# KINETICS AND PHYSICO-CHEMICAL PROPERTIES OF WHITE-ROT FUNGAL LACCASES

By

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# List of Abbreviations

| | |
|---|---|
| ε | extinction coefficient |
| $A_{280}$ | absorbance at 280 nm |
| ABTS | 2,2'azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) |
| BCA | Bicinchoninic acid |
| BSA | Bovine Serum Albumin |
| Cu | copper |
| DEAE | diethylaminoethyl |
| DMP | 2.6-dimethoxyphenol |
| EDTA | Ethylenediaminotetraacetic acid |
| g | centrifugal force |
| HBT | 1-Hydroxybenzotriazole |
| IEF | Iso-electric focussing |
| kDa | kilodalton |
| MEA | malt extract agar |
| Mr | Molecular mass |
| PAGE | polyacrylamide gel electrophoresis |
| PHB | p-hydroxybenzohydrazine |
| pI | iso-electric point |
| SDS | sodium dodecyl sulphate |
| Syr | syringaldazine |
| T1 | Type 1 (copper site) |
| T2/T3 | Type 2/ Type 3 (copper site) |
| XL | 6-Cross linked |

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# CHAPTER 1

# A LITERATURE REVIEW - FUNGAL LACCASES

The japanese lacquer tree (*Rhus vernicifera*), from which

laccase was first isolated in 1883.

(www.jgc.co.jp/waza/b5_lacquer/ lacquer01.htm)

## 1.1. Introduction

Laccase (EC 1.10.3.2, p-diphenol oxidase) is one of a few enzymes that have been studied since the nineteenth century. Yoshida first described laccase in 1883 when he extracted it from the exudates of the Japanese lacquer tree, *Rhus vernicifera*. (Thurston, 1994; Levine, 1965). In 1896 laccase was demonstrated to be a fungal enzyme for the first time by both Bertrand and Laborde (Thurston, 1994; Levine, 1965). Laccase is a member of the large blue copper proteins or blue copper oxidases, which comprise a small group of enzymes. Other enzymes in this group are the plant ascorbate oxidases and the mammalian plasma protein ceruloplasmin (Thurston, 1994; Xu, 1996; Ducros *et al.*, 1998).

Laccases are either mono or multimeric copper-containing oxidases that catalyse the one-electron oxidation of a vast amount of phenolic substrates. Molecular oxygen serves as the terminal electron acceptor and is thus reduced to two molecules of water (Ducros *et al.*, 1998). The ability of laccases to oxidise phenolic compounds as well as their ability to reduce molecular oxygen to water has lead to intensive studies of these enzymes (Jolivalt *et al.*, 1999; Xu, 1996; Thurston, 1994). The biotechnological importance of these enzymes can also be attributed to their substantial retention of activity in organic solvents with applications in organic synthesis.

Laccases have widespread applications, ranging from effluent decolouration and detoxification to pulp bleaching, removal of phenolics from wines and dye transfer blocking functions in detergents and washing powders, many of which have been patented (Yaver *et al.*, 2001). The biotechnological application of laccase has been expanded by the introduction of laccase-mediator systems, which are able to oxidise non-phenolic compounds that are otherwise not attacked and are thus able to degrade lignin in kraft pulps (Bourbonnais and Paice, 1990).

## 1.2.    Occurrence and location

Laccase is the most widely distributed of all the large blue copper-containing proteins, as it is found in a wide range of higher plants and fungi (Leontievsky *et al.*, 1997) as well as in bacteria (Diamantidis *et al.*, 2000).  Laccases in plants have been identified in trees, cabbages, turnips, beets, apples, asparagus, potatoes, pears, and various other vegetables (Levine, 1965).   Laccases have been isolated from Ascomyceteous, Deuteromyceteous and Basidiomyceteous fungi (Assavanig *et al.*, 1992).  In the fungi, Ascomycetes and Deuteromycetes have not been a focus for lignin degradation studies as much as the white-rot Basidiomycetes.  Laccase from *Monocillium indicum* was the first laccase to be characterised from an ascomycete showing peroxidative activity (Thakker *et al.*, 1992).  In this review I will focus on laccases isolated from Basidiomycetes with emphasis on the white-rot fungi.

The white-rot basidiomycetes are the most efficient degraders of lignin and also the most widely studied.   The enzymes implicated in lignin degradation are: lignin peroxidase, which catalyses the oxidation of both phenolic and non-phenolic units, manganese-dependant peroxidase and laccase, which oxidises phenolic compounds to give phenoxy radicals and quinines; glucose oxidase and glyoxal oxidase for $H_2O_2$ production and cellobiose-quinone oxidoreductase for quinone reduction (Kirk and Farrell, 1987; Thakker *et al.*, 1992).  The different degrees of degradation of lignin with respect to other wood components depend on the environmental conditions and the fungal species involved.   It has been demonstrated that there is no unique mechanism to achieve the process of lignin degradation and that the enzymatic machinery of the various microorganisms differ.  *Pleurotus ostreatus,* for instance, belongs to a subclass of lignin–degrading microorganisms that produce laccase, manganese peroxidase and veratryl alcohol oxidase but no lignin peroxidase (Palmieri *et al.*, 1997).  *Pycnoporus cinnabarinus* has been shown to produce laccase as the only ligninolytic enzyme (Eggert *et al.*, 1996) and *P. sanguineus* produces laccase as the sole phenol oxidase (Pointing and Vrijmoed, 2000).

In plants, laccase plays a role in lignification and in fungi, laccases have been implicated to be involved in many cellular processes, including, delignification, sporulation, pigment production, fruiting body formation and plant pathogenesis

(Thurston, 1994; Yaver *et al*., 2001). Only a few of these functions have been experimentally demonstrated (Eggert *et al*., 1998).

Ligninolytic enzymes have mostly been reported to be extracellular but there is evidence in literature of the occurrence of intracellular laccases in white–rot fungi (Schlosser *et al.*, 1997). Intracellular as well as extracellular laccases were identified for *Neurospora crassa* (Froehner and Eriksson, 1974). Froehner and Eriksson suggested that the intracellular laccase functioned as a precursor for extracellular laccase as there were no differences between the two laccases other than their occurrence.

## 1.3. Laccase-catalysed reactions

Substrate oxidation by laccase is a one-electron reaction generating a free radical. As one electron oxidation of a substrate is coupled to a four-electron reduction of oxygen the reaction mechanism cannot be straightforward (Thurston, 1994). The initial product is typically unstable and may undergo a second enzyme-catalysed oxidation or otherwise a non-enzymatic reaction such as hydration, disproportionation or polymerisation. The bonds of the natural substrate, lignin, that are cleaved by laccase include, $C_{\alpha}$ - oxidation, $C_{\alpha}$-$C_{\beta}$ cleavage and aryl-alkyl cleavage (figure 1.1).
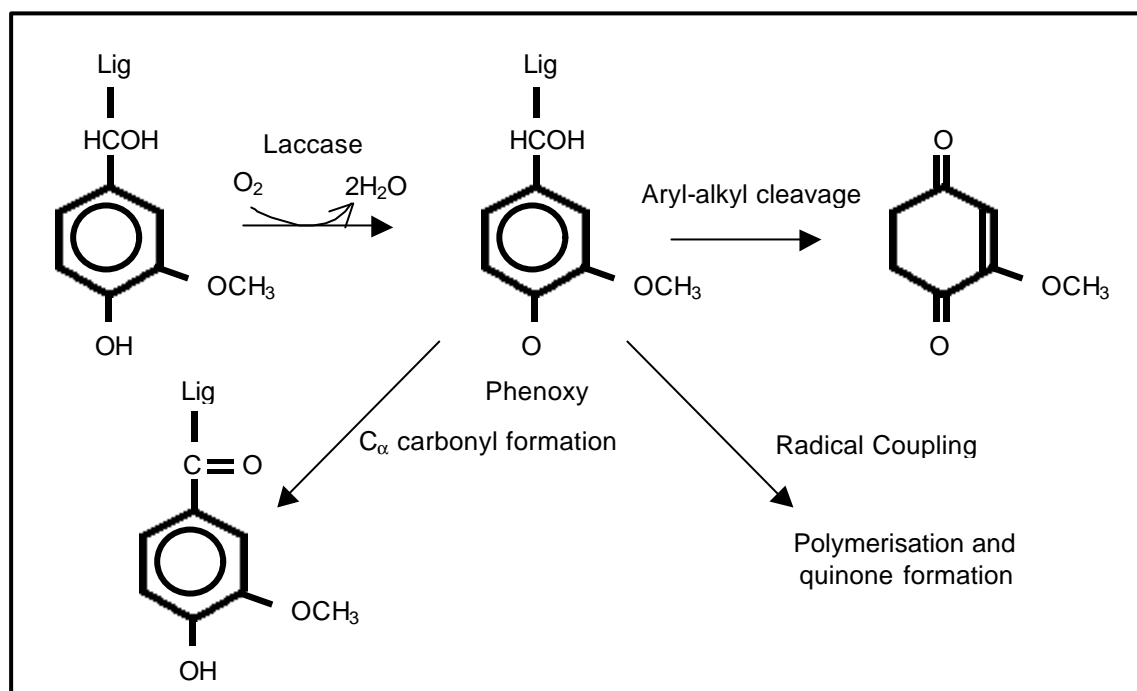
**Figure 1.1.** Laccase-catalysed oxidation of phenolic groups of lignin (taken from Archibald *et al*., 1997).

## 1.4. Classification according to substrate specificity

Laccase (EC 1.10.3.2) as mentioned in the introduction, is a blue copper protein, but also falls within the broader description of polyphenol oxidases. Polyphenol oxidases are copper proteins with the common feature that they are able to oxidise aromatic compounds with molecular oxygen as the terminal electron acceptor (Mayer, 1987). Polyphenol oxidases are associated with three types of activities:

Catechol oxidase or *o*-dipenol: oxygen oxidoreductase (EC 1.10.3.1)

Laccase or *p*-diphenol: oxygen oxidoreductase (EC 1.10.3.2)

Cresolase or monophenol monooxygenase (EC 1.18.14.1)

(Mayer, 1987)

These different enzymes can therefore be differentiated on the basis of substrate specificity (Walker and McCallion, 1980). There is, however, difficulty in defining laccase according to its substrate specificity, because laccase has an overlapping range of substrates with tyrosinase. Catechol oxidases or tyrosinases have *o*-diphenol as well as cresolase activity (oxidation of L-tyrosine). Laccases have ortho and para-diphenol activity, usually with more affinity towards the second group. Only

tyrosinases possess cresolase activity and only laccases have the ability to oxidise syringaldazine (Thurston, 1994; Eggert *et al.*, 1996). There has only been one report of an enzyme exhibiting both tyrosinase and laccase activity (Sanchez-Amat and Solano, 1997).

The second difficulty in defining laccase according to substrate specificity is that laccases are not specific for their substrate range, as it varies from one organism to another. Thurston (1994) stated in a review that hydroquinone and catechol are good laccase substrates, but that guaiacol and 2,6-dimethoxyphenol (DMP) are often better, but not always. Para-phenylenediamine is a common substrate and syringaldazine is a unique substrate for laccase only. Thus, laccase oxidises polyphenols, methoxy–substituted phenols, diamines and a vast range of other compounds (Thurston, 1994). *Neurospora crassa* laccase (Germann *et al.*, 1988) only effectively oxidises para and ortho-diphenols – with the exception of phloroglucinol. Laccase from *Pyricularia oryzae* preferred phloroglucinol as a substrate above other substituted monophenols (Alsubaey *et al.*, 1996). Laccases from *Cerrena unicolor* and *Trametes versicolor* oxidise meta-substituted phenols but to varying degrees. Laccase from *Cerrena unicolor* oxidises para-substituted phenols to the greatest extent (Filazzola *et al.*, 1999) while *Trametes versicolor* laccase oxidises ortho-substituted phenols to the greatest extent (Jolivalt *et al.*, 1999). An immobilised commercial laccase was shown to be able to degrade meta, ortho and para-substituted methoxyphenols, chlorophenols and cresols, but the substituted phenols from these three types of phenols are oxidised in different orders and to different extents (Lante *et al.*, 2000).

Many different reactions have been reported to be catalysed by laccases from different fungi. A comparative study concerning properties of fungal laccases indicated that all the laccases in the study had the ability to oxidise methoxyphenolic acids but to different degrees. The oxidation efficiencies of the laccases were also dependant on pH (Bollag and Leonowicz, 1984). Laccases were also shown to be able to decarboxylate vanillic acid to methoxyquinone (Ander and Eriksson, 1978). Two lignin derived hydroquinones, namely 2-methoxy-1,4-benzohydroquinone and 2,6-dimethoxy-1,4-benzohydroquinone were oxidised by laccase from *Pleurotus eryngii* (Guillen *et al.*, 2000). The auto oxidation of the semiquinones produced by the laccase-catalysed reaction leads to the activation of oxygen. 2,6-dimethoxy-1,4-

benzohydroquinone was oxidised more efficiently than 2-methoxy-1,4-benzohydroquinone by laccase (Guillen *et al.*, 2000). This correlates to the higher affinity of laccase for DMP than for guaiacol.

Leonowicz *et al.* (1985) used fractionated lignosulphonates (peritan Na) to prove that laccases have the ability to polymerise and depolymerise certain substrates. The products of laccase-catalysed reactions often lead to polymerisation through oxidative coupling. Oxidative coupling reactions of such products result from C-O and C-C coupling of phenolic substrates and from N-N and C-N coupling of aromatic amines (Medvedeva *et al.*, 1995; Hublik and Schinner, 2000). Laccase from *Rhizoctonia practicola* was used to demonstrate the ability of laccase to catalyse the coupling of two differently halogenated phenols, 2,4-dichlorophenol and 4-bromo-2-chlorophenol. The laccase catalysed reaction led to the formation of three dimers with asymmetric formation (Bollag *et al.*, 1979).

Industrial processes such as paper bleaching produce organochlorine compounds. These compounds include chlorinated phenols, catechols and guaiacols. Laccase from *Coriolus versicolor* has been shown to dechlorinate tetrachloroguaiacol and release chloride ions (Iimura *et al.*, 1996).

Laccases from *Trametes villosa* and *Trametes hirsuta* have the ability to modify fatty and resin acids to a certain degree. The amount of linoleic, oleic and pinolenic acids were reduced for fatty acids and the amount of conjugated resin contained in resin acids was decreased (Karlsson *et al.*, 2001). Laccase also has the ability to cleave an etheric bond of the substrate, glycol-$\beta$-guaiacyl ether (a model lignin compound).

Phenolic compounds that are oxidised very slowly by laccase have recently been used to increase the storage stability of laccase activity for *Trametes versicolor* (Mai *et al.*, 2000). The increased stability of laccase could have technological importance, as there are so many potential applications for laccase.

## 1.5. Laccase mediator system

Biobleaching techniques have been intensively investigated as a possible alternative for chlorine bleaching of pulp. The laccase mediator system (LMS) was originally developed to solve problems in bio-bleaching of wood pulps and was first described by Bourbonnais and Paice (1990) with the use of ABTS as the first mediator. Laccases were thought to play a role in the biodegradation of lignin but it was restricted to phenolic compounds because of the low oxidation potentials of these enzymes (Reid and Paice, 1994). Application of these enzymes in the presence of so-called mediator compounds resulted in a high oxidation capability leading to the oxidation of nonphenolic lignin model compounds (figure 1.2). The application of the LMS on hardwood kraft pulp resulted in a reduction of kappa number, demethylation and depolymerisation of kraft lignin (Paice *et al.*, 1995; Archibald *et al.*, 1997; Reid and Paice, 1994).
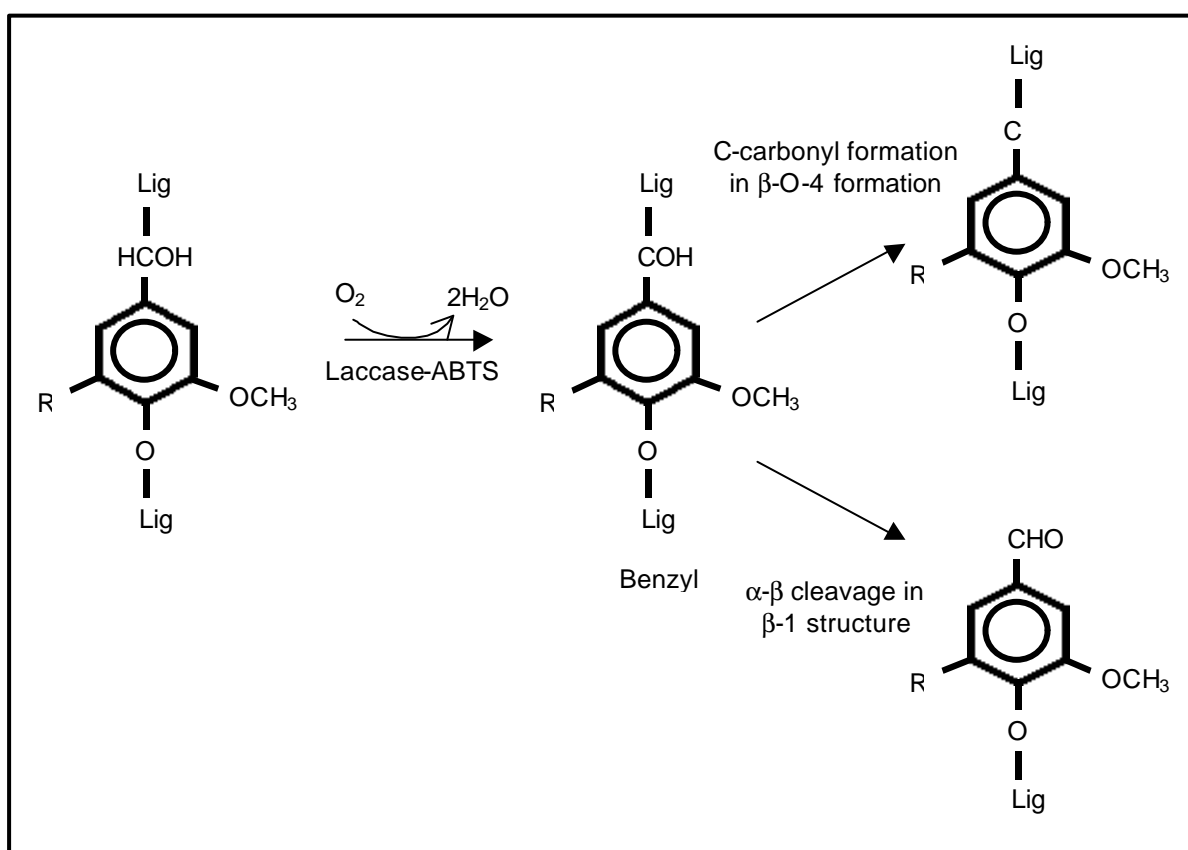


**Figure 1.2**. The oxidation of nonphenolic lignin model compounds by a LMS (taken from Archibald *et al.*, 1997).

The LMS was successfully applied to the oxidation of aromatic methyl groups, benzyl alcohols (Johannes *et al.*, 1998), polycyclic aromatic hydrocarbons (Johannes *et al.*, 1998; Majcherczyk *et al.*, 1998; Johannes and Majcherczyk, 2000) and bleaching of textile dyes (Hardin *et al.*, 2000)

Various polycyclic aromatic hydrocarbons, which closely correlate to the 16 compounds selected by the Environmental Protection Agency (USA) and other national institutions as compounds of toxicological relevance were removed by a LMS. Polycyclic aromatic hydrocarbons that were removed included acenaphtylene, anthracene benzo(a)pyrene acenaphthene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene and perylene (Collins *et al.*, 1996; Majcherczyk *et al.*, 1998; Johannes *et al.*, 1998). Polycyclic aromatic hydrocarbon quinones were formed to differing degrees as oxidation products.

The activity of a LMS towards lignin is dependant on two main factors. Firstly, the redox potential of the enzyme and, secondly, the stability and reactivity of the radical resulting from the oxidation of the mediator. It has been shown that laccases from different organisms react variably with different mediators and different substrates (Bourbonnais *et al.*, 1997a). It is thus imperative that different laccases as well as different mediator compounds be investigated.

Approximately 100 different potential mediator compounds have been described for the LMS, but ABTS and HBT (1-Hydroxybenzotriazole) remain the most commonly used (Bourbonnais *et al.*, 1997a; Johannes and Majcherczyk, 2000). Recently, further studies have revealed the delignifying action of natural mediators in the LMS. Natural mediators include phenol, aniline, 4-hydroxybenzoic acid and 4-hydroxybenzyl alcohol. The use of natural mediators proved to be as efficient as the commonly used ABTS and HBT (Johannes and Majcherczyk, 2000). A method using transition metal complexes in combination with laccase for the delignification of chemical pulp was described by Bourbonnais *et al.* (2000). The metal complex is able to be recycled.

## 1.5.1. ABTS as mediator

Laccase oxidises ABTS to form a stable cation radical (figure 1.3). The role of ABTS in pulp delignification is not yet fully understood but it has been suggested that the ABTS cation radical functions as an electron carrier (figure 1.4) between residual lignin in the Kraft pulp fibre wall and the large laccase molecule that is unable to enter the fibre wall (Paice *et al.*, 1995; Archibald *et al.*, 1997). Much research and comparative studies have been conducted for the laccase/ABTS system.
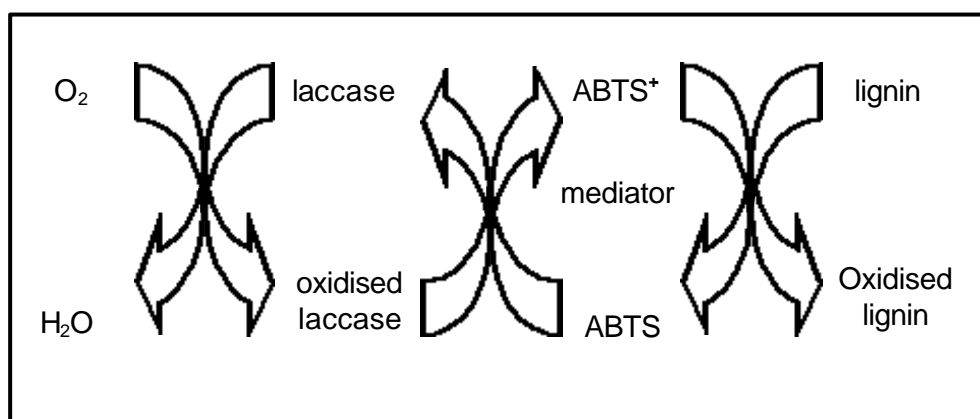


**Figure 1.3.** A suggested pathway for the oxidative catalytic action of laccase on lignin (taken from Paice *et al.*, 1995).
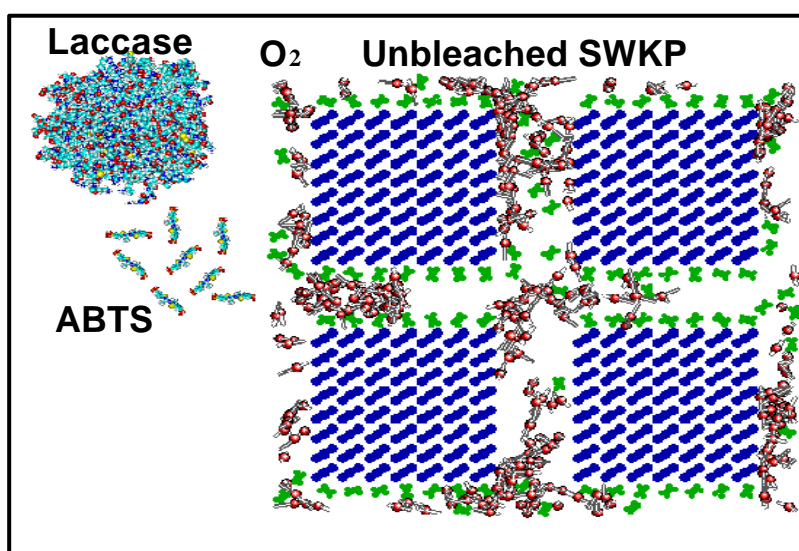


**Figure 1.4.** A computer simulation of a cross-section of the secondary wall of an unbleached kraft pulp fibre. The relative sizes of laccase and manganese peroxidase are shown in comparison to sizes of the cell wall fibres (taken from Paice *et al.*, 1995).

### 1.5.2. HBT as mediator

HBT is oxidised by laccase to form a nitroxide cation radical (Call and Mucke, 1994; Bourbonnais *et al.*, 1997b). The laccase/HBT system has given good results in trials done for the bleaching of pulp and has the ability to oxidise the nonphenolic $\beta$-O-4-linked subunits that are predominant in lignin as well as $\beta$-1 linked dimers (Bourbonnais *et al.*, 1997b, Srebotnik and Hammel, 2000; Xu *et al.*, 1997; Ander and Messner, 1998). This mediator however is unable of acting as a recyclable mediator (Li *et al.*, 1998). Delignification by the laccase/HBT system is not fully understood but as in the case of ABTS, HBT is small enough to access lignin.

### 1.6. Structure of laccase enzymes

### 1.6.1. Studies of purified enzymes

Laccases are monomeric or multimeric copper-containing enzymes. An example of a multimeric enzyme is the laccase produced by *Podospora anserina*, which has a tetrameric structure with identical subunits. Table 1.1 shows a list of purified laccases from basidiomycetes with their individual properties. The typical laccase has a relative molecular mass ($M_r$) of 60 000 to 80 000 and is 15-20 % glycosylated (Thurston, 1994; Luisa *et al.*, 1996), there are many exceptions, however. Exceptions include laccases produced by *Monocillium indicum* ($M_r$ = 100 000), *Agaricus bisporus* ($M_r$ = 100 000), and *Aspergillus nidulans* ($M_r$ = 110 000) (Thakker *et al.*, 1992; Perry *et al.*, 1993a; Thurston, 1994).

There are many reported cases that show that a single fungal species may express more than one laccase enzyme (table 1.1). Different culture conditions may also lead to the production of different isozymes by the same fungus (Bollag and Leonowicz, 1984; Wahleitner *et al.*, 1996; Palmieri *et al.*, 1997; Farnet *et al.*, 2000).

**Table 1.1.** Properties of selected purified laccases

| Organism | Number of isozymes | $M_r$ (Da) | Reference |
|---|---|---|---|
| Podospora anserina | 3 | 70 000 | Thurston, 1994 |
| | | 80 0000 | |
| | | 390 000 | |
| Neurospora crassa | 1 | 65 000 | Germann *et al.*, 1988 |
| Agaricus bisporus | 2 | 100 000 | Perry *et al.*, 1993b |
| | | 65 000 | |
| Botrytis cinerea | 2 | 72 000 | Thurston, 1994 |
| | | 72 000 | |
| Phlebia radiata | 1 | 64 000 | Saloheimo *et al.*, 1991 |
| Armillaria mellea | 1 | 80 000 | Curir *et al.*, 1997 |
| Monocillium indicum | 1 | 72 00 | Thakker *et al.*, 1992 |
| Pleurotus ostreatus | 2 | 54 000 | Palmieri *et al.*, 1997; |
| | | 59 000 | |
| | | 57 000 | |
| Phanerochaete flavido-albans | 1 | 94 000 | Perez *et al.*, 1996 |
| Rhizoctonia solani | 4 | 50 000 to100 000 | Wahleitner *et al.*, 1996 |
| Pleurotus ostreatus RK 36 | 1 | 67 000 | Giardina *et al.*, 1999 |
| Ceriporiopsis subvermispora | 2 | 71 000 | Fukishima and Kirk, 1995 |
| | | 68 000 | |
| Pycnoporus cinnabarinus (a) | 1 | 81 000 | Eggert *et al.*, 1996 |
| Coriolus hirsutus | 1 | 80 000 | Shin and Kim, 1998 |
| Pycnoporus cinnabarinus (b) | 1 | 63 000 | Schliephake *et al.*, 2000 |
| Trametes villosa | 1 | 63 000 | Yaver *et al.*, 1996 |
| Trichoderma | 1 | 71 000 | Assavanig *et al.*, 1992 |
| Marasmius quercophilus | 2 | 61 000 | Farnet *et al.*, 2000 |

## 1.6.2. Active site

Other than laccase that typically contains four copper atoms per monomeric molecule, ceruloplasmin and ascorbate oxidase contain more than four copper atoms per molecule. Therefore, laccase provides the simplest system to study active site structure and reactivity of multicopper oxidases (Germann and Lerch; 1986).

Three types of copper can be distinguished using UV/visible and electroparamagnetic resonance (EPR) spectroscopy. Type 1 copper is responsible for the blue colour of the protein at an absorbance of approximately 600 nm and is EPR detectable, Type 2 copper does not confer colour but is EPR detectable and Type 3 copper consists of a pair of copper atoms in a binuclear conformation that give a weak absorbance in the near UV region but no detectable EPR signal (Thurston, 1994).

The Type 1 Cu site is present as a Cu (II) species for the resting enzyme (Ducros *et al*., 1998). The Type 1 Cu is usually coordinated to two nitrogens from two histidines and sulphur from cysteine (figure 1.5). It is the bond of Type 1 Cu to sulphur that is responsible for the characteristic blue colour of typical laccase enzymes. The geometry is described as a distorted trigonal bipyramidal coordination with a vacant axial position where the substrate docks (Ducros *et al*., 1998). The coordination is unusual as it is intermediate between the preferred coordination states for Cu (I) and Cu (II) species. A leucine residue is present but is too far away to be directly coordinated. The Cu is therefore only coordinated to three atoms (Ducros *et al.*, 1998).
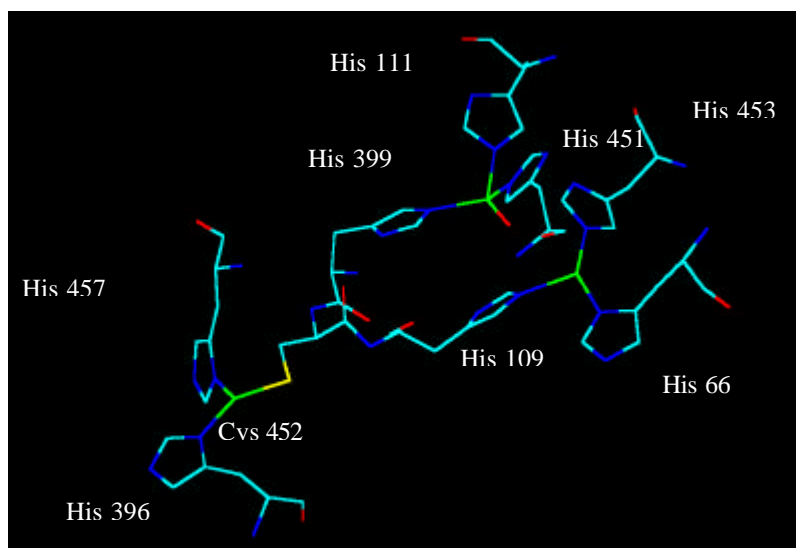
**Figure 1.5.** A ball-and-stick model depicting the coordination of copper atoms and ligands in the active site of Cu-2 depleted *Coprinus cinereus* laccase (taken from Ducros *et al.*, 1998). Copper atoms are in green, sulphur in yellow and oxygen in red.

The sequences of several cloned laccase genes were compared with that of ascorbate oxidase for which a crystallographic structure was already known at the time of the particular study (Leontievsky *et al.*, 1997). The structure showed that type 2 and type 3 coppers are close together in a trinuclear centre (Leontievsky *et al.*, 1997). In 1998 a crystallographic structure of laccase (figure 1.6) was reported for the copper-2 depleted laccase from *Coprinus cinereus* (Ducros *et al.*, 1998). The comparitive studies (Leontievsky *et al.*, 1997) and the findings by Ducros and co-workers coincided. The copper atoms of the T2/T3 sites are coordinated to eight histidines, which are conserved in four His-X-His motifs. The two T3 atoms are coordinated to six of the histidines (figure 1.5) while the T2 atom is coordinated to the remaining two. A hydroxide ligand bridges the pair of T3 atoms (figure 1.5 in red), and because of its strong anti-ferromagnetic coupling it is responsible for the phenomenon of the T3 pair being EPR silent (Ducros *et al.*, 1998).

**Figure 1.6.** Crystallographic structure of the Cu-2 depleted laccase for *Coprinus cinereus* (taken from Ducros *et al*., 1998).

The cloned sequences of various laccases also show that the 10 histidine and 1 cysteine residues that are copper ligands in ascorbate oxidase are conserved in all laccase sequences known to date except one from *Aspergillus nidulans* that has a methionine ligand of type 1 copper (Leontievsky *et al.*, 1997). These conserved cysteine and histidine residues serve as a pathway for the transport of electrons from the T1 Cu site where electrons are extracted from phenolic substrates to the trinuclear site that serves as the binding site of dioxygen where the electrons are required for dioxygen reduction (Ducros *et al*., 1998).

The crystal structure of laccase (Ducros *et al*., 1998) shows that laccase is a monomeric molecule that consists of three cupredoxin-like domains (figure 1.6) that results in a globular structure. The molecular architecture, as for all blue copper oxidases, contains β-barrel domains (figure 1.7a). The third domain has a β-sandwich conformation (figure 1.7b) as well as four short helical regions.

**(a)**                                    **(b)**
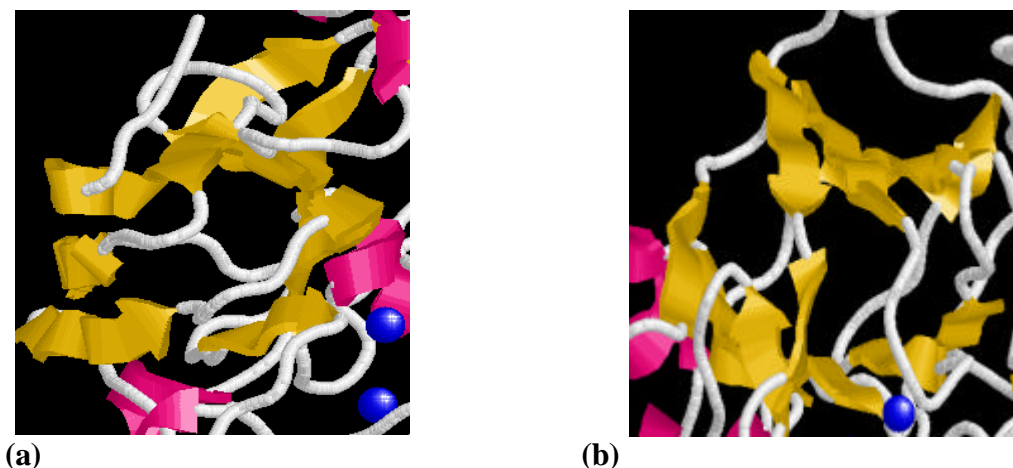
**Figure 1.7.**    β-barrel (a) and β-sandwich (b) conformations from the cupredoxin-like domains described by Ducros and co-workers for *C. cinereus* laccase (Cu-2 depleted). (Taken from Ducros *et al.*, 1998).

The exact nature of the reaction mechanism of laccases that involves the reduction of dioxygen to water and the concomitant four one-electron oxidations of reducing substrates remains controversial. 'Two-site-ping-pong-bi-bi'-kinetics has been proposed. This involves a multi-product, multi-substrate reaction, where the release of initial products is necessary before new substrates are bound by the enzyme (Ducros *et al.*, 1998).

Xu (1996) did a comparative study with different fungal laccases and a range of substrates including, phenols, anilines and benzenethiols. He proved that the first transfer of one electron between substrate and enzyme was governed by the outer-sphere mechanism. The steric effect of small ortho-substituents such as methyl or methoxy groups was found to be of little importance when compared to the electronic effect (Xu, 1996). Xu (1996) estimated the type 1 copper site of laccase to be approximately 10 Å in depth.

## 1.6.3. Active site exceptions

Although most laccases adhere to these phenomena there are certain highly purified laccases that do not show these typical characteristics. Not all laccases are reported to possess four copper atoms (Thurston *et al.*, 1994) per monomeric molecule. One of

the laccases from *Pleurotus ostreatus* is said to confer no blue colour and was described by the author to be a white laccase (Palmieri *et al*., 1997). It was determined by atomic absorption that the laccase consisted of 1 copper atom, 1 zinc atom and 2 iron atoms instead of the typical four coppers.

Certain laccases have been found to be yellow or yellow-brown rather than the blue colour that is expected for laccases (Leontievsky *et al.*, 1997). Yellow laccases and blue laccases from the same organism had similar copper contents. It was proposed that yellow laccases, under normal aerobic conditions, did not maintain their copper centres in the oxidised state of resting enzymes. The binding of low molecular mass phenolic material from lignin degradation could contribute to such a change of enzymatic property. The explanation has not yet been verified (Leontievsky *et al.*, 1997).

## 1.6.4. cDNA and gene sequences

The first gene and/or cDNA sequences were recorded for laccase from the Ascomycete fungus, *Neurospora crassa* (Germann and Lerch, 1986). Further sequences were published from 1990 onwards. These included laccases from *Aspergillus nidulans* (Aramayo and Timberlake, 1990), *Coriolus hirsutus* (Kojima *et al*., 1990), *Phlebia radiata* (Saloheimo *et al*., 1991), *Agaricus bisporus* (Perry *et al*., 1993b), *Pycnoporus cinnabarinus* (Eggert *et al*., 1998), *Coriolus versicolor* (Mikuni and Morohoshi, 1997), *Trametes versicolor* (Jönsson *et al*., 1997), *Podospora anserina* (Fernandez-Larrea and Stahl, 1996), *Coprinus congregatus* (Leem *et al*., 1999), *Ganoderma lucidum, Phlebia brevispora, Lentinula edodes, Lentinus tigrinus* (D'Souza *et al*., 1996).

The sequences mostly encoded polypeptides of approximately 520 to 550 amino acids (including the N-terminal secretion peptide). The one cysteine and ten histidine residues involved in the binding of copper atoms were conserved for laccases and this is also similar to what is found for sequences from ascorbate oxidase. The difference between laccases and ascorbate oxidase in the copper-binding region is that ascorbate oxidase exhibits the presence of a methionine ligand, which is not present in the laccase sequences. The absence and presence of the methionine ligand has led to

interesting studies of mutagenesis conducted by Xu and coworkers (Xu *et al.*, 1998; Xu *et al*., 1999).

## 1.6.5. Mutagenesis of the active site

Various models have been generated to correlate the Cu site structure and the molecular properties of laccase. In particular, it has been postulated that the co-ordination geometry and ligands of the type-I Cu might determine the redox potential of this site. Many laccases were shown to have a leucine or methionine residue at the position corresponding to that of the T1 Cu site (Thurston, 1994; Ducros *et al*., 1998; Aramayo and Timberlake; 1990; Leontievsky *et al.*, 1997). Xu *et al.* (1998) observed that *Trametes versicolor* laccase that has a high redox potential (0.8 V) presented a phenylalanine residue instead of methionine or leucine and predicted that it might be responsible for the high redox potential.

In 1996 Xu and his co-workers showed that three high redox laccases had a leucine-glutamate-alanine tripeptide, rather than the valine-serine-glycine tripeptide found in low redox laccases. The position of the tripeptide corresponds to the T1 pocket and serves as part of the substrate-binding pocket (Xu *et al.*, 1996). The effects of the triple mutation on the redox potential, suggest that the substrate binding pocket and the electron transfer pathway from the substrate to the T1 Cu were affected. They thus proved that it might be possible to regulate laccase catalysis by targeted engineering (Xu *et al.*, 1998).

A pentapeptide was also targeted in the vicinity of the T1 copper site of a low and a high redox laccase. A leucine residue was replaced by a phenylalanine residue at the position corresponding to the T1 Cu axial ligand. No significant effects could be elucidated (Xu *et al.*, 1998).

## 1.7.  Applications of laccase in biotechnology

A vast amount of industrial applications for laccases have been proposed and they include paper processing, prevention of wine decolouration, detoxification of environmental pollutants, oxidation of dye and dye precursors, enzymatic conversion

of chemical intermediates and production of chemicals from lignin. Before laccases can be commercially implemented for potential applications, however, an inexpensive enzyme source needs to be made available (Yaver *et al.*, 2001). Two of the most intensively studied areas in the potential industrial application of laccase are the delignification or biobleaching pulp and the bioremediation of contaminating environmental pollutants (Schlosser *et al.*, 1997).

## 1.7.1. Biobleaching

Environmental considerations have led to a search for alternative methods to chlorine bleaching in kraft pulp mills. Biological bleaching has been investigated with fungal lignin-degrading enzymes including laccase and manganese peroxide. Both enzymes have been shown to increase pulp brightness. The use of the laccase mediator system has been receiving increasing attention since the initial discovery that non-phenolic lignin model compounds can be oxidised by laccase in the presence of a mediator such as ABTS or HBT (Bourbonnais *et al.*, 1997b).

Xylanase prebleaching is now in use in a number of mills around the world but this technology has only led to a maximum saving of 20 % of bleach chemicals. Laccase is produced during bleaching by the fungus *Trametes versicolor*. Laccase can partially delignify kraft pulp and is effective within a short reaction time but slight pressure of oxygen and a low molecular weight electron carrier are required to drive their oxidative cycles (Paice *et al.*, 1995).

## 1.7.2. Detoxification and decolourisation of waste water

Removal of phenolics from industrial water effluents is an important practical problem, since many of these compounds are toxic and their presence in drinking and irrigation water is a health hazard. Phenolic pollutants can originate from agricultural activities such as phenoxy herbicides or wood preservatives, or industrial activities including generation of wastes by pulp and paper or petrochemicals or by dyeing and other organic chemicals and the textile industries (Duran and Esposito, 2000; Hardin *et al.*, 2000).

The main drawback of the use of free enzymes to detoxify wastewater is their susceptibility towards denaturation by pH, temperature and proteolysis (D'Annibale *et al*., 2000; Hardin *et al*., 2000). Laccase has an advantage in the removal of phenolics over other ligninolytic enzymes because its function does not require the presence of hydrogen peroxide as for lignin peroxidase and it has broader substrate specificity than tyrosinase. Free laccase has therefore been immobilised on various materials to potentially improve the stability of the enzyme (Krastanov, 2000). Laccase from *Lentinula edodes* was immobilised on Eupergit C (epoxy-activated polyacrylic matrix) and in another case laccase was immobilised on a spiral-wound asymmetric polyethersulphone (D'Annibale *et al*., 2000).

Azo- and triphenylmethane dyes are widely used in industry but are not easily biodegradable and are therefore commonly found in dye-containing wastewaters. The white–rot fungi are the only group of organisms capable of significantly decolourising dyes. In most cases the decolourisation can be attributed to the activities of lignin peroxidase and manganese peroxidase. *Pycnoporus sanguineus* (Pointing and Vrijmoed, 2000) produces laccase as the sole lignin-degrading enzyme and thus proved that laccase is successful in dye decolorisation. Laccase from a different strain of *Pycnoporus sanguineus* was efficient in the decolourisation of alkaline extract effluent from a Kraft pulp mill (Esposito *et al*., 1993).

Laccase from *P. cinnabarinus* has been shown to be able to degrade a variety of dye compounds including, C.I. Reactive Blue 19 (Schliephake and Lonergon, 1996), Direct Red 16 and Acid Blue 113 (Hardin *et al.*, 2000). The decolourisations of the latter two dyes lead to the formation of a slight orange colour.

## 1.8. Conclusions

Laccases are widespread in nature, being produced by a wide variety of plants, fungi and also bacteria. The functions of the enzyme differ from organism to organism and typify the diversity of laccase in nature. Laccases catalyse the oxidation of phenolic compounds whilst simultaneously reducing molecular oxygen to water. The catalytic ability of laccases has, not surprisingly, led to diverse biotechnological applications of this enzyme.

The introduction of the laccase-mediator system led to the further application of laccase in biobleaching and catalysis of nonphenolic compounds such as polycyclic aromatic hydrocarbons. Potential applications for laccase include the removal of phenolics from wastewater as well as the decolourisation of certain phenol-containing dyes. It is therefore not surprising that this enzyme has been studied intensively since the nineteenth century and yet remains a topic of intense research today.

# CHAPTER 2
# INTRODUCTION TO THE PRESENT STUDY



The white-rot fungus - *Pycnoporus sanguineus*

(picture kindly supplied by Forest Products Biotechnology)



The white-rot fungus – *Coprinus micaceus*

(picture kindly supplied by Forest Products Biotechnology)

Laccase (EC 1.10.3.2; benzenediol:oxygen oxidoreductase) has been the subject of study since the nineteenth century (Thurston, 1994). It is of interest in biotechnology mainly because of its ability to oxidise a wide variety of aromatic compounds and because of the diversity of this enzyme. Current and potential applications of laccase include: textile dye or stain bleaching (Pointing and Vrijmoed, 2000; Kirby *et al.*, 2000; Fu and Viaraghavan, 2001), the detoxification of contaminated soil and water (Filazzola *et al.*, 1999) and even stonewashed denims (Denilite$^®$, Novozyme; 2000). The ability of laccases to oxidise mediator compounds such as ABTS (Bourbonnais and Paice, 1990) and 1-HBT enables laccase to delignify pulp in a process referred to as biobleaching. The use of the laccase mediator system results in larger savings of bleach chemicals than obtained with hemicellulases (Reid & Paice, 1994). Laccases, mediators and pulp interact variably (Bourbonnais *et al.*, 1997a and b) and it is, therefore, important to study different laccases from different sources.

Screening of white-rot fungi from the Sappi Culture Collection maintained at the Department of Microbiology and Biochemistry, University of the Free State, was conducted to identify fungi able to produce laccase with novel properties (van der Merwe *et al.*, 1999). *Pycnoporus sanguineus* (SCC 108) was identified as a fungus with the ability to produce relatively high titres of apparently thermostable laccase and *Coprinus micaceus* (SCC 389) was identified as a fungus with the ability to produce laccase that is able to function at neutral to alkaline pH values.

The aim of this project was to purify and characterise laccases from these fungi. The importance of enzyme purification is described by Dixon and Webb (1979) as follows: "Enzymes are found in nature in complex mixtures, usually in cells which perhaps contain a hundred or more different enzymes, and in order to study a given enzyme properly it must be purified." Laccase enzymes have been purified from a number of different organisms, mainly white-rot fungi (Farnet *et al.*, 2000, Luisa *et al.*, 1996, Palmieri *et al.*, 1997, Thurston, 1994). These enzymes are mainly found extracellularly, excreted into the growth medium from where it can readily be harvested for purification (Levine, 1965).

Properties investigated in this study will include: molecular mass, iso-electric point, the production of laccase isozymes, the effect of pH and temperature on enzyme activity, thermal stability, substrate specificity and effects of various inhibitors. By studying these various properties it will be possible to conclusively identify the enzymes under study to be laccases as well as to identify novel properties after comparison with characterised laccases reported in the literature.

# CHAPTER 3
# MATERIALS AND METHODS

In theory there is no difference between theory and practice,
but in practice there is

(L.A. van de Snepscheut)

## 3.1.  Chemicals and chromatography media

Chemicals used as buffers, substrates and growth media were commercially available products of analytical grade, if not otherwise indicated, and were used without further purification.  Chemicals were obtained from Merck or Sigma unless stated otherwise.  Molasses was obtained from Transvaal Sugar Limited (Malelane, South Africa).  Chromatography media were supplied by Whatman Limited (DE52), Tosohaas (DEAE Toyopearl, Phenyl Toyopearl and HW50F Toyopearl) and Affinity Chromatography Limited (MIMETIC dye adsorbent 6XL resins Piksi kit, Hydrophobic interaction chromatography 4XL and 6XL agarose resins Piksi kit, MIMETIC Red 1 and MIMETIC Yellow II).  SDS-PAGE and IEF calibration proteins were from Pierce (Blueranger; prestained) and Bio-Rad (broad range).

## 3.2.  Micro-organisms

Laccases were isolated from two white-rot fungi.  Strains were obtained from the Sappi Culture Collection (Department of Microbiology and Biochemistry, University of the Free State).  *Pycnoporus sanguineus* (SCC 108) was identified as a fungus that is able to produce thermostable laccase at high titres of activity (van der Merwe et al., 2001).   During a second screening trial for alkaline laccase producers, *Coprinus micaceus* (SCC 389) was identified as having the potential to produce laccase that functions at pH 7 (Coetzee, unpublished results).

## 3.3.  Cultivation conditions for *Pycnoporus sanguineus* (SCC 108)

The *P. sanguineus* master culture was maintained on Malt Extract Agar (MEA) slants at 4 °C.  Pre-inoculum was cultivated for 10 days at 25 °C as stationary cultures in 500-ml conical flasks containing 100 ml diluted molasses (4 %) at pH 5.5.  Molasses is obtained from the sugar industry as a waste product.  It is a complex mixture of sugars and also contains nitrogenous material.  Johnson *et al*. (1995) described it as an ideal economical substrate or medium.  The mycelium was briefly macerated with a Heidolph Diax 600 homogeniser (Germany) that was added at a ratio of 10 % of the

work volume to diluted molasses. The cultures were grown at 25 °C with agitation (Labotec orbital shaker) for 10 days or until laccase activity in the medium had reached a maximum. The mycelium was removed from the supernatant by centrifugation at 22 100 x g for 30 minutes (Beckman J2-MC Centrifuge, UK).

## 3.4. Cultivation conditions for *Coprinus micaceus* (SCC 389)

The cultivation conditions were conducted in a similar manner as described for *P. sanguineus,* except that the laccase activity reached a maximum after 4 days and mycelium was removed from the culture by filtration.

## 3.5. Optimisation of laccase production by *C. micaceus* (SCC 389)

A factorial experiment with a completely randomised design was done to optimise laccase production by *C. micaceus*. Three factors (carbon source, inducer and pH for assay) were replicated three times. Data were subjected to analysis of variance and means tested for significant differences with Tukey's test (Winer, 1971).

Molasses (4 %) and malt extract (20 g/l) (Perry *et al.*, 1993a; Farnet *et al.*, 2000) were evaluated for laccase production. Two inducers were evaluated: 500 mg/l $CuSO_4$ and 5 mg/l p-hydroxybenzohydrazine (Tagger *et al.*, 1998; Farnet *et al.*, 2000). Assays for laccase activity were conducted at three different pH values (pH 4, pH 6, pH 7) with 2,6-dimethoxyphenol as substrate.

## 3.6. Enzyme and protein assays

### 3.6.1. ABTS assay method for laccase

This assay method is commonly used and is described by Bourbonnais and Paice (1990). The nonphenolic dye 2,2'-azinobis-bis-(3-ethylbenzthiazolinesulphonate) (ABTS) is oxidized by laccase to the more stable and preferred state of the cation radical (figure 3.1). The concentration of the cation radical responsible for the intense

blue-green colour can be correlated to enzyme activity (Macherczyk *et al.*, 1995) and is most often read between 415 nm and 420 nm.
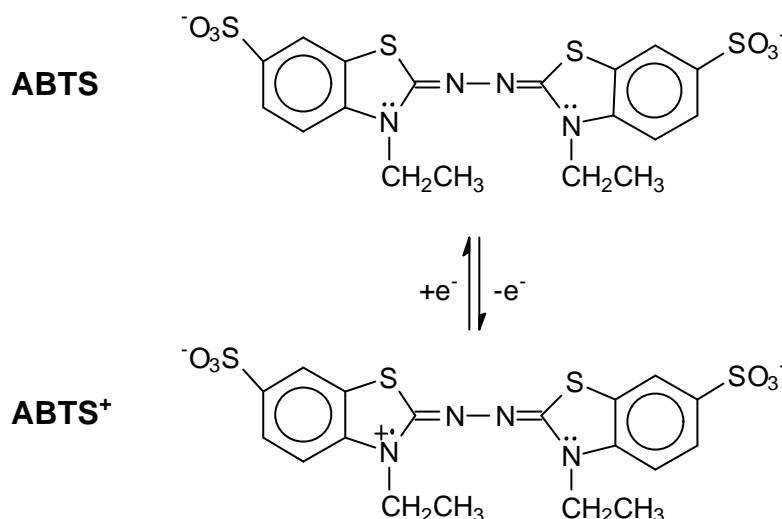


**Figure 3.1.**     The laccase-catalysed oxidation of ABTS to a cation radical (ABTS$^+$) (taken from Macherczyk *et al.*, 1998).

ABTS (0.4 mM) was dissolved in sodium acetate buffer (pH 4.5; 25 °C).  The absorbance of the cation radical was monitored at 420 nm ($\varepsilon_{mM} = 36$ mM$^{-1}$cm$^{-1}$) and 25 °C (Palmieri *et al.*, 1997) using a Beckman DU-650 spectrophotometer fitted with an Auto-6-sampler (water-regulated) connected to a circulating water bath.  Enzyme activity was expressed as international units (IU) where 1 IU is defined as the amount of enzyme forming 1 ìmole of product per minute.  The reaction mixture contained 580 µl of substrate and 20 µl of enzyme or sample.

The laccase activity in U/ml ($\mu$mol cation radical released.min$^{-1}$.ml$^{-1}$ enzyme) was calculated as follows:

$$U / ml = 2\left( \frac{V}{v \times \boldsymbol{e} \times d \times \Delta A.\min^{-1}} \right)$$

$$U / ml = 2\left( \frac{0.6}{0.02 \times 36 \times 1 \times \Delta A.\min^{-1}} \right)$$

$$= 1.667 \times \Delta A.\min^{-1},$$

where

| | | |
|---|---|---|
| $V$ | = | Total reaction volume (ml) |
| $v$ | = | Enzyme volume (ml) |
| $\boldsymbol{e}$ | = | Extinction coefficient of ABTS at 420 nm = 36 mM$^{-1}$cm$^{-1}$ |
| | | (Bourbonnais and Paice, 1990) |
| $d$ | = | Light path of cuvette (cm) |
| $\boldsymbol{D}$A.min$^{-1}$ | = | Absorbance change per minute at 420 nm |

### 3.6.2.  Syringaldazine assay method for laccase

This assay method is adapted from a method described by Harkin and Obst (1973).  It is based on the oxidation of 4,4'-[azinobis(methanylylidene)]bis(2,6-dimethoxyphenol) (syringaldazine) to the corresponding quinone, 4,4'-[azinobis(methanylylidene)]bis(2,6-dimethoxycyclohexa-2,5-diene-1-one)     (figure 3.2).  An increase in absorbance at 530 nm is followed at 25 °C to determine laccase activity in international units (IU) where 1 IU is defined as the amount of enzyme forming 1 ìmole of product per minute.
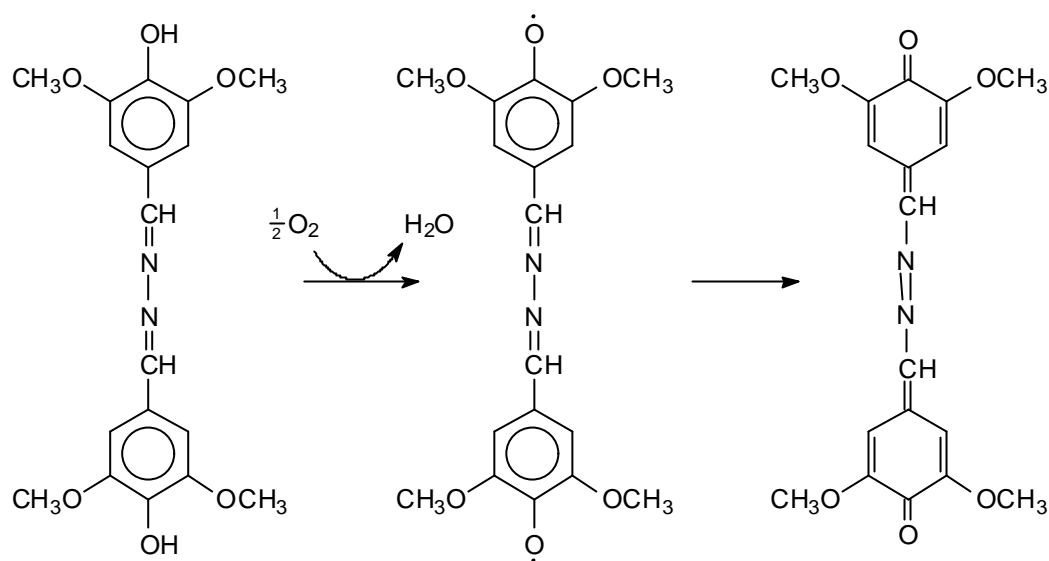
**Figure 3.2.** The laccase-catalysed oxidation of syringaldazine to its corresponding quinone (taken from Sanchez-Amat and Solano, 1997).

A stock solution of syringaldazine was prepared by dissolving the substrate over a period of 3 hours in 96 % ethanol. Syringaldazine for analysis was prepared by adding deionised water to the stock solution until the desired concentration of 0.28 mM was achieved (Felby, 1998). The reaction mixture consisted of 1 ml buffer (Sodium phosphate at the desired pH), 0.075 ml substrate and 0.025 ml enzyme.

Laccase activity in U/ml ($\mu$mol cation radical released.$min^{-1}$.$ml^{-1}$ enzyme) was calculated as follows:

$$U/ml = \left( \frac{V}{v \times \boldsymbol{e} \times d \times \Delta A.\min^{-1}} \right)$$

$$U/ml = \left( \frac{1.1}{0.025 \times 65 \times 1 \times \Delta A.\min^{-1}} \right)$$

$$= 0.667 \times \Delta A.\min^{-1},$$

where

| | | |
|---|---|---|
| $V$ | = | Total reaction volume (ml) |
| $v$ | = | Enzyme volume (ml) |
| $e$ | = | Extinction coefficient of ABTS at 420 nm = 65 mM$^{-1}$cm$^{-1}$ |
| | | (Felby, 1998) |
| $d$ | = | Light path of cuvette (cm) |
| $DA$.min$^{-1}$ | = | Absorbance change per minute at 420 nm |

### 3.6.3. DMP assay method for laccase

2,6-Dimethoxyphenol (DMP) is oxidized to its quinone, 2,6-dimethoxycyclohexa-2,5-diene-1-one, by a laccase catalysed reaction (figure 3.3). The yellow-orange colour formed by the quinone is measured spectrophotometrically and can be correlated to enzyme activity (Palmieri *et al.*, 1997).
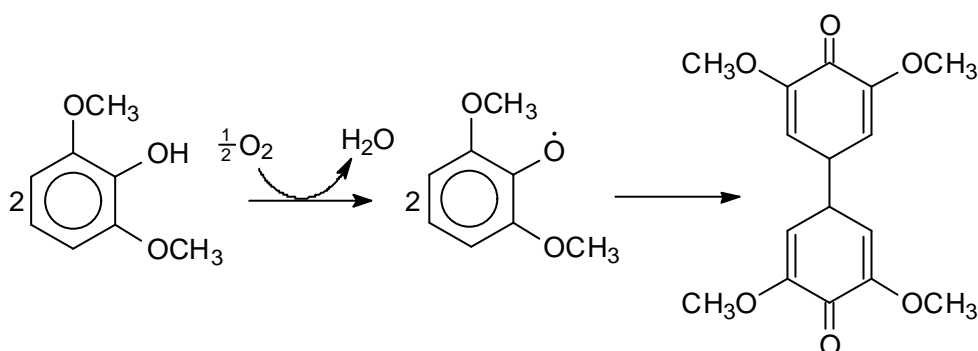


**Figure 3.3.**   The laccase-catalysed oxidation of 2,6-dimethoxyphenol to its corresponding quinone (taken from Sanchez-Amat and Solano, 1997).

DMP (1 mM) was dissolved in McIlvaine buffer (pH 7, 25 °C). The McIlvaine buffer was prepared by adding 0.1 M citric acid to 0.2 M Na$_2$HPO$_4$ until the desired pH was achieved. The oxidation of DMP was followed spectrophotometrically at 477 nm at 25 °C (Palmieri *et al.*, 1997). The reaction mixture contained 0.58 ml substrate and 0.02 ml enzyme.

The DMP assay was found to be effective over a wide pH range. It was therefore the assay of choice used during the characterisation of these enzymes.

Laccase activity in U/ml ($\mu$mol cation radical released.min$^{-1}$.ml$^{-1}$ enzyme) was calculated as follows:

$$U/ml = \left( \frac{V}{v \times e \times d \times \Delta A.\min^{-1}} \right)$$

$$U/ml = \left( \frac{0.6}{0.02 \times 14.8 \times 1 \times \Delta A.\min^{-1}} \right)$$

$$= 2.027 \times \Delta A.\min^{-1},$$

where

| | | |
|---|---|---|
| $V$ | = | Total reaction volume (ml) |
| $v$ | = | Enzyme volume (ml) |
| $e$ | = | Extinction coefficient of ABTS at 420 nm = 14.8 mM$^{-1}$cm$^{-1}$ |

(Palmieri *et al.*, 1997).

| | | |
|---|---|---|
| $d$ | = | Light path of cuvette (cm) |
| $\Delta A.\min^{-1}$ | = | Absorbance change per minute at 420 nm |

### 3.6.4. Assays with other substrates

Assays with substrates other than the above-mentioned were conducted in the same manner as for the DMP assay, except that DMP was substituted by the substrate in question at the desired concentrations. The oxidation of these substrates was then followed at the wavelength of maximum absorbance, which was determined using wavelength scans during the course of the reaction.

### 3.6.5. Protein assays

The protein contents of column effluents were estimated by spectrophotometric measurement at 280 nm. Most proteins have absorption at 280 nm ($A_{280}$), due to the presence of aromatic groups in tyrosine and tryptophan residues (Boyer, 1993).

Protein concentration (mg/ml) was determined using the BCA (bicinchoninic acid) protein assay kit as well as the MicroBCA protein assay kit from Pierce (Smith *et al.*, 1985). This method of protein determination is described as a highly sensitive method (Boyer, 1993). The MicroBCA method has sensitivity in the range of 0.5 µg to 20 µg of protein. The assays were carried out according to the manufacturers instructions. Standards (bovine serum albumin) were supplied by the manufacturer and used to construct standard curves (figure 3.4 and 3.5) from which the protein concentrations of unknown samples were determined.
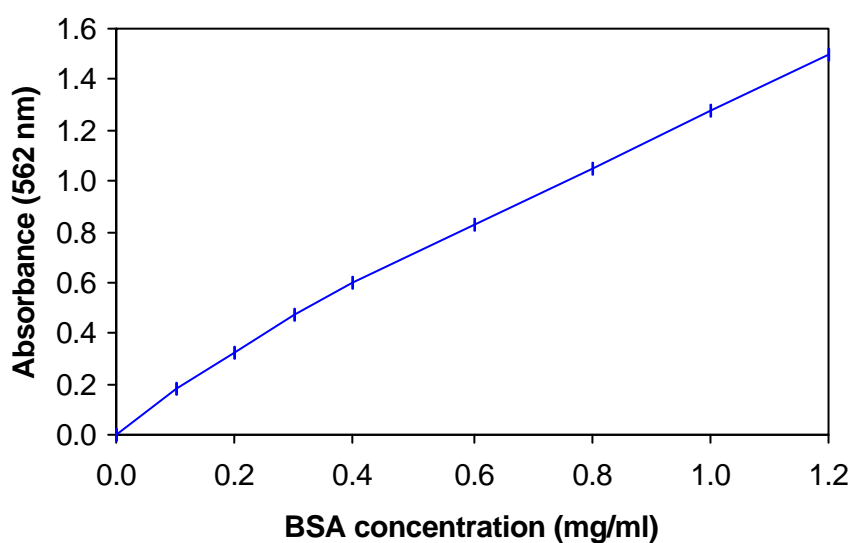


**Figure 3.4.**     Standard curve for BCA protein assay with BSA as protein standard.
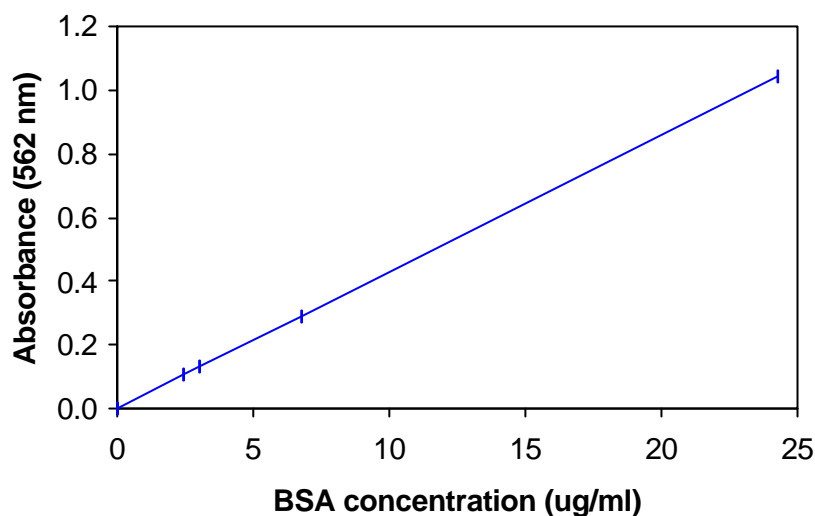
**Figure 3.5.**    Standard curve for Micro BCA protein assay with BSA as protein standard.

### 3.7.    Purification of laccase from *Pycnoporus sanguineus*

The method for the purification of laccase from *P. sanguineus* was adapted from a protocol described by Fukishima and Kirk (1995).  The culture was centrifuged (22100 x g; 30 min) to remove the mycelial mass from the supernatant.  The supernatant was frozen overnight at -20 °C, thawed and centrifuged at 22 100 x g for 30 minutes (Beckman J2-MC Centrifuge)  to  remove  unwanted  long-chain polysaccharides.  The supernatant was then fractionated with ammonium sulphate in order to remove unwanted proteins.  Different proteins have varying amino acid compositions, the degree of water solvation between different proteins vary (Boyer, 1993).    Different  proteins  therefore  precipitate  at  different  concentrations  of precipitating agent (ammonium sulphate).

An experiment was conducted to determine the degree of ammonium sulphate saturation required to precipitate laccase from the supernatant.  Ammonium sulphate was dissolved in 5 ml of supernatant and centrifuged (7300 x g; 10 minutes).  The amount of ammonium sulphate was increased by 5 % intervals ranging from 30 % to 95 % saturation.  The amount of laccase activity remaining in the supernatant was

determined spectrophotometrically with the ABTS assay. The amounts of solid ammonium sulphate required (grams per 100 ml of supernatant) to obtain different levels of saturation were obtained from 'Methods in protein purification' (Harris and Angal, 1989).

After fractionation, the supernatant containing the laccase activity was centrifuged (22100 x g; 30 minutes) and dialysed, using a dialysis tube with a molecular weight cut-off of 10 000 Da, against 0.01 M sodium phosphate buffer (pH 6) at 4 °C. The buffer was changed at least three times.

### 3.7.1. First isolation

The dialysate was loaded onto a pre-equilibrated DE52 anion exchange chromatography column. The column was washed with 0.01 M sodium phosphate buffer, pH 6, (running buffer) until the $A_{280}$ reading was less than 0.02. Bound protein was eluted with a linear salt gradient (0 to 1 M KCl in 200 ml running buffer). The flow rate was 0.5 ml/min and 5 ml fractions were collected with a RediFrac fraction collector (Pharmacia Biotech.). The eluted fractions were assayed for laccase activity and the $A_{280}$ monitored. The active fractions were pooled and dialysed against running buffer to rid the solution of excess salt.

The dialysate was loaded onto a pre-equilibrated DEAE Toyopearl anion exchange chromatography column. The column was washed and bound protein eluted with a linear gradient (0 to 1 M KCl in 200 ml running buffer). The flow rate was 1 ml/min and 2 ml fractions were collected. The fractions were assayed for laccase activity and the $A_{280}$ monitored. The active fractions were pooled and adjusted to 1.5 M ammonium sulphate.

A pre-equilibrated Phenyl Toyopearl hydrophobic interaction chromatography column was loaded with the pooled active fraction from the second chromatography step. The column was washed with the running buffer (0.01 M sodium phosphate buffer supplemented with 1.5 M ammonium sulphate). The enzyme was eluted with a

decreasing linear gradient of 1.5 to 0 M ammonium sulphate in 200 ml running buffer. The flow-rate was 1 ml/min and 2 ml fractions were collected, assayed for laccase activity and the $A_{280}$ monitored.

### 3.7.2. Second isolation

The protocol described for first isolation (freeze/thaw, $(NH_4)_2SO_4$ fractionation, dialysis, DE52 anion exchange chromatography, DEAE chromatography and Phenyl Toyopearl hydrophobic chromatography) was repeated with the following changes: the linear gradient for the DEAE anion exchange chromatography was altered to 0 to 0.5 M KCl in 400 ml running buffer. The linear gradient for the Phenyl Toyopearl step was altered to 1.5 M to 0.75 M ammonium sulphate in 300 ml running buffer.

### 3.7.3. Third isolation

The purification protocol described in the second isolation (freeze/thaw, $(NH_4)_2SO_4$ fractionation, dialysis, DE52 anion exchange chromatography, DEAE chromatography and Phenyl Toyopearl hydrophobic chromatography) was followed, but instead of using the Phenyl Toyopearl column as a third chromatography step, alternative chromatographic steps were evaluated as possible substitutions. The binding of *P. sanguineus* laccase to MIMETIC 6XL dye adsorbent ligands and hydrophobic interaction resins was assessed using Piksi kits according to the manufacturer's instructions

### 3.7.4. Fourth isolation

The protocol developed in isolation experiment 2 (freeze/thaw, $(NH_4)_2SO_4$ fractionation, dialysis, DE52 anion exchange chromatography, DEAE chromatography and Phenyl Toyopearl hydrophobic chromatography) was followed, but Phenyl Toyopearl hydrophobic interaction chromatography was replaced with MIMETIC Red I from the Piksi kit trials. The pooled active fraction from the second anion exchange chromatographic step was dialysed against the running buffer (0.02 M

sodium acetate buffer, pH 5), in order to change buffers and rid the solution of excess salt. Three buffer changes were necessary. The dialysate was loaded onto the pre-equilibrated MIMETIC Red I column followed by washing with the running buffer until the $A_{280}$ readings were less than 0.02. Bound protein was eluted with a linear salt gradient (0 to 0.5 M NaCl in 200 ml). A flow speed of 1.5 ml/min was maintained and 3 ml fractions were collected and assayed for laccase activity and monitored for $A_{280}$.

### 3.7.5. Fifth isolation

The protocol described in the fourth isolation (freeze/thaw, $(NH_4)_2SO_4$ fractionation, dialysis, DE52 anion exchange chromatography, DEAE chromatography and MIMETIC Red I affinity chromatography) was followed but the MIMETIC Red I column was substituted with a MIMETIC Yellow II column, identified during the assessment of alternative resins (third isolation). The active fraction from the second anion exchange chromatography step was dialysed against 0.01 M sodium phosphate buffer (pH 6). The dialysate was loaded onto a pre-equilibrated MIMETIC Yellow II affinity chromatography resin. The column was washed and the enzyme eluted with a linear gradient of 0 – 0.5 M NaCl in 200 ml running buffer. The flow speed was 2 ml/min and 2 ml fractions were collected and assayed for laccase activity and monitored for $A_{280}$.

### 3.7.6. Sixth isolation

The protocol described in isolation experiment 2 was followed to the second chromatography step (freeze/thaw, $(NH_4)_2SO_4$ fractionation, dialysis, DE52 anion exchange chromatography, DEAE anion exchange chromatography). The active fraction was then dialysed against 0.01 M sodium phosphate buffer (pH 6). The sample was then concentrated to 9 ml on an Amicon ultrafiltration system to a volume less than 10 % of the total column volume and loaded onto a Toyopearl HW50F gel filtration column (100 cm x 2.5 cm). The flow rate was 0.2 ml/min and 2-ml fractions were collected and assayed for laccase activity and monitored for $A_{280}$. The active

fractions were pooled and loaded onto the mimetic yellow II affinity chromatography resin.

### 3.7.7. Seventh isolation

The final and most successful protocol included the freezing and thawing of the supernatant, $(NH_4)_2SO_4$ fractionation at a saturation of 75 %, dialysis, DE52 anion exchange chromatography, DEAE anion exchange chromatography and MIMETIC Yellow II affinity chromatography.

### 3.8.    Purification of laccase from *Coprinus micaceus*

The initial purification protocol was adapted from the protocol established for laccase from *P. sanguineus.* The biomass obtained after three to four days of cultivation was separated from the culture by vacuum filtration on a Whatman 113 filter paper. Ammonium sulphate fractionation (75 % saturation) was then carried out on the filtrate.  After centrifugation (22100 x g; 30 min) in a Beckman J2-MC Centrifuge, the precipitate was dissolved in 0.01 M sodium phosphate buffer (pH 6) and dialysed against the same buffer with at least three buffer changes to remove excess salts.  The following experiments were conducted to optimise a protocol for the  isolation of laccase from *C. micaceus*:

### 3.8.1. First isolation

The supernatant was loaded onto a pre-equilibrated anion exchange chromatography column (DE 52).  The column was washed with running buffer (0.01 M sodium phosphate buffer, pH 6) until the $A_{280}$ reading was less than 0.02.  The enzyme was eluted with a linear salt gradient (0 – 1 M KCl dissolved in 200 ml of running buffer).  The flow rate was 0.5 ml/min and 5 ml fractions were collected with an automated RediFrac fraction collector (Pharmacia; Biotech.).   The absorbance of collected fractions was monitored for $A_{280}$ and assayed for laccase activity with DMP as substrate at pH 7.  Three active fractions corresponding with to three different protein

peaks were pooled, dialysed individually and each pooled fraction was then loaded onto a pre-equilibrated DEAE Toyopearl column.

The column was washed with running buffer until the $A_{280nm}$ was less than 0.02. The enzyme was then eluted with a salt gradient (0 – 1 M KCl dissolved in 400 ml of running buffer). The flow rate was 1 ml/min and 2 ml fractions were collected with an automated RediFrac fraction collector (Pharmacia; Biotech). The $A_{280nm}$ of each fraction was monitored and assayed for laccase activity. Active fractions for each laccase were pooled and adjusted to 1.5 M ammonium sulphate. The pooled fractions were loaded onto individual pre-equilibrated Phenyl Toyopearl hydrophobic interaction chromatography columns. The columns were washed with the running buffer (0.01 M sodium phosphate buffer supplemented with 1.5 M ammonium sulphate, pH 6) until the $A_{280}$ reading was less than 0.02. Bound protein was eluted with a linear salt gradient (1.5 – 0 M ammonium sulphate in 200 ml of running buffer) and washed with a further 50 ml of buffer without ammonium sulphate. The flow rate was 1 ml/min and 2 ml fractions were collected with an automated fraction collector. Each fraction was monitored for $A_{280}$ and then assayed for laccase activity.

## 3.8.2.  Second isolation

The purification protocol from the first isolation (($NH_4$)$_2SO_4$ fractionation, dialysis, DE52 anion exchange chromatography, DEAE anion exchange chromatography and Phenyl Toyopearl hydrophobic interaction chromatography) was followed up to the end of the second chromatographic column. The three pooled enzymes bound extremely strongly to the Phenyl Toyopearl resin. As an alternative to the Phenyl Toyopearl resins, HW50F Toyopearl size exclusion chromatography was used as a final step in the purification protocol for all three pools of laccases from *C. micaceus*. The pooled fractions were concentrated on an Amicon ultrafiltration system (Amicon 8050) so as not to exceed 10 % of the total column volume. The flow rate was 0.2 ml/min and 2 ml fractions were collected automatically and assayed for laccase activity and monitored for $A_{280}$.

### 3.8.3. Third isolation

The purification protocol described in the second isolation isolation ($(NH_4)_2SO_4$ fractionation, dialysis, DE52 anion exchange chromatography, DEAE anion exchange chromatography and HW50F size exclusion chromatography) led to homogeneous preparations for pool 1 and 2 laccases. Pool 3 however remained heterogeneous. An alternative chromatographic step was explored for the purification of pool 3 laccase.

The pool 3 laccase from *C. micaceus* was assessed for its binding affinity towards hydrophobic interaction resins using an H-Piksi kit. Piksi kits were used as described by the manufacturers instructions.

### 3.9. Electrophoretic analysis

### 3.9.1. SDS-PAGE

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), or denaturing gel electrophoresis, was used to monitor the development of the purification process, to determine homogeneity and to determine the relative molecular mass of the laccase enzymes. SDS-PAGE was carried out on a 10 % resolving gel and a 5 % stacking gel according to a method adapted from Laemmli *et al.* (1970). SDS-PAGE was performed using a Hoefer Mini-Vertical Electrophoresis system (Hoefer scientific instruments) and proteins were separated at 20 mA per gel.

Proteins were visualised by staining for 3 hours with Coomassie Brilliant Blue-R250. Gels were then destained with a mixture of acetic acid and ethanol (40 %: 10 %). Proteins at low concentrations were stained with a silver stain kit (Quicksilver) supplied by Amersham or by a method adapted from Switzer *et al.* (1979).

The determinations of the relative molecular masses of the denatured laccase enzymes were based on the relative distance of migration of either of two sets of molecular

standards. The BlueRanger prestained protein molecular weight marker mix (Pierce; USA) included (sizes in Dalton): myosin (215 000), phosphorylase b (120 000), serum albumin (84 000), ovalbumin (60 000), carbonic anhydrase (39 200), trypsin inhibitor (28 000) and lysozyme (18 300). The second set of standards was the broad range SDS-PAGE molecular weight standards from Bio-Rad (Hercules, Calif.) that included: myosin (200 000), β-galactosidase (116 500), phosphorylase b (97 400), serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), trypsin inhibitor (21 500) and lysozyme (14 400). Protein samples that were too dilute to be loaded directly were concentrated by the use of an Amicon ultrafiltration unit or microspin vectorettes (Whatman).

### 3.9.2. Native-PAGE

Native PAGE or non-denaturing PAGE was by the same protocol used for SDS-PAGE with the exception that the anionic detergent, SDS, was omitted from gels and samples and the samples were not incubated prior to the loading of the gel.

### 3.9.3. Zymogram analyses

Activity staining of laccases was performed after denaturing or native gel electrophoresis (Candussio, personal communication). After completion of the electrophoresis, the gel was soaked in 17.3 mM DMP in 0.2 M McIlvaine buffer at room temperature. After approximately 5 to 20 minutes, depending on the amount of laccase activity present, a yellowish-brown colour appeared at the position of the laccase. Zymogram analysis was done at pH 4.5 for *P. sanguineus* laccase and pH 7 for laccases from *C. micaceus*.

### 3.9.4. Iso-electric focussing

Iso-electric focussing (IEF) analyses were performed using the Hoefer mini-Vertical Electrophoresis system (Hoefer Scientific Instruments) according to a method by

Robertson *et al.* (1987) over a pH range of 3 to 10. The standards used were purchased from Bio-rad. Proteins were visualised by silver staining.

## 3.10. pH optimum

The influence of pH on laccase activity was studied spectrophotometrically. Four different substrates were used to determine the optimum pH of laccases, because it is known that laccases have different optimum pH values for different substrates (Xu, 1996). The four substrates used were: ABTS, syringaldazine, DMP and guaiacol. The pH optima were determined over a range of pH 3 to 8. A 0.1 M Britton-Robinson buffer (Xu, 1996) was used for the entire pH range. The Britton-Robinson buffer was made by mixing 0.1 M boric acid, 0.1 M acetic acid and 0.1 M phosphoric acid and adjusting the pH with 0.5 M NaOH. The different assays for the different substrates were conducted as described under assay methods. All assays were done in triplicate.

## 3.11. Temperature optimum

The effect of temperature on laccase activity was determined by spectrophotometrically following the laccase-catalysed oxidation of 1 mM DMP for 2 minutes at temperatures ranging 25 °C to 80 °C at 5 °C intervals. Britton Robinson buffer (0.1 M) was used for all the reactions, at the optimum pH for DMP of each laccase: *Pycnoporus sanguineus* laccase at a pH of 4 and pool 1, 2 and 3 laccases from *C. micaceus* at pH 4, 7.5 and 6.5 respectively. The substrate was incubated for at least 20 minutes at the different temperatures before the enzyme was added to start the reaction.

## 3.12. Thermostability

The thermal stability of the different laccases was determined by following the oxidation of 1 mM DMP for 2 minutes at 25 °C after pre-incubation of laccase for 1,

3, 5, 10, 20, 30, 45, 60, 120, 180 and 240 minutes at 45 °C, 55 °C, 65 °C and 75 °C. The reaction was started by the addition of substrate.

## 3.13. Substrate specificity

The specificity of laccases toward different phenolic compounds was investigated. Substrates were chosen according to the positions of substituents on the phenolic ring and the type and/or length of the substituents. Four of the most commonly used substrates (ABTS, DMP, syringaldazine and guaiacol) (Palmieri *et al*., 1997; Giardina *et al*., 1999; Xu, 1996) for laccase determination were evaluated for all four of the isolated laccase enzymes. Fourteen other phenolic compounds (pyrocatechol, resorcinol, hydroquinone, pyrogallol, vanillic acid, gallic acid, tannic acid, o-cresol, p-cresol, m-cresol, 2,4-benzenetriol, phloroglucinol, ferulic acid and tyrosine) were evaluated for laccase isolated from *P. sanguineus* and the pool-2 laccase isolated from *C. micaceus* only.

The substrates were dissolved in 0.1 M Britton-Robinson buffer at concentrations of 1 mM and 5 mM. The reaction mixtures contained 20 $\mu$l of enzyme that was added to 580 $\mu$l of substrate to start the reaction. The oxidation of the substrates was followed during a wavelength scan on the Beckman DU-650 spectrophotometer in the visible region (300 nm to 700 nm). The wavelength where maximal absorbance increase over time was observed was recorded and used to measure the oxidation rate during kinetic studies. If no reaction was found for a substrate during the wavelength scans the experiment was repeated with a 20 mM concentration of substrate.

Kinetic studies were conducted for each substrate that could be oxidized by the laccase enzymes. At least eight different substrate concentrations for each substrate were assayed for each laccase at the optimal pH for each enzyme. Triplicates of each assay were done. The data was subjected to nonlinear regression analysis (Graph Pad Prism Software) using the Michaelis-Menten equation and the kinetic parameters ($K_m$, $V_{max}$ and $k_{cat}$) were determined.

## 3.14.  Inhibition studies

Six potential inhibitors were evaluated to test the inhibition properties of laccases. These inhibitors included: sodium azide ($NaN_3$) that complexes to the coppers in the active site, cysteine that is a sulfhydryl organic compound with a reducing effect on the copper-containing active site of laccase, EDTA that exhibits metal chelating properties and three halides ($I^-$, $CI^-$, $F^-$) of different sizes.  Halides are known to inhibit laccase at the Type 2/3 trinuclear copper site.  It is at this site that molecular oxygen is reduced to two molecules of water.  The inhibition therefore entails that the oxygen is prohibited from being reduced, causing a break in the terminal electron acceptance, and leading to a decrease in redox potential difference between the two copper sites.

Different concentrations of these inhibitors were added to the substrate (DMP) before addition of the enzyme (table 3.1).  The percentage of inhibition was then calculated.

**Table 3.1.** Inhibitor concentrations used to determine the percentage of laccase inhibition.

| Inhibitors | Inhibitor concentrations (mM) |
|---|---|
| Sodium azide | 0 |
| | 0.01 |
| | 0.02 |
| | 0.05 |
| | 0.1 |
| EDTA | 0 |
| | 0.1 |
| | 1 |
| | 2 |
| | 3 |
| | 5 |
| Cysteine | 0 |
| | 0.01 |
| | 0.05 |
| | 0.07 |
| | 0.1 |
| | 0.2 |
| $F^-$ | 0 |
| | 10 |
| | 50 |
| | 100 |
| | 150 |
| | 200 |
| Cl- | 0 |
| | 10 |
| | 50 |
| | 100 |
| | 150 |
| | 200 |
| $I^-$ | 0 |
| | 10 |
| | 100 |
| | 150 |
| | 250 |
| | 500 |

The inhibition constant ($K_i$) and the type of inhibition were determined by adding four different concentrations of each inhibitor to each of six different concentrations of DMP, which was used as substrate for the reactions. These experiments were done in triplicate for laccase from *P. sanguineus* and pool-2 laccase from *C. micaceus*. $K_m$ and $V_m$ values, determined by nonlinear regression (Graph Pad Prism Software) were

used to calculate the vertical intercepts and slopes of the curves. The correlations obtained when the slopes and vertical intercepts of Lineweaver-Burke plots were plotted against the inhibitor concentrations, determined if the inhibition was partial (convex correlation) or full (linear correlation).

The inhibitor constant ($K_i$) is a quantitative measure of inihibitor potency for reversible inhibitors. The effectiveness of inhibitors in our study is therefore expressed by $K_i$, which is the reciprocal of the enzyme-inhibitor affinity. Inhibition constants were determined graphically as described by Dixon and Webb (1979). Slopes and vertical intercepts of the Lineweaver-Burke plots calculated as described previously were plotted against inhibitor concentrations. Inhibitor constants were read from the intercept on the base line.

# CHAPTER 4
# RESULTS AND DISCUSSION
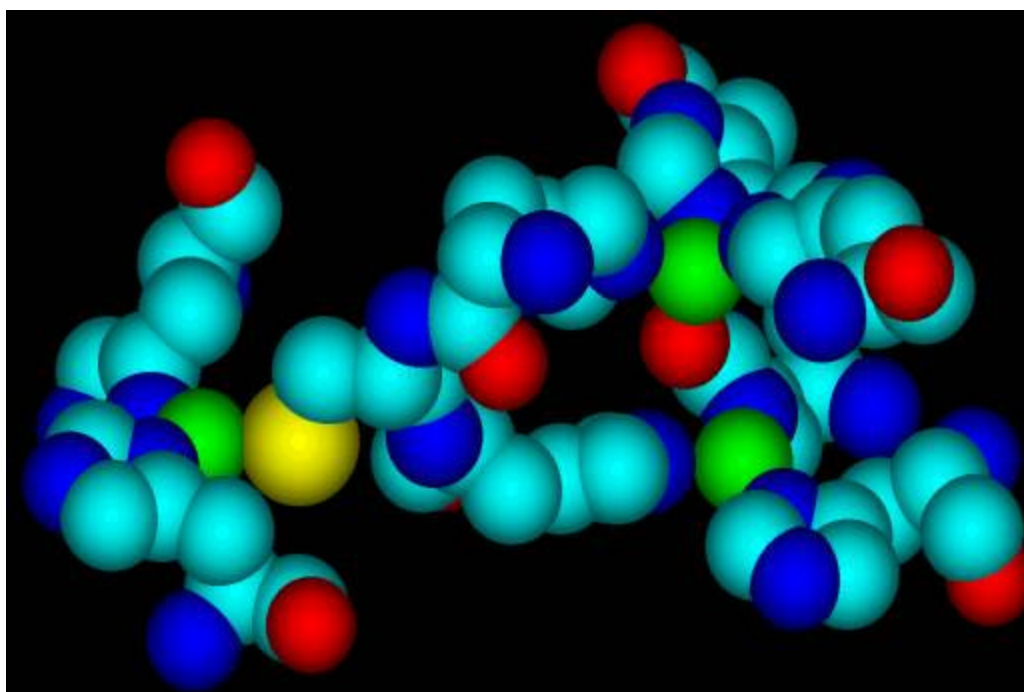


A representation of the active site of copper-2 depleted laccase from

*Coprinus cinereus*

(taken from Ducros et al., 1998)

## 4.1. Cultivation conditions for *Pycnoporus sanguineus*

*Pycnoporus sanguineus* culture was grown in diluted molasses (4 %) for 11 days. Laccase activity in the medium reached a maximum after ten days of cultivation. The supernatant was therefore routinely harvested after 10 days of growth to retrieve maximal laccase activity.

## 4.2. Cultivation conditions for *Coprinus micaceus*

Initially diluted molasses (4 %) was used to culture *C. micaceus*. The laccase activity in the medium reached a maximum after four days of cultivation and the supernatant was thus harvested at this time (figure 4.1). The highest laccase activity was detected when the assay was conducted at a pH of 7.
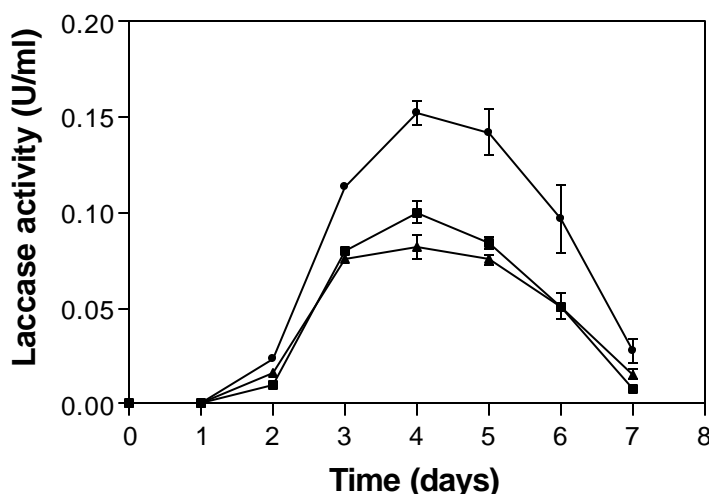


**Figure 4.1.**    Production of laccase activity by the white-rot fungus *Coprinus micaceus* in 4 % molasses medium in shake flasks. Laccase activity was assayed at pH 4, 6 and 7. Plotted values are averages of triplicates shown as error bars.  ■ = pH 4,  ▲ = pH 6,  • = pH 7.

Very low activity titres were produced by *C. micaceus* during cultivation in molasses alone. Although it would have been sufficient for the preliminary comparison of laccase enzymes produced by the two different fungi under the same conditions, production would be too low for comparative kinetic studies of the enzymes. As an attempt to improve the amounts of laccase activity produced by *C. micaceus* an experiment in which

different culture conditions were varied was performed. Results from this experiment indicated that laccase produced by *C. micaceus* under any method of cultivation performed better at a neutral to alkaline pH. The molasses produced significantly higher laccase activity at pH 7 than did 20 g/l of malt extract. Higher laccase activity titres were also achieved by adding copper sulphate and p-hydroxybenzohydrazine (pHB) to the molasses medium as potential inducers. Addition of p-hydroxybenzohydrazine alone as well as the addition of p-hydroxybenzohydrazine and copper sulphate together differed significantly from unsupplemented molasses medium. There was no significant difference between the two supplemented media. Hence, diluted molasses (4 %), supplemented with 0.5 mg/l p-hydroxybenzohydrazine, was used as medium for the cultivation of *C. micaceus* and laccase activity was assayed at pH 7.

**Table 4.1.** Laccase activity produced by *C. micaceus* after 4 days of cultivation in different media. Data shown are averages of three replications. Laccase activity was assayed at a pH of 7. Letters (a to c) indicate the level of significant difference between different additives for each individual medium. The letters x and y indicate a significant difference between the averages of growth conditions for the two media. pHB = p-hydroxybenzohydrazine, Cu = coppersulphate, control = medium without any form of supplementation.

| Additives | Molasses | Malt extract |
|-----------|----------|--------------|
| pHB; Cu   | 0.25 a   | 0.23 a       |
| pHB       | 0.22 a   | 0.19 a       |
| Cu        | 0.20 b   | 0.15 b       |
| Control   | 0.16 c   | 0.11 c       |
| Averages  | 0.21 x   | 0.17 y       |

## 4.3. Purification of laccase from *P. sanguineus*

After removal of the biomass, the supernatant was frozen and thawed. This was necessary in order to remove long-chain polysaccharides from the supernatant, which were produced during growth of the fungus. Ammonium sulphate precipitation was used to remove unwanted proteins in the next step. It was determined that for the complete

precipitation of laccase from the supernatant an amount of ammonium sulphate equal to at least 75 % saturation of the supernatant is required (figure 4.2).
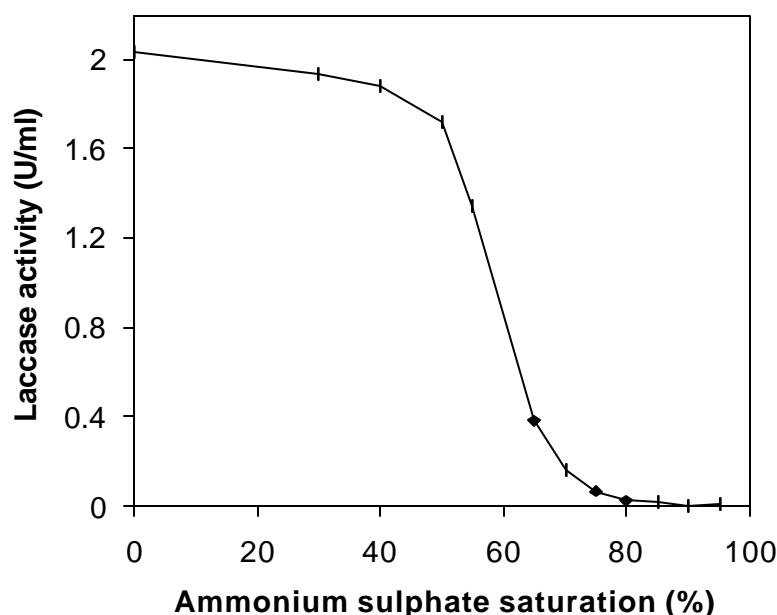


**Figure 4.2.**     Remaining laccase activity in the supernatant after addition of ammonium sulphate.

Dialysis against the running buffer (0.01 M sodium phosphate) was done to remove excess ammonium sulphate from the supernatant prior to column chromatography.  Three buffer changes were necessary to achieve a suitable removal of unwanted salt.

The DE 52 anion exchange chromatography resin successfully  separated the laccase enzyme from the contaminating dark brown colour present in the supernatant (figure 4.3). The column step resulted in the separation of various protein peaks that could be separated from the laccase enzyme.  The use of this column chromatography step caused an average increase of 55 in the degree of purification over seven purifications (table 4.4).
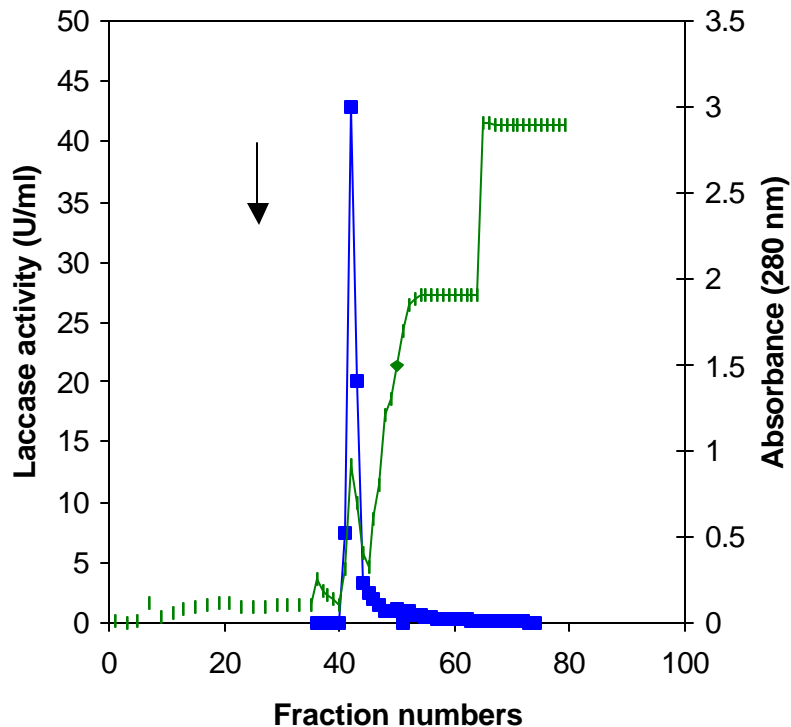
**Figure 4.3.**     A typical elution profile of laccase from *P. sanguineus* on DE 52 anion exchange chromatography.    The arrow indicates the start of the salt gradient.  ■ = enzyme activity; ♦ = $A_{280}$

The second anion exchange resin (DEAE Toyopearl) served to further separate the laccase enzyme, free of contaminating brown colour, from other proteins present in the fraction (figure 4.4).  The purification fold increased by a factor of 1.5 to 2.5 during this step.  A typical elution profile for this purification step indicates a single laccase activity peak corresponding to the position of a shoulder in the peak representing protein content (figure 4.4).
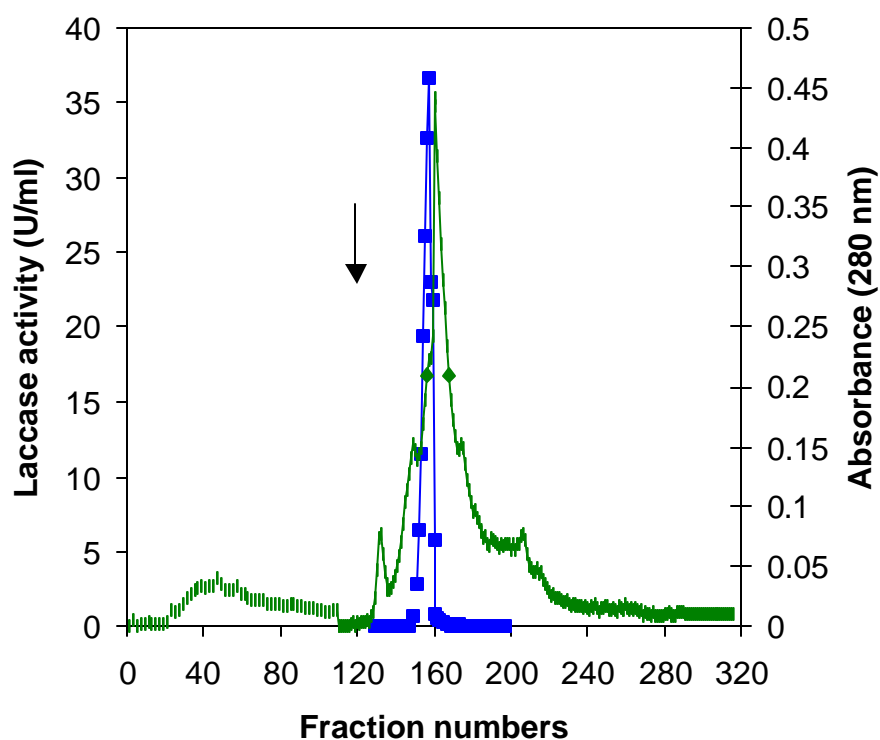
**Figure 4.4**.     A typical elution profile of laccase from *P. sanguineus* on DEAE Toyopearl chromatography.   The arrow indicates the start of the salt gradient.  ■ = enzyme activity;  ♦ = A$_{280}$

For the final column chromatography step, Phenyl Toyopearl, MIMETIC Red I, HW50F Gel filtration and MIMETIC Yellow II resins were assessed.  The use of the MIMETIC and Hydrophobic resins was decided upon after results with Piksi kits were evaluated. The MIMETIC dye binding kit showed that *P. sanguineus* laccase had an affinity for all of the ten MIMETIC resins (table 4.2).  The MIMETIC Red I and MIMETIC Yellow II resins were, therefore, used as purification steps in following isolation experiments.

The H-Piksi kit or hydrophobic interaction piksi kit indicated that only the resins with phenolic functional groups were effective in binding the laccase enzyme from *P. sanguineus* (table 4.3).

**Table 4.2.**  Assessment of the binding affinity of *P. sanguineus* laccase toward different MIMETIC dye affinity resins from the MIMETIC Piksi Kit.

| MIMETIC Resins | Relative Activity (% of maximum) | |
| --- | --- | --- |
| | Non-Binding Fraction | Binding Fraction |
| Red I | 0.36 | 86.7 |
| Red II | 1.3 | 83.9 |
| Orange I | 1 | 83.6 |
| Orange II | 1.3 | 85.1 |
| Orange III | 0.33 | 81.1 |
| Yellow I | 1.4 | 76.3 |
| Yellow II | 0.2 | 78.2 |
| Green | 0.1 | 82.6 |
| Blue I | 0.2 | 85.2 |
| Blue II | 0.1 | 79.6 |

**Table 4.3.**  Assessment of the binding affinity of *P. sanguineus* laccase toward different hydrophobic interaction chromatography resins from the H-Piksi Kit.

| Hydrophobic Resins | Relative Activity (% of maximum) | |
| --- | --- | --- |
| | Non-Binding Fraction | Binding Fraction |
| Butyl 4XL agarose | 37 | 0.01 |
| Butyl 6XL agarose | 22 | 0.7 |
| Hexyl 4XL agarose | 58 | 0.03 |
| Hexyl 6XL agarose | 60 | 0.02 |
| Octyl 4XL agarose | 58 | 0.17 |
| Octyl 6XL agarose | 42 | 0.32 |
| Decyl 4XL agarose | 54 | 0.09 |
| Decyl 6XL agarose | 59 | 0.45 |
| Phenyl 4XL agarose | 3.2 | 67 |
| Phenyl 6XL agarose | 0.2 | 68 |

Initial use of Phenyl Toyopearl hydrophobic interaction chromatography (figure 4.6) did not yield a homogenous product. The scatter in the $A_{280}$ is a result of the low absorbance values measured. After the gradient volumes of the anion exchange columns had been increased, laccase was still not homogenous, but from denaturing electrophoresis only one contaminating protein was visible in addition to the laccase protein (figure 4.5). The position of the laccase enzyme was determined by zymogram analysis with DMP as substrate.
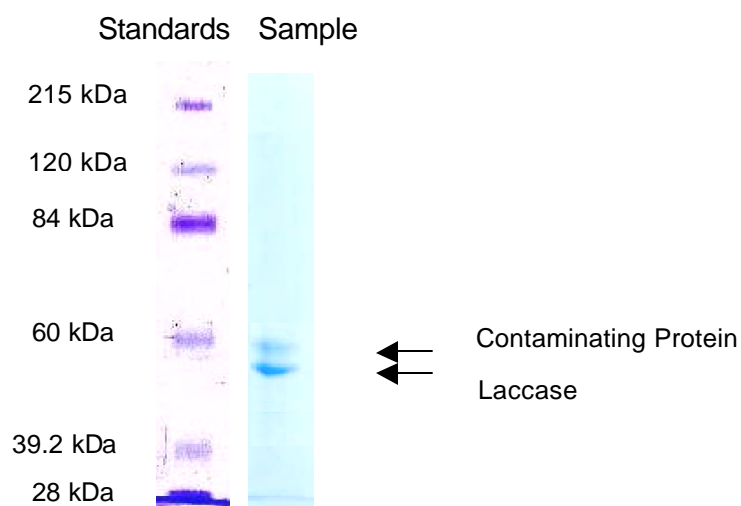


**Figure 4.5.**   Results of SDS-PAGE showing the presence of *P. sanguineus* laccase and the single contaminating protein.
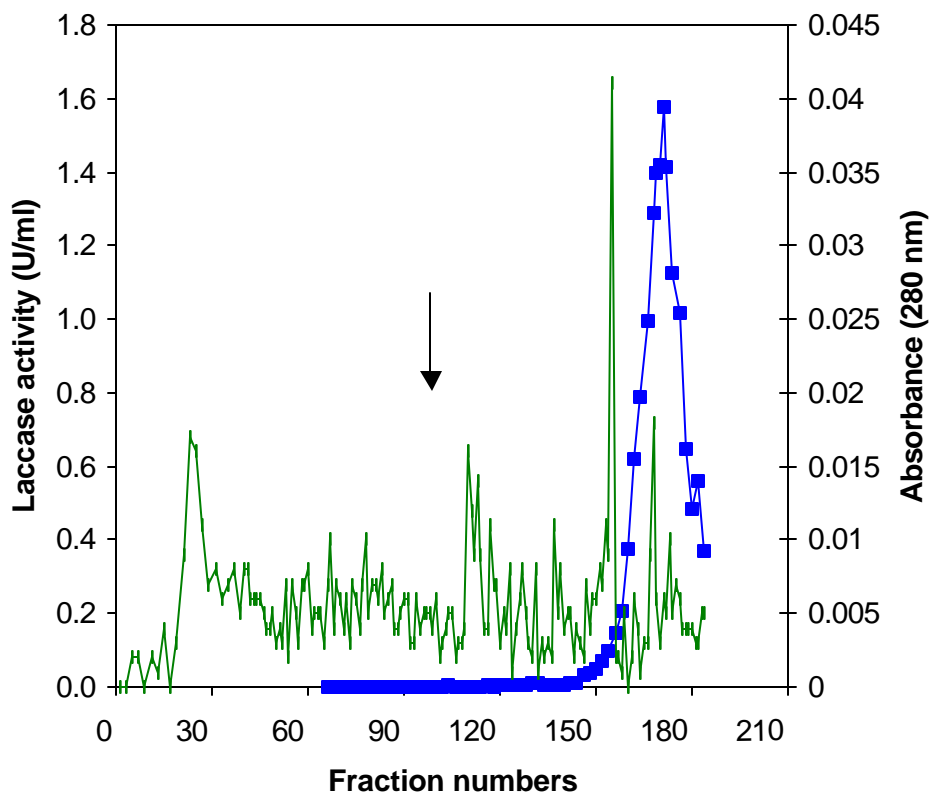
**Figure 4.6.** A typical elution profile of laccase from *P. sanguineus* on Phenyl Toyopearl hydrophobic interaction chromatography resin. The arrow indicates the start of the salt gradient. ■ = enzyme activity; ♦ = $A_{280}$

When HW50F Toyopearl size exclusion chromatography (figure 4.7) was used an extremely high purification fold was obtained, unfortunately, it did not succeed in separating the two protein bands visible on SDS-PAGE (figure 4.5).
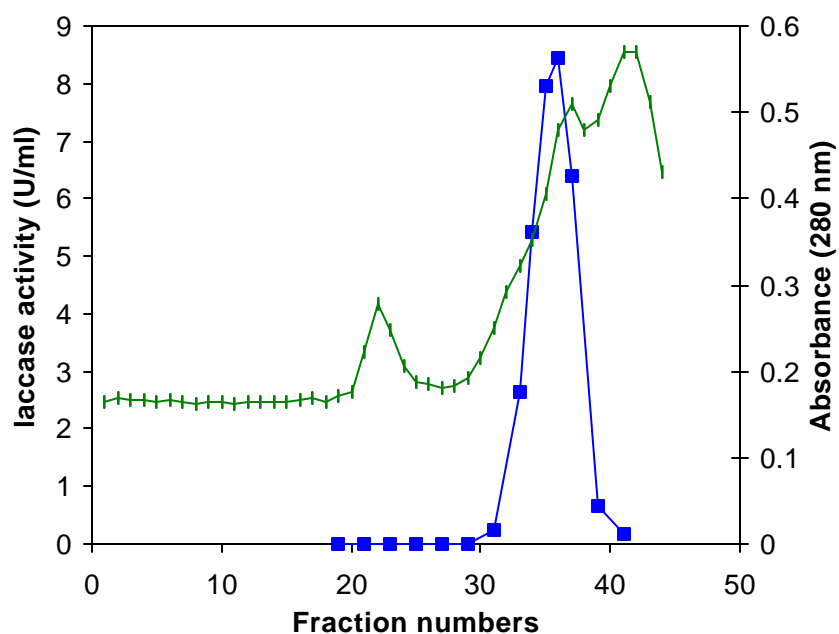
**Figure 4.7.** A typical elution profile of laccase from *P. sanguineus* on HW50F Toyopearl size exclusion chromatography. ■ = enzyme activity; ◆ = A$_{280}$

MIMETIC Red I (graph not shown) and MIMETIC Yellow II (figure 4.8) affinity resins were evaluated. Only MIMETIC Yellow II worked particularly well, as a homogenous product was obtained. The purification fold, yield and protein concentration was higher than for the other resins (table 4.4).
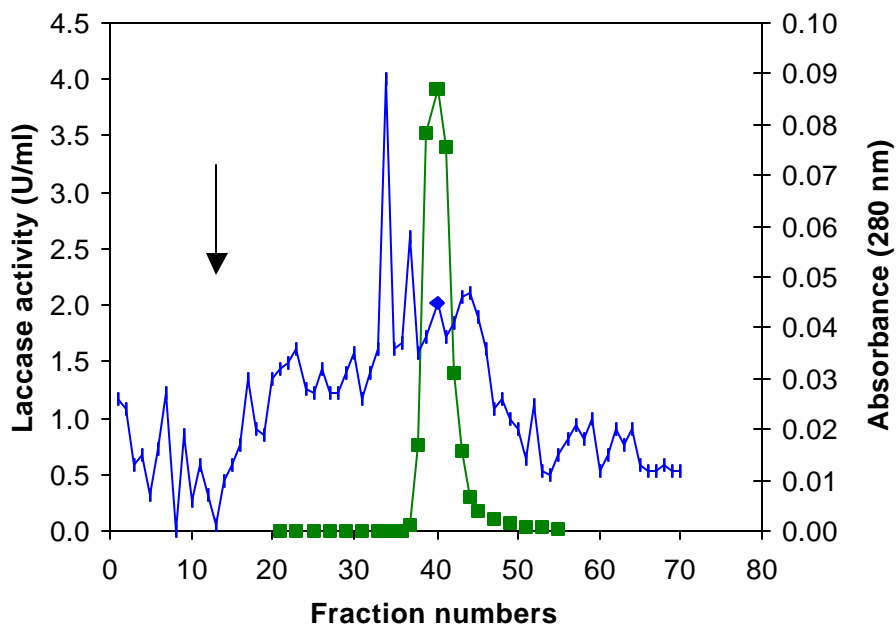
**Figure 4.8.** A typical elution profile of laccase from *P. sanguineus* on MIMETIC Yellow II affinity chromatography resin. The arrow indicates the start of the salt gradient. ■ = enzyme activity; ♦ = $A_{280}$

Various attempts to purify laccase from *P. sanguineus* were made to establish a suitable protocol.

**Table 4.4.** Enrichment table for the various isolation experiments of laccase from *P. sanguineus*.

| Purification steps | Total Act. (Units) | Total Yield (%) | Specific act. (U/mg) | Purification Fold |
|---|---|---|---|---|
| First Isolation | | | | |
| Culture | 300 | 100 | 0.16 | 1 |
| Freeze/Thaw | 185.6 | 62 | 0.10 | 0.6 |
| $(NH_4)_2SO_4$ | 346.8 | 116 | 1.03 | 6.5 |
| Dialysis | 403.2 | 134 | 1.24 | 7.8 |
| DE 52 | 259.2 | 86 | 67.50 | 423.5 |
| DEAE Toyopearl | 239.8 | 80 | 178.69 | 1121.2 |
| Phenyl Toyopearl | 163.8 | 55 | 160.27 | 1005.6 |
| Second Isolation | | | | |
| Culture | 306 | 100 | 0.2 | 1 |
| Freeze/Thaw | 357 | 116.7 | 0.24 | 1.2 |
| $(NH_4)_2SO_4$ | 289.12 | 94.5 | 1.3 | 6.5 |
| Dialysis | 501.42 | 163.9 | 3.2 | 16 |
| DE 52 | 739.6 | 241.7 | 220.1 | 1100.5 |
| DEAE Toyopearl | 80.22 | 26.2 | 230.5 | 1152.5 |
| Phenyl Toyopearl | 19.2 | 6.3 | 40 | 200 |
| Third Isolation | | | | |
| MIMETIC-dye and H-Piksi Kit Assessments | | | | |
| Fourth Isolation | | | | |
| Culture | 306 | 100 | 0.18 | 1 |
| Freeze/Thaw | 275 | 90.1 | 0.2 | 1.1 |
| $(NH_4)_2SO_4$ | 135.2 | 44.2 | 0.5 | 2.8 |
| Dialysis | 606.5 | 198.1 | 2.8 | 15.6 |
| DE 52 | 326.9 | 106.7 | 155.6 | 864 |
| DEAE Toyopearl | 91.6 | 29.9 | 176 | 978 |
| Mimetic Red I | 12.7 | 4.2 | 13.4 | 74.4 |
| Fifth Isolation | | | | |
| Culture | 300 | 100 | 0.16 | 1 |
| Freeze/Thaw | 185.6 | 62 | 0.10 | 0.6 |
| $(NH_4)_2SO_4$ | 346.8 | 116 | 1.03 | 6.5 |
| Dialysis | 403.2 | 134 | 1.24 | 7.8 |
| DE 52 | 259.2 | 86 | 67.50 | 423.5 |
| DEAE Toyopearl | 239.8 | 80 | 178.69 | 1121.2 |
| Mimetic Yellow II | 163.8 | 55 | 160.27 | 1005.6 |
| Sixth Isolation | | | | |
| Culture | 200 | 100 | 0.067 | 1 |
| Freeze/Thaw | 181.08 | 90.54 | 0.069 | 1.03 |
| $(NH_4)_2SO_4$ | 243.8 | 121.9 | 0.62 | 9.3 |
| Dialysis | 353.4 | 176.7 | 1.39 | 20.7 |
| DE 52 | 297.5 | 148.75 | 43 | 641.8 |
| DEAE Toyopearl | 252 | 126 | 60 | 895.5 |
| HW50F | 59.4 | 29.7 | 55 | 820.9 |
| Seventh Isolation | | | | |
| Culture | 165 | 100 | 0.034 | 1 |
| Freeze/Thaw | 110 | 67 | 0.021 | 0.622 |
| $(NH_4)_2SO_4$ | 142 | 86 | 0.201 | 5.923 |
| Dialysis | 170 | 103 | 0.256 | 7.518 |
| DE 52 | 143 | 87 | 8.367 | 245.929 |
| DEAE Toyopearl | 91 | 55 | 22.235 | 653.583 |
| Mimetic Yellow II | 62 | 38 | 32.911 | 967.395 |

## 4.4.    Purification of laccase from *C. micaceus*

The biomass was separated from the supernatant by vacuum filtration on Whatman 113 filter paper.  The supernatant was not frozen and thawed, as this made no difference to the consistency of the liquid.  Ammonium sulphate fractionation to remove *C. micaceus* laccase from the supernatant was done at a saturation of 75 %.  No laccase activity remained in the supernatant after centrifugation.  Dialysis against the running buffer (0.01 M sodium phosphate) was done to remove excess ammonium sulphate from the supernatant prior to column chromatography.  Three buffer changes were necessary to achieve complete removal of unwanted salt.

The DE 52 anion exchange chromatography successfully separated the dark brown contaminating colour of the supernatant from the laccase enzyme.  Three activity peaks were resolved (figure 4.9).  Active fractions from the three identified peaks were pooled separately and labelled pool 1, pool 2 and pool 3.  The third activity pool contained high concentrations of the coloured compounds and had very high absorbance at 280 nm.
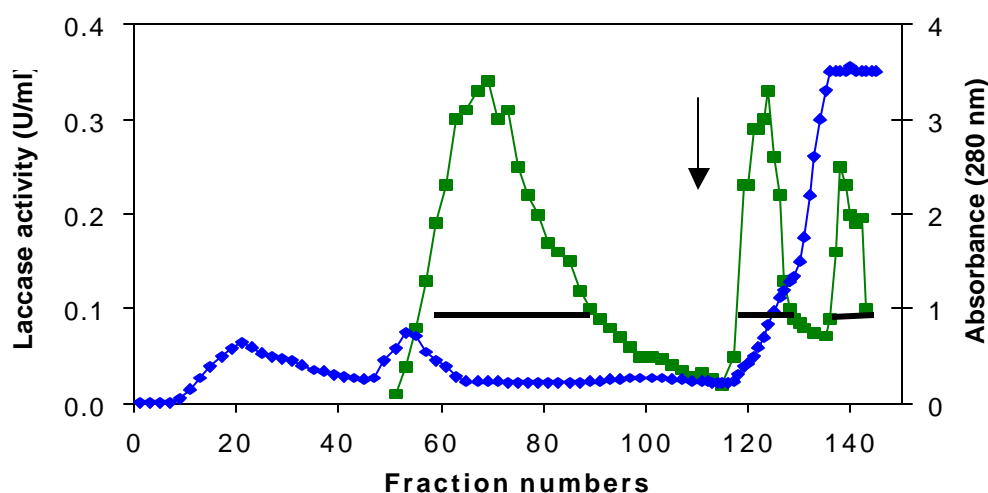


**Figure 4.9.**    Typical elution profile for laccase from *C. micaceus* separated on DE 52 anion exchange chromatography.  The arrow indicates the start of the salt gradient and the horizontal lines indicate the approximate fractions that were pooled during different experiments.  ■ = enzyme activity; ♦ = $A_{280}$

DEAE anion exchange chromatography further separated the laccase proteins from proteins not containing laccase activity. This step was eventually only used for pool 1 laccase (figure 4.10) and the fraction was loaded without prior dialysis. For the second and third pools (figures 4.11 and 4.12) the DEAE Toyopearl chromatography step was omitted as little or no separation of proteins resulted from the use of this resin. No significant effect on the homogeneity of these two laccase fractions could be distinguished.
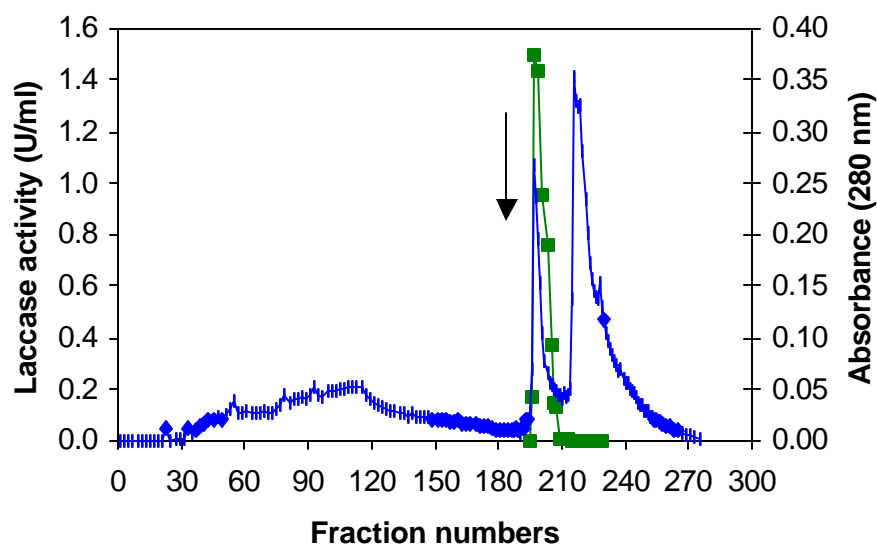


**Figure 4.10.** Typical elution profile of pool 1 laccase from *C. micaceus* on DEAE Toyopearl anion exchange chromatography. The arrow indicates the start of the salt gradient. ■ = enzyme activity; ♦ = $A_{280}$
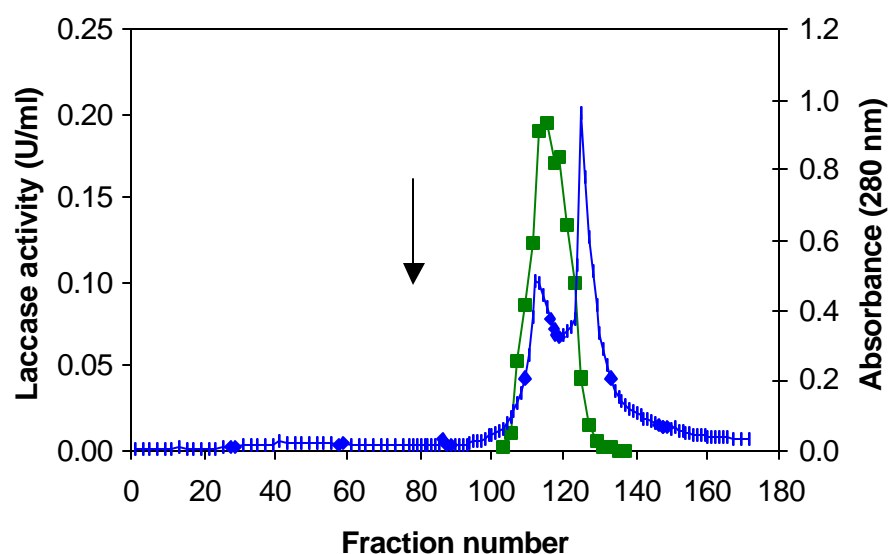
**Figure 4.11.** Typical elution profile of pool 2 laccase from *C. micaceus* on DEAE Toyopearl anion exchange chromatography. The arrow indicates the start of the salt gradient. ■ = enzyme activity; ◆ = $A_{280}$
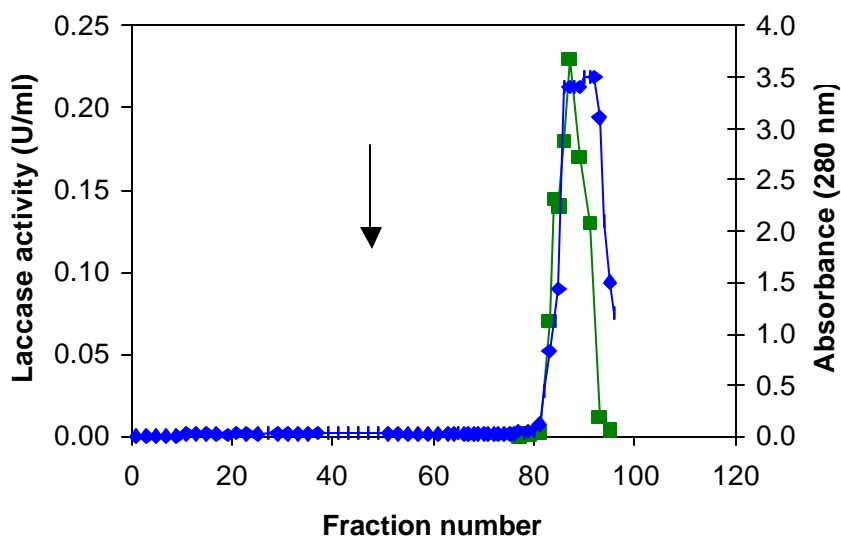


**Figure 4.12.** Typical elution profile of pool 3 laccase from *C. micaceus* on DEAE Toyopearl anion exchange chromatography. The arrow indicates the start of the salt gradient ■ = enzyme activity; ◆ = $A_{280}$

From denaturing gel electrophoresis it appeared that the contaminating proteins and the laccase proteins differed remarkably in size (figure 4.13). Laccase proteins were identified by zymogram analysis with DMP as substrate.
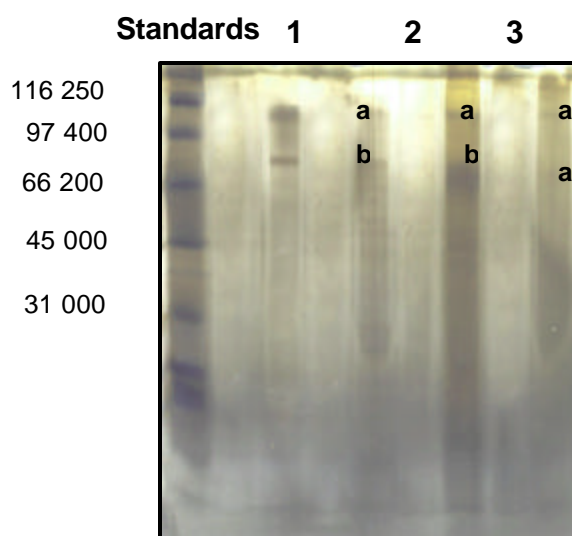


**Figure 4.13.** Results of SDS-PAGE for pool 1, 2 and 3 laccases from *C. micaceus* after DEAE anion exchange chromatography for all the different pools. The letter, **a** represents the different laccases and the letter, **b** represents contaminating proteins. 1 = Pool 1 laccase; 2 = Pool 2 laccase; 3 = Pool 3 laccase.

Size exclusion chromatography was attempted as purification step (figures 4.14 to 4.16) to separate laccase from the contaminating proteins.
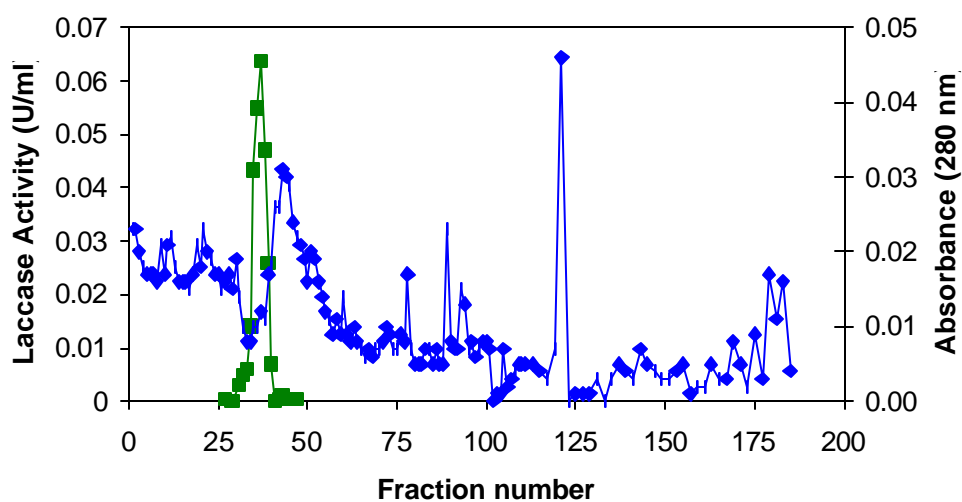
**Figure 4.14.** Elution profile of pool 1 laccase from *C. micaceus* on HW50F Toyopearl gel filtration. ■ = enzyme activity; ♦ = $A_{280}$
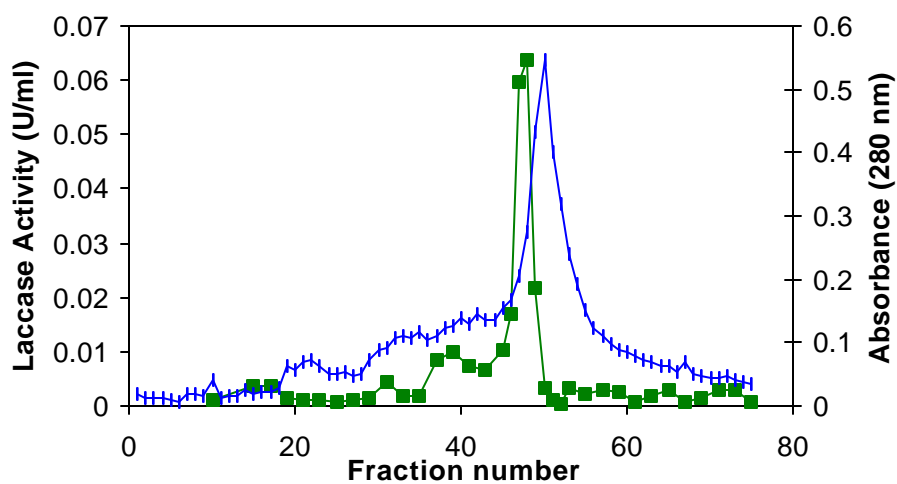


**Figure 4.15.** Elution profile of pool 2 laccase from *C. micaceus* on HW50F Toyopearl gel filtration. ■ = enzyme activity; ♦ = $A_{280}$
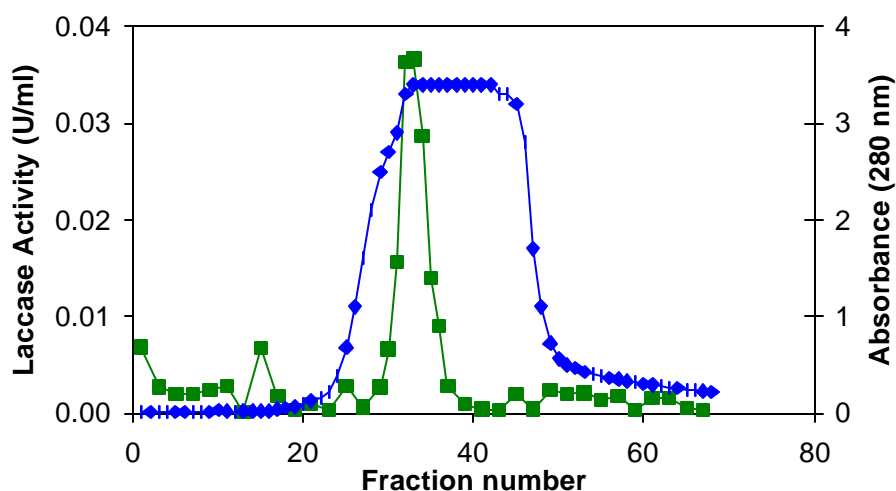
**Figure 4.16.** Elution profile of pool 3 laccase from *C. micaceus* on HW50F Toyopearl gel filtration. ■ = enzyme activity; ♦ = A$_{280}$

Homogeneity was achieved for pool 1 and pool 2, but pool 3 remained contaminated. The protein concentrations of the purified laccases were extremely low. The purification fold of pool 1 laccase was 2738. This is a decrease from the previous purification step (table 4.5) but a homogenous product was obtained. The purification fold of the homogenous pool 2 laccase was approximately 312 (table 4.5). Pool 3 laccase could not be purified to homogeneity by gel filtration.

No other purification steps were successful in purifying the pool 3 laccase and, due to the low yields; further purification of this fraction was abandoned. For each purification attempt a purification table is constructed, to annotate the various informative parameters. Table 4.5 is the purification table constructed to summarise the successful purification of pools 1 and 2 laccase using the second isolation protocol.

**Table 4.5.** Enrichment table for the isolation of *C. micaceus* laccases (second isolation).

| Purification steps | Total Act. (Units) | Total Yield (%) | Specific act. (U/mg) | Purification Fold |
|---|---|---|---|---|
| Culture | 12.4 | 100 | 0.01 | 1 |
| $(NH_4)SO_4$ | 15.1 | 122 | 0.03 | 2.51 |
| Dialysis | 16.6 | 134 | 0.04 | 3.05 |
| DE52 pool 1 | 5.41 | 44 | 2.36 | 179 |
| DE52 pool 2 | 1.74 | 14 | 0.71 | 53.6 |
| DE52 pool 3 | 0.85 | 7 | 0.04 | 2.91 |
| DEAE pool 1 | 3.92 | 32 | 56.2 | 4245 |
| HW50F pool 1 | 0.78 | 6 | 36.1 | 2737 |
| HW50F pool 2 | 0.36 | 3 | 4.11 | 311 |
| HW50F pool 3 | 0.21 | 1 | 0.06 | 2.42 |

## 4.5. Electrophoresis

### 4.5.1. Laccase from *P. sanguineus*

Laccase from *P. sanguineus* was shown to be homogenous according to size with SDS-PAGE (figure 4.17 a) as well as according to charge with Native-PAGE (figure 4.17 b). The relative molecular mass of the laccase protein was determined to be approximately 58 000 relative to the molecular mass markers (figure 4.18). Zymogram analyses were done for laccase activity on both the denaturing as well as the non-denaturing electrophoresis gels. Activity staining of the laccase, with DMP as substrate, revealed that the single protein band corresponded with the position of the laccase activity.
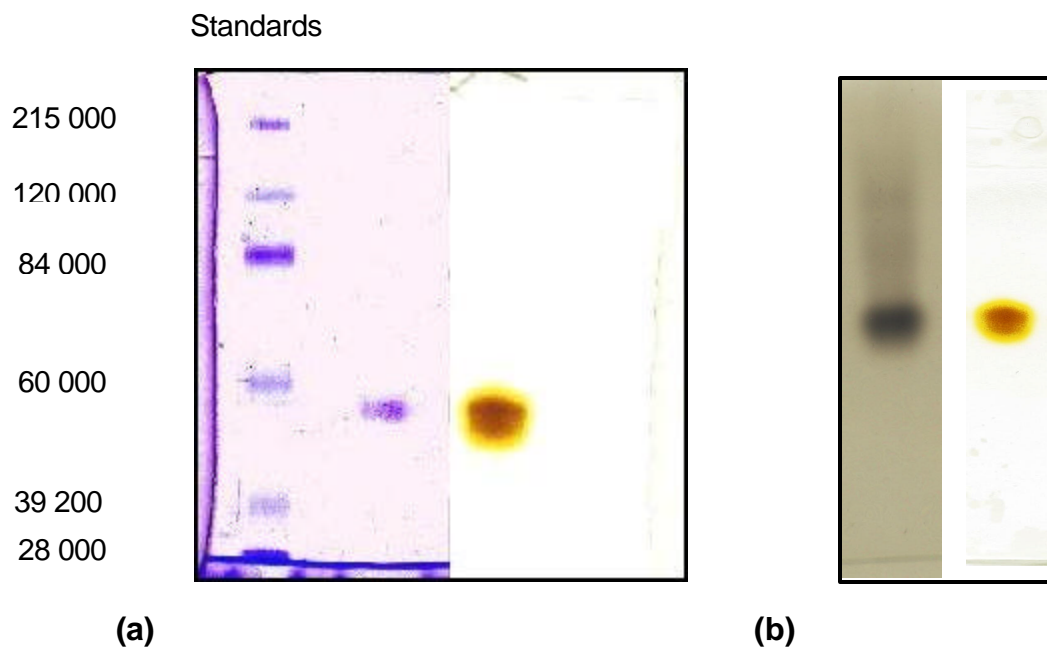


**Figure 4.17**.   (a)Results of SDS-PAGE and zymogram analysis on SDS-PAGE for laccase from *P. sanguinueus* (b) Results of Native PAGE and zymogram analysis on Native PAGE for laccase from *P. sanguineus*.

The molecular mass obtained for laccase from *P. sanguineus* compares well to that expected for typical laccase enzymes (Luisa *et al.*, 1996, Thurston, 1994).  Molecular masses for laccases typically range between 60 000 and 80 000 Da (Palmieri *et al.*, 1997; Farnet *et al.*, 2000; Saloheimo *et al.*, 1991; Fukishima and Kirk, 1995).
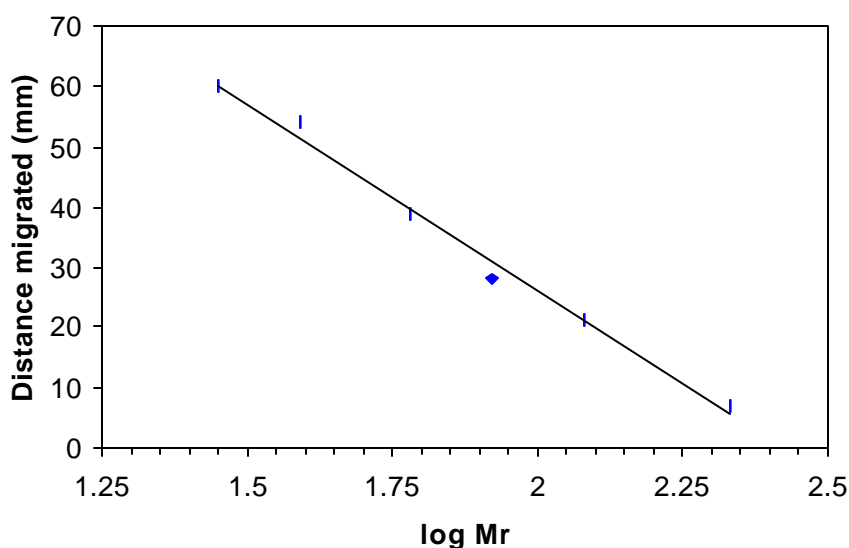


**Figure 4.18.**   Calibration curve for the determination of molecular mass for laccase from *P. sanguineus* relative to the migration of protein standards on denaturing gel electrophoresis.

Homogeneity of the *P. sanguineus* laccase can further be confirmed by iso-electric focussing (figure 4.19).  The apparent iso-electric point (pI) of this enzyme was determined to be 6.7 relative to the standards.

Standards    Laccase

9.3

8.2

6.8

5.9

5.1

4.6
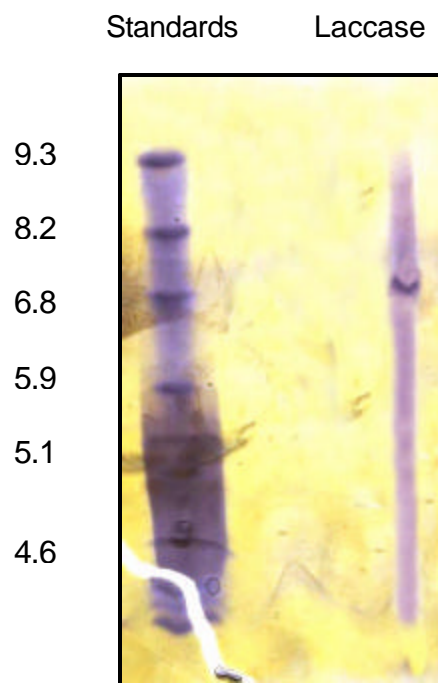
**Figure 4.19.** Results of iso-electric focussing of purified laccase from *P. sanguineus*.

### 4.5.2. Laccase from *C. micaceus*

Three laccase isozymes produced by the fungus *C. micaceus* were identified and separated during anion exchange interaction chromatograpy (figure 4.9). Of the three pools only two were purified to homogeneity (figure 4.20) and their size estimated by comparison with molecular mass standards (figure 4.21). It was determined that both laccase from pool 1 and laccase from pool 2 had an apparent molecular mass of 108 000.
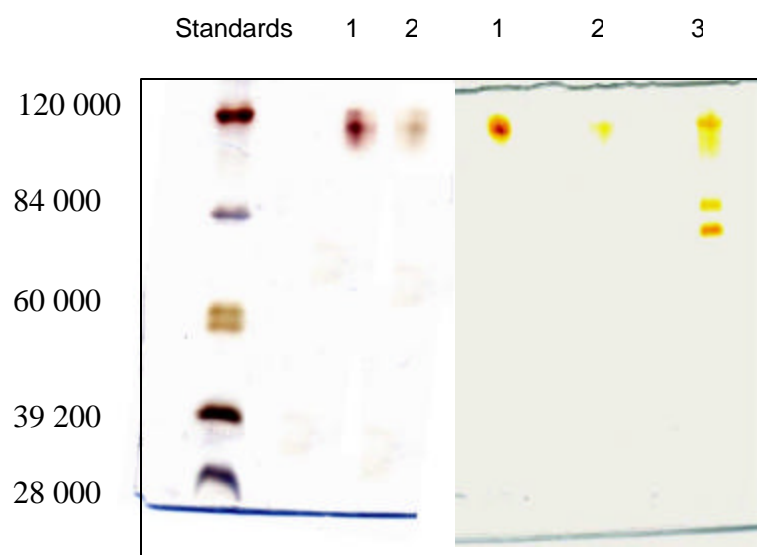
**Figure 4.20.** Homogeneity of laccases from *C. micaceus* on SDS-PAGE and zymogram analysis on denaturing electrophoresis. 1 = pool 1 laccase, 2 = pool 2 laccase, 3 = pool 3 laccase.
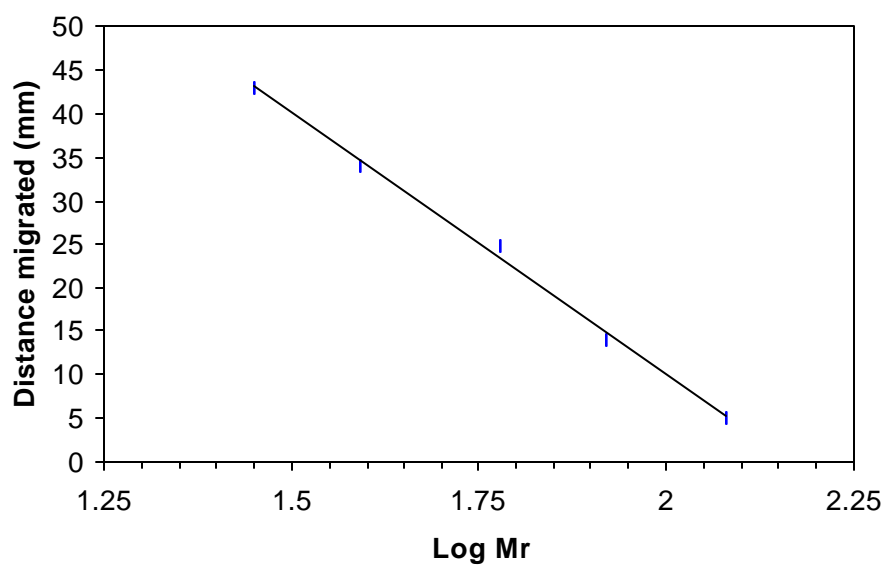


**Figure 4.21.** Calibration curve for the determination of molecular mass of laccases from *C. micaceus,* relative to the migration of protein standards on denaturing gel electrophoresis.

Even though it has been reported that the typical molecular masses for laccases range between 60 000 and 80 000 Da there are many reported cases, which do not adhere to this typical size (Thurston, 1994). Such laccases are produced by *Phanerochaete flavido-albans,* 100 000 Da (Perez *et al*., 1996), *Agaricus bisporus*, 100 000 Da (Perry *et al*., 1993a) and *Aspergillus nidulans*, 110 000 Da (Thurston, 1994).

A total of five laccase isozymes were identified by zymogram analysis for the isolated fractions from *C. micaceus* (figure 4.22). Three bands of laccase activity were identified for the third pool of *C. micaceus* laccase (figure 4.20 and 4.22). Zymogram analysis on denaturing electrophoresis revealed that the laccase from pool 1, 2 and the largest laccase from pool 3 appear to be the same size (figure 4.20). Non-denaturing zymograms show that the five isozymes migrate differently and therefore have different charge properties (figure 4.22). This proves that there are indeed at least five different isozymes of laccase produced by *C. micaceus* under the current growth conditions.
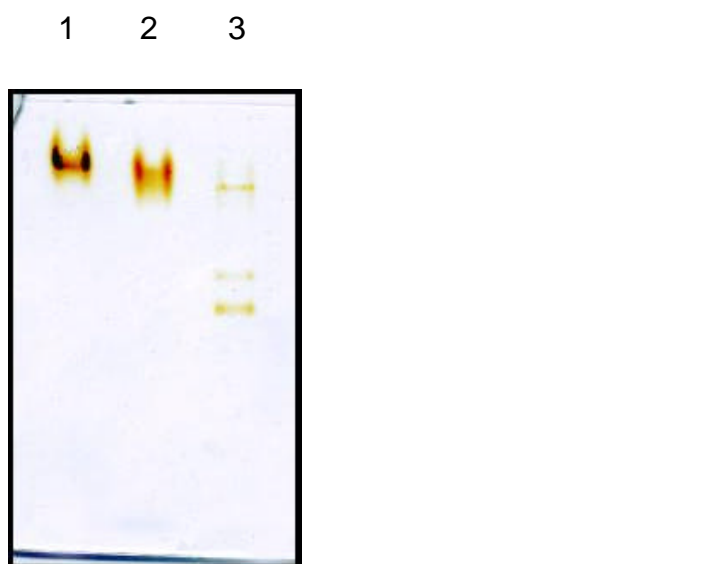


**Figure 4.22.** Results of zymogram analysis of laccases from *C. micaceus* on non–denaturing gel electrophoresis. (1 = pool 1 laccase, 2 = pool 2 laccase, 3 = pool 3 laccase).

There are many reported cases that show a single fungal species able to express more than one laccase enzyme. Different culture conditions, for instance, may lead to the production of different isozymes by the same fungus (Bollag and Leonowicz, 1984, Wahleithner *et al.*, 1996, Palmieri *et al.*, 1997, Farnet *et al.*, 2000). Examples of organisms able to produce more than one laccase isozyme include, *Podospora anserina*, 3 isozymes (Thurston, 1994), *Agaricus bisporus*, 2 isozymes (Perry *et al.*, 1993b), *Pleurotus ostreatus*, 3 isozymes (Palmieri *et al.*, 1997) and *Rhizoctonia solani*, 4 isozymes (Wahleitner *et al.*, 1996).

## 4.6. Optimum temperature

The optimum temperature of laccase from *P. sanguineus* was determined to be approximately 55 °C (figure 4.23). The temperature range where the enzyme is active is remarkably wide. Significant activity is detected as low as 25 °C and approximately 95% of activity is maintained at temperatures as high as 70°C. Optimum temperatures are significantly affected by the assay used so this data should be interpreted with care. Temperature stability studies are usually more informative. According to literature the typical optimum temperature range for laccases are 50 °C to 60 °C (Luisa *et al.*, 1996). *P. sanguineus* laccase therefore exhibits a typical optimum temperature.
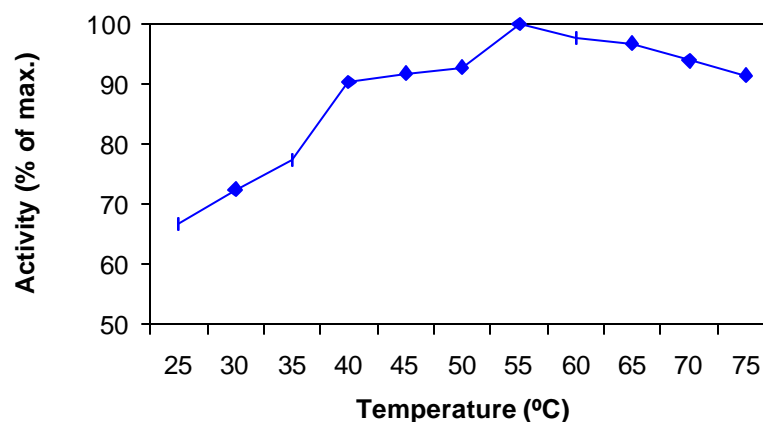


**Figure 4.23.** The effect of temperature on the activity of *P. sanguineus* laccase.

The three pools of laccase, isolated from *C. micaceus*, reacted similar to *P. sanguineus* laccase with respect to the effect of temperature. Optimum temperature of 65 °C, 60 °C and 65 °C were obtained for pool 1, 2 and 3 laccases, respectively (figure 4.24). Once again the wide temperature range over which the enzyme is active is remarkable. These values obtained for laccases from *C. micaceus* are slightly higher than the typical optimum temperatures expected for laccases (Luisa *et al.*, 1996). Examples of other laccases with higher temperature optima than what is commonly expected were produced by the fungus, *Marasmius quercophilus* strain 17 (Farnet *et al.*, 2000) and *Coriolus hirsutus* (Shin and Kim, 1998). The profile of the effect of temperature on *M. quercophilus* laccases (Farnet *et al.*, 2000) for both the constitutive and induced forms corresponds to what was found for laccases from *C. micaceus*.
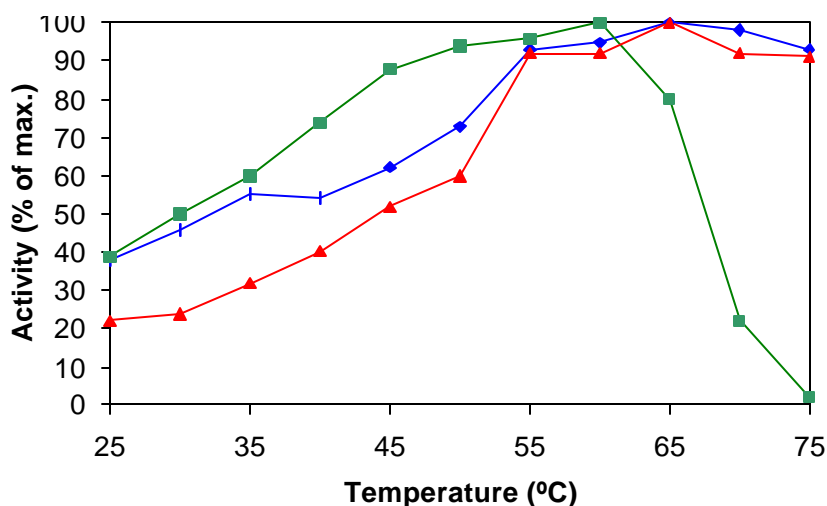


**Figure 4.24.** The effect of temperature on the activity of laccases isolated from *C. micaceus*. ♦ = pool 1 ; ■ = pool 2 ; ▲ = pool 3

## 4.7. Thermostability

The temperature stability of the various laccases under study (table 4.6) was obtained by incubating the enzyme for different lengths of time at different temperatures as described

in section 3.12. Laccase from *P. sanguineus* is more thermostable than what is usually expected for typical laccases (Luisa et al., 1996, Shin and Kim, 1998, Thakker *et al.*, 1992). For temperatures 35 °C, 45 °C and 55°C a $t_{1/2}$ cannot be determined from our results as half of the laccase activity had not been lost after the allocated 240 minutes.

**Table 4.6.** Half-lives ($t_{1/2}$) determined for different laccases at different temperatures.

| Temperatures | $t_{1/2}$ (minutes) | | | |
| --- | --- | --- | --- | --- |
| | *P. sanguineus* | *C. micaceus* pool 1 | *C. micaceus* pool 2 | *C. micaceus* pool 3 |
| 45 °C | > 240 | > 240 | > 240 | > 240 |
| 55 °C | > 240 | 10 | 32 | 220 |
| 65 °C | 200 | 5 | 2 | 4 |
| 75 °C | 170 | < 15 seconds | < 15 seconds | < 15 seconds |

Pool 1, 2 and 3 laccases from *C. micaceus* were stable at 45 °C (table 4.6). At 75 °C and 85 °C no activity was detected after the first incubation period. Compared to the thermal stability reported for *P. sanguineus* laccase, the thermal stability data for laccases from *C. micaceus* is rather poor, but it compares well to the thermostabilities reported for laccases from other white-rot fungi (Farnet *et al.*, 2000).

## 4.8. The effect of pH on laccase activity

Four substrates were used to determine the effect of pH on laccase activity, as laccase enzymes tend to react differently to pH with different substrates (Palmieri *et al.*, 1997, Fukishima and Kirk, 1995, Xu, 1997).

Pool 2 and pool 3 laccases from *C. micaceus* had pH optima in the neutral range (table 4.7), which should arouse much interest in the biobleaching industry as their processes require neutral to alkaline conditions of pH (Heinzkill *et al*., 1998). The pH optima for these two laccases (table 4.8) are also somewhat higher than the expected pH 3 to 5 of typical laccases (Luisa *et al.*, 1996). The pH optima obtained for pool 1 laccase from *C. micaceus* and *P. sanguineus* laccase were more acidic and representative of typical laccases (Luisa *et al.*, 1996).

**Table 4.7.**   Optimal pH values obtained with the four different isolated enzymes for four different substrates (ABTS, DMP, Syringaldazine, Guaiacol).

| Substrates | *P. sanguineus* | *C. micaceus* pool 1 | *C. micaceus* pool 2 | *C. micaceus* pool 3 |
|---|---|---|---|---|
| ABTS | 3 | 3 | 3 | 3 |
| DMP | 4 | 4 | 7.5 | 6.5 |
| Syringaldazine | 5 | 5 | 7 | 7 |
| Guaiacol | 4 | 4 | ND | ND |

(ND = Not Detected)

In 1996 a study was done by Feng Xu to elucidate the reason for the different pH dependencies of laccases with different substrates. The dependence of laccase on pH usually renders a bell-shaped profile as can be seen for DMP, syringaldazine and guaiacol (figure 4.25). This bi-phasic profile is the result of two opposing effects. The first is due to the redox potential difference between a reducing substrate and the Type 1 copper centre of laccase, where the substrates dock. Here the electron transfer rate is favoured for phenolic substrates at a high pH. The second is generated by the binding of a hydroxide anion to the Type 2/ Type 3 copper centre of laccase, which inhibits the binding of $O_2$, the terminal electron acceptor, and therefore inhibits the activity at a higher pH because of the increased amount of OH$^-$ ions.
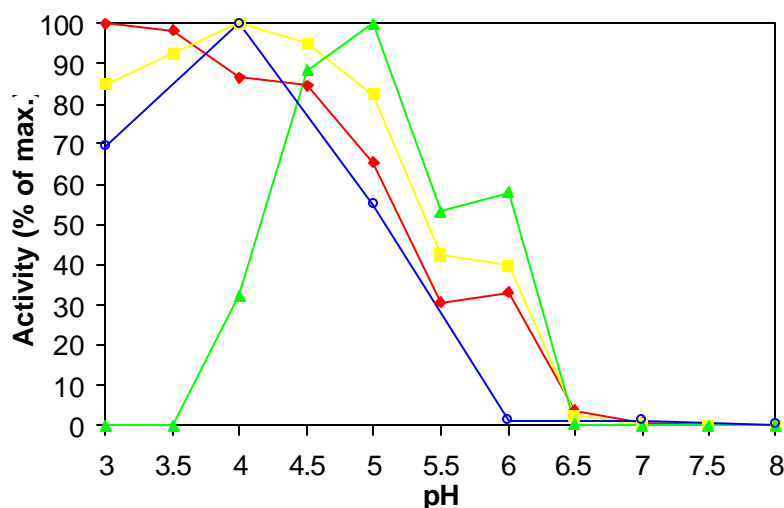
**Figure 4.25.** pH profiles of laccase from *P. sanguineus* with different substrates. This graph is representative of the shape of profiles obtained for different substrates. ♦ = ABTS ; ■ = DMP ; ▲ = syringaldazine; ● = guaiacol.

ABTS is the only substrate in our studies that does not adhere to this principle. With ABTS there is rather a monotonic decline than a bell-shaped profile (figures 4.25). ABTS is regarded as a non-phenolic substrate in contrast to the three phenolic substrates discussed in the previous paragraph. The oxidation of ABTS to the cation radical by laccase does not involve protons and its redox potential is therefore independent of pH (Xu *et al*., 1996). The contribution of the hydroxide anion therefore leads to a monotonic decline (Xu, 1997).

## 4.9. Substrate specificity

The ability of laccase to oxidise aromatic compounds (especially phenols) is of interest in delignification (Archibald *et al.*, 1997; Reid and Paice, 1994; Bourbonnais *et al.*, 1995), textile dye or stain bleaching (Pointing and Vrijmoed, 2000; Fu and Viaraghavan, 2001) and the detoxification of contaminated soil and water (Filazzola *et al.*, 1999). Laccase is also regarded as the simplest enzyme to use in defining the structure-function relationships of copper containing oxidases (Xu, 1996; Germann and Lerch, 1986; Ducros *et al.*, 1998).

Polyphenol oxidases are copper proteins with the common feature that they are able to oxidise aromatic compounds with molecular oxygen as the terminal electron acceptor. Polyphenol oxidases are associated with three types of activities:

Catechol oxidase or o-dipenol:oxygen oxidoreductase (EC 1.10.3.1)

Laccase or p-diphenol:oxygen oxidoreductase (EC 1.10.3.2)

Cresolase or monophenol monooxygenase (EC 1.18.14.1)

(Mayer, 1987)

These different enzymes can therefore be differentiated on the basis of substrate specificity. Catechol oxidases or tyrosinases have o-diphenol as well as cresolase activity. Cresolase activity refers to the oxidation of L-tyrosine. Laccases have o- and p-diphenol activity, usually with more affinity towards the p-diphenols. Catechol oxidases and laccases therefore catalyse an overlapping range of substrates. Only tyrosinases possess cresolase activity and only laccases have the ability to oxidise activated methoxyphenols such as syringaldazine (Thurston, 1994; Eggert *et al.*, 1996). There has only been one reported incidence of an enzyme exhibiting both tyrosinase and laccase activity (Sanchez-Amat and Solano, 1997).

The experimental kinetic data (averages of triplicate data) was fitted mainly to single-substrate (Michaelis-Menten) kinetics by nonlinear regression analysis (figure 4.26). The data observed for Michaelis-Menten kinetics with different substrates is illustrated with DMP as substrate in figure 4.26(a-d).
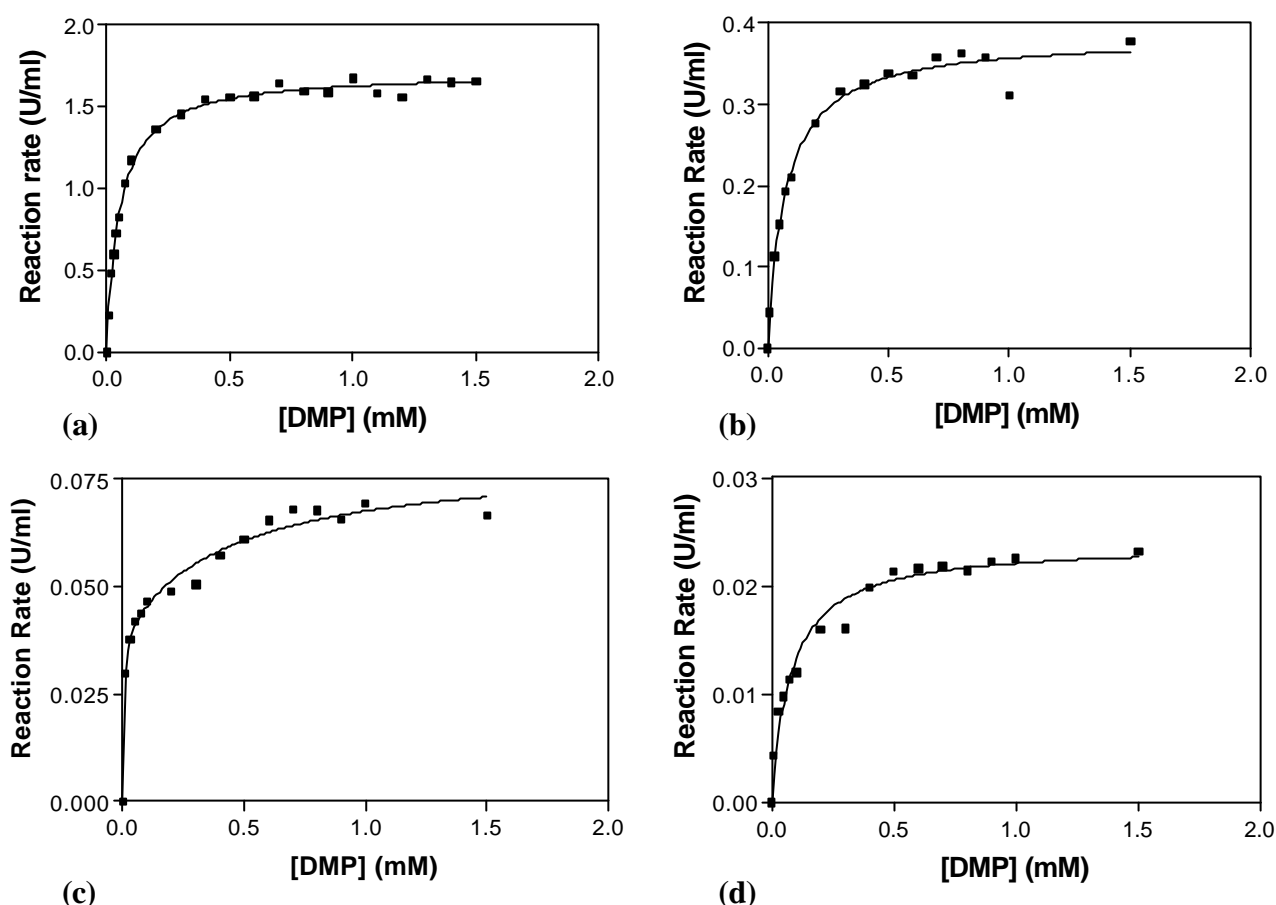
**Figure 4.26.** Illustration of Michaelis-Menten kinetics of the laccase-catalysed oxidation of DMP as substrate for (a) *P. sanguineus* laccase, (b) *C. micaceus* pool 1 laccase, (c) *C. micaceus* pool 2 laccase and (d) *C. micaceus* pool 3 laccase.

Kinetic parameters for the four identified laccase fractions were evaluated for three substrates (ABTS, syringaldazine and DMP) (Palmieri *et al.*, 1997; Giardina *et al.*, 1999). In order to enable comparison of the specificities of the enzymes to different substrates, the $V_{max}$ values of the *C. micaceus* laccases were normalised to the $V_{max}$ value of laccase from *P. sanguineus* with DMP as substrate (table 4.8).

The $K_m$ values (table 4.8) of pool 1 and pool 2 laccases from *C. micaceus* toward the various substrates indicated that the binding affinities toward the different substrates were in the same order: ABTS> DMP> syringaldazine. The binding affinities ($K_m$ values) of pool 3 laccase from *C. micaceus* for the different substrates was in the order DMP> ABTS> syringaldazine and for laccase from *P. sanguineus* the order was DMP> syringaldazine> ABTS (table 4.8). Giardina *et al.* (1999), using the same three substrates, reported the highest binding affinity for laccase from

*Pleurotus ostreatus* towards syringaldazine. The order of binding preference of substrates therefore differs significantly between different laccases.

The values reported for the parameter, $k_{cat}$, is a representation of the rate of the catalytic process. The ratio $k_{cat}/K_m$ gives an indication of the efficiency of each enzyme and therefore allows us to compare the efficiencies the different enzymes. Table 4.8 shows that laccase from *C. micaceus* pool 1 is the most efficient enzyme as it exhibits the highest values for the $k_{cat}/K_m$ ratio for all the substrates studied. Pool 2 laccase from *C. micaceus* is the second most efficient for the oxidation of ABTS and syringaldazine but is less reactive towards DMP than is laccase from *P. sanguineus*. The overall order of efficiency of the different laccases studied is: *C. micaceus* pool 1 laccase> *C. micaceus* pool 2 laccase> *P. sanguineus* laccase> *C. micaceus* pool 3 laccase.

**Table 4.8.**    Kinetic parameters for laccases from *P. sanguineus* and pool 1,2 and 3 from *C. micaceus* for three different substrates.

| Substrates | Kinetic Parameters | *P. sanguineus* | *C. micaceus* Pool 1 | *C. micaceus* Pool 2 | *C. micaceus* Pool 3 |
|---|---|---|---|---|---|
| ABTS | $K_m$ (mM) | 0.13 | 0.022 | 0.008 | 0.16 |
| | $k_{cat}$ (sec$^{-1}$) | 48 | $6.4 \times 10^3$ | $1.2 \times 10^3$ | 22 |
| | $k_{cat}/K_m$ (mM$^{-1}$. sec$^{-1}$) | 369 | $2.9 \times 10^5$ | $1.5 \times 10^5$ | 137.5 |
| DMP | $K_m$ (mM) | 0.052 | 0.075 | 0.025 | 0.08 |
| | $k_{cat}$ (sec$^{-1}$) | 21 | 1750 | 414 | 12 |
| | $k_{cat}/K_m$ (mM$^{-1}$. sec$^{-1}$) | 404 | $2.3 \times 10^4$ | $1.7 \times 10^4$ | 150 |
| Syr | $K_m$ (mM) | 0.083 | 0.42 | 0.26 | 0.25 |
| | $k_{cat}$ (sec$^{-1}$) | 7.4 | $1.3 \times 10^3$ | 251 | 4.4 |
| | $k_{cat}/K_m$ (mM$^{-1}$. sec$^{-1}$) | 89 | $2.97 \times 10^3$ | 965 | 18 |

Nineteen different aromatic compounds (figure 4.27) were chosen to study the substrate specificity (figure 4.28) of the isolated pool 2 laccase from *C. micaceus* and the *P. sanguineus* laccase. Pool 2 laccase from *C. micaceus* was chosen because its ability to function at a neutral pH and because of its homogeneity. The substrates were chosen according to the nature and position of substituents on the phenolic ring. ABTS, a non-phenolic compound and common substrate as well as mediator, was included in the study. ABTS has a higher redox potential than laccase itself and the

mechanism by which laccase-catalysed oxidation of ABTS occurs is not yet fully understood (Bourbonnais *et al.*, 1997a and b). $V_m$ values were normalised as described above.
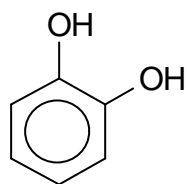


**Pyrocatechol**
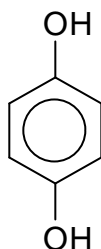
**Hydroquinone**
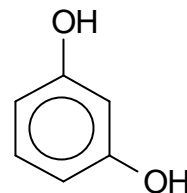
**Resorcinol**
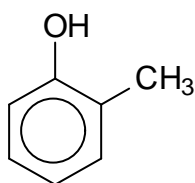
**o-Cresol**
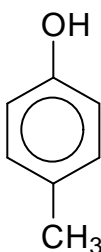
**p-Cresol**
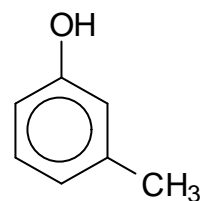
**m-Cresol**
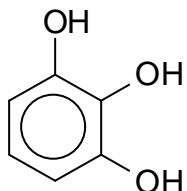
**Pyrogallol**
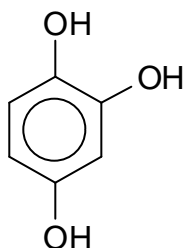
**1,2,4-Benzenetriol**
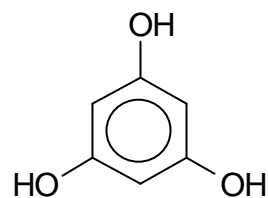
**Phloroglucinol**
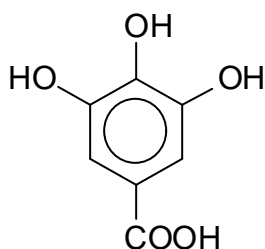
**Gallic acid**
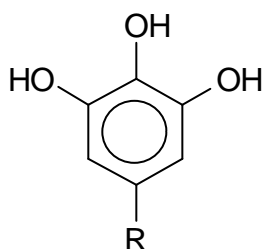
**Tannic acid**
**(functional group)**
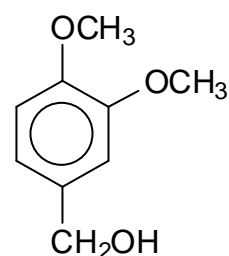
**Veratryl alcohol**
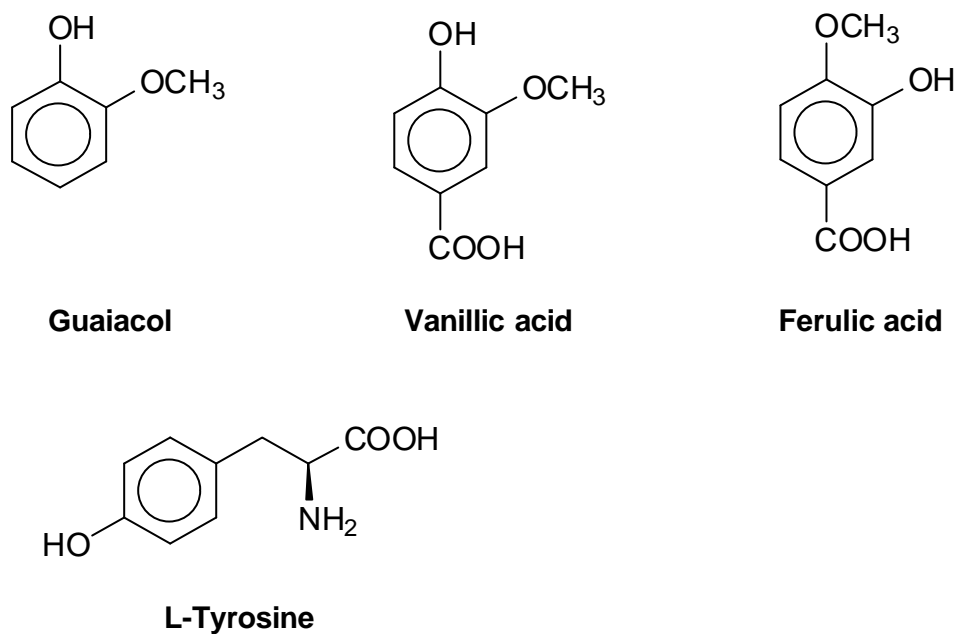
**Figure 4.27.** Structures of phenolic substrates used in the evaluation of substrate specificity

The wavelengths of maximum absorbance for the oxidised substrates by the isolated laccases are reported in table 4.9. The wavelength of maximal change was mainly approximately 390 nm, which is the wavelength commonly used to detect the formation of quinone structures.

**Table 4.9.**     Wavelengths of maximal absorption of laccase-oxidisable aromatic compounds.

| Substrates | $\lambda$ (max. change, nm) |
|---|---|
| ABTS | 420 |
| 2,6-DMP | 477 |
| Syringaldazine | 525 |
| Guaiacol | 468 |
| Catechol | 390 |
| Pyrogallol | 390 |
| Hydroquinone | 390 |
| 1,2,4-Benzenetriol | 480 |
| Tannic acid | 458 |
| Gallic acid | 388 |
| Vanillic acid | 390 |
| o-Cresol | 410 |
| p-Cresol | 390 |

$V_m/K_m$ ratios were expressed for the reactions of laccase towards the different substrates, as extinction coefficients are not available for all of the substrates studied. Laccase activity toward the substituted phenols was in the following order: meta< para< ortho substituted phenols (table 4.10).   Catechol (-OH in ortho-position), as revealed by the $V_m/K_m$ ratios (table 4.10) was more readily oxidised than its structural isomers hydroquinone (-OH in para-position) and resorcinol (-OH in meta-position). Of the three isomers of cresol, *o*-cresol (-CH$_3$ in ortho position) was oxidised to the greatest extent (table 4.10) while *m*-cresol was the least oxidised.   For both hydroquinone and *p*-cresol (para-substitutions) very low oxidation by the laccase was noted during wavelength scans (table 4.9), but the activity was too low to quantify reliably (Ranocha *et al.*, 1999).
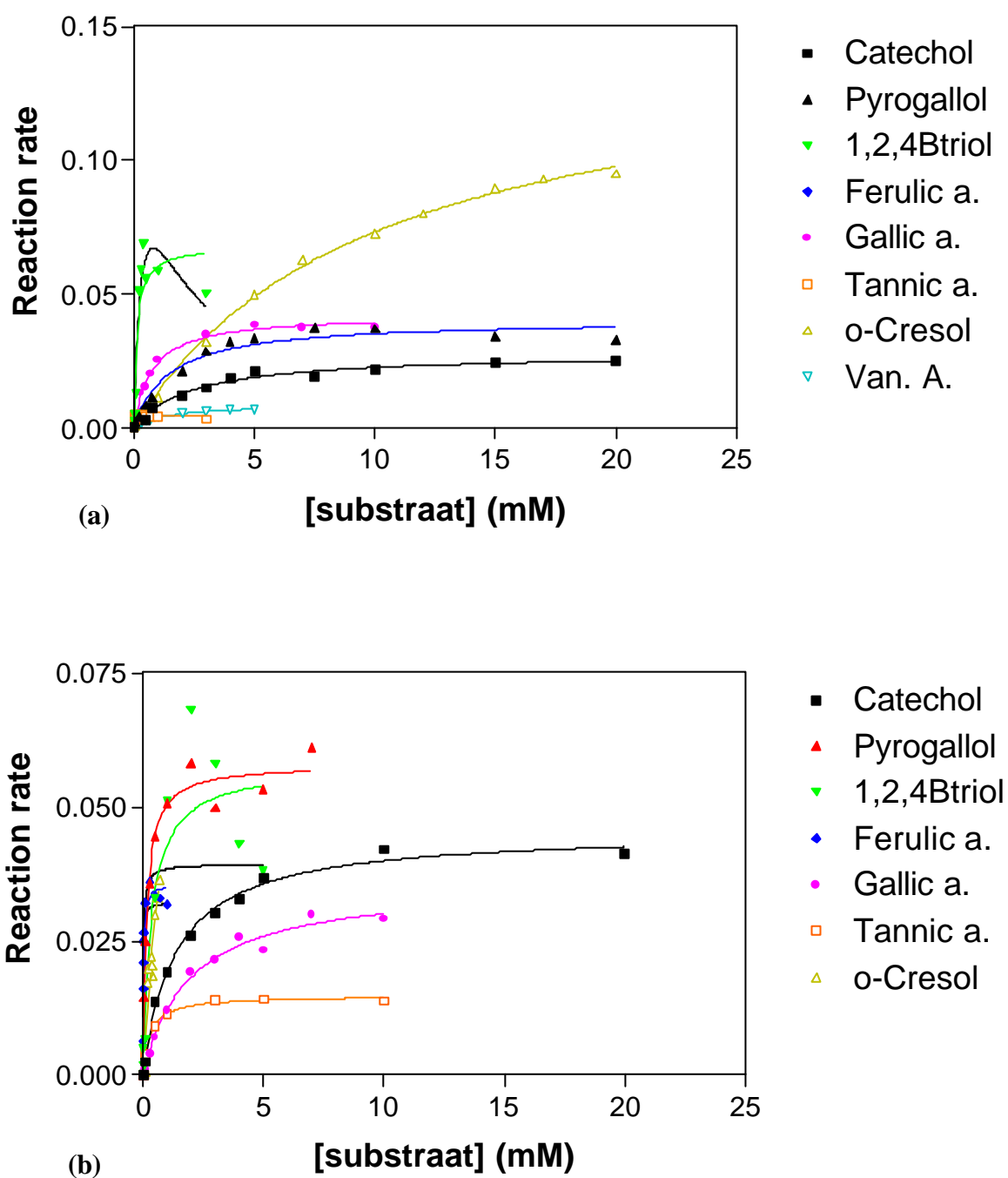
**Figure 4.28.** Michaelis-menten kinetics of various substrates oxidised by laccases from (a) *P. sanguineus* and (b) *C. micaceus* (pool 2).

**Table 4.10.** Kinetic parameters for the oxidation of various substrates by laccases from *P. sanguineus* and *C. micaceus*.

| Substrates | *P. sanguineus* laccase | | | *C. micaceus* laccase (pool 2) | | |
|---|---|---|---|---|---|---|
| | $K_m$ (mM) | $V_m$ | $V_m/K_m$ | $K_m$ (mM) | $V_m$ | $V_m/K_m$ |
| ABTS | 0.13 | 3.88 | 29.6 | 0.0084 | 5.1 | 610 |
| 2,6-DMP | 0.05 | 1.7 | 32.9 | 0.025 | 1.7 | 67 |
| Syringaldazine | 0.08 | 0.59 | 7.2 | 2.2 | 26 | 12 |
| Guaiacol | 0.35 | 0.03 | 0.089 | - | ND | - |
| Catechol | 2.4 | 0.028 | 0.012 | 1.37 | 1.2 | 0.9 |
| Pyrogallol | 1.48 | 0.04 | 0.027 | 0.15 | 1.5 | 10.1 |
| Resorcinol | - | 0 | - | - | 0 | - |
| Hydroquinone | - | >0 | - | - | 0 | - |
| 1,2,4-Benzenetriol | 0.2 | 0.08 | 0.4 | 1.27 | 3 | 2.3 |
| Phloroglucinol | - | 0 | - | - | 0 | - |
| Tannic acid | 0.01 | 0.0047 | 0.38 | 0.29 | 0.39 | 1.3 |
| Gallic acid | 0.7 | 0.04 | 0.057 | 1.96 | 0.9 | 0.48 |
| Vanillic acid | 1.36 | 0.0088 | 0.0065 | - | 0 | - |
| Ferulic acid | - | 0 | - | 0.02 | 0.9 | 43 |
| Veratryl alcohol | - | 0 | - | - | 0 | - |
| *o*-Cresol | 9.97 | 0.15 | 0.015 | 1.46 | 2.9 | 2 |
| *p*-Cresol | - | 0 | - | - | 0 | - |
| *m*-Cresol | - | 0 | - | - | 0 | - |
| Tyrosine | - | 0 | - | - | 0 | - |

No activity was noted for meta-substituted phenols whatsoever. The lack of reaction towards such 3-substituted phenolic compounds could be due to the fact that an aryloxy radical is formed during the initial step by the removal of one electron and one hydrogen atom from the hydroxyl group. Aryloxy radicals generate resonance structures that are stabilised in para and ortho positions. Meta substituents do not allow stabilisation and this implies a low reactivity of laccases toward meta-substituted phenols (Jolivalt *et al.*, 1999). Groups that are meta-orienting reduce the electron density at the hydroxyl group.

Laccases are known to have *p*-diphenol as well as *o*-diphenol activity, but they mostly have a higher affinity and activity toward the *p*-diphenols (Xu, 1996; Filazzola *et al.*,

1999; Sanchez-Amat and Solano, 1997). This is not the case with the two laccases under current investigation. The correlation between the position of the substituent groups and laccase activity found for laccases from *P. sanguineus* and *C. micaceus* is supported by a report by Jolivalt *et al.* (1999).

From the $V_m/K_m$ ratios reported in table 4.10 it was apparent that syringaldazine was oxidised efficiently by both laccases but there was no activity towards L-tyrosine. Laccases from both *C. micaceus* and *P. sanguineus* therefore classify as true laccases according to substrate specificity.

Compounds typified by three hydroxyl groups (pyrogallol, tannic acid, gallic acid) were oxidised efficiently (table 4.10). Laccases had higher affinities (according to $K_m$ values) toward these compounds than towards the compounds with two adjacent hydroxyl groups such as catechol.

1,2,4-Benzenetriol consists of one *o*-orienting group and one *p*-orienting group (-OH) (figure 4.27). Laccase from *P. sanguineus* had a higher binding affinity ($K_m$ values, table 4.10) for 1,2,4-benzenetriol than for pyrogallol (containing ortho and meta-substituents). This supports the supposition that a meta-substituent reduces the electron density of the oxidisable group. *Coprinus micaceus* laccase however had a higher binding affinity (lower $K_m$) for pyrogallol than for 1,2,4-benzenetriol. It could be because with pyrogallol as substrate the enzyme can bind the substrate in two possible orientations.

Dimethoxy substituted phenols such as DMP were more readily oxidised (table 4.10) than phenols with only one methoxy substitution, such as ferulic and vanillic acids. The same findings were reported by Palmieri *et al.* (1997). Ferulic acid and vanillic acid are structural isomers, each containing one meta-substitution and one para-substitution (figure 4.27). Laccase from *P. sanguineus* was able to oxidise vanillic acid and laccase from *C. micaceus* was able to oxidise ferulic acid but not vice versa (table 4.10). This could be an indication of the difference in the formation of the active sites of these two laccases. Phloroglucinol could not be oxidised by either of the laccases.

Guaiacol, catechol and *o*-cresol are ortho-substituted phenols with different groups in the ortho position (figure 4.27). It seems that groups such as methoxy groups ($OCH_3$) and hydroxyl groups (-OH) allow the adjacent hydroxyl group to be oxidised efficiently by the enzyme. But groups such as methyl groups (-$CH_3$) in the same position, which do not have a lone electron pair, have a weaker effect (Garzillo *et al.*, 1998).

*Coprinus micaceus* laccase exhibits very low $K_m$ values for ABTS, DMP and tannic acid, indicating very high binding affinity toward these substrates (table 4.10). Laccase from *P. sanguineus* has high binding affinities towards DMP and syringaldazine (table 4.10). In all instances *C. micaceus* laccase was more reactive towards substrates than laccase from *P. sanguineus* as can be seen from the $V_m/K_m$ values in table 4.10.

The size of substituents could have a steric effect, but the substituents involved in the current experiment were not large enough to cause sufficient hindrance. We can therefore deduce from our results that for substrates with small substitutions that are oxidisable by laccase, electronic contribution plays a more important role than steric effect when determining activity (Xu, 1996).

All substrates that were oxidised by the laccases had a common motif of two adjacent substituents (figure 4.29). In the case of pyrogallol and tannic acid, where there are three adjacent substituents, the $K_m$ values (table 4.10) decreased indicating an increased binding affinity. It could be possible that this motif is necessary for the substrate to bind the active site. When there are three adjacent substituents there are two possible ways in which the active site of the enzyme can bind to the substrate (figure 4.29) leading to an apparent increase in the binding affinity.
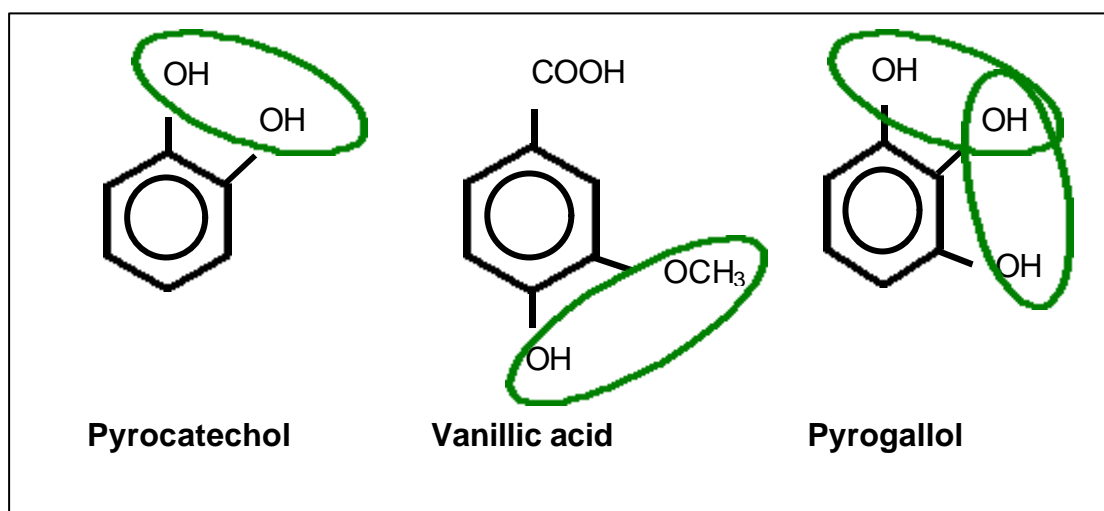
**Figure 4.29.** Schematic representation of the hypothetical motif presented by phenolic substrates, necessary for binding to the laccase active site.

## 4.10. Inhibition studies

Six potential inhibitors (sodium azide, cysteine, EDTA, $F^-$, $Cl^-$, and $I^-$) were evaluated to test the inhibition properties of laccases. Sodium azide was the most efficient inhibitor (figure 4.30). EDTA and cysteine inhibited the laccases to a lesser extent. The two laccases were inhibited in the same relative order by the various inhibitors, but the degree of inhibition between the two enzymes differed remarkably. Laccase from *P. sanguineus* was much more susceptible to inhibition than laccase from *C. micaceus* (figure 4.30). The different kinetic parameters will be discussed at a later stage.
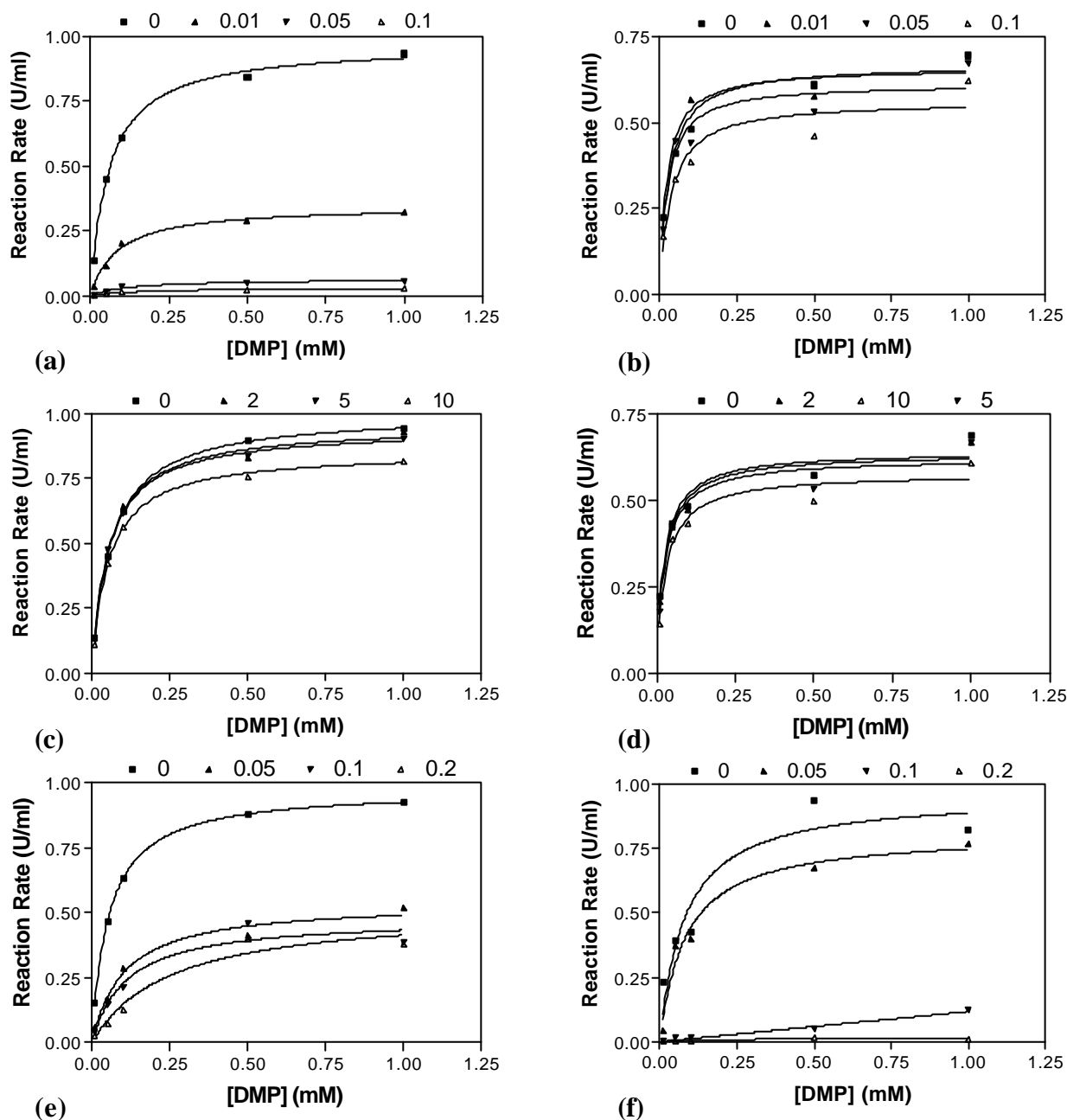
**Figure 4.30.** Inhibition of laccases from *P. sanguineus* (a, c, e) and *C. micaceus* (b, d, f) by sodium azide (a and b), EDTA (c and d) and cysteine (e and f) in the presence of DMP as substrate. The legend above each graph depicts the inhibitor concentrations specific to each inhibitor.

Halides are known laccase inhibitors (Xu, 1996; Garzillo *et al.*, 1998). The effects of three halides (F⁻, Cl⁻ and I⁻) were investigated for laccases from *P. sanguineus* and *C. micaceus* (figure 4.31).

Fluoride was found to be an effective inhibitor for both laccases (figure 4.31), although *C. micaceus* required higher concentrations of fluoride before total inhibition was reached. Chloride was less effective than fluoride for the inhibition of *P. sanguineus* laccase and had no inhibitory effect on laccase from *C. micaceus*. Iodide had no inhibitory effect on either of the laccases. On the contrary it seemed to react as a substrate (table 4.11). The order of inhibition of laccases by halides is F> Cl> I⁻. This phenomenon has been attributed to the limited accessibility of the T2/T3 copper site by larger halides (Xu, 1996, Garzillo *et al.*, 1998).
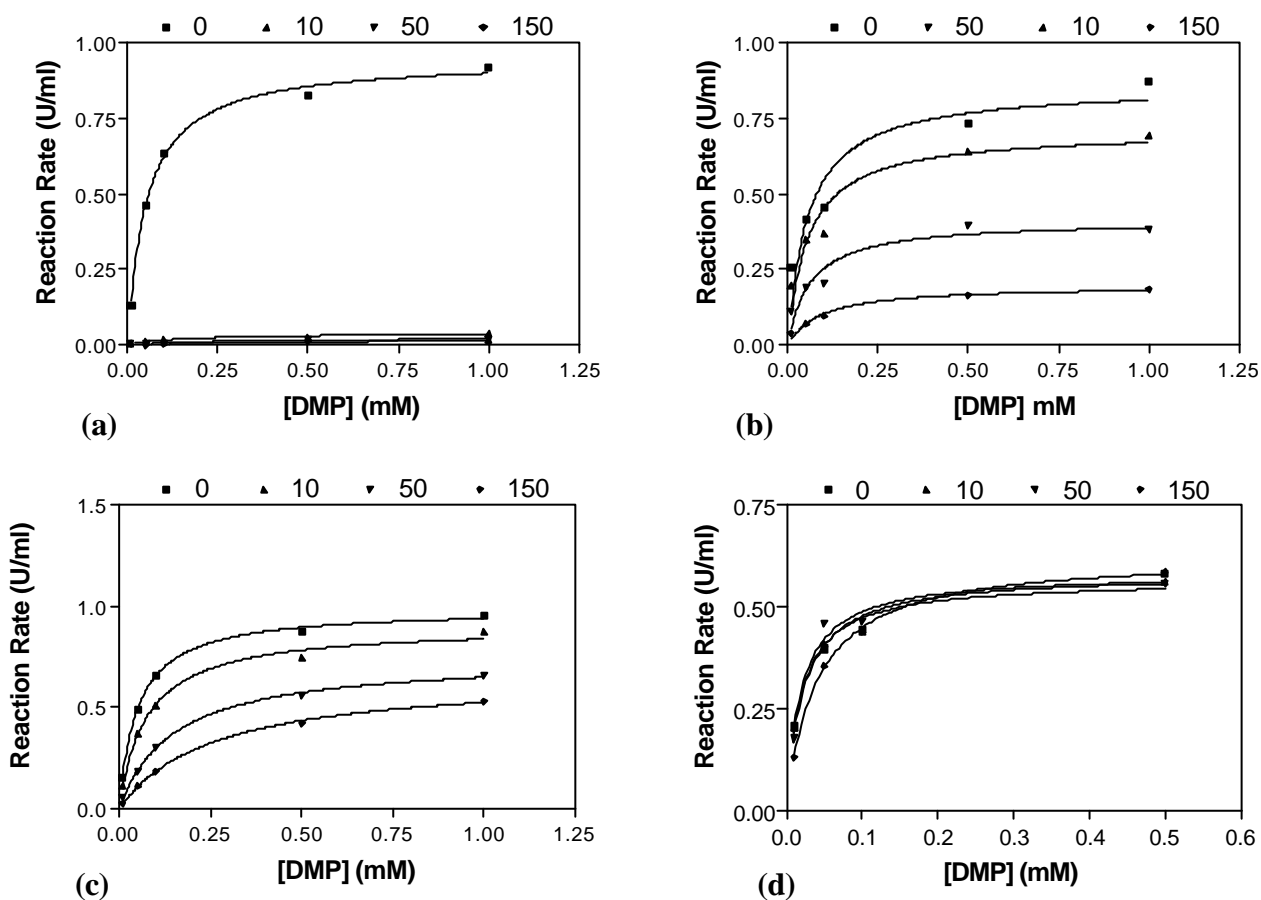


**Figure 4.31.**     Inhibition of laccases from *P. sanguineus* (a and c) and *C. micaceus* (b and d) by halides in the presence of DMP as substrate. In figures a and b laccase is inhibited by NaF. In figures c and d, laccase is inhibited by NaCl. The legend above each graph depicts the inhibitor concentrations specific to each inhibitor.

Potassium iodide was dissolved in Britton-Robinson buffer at the optimal pH values for the two different enzymes. This was then used as substrate without the addition of DMP. A reaction rate was visible at 410 nm. The product tested positive for iodine

with a 1 % soluble starch test, thus proving that laccase catalyses the oxidation of iodide (I⁻) to iodine (I₂). If a closer look is taken at the redox potential of the halides in comparison to the typical redox potential expected for laccases (figure 4.32), it is possible for iodide to be oxidised by a typical laccase enzyme. This would not be possible for chloride and fluoride as their redox potentials are significantly higher than that of iodide. The concept that iodide acts as a substrate is confirmed by Xu (1996).

$$I_2 \quad + \quad 2e^- \quad \longrightarrow \quad 2\,I^- \quad (E_0: 0.535\ V)$$

$$F_2 \quad + \quad 2e^- \quad \longrightarrow \quad 2\,F^- \quad (E_0: 2.87\ V)$$

$$Cl_2 \quad + \quad 2e^- \quad \longrightarrow \quad 2\,Cl^- \quad (E_0: 1.383\ V)$$

Typical $E_0$ for laccase: 0.47 V-0.74 V (Xu *et al.*, 1998)
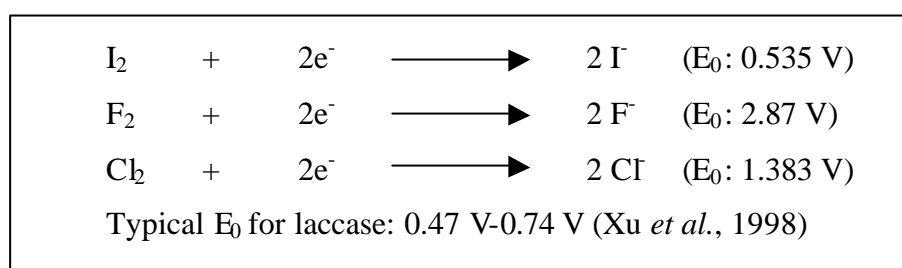
**Figure 4.32.** Redox potentials of redox reactions of the three halides used as potential laccase inhibitors.

Figure 4.33 shows the reaction schemes of the different types of inhibition noted in our data (table 4.13). Mixed inhibition is classified by a change in $K_m$ as well as $V_m$ values (table 4.12). One can further distinguish between fully mixed inhibition and partially mixed inhibition. Fully mixed inhibition is typified by the fact that only the enzyme-substrate complex (ES) breaks down to form products. For partially mixed inhibition however, the EIS complex can also break down although at a rate less than that of the ES complex (Dixon and Webb, 1979). For non-competitive inhibition the inhibitor does not affect the formation of the enzyme-substrate complex but only affects V. For fully non-competitive inhibition the EIS complex does not break down at all and therefore the velocity is only dependent on the breakdown of ES (Dixon and Webb, 1979).
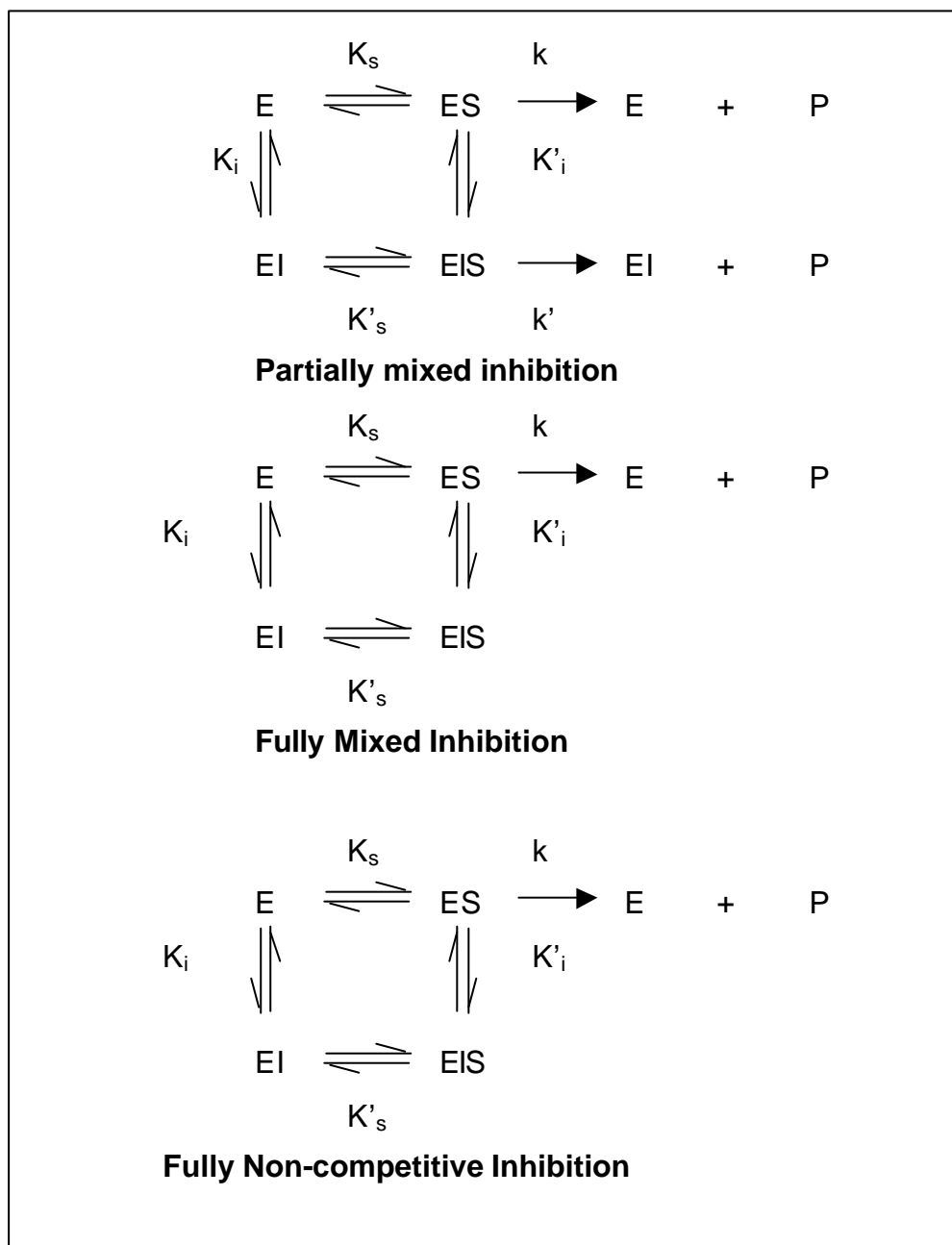
**Figure 4.33.** Schematic representations of partially mixed, fully mixed and fully non-competitive types of inhibition (taken from Dixon and Webb, 1979).

Characteristics of linear mixed type inhibition were mostly observed (table 4.11 and 4.12). A convex type correlation, indicating partial inhibition, was observed for inhibition by $F^-$ and cysteine toward *P. sanguineus* laccase. These results are supported by the work of Xu (1996) and Xu *et al*, (1996).

**Table 4.11.** $K_m$ and $V_m$ values determined for the oxidation of DMP at different concentrations of various inhibitors. Standard deviations are shown.

| Inhibitor | Inhibitor concentration | P. sanguineus | | C. micaceus | |
|---|---|---|---|---|---|
| | | $K_m$ (mM) | $V_m$ | $K_m$ (mM) | $V_m$ |
| NaN$_3$ | 0 | 0.06±0.004 | 0.97±0.016 | 0.03±0.008 | 0.67±0.04 |
| | 0.01 | 0.09±0.01 | 0.35±0.012 | 0.022±0.007 | 0.66±0.04 |
| | 0.05 | 0.13±0.02 | 0.06±0.003 | 0.024±0.009 | 0.61±0.048 |
| | 0.1 | 0.19±0.02 | 0.03±0.002 | 0.035±0.01 | 0.56±0.05 |
| EDTA | 0 | 0.06±0.001 | 1±0.004 | 0.024±0.006 | 0.64±0.04 |
| | 2 | 0.05±0.005 | 0.95±0.02 | 0.025±0.006 | 0.63±0.03 |
| | 5 | 0.05±0.003 | 0.94±0.02 | 0.025±0.008 | 0.62±0.04 |
| | 10 | 0.05±0.004 | 0.85±0.016 | 0.028±0.007 | 0.58±0.03 |
| Cysteine | 0 | 0.05±0.0004 | 0.97±0.001 | 0.08±0.02 | 0.96±0.1 |
| | 0.05 | 0.1±0.02 | 0.54±0.03 | 0.08±0.02 | 0.8±0.05 |
| | 0.1 | 0.11±0.04 | 0.48±0.05 | 0.1±0.001 | 0.5±0.002 |
| | 0.2 | 0.25±0.08 | 0.52±0.07 | 0.16±0.02 | 0.01±0.004 |
| NaF | 0 | 0.05±0.005 | 0.95±0.02 | 0.057±0.02 | 0.86±0.09 |
| | 10 | 0.15±0.04 | 0.04±0.003 | 0.057±0.02 | 0.7±0.06 |
| | 50 | 0.27±0.1 | 0.025±0.003 | 0.063±0.02 | 0.4±0.04 |
| | 150 | 0.27±0.18 | 0.016±0.005 | 0.09±0.02 | 0.2±0.01 |
| NaCl | 0 | 0.05±0.003 | 0.99±0.01 | 0.01±0.005 | 0.4±0.03 |
| | 10 | 0.08±0.01 | 0.9±0.03 | 0.01±0.004 | 0.4±0.026 |
| | 50 | 0.15±0.008 | 0.75±0.01 | 0.012±0.005 | 0.47±0.036 |
| | 150 | 0.66±0.02 | 0.26±0.018 | 0.02±0.0028 | 0.46±0.012 |

**Table 4.12.**   Types of inhibition obtained for laccases from *P. sanguineus* and *C. micaceus* with six potential inhibitors.

| | Type of Inhibition | |
|---|---|---|
| **Inhibitors** | ***P. sanguineus* laccase** | ***C. micaceus* laccase** |
| Sodium Azide | Mixed | Mixed |
| EDTA | Non-Competitive | Mixed |
| Cysteine | Partially Mixed | Mixed |
| NaF | Partially mixed | Mixed |
| NaCl | Mixed | No inhibition |
| KI | Acts as substrate | Acts as substrate |

Inhibition constants were determined graphically as described in section 3.14. Laccase from *P. sanguineus* was inhibited with a high degree of affinity by sodium azide (table 4.13).  Fluoride also had a high inhibitory affect towards this enzyme, but the inhibitor constant was not determined as the type of inhibition made it impossible with the available data.

**Table 4.13.**   Inhibition constants for the inhibition of laccase from *P. sanguineus* and *C. micaceus* by various inhibitors.

| **Parameter** | **Sodium azide** | | **EDTA** | | **Cysteine** | |
|---|---|---|---|---|---|---|
| | ***P. san.*** | ***C. mic.*** | ***P. san.*** | ***C. mic.*** | ***P.san.*** | ***C. mic.*** |
| $K_i$ (mM) | 0.005 | 0.16 | 242.2 | 31.5 | ND | 4.5 |
| $K'_i$ (mM) | 0.0015 | 0.52 | 60.5 | 88.6 | ND | 0.21 |
| $K'_i/K_i$ | 0.3 | 3.3 | 0.25 | 2.8 | - | 0.05 |

| **Parameter** | **F$^-$** | | **Cl$^-$** | | **I$^-$** | |
|---|---|---|---|---|---|---|
| | ***P. san.*** | ***C. mic.*** | ***P. san.*** | ***C. mic.*** | ***P.san.*** | ***C. mic.*** |
| $K_i$ (mM) | ND | 18.5 | 28.8 | NI | NI | NI |
| $K'_i$ (mM) | ND | 44.3 | 350.4 | NI | NI | NI |
| $K'_i/K_i$ | - | 2.39 | 12.17 | - | - | - |

(ND = Not Determined; NI = No Inhibition)

*C. micaceus* laccase was inhibited to large degrees by sodium azide and cysteine (table 4.14). A $K'_i/K_i$ ratio of more than 1 means that the inhibitor preferentially binds to the free enzyme. Thus sodium azide, $F^-$ and $Cl^-$ preferentially bind to free *C. micaceus* laccase enzyme whilst for P. sanguineus only $Cl^-$ preferentially binds the free enzyme.

# CHAPTER 5
# GENERAL CONCLUSIONS

---

At least five novel laccase enzymes were identified for the two white-rot fungal species studied: *P. sanguineus* (SCC 108) and *C. micaceus* (SCC 389). Three of these enzymes were purified to apparent homogeneity. Laccase from *P. sanguineus* produces high titres of laccase activity and has a high specific activity. This enzyme also showed remarkable stability at high temperatures. Pool 2 and 3 laccases from *C. micaceus* functioned at a suitably high pH for use in certain pulp delignification operations. *Coprinus micaceus* laccase (pool 2) showed high binding affinities for various phenolic substrates and exhibited high resistance against typical laccase inhibitors when compared to the inhibition of *P. sanguineus* laccase. The different laccases that were identified and characterised, differed remarkably in their characteristics and also diffred from laccases previously described from other sources.

Problems that occurred during this study were all associated with the extremely low protein concentrations obtained during fungal growth and purification procedures. This problem could be overcome by laccase production on large scale, for instance in bioreactors or by improving the cultivation conditions to a further extent by exploring the use of various potential inducers.

If the protein concentrations could be improved significantly it would be most interesting to investigate the structural properties of these novel enzymes. This would be of interest because all of the isolated laccases lack the characteristic blue colour of the typical laccase, possibly due to the lack of copper atoms in the active site. It has been found that laccases, which do not have the blue colour, can have copper, zinc and iron atoms instead of the classical four copper atoms (Palmieri *et al*., 1997). They are referred to as 'white' laccases. The other possibility is that the laccases could be 'yellow' instead of 'blue'. Leontievsky *et al*. (1997) propose that yellow laccases are a result of the modification of

blue laccases by products of lignin degradation. Possible experiments to elucidate structural properties of the laccases in question would include: atomic absorption, EPR spectroscopy, UV/Visible spectroscopy and the determination of the redox potentials of these enzymes by cyclic voltammetry.

# SUMMARY

Laccases (EC 1.10.3.2; benzenediol: oxygen oxidoreductases) are either mono or multimeric copper-containing oxidases that catalyse the one-electron oxidation of a vast number of phenolic substrates. Molecular oxygen serves as the terminal electron acceptor and is thus reduced to two molecules of water. The ability of laccases to oxidise phenolic compounds and reduce molecular oxygen to water has led to intensive study of these enzymes. Laccase is of interest in biotechnology because of the reactions that it is able to catalyse. A number of industrial applications for laccases have been proposed and they include, paper processing, prevention of wine decolouration, detoxification of environmental pollutants, oxidation of dye and dye precursors, enzymatic conversion of chemical intermediates and the production of valuable compounds from lignin. The ideal laccases for industrial use would exhibit stability at high temperature and pH conditions.

*Pycnoporus sanguineus* (SCC 108) and *Coprinus micaceus* (SCC389) were identified with suitable characteristics for biotechnological applications. *Pycnoporus sanguineus* laccase was produced at high titres and with considerable stability at high temperatures and *C. micaceus* laccase was able to function at neutral pH.

One laccase enzyme was identified and isolated from *P. sanguineus*. The final purifcation protocol consisted of freezing and thawing of the supernatant, precipitation with ammonium sulphate at 75 % saturation, dialysis, anion exchange chromatography with two different resins and affinity chromatography. The final purification (967-fold) led to a homogenous product with a specific activity of 32 and a purification yield of 38 %.

Laccase from *P. sanguineus* has a molecular mass of approximately 58 000 and an iso-electric point of approximately 6.9. The protein exhibits a temperature optimum of 55 °C and has a half-life of 170 minutes at 75 °C. The optimum pH differed for different substrates and were evaluated for ABTS (pH 3), DMP and guaiacol (pH 4) and syringaldazine (pH 5).

Five isozymes produced by *C. micaceus* under the specified growth conditions could be identified. Two of the five isozymes were purified to homogeneity. The final protocol for pool 1 laccase from *C. micaceus* consisted of precipitation with ammonium sulphate at 75 % saturation, dialysis, anion exchange chromatography using two separate resins and size exclusion chromatography. The final protocol delivered a 2737-fold purified protein with a specific activity of 36 U/mg and a purification yield of 6 %.

Pool 2 laccase from *C. micaceus* was purified to homogeneity with a protocol consisting of 75 % ammonium sulphate precipitation, dialysis, anion exchange chromatography and size exclusion chromatography. The laccase enzyme was purified 311-fold. The specific activity was 4 and the purification yielded 3 %. Pool 3 laccase from C. micaceus consisted of three laccase isozymes and these isozymes were not purified to homogeneity.

The three pools of laccase from *C. micaceus* migrated similarly on denaturing electrophoresis gels. The approximate size for pool 1 and 2 laccases as well as the largest laccase contained in pool 3 was 108 000. The optimum temperatures for the three laccases were 60 °C for pool 2 laccase and 65 °C for both pool 1 and 3. The laccases were stable at 45 °C, but lost all activity at 75 °C. Pool 1 laccase showed the same pH optima as the *P. sanguineus* laccase. Pool 2 and 3 laccases had higher pH optima.

Kinetic analysis for *P. sanguineus* and pool 2 laccase from *C. micaceus* included evaluation of 19 different phenolic substrates. *C. micaceus* laccase was more reactive toward the substrates than *P. sanguineus* laccase. The order of reaction toward phenolics was in the order: ortho> para> meta-substituted phenols for both enzymes.

Inhibition studies for the same two enzymes included kinetic analysis with six different inhibitors (sodium azide, EDTA, cysteine, $F^-$, $Cl^-$ and $I^-$). *Pycnoporus sanguineus* laccase was more susceptible to inhibition by the inhibitors than laccase from *C.* micaceus and a linear mixed type inhibition was mostly exhibited.

# OPSOMMING

Lakkases (EC 1.10.3.2; benzeendiol: suurstof suurstofreduktase) kan of as mono- of multimeriese koper-bevattende oksidases voorkom. Lakkases kataliseer die enkel-elektron oksidasie van 'n wye verskeidenheid fenoliese substrate. Molekulêre suurstof dien as die terminale elektron ontvanger en word gereduseer om twee water molekules op te lewer. Die vermoë van lakkase om fenoliese substrate te oksideer en molekulêre suurstof te reduseer het daartoe gelei dat hierdie ensieme intensief bestudeer is. Lakkase is van biotegnologiese belang as gevolg van die reaksies wat die ensiem kan kataliseer. 'n Wye verskeidenheid industriële toepassingsmoontlikhede is al vir lakkases voorgestel wat die volgende insluit: papier prosessering, voorkoming van die verkleuring van wyn, detoksifisering van omgewingsbesoedelingstowwe, oksidasie van kleurpigmente, ensimatiese omskakelings van chemiese tussengangers en die produksie van waardevolle stowwe vanuit lignien. Ideale lakkases vir industriële gebruik is stabiel by hoë temperature en hoë pH.

Die fungi, *Pycnoporus sanguineus* (SCC 108) en *Coprinus micaceus* (SCC389) is geïdentifiseer as fungi met die vermoë om lakkases te produseer wat eienskappe toon met biotegnologiese toepassingsmoontlikhede. *Pycnoporus sanguineus* produseer lakkase in hoë konsentrasies wat stabiel is by hoë temperature en *C. micaceus* lakkase beskik oor die vermoë om te funksioneer by 'n neutrale pH.

Slegs een lakkase-ensiem is geïdentifiseer en geïsoleer vanaf *P. sanguineus*. Die finale suiweringsprotokol bestaan uit die vries en ontdooi van die medium waarin die lakkase teenwoordig is, ammonium sulfaat presipitasie teen 'n 75 % versadigingsvlak, dialise, anioon uitruilingschromatografie met twee verskillende harse en affiniteitschromatografie. Die finale suiwering (976-voudig) het gelei tot 'n homogene produk met 'n spesifieke aktiwiteit van 32 U/mg en 'n suiweringsopbrengs van 38 %.

Lakkase geproduseer deur *P. sanguineus* het 'n molekulêre massa van ongeveer 58 000 en 'n iso-elektriese punt van ongeveer 6.9. Die proteïen funksioneer optimaal by 'n

temperatuur van 55 °C en het 'n halfleeftyd van 170 minute by 75 °C. Die optimale pH van die ensiem verskil tussen substrate en is pH 3 vir ABTS, pH 4 vir DMP en guaiacol en pH 5 vir syringaldasien as substraat.

Vyf lakkase isoensieme word deur *C. micaceus* geproduseer en twee van hierdie isoensieme is gesuiwer. Die finale suiweringsprotokol vir poel 1 lakkase het bestaan uit ammonium sulfaat presipitasie teen 'n 75 % versadigingsvlak, dialise, anioon uitruilingschromatografie met twee verskillende media en jelfiltrasie. Die finale protokol het 'n 2737-voudig gesuiwerde proteï en gelewer met 'n spesifieke aktiwiteit van 36 U/mg en 'n suiweringsopbrengs van 6 %.

Poel 2 lakkase is gesuiwer deur 'n suiweringsprotokol wat bestaan uit, ammonium sulfaat presipitasie teen 'n 75 % versadigingsvlak, dialise, anioon uitruilingschromatografie en jelfiltrasie. Die ensiem is 311-voudig gesuiwer en het 'n spesifieke aktiwiteit van 4 U/mg en 'n suiweringsopbrengs van 3 %. Poel 3 lakkase bevat drie isoensieme en hierdie isoensieme is nie gesuiwer gedurende die studie nie.

Die drie lakkase fraksies het min of meer dieselfde groottes op SDS gel elektroforese (108 000). Die temperature waar die lakkases optimal aktief was, was 60 °C vir poel 2 lakkase and 65 °C vir beide poel 1 en 3 lakkases. Die lakkases was stabiel by 45 °C, maar was heeltemaal onaktief by 75 °C. Die optimale pH waardes van poel 1 lakkase was soortgelyk aan die waardes van *P. sanguineus* lakkase, maar poel 2 en 3 lakkases het aansienlike hoër optimum waardes getoon.

Kinetiese analises is uitgevoer vir *P. sanguineus* lakkase en poel 2 lakkase van *C. micaceus*. Die analise het die studie van 19 substrate behels. *Coprinus micaceus* lakkase was meer reaktief teenoor die substrate. Die orde van reaksie ten opsigte van fenoliese substrate was in die orde: orto-> para-> meta-gesubstitueerde fenole vir beide ensieme.

Inhibisie studies vir dieselfde twee ensieme het die kinetiese analise van ses verskillende inhibitors behels (natriumazied, EDTA, sisteien, F⁻, Cl⁻ en I⁻). *Pycnoporus sanguineus* lakkase was makliker geinhibeer deur die verskeie inhibeerders as lakkase van *C. micaceus* en 'n lineêre, gemengde tipe inhibisie kon vir byna al die inhibitors beskryf word.

# CHAPTER 6
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