-CLEA's aantrekkelijk voor biokatalytische toepassingen.

1

Laccases – from biochemical studies to chemical and industrial applications

Introduction

The conversion of an alcohol into an aldehyde is a pivotal reaction in organic chemistry. Alcohols are widely available i.e. from natural resources, and their one-step selective conversion provides a straightforward pathway to the production of aldehydes. In contrast to low molecular weight aldehydes (formaldehyde and acetaldehyde have sharp unpleasant odors) higher molecular weight aldehydes have pleasant, often flowery, odors and can be found in essential oils of certain plants. Their eco-friendly production is therefore directly relevant to the flavour industry, where they can be used as ingredients for perfumes and flavouring products. In addition, aldehydes are key intermediates for the manufacture of e.g. synthetic resins, dyes, disinfectants and preservatives. In this thesis we focus on the catalytic eco-friendly conversion of linear aliphatic alcohols into aldehydes, the so-called green notes.

Traditional text book examples towards alcohol oxidation, involve the use of high-valent chromium as oxidant (pyridinium chlorochromate and Jones reagent H_2CrO_4), permanganate, hypervalent iodine and the use of hypochlorite in combination with various co-oxidants such as TEMPO [1-3]. These oxidants generate copious amounts of waste, often toxic to the environment. Therefore there is a definite need to replace these classic stoichiometric methodologies with green alternative catalytic methods [4-6].

Considerable progress has been made in the development of metal-based methodologies using molecular oxygen as the oxidant, for the

conversion of alcohols into aldehydes. Pivotal examples include the use of both homogeneous and heterogeneous Pt, Pd, Ru, Cu or Au-based catalysts. All these catalysts are able to coordinate to alcohols. In general a β-elimination of aldehyde occurs after the first initial step, after which the metal-ligand assembly is reoxidized by oxygen [8-12].

The use of metal-based catalysts however, still involves the use of permanent natural resources, the metals, which can not completely be recycled in most cases, and which are still rather costly. In addition, for eco-friendly production of aldehydes in the flavour industry the use of metal-catalysts is under pressure because of the possibility of metal contaminating the final product. The use of enzymes as biocatalysts fulfils all the requirements listed above, and still offers us the possibility to use clean and cheap molecular oxygen as the oxidant. Nowadays, over 134 industrial chemical processes based on enzymes have been reported, which demonstrates the increasing importance for this field [5].

In the field of enzymes, oxidations are performed by the oxidoreductases. Within this category the alcohol oxidases perform alcohol oxidations with molecular oxygen (in contrast to alcohol dehydrogenases which require an additional cofactor, NAD^+, to scavenge the protons and electrons from the alcohol) [13, 14]. In general two categories of oxidases can be distinguished:

1. *The flavin-dependent alcohol oxidases* such as glucose oxidase, vanillyl alcohol oxidase [15, 16] and alcohol oxidase [17].

2. *The metal-dependent alcohol oxidases*. Pregnant examples are galactose oxidase, which is a mono-copper dependent oxidase, with an additional modified tyrosine in the active site. Galactose oxidase is highly selective for galactose and galactose units [18]. The second notable example is that of laccase. Laccase belongs to the family of blue multi-copper enzymes, which are capable of oxidizing electron-rich alcohols. Their great advantage lies in their wide substrate scope, good availability, and relatively high stability.

An excellent comparison of the substrate scope of alcohol oxidase and galactose oxidase was presented by Siebum and co workers [19]. The paper illustrates that the overall activity for linear aliphatic alcohols is rather low for alcohol oxidases. Therefore in our studies we focused on the use of laccase as starting point for clean and selective alcohol oxidation using O_2 as oxidant.

Laccase

Laccases are glycosylated multi-copper containing oxidases (p-dihydroxybenzene dioxygen oxidoreductases, EC 1.10.3.2), which belong to the small family of blue multi-copper oxidases. These enzymes catalyze the one-electron oxidation of substrate concomitant with the four-electron reduction of molecular oxygen to water (Figure 1):

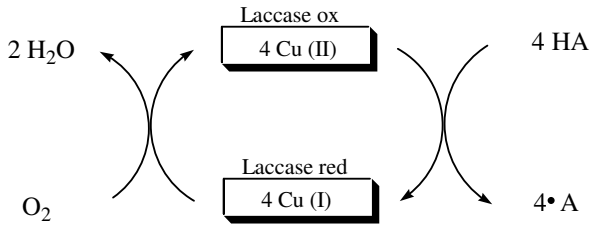

Figure 1 Oxidation reaction catalyzed by Laccase

Laccases oxidize a broad range of substrates such as polyphenols, diamines, methoxy-substituted phenols and some inorganic compounds. Laccase is widely distributed in plants and fungi. In addition, homologues of laccase occur in bacteria [20]. The first laccase was discovered as early as 1883 by Yoshida. He was able to prepare from the latex of the Japanese lacquer tree (*Rhus*) a thermostable substance, a catalyst, which could oxidize urushiol by dioxygen. Urushiol (3-pentadecadienyl catechol) is a toxic substance present in the resin or on the surface of plants of the genus *Rhus*. Later Bertrand and coworkers around 1895 purified and characterized this catalyst and gave it the name "laccase" [21]. They also claimed that the laccase contained manganese and introduced for the first time the concept of metalloenzymes. The crude latex indeed contains large amount of manganese, which explains Bertrand's findings, but later studies by Keilin and Mann showed that the enzyme contains copper, not manganese [21]. The function of the laccase from lacquer trees is to heal injuries of the bark. A white sap (lacquer) seeps out the bark upon injury and in the presence of dioxygen the laccase oxidizes phenols present in the sap to their conjugate radicals, which spontaneously polymerize to form a protective structure, somewhat similar to the blood clotting system in animals. The lacquer has been used for about 6000 years as the first sort of plastic to paint wooden articles and beautiful artistic objects have been produced over the ages [22].

Laccase catalyses the oxidation of phenolic compounds (for example lignin), by one electron transfer leading to the formation of the aryloxy radical, which via a second one-electron transfer yields a variety of

products. This oxidation process is coupled to the reduction of dioxygen to water by a four-electron transfer. Non-phenolic substrates are more resistant to monoelectronic oxidation and are not oxidized by laccase directly. However, the employment of so-called mediators, low molecular weight electron transfer agents, makes a wide range of non-phenolic derivatives suitable substrates for laccase as well [23]. The wide choice of mediators, in combination with the wide range of substrates which can thus be oxidized is the origin behind the wide applicability of laccase. Applications range from water-purification, to the bleaching of indigo-blue jeans [6]. At the end of this chapter a more detailed overview will be given. First we shall focus on the mechanistic aspects of the laccase mediator interaction.

Laccase/Mediator system

The Laccase/Mediator system is based on the one-electron oxidation of the mediator by laccase. The mediator needs to be a stable oxidized intermediate that has the capacity to diffuse into solution and interact with the substrate of choice. In this way the actual oxidation can take place outside the enzyme and possible steric and polar inhibiting interactions are eliminated.

The thus formed mediator can interact with the substrate in basically two ways [24, 25]:

1. The mediator can abstract an electron leading to a radical cation intermediate which can undergo rearrangement and cleavage reactions. A common example of this type of mediator is 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).

Thermodynamically this reaction is favoured due to the higher redox potential of ABTS dication [26].

2. The mediator can directly abstract a hydrogen atom leading to a radical. This is the mechanism in the case of N-OH type mediators, like HBT (hydroxybenzotriazole), HPI (N-hydroxyphthalimide) and VLA (violuric acid).

In Figure 2 the two routes are illustrated for the oxidation of alcohols.

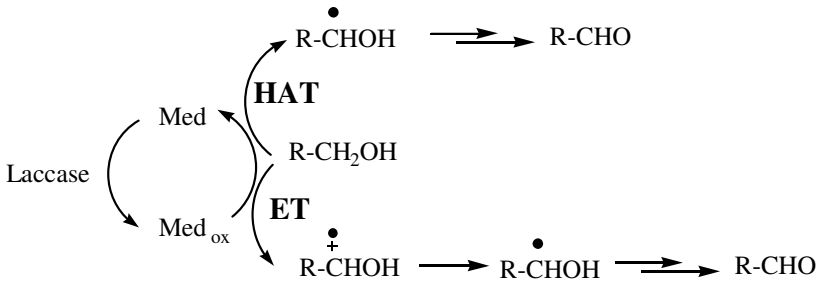

Figure 2 Oxidation by electron transfer (ET) and hydrogen atom transfer (HAT).

In addition, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) and its derivatives have been successfully used as mediators in the laccase catalyzed oxidation of alcohols. Indeed, TEMPO and its derivatives were more effective than the other mediators mentioned above. TEMPO is also unique from a mechanistic viewpoint, in that it involves either an electron transfer mechanism or a hydrogen abstraction mechanism in its reaction with the substrate. TEMPO first undergoes a one-electron oxidation to form the oxoammonium cation, which forms an adduct with the alcohol which subsequently decomposes to the aldehyde and the hydroxylamine in a two-electron oxidation (Figure 3). The oxoammonium can also be generated by chemical oxidation of TEMPO, usually by

hypochlorite, which is a practically applied method for the oxidation of alcohols [27, 28].

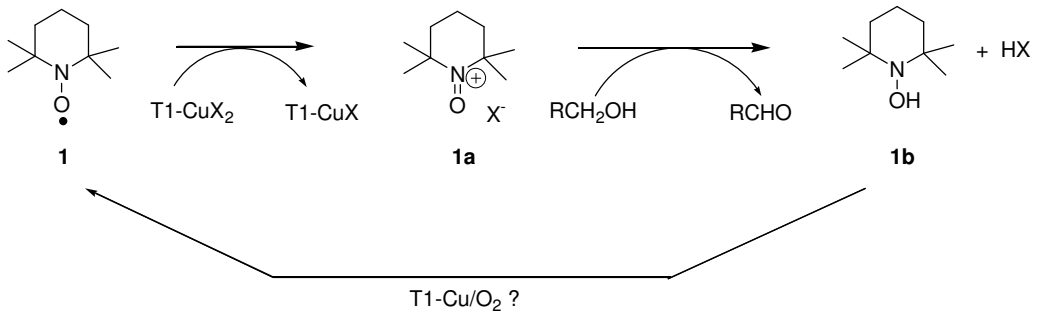

Figure 3 Postulated route for the oxidation cycle of alcohols by the Laccase/TEMPO system

The regeneration of TEMPO in the above scheme is not clear and *a priori* two routes can be proposed: oxidation of hydroxylamine catalyzed by laccase, or disproportionation between oxoammonium and hydroxylamine back to two molecules of TEMPO. The latter reaction generally occurs only at low pH (<3). The direct interaction of laccase both TEMPO as well as with hydroxylamine has never been studied. Thus one of the targets in this thesis was to study this interaction more closely.

Applications of Laccase

Laccase is presently used in several industrial applications [6]. For textile and other technical applications laccase is known under the names DeniLite and Novozym 51003 (used for pilot/large – scale trials). The application range of laccase can be divided in six main areas (see

Figure 4). In most applications the co-presence of mediators is required. Most applications are well documented and will not be mentioned in detail here. The key examples are treatment of dye waste streams and the bleaching of indigo jeans. Another example previously investigated by TNO, involves the cleaning of brew filters [29].

Presently, several multidisciplinary research efforts (genetic engineering, enzyme evolution etc.) are aimed at obtaining improved versions of laccase as a biocatalyst, i.e. to widen its application in industrial processes. One of such applications is in biofuel cells. A combination of a carbohydrate oxidase as anode and laccase as cathode can lead to a very attractive energy source with a small size, a potentially higher energy efficiency and a lower environmental pollution [30].

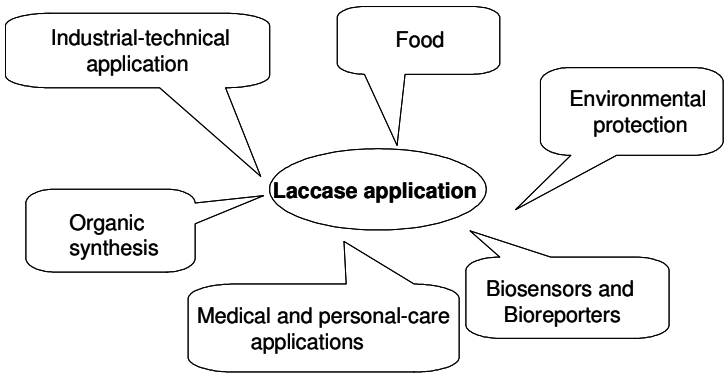

Figure 4 Applications of Laccase

Laccase can also be used for the upgrading of fossil fuels. Laboratory scale experiments showed that inorganic and organic sulfur compounds in coal slurry can be oxidized by laccase, solubilized in water and separated from solid coal particles [31].

Recently, some unexpected medical applications have been reported. Laccase from the fungus *Tricholoma giganteumis* has been reported to possess significant HIV-1 reverse transcriptase inhibitory activity [32]. Another laccase was shown to be capable of fighting aceruloplasminemia, a medical condition in which ceruloplasmin is lacking [33].

Biochemistry of Laccases

Occurrence and function of laccases

Laccases are found in plants, fungi and some bacteria [20, 34-37]. While present in many plants (peach, Japanese lacquer tree, tobacco), the plant laccases have not been used extensively in applications due to their difficult detection - crude plant extracts contain a large variety of oxidative enzymes with broad substrate specificity - and purification. The majority of the characterized laccases are from fungi (~ 100), especially from the white-rot basidiomycetes species (e.g. *T.versicolor, P.cinnabarinus*) in which they may be involved in lignin degradation. So far only a few bacterial laccases have been purified and characterized (*A.lipoferum*, *M.mediterranea, EpoA from Strep.griseus, cotA B. subtilis*). The specific physiological functions of laccase in plants, fungi and bacteria are not completely clear and may depend on their cellular location. For example, laccases associated with the cell wall or spores could be responsible for the formation of melanin and other protective cell wall compounds [1]. Intracellular fungal or periplasmic bacterial laccases might, however, be involved in the conversion of low molecular weight phenolic compounds. Indeed, a comprehensive screening of 44

possible lignin-derived compounds identified 10 phenolic compounds (among which were syringaldehyde, acetosyringone, vanillin, methyl vanillate, p-coumaric acid), which showed high activity with the laccases from *P.cinnabarinus* and *T.villosa*.

Structure of the Laccase from Trametes versicolor

Based on comparative sequence analysis and 3D-structures, laccases (and ascorbate oxidases and ceruloplasmin) are proposed to have evolved from electron-transferring small blue-copper proteins (e.g. azurin, plastocyanin) by several domain duplication events. Fungal laccases consist of approximately 500 to 700 amino acid residues including an N-terminal signal peptide and are glycosylated to various degrees, a feature which has long hampered their purification and crystallization. The mature laccase carries four copper atoms arranged in three copper centers: T1, T2 and T3. The T1, or blue, Cu site is distinguished by an intense ($\varepsilon \approx 5000\ M^{-1}cm^{-1}$) absorption feature around 600 nm and a small ($<100\ x10^{-4}\ cm^{-1}$) g-parallel hyperfine coupling in electron paramagnetic resonance (EPR). The T2, or non-blue, Cu site displays a parallel hyperfine coupling ($>160\ x\ 10^{-4}cm^{-1}$) typical of tetragonal Cu. The T3, or coupled binuclear, Cu site is comprised of two Cu^{2+} ions that are antiferromagnetically coupled through a bridging hydroxide. The resulting diamagnetic ($S_{total}=0$) T3 Cu site lacks an EPR signal, but it displays an absorbance feature around 330 nm ($\varepsilon \approx 5000\ M^{-1}cm^{-1}$) [2]. Upon reduction, the blue color, the absorbance around 330 nm and the EPR signals all disappear.

The T1 Cu is ligated by three strong ligands, two His and one Cys residue, all of which are conserved among the laccases. Likewise, the T2 Cu binds two His and each Cu of the T3 site binds three His residues. Since the Cu atoms of the T2 and T3 sites are very close, they form a single trinuclear T2/T3 Cu site. In addition to 10 conserved His and one Cys ligating the coppers, laccases contain a very strongly conserved His-Cys-His tripeptide sequence, which might be important in mediating electron transfer between the T1 and T2/T3 Cu sites (see Figure 5).

In 2002, two papers describing the crystal structure of *Trametes versicolor* laccase were published (Bertrand *et al*. [3, 4] and Piontek *et al*. [5]). The *T.versicolor* laccase (TvL) structure shows a monomeric enzyme with dimensions of about 65 x 55 x 45 $Å^3$, and is organized in three sequentially arranged domains (Figure 6).

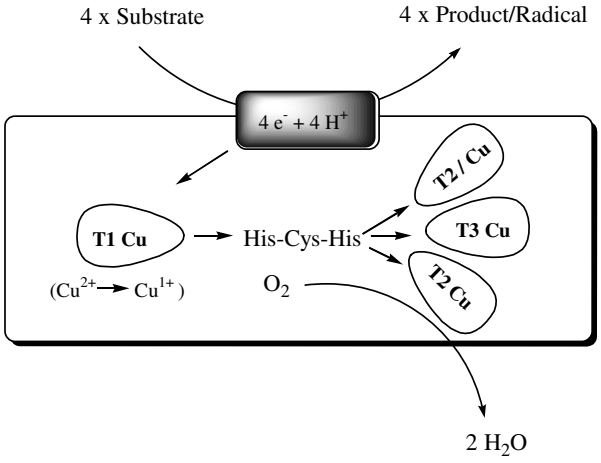

Figure 5 Schematic structure and general catalytic scheme of laccases

Each of these domains has a *β*-barrel type architecture, similar to that in the small blue copper proteins azurin and plastocyanin. *Domain 1* comprises two four-stranded *β*-sheets and four 3_{10} – helices and is like

Domain 2 devoid of Cu centers. The T1 Cu site is located in *Domain 3*, the T2/T3 trinuclear center is located at the interface of *Domain 1* and *Domain 3*, with both domains providing residues for the coordination of the coppers. The acidic p*I* of about 3.5 of the TvL is consistent with a net negative charge of the surface amino acids. In terms of its structure, the TvL structure is most similar to that of *C. cinereus* [41].

The trinuclear copper center T2/T3 is situated between *Domain 1* and *3* and buried about 12 Å deep within the enzyme (Figure 6). The three coppers are arranged in an almost perfect regular triangle with a mean distance of 3.85 Å (Figure 7). Cu2 and Cu3 of the T3 site are 3.9 Å apart; the distances of Cu2 and Cu3 to the T2 copper (Cu4) are 3.8 Å each.

The two T3 coppers are bridged by either an OH^- or O^{2-} unit with bond lengths of 2.19 and 2.08 Å to Cu2 and Cu3, respectively, making an angle of 133°. A total of six His coordinate to the two T3 coppers symmetrically with a mean distance of 2.16 Å resulting in a four-fold coordination for each copper, best described as a distorted tetrahedron.

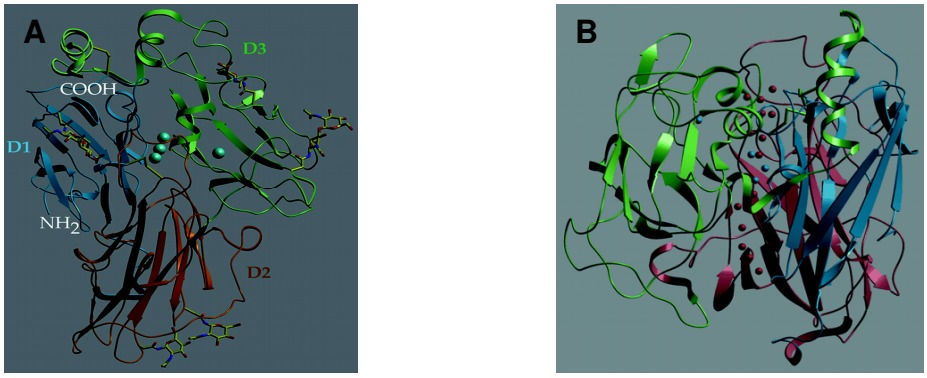

Figure 6 Ribbon diagram of laccase *Trametes versicolor* [41]

A – diagram of the three domains (*D1*, *D2*, *D3*) and the Cu sites; B - the two channels leading to the T2/T3 cluster. H_2O molecules are depicted as red spheres and Cu ions are depicted as blue spheres

Oxygen reduction to water occurs at the T2/T3 cluster. This copper cluster is accessible to water and O_2 through two channels, which lead to the T3 Cu sites and to the T2 Cu site (Figure 6B) [41, 42]. Water molecules found in the two channels are well defined in the electron density and form numerous hydrogen bonds with the surrounding amino acid residues. Superposition of the TvL structure with that of other laccases shows that these water molecules and the amino acids that form the channels are highly conserved.

The mononuclear copper of the T1 center is embedded in *Domain 1*, about 6.5 Å below the surface of enzyme. This copper occupies a depression of the enzyme surface, delimited by a *β*-turn, belonging to *Domain 1*, and two *β*-turns of *Domain 3*. These *β*-turns have been implicated for the *B. subtilis* endospore coat laccase, cotA, as the ABTS binding site [42]. ABTS binds in a small negatively charged cavity near the copper T1 site. A similar negatively charged stretch of amino acids is

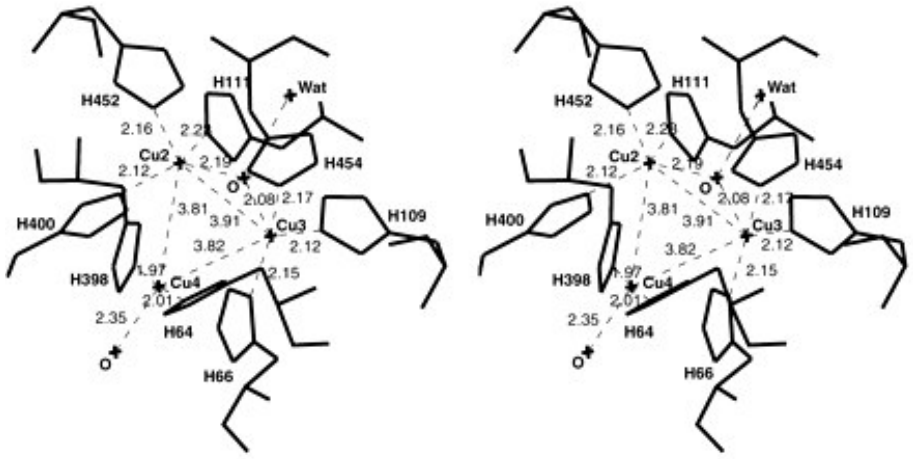

Figure 7 Stereo view of the T2/T3 coppers and their ligands in the Tv laccase [41]

found the TvL laccase potentially stabilizing ABTS or TEMPO, the mediator used in the alcohol conversion studies described in this thesis (Chapters 2-6). This position of the substrate (ABTS/TEMPO) makes the T1 copper the most likely primary electron acceptor site.

Between the T1 copper and the trinuclear cluster lays the His-Cys-His tripeptide, which is highly conserved among blue multicopper oxidases. The closest distance between T1 and T2/T3 coppers is about 12Å enabling fast (in the µs) intramolecular electron transfer possibly involving the His-Cys-His tripeptide contributing via a through-bond electron transfer pathway.

Interestingly, the TvL crystal structure indicates that the blue T1 Cu is coordinated by only three strong ligands His (2x) and Cys. In the 'classical' blue copper proteins azurin, plastocyanin and ascorbate oxidase the T1 Cu is further weakly coordinated by a conserved Met

residue. Such a Met residue is absent in the TvL sequence and 'replaced' by a non-coordinating Phe. In other laccases (e.g. *Nicotiana tabacum*, *Neurospora crassa*) a non-coordinating Leu rather than Phe is present, whereas in some laccases (e.g. *Aspergillus nidulans*) a Met residue is present, which presumably coordinates to the T1 Cu. The presence of Met, Phe or Leu has led to a subdivision of the laccases into Class 1, Class 2 and Class 3 laccases, respectively. Importantly, the T1 Cu reduction potential in Class 2 and Class 3 laccases, having a three-fold coordination, is > 700 mV, while that of the T1 Cu in Class 1 is much lower, 300-400 mV. Indeed, mutation of Met to Leu in azurin led to an increase of 100 mV in the T1 Cu reduction potential [43]. The high reduction potential of their T1 Cu makes the Class 2 and Class 3 laccases potentially more useful to carry out catalytic oxidations of highly oxidizing substrates. For this reason this thesis deals with the laccase form the fungus *Trametes versicolor* rather than the *Rhus vernicifera* laccase. The latter is known to have a low redox potential T1 Cu that adversely affects [44] the oxidation reactions of interest in this thesis. On the other hand, Class 1 laccases that would be suitable e.g. in terms of stability or pH optimum for a particular application might be simply optimized using genetic engineering by replacing Met with Leu or Phe.

Catalytic characteristics of fungal laccases

Laccases catalyze the oxidation of various compounds, including *o*- and *p*-diphenols, aminophenols, polyphenols, polyamines, aryldiamines and lignin. They are generally most active with ortho-substituted compounds, followed by their para-substituted isomers and quite inactive towards

meta-substituted compounds. Some inorganic salts such as $[Mo(CN)_8]^{4-}$, $[Fe(CN)_6]^{4-}$, $[Os(CN)_6]^{4-}$ and $[W(CN)_8]^{4-}$ are also oxidized by laccases [6]. Many laccases show a high affinity and activity towards ABTS and syringaldazine, while the naturally occurring substrate 2,6-dimethoxyphenol usually displays lower activity. Given their wide range of substrate specificities and the large amount of catalytic data available on purified laccases from diverse sources, a direct comparison between them is difficult, but several trends emerge from the data in Table 1, which lists the activities obtained with the (artificial) substrates ABTS, guaiacol, syringaldazine and 2,6-dimethoxyphenol.

Table 1 Properties of fungi laccases [20]

Property	N	Medium	Min	Max
Molecular weight (*Da*)	103	66000	43000	383000
p*I*	67	3.9	2.6	6.9
Temperature optimum (°C)	39	55	25	80
pH optimum				
ABTS	49	3.0	2.0	5.0
2,6-Dimethoxyphenol	36	4.0	3.0	8.0
Guaiacol	24	4.5	3.0	7.0
Syringaldazine	31	6.0	3.5	7.0
K_M, (μM)				
ABTS	36	39	4	770
2,6-Dimethoxyphenol	30	405	26	14720
Guaiacol	23	420	4	30000
Syringaldazine	21	36	3	4307
k_{cat} (s^{-1})				
ABTS	12	24050	198	350000
2,6-Dimethoxyphenol	12	3680	100	360000
Guaiacol	10	295	90	10800
Syringaldazine	4	21500	16800	28000

ABTS – 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid; n – number of observations; Min and Max values;

Turnover values for the best substrates exceed 3.10^5 s^{-1}, making laccases belonging to the most active enzymes. While K_M values of some enzymes may be in the low µM range for laccase, they are usually in the tens to few hundred µM range.

Nevertheless, laccases appear to display specificity constants (k_{cat}/K_M) exceeding 10^{8-9} $M^{-1}s^{-1}$ for both the natural organic substrate and O_2 and even for non-natural substrates. Such high specificities are commonly found for evolutionary optimized 'enzyme/substrate' couples.

The pH optimum for the majority of fungal laccases is in the range 3-6. The catalytic activity of laccases converting e.g. phenols or more generally organic donors with oxidizable H-atoms is strongly dependent on pH.

This is due to two effects. Firstly, the solution reduction potential of phenolic compounds decreases upon increasing the pH, thus increasing the redox potential difference between the phenol and the T1 Cu, the primary electron acceptor. The greater driving force obtained at higher pH leads to an activity increase at higher pH values. However, at elevated pH values a hydroxide ion binds to the T2/T3 trinuclear center, apparently interrupting internal electron transfer from the T1 to the T2/T3 Cu. These two opposing effects of the pH lead to the experimentally observed pH optima.

While the temperature profiles for laccases are similar to those of other extracellular lignolytic enzymes (50-70 °C), the optimum temperature in biocatalytic conversions by laccases is approximately 25-40 °C, due to– in general- their limited thermal stability. However, the *M. albomyces*

laccase has a half-life of 5 h at 60 °C and is applied in biotechnological applications based on its thermostability.

Catalytic mechanism of laccase

For mechanistic studies mainly the *Rhus vernicifera* laccase has been employed. To gather detailed insights into the molecular mechanism a great variety of spectroscopic techniques have been applied as well as stopped-flow analysis and RFQ (Rapid Freeze Quench) to capture reaction intermediates. Copper in its oxidized state can be studied by EPR (Electron Paramagnetic Resonance), MCD (Magnetic Circular Dichroism), UV-Vis spectroscopy and resonance Raman spectroscopy, while EXAFS (Extended X-ray Absorption Fine Structure) can be applied to study the ligand coordination in both cupric and cuprous redox states. The combination of all these spectroscopic techniques in conjunction with pre-steady state kinetics, inhibitor effects and X-ray structural analyses have led to a detailed picture of the laccase catalytic mechanism.

Exogenous ligands (F^-, CN^-, N_3^-, O_2^{2-}) have been shown to bind to the T2 Cu site and in some cases (N_3^-, and O_2^{2-}) to bridge the T2 and T3 Cu centers [7]. The T1 Cu is unaffected by exogenous ligands. The T1 Cu can, however, be replaced by a redox inactive Hg^{2+} ion, and since the Hg-laccase still contains an intact T2/T3 trinuclear center, the three electron reaction mechanism can be monitored. Other inhibitors, e.g. dithiothreitol and thioglycolic acid may act as inhibitors by reduction of the S-S bridge(s) in the enzyme leading to unfolding, while di-ethyldithiocarbamic acid extracts the T2 Cu. The T2 Cu depleted

reduced enzyme is unreactive towards O_2, indicating its participation in O_2 binding/reduction.

Already in the nineteen seventies it was shown that electrons enter the laccase *via* the T1 Cu site [8, 9] and further that reoxidation of the reduced Cu sites is much faster than their reduction by a substrate (*i.e.* hydroquinone [10]). Moreover, the oxidation state of the T3 Cu site did not appear to affect the reduction rate of the T1 Cu, and *vice versa*.

The studies of the past twenty years were able to answer the question of whether O_2 reduction occurred in a single four-electron reaction (plus a proton), after all the reduced enzyme contains precisely the four required electrons, or whether reduction takes place in two consecutive two-electron transfer steps with a peroxide intermediate. The Hg-modified laccase turned out to be essential to distinguish between these two possible reaction paths, because it contains only three electrons, not sufficient to break the O-O bond, unless the enzyme would be blue to provide the 'missing' fourth electron via a redox active amino acid such as Tyr, Trp or Cys.

Figure 8 shows our current understanding of the mechanism. The fully reduced T2/T3 cluster site reacts with O_2 to generate the peroxy intermediate as determined with the T1 Hg derivative of laccase [52-54]. In this derivative, the peroxy intermediate decays slowly to the resting oxidized T1 Hg laccase via a species similar to the native intermediate; however this latter intermediate cannot be a reaction intermediate in the normal laccase. Somewhat surprisingly, the O_2 binding rate in the T1 Hg laccase is ~10^3 M^{-1} s^{-1}, in contrast to 2 x 10^6 $M^{-1}s^{-1}$ for the normal enzyme (Figure 8).

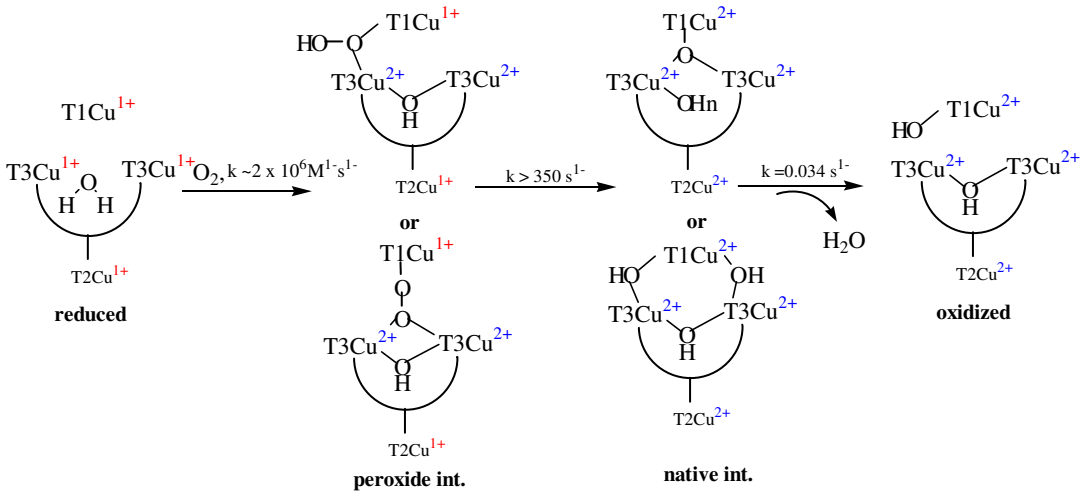

Figure 8 Concept of intermediates formation in laccase oxidation

Spectroscopic studies indicated that in the peroxy intermediate the T2 Cu remains reduced, while the T3 Cu site had transferred the two electrons. Both T3 Cu_s are antiferromagnetically coupled indicating that they are bridged by hydroxide, but not by peroxide, because the distance of 3.4 Å is too small to accommodate peroxide. The peroxide binds as a μ-1,1-hydroperoxide.

When the reduced laccase –without excess reductant - is reacted with O_2, the peroxy intermediate does not accumulate, but a stable (final) 'native intermediate' is formed (Figure 8). Given the dead times of the stopped-flow and rapid freeze quench set up, the conversion rate from the peroxy state to this 'native intermediate' is estimated as > 350 s^{-1} (Figure 8) [53]. In Chapter 4 we estimate a value > 25.000 s^{-1} for this step.

In the native intermediate all copper centers are oxidized, but e.g. the EPR spectrum is different from the oxidized laccase. EPR studies of the

native intermediate indicated a magnetically coupled trinuclear Cu center comprised of the three copper atoms of the T2/T3 cluster coupled via hydroxide- or oxo- bridge(s), as indicated in Figure 9.

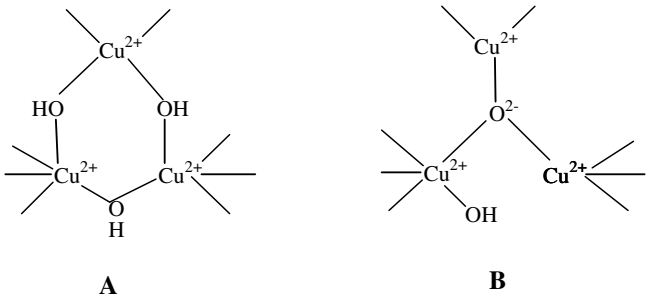

Figure 9 Possible structure models for native intermediates [53]

In the absence of reducing agents, the native intermediate is quite stable, $t_{1/2}$ = 104 s at 4°C. This slow decay of the native intermediate, k = 0.034 s^{-1}, to the resting oxidized laccase can be understood in terms of a large structural change –including rearrangement (+ protonation) of one of the active site metallo-hydroxides from the interior to the exterior of the trimer- finally leading to dissociation of water. Given the high k_{cat} values for laccase (Table 1) the transition from 'native intermediate' to fully oxidized enzyme cannot in general be part of the normal catalytic cycle. In addition, electron transfer from the T1 Cu to the trinuclear site in the oxidized laccase is very slow, ~ 1 s^{-1}, in particular to the (magnetically) isolated T2 Cu. It has therefore been proposed that the oxidized laccase does not form part of the catalytic cycle, but that the native intermediate is rapidly (re)reduced by substrate to initiate the next turnover. Arguments favouring this view are the strong coupling between the three coppers in the native intermediate allowing fast electron transfer between the T2 and T3 coppers, and the higher reduction

potential of the T2/T3 Cu in the native intermediate compared to the oxidized enzyme providing a larger driving force. The different single electron reduction steps occurring in oxidized laccase and the native intermediate, the latter representing the catalytically active laccase, are shown in Figure 10.

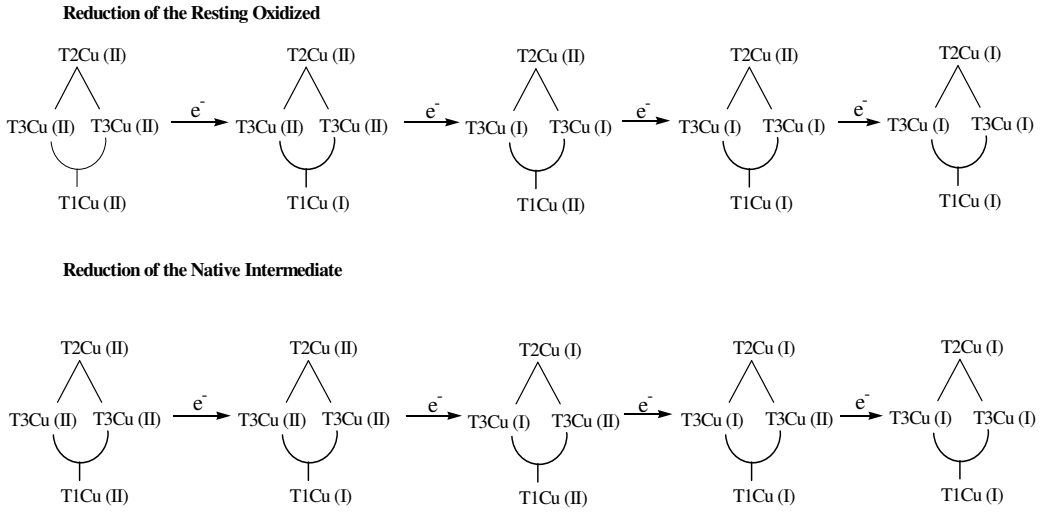

Figure 10 Electron transfer mechanisms for oxidized laccase and the native intermediate

The reducing substrate delivers the first electron to the active site via the T1 Cu center. In the oxidized laccase, the T2 Cu is the last to be reduced. However, in the native intermediate the T2 and T3 copper are quite similar owing to their strong coupling. After injection of two electrons the T2 Cu and one of the T3 copper atoms would be reduced, predicting formation of a mixed valence T3 copper center. Such a mixed-valence center has been detected experimentally [55, 56]. Further reduction of the remaining T3 center in the native intermediate should

be fast via the His-Cys-His pathway between the T1 and T3 centers. Thus the strong coupling between the T2 and T3 coppers in the native intermediate is proposed to enable fast intramolecular electron transfer providing the high catalytic rates observed in laccases.

Scope of the thesis

The research described in this thesis deals with aerobic alcohol oxidation catalyzed by laccase from *Trametes versicolor* and mediated by TEMPO. In a previous thesis Li [57] reported preliminary studies on the use of different TEMPO derivatives and sources of laccase for the oxidation of benzyl alcohol and furfuryl alcohol. His results indicated a critical influence of the amount of mediator. Our aim was to improve the performance of the laccase/TEMPO system for aerobic alcohol oxidation based on detailed kinetic studies for all individual components in the system. We started out work by an overall inventory of the reaction conditions (*i.e.* pH, temperature, concentrations) required to acquire reasonable conversions on a synthetic scale for a variety of primary and secondary aromatic alcohols (Chapter 2). Chapter 3 deals with the development of the laccase/TEMPO system in the selective aerobic oxidation of aliphatic alcohols to the corresponding aldehydes. In particular, the reaction was studied under mono- and biphasic conditions. As a central point in the work the laccase-TEMPO interaction was dealt with in great detail using purified samples of laccase *Trametes versicolor* (Chapter 4). Furthermore, kinetic measurements were performed for each separate step of the overall oxidation reaction taking benzyl alcohol as a model. Modeling in-time was performed by

mimicking the steady-state concentration of TEMPO in the system (Chapter 5). To make the system more economically attractive the cross-linked enzyme aggregates of laccase were prepared in order to replace soluble enzyme (Chapter 6).

This work was financed by the ACTS (Advanced Catalytic technologies for a Sustainable Society) – NOW program: Integration of Bio-and Organic Synthesis (IBOS) and was conducted in parallel with another approach towards the oxidation of alcohols: A study on the polymer-immobilized hypervalent iodine mediated oxidation of alcohols (A.Kotlewska). Both projects demonstrate the quest for novel and broadly applicable catalytic methodologies in this area.

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2

Selective oxidation of aromatic alcohols catalyzed by the Laccase/TEMPO system

Abstract

The reaction parameters for the Laccase/TEMPO system in the aerobic oxidation of aromatic alcohols were investigated. The optimized concentrations of laccase and alcohol were found to be 10 U/ml and 160 mM respectively. Under these conditions the use of 9 mol % TEMPO led to good conversions within 4 h. The optimum temperature for the reaction was found to be above room temperature (around 30 °C).

Introduction

Organocatalytic oxidations of alcohols mediated by nitroxyl radicals are well explored in organic chemistry [1]. Two kinds of reactivities can be distinguished: stable dialkylnitroxyls (TEMPO and its derivatives) and reactive diacylnitroxyls (N-hydroxylimide derivatives, such as NHPI). These mediators in combination with single oxygen donors (hypochlorite, organic peracids) or in combination with molecular oxygen and catalytic amounts of transition metals (Pd, Cu, Ru) very efficiently catalyze oxidation reactions [2, 3]. In particular, the TEMPO/hypochlorite/bromide system is widely used in organic synthesis (Figure 1).

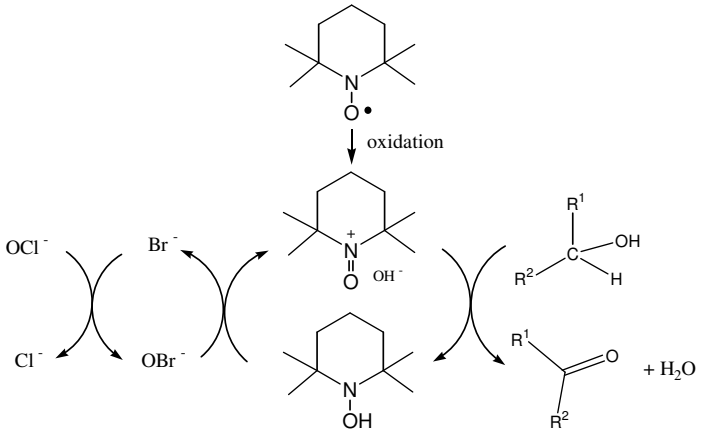
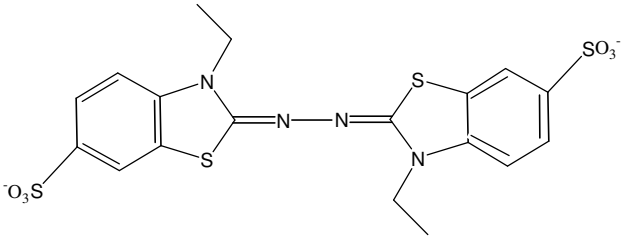


Figure 1 Alcohol oxidation by the TEMPO/NaOCl/bromide system

The oxidation of primary alcohols was performed in high yields (> 95 %) using 1 mol % TEMPO in combination with 10 mol % sodium bromide as co-catalyst in dichloromethane-water [4]. The catalytic cycle involves alternating oxidation of alcohol by the oxoammonium cation and regeneration of the latter by reaction of hydroxylamine with the primary

oxidant (hypochlorite). This oxidation protocol is synthetically very efficient, but has many environmental and economical drawbacks such as the use of dichloromethane as solvent, the possible formation of chlorinated by-products in addition to stoichiometric amounts of sodium chloride waste, and the use of 10 mol % of bromide as a co-catalyst.

In 1996, Potthast and co-workers [5], reported a novel method for the aerobic oxidation of alcohols catalyzed by the laccase from *Trametes versicolor* in combination with ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) as a mediator (ABTS structure is shown in the scheme):



The ABTS/laccase combination catalyzed the oxidation of a series of benzylic alcohols to the corresponding aldehydes in yields > 95 % within 24 h.

Laccases are multicopper oxidases having 4 copper atoms. Three closely spaced coppers constitute the active site where reduction of oxygen to water occurs; the fourth copper, a Type I copper, mediates electron transfer between the electron donor and the active site. Native laccases can catalyze the oxidation of phenol derivatives coupled to the reduction of molecular oxygen to water. The oxidation of non-phenolic derivatives, however, requires the presence of a mediator, which acts as

the oxidant for the respective alcohols. In the case of ABTS, two subsequent one-electron transfers generate $ABTS^+$ as the final oxidant. $ABTS^+$ is a strong oxidant with a redox potential of 0.7 V [6] and can oxidize also electron-poor alcohols. This method however required 1.5 mol of mediator (ABTS) relative to 1 mol of alcohol. Moreover, the authors pointed out that the pH of the reaction medium is important and that the influence of solvent and aeration of the reaction solution due to heterogeneity of the mixture should be carefully studied. Subsequently, Galli and co-workers reported that the combination TEMPO/Laccase (*Trametes villosa*) is capable of catalyzing the oxidation of primary benzylic alcohols [7].

Laccases have a pH optimum between 3.5 and 6.0, depending on the source [8]. In contrast, the reaction of the oxoammonium cation, derived from TEMPO, with an alcohol is fast under basic conditions, at pH > 8 in organic solvents [9]. Nevertheless, the pH should be kept in the acidic pH range with respect to the poor stability of laccase in solutions at basic pH.

In Chapter 1 we discussed that, depending on the specific steric and stereo electronic characteristics of the mediators, the reaction of the oxidized mediator with the alcohol substrate follows either a hydrogen atom transfer (HAT) or an electron transfer (ET) mechanism. The case of TEMPO is rather unique in that reaction of the oxidized mediator with the substrate involves a two-electron oxidation via a mediator-alkoxy intermediate which subsequently decomposes to aldehyde and the

hydroxylamine via an inter- or intramolecular proton abstraction (Figure 2) [10-12].

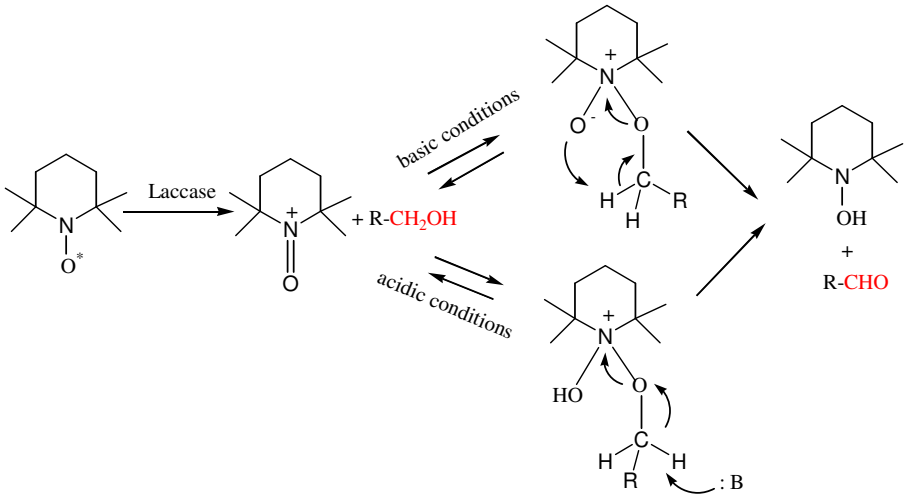


Figure 2 Oxidation of alcohols by oxoammonium under basic and acidic conditions

Another important issue in the laccase/mediator system is the reaction medium. In many cases a mixed solution of water (buffer) and organic solvent is needed to increase the solubility of poorly soluble hydrophobic substrates, at the expense of a moderate reduction of the enzyme activity [13]. In organic solvents, the reactivity of the TEMPO/NaOCl system was even lower in the presence of water compared to organic solvents [4]. In addition, salt solutions may cause not only denaturation of the enzyme by destruction of the protein hydration shell, but also influence the catalytic behaviour of the enzyme [13-15]. Thus, the choice of water versus organic solvent is not straightforward.

The advantages of the laccase/TEMPO system are its high selectivity (usually no by-products are obtained) and its low environmental burden

(no chlorinated waste compounds). The disadvantages of the system are the long reaction time and the large amounts of TEMPO required (up to 30 mol %) to generate suitable rates.

In this chapter two main issues will be addressed: how to increase the overall activity of the reaction system and which TEMPO loading is required to achieve this activity. For that purpose, the reaction parameters (temperature, concentrations of reaction components, etc.) required for optimum performance in the aerobic oxidation of alcohols using the laccase/TEMPO system will be investigated.

Results and Discussion

General parameters of the system

Alcohol oxidation by the laccase/mediator (TEMPO) system follows the scheme:

$$\text{R-CH}_2\text{OH} \xrightarrow[\text{O}_2\text{, buffer}]{\text{Laccase / Mediator (TEMPO)}} \text{R-CHO} + \text{H}_2\text{O}$$

Laccase from the fungus *Trametes versicolor*, which is commercially available, was applied for this study. Laccases from other sources (*Polyporus pinsitus*, *Biofresh L*) were tested as well, but they showed lower activities in the laccase/TEMPO catalyzed oxidation of benzyl alcohol. Therefore, only laccase from *T.versicolor* was applied for our oxidation reactions. All parameters, such as concentrations of substrate, mediator, and enzyme; the temperature of the reaction and the efficiency of aeration of the reaction solution are of great importance. They need to be optimized to obtain sufficient conversion of alcohols,

but before studying these parameters we wanted to check whether some substrates can also be oxidized without mediator [19]. Therefore, the blank reactions were performed as described in Table 1.

Table 1 Blank reactions with aeration of reaction mixture

Substrate	**Omitted from the reaction**	**Conversion** (%)
Veratryl alcohol	TEMPO	0
Veratryl alcohol	Laccase	1.0
4-Methoxybenzyl alcohol	TEMPO	0
4-Methoxybenzyl alcohol	Laccase	0

Conditions: [Subs] 160 mM, [Lacc] 12.5 U/ml, [TEMPO] 15 mM, 38 ^{o}C, 4 h, O_2.

Veratryl alcohol (3,4-dimethoxybenzyl alcohol) and 4-methoxybenzyl alcohol could not be oxidized by TEMPO and O_2 as expected. For the experiments with laccase, but without mediator (Table 1), only veratryl alcohol showed some conversion after 4 h, which is in line with its electron rich nature.

Purging of O_2 – gas

The need for purging O_2 during the reaction time becomes relevant when higher concentrations of substrate are used and more O_2 is required [20]. Moreover, the solubility of oxygen is lower at higher temperatures. We performed oxidation reactions with 1.6 mmoles of substrate and 125 U of enzyme in 10 ml reaction volume. The experiments in Table 2 demonstrate that aeration of the reaction solution is essential for effective oxidation.

Table 2 The influence of aeration on the oxidation reactions

Alcohol	Conversion (%)		
	Without purging * (O_2/air)	Purging O_2	Purging Air
3-Methoxybenzyl alcohol	23	100	100
4-Methoxybenzyl alcohol	13	100	100
Veratryl alcohol	14	100	100
1-Phenylethanol	14	35	34
Furfuryl alcohol	3	66	65

Conditions: [Subs] 160 mM, [Lacc] 12.5 U/ml, [TEMPO] 15 mM, 38 °C, 4 h, O_2
* 1 atmospheric static O_2 on top of solution.

As shown in Table 2, the use of compressed air yields a similar conversion of alcohol into aldehydes as pure oxygen. 3-Methoxy- and 4-methoxybenzyl alcohol derivatives were oxidized with 100 % conversion in 4 hours, while secondary aromatic alcohol (1-phenylethanol) and furfuryl alcohol were converted in the range of 35 % and 65 %, respectively, under aeration. This observation makes the oxidations economically more attractive, due to the price difference between pure oxygen and compressed air. For future experiments therefore compressed air was used as the oxidant.

Influence of the reaction vessel

The oxidation reactions were carried out in a Prosense 10 place Omni Reaction Station. Some of the oxidation reactions were carried out in reactors that were delivered with the apparatus. However, the conversions, which were previously around 100%, were found to decrease to 45%. This led to the idea of testing reactors with different lengths and diameters: long test tubes, Omni reactor tubes and round-

bottomed flasks were compared. Moreover, the mode of aeration was compared to obtain the best method for the oxidation reactions. The rate of oxygen mass transfer from the gas (air bubbles) to the liquid phase (water/reaction mixture) is governed by bubble surface, water (reaction) volume and oxygen concentration. Small bubbles afford greater surface area and lead to more efficient gas transfer. Also, a specific interfacial area is important where air bubbles interface with the water area (volume). Two different methods for aeration were tested: a needle and steel filter stones. Fine sized air bubbles are formed using the steel filter stone. Aeration by needle results in a lower oxygen concentration which might be beneficial in some cases, *i.e.* to overcome overoxidation of long-chain alcohols to their corresponding acids. Three sets of reactions were performed under standard reaction conditions using 4-methoxybenzyl alcohol as a substrate. In the first set, original reaction tubes from Omni Reaction Station were employed using both: aeration by needle and by steel filter stones (Table 3). These tubes have an inner deflection on the bottom of the tube. In the second set of experiments round-bottomed flasks were used. For this experiment only the needle was tested. Because of the small volume of the flask, it was technically difficult and inefficient to use a filter stone. For the third set, long test tubes were used. The results from Table 3 clearly indicate that the Omni reactor tubes are not sufficient for performing the oxidation reactions. It resulted in approximately 85 % conversion with filter stones, while with a needle the obtained conversion was even lower - 42 %.

Table 3: Comparison of aeration systems of different reactors

Type of reaction vessel and aeration		Conversion %
Original reaction tube of Omni Reacto Station (needle)		42
Original reaction tube of Omni Reacto Station (steel filter stone)		85
Round- bottomed flask (10ml) (needle aeration)		79
Long test tube (steel filter stone)		100
Long test tube (needle aeration)		65

Reaction conditions: [4-methoxybenzyl alcohol] 160 mM, [TEMPO] 15 mM, [Lacc] 10 U/ml, 4 h, air.

Using a round-bottomed flask 79 % conversion was reached, but it was technically not possible to use them in a multi-reaction setup. To perform multiple parallel reactions, the long test tubes were chosen, which gave 100 % conversion, when used in combination with a steel filter stone. Only for long-chain alcohols a needle was applied in order to circumvent overoxidation to carboxylic acids.

Purified laccase

The influence of purified *versus* non-purified laccase was also addressed: during the growth, fungi are expressing not only laccase, but also other enzymes, particularly, lignin peroxidase (LiP) and manganese peroxidase (MnP) [16]. These enzymes are also involved in the

degradation of biopolymers in woody tissues. According to the literature six types of LiP and three types of MnP are expressed in fungi *Trametes versicolor*. With respect to laccase, LiP and MnP are more powerful oxidants due to their higher reduction potential but H_2O_2 is needed for their catalytic activity. The commercially available sample of laccase from Fluka was a deep brown coloured powder. Therefore, it was decided, to check the activities of the peroxidase enzymes as well. Total peroxidase activity was measured using ferulic acid and H_2O_2, at 350 nm (*see Materials and methods*). A total peroxidase activity of 0.01 U/g was determined, which was rather low. The activity assay for laccase was based on the reaction with metol (see Chapter 3). No other enzymes with catalytic activities were found in the sample (see also Chapter 4). Purified laccase that was prepared according to the procedure developed and described in Chapter 4 had no peroxidase activity. The purified and non-purified laccase were tested for oxidation of two substrates under standard conditions (Table 4).

Table 4: Oxidation reactions using purified and non-purified laccase

Substrate	Conversion %	
	Pure Lacc	Non-pure Lacc
4-Methoxybenzyl alcohol	100	100
1-Octanol	29	26

Conditions: [Subs] 160 mM, [Lacc] 6.3 U/ml, [TEMPO] 15 mM, 38 ^{o}C, 4 h, air.

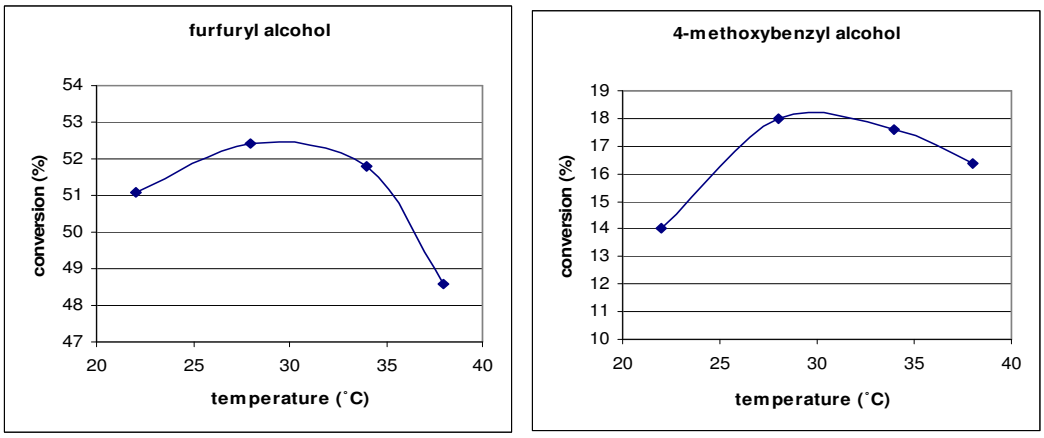
The amount of enzyme used for this experiment is low compared to the 10 U/ml concentration of laccase usually applied for oxidation reactions. Nevertheless, the obtained conversions were 100 % and ~ 28 % for 4-methoxybenzyl alcohol and 1-octanol, respectively. The purification of

the enzyme increases the cost of it and moreover, increases the cost of the oxidation reaction in total. Therefore, given the good agreement between purified and non-purified laccase we conclude that it is not necessary to apply purified enzyme for efficient oxidation reactions.

Effect of temperature

Laccase has a temperature optimum of 38 ˚C, and rapidly loses its activity above 60 ˚C [16]. We wanted to determine the optimum temperature in our system, which depends on more parameters than just the laccase activity. For this purpose, the oxidation reactions were performed between 22˚C (room temperature) and 38˚C. Furfuryl alcohol and 4-methoxybenzyl alcohol were used as substrates (Figure 3).

Figure 3: Oxidation reactions of furfuryl and 4-methoxybenzyl alcohol at different reaction temperatures (note the expanded conversion scales)



Reaction conditions: [Subs] 160 mM, [Lacc] 12.5 U/ml, [TEMPO] 15 mM, 2 h, air.

For both substrates the optimum temperature is approximately 30 ℃. Lower conversions at 38 ℃ could be related to lower solubility of

oxygen in water at higher temperatures and lower laccase stability. For further experiments a temperature of 30 °C was used.

Enzyme concentration

The overall conversion rate is dependent on the amount of oxidant, oxoammonium, which is generated by the laccase. Therefore, our aim was to perform the conversion under such conditions that the amount of laccase was not rate-limiting during an average reaction time of 4 h. Previous studies [17] had shown that approximately 12 units (U) of enzyme per ml of reaction volume were required or 120 units in 10 ml, which is a relatively large amount of enzyme. Oxidation reactions were performed with between 25-200 units of enzyme, to see if the amount of enzyme could be lowered. 4-Methoxybenzyl alcohol was used as a substrate. As shown in Figure 4, only small differences in conversion were observed after four hours with enzyme concentrations between 2.5 U/ml and 20 U/ml.

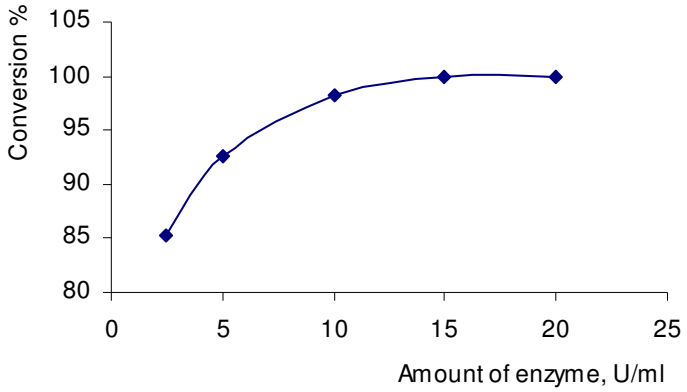


Figure 4 Oxidation of 4-methoxybenzyl alcohol with different amounts of enzyme
Reaction conditions: [Subs] 160 mM, [TEMPO] 15 mM, 4 h, air.

Further mechanistic studies in this thesis will evaluate how these enzymatic catalytic oxidations depend on the amount of laccase (Chapter 5). Figure 4 indicates that the amount of laccase can be reduced to 10 units per 1 ml of reaction volume without loss of final conversion. Therefore, the reactions were performed with 10 U/ml.

Mediator concentration

Up till now many investigations have been directed towards finding the appropriate mediator for alcohol oxidations [18-22]. The studies indicated that 30 mol % or more of TEMPO in combination with laccase was needed to obtain sufficient alcohol oxidation [7]. This high amount would surely hamper the possible application of the laccase/TEMPO system in industry. We have been performing the reactions with 9.4 mol % of TEMPO relative to substrate. However, this is still a relatively high amount for a catalyst. The reaction was performed under standard conditions using furfuryl alcohol as water-soluble substrate and TEMPO between 3.1- 9.4 mol % (5-15 mM). The results show that the reaction efficiency is depending strongly on the amount of mediator relative to substrate and enzyme (Figure 5).

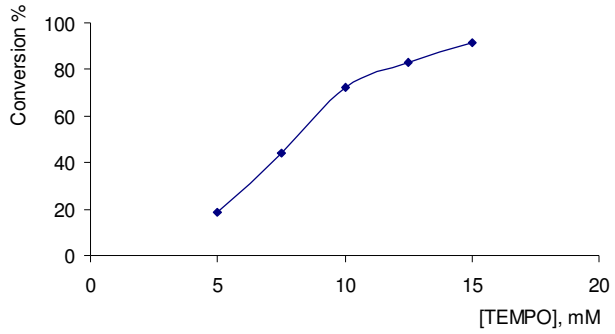


Figure 5 Influence of the amount of TEMPO on the oxidation of furfuryl alcohol
Reaction conditions: [Subs] 160 mM, [Lacc] 10 U/m , 4 h, air.

A decrease of the TEMPO concentration below 15 mM results in lower conversions. This is consistent with a reaction cycle in which the reaction between the oxoammonium cation and the alcohol is rate limiting. In other words, 15 mM of TEMPO is needed to convert the alcohol via a bimolecular reaction in which an oxoammonium – alcohol adduct is an intermediate. The quantitative details will be discussed in more detail in Chapter 5. For further experiments the TEMPO concentration was kept at 15 mM.

Substrate concentration

The influence of the substrate concentration was investigated to determine which concentration of substrate yields optimal conversion. The reactions were performed with furfuryl alcohol as water-soluble substrate and the alcohol conversion after 4 h was determined as a function of alcohol concentration under standard conditions (Figure 6).

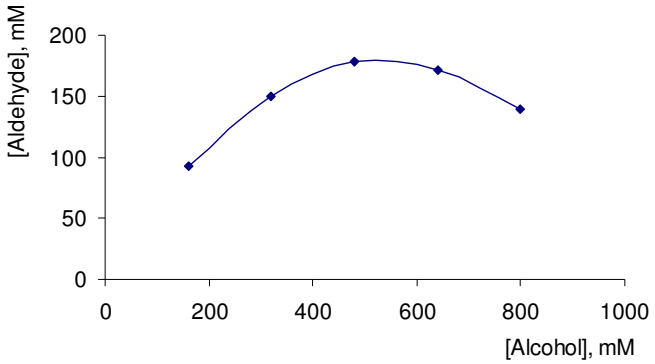


Figure 6 Absolute amount of aldehyde (furfural) obtained varying amounts of furfuryl alcohol

Reaction conditions: [TEMPO] 15 mM, [Lacc] 10 U/ml, 4 h, air.

It can be seen that at > 480 mM alcohol the absolute amount of conversion even drops. A maximum conversion, of ca. 50 %, was obtained at starting concentrations of 200-250 mM. High alcohol/aldehyde concentrations probably lead to inactivation of the enzyme.

Buffer effect

The phosphate buffer was chosen for several reasons. Firstly, it was reported that laccase loses activity (~ 90 % in 23 min) in contact with acetate at pH 4.0 [23]. Secondly, previous work indicated a possible mechanism of nitroxyl decomposition in acetate buffer which leads to loss of its oxidizing ability [17]. Moreover, preliminary studies of alcohol oxidation in acetate buffer resulted in approximately 10 % lower conversions compared to phosphate buffer. The stability of TEMPO in 0.1 M phosphate buffer pH 4 was confirmed by EPR measurements and UV-Vis spectroscopy. Both experiments indicated that TEMPO is stable.

Different mediators

Since 1990, when ABTS was found to serve as a mediator in laccase catalyzed conversions, several comparative studies on the effect of different mediators in the oxidation of alcohols have been reported [7, 15, 24, 25]. The ideal redox mediator should be good substrate for laccase, its oxidation product should not inhibit the enzyme and its redox conversion should be reversible. So far, the best mediator seems to be TEMPO, which is also a well known catalyst in synthetic organic chemistry. The oxoammonium cation is formed by one-electron

oxidation of the nitroxyl radical, but a direct 2-electron oxidation of the hydroxylamine may also be possible. The reduction potential of the mediators is also important and a range of TEMPO-derivatives and a few hydroxyl amines were tested: dimethylglyoxim, N-hydroxy-1,2,4-benzotriazole (HBT), N-hydroxyphthalimide (NHPI), 4-acetamido TEMPO, 4-amino-4-carboxylic TEMPO, 4-hydroxy TEMPO, 4-hydroxy-TEMPO-benzoate, 4-oxo TEMPO, 4-methoxy TEMPO, 4-amino-2,2,6,6,-tetramethylpiperidine, 4-amino TEMPO and TEMPO. The structures of these mediators are presented in Figure 7. Not all of the mediators gave a good conversion of non-natural alcohols, therefore it is essential to find the most suitable mediator for a given substrate (in our case by alcohol).

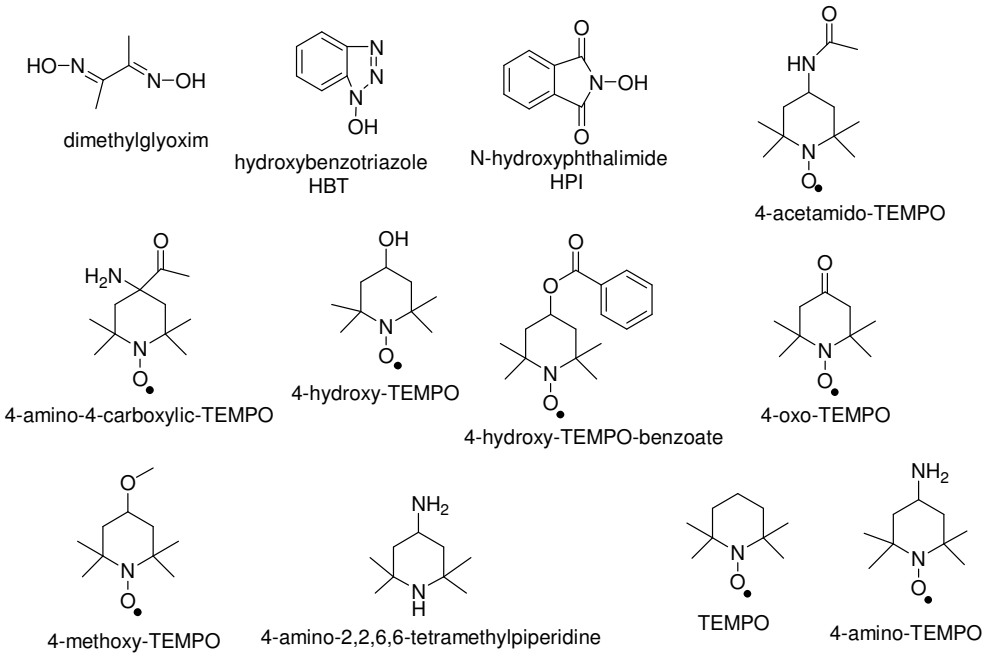


Figure 7 Structures of the mediators that were tested for the enzymatic oxidation of 4-methoxybenzyl alcohol

The aim was therefore to compare these known mediators under optimized conditions - at least employing TEMPO - described in section 2.1. 4-Methoxybenzyl alcohol was chosen as the alcohol substrate. The results are shown in Table 5.

Table 5 Oxidation reactions of 4-methoxybenzyl alcohol with different mediators

Mediator	**Conversion (%)**
Dimethylglyoxim	1
1-hydroxybenzotriazole (HBT)	78
N-hydroxyphthalimide (HPI)	67
4-acetamido-TEMPO	100
4-amino-4-carboxylicacid-TEMPO	20
4-hydroxy-TEMPO-benzoate	17
4-hydroxy-TEMPO	94
4-amino-TEMPO	33
4-methoxy-TEMPO	84
4-amino-2,2,6,6-tetramethylpiperidine	0
4-oxo-TEMPO	25
TEMPO	100

Reaction conditions: [Subs] 160 mM [TEMPO] 15 mM, [Lacc] 10 U/ml, 4 h, 30 °C, air.

Only TEMPO and 4-acetamido TEMPO yielded 100% conversion. The difference between the different mediator results cannot be explained solely on the basis of the reduction potential of the nitroxyl radicals. Actually, both TEMPO and 4-acetamido TEMPO have lower reduction

potentials than other derivatives [15]. Also, for application purposes the price difference of the derivatives should not be neglected. Therefore, it was decided to use TEMPO as mediator in the oxidation reactions.

Oxidation of alcohols under optimized conditions

In nature laccase is responsible for lignin degradation. The best substrates for laccase are aromatic compounds, also in mediated alcohol oxidations. It was shown that electron rich *ortho*-, *meta*- or *para*-substituted aromatic compounds with a lone electron pair are the best substrates in a non-mediated system [27]. Substrates studied are (Table 6): primary benzylic alcohols (benzyl alcohol, 4- and 3-methoxybenzyl alcohols, furfuryl alcohol, and 3-(hydroxymethyl) pyridine), primary allylic alcohols (cinnamyl alcohol and geraniol) and two non-activated alcohols (citronellol and cyclohexanol).

Table 6 Oxidation of benzylic, two non-activated alcohols and allylic primary alcohols

Alcohol	**Conversion %**	
	4 h	**7 h**
Benzyl alcohol	90	98
4-Methoxybenzyl alcohol	100	100
3-Methoxybenzyl alcohol	100	100
Furfuryl alcohol	89	98
3-(Hydroxymethyl) pyridine	98	-
Geraniol	70	98
Cinnamyl alcohol	72	97
Citronellol	31	42
Cyclohexanol	16	18

Reaction conditions: [Subs] 160 mM, [TEMPO] 15 mM, [Lacc] 10 U/ml, 4 h, 30 °C, air.

It should be noted that some of the alcohols are not soluble in aqueous buffer (4-methoxybenzyl alcohol, geraniol) or are only partially soluble (cyclohexanol, citronellol); so, formation of two phases will take place in some cases, which will influence the activity. 3-(Hydroxymethyl)pyridine and the electron rich aromatic alcohols like 4-methoxybenzyl alcohol and 3-methoxybenzyl alcohol were completely oxidised after 4 h. In the case of cinnamyl and benzyl alcohols 7 h were needed to obtain (close to) full conversion. The allylic alcohols, geraniol and cinnamyl alcohol were essentially completely converted after 7 h. Probably, these oxidation efficiencies are dependent on the relative rates of oxoammonium ions with individual alcohols. Thus the formation and decomposition of the oxoammonium-alcohol intermediate is the rate limiting step (see Chapter 5).

Oxidation of secondary alcohols

It is known that the TEMPO/NaOCl system is suitable for oxidation not only of primary alcohols but also of secondary alcohols [36]. We here describe the performance of the laccase/ TEMPO system for the oxidation of secondary alcohols, stimulated by the observation that for 1-phenyl ethanol a reasonable conversion of 50 % was obtained. Therefore, besides 1-phenylethanol, also non-activated secondary alcohols were tested. As can be concluded from Table 7, the reactivities of secondary linear aliphatic alcohols is negligible, while for cyclohexanol a low conversion is observed.

Table 7 Secondary alcohol oxidation

Alcohol	**Conversion** (%)
2-Octanol	0
3-Octanol	0
1-Phenylethanol	35
Cyclohexanol	16

Reaction conditions: [Subs] 160 mM, [TEMPO] 15 mM, [Lacc] 10 U/ml, 4 h, 30 °C, air.

It appears that only aromatic secondary alcohols can be oxidised by the laccase/TEMPO system, although the % conversion (and likely the rate) is lower than for primary aromatic alcohols. Linear secondary alcohols did not show any ketone formation. To test possible steric effects in the laccase/TEMPO system competition reactions were performed with primary versus secondary alcohols (see Chapter 3, Figure 9).

Conclusions

In conclusion, in this chapter we have identified the optimum conditions for the oxidation of primary alcohols using the laccase/TEMPO system. For alcohol oxidation in aqueous buffer the effect of the amount of enzyme, substrate, TEMPO and reaction temperature were tested. It was shown that crude laccase performed as well as purified laccase; the amount of enzyme used could be reduced to 10 U/ml, while TEMPO concentrations below 15 mM led to lowered conversions and yields. Moreover, we have demonstrated that aeration of the reaction is essential to overcome oxygen mass transfer limitations, but that mass

transfer by air rather than by pure oxygen is sufficient. We also confirmed that TEMPO, next to 4-acetamido-TEMPO, is the most effective mediator for conversion of primary benzylic alcohols. Under these conditions the mediator – TEMPO – displays a high selectivity for primary alcohols. Of the secondary alcohols only 1-phenylethanol and cyclohexanol could be converted to the corresponding ketone.

Materials and methods

The substrates and reagents were of analytical grade and obtained from Fluka and Sigma Aldrich. Laccase [E.C. 1.10.3.2] from *Trametes versicolor* was purchased from Fluka. 2,2,6,6-tetramethylpiperidinyl-1-oxy (TEMPO) was received from Acros. The reactions were carried out in stationary Omni Reaction Station with comprehensive heating/cooling/stirring/aeration control. The conversions were followed by GC analysis.

1. Laccase activity assay

Laccase activity was measured spectroscopically using metol as a substrate. The reaction mixture contained 1 ml 0.05 M metol solution in water. The reaction was initiated by addition of 0.5 ml enzyme solution in 0.1 M acetate buffer pH 4.5 and the increase in absorbance was monitored at 540 nm (ε = 2000 $M^{-1}cm^{-1}$). One unit (U) of the laccase was defined as 1 µmol metol oxidised per minute under the stated assay conditions.

2. Peroxidase activity assay

Total peroxidase activity was measured using a reaction mixture consisting of 2.85 ml of 0.04 % (w/v) ferulic acid and 4 % H_2O_2. The reaction was initiated by adding 150 µl enzyme solution in 0.1 M citrate buffer pH 7.0 and the increase in absorbance was monitored at 350 nm at room temperature. One unit (U) of the peroxidase was defined as 1 µmol ferulic acid oxidised per minute under stated assay conditions.

3. Oxidation procedure by laccase/TEMPO system

For the standard oxidation reaction procedure the following amounts were used: 1.6 mmoles alcohol, 0.15 mmoles TEMPO, 0.25 mmoles IS (anisole) and 100 U laccase in 0.1 M phosphate buffer pH 4 to 10 ml total reaction volume. The vials with reaction mixture were aerated continuously. The reactions were carried out in the Omni Reaction Station at certain temperature for certain time. After reaction, the reaction products and unreacted substrates were extracted with ethyl acetate (3 x 10 ml), dried with anhydrous magnesium sulphate and analysed with GC. The blank reactions were performed eliminating one of the components mentioned above.

Analysis methods

Alcohol conversions were analyzed by GC with column WAX 52 CB (50m x 0.53 mm). The method for GC was programmed as followed: 80°C for 5 minutes, then at the rate of 7°C per minute to 235°C. Anisole was used as internal standard.

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3

Studies on the oxidation of aliphatic alcohols by the Laccase/TEMPO system

Abstract

The laccase catalyzed aerobic oxidation of long-chain alcohols in the presence of TEMPO was studied. The optimized conditions established in Chapter 2 were found to be suitable here as well, except for the temperature optimum which was around 35°C. The initial oxidation rates were determined for a wide range of aliphatic alcohols (C5-C12). No reactivity for secondary alcohols could be detected. In order to improve conversions, the importance of laccase deactivation during the reaction was studied. The use of a co-solvent leading to a true monophasic system proved useful for some alcohols, although in general the two-phase buffer-alcohol system turned out to give the best performance.

Introduction

Stable nitroxyl radicals such as 2,2,6,6-tetramethyl-piperidyl-1-oxy (TEMPO, I) are well known as catalysts for the selective oxidation of alcohols to corresponding aldehydes or ketones using sodium hypochlorite (NaOCl) as the primary oxidant [1, 2]. The stability can be mainly ascribed to a lowering of the ground state energy of the molecule as a result of the intrinsic stability of a N-O three electron bond [3]. Recent electrochemical studies indicated that the N-O bond has a partial *π-bond* character [4].

As shown in Figure 1, the oxoammonium cation(II) is formed by one-electron oxidation of (I), while one – electron reduction affords the hydroxylamine (III).

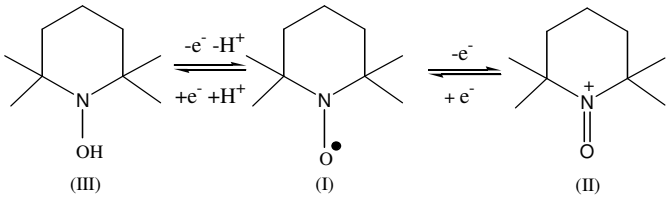


Figure 1 Redox system of TEMPO

TEMPO is orange while the oxoammonium cation has a bright yellow colour and the hydroxylamine is white [5]. The hydroxylamine undergoes facile oxidation by air to give TEMPO. TEMPO is a weak oxidant and can only oxidize relatively strong reducing agents such as *L*-ascorbic acid and phenylhydrazine. The oxoammonium cation(II) is a much stronger oxidant capable of selective oxidation of a variety of substrates (Figure 2), the most important of which are alcohols [6].

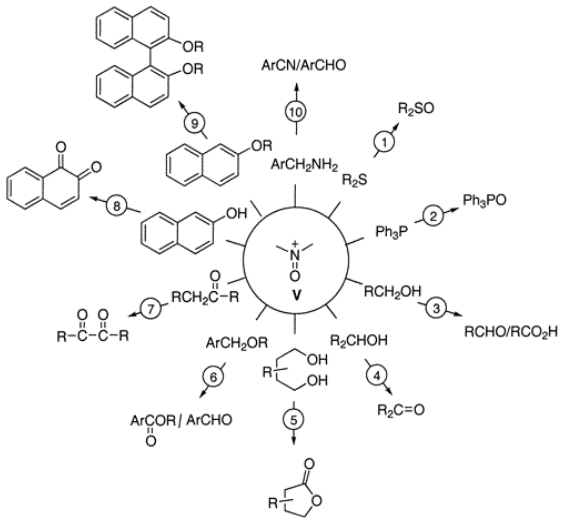


Figure 2 A rosette of oxidation reactions catalyzed by oxoammonium [6]

The catalytic cycle for alcohol oxidation by TEMPO is shown in Figure 3.

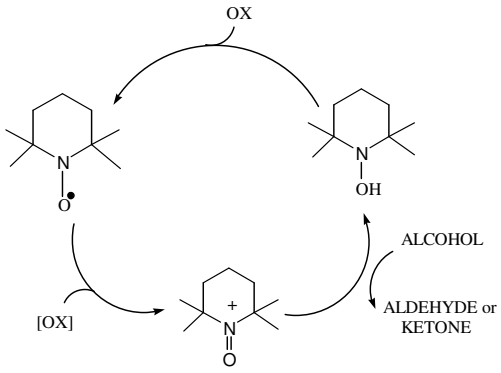
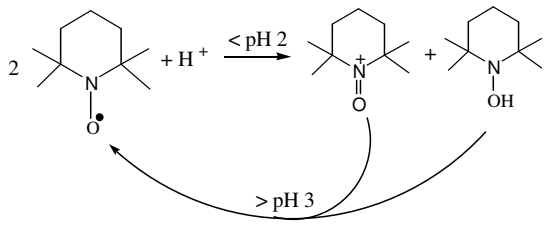


Figure 3 Catalytic cycle for oxidation reaction by TEMPO

The interconversion of TEMPO with the oxoammonium cation and the hydroxylamine is pH dependent. Below pH 2 the acid catalyzed disproportionation of two molecules of TEMPO to oxoammonium and hydroxylamine occurs, while the reverse reaction is favoured at a pH above 3 [1, 7].



In addition to hypochlorite (household bleach), oxone, bromine, chlorine and iodate have been used as the primary oxidant [8]. Molecular oxygen has been used as the primary oxidant in conjunction with TEMPO and transition-metal salts, such as copper, ruthenium and palladium [2]. The oxoammonium salt can also be generated in situ by electrochemical oxidation [9, 10] and is very stable in acetonitrile [11, 12]. Electrochemical studies indicated that the oxoammonium cation is stabilized by dipolar attraction in a polar medium [11]. However, its properties are very dependent on the counter ion [12]. Counter ions such as $[BF_4]^-$ render oxoammonium salts extremely thermostable (decomposition initiates at >195 °C). While perchlorates are well known, they are not recommended owing to their ability to detonate [13]. Bromide ions accelerate oxidations using hypochlorite as the primary oxidant possibly via the formation of hypobromite [8]. The TEMPO/NaOCl system is selective for primary alcohols but is not very suitable for oxidation of electron rich substrates like methoxy substituted benzyl alcohols [14].

Despite the considerable effort that has been devoted to elucidate the mechanism of the reaction between the oxoammonium salt and alcohol substrates (Figure 4), it is still unclear. Semmelhack and co-workers suggested the cyclic, intramolecular elimination mechanism [16]. Bobbitt

and co-workers on the other hand, proposed an acyclic, intermolecular elimination based on their observation of few steric effects [7], Kishioka *et al*. also favoured an intermolecular mechanism based on their electrochemical studies [9]. All authors agreed that the dissociation of the intermediate to aldehyde or ketone is the rate limiting step.

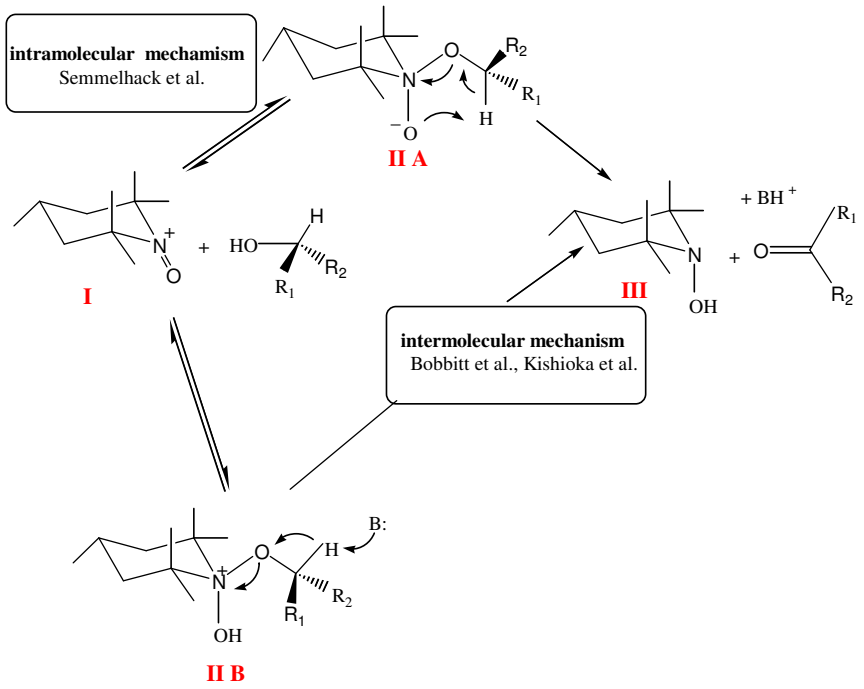


Figure 4 Reaction mechanism of oxoammonium salt with alcohol substrate

In our studies we were particularly interested in oxidation of long-chain alcohols (C5-C12). The carbonyl products of these compounds are of commercial interest as fragrances and flavours. For example, hexanal is used for its oily-green odour and, at high dilution; it adds an effect of freshly cut grass and unripe fruit. Nonanal boosts freshness in floral

compositions such as rose and jasmine. Decanal has an “orange character” and is an extremely important component in perfumery. It is used in floral blends such as rose, jasmine and iris, in aldehydic bouquets, and in citrus fragrances, where it enhances power and diffusion. Dodecanal is widely used in all types of perfumes as its versatile character enables its application in a variety of odour types: cologne, violet, pine, chypre. All these above mentioned aldehydes are also used widely in flavours. Consequently, there is substantial interest in developing “green” processes for their industrial scale synthesis. Initial results reported by Fabbrini and co-workers indicated that hydrophobic aliphatic alcohols could be selectively oxidized by dioxygen (air) in the presence of TEMPO in combination with the copper-dependent oxidase, laccase [17]. Therefore, our interest was to explore the laccase/TEMPO system in the aerobic oxidation of a broader range of linear aliphatic alcohols, particularly long-chain alcohols.

Results and Discussion

The oxidation of benzylic alcohols was discussed in Chapter 2, while in this chapter we will present an investigation of the laccase/TEMPO system in the aerobic oxidation of aliphatic alcohols. 1-Octanol was chosen as a reference substrate from the range of aliphatic alcohols (C5 – C12). Commercially available laccase from the fungus *Trametes versicolor* was employed as received, without any additional purification. The reactions were performed in 0.1 M potassium phosphate buffer solution at pH 4. In practice, the reactions were performed under biphasic conditions as a result of the low solubility of linear fatty alcohols

(partition coefficient values for the substrates are given in Table 1). The C5 - C7 alcohols are partially soluble in water, while C8 – C12 are insoluble. In order to compare initial rates the reactions were stopped after 2 hours which corresponded to ~ 20 % conversion.

Table 1 Partition coefficients for the substrates used for oxidation reactions [18]

Substrate	Partition coefficient (log $P_{w/o}$)
1-Pentanol	1.42
1-Hexanol	1.95
1-Heptanol	2.62
1-Octanol	3.02
1-Nonanol	3.56
1-Decanol	4.10
1-Dodecanol	5.17

The influence of various parameters (amount of enzyme, substrate and TEMPO and the temperature on the course of reaction were studied and the results are shown in Figure 5. A minimum of 10 U/ml of laccase was required for a reasonable rate of reaction. The influence of temperature was studied in the range 25 to 45 °C, the best result being obtained at 35 °C. TEMPO and substrate concentrations were investigated in the range of 5 – 20 mM, and 50 – 500 mM, respectively. Although the use of higher concentrations of TEMPO afforded a higher rate we decided to use a maximum of 10 % of TEMPO respective to substrate concentration, in order to maintain a ‘catalytic amount’ of mediator.

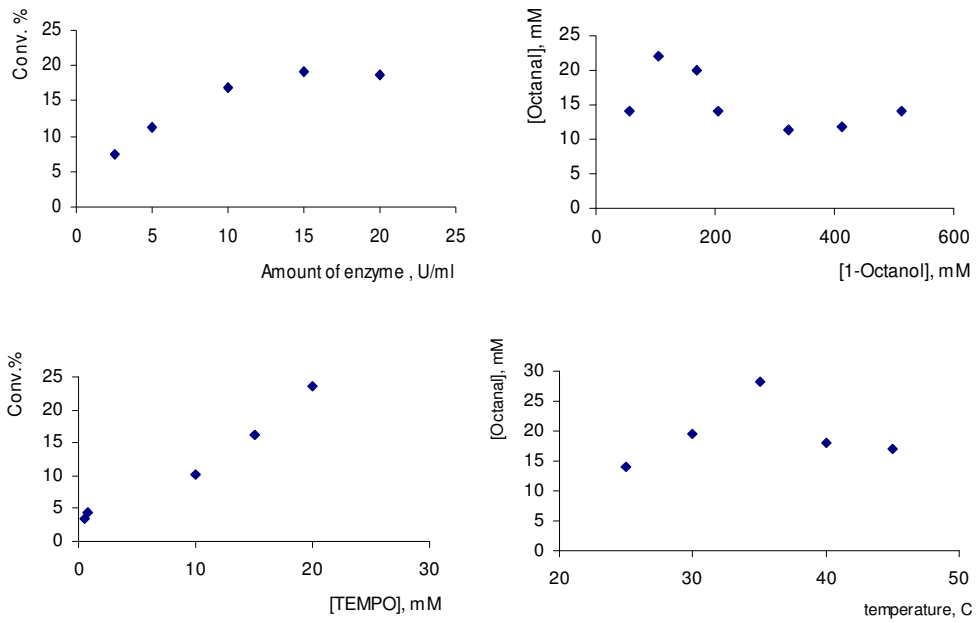


Figure 5 Influence of reaction parameters on conversion in laccase/TEMPO catalysed oxidation of 1-octanol at standard conditions

A decrease in rate was observed at substrate concentrations higher than 180 mM. Based on these results we settled for the following general conditions for the oxidation of alcohols: 1.6 mmol of alcohol with 0.15 mmol of TEMPO and 100 U of laccase in a total volume of 10 ml at 35 ℃ temperature. Efficient stirring and aeration of the reaction solutions was crucial, as was also observed by others [19].

The conversion of 1-octanol plotted against time, under these conditions, is shown in Figure 6.

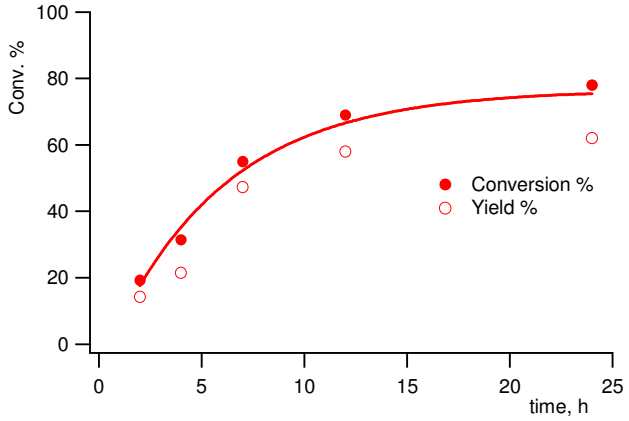


Figure 6 Time profile for oxidation of 1-octanol

Octanal was obtained in 62 % yield after 24 hours with a turnover frequency (TOF) of 1.7 x 10^{-3} min^{-1}. No formation of acid was observed until the alcohol substrate had been completely converted to aldehyde. It was previously shown that TEMPO inhibits the autooxidation of aliphatic aldehydes to the corresponding carboxylic acids [20, 21]. Nonetheless, it is preferable not to reach full conversion since overoxidation to the acid is facile in an aqueous environment, probably proceeding via the oxidation of the aldehyde hydrate. Thus, after 48 h no aldehyde was observed anymore, only the formation of acid. Similarly, 1-pentanol afforded 56 % aldehyde in 24 h and 1-decanol gave 20 % decanal in 4 h.

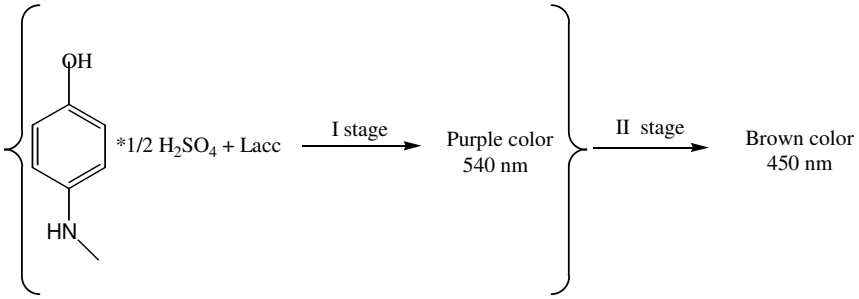
Subsequently, a whole range of C5 to C12 (except C 11) alcohols were tested under optimized conditions and the initial rates are presented in Table 2. 1-Decanol has the lowest oxidation rate while 1-heptanol and 1-octanol have the highest rates. One might have expected the more hydrophilic C5-C7 alcohols to have higher oxidation rates but this was not the case.

Table 2 Initial oxidation rates for aliphatic alcohols

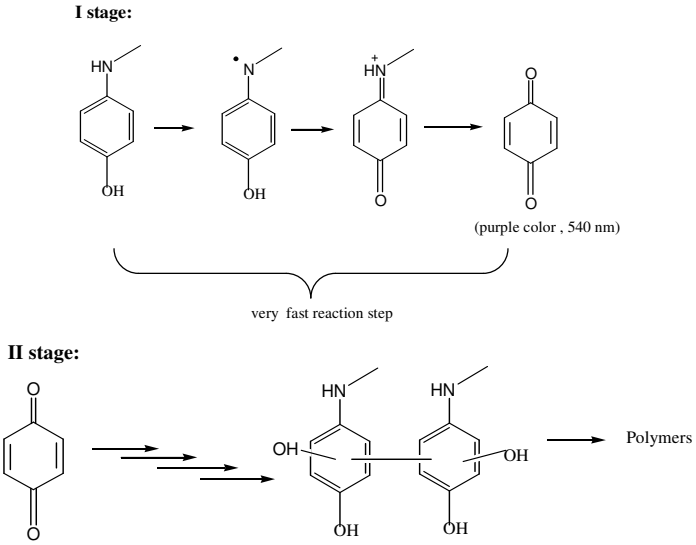
Alcohol	Initial rate, µmol/min
1-Pentanol	5.9
1-Hexanol	3.9
1-Heptanol	9.5
1-Octanol	9.3
1-Nonanol	3.5
1-Decanol	0.8
1-Dodecanol	2.8

Niku-Paavola and co-workers [22] reported rather low (at most 5%) conversions of short chain alcohols like 1-hexanol, 1-butanol, and 1-propanol with the TEMPO/Laccase system. Similarly, with the TEMPO/NaOCl system lower yields were observed owing to overoxidation when the substrate or product was highly hydrophilic. Furthermore, analogous to our results, water insoluble alcohols such as undecanol were oxidized in slightly higher yield than 1-decanol, both without overoxidation [21].

We also followed the laccase activity in time, using the metol assay under the standard conditions. The metol (N-methyl-p-aminophenol sulfate) assay is based on the laccase mediated oxidation of both the amino and phenolic moieties of metol to afford a mixture of products. So far, in the literature it is not indicated what is the exact pathway for metol oxidation [23, 24]. Nevertheless, the reaction scheme can be written as follows:



The mechanism can be roughly divided into two stages: in the first stage the formed product(s) has an intensive purple colour which gives absorbance at 540 nm, and in the second stage polymerization products are formed. Schematically it can be showed:



The first stage consists of several side-reactions which occur rapidly. We were following the initial rate of the first stage of oxidation of metol by laccase (for procedure see materials and methods).

1- Octanol and 1-nonanol were chosen as substrates and various blanks were performed in the metol assay: with TEMPO, substrate (alcohol) and product (aldehyde) and the oxoammonium cation. Only oxoammonium showed a slight increase in absorbance, as would be expected, because it is an oxidant and can oxidize the metol.

The stability test for laccase under the reaction conditions was performed by incubation of the enzyme in phosphate buffer for approximately 4 h (usual reaction time). Similarly, laccase was incubated separately with each of the reaction components: TEMPO, substrates and products for 4 h to determine if it is deactivated by the nitroxyl radical. No inactivation of the laccase was observed. Furthermore, the activity of laccase was followed during oxidation of 1-octanol and 1-nonanol under standard reaction conditions when all reaction components are present (Figure 7).

A slight increase of initial rates was observed after approximately two hours with both substrates. This variation could possibly be due to the oxidation of metol by the oxoammonium cation (see the discussion of blank experiments above). In any case, the results shown in Figure 7 indicate that the activity of laccase is still intact after 4.5 h.

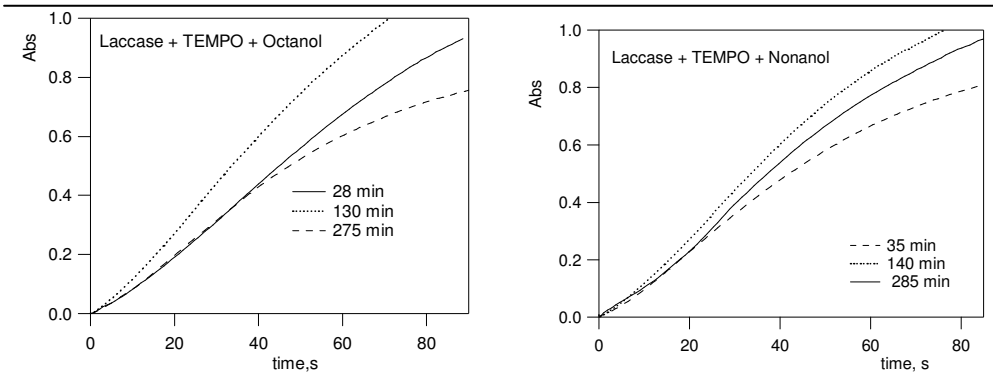


Figure 7 Metol activity assay under standard reaction conditions.

The low solubilities of long chain alcohols in water are another concern. These substrates are more soluble in organic solvents but the laccase prefers an aqueous environment and we assume that the oxidation of TEMPO to the oxoammonium cation by the laccase takes place in the water phase. Moreover, Anelli and co-workers demonstrated that using the TEMPO/NaOCl system for the oxidation of long-chain alcohols, full conversion could be reached in several minutes in organic media, but in the presence of water the reaction rates decreased drastically [25]. In order to establish where the different components are during the course of the reaction we performed some simple tests. Ethyl acetate was chosen as a water immiscible solvent in order to have a biphasic system. Phosphate buffer, (0.1 M, pH 4 as used for reactions) was applied as the water phase. Two identical volumes of water and organic phase mixed in a ratio 1:1 (total 10 ml) were prepared. 0.15 mmol of TEMPO was added in one volume and the same amount of synthetically prepared oxoammonium in another. After mixing, the phases were allowed to separate (Figure 8).

Figure 8 visibly indicates that TEMPO is soluble in the organic phase, while oxoammonium dissolves in the water phase. The solubility of oxoammonium in water can also be influenced by the counter ion, in this case phosphate (from the buffer).

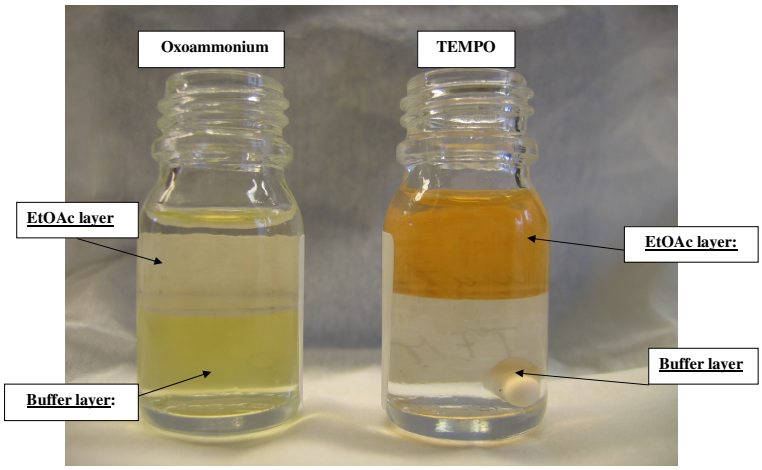
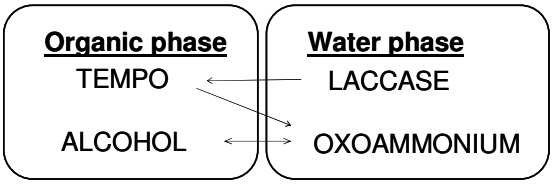


Figure 8 Solubility of TEMPO and Oxoammonium in biphasic system

Hence, we conclude that the distribution of the various components in the oxidation reactions would be as shown below:



TEMPO and alcohol (as well as aldehyde) are to be found in the organic phase while laccase and oxoammonium are in the water phase. Laccase needs to react with TEMPO in order to oxidize it to the oxoammonium cation and we assume that this takes place in the water phase. Similarly, we assume that the oxidation of the alcohol by the

oxoammonium cation takes place in the water phase. We assume that the hydroxylamine is in the protonated form at pH 4 and thus stays in the water layer. Hence, we conclude that the use of a biphasic system is probably not beneficial for the rate but could be useful in suppressing overoxidation by removing the aldehyde product from the water phase.

Furthermore, we have compared the use of a water miscible (dioxane) and water immiscible solvent (ethyl acetate) in these reactions. We showed that laccase is stable when it is incubated in a mixture of buffer and these solvents in a 9:1 v/v ratio. Laccase was dissolved in buffer and the alcohol and TEMPO in the co-solvent. Subsequently, these two solutions were mixed and allowed to react at 35 °C under intensive aeration. The results are shown in Table 3.

Table 3 Initial oxidation rates for aliphatic alcohols

Aliphatic alcohol	**Yield %**			
	Dioxane		**Ethyl acetate**	
	2 h	**4 h**	**2 h**	**4 h**
1- Octanol	8.9	18.7	3.8	17.6
1- Nonanol	6.2	17.6	3.1	8.4
1-Decanol	4.5	16.9	7.9	12.8
1- Dodecanol	4.1	9.8	3.2	6.5

Reaction conditions: [subs] 1.6 mmol, [TEMPO] 0.15 mmol, 100 U laccase, 0.1 M phosphate buffer pH 4, 10 ml volume, buffer/co-solvent ratio 9:1.

When dioxane was used (monophasic) the yield decreased with increasing chain length of the substrate. Yields were lower than those observed in buffer alone. When ethyl acetate was used as a co-solvent

(biphasic system) the yields were even lower than those obtained in dioxane. Moreover, the same trend that the yield decreased with increasing chain length was observed, with the exception of 1-decanol. Experiments were performed in triplicate, thus the results for 1-decanol is not a matter of experimental error. We also tested acetonitrile as a water miscible co-solvent, with 1-octanol and 1-hexanol. After 4 h the aldehyde yields were similar to those obtained in dioxane/water, 9.3 % and 6.2 %, respectively. It is mentioned in the literature that acetonitrile stabilizes the nitroxyl radicals [11]. We conclude from the above results that the use of a co-solvent, in either a monophasic or biphasic system, does not result in any improvement compared to the use of buffer alone.

Secondary linear unsaturated alcohols are also of great importance in the fragrance and flavour industry. Their oxidized products such as 1-octen-3-one (mushroom flavour), 1-octen-4-one (tropical fruit taste) and methyl 3-nonenoate are of commercial interest. According to literature reports, primary alcohols are generally oxidized faster than secondary alcohols with TEMPO-based systems [1, 25]. Recently, Bailey and co-workers reported that computational studies indicated that the oxidation of alcohols by oxoammonium cation in basic media is more selective for primary alcohols than for secondary ones, whereas in a neutral or acidic medium, the relative activities are the reverse [26]. In contrast, we observed the opposite activity trend using laccase/TEMPO in buffer at pH 4. Two pairs of primary and secondary alcohols were compared: benzyl alcohol *versus* 1-phenyl ethanol and 1-octanol *versus* 2-octanol. The results are shown in Figure 9.

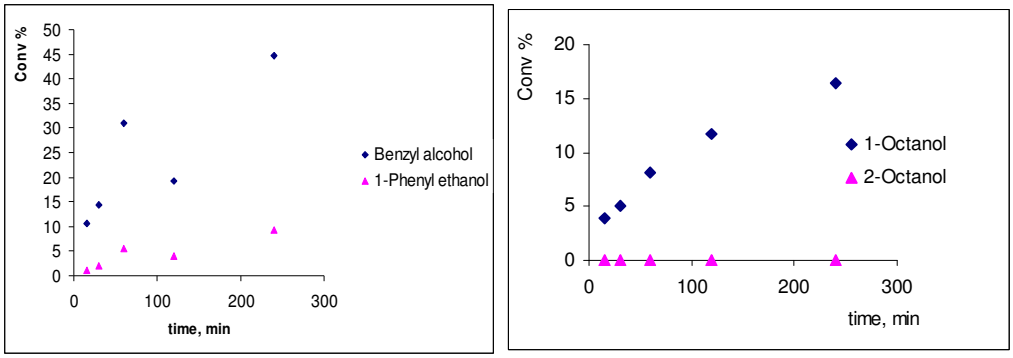


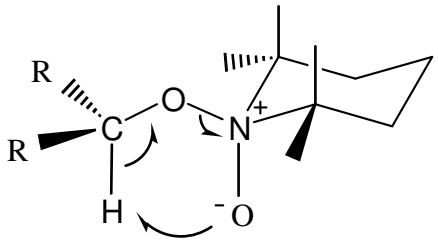
Figure 9 Competition oxidation reaction: primary alcohol *versus* secondary alcohol

Clearly, the laccase/TEMPO system shows a higher preference towards primary alcohols in aqueous solution. The secondary benzylic alcohol, 1-phenylethanol, was oxidized by the laccase/TEMPO system, albeit at a 2-fold lower rate than the primary benzyl alcohol. (0.011 min^{-1} and 0.022 min^{-1}, respectively). In contrast, the secondary aliphatic alcohol was completely unreactive towards the laccase/TEMPO system. The rate constant for 1-octanol was calculated to be 0.0076 min^{-1}. Such results are not really surprising. In a competition experiment with the TEMPO/NaOCl system in organic media, the relative rates of oxidation of 1-nonanol and 2-nonanol were 9:1 [25].

Similarly, Einhorn and co-workers investigated the TEMPO/*N*-chlorosuccini-mide system for primary and secondary alcohols with different functional groups [20]. In the case of benzylic alcohols, electron donating groups (methoxy) slowed down the oxidation reaction, whereas electron withdrawing groups (NO_2) accelerated it. In competition experiments with 1-octanol and 2-octanol the former gave complete conversion, while virtually no ketone formation was detected with the

latter. The competitive oxidation of benzyl alcohol and 1-phenylethanol exhibited similar selectivity: after complete transformation of primary alcohol into aldehyde, the amount of ketone remained under GC detection level [20].

The observed relative activities of primary and secondary alcohols can be understood by considering the reaction intermediate shown below [19]. The rate determining step is probably decomposition of this intermediate to the aldehyde or ketone product as shown.



In the case of secondary alcohols the formation of this intermediate is probably highly reversible or prevented because of steric interference of the alkyl groups. Only in the case of an activated α-C-H bond (as for benzylic alcohols), β-elimination is feasible and formation of the corresponding aldehyde occurs. In case of primary alcohols the equilibrium for adduct formation is much more favorable; therefore, the reaction takes place for both activated and non-activated alcohols.

Conclusions

In this chapter we have identified the optimum conditions for the oxidation of primary aliphatic alcohols. For long-chain alcohols the optimum concentrations of TEMPO, substrate, enzyme and reaction temperature were determined in aqueous medium. Similar to the results

for benzylic alcohols described in Chapter 2, the optimum concentrations for effective conversion were 10 U/ml of enzyme, 160 mM substrate and a TEMPO concentration of 15 mM. A TEMPO concentration below 10 mM led to lower conversions. In contrast to benzylic substrates the oxidation of long-chain alcohols requires somewhat higher temperature (35 °C).

The range of long-chain alcohols (C5-C12) were tested at optimized reaction conditions. The observed initial rates (Table 2) indicated that the oxidation rate does not depend on solubility or length of alcohol substrate. For example, 1-heptanol and 1-octanol showed higher initial rates in comparison with 1-pentanol or 1-hexanol. Moreover, no formation of acid was observed over long reaction times (24-48 h) until the alcohol substrate had been completely converted to aldehyde. The metol activity tests performed during the oxidation reaction did not show any deactivation of enzyme. The use of co-solvent to increase solubility of the substrate, either in the monophasic or biphasic system, did not result in any improvement compared to the buffer alone. Additionally, the results demonstrated that the laccase/TEMPO system is highly selective for primary alcohols [1].

Materials and methods

The substrates and reagents were of analytical grade and obtained from Fluka and Sigma Aldrich. Laccase [E.C. 1.10.3.2] from *Trametes versicolor* was purchased from Fluka. 2,2,6,6-tetramethylpiperidinyl-1-oxy (TEMPO) was received from Acros. The reactions were carried out in a stationary Omni Reaction Station with comprehensive

heating/cooling/stirring/aeration control. The conversions were followed by GC analysis.

1. Laccase activity assay

Laccase activity was measured spectroscopically using metol as a substrate [24]. The reaction mixture contained 1 ml 0.05 M metol solution in water. The reaction was initiated by addition of 0.5 ml enzyme solution in 0.1 M acetate buffer pH 4.5 and the increase in absorbance was monitored at 540 nm (ε = 2000 $M^{-1}cm^{-1}$). One unit (U) of the laccase was defined as 1 µmol metol oxidised per minute under the stated assay conditions.

2. Oxidation procedure by laccase/TEMPO system

In the standard oxidation reaction protocol the following amounts were used: 1.6 mmol of alcohol, 0.15 mmol of TEMPO and 0.25 mmol of internal standard (anisole) together with 100 U of laccase in 0.1M phosphate buffer (pH 4) in a total of 10 ml reaction volume. In the case of 1-hexanol, dodecane was used as internal standard at the same concentration. The vials with reaction mixture were aerated with steel filter stones during reaction. The reactions were carried out in the Omni Reaction Station at certain temperature for certain time. After reaction, the reaction products and unreacted substrates were extracted with EtOAc (3 x 10 ml), dried with anhydrous magnesium sulphate and analysed with GC. The blank reactions were performed deleting one of the components mentioned above.

3. Metol activity assay during oxidation reaction

Oxidation reactions were carried out under standard reaction conditions (method 2), samples were taken from the reaction mixture and the activity of laccase was measured according to method 1 (laccase activity assay).

4. Analysis methods

Alcohol conversions were analyzed by GC with column WAX 52 CB (50m x 0.53 mm). The method for GC was programmed as follows: 80℃ for 5 minutes, then at the rate of 7℃ per minute to 235℃. Anisole was used as internal standard.

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4*

Pre-steady state kinetic studies on the microsecond time scale of the laccase from *Trametes versicolor*

Abstract

The catalytic mechanism of the enzyme was studied with three substrates (TEMPO, hydroquinone and phenol using the stopped-flow and our newly developed rapid-mixing rapid sampling method, which has an experimental dead time of 75 ± 15 μs. Moreover, the optimized purification method for laccase from T.versicolor is presented. Equilibrium and kinetic analyses yielded a reduction potential of 717 ± 5 mV for Type 1 copper center. EPR and low-temperature UV-Visible spectroscopy indicated that oxidation of the blue copper center and O=O bond cleavage occurs within 100 μs, without detectable formation of a peroxide intermediate. These results indicate a rapid internal electron transfer between the various copper centers (> 25.000/s) and rapid binding of O_2 ($k_{on} > 5 \times 10^7 M^{-1}s^{-1}$). Mechanistic aspects of the catalytic cycle are briefly discussed.

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Introduction

Nowadays only a few examples of biocatalytic oxidations are known, while there is a serious need to replace classic stoichiometric reagents with green catalytic alternatives. In this respect, laccase-mediator systems that catalyze selective oxidation of alcohols have drawn increasing attention in organic synthesis. Nitroxyl radical 2,2,6,6-tetramethylpiperidinyloxy (TEMPO) was shown to be the most effective mediator for alcohol oxidation [1]. It seems likely that the oxoammonium ion, which is formed *in situ,* is the actual oxidant. However, for efficient synthesis under optimized conditions, the precise role of laccase must be determined.

Currently, there is considerable interest in the use of laccase [E.C. 1.10.3.2] in biocatalysis and, in combination with hydrogenases, in a new generation of biofuel cells [2]. For both applications a detailed understanding of the catalytic mechanism of laccase would be beneficial.

The most challenging aspect to understanding laccases is how the unique structural and spectral features of the four catalytic copper atoms of laccase are related to its efficient catalysis. The T1 Cu, or blue copper, can be distinguished by an intense absorbance ($\varepsilon \approx 5000\ M^{-1}cm^{-1}$) at approximately 600 nm and a small ($<100 \times 10^{-4}\ cm^{-1}$) g-parallel hyperfine coupling in electron paramagnetic resonance (EPR). The colorless T2 Cu site displays a large g-parallel hyperfine coupling (>160

x $10^{-4}cm^{-1}$) typical of tetragonal Cu. The T3, or coupled binuclear, Cu site is comprised of two Cu^{2+} ions that are antiferromagnetically coupled through a bridging hydroxide. The resulting diamagnetic ($S_{total}=0$) T3 Cu site lacks an EPR signal, but it displays an optical absorbance around 330 nm ($\varepsilon \approx 5000\ M^{-1}cm^{-1}$) [3].

Using a combination of spectroscopic techniques it was shown that the electrons enter the laccase *via* the T1 Cu site, while oxygen reduction to water occurs at the T2/T3 cluster [4-7]. Reduced wild type *Rhus vernicifera* laccase reacts rapidly with oxygen to form a 'native intermediate' within ~ 1 ms in which the O=O bond has been broken. The native intermediate is stable for minutes in the absence of reductant. In the native intermediate the three Cu of the T2/T3 cluster are proposed to form a single trinuclear exchange coupled system in which all three Cu are bridged by hydroxide or by a single μ_3-oxo. In laccase from *Rhus vernicifera* in which the T1 Cu had been replaced by Hg, the three-electron reduced enzyme forms a peroxide intermediate in reaction with oxygen [8-11]. These findings suggest that the catalytic cycle of laccase comprises initial formation of a peroxide intermediate – the two-electron reduced state- followed by O=O bond cleavage yielding a native intermediate like species. Accumulation of a peroxide intermediate could not be established so far in the wild type enzyme using e.g. stopped-flow techniques.

Recently, a rapid freeze-quench mixing and sampling methodology was developed, so-called microsecond freeze-hyperquenching (MHQ), which

enables UV-Visible and EPR spectroscopic analyses on samples aged for only ~ 60-80 µs [12,13]. We have used the MHQ technique, in conjunction with the stopped-flow technique, to gather information on the early events in oxygen binding and reduction by the laccase from *Trametes versicolor.*

Results and Discussion

Commercially obtained laccase from *T. versicolor* is brownish and contained a precipitate, which encumbers spectroscopic measurements. The enzyme was purified in two steps employing Q-Sepharose and Superdex-75 column chromatography. Pure laccase was obtained in 60 % purification yield with a four-fold purification factor (Table 1).

Table 1 Production of purified laccase from *Trametes versicolor*

	Activity		Protein conc.		Spec.activity U/mg	Purification factor	Yield %
	U/ml	U	mg/ml	mg			
Batch wise	245	2450	24.7	247	9.92	1	100
Q-Sepharose	296	1480	13.4	67	22.1	2.23	60.4
Superdex-75	208	1456	5.04	35.5	41.2	4.15	59.4

The purity and homogeneity of the enzyme was confirmed by SDS-PAGE resulting in one band of approximately 67 kDa (Figure 1).

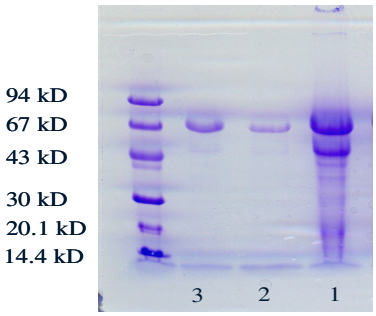
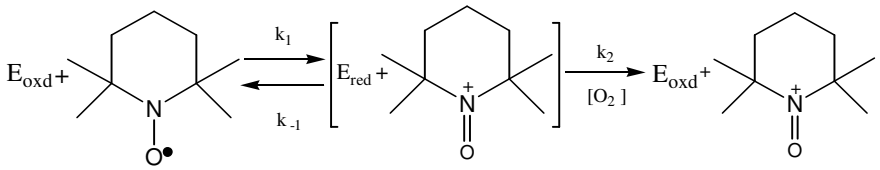


Figure 1 SDS – PAGE of laccase. Lane 1: Starting material; lane 2: fraction of enzyme after Q-Sepharose column, lane 3: purified enzyme. The left lane contains the molecular mass standards with indicated molecular mass.

The molecular weight determined for pure laccase is in excellent agreement with that reported by M. Antorini *et al*. [14].

The steady-state activity of laccase was determined for three substrates, hydroquinone, TEMPO and phenol. A full turnover of laccase reduction, for example by TEMPO, can be described by the simplified reaction scheme:



In the first step, represented by k_{-1}/k_1, four TEMPO molecules binding consecutively are needed to fully reduce the four coppers of laccase, represented by k_{-1}/k_1. The reduced laccase is subsequently oxidised by molecular oxygen, given by k_2 or k_{cat}. For the two-electron donors

hydroquinone and phenol, substrate-based radical intermediates may be formed.

Steady-state polarographic experiments with the Clark electrode yielded maximal (O_2) turnover rates (corresponding to k_{cat} or k_2) of 42 s^{-1}, 28 s^{-1}, and 2 s^{-1} for hydroquinone, TEMPO and phenol, respectively (Table 2). Hydroquinone had the highest rate and specificity constant (Table 2).

Table 2 Turnover rates for O_2 of laccase with various substrates

Substrate	**K_m (app), mM**	**Turnover (k_2), s^{-1}**	**k_{cat}/K_m ($M^{-1}.s^{-1}$)**
Hydroquinone	1	42	4.2×10^4
TEMPO	1.8	28	1.6×10^4
Phenol	21	2	0.1×10^3

To determine values for k_1 and k_{-1} pre-steady state kinetic experiments for the reduction of laccase by TEMPO were performed with the stopped-flow spectrofluorimeter under anaerobic conditions. The reduction of laccase was followed at 600 nm monitoring the reduction of the T1 Cu only. The rate constants were derived from the equation $k_{obs} = k_{-1} + k_1$ [TEMPO], by varying the TEMPO concentration (Figure 2).

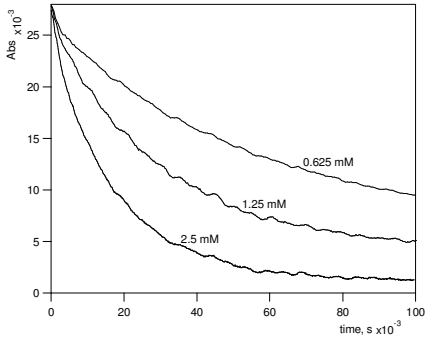


Figure 2 Stopped-flow traces with different concentrations of TEMPO measured at 600 nm monitoring reduction of the Type 1 Cu at 20 °C

The slope in the linear plot (Figure 3) yielded the value for k_1 of 19.9 10^3 $M^{-1}s^{-1}$ and 5.0 s^{-1} for the reverse rate constant k_{-1}.

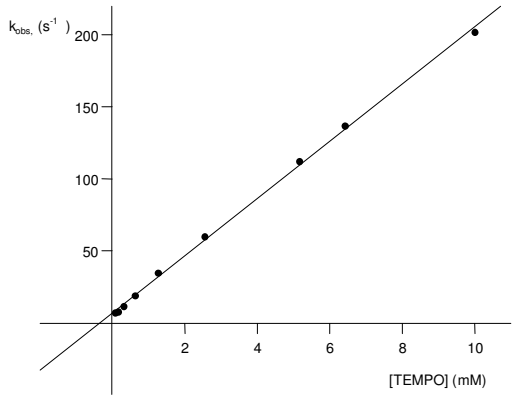


Figure 3 Plot of k_{obs} versus [TEMPO] for the reduction of laccase at 20 °C

These steady state and pre-steady state kinetics clearly implicate a direct interaction between laccase and TEMPO. Similar results were obtained using Hydroquinone or Phenol as substrate (data not shown).
The traces in Fig. 2 indicate partial reduction of the T1 Cu at low [TEMPO] and long reaction times, allowing the determination of the midpoint potential (E_m) of the Type 1 Cu using the Nernst equation and a TEMPO E_m-value of 750 mV [15]. A value for E_m of 717 mV ± 5 mV for T1 Cu was calculated, which is close to the value of 714 mV obtained from the k_1/k_{-1}.

The microsecond freeze-hyper quenching technique [13] (MHQ) was applied for determination of possible reaction intermediates, such as a peroxide adduct or the native intermediate. The freeze-quenched samples were studied by low-temperature UV-Visible and EPR spectroscopy. MHQ experiments (100 µs – 10 ms) in which TEMPO-

reduced or phenol-reduced laccase was rapidly mixed with an oxygen-saturated buffer solution yielded the following results. The Type 1 Cu was (almost) completely oxidized after 100 µs (Figure 4) as evidenced from both EPR and low temperature UV-Vis spectroscopy.

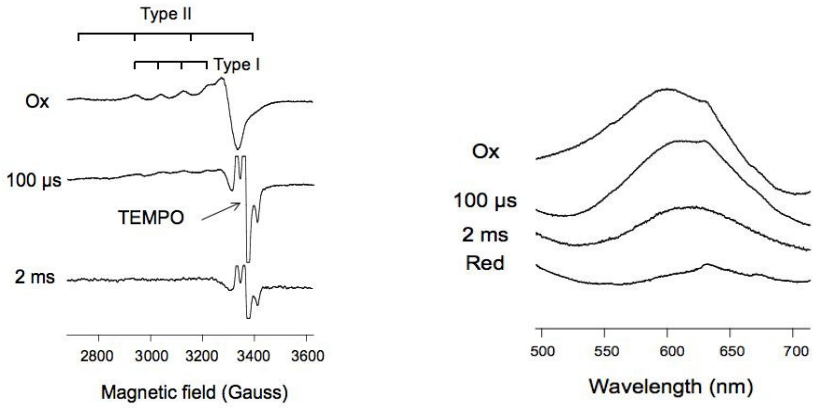


Figure 4 X-band EPR (left) and Low-temperature UV-Visible spectra (right) of MHQ samples. Laccase prereduced with TEMPO was reacted for the indicated times with a saturated oxygen solution

Partial rereduction of the Type 1 Cu by TEMPO is indicated by the disappearance of the Type I Cu EPR signal, decrease of the TEMPO EPR signal and decrease of absorbance at 600 nm (Figure 4). The rereduction was completed in ~ 2-10 ms in accordance with the second order rate constants derived from stopped-flow experiments. In the same time window transient formation of a (presumably) phenoxy radical was observed when phenol was used as a substrate (results not shown). Our results obtained so far with either TEMPO or phenol as a reductant did not provide direct evidence for a copper EPR signal similar or identical to the so-called “native intermediate” [13] nor could we find

indication of a peroxy intermediate in the UV-Vis spectra in the spectral range between 300-400 nm.

Conclusions

The obtained results permit us to conclude that TEMPO interacts directly with laccase, indicated by the rapid electron transfer to the Type 1 Cu and from there to the other copper sites resulting in a steady-state oxidation of the substrate.

The complete oxidation of the reduced T1 Cu within ~ 100 µs after mixing with oxygen indicates a fast internal electron transfer (> 25.000/s) and rapid binding of O_2 ($k_{on} > 5 \times 10^7$ $M^{-1}s^{-1}$). Lack of detection of a peroxide intermediate could be due to a very short life-time (< 10 µs). Alternatively, it is possible that such an intermediate is not formed and the O=O bind is cleaved in a concerted four-electron transfer reaction. We can at present not define the state of the enzyme after a single turnover, but the lack of a T2 Cu EPR signal suggests a 'native intermediate' like structure, i.e. in which the T2 and T3 Coppers are magnetically coupled. The results further suggest that neither internal electron transfer nor binding of oxygen are limiting the turnover rate of the enzyme.

Materials and methods

All reagents were of analytical grade. Laccase [E.C. 1.10.3.2] from *T. versicolor* was obtained from Fluka. The enzyme obtained after the last purification step was used for the indicated experiments without further

changes of buffer. 2,2,6,6-Tetramethylpiperidinyloxy (TEMPO) was purchased from ACROS and was applied as received. Kinetic measurements were performed on a SX.18MV Stopped-flow spectrofluorimeter. EPR spectra were obtained as reported in [16] using a Bruker 200D ERP spectrophotometer. The microsecond freeze-hyper quenching experiments were performed as described in [12, 13]. All the buffer and substrate solutions were made up in Milli Q water (18.2 $M\Omega \cdot cm^{-3}$).

1. Enzyme purification

Purification encompassed two chromatography steps. First, ion-exchange chromatography on Q-Sepharose was used, and then gel filtration chromatography on Superdex-75 column was applied. The Q-Sepharose chromatography column was pre-equilibrated with 10 mM Tris–HCl buffer, pH 7.5. The laccase was eluted with the same buffer with a gradient of 0-1 M NaCl. The flow rate was 10 ml/min. Fractions containing active enzyme were collected and concentrated by ultrafiltration with 50 kDa cutoff filter. Next, concentrated enzyme was applied to a Superdex-75 column and eluted with 50 mM MES (4-morpholinoethanesulfonic acid) buffer, pH 6 at 4 ml/min. The purity and homogeneity of the enzyme was confirmed by SDS-PAGE.

The protein concentration was determined measuring the absorbance of the BCA reagent (commercial source Uptima) after 30 min incubation with enzyme. Laccase activity was measured spectroscopically using metol as a substrate. The reaction mixture contained 1 ml 0.05 M metol solution in water. The reaction was initiated by addition of 0.5 ml enzyme

solution in 0.1 M acetate buffer pH 4.5 and the increase in absorbance was monitored at 540 nm.

2. Voltammetric experiments

Hydroquinone, TEMPO and phenol were used as electron donors. The experiments were carried out in 0.1 M phosphate buffer pH 4 at 22 ˚C. The substrate concentrations were used in the range of 0.2 – 4 mM for hydroquinone, 4 - 44 mM for phenol and 0.2 – 5 mM for TEMPO. Laccase ([1.008 µM]) in 50 mM MES buffer was applied in each experiment. The reported values for the rates were averaged from 3 measurements. Before the experiments the electrode was polished with aluminium oxide powder (grain size down to 0.05 µm) on a wet pad, rinsed with water and than dried.

3. Stopped-flow experiments

Laccase concentration was 19 µM (in 50 mM MES buffer). TEMPO solutions were prepared at a concentration of 50 mM as a stock solution in 0.1 phosphate buffer pH 4. Further dilutions were made to concentrations of 0.02-10 mM. The experiments were performed at 20 ± 0.2 ˚C. For the stopped-flow measurements, solutions that have to be mixed were contained in glass Hamilton syringes submerged in a circulating water bath for temperature control. Anaerobic samples of laccase and TEMPO solutions were deoxygenated by five cycles of purging the solution with a stream of argon and evacuation.

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5

Kinetic survey of the reaction cycle followed by Laccase and TEMPO in alcohol oxidation

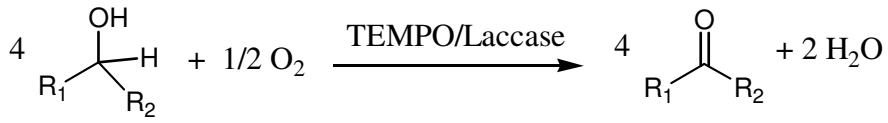
Abstract

Following the work in Chapter 2 in which optimum conditions for the Laccase/TEMPO system were established, this chapter aims to develop a kinetic model of benzyl alcohol oxidation and measure the various individual reaction rates. The complete reaction cycle was broken down into five steps for which rate constants were determined, including the rate of TEMPO and hydroxylamine oxidation by laccase, the hydroxylamine/oxoammonium disproportionation, the hydroxylamine oxidation by O_2 and the oxidation of benzyl alcohol by oxoammonium. Further, steady state TEMPO concentrations were determined by EPR under process conditions as this can provide insights into the nature of the rate limiting step(s).

The kinetic model indicates that the conversion cycle runs at only ~ 10% of the added laccase activity. The model predicts the same steady state TEMPO concentrations irrespective of whether the reaction is initiated with TEMPO, hydroxylamine or oxoammonium. However this was not corroborated experimentally. In addition, when the reaction was started with oxoammonium, the conversion obtained after one hour was only marginally higher than that obtained in the absence of laccase. The complete system contains a quasi-irreversible step that could not be identified.

Introduction

Optimum conditions for the oxidation of two classes of alcohols (benzylic and long-chain aliphatic) using the Laccase/TEMPO system were identified in Chapters 2 and 3:



To obtain suitable conversion of 160 mM alcohol within four hours at pH 4 in aqueous buffer required 10 U/ml of laccase and 15 mM TEMPO. The only apparent difference between benzylic and aliphatic alcohols was the optimum temperature, 30 °C for benzylic alcohols and 35 °C for aliphatic alcohols.

Since the first report was published on benzyl alcohol oxidation catalyzed by laccase and mediated by TEMPO [1] many publications appeared in which the mechanism of the laccase-mediator interaction was discussed [2-11]. Among the various mediators, TEMPO is rather unique, because its does not undergo a direct electron transfer or hydrogen atom transfer with the alcohol substrate. It first undergoes a one-electron oxidation by the oxidized laccase (Cu^{2+}) to afford the corresponding oxoammonium cation and the latter oxidizes the alcohol in a two-electron oxidation involving an intermediate oxoammonium-alkoxide complex (Figure 1, reactions 3a and 3b). The overall reaction cycle can be divided into five steps: 1) the reduction of laccase by TEMPO, 2) the oxidation of reduced laccase by molecular oxygen,

3) the oxidation of alcohol by the oxoammonium (the oxidized form of TEMPO), 4) the oxidation of hydroxylamine (the reduced TEMPO form) by laccase or by molecular oxygen, 5) the disproportionation reaction between oxoammonium and hydroxylamine

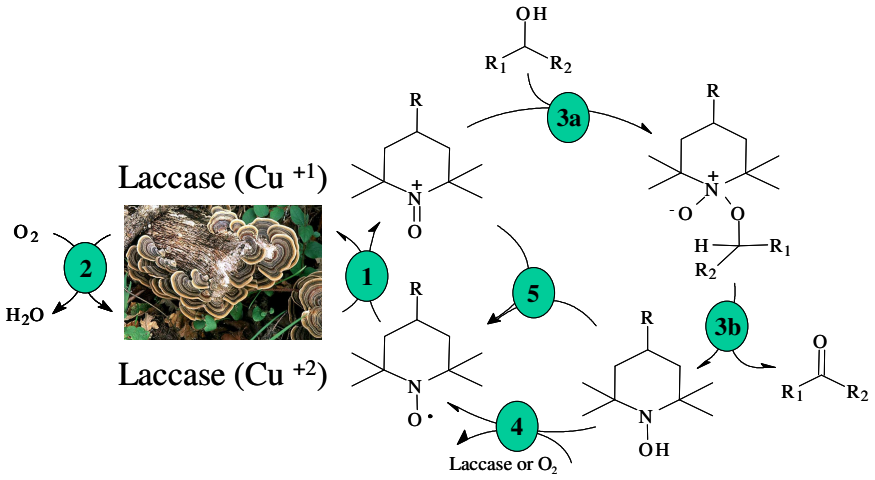


Figure 1 Proposed alcohol oxidation mechanism catalyzed by laccase and mediated by TEMPO

Steps 4 and 5 are TEMPO regeneration steps, which should drive the cycle until 100 % conversion is reached. The rates of some of these steps are not known and their determination is detailed below.

The aim of this chapter is to quantitatively model the observed rates of benzyl alcohol conversion by the complete laccase/mediator system on the basis of the rates of the individual steps shown in Figure 1. A quantitative analysis could help to identify the major limiting step of the cycle and provide hints to improve the present performance.

Results and discussion

Determination of the rate constants of the individual steps of alcohol oxidation by the laccase/TEMPO system

pH dependence of the laccase from Trametes versicolor

The robustness of the TEMPO/laccase system depends –among other factors- on the stability and activity of the laccase during the four hour reaction period. The preliminary catalytic oxidation experiments indicated that the best conversions with the *T. versicolor* laccase were obtained at pH 4.5 – 4.8 in phosphate buffer rather than in acetate buffer [12]. The enzyme appeared quite unstable in acetate buffer. Therefore, the pH optimum was determined in phosphate buffer with TEMPO as a substrate. The enzyme shows a relatively broad pH optimum with a maximum around pH 4 (Figure 2), while good (> 80%) activity is retained at pH 5 and pH 3 (~ 70%). Above pH 5, the enzyme activity appears to drop sharply.

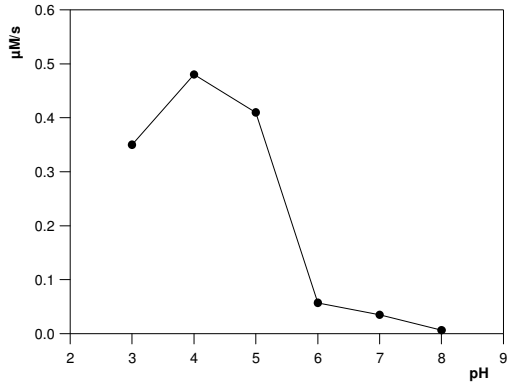


Figure 2 pH profile of laccase activity with TEMPO as substrate

To test the laccase stability during benzyl alcohol conversion, samples of several catalytic oxidations were taken between 1-4 h reaction time and analysed with the standard activity test (metol) or in the oxygraph with TEMPO (8-15 mM), sometimes in the further presence of oxoammonium (5-15 mM). The overall conclusion (data not shown) is that the laccase retains full (80-100%) metol and TEMPO conversion activity over time irrespective of the presence of oxoammonium, benzyl alcohol or the product benzaldehyde. Thus the results indicate that laccase is not irreversibly inactivated by any of the components of the TEMPO/laccase system.

Determination of the pH during conversion by the laccase/TEMPO system indicated that the pH was not held constant by the presence of 0.1 M phosphate. At t = 0, after addition of all the reaction components, the pH was 4.6 – 4.7, which value increased in the first hour to pH 5.0 – 5.1. Thereafter, in 1 – 4 h, the pH dropped to ~ pH 4.8.

Hydroxylamine oxidation by laccase (step 4)

According to Figure 1, hydroxylamine is formed during the reaction (step 4), which has to be converted to TEMPO and finally to the oxoammonium by the laccase. Hydroxylamine (TEMPO-H) was obtained by chemical synthesis and allowed to react with laccase in the stopped-flow analogous to that described in Chapter 4 for TEMPO. The laccase reduction of the T1 Cu by hydroxylamine was followed by UV-Vis spectroscopy (600 nm) at 20 °C under anaerobic conditions (Figure 3):

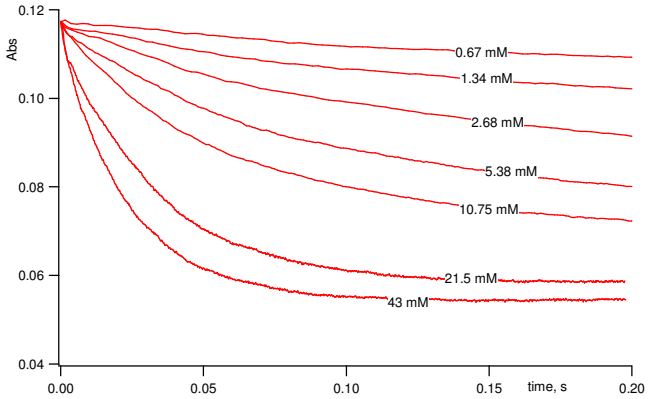


Figure 3 Stopped -flow traces with different concentrations of hydroxylamine monitored at 600 nm measuring the reduction of the Type 1 Cu.

The rate constants were determined by simulation of the kinetic traces recorded at different TEMPO-H concentrations and application of the equation $k_{obs}= k_{-4} + k_4*[TEMPO-H]$. The slope of the line in Figure 4 yields a value for k_4 of 1.76 x 10^3 $M^{-1}s^{-1}$ and 4.1 s^{-1} for the reverse rate constant.

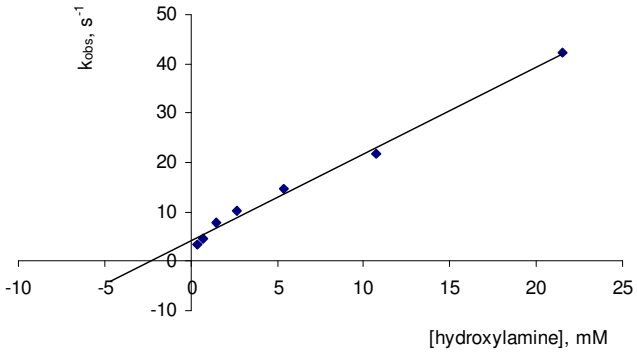


Figure 4 Plot of k_{obs} versus [Hydroxylamine] for the reduction of laccase at 22 ℃

The rate constant for the initial reduction by hydroxylamine is about 11 times smaller than for TEMPO, but the maximum rate for TEMPO-H is somewhat higher than for TEMPO (see Table 4, steps 1 and 4). The data show for the first time that both TEMPO and TEMPO-H, or rather TEMPO-H_2^+, serve as a substrate for laccase, underscoring the regeneration pathway of the catalytic conversion cycle (Figure 1).

Direct oxidation of hydroxylamine by molecular oxygen yielding TEMPO and/or oxoammonium is also a possible regeneration pathway. While TEMPO is very stable with respect to oxidation by oxygen and hydroxylamine oxidation by molecular oxygen in organic solvents is rapid, the stability of TEMPO-H in aqueous solution at pH 4 is not known. By monitoring the oxidation of 68 mM TEMPO-H while stirring to maintain aerobiosis over 24 h a very low oxidation rate of 2.2 x 10^{-3} mM/min was obtained – *i.e.* low compared to 0.4 – 0.65 mM benzyl alcohol/min for the complete reaction (Table 4) - yielding a rate constant (k_4*) of 2.7 x 10^{-3} $M^{-1}s^{-1}$ (Table 4 step 4). As a consequence, oxidation of TEMPO-H by molecular oxygen is eliminated as an important regeneration pathway in the laccase/TEMPO conversion system.

Disproportionation reaction of TEMPO

According to the literature the TEMPO disproportionation reaction (step 5) occurs readily in organic solvents. Anelli and co-workers showed that small amounts of water added to the organic solvent slowed down the disproportionation reaction [13]. In our case, we have a pure aqueous solution. The oxoammonium salt was synthesized with phosphate as counter ion and reacted in equimolar amounts with hydroxylamine

solutions in 0.1 M phosphate buffer, pH 4. Hydroxylamine is colourless and the reaction progress can be monitored spectrophotometrically by the appearance of TEMPO (420 nm) and disappearance of oxoammonium (465 nm at pH 4) (Figure 5), or by EPR spectroscopy through the formation of TEMPO (data not shown).

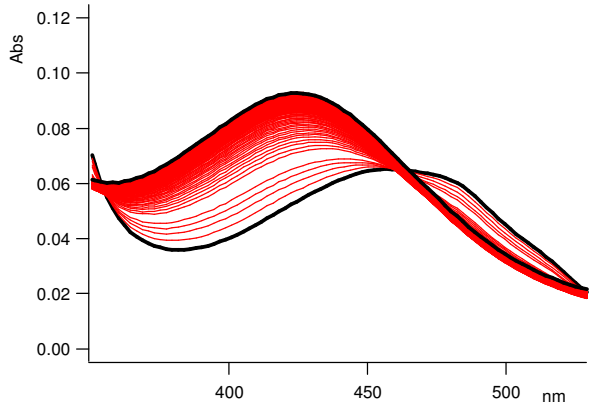


Figure 5 Comproportionation reaction of TEMPO-OH with oxoammonium for 499 s monitored in the stopped-flow experiments. Black spectra represent the initial oxoammonium (465 nm) and the final TEMPO (425 nm).

Analysis of the kinetics obtained by UV-Vis and EPR yielded a rate constant of 0.5 ± 0.2 $M^{-1}s^{-1}$ at pH 4. This value is 5-10 times higher than determined by Israeli and co-workers [14]. They found that the reaction rate is proportional to $[H^+]$ between pH 4 - 6.5 and at pH 4 their value corresponds to 0.06 $M^{-1}s^{-1}$. We cannot rule out that in fact a higher pH (4.6 - 4.7) was present in our experiments because the hydroxylamine reacts in its non-protonated form (pK_a for hydroxylamine is around 7.5, see ref. 14).

Direct oxidation of alcohols by oxoammonium

Alcohol oxidation to the corresponding aldehyde by oxoammonium was monitored optically by UV-Vis spectrophotometry. The alcohols tested were benzyl alcohol, 4-methoxybenzyl alcohol and 1-octanol each at 160 mM at various pH values (4, 6 and 8 in 0.1 M phosphate buffer). The UV-Vis spectrum of the oxoammonium salt was dependent on pH showing maxima at 465 nm for pH 4, at 455 nm for pH 6 and at 440 nm for pH 8. The calculated rate constants at different pHs are shown in Table 1.

Table 1 Rate constants for the alcohol oxidation by oxoammonium at different pHs

Alcohol	**k, M^{-1} s^{-1}**		
	pH 4	**pH 6**	**pH 8**
Benzyl alcohol	1.73×10^{-2}	0.45×10^{-2}	1.10×10^{-2}
4-Methoxybenzyl alcohol	0.60×10^{-2}	0.40×10^{-2}	0.80×10^{-2}
1-Octanol	1.35×10^{-2}	0.17×10^{-2}	0.80×10^{-2}

A general observation is that all rates are the same within a factor of 10 (Table 1). The rates observed at pH 4 and at pH 8 are always higher than those at pH 6. This could be explained if two different intermolecular reaction mechanisms between oxoammonium and alcohol occur under acidic conditions (pH 4) and basic conditions (pH 8), which is in accordance with literature (Figure 6). The latter two mechanisms were discussed by de Nooy *et al*. in their review [15]. The reason why the reaction at pH 4 is lower than that at pH 6 is not directly clear from this picture, but apparently under non-basic conditions the

conversion is proportional to pH. For both benzyl alcohol and 1-octanol the highest rate is obtained at pH 4 while for 4-methoxybenzyl alcohol the rate at pH 8 is slightly higher than that at pH 4.

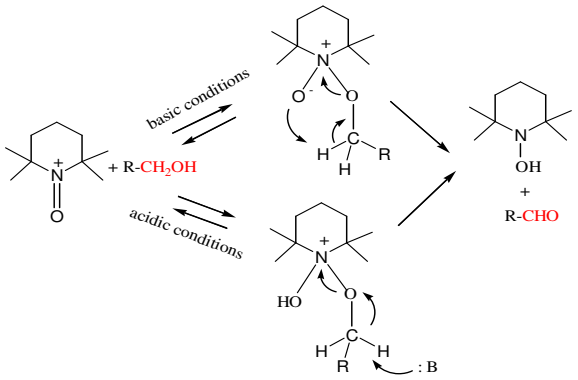


Figure 6 Oxidation mechanisms under basic and acidic conditions [15]

In comparison with literature (Table 2) the rate of benzyl alcohol oxidation is quite low compared to that of methanol at pH 4. Under these conditions, the benzyl alcohol oxidation rate is approximately 10-fold higher than that of methanol. This could be due to steric hindrance. A comparison with other data for other alcohols measured in buffered solutions is not readily available from literature, but some scatter seems justified.

Table 2 Rate constants for benzyl alcohol oxidation by oxoammonium

Literature source	**Alcohol + Oxoammonium k ($M^{-1}s^{-1}$)**
This study, pH 4 *	1.73×10^{-2} (benzyl alcohol)
Goldstein *et al*. [16] pH 6.9 *	0.48 (CH_3OH)
Chmielewska *et al*. [17] pH 4 **	3.13×10^{-4} (CH_3OH)
Golubev *et al*. [18] pH 4.6 * and **	0.27×10^{-2} (CH_3OH)

*phosphate buffer ** acetate buffer

Steady state TEMPO concentration

The scheme in Figure 1 predicts that the steady state conversion of alcohol to aldehyde could be reached by starting with oxoammonium or hydroxylamine instead of TEMPO. When initiated by oxoammonium, the reaction with the alcohol would produce the corresponding aldehyde and hydroxylamine, the latter being oxidized in two steps by laccase with TEMPO as the intermediate. Likewise, when starting with the hydroxylamine, TEMPO and oxoammonium would be formed rapidly by laccase and the oxoammonium would subsequently oxidize the alcohol. The laccase/TEMPO cycle of Figure 1 predicts (see Figure 10 below) that the same steady-state concentrations of TEMPO, oxoammonium and hydroxylamine would be obtained as well as similar rates for the alcohol conversion irrespective of the initial redox state of the mediator.

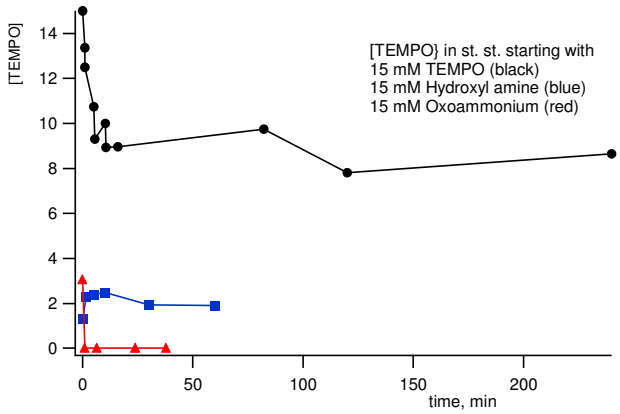


Figure 7 Steady state TEMPO concentrations in the presence of laccase for benzyl alcohol conversion initiated by TEMPO (black), hydroxylamine (blue) and oxoammonium (red). *NB: the oxoammonium contained ~ 3 mM TEMPO at t=0, which was rapidly converted yielding a steady state TEMPO concentration < 20 µM.*

To test this, reactions were initiated with 15 mM TEMPO, oxoammonium or hydroxylamine; the steady state TEMPO concentration was determined by EPR.

Figure 7 indicates that the initial TEMPO concentration of 15 mM drops rapidly (< 6 min) to a value of ~ 9 mM, as expected on basis of the high laccase activity. Surprisingly, starting the reaction with hydroxylamine yielded a different (~ 2-2.5 mM) steady state TEMPO concentration, while no TEMPO intermediate whatsoever (<20 µM) was formed when the reaction was started with the oxoammonium. Table 3 indicates that the conversion in this latter case was marginally above the background without laccase (7% versus 9%). When starting with hydroxylamine approximately 72% of the conversion rate obtained with TEMPO was achieved. These results are difficult to interpret within the scheme of Figure 1 in particular with respect to the lack of reactivity when starting with oxoammonium. Concerning the hydroxylamine, though the initial concentration might have been lower than 15 mM, it seems unlikely that its concentration was as low as ~ 4 mM as suggested by the TEMPO concentration (2 - 2.5 mM) during the steady state.

Table 3 Benzyl alcohol conversion in 60 minutes varying the starting form of TEMPO

"TEMPO form"	**Conversion %**	**Benzaldehyde, mM**
15 mM TEMPO	25	40
15 mM TEMPO-H_2^+	18	29
15 mM Oxoammonium	9	14
15 mM Oxoammonium without laccase	7	11

Conditions: 160 mM benzyl alcohol, 100 U laccase, 15 mM each of the TEMPO form, room temperature, aeration. TEMPO-H_2^+ - protonated form of hydroxylamine.

Kinetic modelling of the laccase/TEMPO system

The reaction cycle shown in Figure 1 was simplified to that of Figure 8. Step 2 (Figure 1), oxidation by molecular oxygen of reduced laccase, was omitted because it so fast that the laccase turnover rate is determined by the reduction rates for TEMPO (k_1) and TEMPO-H (k_4) oxidation. Furthermore, alcohol oxidation by oxoammonium is described by one reaction (k_3) omitting the oxoammonium-alkoxy intermediate. The disproportionation reaction is given by k_5.

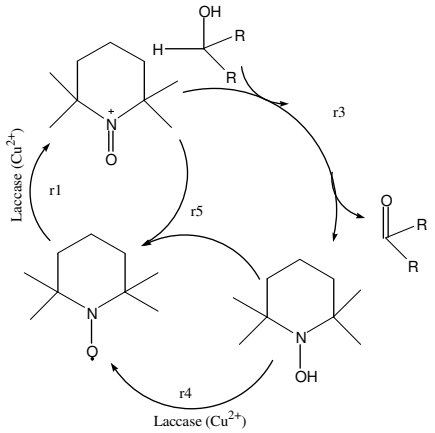


Figure 8 Catalytic cycle of the laccase/TEMPO system used in kinetic modelling

The equations used for the kinetic model are listed in the Appendix to this chapter. Simulations were performed with the rate constants shown in Table 4 further including the laccase concentration (3.6 µM), corresponding to 10 U/ml. The kinetics of benzaldehyde formation obtained from five different initial benzyl alcohol concentrations (10 – 160 mM) over a two-hour conversion period was simulated to test the model. In addition, the steady state TEMPO formation at 160 mM initial

benzyl alcohol was monitored by EPR in time (see section "Steady state TEMPO concentration").

Table 4 Reaction rate constants for laccase/TEMPO benzyl alcohol oxidation system

Parameter		
Benzyl alcohol:	$V_{overall}$ 0.4 – 0.65 mM /min or ~ 1.0 - 1.6 O_2/laccase.s	**RCH_2OH + TEMPO + Lacc +O_2**
Step 1	K_{M1} 1.8 mM (TEMPO) k_{cat} 28 s^{-1} (7 O_2 s^{-1}) k_1 1.99 x 10^4 M^{-1} s^{-1} k_{-1} 5 s^{-1}	**TEMPO + Laccase**
Step 2	k_2 > 5 x 10^7 M^{-1} s^{-1} or > 10.000 s^{-1} (0.2 mM O_2)	**Laccase + O_2**
Step 3	k_3 0.45 -1.73 x 10^{-2} M^{-1} s^{-1}	**Oxoammonium + Alcohol**
Step 4 by laccase by oxygen	K_{M4} 11 mM (TEMPO-H) k_{cat} 46 s^{-1} (11.5 O_2 s^{-1}) k_4 1.77 x 10^3 $M^{-1}s^{-1}$ k_{-4} 4.1 s^{-1} k_4* 2.7 x 10^{-3} $M^{-1}s^{-1}$	**TEMPO-H + Laccase** **TEMPO-H + O_2**
Step 5	k_5 0.06 - 0.5 M^{-1} s^{-1}	**Oxoammonium + TEMPO-H**

Conditions: the parameters were determined at 20 °C

The experimental results and the best simulations combining both the data for benzyl aldehyde formation and the steady state TEMPO concentration are shown in Figures 9 and 10. The simulations consistently underestimated either the rate for the lowest benzyl alcohol

concentration, which is mainly non-enzymatic, or when this was fitted correctly (with the high value k_3= 1.17 $M^{-1}s^{-1}$), neither the TEMPO steady state concentration nor the benzyl alcohol conversion rate were predicted correctly.

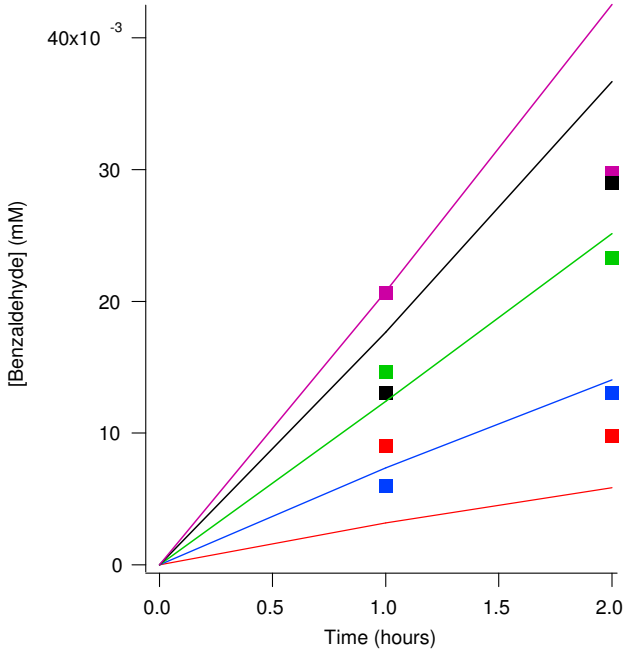


Figure 9 Simulation of benzyl aldehyde formation by the laccase/TEMPO system, actual pH= 4.7. *Simulation parameters: [active laccase] = 0.12*3.6= 0.43 µM; k_3= 9.4x10^{-3} $M^{-1}s^{-1}$; k_5= 0.5 $M^{-1}s^{-1}$ (see Appendix for the other parameters). Dots – experimental data, lines – data obtained from the model; [benzyl alcohol] – 160 mM (purple), 100 mM (black), 50 mM (green), 25 mM (blue), 10 mM (red);*

While the enzymatic parameters shown in Table 4 contain an error of ± 15%, the values for k_3 and k_5 show a larger variation, most likely due to uncertainties of the pH. With respect to k_5, the value of 0.5 $M^{-1}s^{-1}$ corresponding to a pH of 4.7 was found to be optimum for the simulations. In spite of these uncertainties and the considerable scatter

in the benzyl aldehyde concentrations the simulations show unequivocally that the laccase activity is only 10 % ± 3% (1 ± 0.3 U/ml) of that added (10 U/ml) consistent with an overall activity of the laccase/TEMPO system of ~1.0 - 1.6 O_2/laccase/s (Table 4). While this might suggest that laccase is a main contributor to the overall conversion rate, addition of more laccase hardly increased the conversion rate (Chapter 2). We established that the laccase is not irreversibly inhibited since samples taken from the reactor displayed close to full activity when measured by metol or TEMPO oxidation assays (see section "*pH dependence of the laccase from T.versicolor*"). The low laccase activity during conversion remains to be explained. The 10% activity of the laccase obtained by simulation appears also to consistent with the relatively slow approach to the steady state of the TEMPO concentration (Figure 10), after 6 min ~ 6 mM TEMPO is converted, indeed to approximately 10 % of the maximal rate (*i.e.* ignoring TEMPO formation via steps 4 and 5).

The simulation of the TEMPO steady state concentration (Figure 10) is satisfactory only when the reaction is started with TEMPO. The model predicts that the TEMPO steady state concentration is the same whether the reaction is started with 15 mM TEMPO, oxoammonium or TEMPO-H, in contrast to the experimental findings.

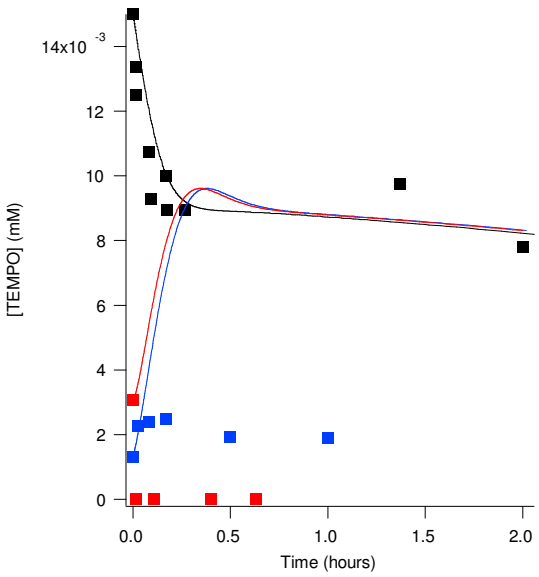


Figure 10 Simulated steady state TEMPO concentrations in the presence of laccase for benzyl alcohol (160 mM) conversion initiated by 15 mM TEMPO (black), 15 mM hydroxylamine (blue) and 12 mM oxoammonium + 3 mM TEMPO (red). *Simulation parameters as in Figure 9*.

However, the model correctly predicts the overshoot in the TEMPO formation in case the reaction is started with hydroxylamine. Other simulations (not shown) indicate that during the steady state similar amounts of oxoammonium and hydroxylamine are formed (each ~ 3 mM), which yields the maximum rate for the disproportionation reaction.

Conclusions

The optimum pH of 4.5 - 4.8 of the laccase/TEMPO system can be understood in terms of the laccase pH optimum and a sufficient rate of the disproportionation reaction (0.5 $M^{-1}s^{-1}$) both vital reactions for the

TEMPO-H regeneration. The model for the laccase/TEMPO conversion system presented in Figure 8 is widely quoted in the literature [3, 4, 8, 19]. The scheme is certainly plausible and amenable to quantitative simulation of the experiments but has several shortcomings. The model does not explain the TEMPO data in Figure 10 and suggests that one of the steps in the system is quasi-irreversible; in particular the finding that the catalytic conversion cycle cannot be initiated by oxoammonium needs further examination. The model does not provide insight as to why the laccase activity is so low, because it lacks parameters directly affecting the laccase activity. Several of the experiments described above were intended to detect possible adverse interactions between the various reaction constituents and laccase, but failed to find a clear culprit. The 'ABTS' binding site of laccase, which is close to the Type 1 Cu, might form a tight complex with the hydroxylamine together with another component (i.e. oxoammonium, TEMPO), preventing a high catalytic activity. Such a hypothetical complex would likely fall apart in the standard enzymatic assays yielding an apparent uninhibited rate.

In the future, catalytic conversions might be performed under tight pH control to determine the optimum pH and to obtain more reliable conversion rates enabling better simulations. A series of steady state and pre-steady state reactions should be carried out with laccase in which the effect of combinations of oxoammonium, hydroxylamine and TEMPO on the laccase activity is systematically determined.

Materials and methods

All reagents were of analytical grade. Laccase [E.C. 1.10.3.2] from *T. versicolor* was obtained from Fluka. The enzyme obtained after the last purification step was used for the indicated experiments without further changes of buffer (the enzyme purification is described in Chapter 4). 2,2,6,6-Tetramethylpiperidinyloxy (TEMPO) was purchased from ACROS and was applied as received. Kinetic measurements were performed on a SX.18MV Stopped-flow spectrofluorimeter or Shimadzu UV-Vis spectrophotometer. EPR spectra were obtained as reported in [17] using a Bruker 200D EPR spectrophotometer.

1. Enzyme activity test

Laccase activity was measured spectroscopically using metol as a substrate. The reaction mixture contained 1 ml 0.05 M metol solution in water. The reaction was initiated by addition of 0.5 ml enzyme solution in 0.1 M acetate buffer pH 4.5 and the increase in absorbance was monitored at 540 nm. Oxygen reduction activities were also determined in the oxygraph.

2. Stopped-flow experiments:

Laccase reduction by hydroxylamine. The laccase concentration was 45 µM (in 50 mM MES buffer, pH 6). Hydroxylamine solutions were prepared at 43 mM as a stock solution in 0.1 M phosphate buffer pH 4. Dilutions were made in the range of 0.67-21.5 mM. The experiments were performed at 20 ± 0.2˚C. Anaerobic samples of laccase and

hydroxylamine solutions were deoxygenated by five cycles of purging with a stream of argon and evacuation.

Disproportionation reaction. Oxoammonium bromide was synthesized analogously to the procedure in the article by Miyazawa *et al*. [20]. Further, the bromide anion was exchanged to the phosphate anion with ion-exchange chromatography over DOWEX 1x8, saturated with 1 M NaH_2PO_4-Na_2HPO_4 solution (pH 4.5). 2 g of oxoammonium bromide was dissolved in 1 L water, and passed through the column. All fractions were collected and the oxoammonium phosphate was segregated from the water layer by evaporation. The oxoammonium yield was 94 %. The product was pure, as analysed by phosphorus NMR. Hydroxylamine was synthesized according to Paleos *et al*. [21] in which TEMPO is reduced with ascorbic acid. The yield of hydroxylamine was 94 %. The NMR analysis showed no traces of ascorbic acid in the final sample. Solutions of hydroxylamine and oxoammonium were prepared in 0.1 M phosphate buffer pH 4. The formation of TEMPO was monitored over time after mixing anaerobic solutions of oxoammonium and hydroxylamine at 20 °C.

3. Alcohol oxidation by means of UV-Vis spectroscopy:

Alcohol oxidation by the oxoammonium salt. Synthetically obtained oxoammonium phosphate salt solutions were prepared in the range 3.3 - 43.5 mM in 0.1 M phosphate buffer pH 4. 160 mM concentration of the alcohols was used for the various measurements. The reduction of oxoammonium to the hydroxylamine was monitored at 465 nm by UV-Vis spectroscopy at 20° C, while stirring.

4. Catalytic oxidation reactions:

In the standard oxidation reaction protocol, with a reaction volume of 10 ml –0.1 M phosphate buffer pH 4 - the following amounts were used: [alcohol] 0.1-1.6 mmol, [TEMPO] 0.15 mmol, internal standard (anisole) 0.25 mmol, laccase, 100 U. The vials with the complete reaction mixture were aerated with steel filter stones during reaction. The reactions were carried out by the Omni Reaction Station at various temperatures for various times. After the reaction, the reaction products and unreacted substrates were extracted with ethyl acetate (3 x 10 ml), dried with anhydrous magnesium sulfate and analysed with GC. The blank reactions were performed by omitting one of the components mentioned above. Alcohol conversions were analyzed by GC with column WAX 52 CB (50m x 0.53 mm). The GC was programmed as follows: 80 °C for 5 min, then at the rate of 7 °C per min to 235 °C.

Appendix

The following component balances for the scheme in Figure 8 were used:

$$\frac{dC_{\text{alcohol}}}{dt} = -r_3$$

$$\frac{dC_{\text{oxoammonium}}}{dt} = -r_3 - r_5 + r_1$$

$$\frac{dC_{\text{aldehyde}}}{dt} = r_3$$

$$\frac{dC_{\text{hydroxylamine}}}{dt} = r_3 - r_4 - r_5$$

$$\frac{dC_{\text{tempo}}}{dt} = r_4 + 2 \cdot r_5 - r_1$$

Further, the reaction equations were expressed as:

$$r_1 = k_1 \cdot C_{\text{laccase\_ox}} \cdot \frac{C_{\text{tempo}}}{K_{m1} + C_{\text{tempo}}}$$

$$r_3 = k_3 \cdot C_{\text{oxoammonium}} \cdot C_{\text{alcohol}}$$

$$r_4 = k_4 \cdot C_{\text{laccase\_ox}} \cdot \frac{C_{\text{hydroxylamine}}}{K_{m4} + C_{\text{hydroxylamine}}}$$

$$r_5 = k_5 \cdot C_{\text{oxoammonium}} \cdot C_{\text{hydroxylamine}}$$

K_{M1} and K_{M4} are the Michaelis-Menten constants for TEMPO and hydroxylamine, respectively. For kinetic modelling the reaction rate constants shown in Table 4 were used. Note that k_3 has been varied between 0.45-1.73 x 10^{-2} M^{-1} s^{-1}. Depending on the experimental results fitted, initial benzyl alcohol concentrations between 0.01 and 0.16 M and initial TEMPO, hydroxylamine and oxoammonium concentrations

between 0 and 0.015 M were used in the simulations. Initial benzaldehyde concentrations were zero in all simulations and initial laccase concentrations were all 3.6 µM. The use of 100 % O_2 did not change the TEMPO concentration in air and did not effect the overall conversion (Chapter 2).
Simulations were performed with the Matlab software package using the "*lsqcurvefit and ode15 s routines*" to optimize the fit parameters and calculate differential equations, respectively.

Fitting only the given reaction constants For these simulations, no fitting routine was applied. The previously determined reaction rate constants were used and the effects of the benzyl alcohol oxidation rate constant k_3, having an uncertainty between $4x10^{-3}$ and $1.7x10^{-2}$ $M^{-1}s^{-1}$, was investigated. It was found that for a reaction rate of $4x10^{-3}$ $M^{-1}s^{-1}$ (step 3) the calculated TEMPO concentration is nearly zero, but the calculated aldehyde concentrations are in the right order of magnitude. For the reaction rate of $1.7{\cdot}10^{-2}$ $M^{-1}s^{-1}$ (step 3) the calculated TEMPO concentration is still too low, but some TEMPO is present. However, especially for the higher initial alcohol concentrations, the alcohol oxidation is expected to proceed much faster than experimentally found.

Fitting the enzyme activity
Possibly, the enzyme is not as active as expected. Therefore, the enzymatic activity (or initial laccase concentration) and k_3 were set as free variables, within the given boundaries. The TEMPO concentrations found by EPR (see Figure 7) were fitted for the initial conditions of 15

mM TEMPO and 160 mM benzyl alcohol. From these simulations, an optimal value for k_3 of 9.4×10^{-3} $M^{-1}s^{-1}$ was found with a corresponding enzymatic activity of only 12 % of the expected value. Without any further fitting, the benzaldehyde and TEMPO concentrations shown in Figures 9 and 10 were obtained.

Parallel reaction rates and units

The disproportionation reaction and hydroxylamine oxidation by laccase are parallel reactions. Starting with 160 mM benzyl alcohol, the enzymatic hydroxylamine oxidation was found through modelling to be roughly 2 times faster than the comproportionation at pseudo steady state, meaning that TEMPO is produced nearly equally fast through both routes. With a relatively low initial benzyl alcohol concentration of 10 mM, enzymatic oxidation of hydroxylamine dominates the TEMPO formation, which is in turn quickly oxidized to oxoammonium.

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6

Preparation and use of cross-linked aggregates of laccases

Abstract

Three sources of laccase, Trametes versicolor, Trametes villosa and Agaricus bisporus, were applied for CLEA preparation. The optimum conditions for precipitation (best precipitant, pH, temperature) and cross-linking (concentration of cross-linker glutaraldehyde, cross-linking time) steps were investigated. The laccase CLEA stability in comparison with free enzyme and the reusability of CLEAs were investigated. Furthermore, the laccase CLEAs were tested for oxidation of aliphatic alcohols in biphasic and monophasic systems.

Introduction

The application of biocatalysts, as whole cells or isolated enzymes, has increased greatly over the years not only on laboratory but also on industrial scale (Straathof, Panke et al. 2002). Enzymes catalyze chemical conversions with high chemo-, regio- and stereoselectivities under mild conditions (ambient temperature, aqueous media). However, the commercialization of enzymatic processes is often hampered by a lack of availability, high price on a commercial scale or limited stability under operational conditions These drawbacks can be overcome by immobilization of the enzyme thereby rendering it more stable and easy to recover and recycle (Sheldon, Schoevaart et al. 2005).

Conceptually, there are three types of methodology for immobilization: binding to a carrier (Figure 1, A), inclusion/entrapment (B) or encapsulation (C) in an organic or inorganic polymer, and cross-linking of protein molecules (D).

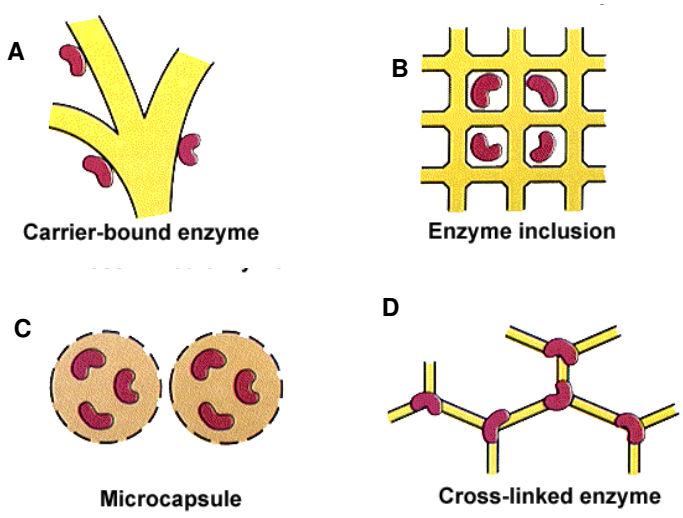


Figure 1 Methodology of immobilization [3]

There is no universal method of enzyme immobilization. A lot of factors effect successful immobilization: a suitable carrier, immobilization conditions (pH, temperature, nature of medium) and the enzyme itself (source, purity, nature of protein). All the methodologies mentioned above have advantages and disadvantages. A major disadvantage of many carrier-bound or encapsulated enzymes is lack of stability towards leaching in aqueous media. Moreover, a high enzyme loading is needed for industrial viability and a compromise has to be made between the pore size and surface area, to reach high enzyme loading and activity retention (Cao 2005).

Biocatalysts with high volumetric activities and catalyst productivities can be obtained by carrier-free immobilization methods, thereby avoiding the need for extra inactive mass as carrier. Alternative methods for making carrier-free immobilized enzymes are shown in Figure 2. The technique of protein cross-linking via the reaction of glutaraldehyde with reactive NH_2 groups of the protein surface was initially developed by Doscher in the 1960s. However, this method of producing cross-linked enzymes (CLEs) has several drawbacks, such as low activity retention, low mechanical stability and difficulties in handling the gelatinous CLEs. Cross-Linked Enzyme Crystals CLECs® are prepared by cross-linking the crystals of enzymes which have been initially purified and crystallized (route A). Cross-Linked Enzyme Aggregates CLEAs® are formed when the soluble enzyme is precipitated from aqueous buffer and the resulting physical aggregates are cross-linked by reaction with a bifunctional reagent, such as glutaraldehyde (route B).

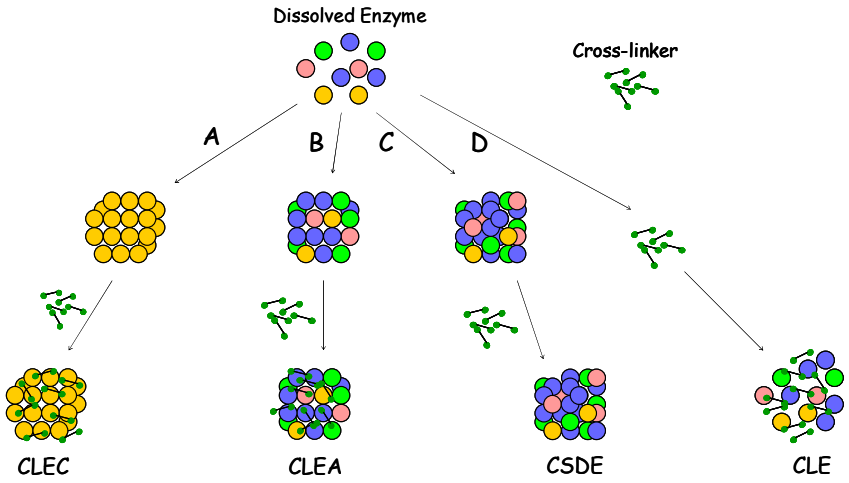


Figure 2 Four different methodological approaches for the carrier-free immobilization

Since precipitation is often used to purify enzymes, the preparation of CLEAs essentially combines purification and immobilization into a single process. Spray-dried enzyme powders can also be used to prepare cross-linked enzymes – CSDEs (route C). Although reasonable activity was obtained, this approach has not been widely exploited due to the fact that the spray–drying process reversibly deactivates the enzymes and CSDEs have relatively low activity. CLEs preparation is based on cross-linking of dissolved enzyme without physical aggregation (route D). All these methodologies of carrier-free immobilization have disadvantages and advantages (see Table 1).

The main drawback of CLECs is the high purity needed to crystallize the enzyme, which translates to high prices. CLEAs, on the other hand, combine the excellent operational performance of the CLECs with ease of preparation and relatively low price.

Table 1 Comparison of methods for obtaining carrier-free immobilized enzymes

Immobilized form	Advantages	Disadvantages
CLECs	Highly enhanced thermostability Mechanically very stable Stability at broad pH and in organic solvents Controllable size	Minimization of crystal size is crucial for high activity retention Very high price
CLEAs	Stability over broad pH range , Enhanced thermal stability Mechanical stability, Widely applicable Highly pure enzymes not needed (combines purification and immobilization) Controllable particle size Low or no allergenicity	Catalytic behaviour differs depending on the properties of the precipitant
CSDEs	Convenient handling	Low activity retention Process deactivates the enzyme reversibly
CLEs	Enhanced thermostability compared with soluble enzyme	Low activity retention Poor reproducibility Low mechanical stability Difficult to handle the gelatinous CLEs and controlling parameters of random cross-linking

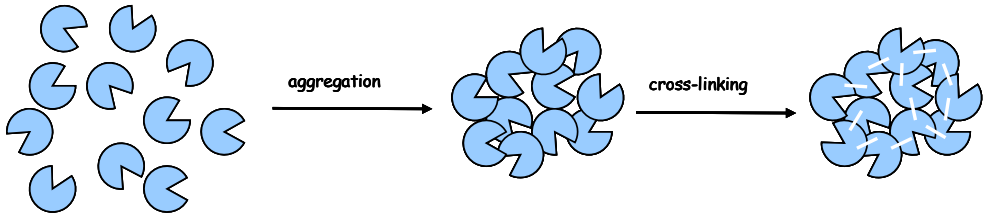
The CLEA methodology is applicable to a wide variety of enzymes: hydrolases (lipases, esterases, amidases, proteases, nitrilases and glycosidases), lyases (nitrile hydratases, oxynitrilases and aldolases) and oxidoreductases (galactose oxidase, glucose oxidase and laccase)

(Lopez-Serrano, Cao et al. 2002; Sheldon, Sorgedrager et al. 2007). An extremely successful example is the preparation of hyperactive lipase CLEAs, with activities higher than the corresponding free enzyme that they were prepared from (Lopez-Serrano, Cao et al. 2002).

The preparation of a CLEA from laccase which is stable in organic solvents was envisaged as a method for improving the stability of laccase under operating conditions, e.g. in combination with TEMPO as a catalyst for the aerobic oxidation of alcohols. Moreover, a recyclable CLEA would decrease the enzyme costs in such oxidation reactions. These possibilities stimulated us to prepare Laccase - CLEAs and investigate their activity in alcohol oxidations. This forms the subject of this chapter.

Results and Discussion

As mentioned above, CLEA preparation consists of two steps: aggregation by precipitation and cross-linking:



Aggregation and precipitation involving the addition of salts, organic solvents and non-ionic polymers to aqueous solutions of proteins is a commonly used method for protein purification (Schoevaart, Wolbers et al. 2004). The resulting physical aggregates of enzyme molecules are

supramolecular structures that are held together by non-covalent binding and can be easily redissolved in water. Cross-linking produces insoluble CLEAs in which the structural properties and catalytic activities of the protein are maintained. Due to the different biochemical and structural properties of proteins the best precipitant and cross-linker can vary from one enzyme to another. Laccase is a highly glycosylated enzyme, which has 4 copper atoms in the active site. We chose laccases from three different sources for our initial studies of CLEA preparation. Laccase from *Trametes versicolor* is commercially available. It is a monomeric enzyme and has 5 lysine and 15 arginine residues which are potential residues for cross-linking. Laccase from *Trametes villosa (Polyporus pinsitus*) was a gift from Givaudan. It is a dimeric protein consisting of two subunits each of which is approximately 61 kDa in size and it has 5 lysine and 12 arginine residues (Yaver, Xu et al. 1996). Laccase from *Agaricus bisporus* also is a dimeric enzyme, but has almost twice the number of lysine (11) and arginine (27) residues (Wood 1980). The selection of the best precipitant for these free laccases was based on precipitation at two different temperatures such as 20 °C and 4 °C. In the case of laccase from *T.versicolor* 8 °C instead of 4° was applied (Table 2). Organic solvents such as ethanol, isopropanol, acetone, acetonitrile, dioxane, and dimethylformamide, a salt such as ammonium sulfate and water miscible polymers with two different molecular weights were chosen as potential precipitants. A ratio of 1:2.5 of enzyme solution to precipitant was used for protein precipitation. The specific activity of the redissolved enzyme was a measure of how effective the precipitation was and is expressed in units

of active laccase (1 U is 1 μmol of metol converted per 1 min, see Chapter 2) on 1 mg of total protein mass. The purification factor (P.F.) stands for a relative specific activity in comparison with control reference. 2-Propanol, dioxane, dimethoxyethane, ammonium sulfate and PEG 3400 were effective precipitants for laccase from *Trametes versicolor* at 20 ℃.

Table 2 Selection of best precipitant *

Precipitant	***T.versicolor***				***T.villosa***				***A.bisporus***			
	20 ℃		**8 ℃**		**20 ℃**		**4 ℃**		**20 ℃**		**4 ℃**	
	U/mg	**P.F**	**U/mg**	**P.F**	**U/mg**	**P.F**	**U/mg**	**P.F**	**U/mg**	**P.F**	**U/mg**	**P.F**
Control buffer	1.04	1	6.8	1	4.5	1	4.9	1	1.12	1	1.4	1
EtOH	2.06	2	2.3	0.3	5.6	1.2	3.2	0.7	0.47	0.4	1.5	1.
2-Propanol	16.5	17	9.4	1.4	1.8	0.4	6.4	1.3	0.4	0.7	1.2	0.9
Acetone	4.1	4	1.3	0.2	4.2	0.9	6.5	1.3	0.54	0.5	1.5	1.0
Acetonitrile	4.5	4	1.5	0.2	3.7	0.8	0.5	0.1	0.45	0.4	0.5	0.4
THF	7.6	7	1.5	0.2	-	-	0.9	0.2	-	-	0.5	0.4
Dioxane	11.1	11	8.1	1.2	5.2	1.2	2.9	0.6	0.58	0.5	1.9	1.4
DMF	-	-	-	-	0.4	0.1	0.3	0.1	-	-	-	-
PEG 1500	5.0	5	1.9	0.3	4.7	1.0	2.4	0.5	-	-	1.4	0.9
PEG 3400	17.6	17	5.5	0.8	-	-	2.5	0.5	1.2	1.1	2.8	1.9
$(NH_4)_2SO_4$	13.6	13	-	-	-	-	9.6	2.0	0.65	0.6	3.6	2.5
DME	7.4	7.1	2.1	0.3	6.4	1.4	2.7	0.6	1.9	1.7	3.6	2.6

* For conditions and procedure see Materials and methods: *4. Precipitation procedure; P.F. – purification factor*

At 4 °C the observed specific activities were lower. In contrast, the laccase from *Trametes villosa* showed higher activities with these precipitants at 4 °C. With ethanol, acetone and dioxane higher activities were obtained at 20 °C. Similarly, laccase from *Agaricus bisporus* showed higher activities when DME, $(NH_4)_2SO_4$, dioxane and PEG 3400 were used as precipitant. Polyethylene imine (PEI) and polyethylene glycol polyacrylamide (PEGA) were also tested as precipitants but no aggregate formation was observed. Although laccases from *T.villosa* and *A.bisporus* gave better precipitation at lower temperatures we decided to perform further preparations at 20 °C. The choice of best precipitant is different for laccases from different sources. Nevertheless, a compromise between best activities and visual observation and handling of formed laccase aggregates led to a choice of four precipitants for further investigation of, for example, pH range.

The four precipitants were: 2-propanol, acetone, dioxane and dimethoxyethane. Addition of water miscible solvents to a solution causes proteins to precipitate. The solvation layer around the protein decreases when organic solvent gradually displaces water from the protein surface and binds it in hydration layers around the organic solvent molecules. With smaller hydration layers, the proteins can aggregate by attractive electrostatic and dipole forces. When the dielectric constant of water decreases, it is easier for protein molecules to come close together. The isoelectric point of proteins (pI) is the pH of a solution at which the net charge of the protein becomes zero. As a result of changes in pH the enzyme can be subjected to repulsive forces

(both electrostatic and dispersive). When pH ≈ pI the electrostatic forces are reduced and dispersive forces dominate which causes the protein to aggregate and precipitate. Although, the pI values for the used laccases are known and are in the range 4-6, we performed precipitation at pH values in the range 4-8 due to interest of CLEA application in processes where a higher pH is required. The control shows the activity of soluble enzyme, with no addition of precipitant, at pH 4, which corresponds to the conditions usually applied in oxidation reactions. The laccase solution was mixed with organic solvent in the v/v ratio 1: 2.5 and allowed to stir for 1 h leading to the formation of a precipitate in the form of pellets. After centrifugation and several washings with the buffer, the pellets were redissolved and activity was determined using the metol assay. The results are presented in Figure 3.

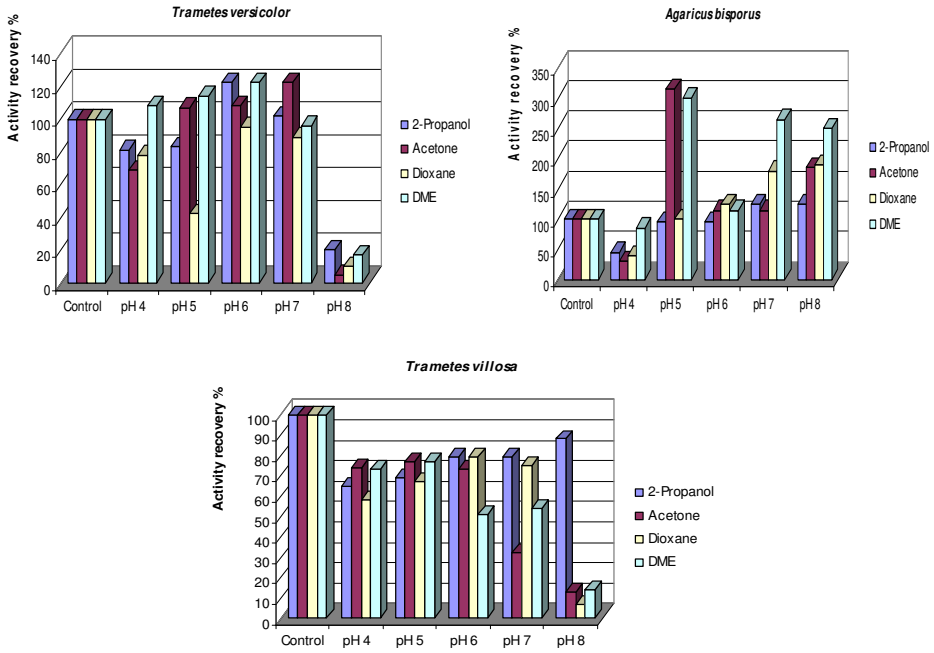


Figure 3 Precipitation of laccases at different pH

Laccase from *T.versicolor* showed better activity with 2-propanol, dioxane and dimethoxyethane at pH 6 and with acetone at pH 7. At pH 8 a drastic decrease in activity was observed. With *A.bisporus* laccase a higher activity recovery was observed with 2-propanol and dioxane at pH 7 and with acetone and DME at pH 6. Similarly, for laccase from *T.villosa*, the activity was better for 2-propanol and dioxane at pH 6 (one pH unit lower), while for acetone and DME at pH 5. The results for laccase from *A.bisporus* were better at higher pH (7 and 8) which could be due to it having more of the strongly basic Arg and Lys residues compared with the other two laccases. In case of laccases *T.villosa* and *T.versicolor* at pH 8 recovery is very low, except for 2-propanol for laccase *T.villosa*. Activity recoveries are higher in comparison with the control for laccase from *T.versicolor* and *A.bisporus*, while laccase from *T.villosa* exhibited lower values. A second set of experiments revealed essentially the same results: the precipitation behaviour of laccases from different sources is different.

In principle the precipitant with the highest activity recovery is the best starting point for cross-linking. However, aggregate activity might differ from the cross-linked aggregates. Therefore, the three best precipitants were included in further experiments. Glutaraldehyde is generally the cross-linking agent of choice as it is cheap and readily available in commercial quantities. It is worth mentioning, however, that not all enzymes give optimum results with glutaraldehyde as cross-linking agent (Cao, Langen et al. 2003; Sheldon 2007; Sheldon, Sorgedrager et al. 2007). Glutaraldehyde is a small, reactive molecule which could

penetrate the internal structure of the protein and react with amino residues that are crucial for enzyme catalytic activity. In such a case other cross-linkers can be used, such as dextran polyaldehyde.

Optimisation of the CLEA procedure involves optimization of the glutaraldehyde/enzyme ratio; too little cross-linker means that the enzyme molecule is still too flexible while too much results in a loss of the flexibility needed for the enzyme's activity. The influence of the amount of glutaraldehyde on the activity recovery is shown in Figure 4 for laccases from three different sources and three precipitants: DME, dioxane and 2-propanol. For laccase from *T.versicolor* pH 6 was used and, for laccase from *T.villosa* pH 6 with the exception of DME at pH 5, and for laccase from *A.bisporus* pH 7 except for DME at pH 5. The activity assay is based on pyrocatechol (1,2-benzenediol) oxidation in which the reaction is stopped by adding 10 % sulfuric acid (see Materials and methods). The choice of activity assay for CLEA is not easy because the formation of aggregates hampers the spectroscopic measurements, especially when the assay is based on kinetic measurements over time.

After one hour, the glutaraldehyde was added in the range of concentrations 2.4 ÷ 40 mM. The mixture was stirred for 24 h at 20 °C to complete the cross-linking process. Subsequently the laccase CLEAs were centrifuged, and washed with buffer. The washing procedure was repeated three times. When no activity was anymore observed in the supernatant, the pellets were suspended in buffer and the recovered activity was measured. The best results for laccase from *T.versicolor*

were obtained with DME using 5 mM glutaraldehyde concentration and with 2-propanol 10 mM (Figure 4). For laccase from *T.villosa* the best activity recovery was found using 20 mM and 5 mM glutaraldehyde concentration with dioxane and DME, respectively.

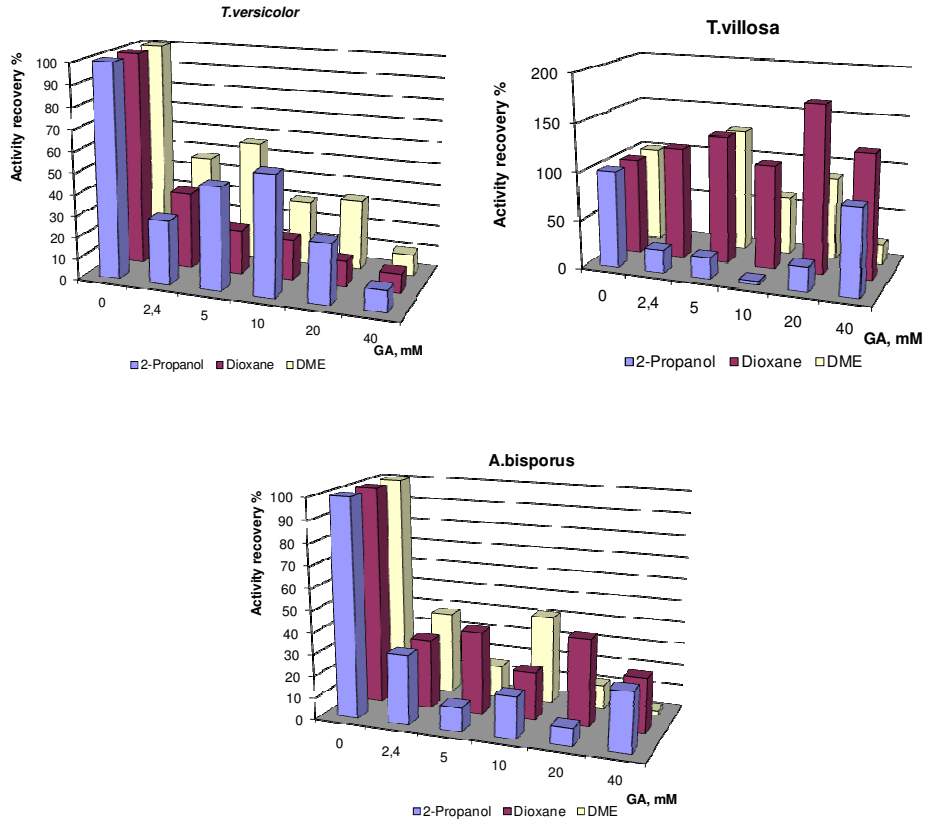


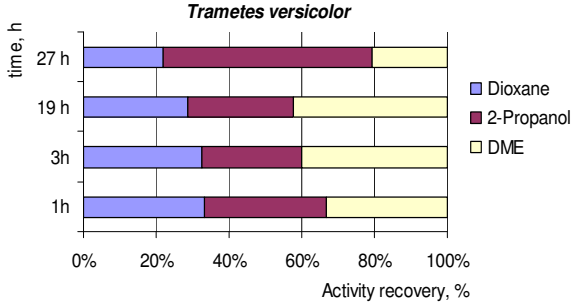
Figure 4 Preparation of CLEAs with different glutaraldehyde (GA) concentration

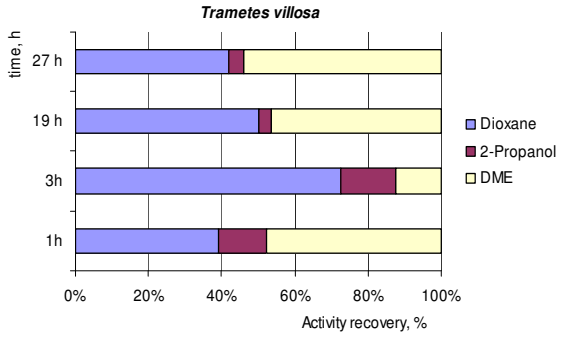
Surprisingly, the activity recovery for the laccase from *A.bisporus*, which has high amounts of the necessary amino acid residues compared with the other two, indicated extremely low values; 40 % activity recovery was reached with 10 mM glutaraldehyde in DME and 30 % with 20 mM

glutaraldehyde in dioxane. It is worth pointing out that that the GA/enzyme molar ratio is varying in these experiments which could influence the cross-linking. If the ratio is too low there is not enough cross-linking and if the ratio is too high there is too much of cross-linking and the enzyme is losing the flexibility which is necessary for its activity. Reviewing the data, we conclude that the concentration of glutaraldehyde is important, but other factors such as the choice of precipitant and enzyme source are important too and can influence the performance of CLEAs.

The initial cross-linking experiments were performed over 24 h. The optimum time for cross-linking can involve a compromise between efficient cross-linking and enzyme stability during the procedure. Therefore, the cross-linking was performed from 1 h till 27 h with the same three precipitants and laccases used in the above experiments. The leaching of enzyme was checked by determining the laccase activity in the supernatant. The used pH and glutaraldehyde concentrations are indicated in *Materials and methods*. The results after cross-linking for 27 h are in good agreement with the ones obtained previously for 24 h of cross-linking. Nevertheless, from enzyme stability and an economic viewpoint it is important to shorten the procedure time. The data indicated that the cross-linking time for laccase from *T.versicolor* should not be shorter than 19 h (Figure 5). When DME was used as precipitant more than two-fold activity loss was observed after 27 h compared to 19 h of cross linking. 3 h was the best cross-linking time for laccase *T.villosa* with dioxane as precipitant and no leaching

was observed after the first washing. No leaching was observed for laccase from *A.bisporus* already after 3 h of cross-linking, in case of precipitation with dioxane. In comparison with other laccases, there is no leaching already after the first washing. We conclude that the higher amount of Lys and Arg residues helps to perform better cross-linking. But the activities measured in pyrocatechol oxidation are relatively low, which could mean that the CLEA is subject to diffusion limitations.





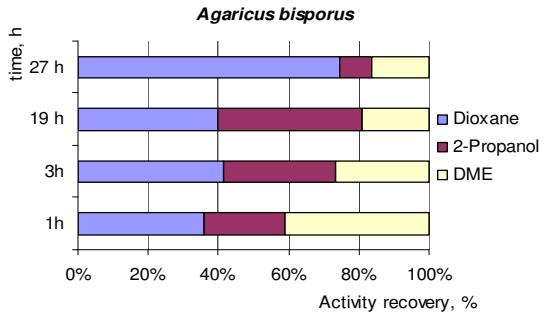


Figure 5 Activity recovery *vs* cross-linking time

From Figure 5 it can be concluded that the best activity recovery from laccase *T.versicolor* could be obtained in 3 h using DME or 27 hours using 2-propanol. The laccase from *T.villosa* gave the best results in dioxane after 3 h while with the laccase from *A.bisporus* the optimum recovery of 70 % was reached after 27 h in dioxane. These results substantiate previous observations that the optimum conditions of precipitation and cross linking (pH, precipitant, concentration of precipitant, amount of cross-linking agent, cross- linking time, and temperature) are different for laccases from different sources.

To test the validity of the parameters found in the small-scale CLEA preparations, we subjected the procedure to a hundred-fold scale up. A compromise was made between the best obtained results and easy handling of the formed aggregates. For the laccase from *T.versicolor* a 5 mM glutaraldehyde concentration was used with DME as precipitant and for laccase from *T.villosa* 20 mM of glutaraldehyde with dioxane. The laccase from *A.bisporus* was no longer commercially available at this point. Additionally, phenyl methyl sulfonyl fluoride (PMSF) was used as a protease inhibitor in the scale-up procedure. 58 % activity recovery was obtained for laccase from *T.versicolor* when 100 µl of 200 mM

PMSF was used and 22 % activity recovery when PMSF was not used. Hence, we conclude that PMSF addition is very important. Surprisingly, for laccase from *T.villosa* the activity recovery was only 16 %, while in a small scale preparation procedure it gave better results in comparison with other laccases. This could be due to the presence of additives in the commercial sample, such as sugar derivatives which stabilize the enzyme. It is also known that the addition of albumins like bovine serum or egg albumins which can form co-aggregates with the enzyme, can form CLEAs with higher residual activity (Cabana, Jones et al. 2007; Cabana, Jones et al. 2007). Therefore, we prepared CLEA samples of laccase with addition of egg albumin (see the preparation procedure in Materials and methods). However, contrary to expectations, the residual activities were even less. In the case of laccase from *T.versicolor* 52 % of residual activity was obtained and even lower for laccase from *T.villosa* - 8 %.

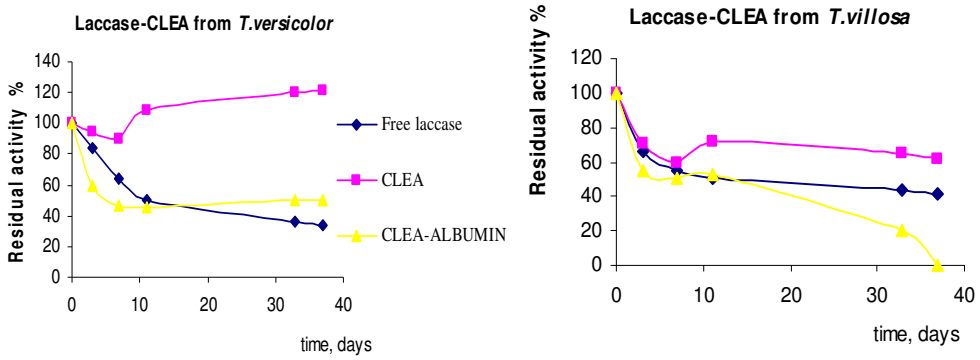


Figure 6 Stability of laccase CLEAs at 5 ℃

The storage stability of these CLEAs suspended in buffer, compared to the free enzyme, was determined at 5 ℃ (Figure 6). The activity of the

CLEA just after preparation was taken as the initial 100 % activity. The samples were suspended in buffer kept in the solution state, not under nitrogen. The results from figure 6 show that the activity of the free enzyme decreases dramatically in the first 10 days of storage. Almost 50 % of initial activity is lost in 10 days with the laccase from *T.villosa*. The CLEAs show the expected increase in stability and the laccase CLEA from *T.versicolor* even showed an increase in activity on storage. The addition of albumin does not improve the stability of the resulting CLEA; the activity decreases in the same range as for free enzyme. The CLEA from laccase from *T.villosa* showed an initial sharp decrease in activity, either with co-aggregation with albumin or without. This could possibly be due to the presence of unknown additives that stabilize the sample.

We subsequently studied the performance and recyclability of CLEA of laccase from *T.versicolor* in alcohol oxidations. 1-Octanol oxidation was performed for 3 h in buffer solution at 30 °C with 50 Units of laccase CLEA. The CLEA was recycled 3 times with a new portion of alcohol and mediator (Figure 7).

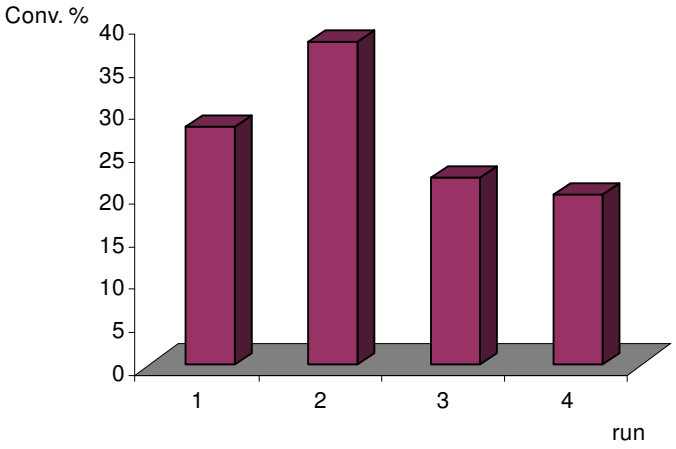


Figure 7 Reusability of CLEA of laccase from *T.versicolor* in 1- octanol oxidation

In the first run 30 % of octanol conversion was obtained. The CLEA was separated from the reaction mixture by centrifugation, washed three times and again applied for the second run of oxidation reaction. Surprisingly, after the second run the obtained conversion was 10 % higher (40%) in comparison with the first run. The conversions in the third and fourth run were in the range of 20-25 %. These results indicate that the CLEA can be reused without appreciable loss of activity. The lower obtained conversions in the run 3 and 4 could be mechanical losses of enzyme aggregates during the washing and centrifugation procedures.

Initial experiments of alcohol oxidation with laccase CLEA from *T.versicolor* showed that a lower amount of enzyme (expressed in units) could achieve the same conversion in comparison with the free enzyme. Usually 100 U of free enzyme was used for oxidation reactions (Chapter 2 and 3). Therefore, the oxidation of octanol was performed using 1 U/ml of free laccase and CLEA. The units for free enzyme and CLEA were determined by pyrocatechol assay. The conversion in 8 hours was 5% for the free enzyme and 45 % for the CLEA (Figure 8).

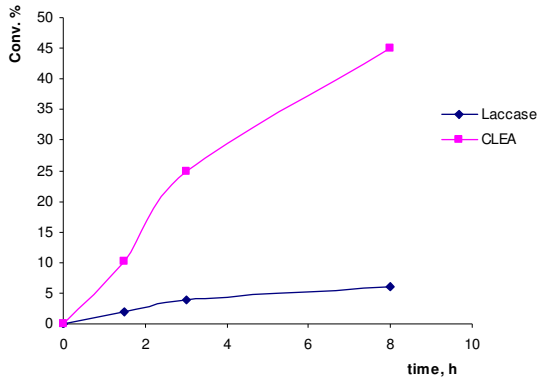


Figure 8 Oxidation of 1-octanol by free enzyme versus CLEA of laccase from *T.versicolor*

These results are quite unexpected. We reasoned that this could possibly be explained by instability of the free enzyme in the reaction mixture, especially when 10 fold less enzyme is used for the reactions. Hence, we checked the activity of the free enzyme and the CLEA after the reaction. Indeed, a drastic activity decrease (almost 80 % in first 2 h) was observed for the free enzyme, while for the CLEA only 20 % of activity was lost (Figure 9).

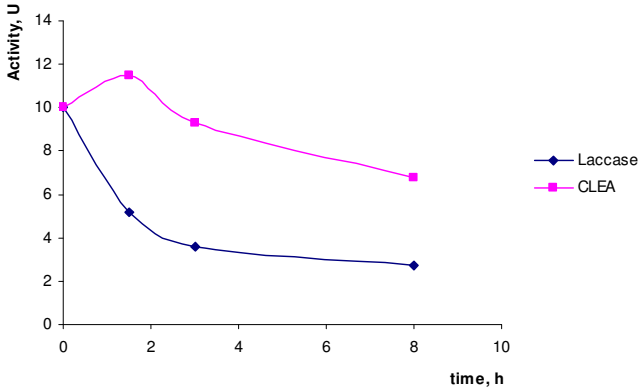


Figure 9 Activity of free enzyme and CLEA during 1-octanol oxidation reaction

In Chapter 3 we showed that the use of a biphasic reaction system leads to lower conversions. Since we expected the laccase CLEAs to be more stable in organic solvents we tried to combine better solubility of aliphatic alcohols and CLEA stability in organic solvents. The results were disappointing: the oxidation of 1-hexanol, 1-heptanol and 1-octanol in a biphasic system with toluene and ethyl acetate did not reach even 6 % of conversion in 4 h. However, these experiments supported the data obtained before (Chapter 3) that the oxidation efficiency in biphasic systems is not depending on enzyme or CLEA stability. Consequently, further experiments were carried out using water miscible organic co-solvents like dioxane and acetonitrile. Conversions of around 15% were obtained for 1-hexanol and 1-octanol using CLEA, compared to 30 % for 1-octanol and 35 % for 1-hexanol in buffer medium in 4 h. Considering these results, further reactions were done only in buffer media. The oxidation of a range of aliphatic alcohols was performed with 10 U of CLEA, 0.15 mmoles of TEMPO and 1.6 mmoles of alcohol substrate in a total reaction volume of 10 ml. For benzyl alcohol and shorter chain alcohols like 1-pentanol and 1-hexanol the reaction time was limited to 5 h in order to avoid aldehyde oxidation to acid when full conversion is reached. For longer chain alcohols like 1-heptanol, 1-octanol, 1-nonanol and 1-decanol the reaction time was 20 h (Table 3).

Table 3 Oxidation of aliphatic alcohols by CLEA of laccase from *T.versicolor*

Alcohol	Reaction time	Conversion %
Benzyl alcohol	5 hours	81
1-Pentanol		7
1-Hexanol		15
1-Heptanol	20 hours	55
1-Octanol		34
1-Nonanol		13
1-Decanol		21

As expected the oxidation of a benzylic alcohol was faster (80 % conversion in 5 h). For heptanol 55 % conversion was obtained in 20 h demonstrating that high conversions of aliphatic alcohols are possible using only 1 U/ml of laccase CLEA. The results can probably be explained by considering that two parameters influence the rate: size and hydrophobicity of the aliphatic alcohols. Moreover, the observed good storage stability and recyclability give promising expectations for further applications.

Conclusions

CLEAs were prepared from three sources of laccase, *Trametes versicolor*, *Trametes villosa* and *Agaricus bisporus*. The effects of various parameters- the nature of the precipitant, pH, temperature, glutaraldehyde concentration, and cross-linking time - on the activity and the storage and operational stability of the resulting CLEAs were investigated. The stability test for the CLEAs showed the expected increase in stability compared to the free enzyme and the laccase CLEA from *T.versicolor* even showed an increase in activity on storage. The

addition of albumin does not improve the stability of the resulting CLEA; the activity decreases in the same range as for free enzyme. The CLEA from laccase from *T.villosa* showed an initial sharp decrease in activity, either with co-aggregation with albumin or without. This could possibly be due to the presence of unknown additives and stabilizers in the sample.

The results of recycling experiments demonstrated that the CLEA can be reused without appreciable loss of activity.

The use of laccase CLEAs for the oxidation of aliphatic alcohols in a biphasic system did not show any improvement because the reaction is not really enzyme dependent (Chapter 3 and 5). Therefore, further performed reactions in buffer produced high conversions of aliphatic alcohols using only 1 U/ml of laccase CLEA, while 10 U/ml soluble enzyme was applied for reactions.

A general conclusion can be drawn on the basis of the observed results: the optimum conditions of precipitation and cross linking (pH, precipitant, and concentration of precipitant, amount of cross-linking agent, a cross-linking time, and temperature) are, as might be expected, different for laccases from different sources. From the results of these small scale experiments it is difficult to draw any definite conclusions regarding the influence of the numbers of surface lysine and arginine residues on the activity recovery in CLEA formation and the storage and operational stability of the resulting CLEA.

Materials and methods

The substrates and reagents were of analytical grade and obtained from Fluka and Sigma Aldrich. Laccase [E.C. 1.10.3.2] from *Trametes versicolor* and *Agaricus bisporus* were purchased from Fluka. Laccase from *Trametes villosa* was kindly donated by Givaudan. 2,2,6,6-tetramethylpiperidinyl-1-oxy (TEMPO) was received from Acros. The reactions were carried out in a stationary Omni Reaction Station with comprehensive heating/cooling/stirring/aeration control.

1. Metol activity assay for laccase

Laccase activity was measured spectroscopically using metol as a substrate. The reaction mixture contained 1 ml 0.05 M metol solution in water. The reaction was initiated by addition of 0.5 ml enzyme solution in 0.1 M acetate buffer pH 4.5 and the increase in absorbance was monitored at 540 nm (ε = 2000 $M^{-1}cm^{-1}$). One unit (U) of the laccase was defined as 1 µmol metol oxidised per minute under the stated assay conditions [14].

2. Pyrocatechol activity assay for laccase

5 mM Pyrocatechol solution (ε = 1260 $M^{-1}cm^{-1}$ (Tyagi, Batra et al. 1999)) was prepared in 0.05 M citrate/phosphate buffer pH 4.5. For the activity assay 1 ml of pyrocatechol solution and 1 ml of total enzyme solution (100µl of enzyme and 900 µl buffer) were incubated at 30 ℃ temperature in a water bath for 90 s. The reaction was stopped by adding 10 % H_2SO_4 solution. The absorbance was measured by a UV-Vis spectrophotometer at 400 nm wavelength.

3. Protein concentration assay

The protein concentration was determined measuring the absorbance of the BCA reagent (commercial source Uptima) after 30 min incubation with enzyme. Bovine serum albumin was used as a standard for the calibration curve.

4. Precipitation procedure

1 ml of enzyme solution was added drop-wise to the chilled 2.5 ml precipitant. Precipitation was processed for 1 h by shaking the solutions at 200 rpm at the given temperature. The mixture was centrifuged and the pellets were washed with the 0.1 M phosphate buffer (pH 4). The washing procedure of the pellets was repeated 3 times, the pellets were redissolved in buffer and activity was measured using the metol assay (method 1) and protein concentration by the BCA method. For pH screening experiments the initial enzyme solution was adjusted to the given pH.

5. Cross-linking procedure

After 1 h of precipitation a particular amount of glutaraldehyde was added drop-wise to the mixture to have the concentration in the range of 2.4 mM-40 mM and shaking at 200 rpm was continued (1-27 h) at 20 °C. The pellets were decanted from the supernatant after centrifugation, washed with 0.1 M phosphate buffer (pH 4) and centrifuged again. The washing procedure was repeated until no traces of activity were determined in the supernatant. The activity of formed CLEAs was measured by the pyrocatechol assay.

6. Scale-up procedure for CLEA preparation

1g of laccase was dissolved in 50 ml 0.1 M phosphate buffer pH 4. The pH was adjusted to pH 6 and 100 µl of 200 mM PMSF solution was added. This solution was added drop-wise to the chilled precipitant (DME or dioxane) and incubated for 1 h by shaking at 200 rpm speed at room temperature. After incubation glutaraldehyde solution (200 mM) was added until the concentration was 5 mM-20 mM and the mixture left to incubate for 19 h at the same shaking speed and temperature. The solutions were centrifuged at 3000 rpm for 20 min at 4 °C. The pellets were washed with 0.1 M phosphate buffer (pH 4) and centrifuged again. The procedure was repeated 3 times. The activity was measured by the pyrocatechol assay.

7. Scaled up procedure for CLEA preparation by co-aggregation with albumin

1g of laccase was dissolved in 50 ml 0.1 M phosphate buffer pH 4(the corresponding initial activity was measured by the pyrocatechol assay). The pH was adjusted to pH 6 and 100 µl of 200 mM PMSF solution was added. 0.66 g of egg albumin was added, corresponding to a 1:1 molar ratio to laccase, and incubated by shaking for 15 min (200 rpm, room temperature). Subsequently, 5 ml of 100 mM PEHA (pentaethylene-hexamine) was added and the mixture was incubated under the same conditions for 30 min. Than 100 ml of chilled DME was added drop-wise and incubated for 1 h by shaking at 200 rpm at room temperature. After 1 h a certain amount of glutaraldehyde solution was added until the concentration was 5 mM-20 mM and left to incubate for 19 h at the same

shaking speed and temperature. The mixtures were centrifuged at 3000 rpm for 20 min at 4 °C. The pellets were washed with 0.1 M phosphate buffer pH 4 and centrifuged again. The procedure was repeated 3 times. The activity was measured by the pyrocatechol assay.

8. Oxidation of alcohol

For the standard oxidation reaction procedure the following amounts were used: 1.6 mmol alcohol, 0.15 mmol TEMPO, 0.25 mmol anisole (internal standard) and 10 U of free laccase or CLEA in 10 ml of 0.1 M phosphate buffer pH 4 . The vials with reaction mixture were aerated with a steel filter stone during the reaction. The reactions were carried out in an Omni Reaction Station at 30 °C for the given time. After reaction, the reaction product and unreacted substrate were extracted with ethyl acetate (3 x 10 ml), dried over anhydrous magnesium sulfate and analysed with GC. The blank reactions were performed omitting one of the components mentioned above.

9. Analysis methods

Alcohol conversions were analyzed by GC with column WAX 52 CB (50m x 0.53 mm). The method for GC was programmed as follows: 80°C for 5 min, then at the rate of 7°C per minute to 235°C. Anisole was used as internal standard

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List of abbreviations:

ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid
Arg	arginine
BCA	bicinchoninic acid
CLEAs	cross-linked enzyme aggregates
CLE	cross-linked enzymes
CLEC	cross-linked enzyme crystals
CSDE	cross-linked spray-dried enzymes
Cys	cysteine
DME	dimethylether
DMF	dimethylformamide
E_m	midpoint potential
ET	electron transfer
EtOAc	ethyl acetate
GC	gas chromatography
HAT	hydrogen abstraction
HBT	hydroxybenzotriazole
HPI	N-hydroxyphthalimide
k_{cat}	a first order rate constant (the turnover number), s^{-1}
k_{on}	an association rate constant
$K_{m(app)}$	an apparent dissociation constant, M
Lacc	laccase
Leu	leucine
Lys	lysine
MHQ	microsecond freeze-hyperquench technique
Met	methionine
MES	4-morpholinoethaneesulfonic acid
NAD^+	nicotinamide adenine dinucleotide
NMR	nuclear magnetic resonance

PEHA	pentaethylenehexamine
PEG	polyethylene glycol
PEGA	polyethylene glycol polyacrylamide
PEI	polyethylene imine
Phe	phenylalanine
PMSF	phenyl methyl sulfonyl fluoride
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel
T	type
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl
TEMPO-H	N-hydroxylamine of TEMPO
Trp	tryptophan
TvL	*Trametes versicolor* laccase
Tyr	tyrosine
VLA	violuric acid