DEGRADATION OF PCP BY LACCASES OF THE WHITE-ROT FUNGUS *TRAMETES* SP. HR577

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ABSTRACT

Pentachlorophenol (PCP) is a biocide used by the NZ forestry industry until 1988. Its use was discontinued due to its toxicity to humans and animals. White-rot fungi have been shown to degrade PCP in laboratory and field trials. New Zealand native white-rot fungi were screened to identify organisms suitable for the clean up of PCP contaminated sites. Four criteria were used for the screening: fungal growth at different temperatures, PCP and creosote resistance and PCP degradation in standard liquid medium. Twenty isolates were identified as potentially useful from over 200 that were screened. One unique isolate, *Trametes* sp. HR577, was chosen for intensive study because it produced the well known laccases previously described from other PCP-degrading white-rot fungi.

The white-rot isolate HR577 was assigned to the genus *Trametes* based on morphological characteristics and gene sequencing studies. The latter showed that the partial laccase gene sequences from *Trametes* sp. HR 577 had high sequence homology to laccases from other *Trametes* species, especially *T. versicolor* and *T. villosa*.

Two laccase isozymes, designated L1c and L2, were purified from *Trametes* sp. HR577. These isozymes had similar biological properties to other *Trametes* species laccase isozymes. Both isozymes had a relatively high temperature optima, however, they were not very stable at elevated temperature. The dependence of laccase on dissolved oxygen for catalysis was demonstrated for isozyme L2. Laccase activity was severely inhibited in the absence of dissolved oxygen. This could be restored by reoxygenation into the assay system.

Whole cultures of *Trametes* sp. HR577 grown in liquid culture removed up to 76% of PCP after 72 hours. PCP removal was mostly due to degradation rather than adsorption of PCP to fungal mycelium. Addition of purified and crude laccase isozymes (100 U mL^{-1}) did not enhance PCP degradation.

6-15% of PCP was removed from solutions containing solely purified isozyme L1c or L2 in acetate buffer over 72 hours. Addition of ethanol or the laccase mediator compound 2,2' azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) resulted in increased PCP disappearance from purified laccase cultures.

These studies show that the white-rot *Trametes* sp. HR577 has potential to be used for the clean up of PCP contaminated sites in NZ.

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ABBREVIATIONS

2,3,4,5-TeCP	2,3,4,5-Tetrachlorophenol
2,3,4,6-TeCP	2,3,4,6-Tetrachlorophenol
2,4,5-TCP	2,4,5-Trichlorophenol
2,4-DCP	2,4-Dichlorophenol
2ClBQ	2-Chlorobenzoquinone
2-CP	2-Chlorophenol
4-CP	4-Chlorophenol
ABTS	2,2'Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
BLASTN	Basic Local Alignment Search Tool Nucleotide
BSA	Bovine Serum Albumin
cDNA	complementary Deoxyribonucleic acid
CP	Chlorophenol
CRI	Crown Research Institute
CYS	Cysteine
DCP	Dichlorophenol
DDT	1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane
DEPC	Diethylpyrocarbonate
DMP	2,6-Dimethoxyphenol
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside Triphosphate
DTT	Dithiothreitol
ECD	Electron-Capture Detector
EDTA	Ethylenediamine Tetraacetic Acid
EPR	Electron Paramagentic Resonance
FA	Ferulic Acid
FPLC	Fast Protein Liquid Chromatography
FRI	Forest Research Institute
GC	Gas Chromatography
gDNA	genomic Deoxyribonucleic acid
GI	GenInfo Identifier

Glu	Glutamic Acid
GSH	Glutathione
GS-TrCHQ	*S*-Glutathionyltrichloro-1,4-hydroquinone
HAA	3-Hydroxyanthranilate
HBA	4-Hydroxybenzoic Acid
HBT	1-Hydroxybenztriazole
His	Histidine
HIV	Human Immunodeficiency Virus
HPI	*N*-Hydroxyphthalimide
HPLC	High Performance Liquid Chromatography
HRCC	HortResearch Culture Collection
HRERM	HortResearch Environment and Risk Management
HSNO	Hazardous Substances New Organisms
IEF	Isoelectric Focusing
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
ITS	Internal Transcribed Spacer
k_{cat}	Turnover Number
K_M	Michaelis-Menton Constant
LB	Luria Broth
LC50	Lethal Concentration 50
Leu	Leucine
LiP	Lignin Peroxidase
MEA	Malt Extract Agar
Met	Methionine
MnP	Manganese Peroxidase
mRNA	messenger RNA
MW	Molecular Weight
NCBI	National Centre for Biotechnology Information Database
NZ	New Zealand
o-chloranil	3,4,5,6-Tetrachloro-3,5-cyclohexadiene-1,2-dione
ORF	Open Reading Frame
PAH	Polyaromatic Hydrocarbon
PCA	Pentachloroanisole

p-chloranil	2,3,5,6-Tetrachloro-2,5-cyclohexadiene-1,4-dione
PCP	Pentachlorophenol
PCR	Polymerase Chain Reaction
Phe	Phenylalanine
pI	Isoelectric Point
PNGase F	Peptide: *N*-glycosidase F
psi	Pounds per Square Inch
rRNA	ribosomal RNA
RNA	Ribosomal Nucleic Acid
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SCS	Sawdust Cornmeal Starch
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
Ser	Serine
T1	Type 1
T2	Type 2
T3	Type 3
TCHD	2,3,5,6-Tetrachloro-2,5-cyclohexadiene-1,4-dione
TCHQ	Tetrachlorobenzohydroquinone
TCP	Trichlorophenol
TeCP	Tetrachlorophenol
TEMPO	2,2,6,6-Tetramethylpiperidin-1-xyloxy
TNT	Trinitrotoluene
TrCHQ	Trichlorohydroquinone
USEPA	United States Environmental Protection Agency
VA	Veratryl Alcohol
VP	Versatile Peroxidase
XYL	2,5-Xylidine

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

Pentachlorophenol (PCP[7], Figure 1.1) is a contaminant in New Zealand (NZ) soils at many sites where sawmilling and logging operations were conducted. PCP was used extensively between the late 1940s until 1988 by the forestry industry for protecting freshly cut *Pinus radiata* (pine) logs against infection by sapstain fungi (Stevenson 1992).

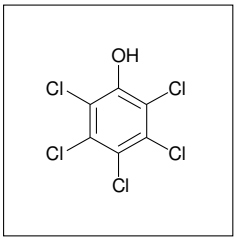


Figure 1.1: Pentachlorophenol

Sapstain fungi cause a permanent blue-black to brown discoloration of logs, especially softwoods like *P. radiata* (Behrendt and Blanchette 1997, Figure 1.2). Although sapstaining does not affect the strength of the wood, it reduces the aesthetic quality and export value of the wood (Kim, Allen *et al.* 2005; Vanneste, Hill, *et al.* 2002). In 2002 sapstain discolouration was estimated to cost the NZ forestry industry $100 M per year in lost revenue (Vanneste, Hill, *et al.* 2002).

[7] The use of the term PCP refers solely to the chemical structure given in Figure 1.1. Not to be confused with the class of compounds known as polychlorinated biphenyls (PCBs). PCP is part of the group of chemicals known as chlorophenols.

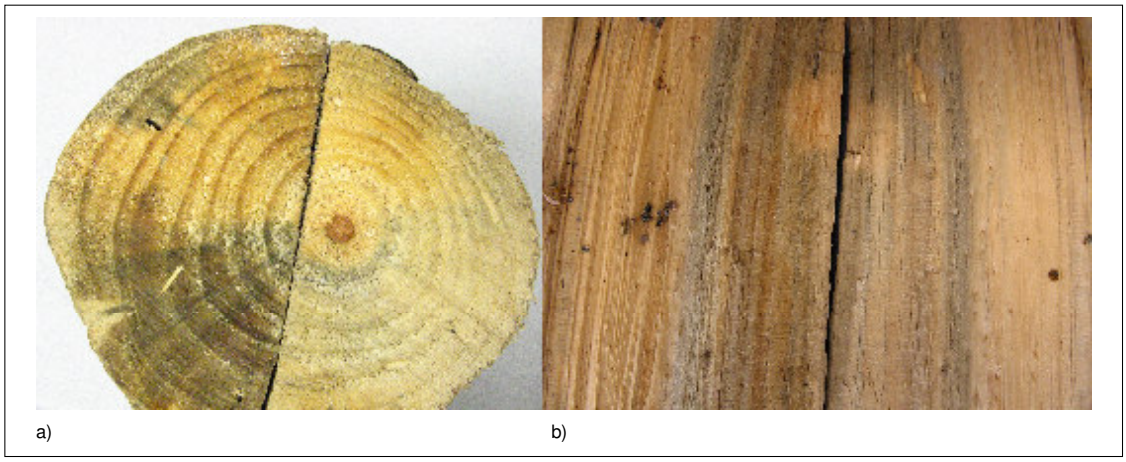


Figure 1.2: Sapstain colonisation of *P. radiata*. a) cross-section of *P. radiata* log showing 'blue' stain bands in several sections. Each half shows different ends of the same log; b) split *P. radiata* log showing penetration of sapstain down the length of the log.

PCP is listed as a priority pollutant by the United States Environmental Protection Agency (USEPA). Classification as a 'priority pollutant' is based on an assessment of the potential exposure of the chemical to human health, the aquatic environment and both human health and aquatic life combined. PCP is a probable human carcinogen and is categorised as highly toxic to freshwater fish (LC50 0.015-0.600 ppm[8]), estuarine/marine fish (LC50 0.240 ppm[2]), Pacific oyster (LC50 0.048 ppm[2]) and shrimp (0.084 ppm[2]).

In this chapter the literature on the use of PCP to control sapstain is first reviewed. Since many of the PCP degrading enzymes described in the literature are natural wood decaying enzymes, the information in the literature on the nature of these enzymes, including the classification of the fungi that produce them, is briefly discussed. The chapter also reviews the literature on the products of PCP biotransformation, including the properties of the ligninolytic enzymes of PCP degrading white-rot fungi.

Sapstain Fungi

Sapstain fungi are all from the phylum Ascomycota and belong to several different genera including *Ophiostoma*, *Aureobasidium*, *Ceratocystis* and *Leptographium* (Schroeder, Kim *et al.* 2001). In NZ there are at least 21 fungi that can cause sapstain

[8] EPA-HQ-OPP-2004-0402-0003, http://www.regulations.gov/fdmspublic/component/main?main=DocketDetail&d=EPA-HQ-OPP-2004-0402 (last accessed 16/07/2007)

wood discolouration. The most common sapstain fungi found in NZ are *Sphaeropsis sapinea*, *Ophiostoma ips*, *Ophiostoma floccosum* and *Ophiostoma piliferum* (Thwaites, Farrell *et al.* 2005). Fungi colonise damp pine logs from the surrounding soil and penetrate the wood where they access simple sugars in the woody matrix. Several NZ *Ophiostoma* species are also carried by *Hylastes ater* (Reay, Thwaites and Farrell 2005), a European bark beetle that feeds on pine seedlings and breeds in stumps and roots of pine and in cut logs.

Methods Used to Control Sapstain Fungi

Different methods have been exploited for the control of sapstain fungi. These include biological, physical and chemical means. Some *Ophistoma* species such as *O. floccosum*, *O. piceae* and *O. pluriannulatum* have been tested as potential biocontrol agents for sapstain (Held, Thwaites *et al.* 2003). These three fungi are albino strains that colonise the wood without causing discolouration of logs. Another potential biological control method is gamma irradiation of the freshly cut logs. Gamma irradiated wood is susceptible to colonisation by mould fungi (a group which includes *Trichoderma*, *Alternaria*, *Cladosporin* and *Penicillium* species) at the expense of sapstain fungi. The mould fungi cause only a superficial discolouration of the wood and can be easily removed by planing or brushing (Strong, Webber and Eaton 2005). Chemical methods have been widely used because they are convenient and easy to apply. The most common chemical method used in NZ was a mixture of PCP and creosote and these two chemicals are discussed further below.

1.2 CREOSOTE

Creosote was used as a timber preservative for many years. It is derived from coal tar distillate and contains a mixture of many different cyclic aromatic compounds (Brooks 1997). Up to 85% of creosote is made up of polyaromatic hydrocarbons (PAH, Brooks 1997). All of these compounds are hydrophobic and have a very low solubility in water (Field, Boelsma *et al.* 1995; Bamforth and Singleton 2005). In general, the more

aromatic rings the compound has, the less soluble and more toxic it is. Several of the PAH found in creosote such as benzo-α-pyrene, benzo[*a*]anthracene and benzo[*k*]fluoranthene are carcinogenic, (Figure 1.3). The PAH mostly found in sediment samples are benzo-α-pyrene, phenanthrene, chrysene and pyrene (Figure 1.3, Brooks 1997). All of these compounds are classified as priority pollutants by the USEPA (Gramss, Kirsche *et al.* 1999).

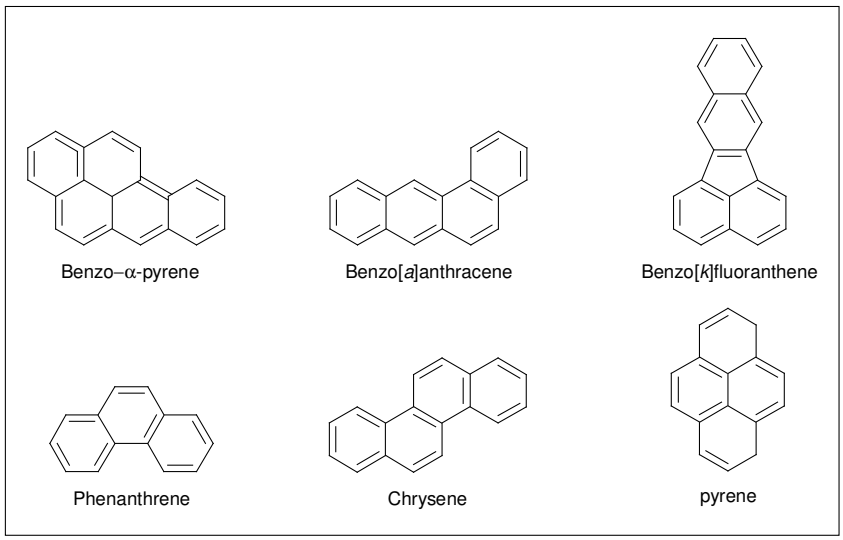


Figure 1.3: Persistent and carcinogenic PAH

1.3 PCP

As mentioned earlier, PCP was used extensively in NZ for control of sapstain fungi. It was synthesised commercially using a temperature controlled reaction of chlorine gas bubbled through phenol with aluminium chloride as the catalyst. The temperature was kept just above the melting temperature of the phenol/chlorophenol mixture to keep it molten (Yu and Savage 2004). The resulting PCP known as "technical grade PCP" was only 85-90% pure. The remaining 10-15% of the mixture contained a range of impurities including monochlorophenols (CP), dichlorophenols (DCP), trichlorophenols (TCP), tetrachlorophenols (TeCP), furans, hexachlorobenzene, and dioxins.

PCP is an effective biocide due to its ability to uncouple oxidative phosphorylation (Cserjesi 1967; Boyd, Mikesell and Lee 1987). However, the same ability makes it

toxic to humans and animals. In addition, the impurities in technical grade PCP have a greater potential to cause chronic effects than PCP alone (Spence 2001).

PCP is soluble in most organic solvents but has limited water solubility depending on the pH (Crosby 1981). PCP has a pK_a of around 4.7, and its addition to pure water at pH 7 results in a drop in pH to about pH 5.1. PCP has a maximum solubility of 10-15 mg PCP per kg water (Arcand, Hawari and Guiot 1995). The low aqueous solubility of PCP meant that other agents were often added in commercial preparations to increase the solubility of PCP. In order to coat timber with PCP, it was often applied as the sodium salt (over 2 x 10^5 mg L^{-1} is soluble at pH 10) or dissolved in oil and/or creosote.

In NZ, technical grade PCP was dissolved in a mixture of water and sodium hydroxide and sold as a 25% aqueous concentrate. The concentrate was then diluted to 0.5-1% for timber treatment. This dilute solution contained 5% 'heavy fuel oil' and 1.5% borax to buffer the solution (McQuire 1997). The timber treatment process involved dipping freshly cut logs in a solution of PCP. Excess PCP dripped off the logs into the soil where it could be washed into nearby streams or groundwater. This has caused several problems in NZ. For example, in 1993, PCP soil and groundwater contamination caused the closure of a holiday camp in Hanmer Springs, which was built on a former forestry site (Jackman, Mills *et al.* 1993; Jackson 1993). Several workers from chemical manufacturing plants that produced PCP have reported toxic side effects from exposure to this compound and its impurities (Spence 2001). Exposure to PCP by humans may occur through contact with contaminated environmental media including inhalation of contaminated air, ingestion of contaminated groundwater and ingestion of contaminated food and soils[9].

There are estimated to be over 600 old forestry sites contaminated with PCP (Figure 1.4, Jackman, Mills *et al.* 1993; Szabo 1993). The location of many of these sites is unknown. Estimates for the cost of cleanup of PCP contaminated sites ranges from US$12-828 per tonne of soil depending on the decontamination method used (Ford 2006).

[9] http://www.ermanz.govt.nz/consultations/ceir/v.pdf

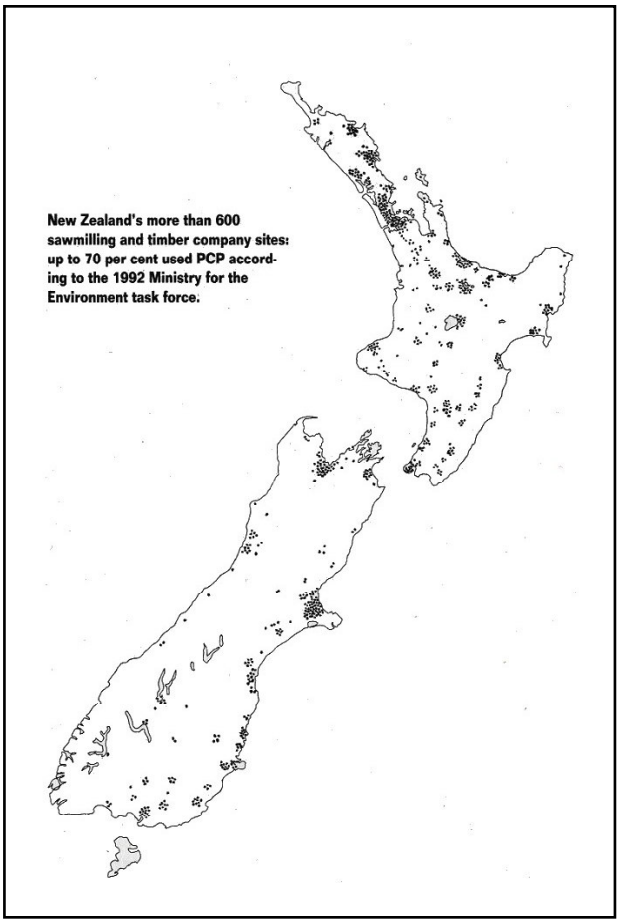


Figure 1.4: Sawmilling and timber company sites in NZ (Jackman, Mills *et al.* 1993)

The Fate of PCP in Soil

PCP degradation in soil depends on many factors. Influences on PCP degradation in soil include moisture, temperature, and organic matter content. Kuwatsuka and Igarashi (1975) studied the effect of soil moisture on the degradation of PCP in rice paddy fields. These researchers found that PCP degraded more rapidly under flooded conditions and in soil with high organic matter content. The major degradation products found (Figure 1.5) were pentachloroanisole (PCA) and 2,3,4,5-tetrachlorophenol (2,3,4,5-TeCP).

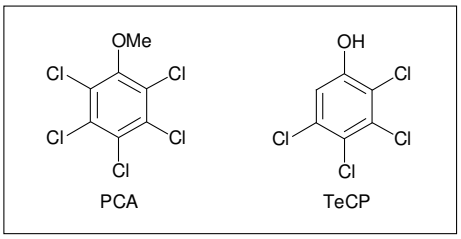


Figure 1.5: PCA and 2,3,4,5-TeCP

Photodegradation of PCP in soil can occur, however the persistence of PCP in the environment suggests that under natural conditions this is a slow process (Boyd, Mikesell and Lee 1987).

Microbial and Fungal Degradation of PCP in Soil

Several species of bacteria have been found to metabolise PCP in soil. Bacteria shown to metabolise PCP include *Flavobacterium* sp. strain ATCC 39723, *Rhodococcus chlorophenolicus, Arthrobacter* sp., and *Sphingomonas* sp. (McAllister, Lee and Trevors 1996).

A number of different fungi can also degrade PCP both *in vitro*[10] and in soil (Cserjesi 1967; Konishi and Inoue 1972; Bollag and Liu 1985; Lamar and Dietrich 1990; Lamar, Glaser and Kirk 1990; Lamar, Larsen and Kirk 1990; Lamar and Dietrich 1992; Lamar, Evans and Glaser 1993; Lamar 1994; Lamar, Davis *et al.* 1994; Alleman, Logan and Gilbertson 1995; Chung and Aust 1995; Glaser and Lamar 1995; Häggblom and Valo 1995; Sims, Sims *et al.* 1999; Tayal, Das and Kaur 1999; Tuomela, Lyytikäinen *et al.* 1999; Cho, Nam *et al.* 2001; Walter, Boyd-Wilson *et al.* 2005a). Wood decay fungi are the most commonly studied for cleanup of PCP-contaminated sites. These fungi produce extracellular ligninolytic enzymes (for degradation of the complex polymeric cell walls in woody tissues) that are believed to play a role in degradation of PCP (Reddy and Gold 2000).

1.4 CLASSIFICATION OF WOOD DECAY FUNGI

The majority of fungi that are capable of degrading wood belong to the phylum Basidiomycota and are in the Hymenomycetes class. Fungi in this phylum produce club-shaped structures used to produce spores known as basidia, hence the name basidiomycota.

[10] The term '*in vitro*' is defined in this thesis as a reaction carried out in the laboratory in defined liquid medium.

The traditional methods used for identifying wood decaying fungi were dependent solely upon the morphological characteristics (growth behaviour and/or fruiting body formation) and the classification systems that were developed coped poorly for identifying the great variety of wood decaying fungi that exist in nature (for a review see Burdsall 1997). In 1965 Nobles introduced the use of biochemical attributes to help classify wood decaying fungi in the Hymenomycetes class[11]. The system compared features of the fruiting body and the vegetative growth of 149 fungi. Her code distinguishes between fungi by using attributes like the presence or absence of extracellular oxidase, colour of hyphae and mycelial mats and odour (Nobles 1965).

With the advent of molecular techniques such as the polymerase chain reaction (PCR), classification of organisms based on DNA sequences has become common. DNA from fungi is extracted; amplified using degenerate primers and the sequences obtained are compared to various gene databanks, such as the National Centre for Biotechnology Information database (NCBI, http://www.ncbi.nlm.nih.gov/). The ribosomal RNA genes (rRNA) are a popular target for PCR amplification and sequence analysis (Bruns, White and Taylor 1991, Gardes and Bruns, 1993, De Koker, Nakasone *et al.*, 2003). These contain highly conserved regions that make ideal targets for universal primers. Between these regions there are highly divergent regions that can be amplified and sequenced to distinguish between fungi.

Currently, wood decay hymenomycetes are arbitrarily divided into two groups, brown- and white-rot fungi, by the colour of the wood decayed by these fungi.

Brown-Rot Fungi

Hymenomycetes fungi that decay the cellulose and hemicellulose in the primary walls of woody tissues leaving behind the brown-coloured lignin are known as brown-rot fungi (Volk 2000; Micales 2001). The mechanisms used by brown-rot fungi to achieve wood decay are believed to involve the formation of reactive oxygen species in Fenton-like reactions[12]. Chelator compounds such as oxalic acid have been proposed to play a

[11] This was an updated version of Nobles' original key (Nobles, 1948).

[12] The Fenton reaction is: (1) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$

(2) $Fe^{2+} + H_2O_2 \rightarrow Fe^{2+} + OOH^{\bullet} + H^{+}$

role in stabilisation of the iron species involved in this reaction. Goodell, Jellison *et al.* (1997) presented evidence that, for the brown-rot fungus *Gloeophyllum trabeum*, low molecular weight chelators react with other metabolites to mediate the degradation of cellulosic and phenolic compounds in wood.

Mineralisation of PCP and 2,4-DCP (degradation to carbon dioxide) by two species of brown-rot fungi (*Gloeophyllum striatum* and *G. trabeum*) has been shown to occur *in vitro* (Fahr, Wetzstein, *et al.* 1999). The reactive oxygen species produced by brown-rot fungi for wood decay are believed to be involved in degradation of xenobiotics such as PCP by these fungi (Rabinovich, Bolobova, and Vasil'chenko 2004).

Genes encoding ligninolytic enzymes (laccases, and peroxidases, see later for a discussion on these enzymes) have been found in some brown-rot fungi. For example, laccase genes have been found in *G. trabeum* and this brown-rot fungus produces laccase *in vitro* (D'Souza, Boominathan and Reddy 1996).

White-Rot Fungi

Hymenomycetes that degrade the lignin leaving behind the cellulose and hemicellulose are known as white-rot fungi due to the bleached appearance of wood decayed by these fungi. Some white-rot fungi can also degrade the cellulose and hemicellulose in wood; however, the lignin is generally degraded first. This group of fungi includes edible mushrooms such as *Agaricus bisporous* (common button mushrooms) and *Pleurotus ostreatus* (oyster mushrooms). Bracket fungi such as *Trametes versicolor* (Turkey tail fungi) and *Pycnoporus coccineus* are also examples of white-rot fungi (Figure 1.6). White-rot fungi do not generally produce conidia. An exception to this is *Phanerochaete chrysosporium*, which has more than one anamorphic state.

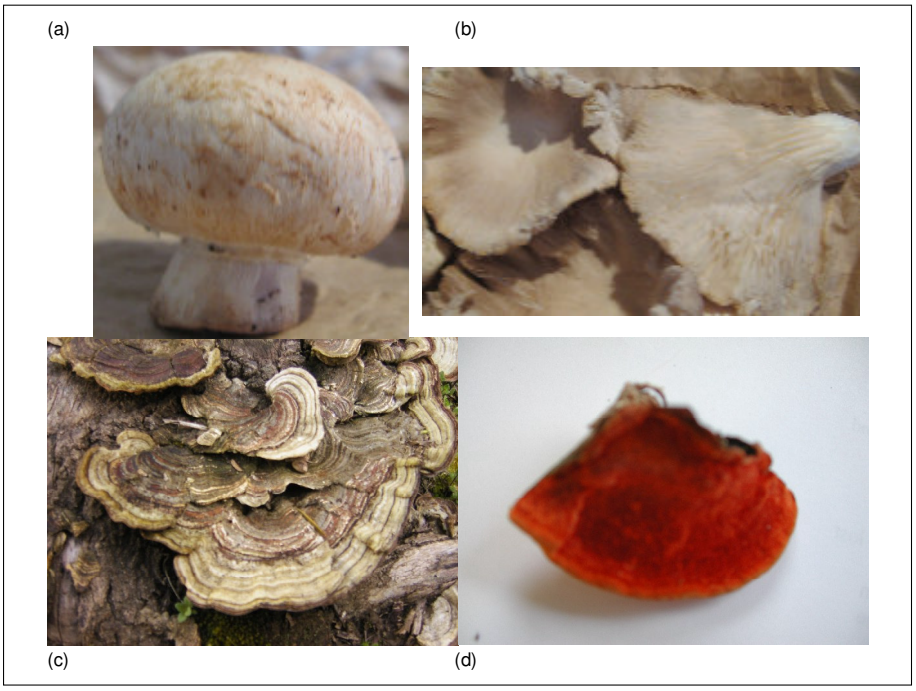


Figure 1.6: Examples of white-rot fungi. a) *A. bisporus*; b) *P. ostreatus*; c) *T. versicolor*[13]; d) *P. coccineus*.

White-rot fungi produce non-selective, ligninolytic, extracellular enzymes capable of complete mineralisation of lignin. Lignin is found in the walls of tracheids (cells involved in water transport in vascular plants) and provides rigidity and strength to wood (Cullen and Kersten 1996). It is a phenylpropanoid polymer synthesised by polymerisation of a mixture of *p*-hydroxycinnamyl alcohols (Figure 1.7a). The proportion of each *p*-hydroxycinnamyl alcohol varies depending on the type of wood.

[13] This photo was taken by S. Sivukumaran from HortResearch and is used with permission.

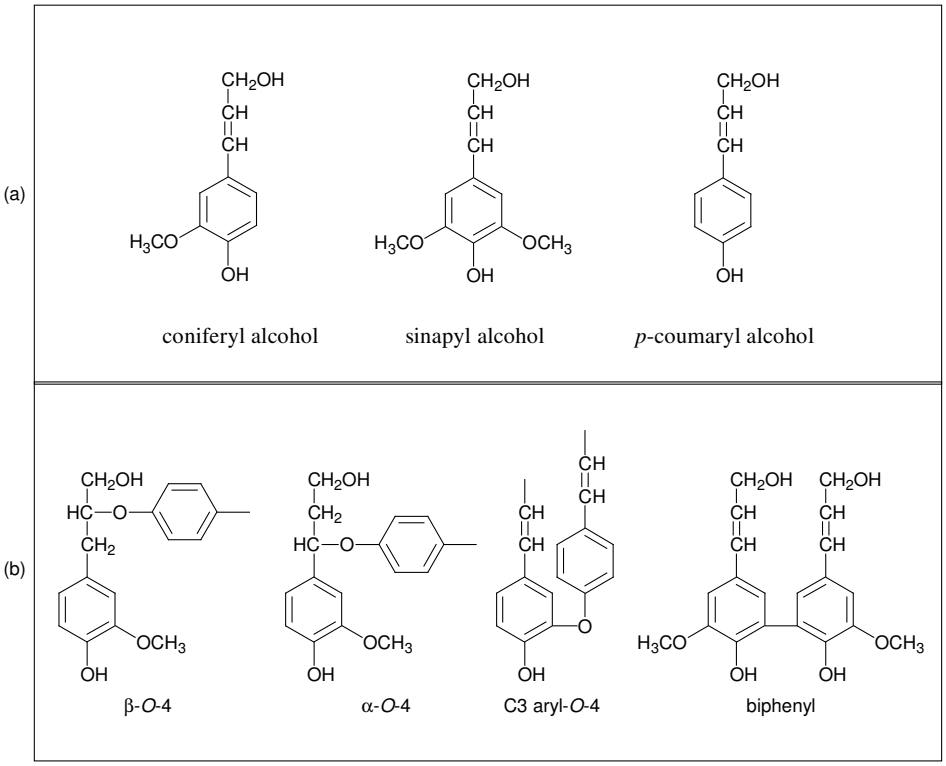


Figure 1.7: a) *p*-Hydroxycinnamyl alcohol subunits of lignin (Betts, Dart *et al.* 1991) **b) Common linkages between monomeric subunits of lignin** (Evans 1991)

The lignin biosynthesis mechanism involves a radical catalysed reaction. The linkages formed between the monomeric units are stable, non-hydrolysable carbon-carbon bonds or relatively inert ether bonds, (Figure 1.7b, Betts, Dart *et al.* 1991). Because the biosynthesis reaction is non-specific, the resulting polymer has an irregular structure containing a mixture of *R*- and *S*-enantiomeric forms. This makes the lignin polymer difficult to degrade.

White-rot fungi are one of the few groups of organisms that are capable of lignin mineralisation (Martinez, Larrondo *et al.* 2004). An example of the degradation of lignin by a white-rot fungus is shown in Figure 1.8. Figure 1.8a shows intact *P. radiata* tracheids. Two hundred and thirty six days after inoculation with *T. versicolor* the lignin has been extensively degraded (Figure 1.8b). As mentioned earlier, because of the complex structure of lignin, the ligninolytic enzymes capable of its degradation are non-stereospecific. It has been proposed that these enzymes could play a role in PCP degradation.

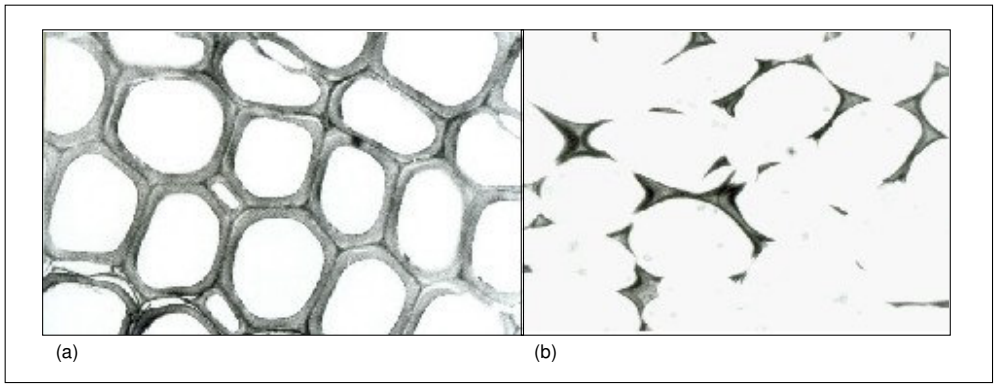


Figure 1.8: Electron microscope (EM) photos showing degradation of lignin by *T. versicolor*: a) Intact *P. radiata* tracheid walls (x 670 magnification) b) *P. radiata* tracheid walls after 236 days incubation with *T. versicolor* (x 670 magnification)[14].

1.5 LIGNINOLYTIC ENZYMES PRODUCED BY WHITE-ROT FUNGI

The ligninolytic enzymes produced by white-rot fungi have been categorised into two groups: peroxidases (EC 1.11.1.13, 1.11.1.14, and 1.11.1.16) and laccases (EC 1.10.3.2). White-rot fungi produce one or more of these types of ligninolytic enzymes, depending on the species and the environmental conditions. There is often a multiplicity in the number of each type of ligninolytic enzyme produced by a particular white-rot fungus. The multiple forms of each type of ligninolytic enzyme are generally referred to as 'isozymes'[15]. Isozymes of the various ligninolytic enzymes produced by many white-rot fungi are encoded by different genes. These isozymes differ in their stability and catalytic properties (Farrell, Murtagh *et al.* 1989; Johansson and Nyman 1993).

Different fungi can produce a number of different isozymes of each type of ligninolytic enzyme. For example, six lignin peroxidases (LiP, EC 1.11.14) and four manganese peroxidases (MnP, EC 1.11.1.13) have been isolated from the white-rot fungus

[14] Photos used with permission (S. Sivakumaran, HortResearch, Palmerston North)

[15] The term 'isozyme' was originally defined as "different variants of the same enzyme having identical functions and present in the same individual" (Hunter, 1957). The original definition included both 'isozymes' and 'allozymes'. However, 'isozymes' have since been defined as variants of an enzyme produced by different genes from different loci. Allozymes have been described as variants of enzymes coded for by different alleles from the same loci. These terms should not be confused with the term 'isoform'. The term isoform is used to define protein variants that differ in posttranslational modifications or in splice variant.

P. chrysosporium (Farrell, Murtagh *et al.* 1989). Isozymes of the various ligninolytic enzymes produced by many white-rot fungi are encoded by different genes. These isozymes differ in their stability and catalytic properties (Farrell, Murtagh *et al.* 1989; Johansson and Nyman 1993). The concentrations of each isozyme in the extracellular culture fluid may vary over time. Some isozymes may be constitutive (that is to say, produced in the same pattern throughout an experiment regardless of what is added to the culture fluid) while others are inducible (produced in greater amounts in the presence of different compounds added to the fungal medium).

Lignin is the natural substrate for peroxidases and laccases. However, lignin is a very complex polymer making it difficult to standardise assays for laccase activity. Therefore, a number of different compounds have been used to assay for different ligninolytic enzymes. For LiP, the substrate most commonly used in assay systems is veratryl alcohol (VA, 3,4-dimethoxyphenol, Tien, and Kirk 1988), a compound that is also produced by some white-rot fungi. For MnP, Mn^{2+} (Paszczynski, Crawford and Huynh 1988; Wariishi, Valli and Gold 1992) or 2,6-dimethoxyphenol (DMP, de Jong, Cazemier *et al.* 1994) are often used as the assay substrates and for laccase several different compounds such as DMP (de Jong, Cazemier *et al.* 1994) and 2,2' azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Wolfenden and Willson 1982) have been used.

1.6 PEROXIDASES

There are several classes of peroxidase based on sequence homology, structural similarity and organism of origin (Welinder 1992). Well known examples include horseradish peroxidase (a class III plant peroxidase) and yeast cytochrome *c* peroxidase (a class I peroxidase). Fungal peroxidases produced by white-rot fungi are monomeric class II peroxidases (secretory fungal peroxidases, Larrondo, González *et al.* 2005).

The peroxidases produced by white-rot fungi are subdivided into three groups: LiP, MnP and versatile peroxidase (VP, EC 1.11.1.16), based on their reactions. LiP oxidises a variety of substrates that have high redox potentials. MnP, as the name

implies, requires manganese to complete its catalytic cycle and its main function appears to be oxidation of chelated Mn^{2+} to Mn^{3+} (Aitken and Irvine 1990). A more recently discovered peroxidase, known as VP, oxidises substrates of both LiP and MnP (Martínez 2002).

Peroxidases require hydrogen peroxide to complete their catalytic cycle (Gold, Kuwahara *et al.* 1984; Tien and Kirk 1984; Higson 1991). White-rot fungi generate hydrogen peroxide with extracellular enzymes such as glyoxyl oxidase, aryl alcohol oxidase and the intracellular enzyme pyranose oxidase (also known as glucose-2-oxidase, Reading, Welch and Aust 2003).

The white-rot secondary metabolite VA has been implicated in lignin degradation by LiP enzymes. A VA binding site has been observed in the crystal structure of a LiP isozyme from *P. chrysosporium* (Choinowski, Blodig *et al.* 1999). Sequence alignment of known LiP enzymes shows that this binding site is conserved in LiP and VP enzymes (Blodig, Doyle *et al.* 1998). The VA binding site is not found in MnP enzymes. Instead, MnP enzymes have a manganese binding pocket where chelated Mn^{2+} is oxidised to Mn^{3+} (Martínez 2002; Canales, Lobos and Vicuña 1998). This manganese binding pocket is also found in VP enzymes (Martínez 2002; Ruiz-Dueñas, Martínez, and Martínez 1999).

1.7 LACCASE

Functions of Laccase in Nature

Extracellular laccases from white-rot fungi have been implicated in lignin degradation. However, other functions have been ascribed to some intracellular laccases from white-rot fungi. Intracellular laccases produced by fruiting bodies of *Lentinus edodes* (Shitake mushroom) are involved in melanin synthesis (Nagai, Kawata *et al.* 2003) whereas laccase from *Volvariella volvacea* has been implicated in fruiting body development (Chen, Ge *et al.* 2004b).

Laccase production is not restricted to white-rot fungi. It was originally discovered in 1883 in the Japanese lacquer tree *Rhus vernicifera* (Nitta, Kataoka and Sakurai 2002). Higher plants that produce laccase include sycamore maple (*Acer pseudoplatanus*, Sterjiades, Dean and Eriksson 1992), Norway spruce (*Picea abies*, Kärkönen, Koutaniemi *et al.* 2002), thale cress (*Arabidopsis thaliana*, McCaig, Meagher and Dean 2005), cotton (*Gossypium arboreum*, Wang, Li *et al.* 2004) and loblolly pine (*Pinus taeda*, Sato, Wuli *et al.* 2001). In plants the major function of laccase appears to be lignin biosynthesis. It has also been implicated in wound healing, pigment formation and detoxification (Nitta, Kataoka and Sakurai 2002).

As mentioned previously, some brown-rot fungi also produce laccase. D'Souza, Boominathan and Reddy (1996) found laccase genes in several brown-rot fungi even though these particular fungi did not express laccase under physiological conditions.

Laccases have also been found in a variety of ascomycete fungi. Most ascomycete laccases only share 20-30% sequence identity to basidiomycete laccases. However, laccase from the ascomycete *Mauginiella* shares much higher sequence identity to two *Trametes* laccase isozymes (*lcc1* from *T. versicolor* and *lcc2* from *Trametes villosa*) at the nucleotide level (Palonen, Saloheimo *et al.* 2003). The reasons for the high sequence identity have not been determined, although it has been suggested this ascomycete fungus may have acquired the gene by horizontal gene transfer.

A number of different functions have been ascribed to ascomycete laccases. Functions in these fungi include pigment synthesis and pathogenicity. For example, *Botrytis cinerea* laccase is involved in plant pathogenicity (Thurston 1994; Yaropolov, Skorobogatko *et al.* 1994), laccase from *Aspergillus nidulans* is believed to be involved in pigmentation of spores (Scherer and Fischer 1998) and laccase from *Mauginiella* sp. can decolourise melanin from sapstain fungi (Palonen, Saloheimo *et al.* 2003).

General Properties of Laccases

Laccases (phenol oxidase, EC 1.10.3.2) are glycosylated phenol oxidases that exist as monomers, homodimers, or homotetramers (Solomon, Sundaram and Machonkin 1996). Most fungal laccases purified to date are monomers with molecular weights ranging

between 50 to 80 kDa (Muñoz, Guillén *et al.* 1997). However, one tetrameric laccase has been purified from the ascomycete *Podospora anserina* (Durrens 1981) and a few homodimeric laccases have been purified from a variety of ascomycetes and basidiomycetes (Marques de Souza and Peralta 2003).

Laccases are part of a group of enzymes known as multicopper oxidases. Examples of other multicopper oxidases include ascorbate oxidase, ceruloplasmin, cytochrome *c* oxidase, rustacyanin, and azurin. Multicopper oxidases catalyse the 4-electron reduction of oxygen to water with four concomitant 1-electron substrate oxidations. Multicopper oxidases have a minimum of four copper atoms per monomeric unit. The copper atoms have been categorised into three groups (type 1-3) based on their spectroscopic and geometric properties. Type 1 (T1) copper atoms are coordinated to a conserved cysteine (Cys) ligand, two histidine (His) ligands and either phenylalanine (Phe), leucine (Leu) (fungal laccases) or methionine (Met) (most multicopper oxidases including plant laccases). The copper-sulphur (Cys) bond has a charge transfer at about 600 nm giving the protein a blue appearance. The EPR spectrum for T1 copper atom displays narrow parallel hyperfine splitting. The Type 2 (T2) copper has a large parallel hyperfine splitting in the EPR spectrum but lacks strong absorption bands in the UV-visible spectrum. Type 3 (T3) copper atoms form a binuclear couple. They are EPR silent but have an intense charge transfer band at 330 nm due to a bridging hydroxide bridge. In laccases, the T2 and T3 copper atoms form a trinuclear copper cluster.

Fungal laccases have a very broad substrate range compared to other multicopper oxidases. Solomon, Sundaram and Machonkin (1996) noted that while most multi copper oxidases are very stereospecific, fungal laccases are not. The Michaelis-Menton constant (K_M) values for fungal laccases are generally in the range of 1-10 mM compared to other multi copper oxidases where the K_M values are generally less than 1 mM. They suggested that this indicates the absence of a substrate-binding pocket in fungal laccases. However, evidence for the presence of a binding pocket has been observed in the crystal structure of a laccase isozyme from *T. versicolor* complexed with xylidine (XYL, Figure 1.9, Bertrand, Jolivalt *et al.* 2002).

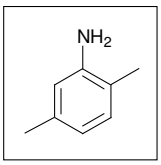


Figure 1.9: XYL

As already discussed, laccases catalyse the 4-electron reduction of oxygen to water with four concomitant 1-electron substrate oxidations. The substrate binds near the T1 copper atom (Bertrand, Jolivalt *et al.* 2002) and an electron is transferred from the substrate to the T1 copper where it is shuttled over a 12-13 Å distance to the T2-T3 trinuclear cluster where oxygen is reduced to water (Piontek, Antorini and Choinowski 2002).

Since the T1 copper site is where oxidation of laccase substrates occurs, the T1 copper redox potential is an important factor in determining the range of substrates that will react with this enzyme (Johannes and Majcherczyk 2000). This also applies for other multicopper oxidases.

The redox potentials of multicopper oxidases vary considerably from 0.184 V (stellacyanin from *R. vernicifera*, Xu, Palmer *et al.* 1999) to over 1.00 V (human ceruloplasmin, Li, Webb *et al.* 2004). This has led to classification of multicopper oxidases as being either 'high' (~0.7-1.0 V) or 'low' (~0.184-0.55 V) redox enzymes. Fungal laccases were once thought to have the highest redox potentials of all multicopper oxidases (~0.7-0.8 V) while plant laccases were classed as 'low' redox laccases (eg *R. vernicifera* laccase has a redox potential of 0.44 V). However, laccases from some white-rot fungi (for example *Coprinus cinereus* and *Trametes* sp. C30) and also from some ascomycete fungi (such as *Myceliophthora thermophila* and *Scytalidium thermophilum*) are also classed as 'low' redox multicopper oxidases. Similarly, some multicopper oxidases such as rustacyanin from the bacteria *Thiobacillus ferrooxidans* and human ceruloplasmin have relatively high redox potentials (0.65 and >1.00 V, respectively).

The axial ligand of the T1 copper has been shown to partially account for the difference in redox potentials between fungal laccases and other multicopper oxidases. Mutating the axial Met of rusticyanin to Leu results in an increase in the T1 copper redox potential by 100 mV (Kanbi, Antonyuk *et al.* 2002). Conversely, substituting the axial

Phe of *Polyporus pinsitus* (*T. villosa*) laccase to a coordinating Met ligand results in a 100 mV decrease in the T1 copper redox potential (Palmer, Randall *et al.* 1999; Xu, Palmer *et al.* 1999). Most 'high' redox fungal laccases have Phe and most 'low' redox fungal laccases have Leu as the axial T1 copper ligand. However, this does not appear to be the cause of the differences in redox potentials between 'high' and 'low' redox fungal laccases (Xu, Berka *et al.* 1998).

Piontek, Antorini and Choinowski (2002) compared the crystal structures of a 'high' redox laccase isozyme (*T. versicolor* isozyme I, E° ~ 0.79 V) with those of a 'low' redox laccase (*C. cinereus* E° ~ 0.5 V, Ducros, Brzozowski *et al.* 2001) and suggested a reason for the high redox potential of the *T. versicolor* laccase compared to that of *C. cinereus*. They noticed that the bond from the T1 copper to one of the His ligands had a longer bond distance in *T. versicolor* (0.17 Å) and looked for something in the structure that could cause this. They found a hydrogen bond between a glutamic acid (Glu-460) that is on the same helix as the His and a serine (Ser-113) that was highly conserved in high E° enzymes. This hydrogen bond pulls the helix away from the T1 site causing a lengthening of the Cu-N (His) bond resulting in an increase in redox potential. The equivalent position to the Glu in *C. cinereus* is a Met residue, which cannot form the hydrogen bond with Ser.

Laccase Cofactors

P. chrysosporium produces a multiplicity of LiP and MnP isozymes but does not produce laccase, although genes encoding multicopper oxidase-like sequences have been found in the *P. chrysosporium* genome (Martinez, Larrondo *et al.* 2004). As this white-rot has been studied extensively, the cofactors involved in MnP and LiP degradation of lignin have been well characterised. For LiP the redox potential is around 1-1.1 V (Call and Mücke 1997) and for the chelated $Mn^{2+/3+}$ couple from MnP oxidation the redox potential is around 0.9-1.2 V (Call and Mücke 1997). Thus, these ligninolytic enzymes can degrade compounds with relatively high redox potentials, such as non-phenolic lignin model compounds, in the presence of the appropriate cofactors.

In contrast, purified laccases can oxidise simple phenolic compounds in buffered aqueous solution without the addition of cofactors such as hydrogen peroxide, VA,

oxalate or manganese. However, the highest redox potential for a laccase is only 800 mV (Li, Xu *et al.* 1999). Therefore, due to its relatively low redox potential compared to LiP and MnP, laccase on its own does not degrade non-phenolic lignin model compounds. Consequently, there has been much debate over its role in degradation of lignin (Evans, 1985; Morohoshi, Fujita, *et al.* 1987), and also in degradation of xenobiotics such as PCP (Ricotta, Unz, and Bollag 1996; Ullah, Bedford, and Evans 2000).

Since the substrate range of LiP and MnP can be increased by addition of mediator compounds, researchers have looked for compounds to mediate laccase reactions. Several synthetic compounds such as ABTS (Figure 1.10) have been shown to act as mediators. As mentioned earlier, ABTS is generally used as a substrate in laccase assays to determine the amount of laccase activity in a sample. Laccase oxidises ABTS to $ABTS^{+\bullet}$, an intensely blue-green coloured cation radical. Bourbonnais and Paice (1990) showed that oxidation of non-phenolic compounds by either laccase or the $ABTS^{+\bullet}$ radical alone does not occur. This suggests that both components are needed for the oxidation reaction.

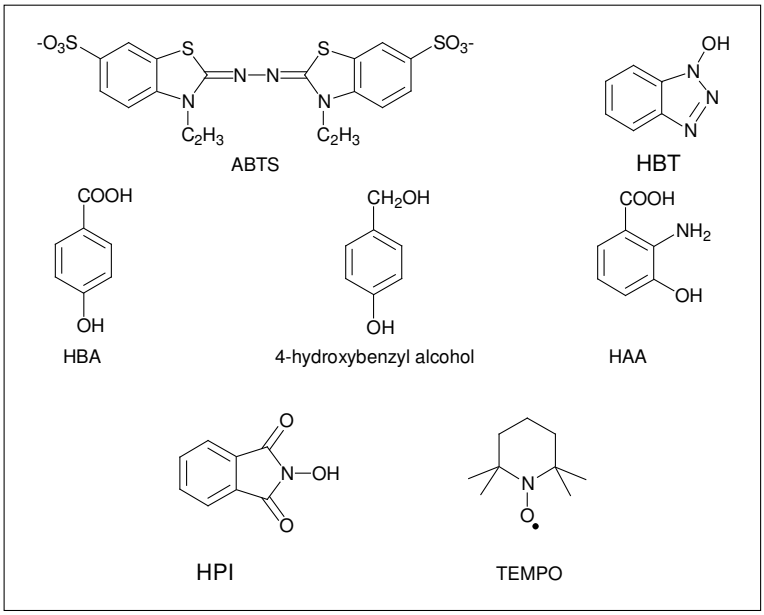


Figure 1.10: Laccase mediator compounds

Collins, Kotterman *et al.* (1996) showed that oxidation of the PAH anthracene by laccase purified from *T. versicolor* was enhanced in the presence of ABTS. No increase in oxidation of anthracene was observed when the ultrafiltrate from the enzyme

purification was used instead of buffer. However, use of the crude enzyme concentrate caused significant oxidation of anthracene. They suggested that the crude enzyme concentrate contained a compound that acted as a mediator in anthracene oxidation by the fungal laccase. They determined that the potential mediator was less than 10 kDa in size and heat stable. Following publication of this paper, Eggert, Temp *et al.* (1996) suggested that 3-hydroxyanthranilate (HAA, Figure 1.10) produced by the white-rot fungus *Pycnoporus cinnabarinus* acted as a natural mediator in lignin degradation. However, more recently this theory was discounted after the discovery that lignin degradation still occurred in the absence of HAA production (Li, Horanyi *et al.* 2001).

Johannes and Majcherczyk (2000) compared several natural mediators in the oxidation of PAH. They found that the most effective natural mediators were 4-hydroxybenzoic acid (HBA) and 4-hydroxybenzyl alcohol (Figure 1.10). Other researchers have suggested that laccases are modified when grown on a solid substrate such as wheat straw to a form known as a 'yellow' laccase. These enzymes do not require a mediator compound in order to oxidise ligninolytic substrates (Leontievsky, Myasoedova *et al.* 1997; Leontievsky, Vares *et al.* 1997). These laccases appear yellow or yellow-brown when purified and lack the characteristic 'blue' absorption at 600 nm. Comparison of *N*-terminal amino acid sequences show high homology between blue and yellow laccase forms for laccases purified from *T. versicolor*, *Panus tigrinus*, *Phlebia radiata*, *Phlebia tremellosa* and *A. bisporus* (Leontievsky, Vares *et al.* 1997).

Applications of Laccase

As already mentioned, the major function of laccase from white-rot fungi is degradation of lignin, a complex polypropanoid structure containing a haphazard mixture of stereocentres. Because of this, fungal laccases are not stereo- or regio-specific. Therefore, several applications for laccases have been suggested and studied. These include delignification of kraft pulp, dye decolourisation, Human Immunodeficiency Virus-1 (HIV-1) transcriptase inhibition and bioremediation.

DELIGNIFICATION OF KRAFT PULP

The most obvious application for white-rot fungal laccases is for use in the pulp and paper industry where deligninfication of wood is necessary for paper production (Micales, 2001). Laccase alone is unable to degrade some of the non-phenolic Kraft pulps. However these components can be deligninfied by laccase in the presence of mediator compounds such as ABTS, 1-hydroxybenzotriazole (HBT), *N*-hydroxyphthalimide (HPI) or 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO, d'Acunzo, Galli *et al.* 2002; Kleen, Ohra-aho and Tamminen 2003, Figure 1.10). Laccase coupled with a mediator compound is also effective at decolourisation of waste effluent from the pulp and paper industry.

SYNTHETIC DYE DECOLOURISATION

The use of synthetic dyes such as Poly R-478 for screening of white-rot fungi has been well established (Lin, Chang *et al.* 1991). Decolourisation of these dyes is used as an indication of the presence of ligninolytic enzymes (Platt, Hadar and Chet 1985). A correlation between degradation of Poly R dyes and PAH degradation has been observed (Field, de Jong *et al.* 1993a). However, there is no apparent correlation between Poly R dye decolourisation and PCP degradation (Walter, Guthrie *et al.* 2003).

There are over 10,000 synthetic dyes used in the textile, pharmaceutical, printing, food, cosmetic, leather and paper industries (Dias, Bezerra *et al.* 2003). It is estimated that up to 20% of dyes are released into waste streams. Of this, 90% is not degraded after activated sludge treatment and is discharged into rivers (Ryan, Schnitzhofer *et al.* 2003). Some dyes are non-toxic when used but will be converted into potentially carcinogenic amines if released into the aquatic environment (Soares, Amorim *et al.* 2002). Other dyes are toxic, mutagenic and carcinogenic (Eichlerová, Homolka *et al.* 2005). White-rot fungi have been shown to be effective at decolourisation of several of these dyes. In some cases the whole organism has been used (Dias, Bezerra *et al.* 2003; Eichlerová, Homolka *et al.* 2005). Other studies have focused on decolourisation by fungal laccase either by itself (Baldrian 2004), or in combination with a range of mediator compounds (Camarero, Ibarra *et al.* 2005). Another related application for laccase is in hair dye.

There are currently over 100 patents that mention the use of laccase as a component of hair dye.

MEDICINAL PROPERTIES OF LACCASE

A research group in China has recently been studying laccases purified from fruiting bodies of several edible mushroom varieties such as *Pleurotus eryngii* (king oyster), *Tricholoma giganteum* (niohshimeji mushroom) and *Hericuium erinaceum* (monkey head mushroom, Wang and Ng 2004a; Wang and Ng 2004b; Wang and Ng 2004c; Wang and Ng 2006). They chose these particular mushrooms to look at their medicinal properties as they are commonly eaten in China. The intracellular laccases expressed and purified from these mushroom fruiting bodies had quite different *N*-terminal sequences to any of the extracellular laccases purified from the same organisms. The intracellular laccase from *P. eryngii* was a homodimer with a monomeric subunit size of 34 kDa. This is low compared to most extracellular laccases (typically 50-80 kDa per monomer). All of the intracellular laccases purified from the Chinese mushrooms exhibited HIV-1 reverse transcriptase inhibition with that of *P. eryngii* being described as a potent HIV-1 reverse transcriptase inhibitor.

BIOREMEDIATION

By far the most studied application of white-rot fungi to date is bioremediation. White-rot fungi have been shown to be effective at degrading a range of different xenobiotic compounds including DDT, TNT, creosote and PCP (Bumpus and Aust 1987; Walter 1992; Lamar, Davis *et al.* 1994; Glaser and Lamar 1995; Hodgson, Rho *et al.* 2000; van Aken, Stahl *et al.* 2000; Wang, Thiele and Bollag 2002).

1.8 DEGRADATION OF PCP BY WHITE-ROT FUNGI

Overseas, several different white-rot fungi have been shown to degrade a range of chlorophenols including PCP in laboratory studies. These include *P. chrysosporium* (Reddy and Gold 2000), *T. versicolor* (Lyr 1963; Konishi and Inoue 1972; Logan, Alleman *et al.* 1994; Ricotta, Unz and Bollag 1996; Ryu, Shim *et al.* 2000; Ullah, Bedford and Evans 2000; Cho, Nam *et al.* 2001), *Rhizoctonia praticola* (Dec and Bollag 1990; Cho, Rogalski *et al.* 1999; Geyer, Richnow and Schlosser 2002), *Cerrena unicolor* (Cho, Nam *et al.* 2001), *Abortiporus biennis* (Cho, Nam *et al.* 2001), *Stropharia rugosannulata* (Geyer, Richnow and Schlosser 2002), a number of *Pleurotus* sp. (Ryu, Shim *et al.* 2000) and various *Ganoderma* sp. (Logan, Alleman *et al.* 1994). In some cases PCP degradation has also been observed in field trials (Lamar and Dietrich 1990; Davis, Glaser *et al.* 1993; Lamar, Evans and Glaser 1993; Lamar 1994; Lamar, Davis *et al.* 1994; Glaser and Lamar 1995; Sims, Sims *et al.* 1999). Extracellular ligninolytic enzymes produced by these white-rot fungi are thought, in most cases, to play a role in this biodegradation process.

The Hazardous Substances New Organisms Act 1996 (HSNO) governs the release of new organisms into NZ (see http://www.ermanz.govt.nz/index.html for more information). The release of non-native white-rot fungi into NZ has not been permitted. However, NZ has a rich variety of native white-rot isolates that may be effective at PCP degradation. To date, three native isolates of *T. versicolor* have been shown to possess excellent PCP and other pollutant degradation *in vitro* and in soil (Walter, Boul *et al.* 2004, Walter, Boyd-Wilson *et al.* 2005a, Walter, Boyd-Wilson *et al.* 2005b). However, no PCP degradation pathways for these or any other NZ white-rot isolates have been elucidated.

The Role of Peroxidases in Chlorophenol Degradation

The degradation pathways of several chlorophenols by the white-rot fungus *P. chrysosporium* have been determined *in vitro* (Valli and Gold 1991; Joshi and Gold 1993; Reddy and Gold 2000). The initial step in this mechanism has been shown to be carried out by peroxidases (Hammel and Tardone 1988; Valli and Gold 1991; Joshi and Gold 1993; Reddy and Gold 2000). Peroxidases catalyse an oxidative dechlorination

reaction at the *para*-position of the chlorophenol to give the corresponding *p*-chloroquinone compound. The resulting chloroquinone compounds are less toxic than the parent chlorophenol (Konishi and Inoue 1972; Ruckdeschel and Renner 1986). However, they are also more lipophilic and tend to bioaccumulate (McAllister, Lee and Trevors 1996; McGrath and Singleton 2000).

Other enzymes are believed to be involved in the chlorophenol degradation pathway. These enzymes are thought to be a combination of cell-bound and intercellular enzymes. For example, two such enzymes have been found in *P. chrysosporium*. A cell-free two component enzymic system can reductively dechlorinate 2,3,5,6-tetrachlorobenzo hydroquinone (TCHQ, Figure 1.11, Reddy and Gold 2001). The two enzymes involved in this pathway are a membrane-bound glutathione *S*-transferase and a soluble glutathione conjugate reductase (Reddy and Gold 1999). The proposed chlorohydroquinone reduction pathway is given in Figure 1.11. In the first step, TCHQ reacts with glutathione *S*-transferase and GSH to give *S*-glutathionyltrichloro-1,4-hydroquinone (TrCHQ-SG). This then reacts with glutathione-conjugate reductase to produce the dechlorinated trichlorohydroquinone (TrCHQ).

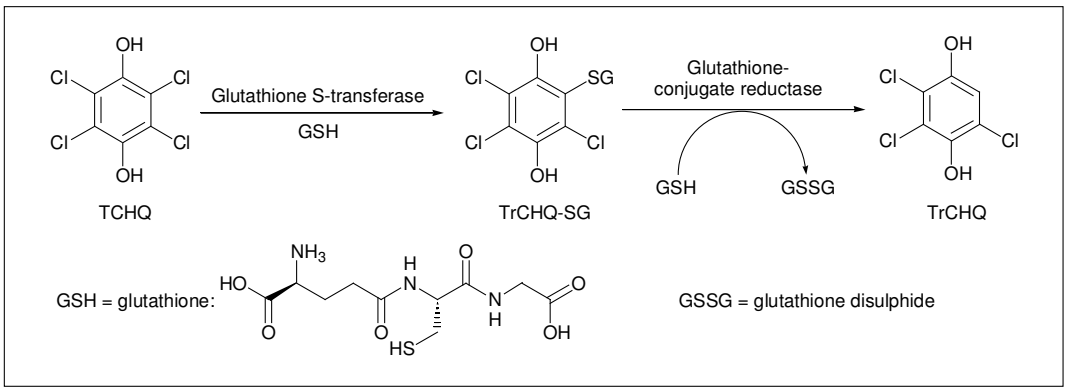


Figure 1.11: Scheme for the two-step reductive dehalogenation of TCHQ to TrCHQ via TrCHQ-GS (Reddy and Gold 2001)

Glutathione *S*-transferase requires glutathione (GSH) as a co-substrate while glutathione conjugate reductase requires either GSH or another thiol containing compound such as dithiothreitol (DTT) or β-mercaptoethanol as a co-substrate.

Reactions of Laccase with Chlorophenols to form Chloroquinone Derivatives

The role of laccase in chlorophenol detoxification appears to be more complicated than that of peroxidases. There has been much debate over what role, if any, laccase plays in chlorophenol detoxification and degradation. This is because researchers have observed a range of different metabolites from reactions of laccase with various chlorophenols. Oxidation of a phenolic compound with laccase gives a resonance-stabilised radical intermediate (Figure 1.12, Roy-Arcand and Archibald 1991). The final product from this reaction depends on the composition of the reaction mixture. In some cases, the major metabolite is a *p*-chloroquinone or *o*-chloroquinone, as has been seen in chlorophenol reactions with peroxidases (Ricotta, Unz and Bollag 1996; Grey, Höfer and Schlosser 1998).

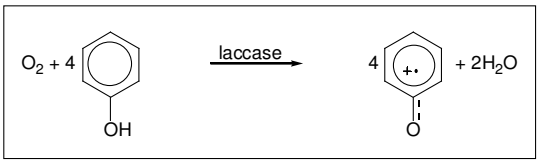


Figure 1.12: The initial reaction of a phenolic compound with laccase (Roy-Arcand and Archibald 1991)

The earliest report of chlorophenol degradation was that of PCP by *T. versicolor*, published in 1962 (Lyr 1962). Lyr observed that chlorinated phenols lose their toxicity after oxidation by fungal oxidases. Lyr proposed that the PCP degradation pathway by *T. versicolor* involved dechlorination by fungal oxidases followed by ring cleavage.

Grey, Höfer and Schlosser (1998) studied the degradation of a simple chlorinated phenol, 2-chlorophenol (2-CP), by 9 day old fungal mycelia and cell-free crude culture liquids of *T. versicolor*. Addition of 2-CP to the fungal medium enhanced the production of laccase. Formation of the metabolite 2-chlorobenzoqinone (2ClBQ, Figure 1.13) was correlated with laccase activity but disappearance of 2-CP was not correlated to laccase activity indicating that other enzymes may be involved in further degradation of the 2ClBQ metabolite.

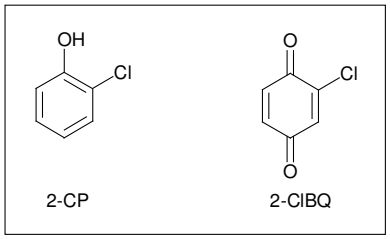


Figure 1.13: 2-CP and 2-ClBQ

Ricotta, Unz and Bollag (1996) investigated the role of laccase produced by *T. versicolor* in PCP degradation. They found that PCP degradation with laccase alone initially resulted in the formation of *p*-chloranil (TCHD) and *o*-chloranil (3,4,5,6-tetrachloro-3,5-cyclohexadiene-1,2-dione, (Figure 1.14). After 10 to 14 days of incubation neither metabolite was observed and 9.2% of the initial radioactivity was present as $^{14}CO_2$.

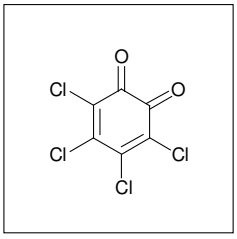


Figure 1.14: *o*-Chloranil

Reactions of Laccases with Chlorophenols to form Polymeric Compounds

The resonance-stabilised phenolic radical shown in (Figure 1.12) can also undergo polymerisation reactions. There have been several reports of formation of polymers from reactions of chlorophenols with laccase. In some cases, the reactions proceed with loss of chloride ions (Roy-Arcand and Archibald 1991), while one group has reported on polymerisation of PCP without loss of chloride (Ullah, Bedford and Evans 2000).

Dec and Bollag (1990) tested purified laccase (from *T. versicolor* and *R. praticola*), horseradish peroxidase and tyrosinase for their ability to polymerise 2,4-DCP, 4-chlorophenol (4-CP), 2,4,5-TCP and PCP. Generally, the efficiency of transformation decreased as the number of chloride atoms increased. The exception to this was that 2,4-DCP was transformed more efficiently than 4-CP. The product of 2,4-DCP transformation was a mixture of high molecular weight polymers. Chloride loss occurred during polymerisation and they postulated that dechlorination would also

occur during transformation of other chlorophenols. The extent of polymerisation of chlorinated phenols depended on the substrate concentration, pH, enzyme concentration, incubation period and temperature. They also compared the effect of substituent position on transformation using mono-substituted bromo-, methoxy-, methyl- and chlorophenols. *Ortho*- and *para*-substituted phenols were transformed more efficiently than *meta*-substituted phenols. Transformation efficiency also decreased with increasing molecular weight of the substituent.

Roy-Arcand and Archibald (1991) demonstrated that purified laccase from *T. versicolor* could partially dechlorinate a number of polychlorinated phenols. For reactions of laccase with PCP, very little degradation was observed in the initial period of the study (30 minutes) but over a longer period of time some PCP consumption and release of chloride ions was observed. A substantial increase in the reactivity of laccase with PCP was observed in the presence of other chlorinated phenols or ABTS compared to reactions with laccase alone. They suggested that the increase in PCP reactivity was due to the presence of free radicals, from the oxidation of other chlorophenols or ABTS, acting on PCP rather than a direct reaction with laccase. This research group did not characterise the degradation products of their reactions. Since they observed an increase in the amount of free chloride detected in solution, the products from PCP degradation may include *o*- or *p*-quinone compounds, as have been observed previously. However, it is likely that the major product from these reactions was formation of polymers of these quinones as a brown dialysis-resistant colour was observed in the treated samples, an observation consistent with formation of insoluble quinonoid polymers (Lundquist and Kristersson 1985; Roy-Arcand and Archibald 1991).

Ullah, Bedford and Evans (2000) examined the reactions of purified laccase with PCP. They varied several different parameters such as pH (pH 3-7), temperature (4-55ºC), amount of laccase (12-200 U) and PCP concentration (25-200 μg mL^{-1}). The product of reactions from 100 μg mL^{-1} PCP with 100 U of laccase[16] gave a high molecular weight product (MW 80 kDa) that was acid stable and stable in aqueous solution over at least 30 days. No free chloride was released during polymer formation. They suggested that this implied laccase catalysed the polymerisation reaction solely through polymerisation

[16] 1 unit of laccase was defined as the amount of enzyme required to oxidise 1 μmol of catechol or syringaldazine per minute per ml at 25ºC.

at the phenolic group of PCP. The structure of the resulting polymer was not identified or discussed.

Copolymerisation of Chlorophenols With Humic Acid Precursors

Some researchers have attempted to exploit the polymerisation process by adding co-substrates to form complex polymers. These experiments are attempts to mimic the humification process observed in the formation of soil. The copolymer substrates used are generally humic acid precursors or lignin-like compounds such as ferulic acid (FA, Figure 1.15). Dec and Bollag (1994) studied oxidative coupling of chlorophenols with either laccase, laccase with humic acid or birnessite (manganous manganite, an inorganic catalyst). They concluded that dechlorination occurred as a result of chlorophenol coupling rather than oxidation by laccase, as birnessite was also able to catalyse the chlorophenol polymerisation reaction.

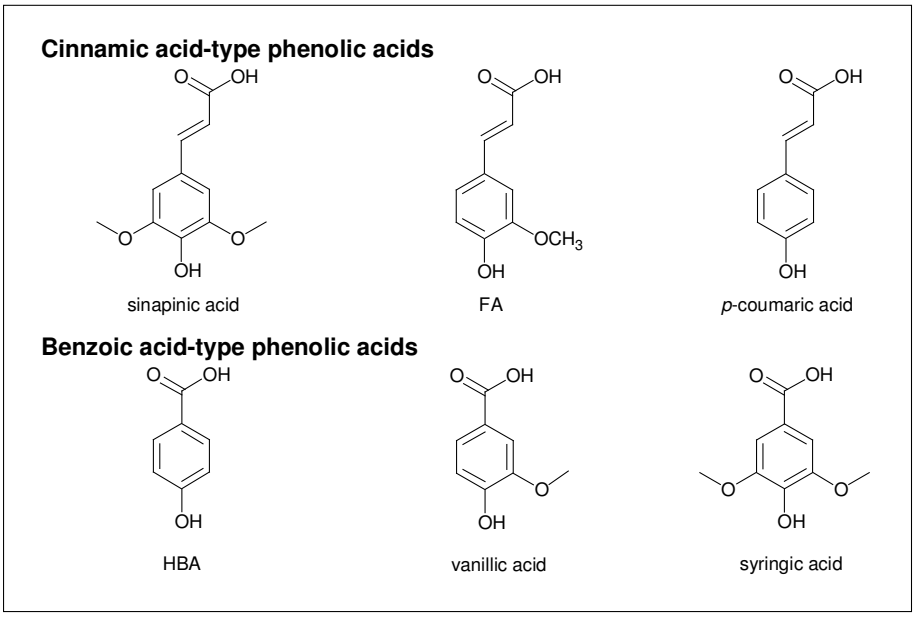


Figure 1.15: Sinapinic acid, FA, *p*-coumaric acid, HBA, vanillic acid and syringic acid

Another group used laccase purified from *C. unicolor* and *R. praticola* to polymerise a range of chlorinated phenols in reactions containing coniferyl alcohol. The major degradation product was a sediment that could be removed from the water (Cho, Rogalski *et al.* 1999).

Itoh, Fujita *et al.* (2000) investigated the effect of six phenolic acids (Figure 1.15) on chlorophenol degradation by purified laccase from *T. versicolor*. They also used bovine serum albumin (BSA) in the enzyme solution to protect it from inactivation by reaction intermediates. The phenolic acids had differing reactivity with the various chlorophenols. In general, the cinnamic acid-type phenolic acids were more reactive with laccase than the benzoic acid-type phenolic acids. FA and sinapinic acid showed opposite effects on chlorophenol degradation. FA acted as an inhibitor in the transformation of 2,4-DCP. Radical intermediates produced by oxidation of FA reacted with laccase causing inactivation of the enzyme. In contrast, sinapinic acid increased transformation of 2,4-DCP. This was postulated to be through reaction of sinapinic acid radicals with 2,4-DCP.

1.9 TOXICITY AND PERSISTENCE OF PCP METABOLITES IN THE ENVIRONMENT

It is important to study the toxicity and persistence of PCP metabolites in soil, as some metabolites are more toxic and/or more persistent in the environment than PCP. For example, the major metabolite from degradation of PCP in soil by *P. chrysosporium* is PCA. This compound is less toxic to fungi than the parent compound (Cserjesi and Johnson 1972; Ruckdeschel and Renner 1986). However, it is more lipophilic and tends to bioaccumulate. It is also resistant to further degradation, although mineralisation of PCA by *P. chrysosporium* has been reported (Chung and Aust 1995). Other white-rot fungi such as *T. versicolor* degrade PCP without the accumulation of PCA (Tuomela, Lyytikäinen *et al.* 1999; Walter, Boul *et al.* 2004).

It is estimated that there are over 30 metabolites possible from PCP degradation. Ruckdeschel and Renner (1986) synthesised several PCP metabolites and used sixteen strains of fungi (including a mixture of sapstain, wood decay, mould and yeast-like fungi) to examine the toxicity of PCP and 31 of its metabolites. Five chlorophenolic metabolites (3,5-DCP, 2,3,4-, 2,3,5-, 2,4,5- and 3,4,5-TCP and 2,3,4,5-TeCP) were more toxic than PCP against all sixteen fungal strains. Eight other PCP metabolites (2,3-, 2,4-, 2,5-, and 3,4-DCP, 2,3,6- and 2,4,6-TCP, and 2,3,4,6- and 2,3,5,6-TeCP) were more toxic than PCP against some, but not all, of the sixteen fungal strains.

Different methods have been used to assess soil toxicity and these give varying results, therefore it is difficult sometimes to compare toxicity studies. For example, two reports into the toxicity of PCP metabolites produced during degradation by *P. chrysosporium* were published in 2000 (McGrath and Singleton 2000; Mendoza-Cantú, Albores *et al.* 2000). In the first study (Mendoza-Cantú, Albores *et al.* 2000) toxicity in soil was measured using the Microtox® bioassay, a bioluminescence assay that assesses the toxicity of a compound using a marine bacteria, *Photobacterium phosphoreum*. Soil toxicity was monitored regularly over an 11 day period. By the end of the experiment, the soil toxicity was almost negligible. In contrast, McGrath and Singleton (2000) carried out a similar experiment over a six week period. They used a soil bacterium, *Bacillus megaterium*, to assess soil toxicity over the course of their experiments. They reported that after six weeks of incubation, although the levels of PCP had decreased considerably, the soil toxicity was still high. They postulated that PCP metabolites present in the soil were responsible for the toxicity to *B. megaterium* cultures.

1.10 SUMMARY OF THE LITERATURE

Pentachlorophenol (PCP), a xenobiotic toxic to humans and animals, was used extensively as a timber preservative in NZ until 1988. Currently over 600 sites throughout NZ remain contaminated by PCP from its use by the forestry industry.

The review of the literature shows that bacteria, and white- and brown-rot fungi are the key organisms in nature that are able to degrade PCP. To date, white-rot fungi have been the preferred organisms because they produce extracellular enzymes that can detoxify PCP. This means that they do not have to ingest PCP in order to begin the degradation process. White-rot fungi have been widely evaluated for their ability to mineralise PCP with a view to their eventual use for the cleanup of PCP contaminated sites.

PCP degradation by white-rot fungi occurs by an initial dechlorination step, followed by further degradation steps to eventually give CO_2. Both intracellular and extracellular enzymes are likely to be involved in the degradation pathway. Ligninolytic enzymes,

i.e. peroxidases and laccases, are reported to be involved in the initial PCP detoxification step. They may also be involved in concert in the later degradation steps that lead to PCP mineralisation.

Laccases are enzymes that are either constitutively present or are inducible. Some white-rot fungi produce a mixture of constitutive and inducible laccases. Whether inducible laccase is expressed or not depends upon the white-rot fungus and the conditions in which they are grown. Laccases can be induced by addition of lignin-like compounds, laccase co-factors or both in the fungal growth medium. Unlike peroxidases, laccases do not require addition of hydrogen peroxide as a cofactor to complete their catalytic cycle. However, addition of mediator compounds such as ABTS can increase the substrate range of laccases.

Peroxidases catalyse an oxidative dechlorination reaction of PCP to give *p*-chloranil. Similarly, laccases either catalyse an oxidative dechlorination reaction of PCP to give *p*-chloranil or *o*-chloranil. Polymer formation has also been observed from reactions of laccase with PCP.

Currently, the only PCP degradation pathway that has been elucidated is that of *P. chrysosporium*. This white-rot fungus is not found in NZ and its importation and release in NZ is not permitted under the HSNO act. However, NZ has a rich variety of native white-rot species and these may be suitable candidates for bioremediation of PCP at contaminated sites in NZ. To date, three native NZ white-rot fungi have been shown to be effective PCP degraders. For effective function in the environment for the clean up of PCP the selected white-rot fungus needs to be able to tolerate high PCP concentrations, be able to degrade PCP *in vitro*, be able to grow at a range of different temperature and be able to produce inducible ligninolytic enzymes.

1.11 AIMS AND OBJECTIVES OF THIS STUDY

The overall aim of these studies was to identify a NZ white rot fungus that may be useful for the biodegradation of PCP and to investigate its PCP handling ability in laboratory studies.

The first objective of this study was to select white-rot isolates with the potential to degrade PCP. This work is described in Chapter 2. This was achieved by screening isolates from the HortResearch culture collection (HRCC) for sensitivity to PCP, sensitivity to creosote and tolerance to a range of different growth temperatures. Isolates that were tolerant to high PCP concentrations were further screened for ligninolytic enzymes and PCP degradation *in vitro*. A preliminary experiment into laccase induction and production by PCP for the chosen white-rot isolate is also described in this chapter.

The second objective of this study was to identify the fungal isolate chosen for these studies so that the gene sequences responsible for laccase isozyme production could be studied in detail. Chapter 3 describes the morphological and genetic techniques used to identify this white-rot. It also describes the gene sequencing studies that allowed a comparison with laccase sequences from other white-rot fungi.

The third objective of this study was to purify and characterise the ligninolytic enzymes from the chosen isolate. Chapter 4 describes this work.

The fourth objective was to study degradation of PCP using crude and partially purified isozymes versus whole cultures to establish whether or not laccase isozymes are involved in PCP degradation by the chosen white-rot isolate. PCP disappearance by whole cultures and purified enzymes is described in Chapter 5.

Chapter 6 provides a summary and a brief discussion of the results presented in this thesis in the context of the literature. This chapter also discusses possible future research that could be carried out using the chosen white-rot fungus.

CHAPTER 2: SCREENING OF WHITE-ROT FUNGI FOR BIOREMEDIATION POTENTIAL

2.1 INTRODUCTION

Bioremediation is potentially an environmentally safe, biologically effective, and low cost method to clean up xenobiotics (man-made pollutants) in the environment (see Chapter 1). It involves the use of bacteria or fungi to breakdown contaminants in soil and water. White-rot fungi have been proposed as good candidates for bioremediation of soil (Glaser and Lamar 1995). These fungi produce extracellular enzymes, namely peroxidases and laccases, believed to be involved in the initial detoxification and may also be involved in breakdown of xenobiotics (Valli and Gold 1991; Field, de Jong *et al.* 1993a; Joshi and Gold 1993; Reddy and Gold 2000). Fungi are preferred over bacteria as they do not need to ingest the xenobiotic in order to carry out the initial detoxification step and therefore can tolerate higher toxin concentrations (Barr and Aust 1994; Glaser and Lamar 1995).

Numerous studies show that white-rot fungi are excellent candidates for the bioremediation of xenobiotics. These studies include both *in vitro* and field trials (Bumpus and Aust 1985; Mileski, Bumpus *et al.* 1988; Lamar and Dietrich 1990; Field, de Jong *et al.* 1992; Bumpus, Kakar and Coleman 1993; Davis, Glaser *et al.* 1993; Lamar, Evans and Glaser 1993; Lamar 1994; Lamar, Davis *et al.* 1994; Glaser and Lamar 1995; Sims, Sims *et al.* 1999). The growth and physiology of many of these fungi has also been examined. Overseas, *P. chrysosporium* is the most extensively studied white-rot fungus for bioremediation, and the degradation pathway used by this organism has been determined *in vitro* (Reddy and Gold 2000). However, this white-rot fungus is not found in NZ. Further, the release of non-native fungi such as *P. chrysosporium* into NZ has not been permitted. Therefore, the use of *P. chrysosporium* as a bioremediation agent is not an option for NZ, and a native NZ white-rot fungus of equal or similar functionality needs to be found for clean up of contaminated sites in NZ.

One of the objectives of this study was to set up a database of NZ white-rot isolates using the information gathered from mass screening experiments. The database

information will eventually be used to select the isolates best suited to decontaminate each site.

This chapter is divided into six sections. For each part the experimental set up and the findings are discussed together, with reference to the published literature relevant to that area. The first part describes mass screening of fungal isolates for temperature tolerance, and sensitivity to PCP and creosote. The aim was to identify 20 isolates for a more detailed study, selection being weighted more towards tolerance to PCP. Part two describes the selection of 20 isolates for further studies and highlights some of the physical properties of these isolates from the mass screening experiments. The third part describes preliminary PCP degradation studies with the chosen 20 isolates and experiments to assay for the presence of laccase. Part four describes further experiments to assay for ligninolytic enzymes in six selected fungi. The fifth part describes criteria used to identify one suitable white-rot isolate for the detailed molecular and biochemical studies described in the rest of this thesis. The final part outlines a brief study examining the influence of PCP on production of laccase by the chosen isolate.

2.2 MASS SCREENING OF NZ WHITE-ROT FUNGI

The white-rot fungus selected for bioremediation at a particular contaminated site should have physical properties suited to that environment (Field, de Jong *et al.* 1993b). Ideally, fungi should be able to grow well over a range of different temperatures and be resistant to high concentrations of xenobiotics likely to be found in contaminated soil. Although contaminated soils often contain a range of xenobiotics, for example creosote is often a co-contaminant in PCP contaminated soil (see Chapter 1), it is much simpler and quicker to study sensitivity to xenobiotics individually.

This part describes the initial mass screening of fungal isolates for temperature tolerance, and sensitivity to PCP and creosote. All of the white-rot fungi chosen for the initial screening experiments were randomly selected from the 367 cultures held in the HRCC. The HRCC[17] contains white-rot isolates collected by three NZ Crown Research Institutes (CRI): HortResearch, Landcare and Forest Research Institute (FRI). The collection includes several strains of overseas isolates kept in quarantine for use as reference isolates. Slightly different total numbers of white-rot fungi were screened in the three different studies: 261 isolates were examined in the temperature tolerance screening study, 253 isolates were examined in the PCP sensitivity study, and 147 isolates were examined in the creosote resistance study. The three screening studies described were conducted concurrently.

Temperature Tolerance

The temperature in most NZ climates rarely gets much above 30ºC. However, when white-rot fungi are used for clean up of contaminated sites a compost-like set up known as a 'biopile' is often used (Walter, Boyd-Wilson *et al.* 2005a). The temperature in biopiles can easily increase above 30ºC. Therefore, it is important to select fungi that can tolerate and grow at higher temperatures. The information from temperature tolerance experiments was included in the bioremediation database to aid in selection of isolates for cleanup of contaminated sites in NZ.

For this part of the study, 261 randomly selected NZ isolates were screened for their ability to tolerate and grow over a range of temperatures (0, 10, 20, 25, 30, 35 and 40ºC, see Chapter 7). Ability to grow was determined by measuring the hyphal extension after seven days incubation at the different temperatures. If cultures did not grow after seven days, they were placed in the 25ºC incubator for a further seven days to observe if fungal growth resumed, to determine if fungi were viable. Because the number of incubators available limited the number of concurrent experiments that could be

[17] The culture collection is kept at HortResearch, PO Box 51, Lincoln, NZ. Three of the *T. versicolor* isolates (HR131, HR154 and HR160) from this collection have also been deposited in the Australian Government Analytical Laboratory, International Depositary Authority, PO Box 385, Pymble, NSW, Australia (accession numbers NM02/27875, NM02/27876 and NM02/27877 for white-rot isolates HR131, HR154 and HR160, respectively)

conducted, it was not possible to confirm whether the best temperatures were in fact true optimum growth temperatures for these fungi.

All 261 isolates survived incubation between the temperatures of 0 to 30°C. However, 18% and 40% did not survive incubation at 35 and 40°C, respectively.

The best temperatures for growth in these studies (20-40ºC) align well with the optimal growth temperatures for a range of American white rot fungi as reported by Lamar's group (Lamar, Larsen and Kirk 1990; Lamar, Dietrich *et al.* 1999). All of the American isolates grew between 14 and 28ºC and the temperature optima for growth varied between 22 and 40ºC[18].

PCP Sensitivity

Resistance to PCP is usually tested by growth on agar plates amended with PCP[19] for fourteen days (Lamar, Larsen and Kirk 1990). Overseas research groups have tested fungal growth on agar amended with different levels of PCP: between 0 and 25 mg L^{-1} PCP (Lamar, Larsen and Kirk 1990), between 0 and 15 mg L^{-1} PCP (Lamar, Dietrich *et al.* 1999), and between 0 and 50 mg L^{-1} PCP (Leontievsky, Myasoedova *et al.* 2000) or 0 and 500 mg L^{-1} PCP concentrations (Cho, Nam *et al.* 2001).

A wide range of PCP concentrations (0, 10, 20, 30, 40 and 50 mg L^{-1} PCP) were therefore used initially in this study to determine the concentration most suitable for screening of the NZ native white-rot fungi. Sixty six randomly selected isolates were tested at these concentrations. Hyphal growth was measured to determine fungal resistance to PCP.

Of the 66 isolates only 9 isolates (14%) produced viable growth at the highest concentration (50 mg L^{-1} PCP) tested. Therefore, it was decided to complete the mass screening by testing the remaining 187 fungi using 50 mg L^{-1} PCP.

[18] The temperature range studied was 0-44ºC in 2ºC increments.

[19] The term 'amended agar' refers to agar that has had a xenobiotic such as PCP added to it while the agar temperature is about 50-55ºC. The xenobiotic is often dissolved in a water miscible solvent such as acetone. For example, PCP amended agar used in these experiments was prepared as follows: PCP was dissolved in acetone and diluted with autoclaved MEA at 50-55°C to give a range of PCP concentrations (0, 10, 20, 30, 40 and 50 mg L^{-1} PCP).

Of the 253 isolates screened for PCP resistance, 95 isolates (38%) were able to grow on 50 mg L^{-1} PCP amended agar. These isolates were then tested for tolerance to a higher PCP concentration in agar (200 mg L^{-1}). Twenty three of the 95 (24% of the white-rots identified as tolerant to 50 mg L^{-1} PCP, and 9% of the original pool of 253 isolates) maintained viable growth at the higher concentration of 200 mg L^{-1} PCP.

In the current study, a total of nine native *T. versicolor* isolates were included in the screening for resistance to PCP. Of the nine isolates tested for resistance to PCP, eight grew on 50 mg L^{-1} PCP amended agar and six of these were able to grow on 200 mg L^{-1} PCP amended agar. The isolates that grew at the highest PCP concentration showed very different resistances to 200 mg L^{-1} PCP in agar. Figure 2.1 shows the resistances of three *T. versicolor* isolates to 200 mg L^{-1} PCP amended agar. These results show that the PCP resistance of a given isolate of *T. versicolor* cannot be taken to be representative of other isolates of *T. versicolor*. It also highlights the need for screening of different strains of fungal isolates from the same species.

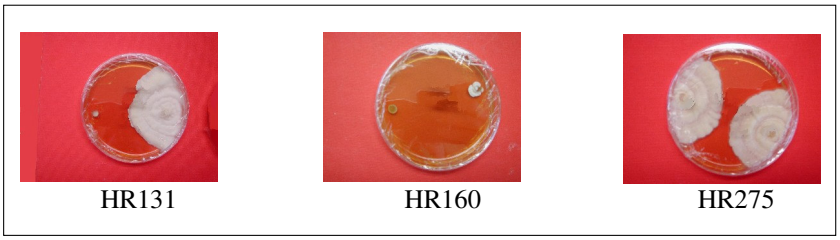


Figure 2.1: Growth of three *T. versicolor* isolates on plates amended with 200 mg L^{-1} PCP. Fungi were incubated at 25ºC in the dark for fourteen days.

The results of this study compare well with overseas studies, which show variations in fungal resistance to different PCP concentrations between different species of white-rot fungi. Differences in resistance amongst strains of the same species have also been observed. In one overseas study, PCP concentrations above 50 mg L^{-1} inhibited fungal growth completely for a Polish strain of *T. versicolor* (Cho, Nam *et al.* 2001) while 75 mg L^{-1} PCP completely inhibited fungal growth of a Russian strain of *T. versicolor* (Leontievsky, Myasoedova *et al.* 2000).

Creosote Resistance

Creosote was sometimes a component of the PCP mixture used to coat logs (McLean 1997; McQuire 1997, see Chapter 1). Because of this, fungal resistance to creosote was tested.

The 147 isolates chosen for the creosote resistance screening were randomly selected. They included isolates tolerant to high concentrations of PCP and isolates with a low resistance to PCP.

Five NZ isolates were used in the initial creosote screening experiment to find a suitable threshold concentration to distinguish between isolates. All five isolates tolerated creosote at 500, 1,000 and 5,000 mg L^{-1} in agar, growing right to the edge of the plates by the end of the two weeks of the experiment. At 10,000 mg L^{-1} some growth inhibition was observed, however all five isolates were still able to grow at this concentration. None of the isolates grew at 100,000 mg L^{-1} creosote. Therefore 10,000 mg L^{-1} was chosen for screening.

One hundred and thirty one (89%) of the 147 white-rot isolates tested produced viable growth on 10,000 mg L^{-1} creosote.

Figure 2.2 shows photos for six examples (of the 147 isolates screened for resistance to creosote in agar) relative to un-amended agar plates. The figure shows that growth of five of the six white-rots was essentially unrestricted in the presence of creosote.

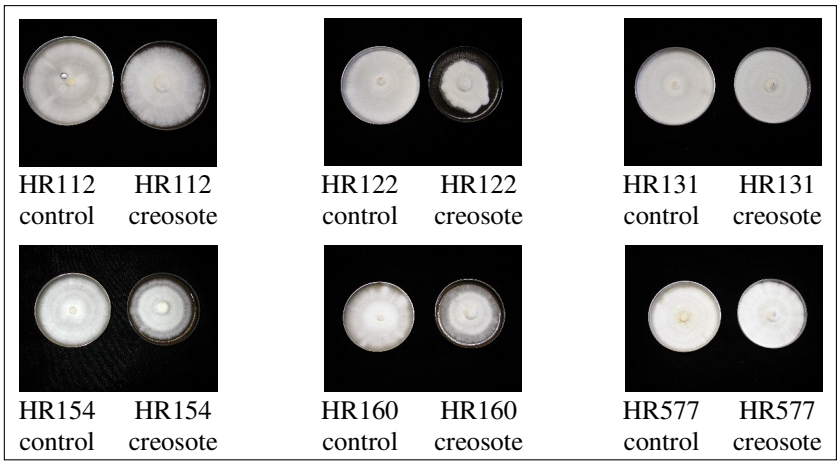


Figure 2.2: Growth of white-rot fungi on agar amended with 10,000 mg L^{-1} creosote compared to no creosote controls. Fungi were incubated at 25ºC in the dark for fourteen days. HR112: unknown basidiomycete sp., HR122: unknown basidiomycete sp., HR160: *T. versicolor*, HR154: *T. versicolor*, HR577 *Trametes* sp.

These results contrast with those of Lamar, Dietrich *et al.* (1999) who found that fewer (5 of 14 American basidiomycetes, i.e. 36%) grew on 1,000 mg L^{-1} creosote in agar. The reasons for the high tolerance of NZ white-rot isolates to creosote compared to the American isolates is unclear. Since PCP degradation was the main focus of this project, creosote resistance was not investigated further.

Summary

PCP and creosote sensitivity on agar and tolerance to a range of growth temperatures was used to screen 367 isolates in the HRCC collection. All 261 isolates tested for temperature tolerance survived incubation between the temperatures of 0 to 30°C. However, 18% and 40% did not survive incubation at 35 and 40°C, respectively. The best temperature for fungal growth varied between 20-40ºC. Of the 253 isolates screened for PCP resistance, 95 isolates (38%) were able to grow on 50 mg L^{-1} PCP amended agar. Twenty three of the 95 (24% of the white-rots identified as tolerant to 50 mg L^{-1} PCP, and 9% of the original pool of 253 isolates) maintained viable growth at the higher concentration of 200 mg L^{-1} PCP. One hundred and thirty one (89% of the 147 isolates tested) produced viable growth on 10,000 mg L^{-1} creosote[20].

Twenty isolates were chosen for further studies[21] based on the results of the mass screening experiments. Selection was weighted towards growth on 200 mg L^{-1} PCP in agar.

2.3 PROPERTIES OF 20 ISOLATES SELECTED FOR FURTHER STUDIES

This part examines the xenobiotic sensitivity and temperature tolerance for growth for the 20 isolates in more detail. The twenty isolates represented a range of different genera: *A. biemmis* (HR145), *Oudemansiella australis* (HR345), *Peniophora sacrata* (HR226, HR235 and HR240), *Stereum faciatum* (HR348), *Trametes* sp. (HR192,

[20] A summary of the temperature, PCP and creosote experiments is in the Appendix for Chapter 2
[21] Due to limitations on available equipment, only twenty isolates could be taken further in the study.

HR196, HR197 and HR577[22]), *T. versicolor* (HR131, HR154, HR160, HR275, HR277 and HR445) and five unknown basidiomycete species, various strains (HR112, HR122, HR152, and HR588).

Nineteen of the twenty were selected based on their ability to tolerate 200 mg L^{-1} PCP in agar. The exception, HR154, did not grow on agar containing 200 mg L^{-1} PCP. It was also included because earlier studies by the HortResearch Environmental Research Management group (HRERM) showed that it could mineralise PCP in soil (Walter, Boul *et al.* 2004).

The temperature tolerance and xenobiotic resistance data (resistance to 200 mg L^{-1} PCP and tolerance to 10,000 mg L^{-1} creosote) for the twenty isolates are shown in Table 2.1. The results show that most fungi grew best at temperatures between 25-30ºC (HR235, HR240, and HR277 were the 3 exceptions) and 8 of the 20 exhibited good growth rates (>10 mm hyphal growth/day) at 25ºC.

These results also show that 5 of the cultures (HR192, HR196, HR197, HR277 and HR445) grew reasonably well in the presence of PCP (i.e. exhibited between 21 and 50% of the growth of the PCP-free control) while two (HR577 and HR275) grew quite well (i.e. exhibited between 51 and 65% of the growth of the PCP-free control) and 12 isolates were tolerant to creosote at 10,000 mg L^{-1}.

Varied Response to Growth in the Presence of Xenobiotics

The ability to resist PCP did not necessarily mean that isolates would also tolerate creosote. Table 2.1 shows that 12 of the 20 isolates (60%) were resistant to creosote when grown in agar plates.

This variation in resistance to different xenobiotics is similar to that observed by Leontievsky, Myasoedova *et al.* (2000) who studied the toxicity of several different chlorophenols to the two white-rot fungi, *P. tigrinus* and *T. versicolor*, when grown in agar. Even though the two fungi were affected in a similar way to most of the

[22] NB: This isolate was subcultured from a fruiting body growing on a birch log. Its identification using morphological features and gene sequences is described in Chapter 3.

chlorophenols, *T. versicolor* was shown to be more resistant to 2-CP and PCP and much more sensitive to 3,5-DCP when compared to *P. tigrinus*.

Table 2.1: Temperature and xenobiotic resistance data for twenty white-rot isolates selected for further studies

Isolate Code	Species	Temperature		200 mg L^{-1} PCP		10,000 mg L^{-1} creosote	
		Maximum growth (ºC)	Growth at 25ºC (mm d^{-1})[23]	Actual growth (mm)[24]	Growth (%) compared to nil control[25]	Actual growth (mm)[26]	Growth (%) compared to nil control[27]
HR112	Unknown	25	18	2	6	36	45
HR122	Unknown	25	18	5	13	31	40
HR131	*T. versicolor*	30	18	7	18	40	51
HR145	*A. biemmis*	25	3	1	8	0	0
HR152	Unknown	25	7	4	13	2	8
HR154	*T. versicolor*	30	18	0	0	32	46
HR160	*T. versicolor*	25-30	15	2	6	14	21
HR192	*Trametes* sp.	25	4	4	22	0	0
HR196	*Trametes* sp.	25	3	6	28	0	0
HR197	*Trametes* sp.	25	3	9	46	0	0
HR226	*P. sacrata*[28]	25	12	3	7	12	16
HR235	*P. sacrata*[22]	20	7	3	7	6	10
HR240	*P. sacrata*[22]	20	8	3	7	13	22
HR275	*T. versicolor*[22]	30	15	24	65	29	42
HR277	*T. versicolor*[22]	20	3	7	31	12	34
HR345	*O. australis*[22]	25	4	3	15	0	0
HR348	*S. fasciatum*[22]	25	3	3	16	0	0
HR445	*T. versicolor*[29]	25	5	9	42	0	0
HR577	*Trametes* sp.	30	16	21	57	26	34
HR588	Unknown	35	7	1	3	0	0

[23] Most rapid growth rate observed at 25ºC
[24] Actual growth (mm) on 200 mg L^{-1} PCP amended agar determined by mycelial extension after 14 days incubation at 25ºC in the dark
[25] Percentage growth compared to nil-control on 200 mg L^{-1} PCP amended agar after 14 days incubation at 25ºC in the dark
[26] Actual growth (mm) on 10,000 mg L^{-1} creosote amended agar determined by mycelial extension after 14 days incubation at 25ºC in the dark
[27] Percentage growth compared to nil-control on 10,000 mg L^{-1} creosote amended agar after 14 days incubation at 25ºC in the dark
[28] Scion proprietary isolate
[29] Landcare Research proprietary isolate

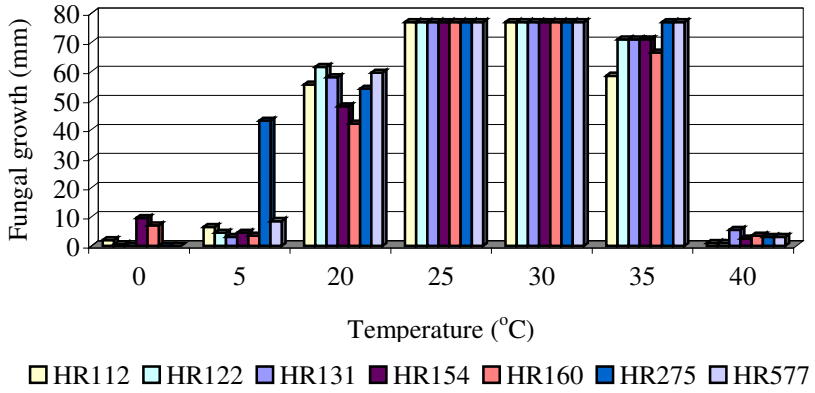
Growth at Different Temperatures for the Twenty Selected Isolates

The twenty fungi in Table 2.1 were grouped on the basis of growth rate at 25ºC. The data is shown in Figure 2.3. Fungi that were able to completely cover the agar plate by day 7 at 25ºC were classed as 'fast growing' (Figure 2.3a) while those that covered between 18-27% were classed as 'slow growing' (Figure 2.3b). There were four white-rot fungi that grew at an intermediate rate (Figure 2.3c). Two of these were *P. sacrata* isolates. These grew best at 20ºC (almost complete plate coverage after 7 days) but did not grow well above 30ºC. One other *P. sacrata* isolate (HR226) was included in the twenty isolates shown in Table 2.1. This isolate had a similar temperature profile to the other two *P. sacrata* isolates but grew slightly better at 25ºC than at 20ºC (Figure 2.3a).

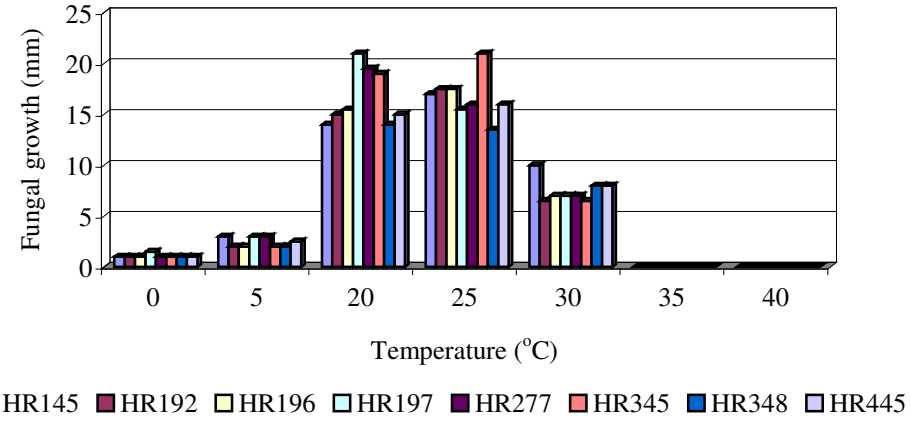
None of the slow growers grew at 35 or 40ºC. Two of these, HR145 and HR348, resumed normal growth when moved to the 25ºC incubator. With the exception of the *P. sacrata* isolate mentioned above (HR226), all of the fast growing white-rot fungi grew at 35ºC and had minimal growth at 40ºC. All of the fast growers (except HR226) resumed normal growth after being moved from the 40ºC incubator to the 25ºC incubator.

The best 10 fungal isolates in terms of percentage growth on PCP-amended agar were all slow growing fungi except for HR275, HR122 and HR577. In contrast, apart from HR277, none of the slow growing isolates were able to grow on creosote-amended agar. The only other isolate that did not grow on creosote-amended agar was HR588, one of the isolates with an intermediate growth rate.

a)



b)



c)

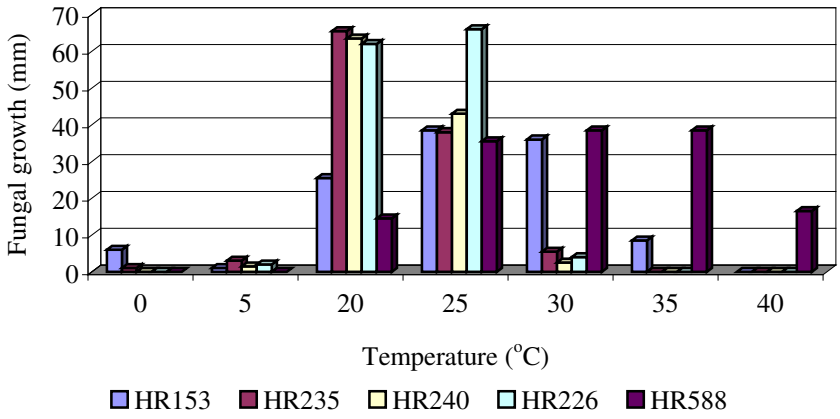


Figure 2.3: Growth at different temperatures for selected white-rot fungi. Fungal growth at each temperature was measured after 7 days growth. Fungal growth of 77 mm indicates that the fungus grew to the edges of the agar plate in the 7 days. Growth over 7 days at 25°C degrees was used to classify fungi as either: a) fungi with a fast growth rate (100% coverage of agar plate in 7 days); b) fungi with a slow growth rate (18-27% coverage of agar plate in 7 days); c) fungi with a moderate growth rate (46-86% coverage of agar plate in 7 days). The identity of each fungal isolate is listed in Table 2.1.

Summary

Most fungi (80%) grew best at temperatures between 25-30ºC. None of the slow growing isolates grew at 35 or 40ºC. All but one of the fast growing white-rot fungi had minimal growth at 40ºC but resumed normal growth after being moved to the 25ºC incubator. Five cultures grew reasonably well in the presence of PCP and two grew quite well in the presence of PCP. The ability to resist PCP did not necessarily mean that isolates would also tolerate creosote. Twelve of the 20 isolates were tolerant to creosote at 10,000 mg L^{-1} in agar.

2.4 PCP DEGRADATION AND LACCASE PRODUCTION *IN VITRO*

This part describes the screening studies performed with the 20 fungal isolates for their ability to degrade PCP and produce extracellular laccase. These features are two criteria considered important in assessing the bioremediation potential of the isolates.

PCP Degradation in vitro

For these studies isolates were grown on a mixture of sawdust cornmeal and starch (SCS) prior to inoculation into the liquid medium. This was to mimic one of the potential methods of introducing the fungi into soil (Boyle 1995; Leštan, Leštan *et al.* 1996; Walter, Boyd-Wilson *et al.* 2005a). A carbon and nitrogen source such as SCS is needed to encourage soil growth because white-rot fungi do not naturally grow in soil. Previous work has shown that the age and composition of the SCS mixture affects both the type and level of enzymes expressed (Schmidt, Chand *et al.* 2005). Therefore the SCS used for the present experiments was prepared fresh prior to fungal inoculation.

In this experiment 50 mg L^{-1} PCP was added to the culture medium. Given their tolerance to 200 mg L^{-1} PCP in agar, the isolates HR577 and HR275 were expected to be the most tolerant to PCP in liquid medium[30]. Isolates HR192, HR196 HR197,

[30] Based on percentage growth on 200 mg L^{-1} relative to growth on un-amended agar.

HR277 and HR445 were expected to be the next most tolerant to PCP in liquid medium and the rest were expected to have a low tolerance to PCP in liquid medium.

Table 2.2 shows that all 20 isolates significantly ($P<0.05$) reduced PCP supplied in the liquid medium over the 42 day incubation period when compared to the PCP control. The level of PCP removal varied between isolates. Four isolates completely removed PCP from the (cell free) liquid medium. These were HR122, HR226, HR235 and HR577. Twelve others removed between 75% and 95% of the PCP supplied and the remaining two (HR196 and HR 131) removed less than 75% of the PCP from the culture medium.

Table 2.2: Comparison of PCP degradation ability, laccase production and growth morphology over six weeks by 20 selected white-rot fungi. PCP remaining in cultures is shown as a percentage compared to the no fungus control cultures. Analysis for residual PCP and laccase activity were performed as described in Chapter 7.

Isolate number	PCP remaining after 42 days (% of control)	Laccase production over 42 days	Formation of a surface mycelial mat
PCP (no fungus) control	100	X	X
HR196	72	X	X
HR131	41	√	X
HR345	27	X	X
HR348	21	X	X
HR160	20	√	X
HR277	14	X	√
HR145	13	X	√
HR192	11	X	√
HR445	8	X	√
HR152	7	√	√
HR154	6	X	X
HR197	3	X	√
HR588	2	X	√
HR240	1	√	√
HR275	0.6	√	X
HR112	0.2	√	√
HR122	0	√	√[a]
HR226	0	√	√
HR235	0	√	√[a]
HR577	0	√	X
HR131 (no PCP) control	0	√	√

[a] Formation of a mycelial mat was observed for one of the replicates but not the other.

Table 2.2 shows that fifteen of the twenty isolates degraded more than 85% of the PCP over the six week period. Of the 15 isolates, 12 isolates produced a surface mycelial mat and six of these also produced laccase.

The PCP degradation ability of the isolates did not correlate to sensitivity to PCP (Table 2.1). PCP degradation appeared to occur irrespective of whether the isolate grew a surface mycelial mat or not (Table 2.2).

Laccase Activity in Culture Medium Amended with PCP

Extracellular laccase activity[31] was measured at regular intervals for all 20 isolates grown in the presence of 50 mg L^{-1} PCP throughout the 42 days in the experiment described above (Table 2.2).

Due to limitations in the number of available culture flasks, "no PCP" control flasks were only grown for one isolate, HR131 (a *T. versicolor* isolate). Figure 2.4 shows a comparison of laccase activity for HR131 in the absence and presence of PCP over the first fourteen days. This figure shows that laccase activity for both treatments was similar over the first three days. However, laccase activity in cultures grown with PCP peaked at day 3 and slowly decreased over the rest of the experiment (see Figure 2.5b) while laccase activity in control cultures increased until day 14 and stayed at similar levels (0.6-0.7 U mL^{-1}) until the end of the 42 day incubation period (see Figure 2.5b).

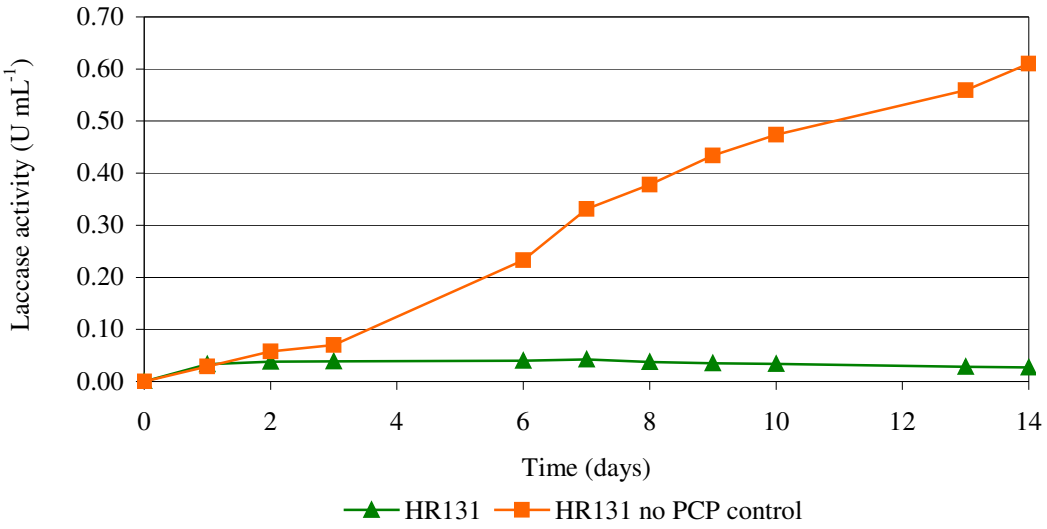


Figure 2.4: Laccase activity over the first 14 days for *T. versicolor* HR131 in the absence and presence of PCP.

[31] Secreted into the medium

Ten isolates (50%) did not produce any detectable laccase over the six week period: HR145, HR152, HR192, HR196, HR197, HR277, HR345, HR348, HR445, and HR588. For laccase producing isolates the laccase production in the cultures varied considerably[32] (Figure 2.5).

[32] For clarity, only data for isolates that produced laccase activity over the 42 day period has been included in the graphs in Figure 2.5

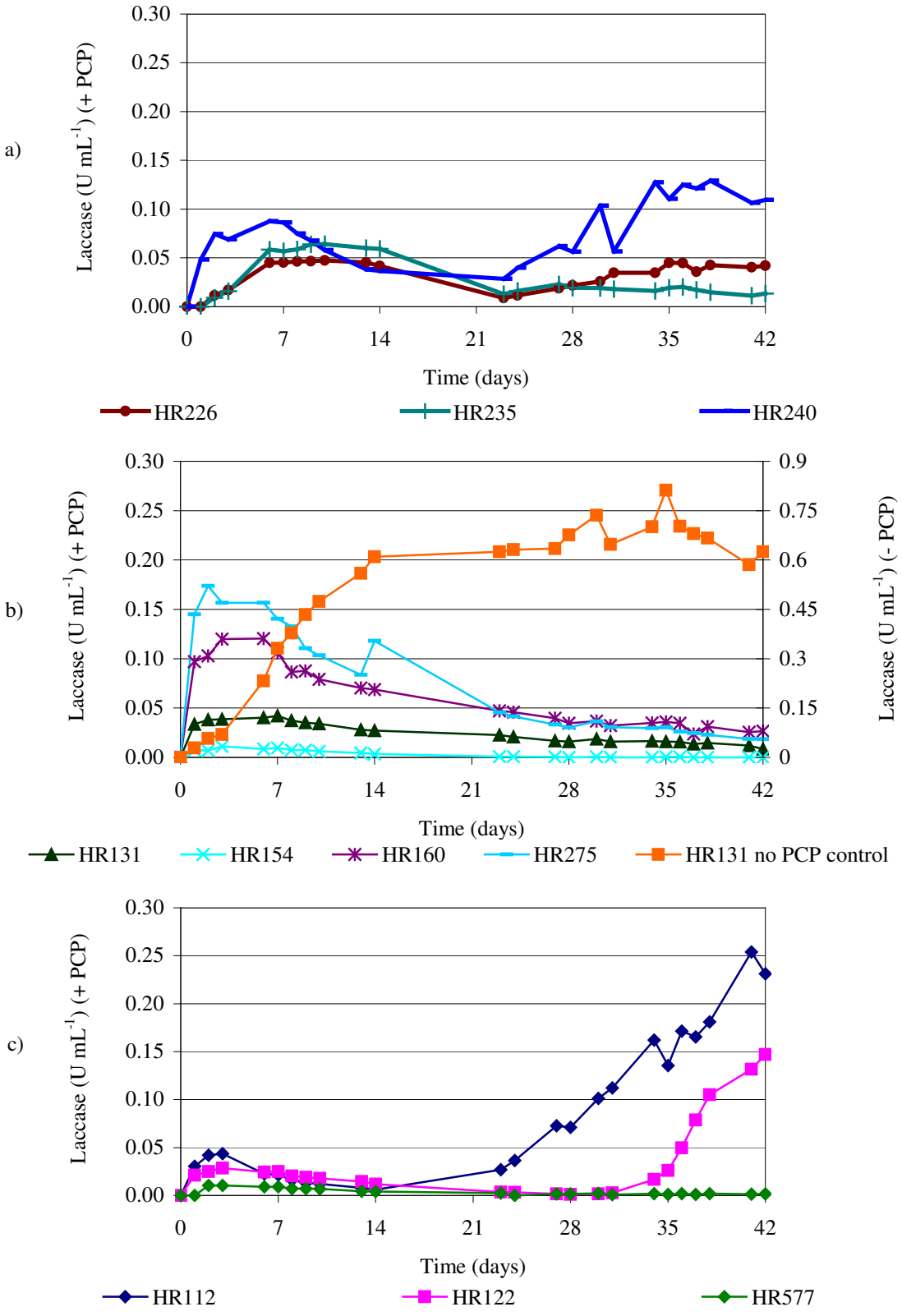


Figure 2.5: Laccase activity over 42 days for a) *P. sacrata* (HR226, HR235 and HR240), b) *T. versicolor* (HR131, HR154, HR160 and HR275) and c) other white-rot isolates (HR112: unknown basidiomycete sp., HR122: unknown basidiomycete sp., and HR577: *Trametes* sp.). The left axis scale was used for all cultures containing PCP. The right axis scale indicates laccase activity for the no PCP control. Data shown is for the average of two replicate cultures. Laccase activities were determined with DMP (de Jong, Cazemier *et al.* 1994). One unit of laccase activity was defined as the amount of enzyme required to oxidise 1 µmol of DMP per minute at 25ºC.

Four *T. versicolor* isolates and three *P. sacrata* isolates produced laccase over the six weeks of the PCP degradation experiment. The pattern of the laccase activity expression for each of these species appeared to be different for the two species but similar within each species. For *P. sacrata* isolates two peaks of laccase activity were detected around days 7 and 35 (Figure 2.5a). In contrast, for the *T. versicolor* isolates, an initial peak of laccase activity was observed in the first 2-3 days and then the laccase activity decreased steadily over the six week period (Figure 2.5b).

The remaining laccase producing isolates were two unknown basidiomycete sp. (HR112 and HR122) and one *Trametes* sp. (HR577, Figure 2.5c). These had mixed patterns of laccase expression: HR577 had early laccase expression only, while HR112 and HR122 both had a small early laccase peak followed by a much larger laccase peak.

Growth morphology and enzyme expression appeared to be linked to some extent in the cultures with late expression of laccase activity. Isolates that grew a surface mycelia mat (e.g. HR240, Figure 2.6) tended to express higher levels of laccase activity after 14 days than those that did not form a surface mat (e.g. HR275, Figure 2.6). For isolates that formed a surface mat, enzyme activity continued to increase thereafter until day 42. Isolates that did not form a mycelial mat, tended to produce their peak laccase activity early, and activity tended to decrease after 14 days (see Figure 2.5). Irrespective of whether a mycelial mat formed on the surface of the liquid, all fungi increased in biomass. However, biomass growth did not correspond to PCP disappearance.

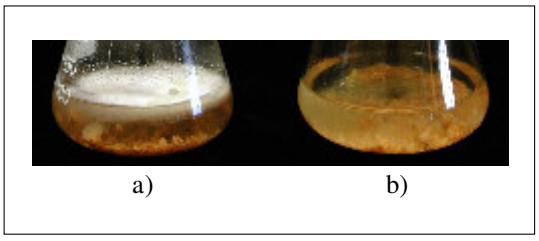


Figure 2.6: Comparison of fungal growth of isolates a) HR240 (*P. sacrata*) and b) HR275 (*T. versicolor*)

Summary

PCP tolerance on agar did not correlate to PCP degradation *in vitro* for the 20 isolates. However, all isolates tolerant to 200 mg L^{-1} PCP in agar significantly reduced PCP in the liquid fraction compared to the PCP control. Half of the isolates produced laccase over the six week experiment. Production of laccase in these isolates after 14 days appeared to be linked to formation of a mycelial mat on the liquid surface. However, formation of a mycelial mat on the liquid surface did not correlate directly to PCP degradation.

2.5 LIGNINOLYTIC ACTIVITIES IN CULTURE MEDIUM FOR SIX SELECTED ISOLATES

This section discusses the results from two parallel 15 day time course experiments that were set up to examine which ligninolytic enzymes were produced *in vitro* for selected white-rot isolates. Here, a total of six isolates were selected for the 'PCP free' (i.e. the first) time course study, and three isolates were used for the 'PCP' (i.e. the second) time course study.

The composition of the liquid medium used for these two experiments was identical to the liquid medium used for the 42 day experiment except that, for one of the time course experiments, PCP was omitted from the liquid medium. In contrast to the 42 day experiment, the inoculum used for the time course experiments was fungi grown on agar rather than on SCS.

The amount of liquid medium used and the number of flasks per isolate was also different. In the 42 day experiment submerged cultures (150 mL of liquid medium per 250 mL flask, 2-3 cm deep) were set up and there were only two replicate flasks per isolate. An aliquot was taken on different days to measure laccase activity. For the time course experiments, shallow cultures (15 mL of liquid per 250 mL medical flat bottle, about 5 mm deep) were set up and three replicate bottles per day were used for each isolate (i.e. a total of 45 medical flat bottles were used per isolate).

Cultures were examined daily for the presence of laccase, LiP and MnP using standard assays for ligninolytic enzymes. Laccase was assayed using ABTS as the substrate, (Wolfenden and Willson 1982); LiP was assayed using VA as the substrate, (Tien and Kirk 1988) and MnP was assayed using Mn^{2+} as the substrate, (Wariishi, Valli and Gold 1992).

Selection of the Six Isolates

The isolates chosen for the 15 day PCP-free time course experiment were HR112, HR122, HR160, HR197, HR240 and HR577. The reasons for their selection were as follows: all except one are very good degraders of PCP (0-4% residual PCP detected in cultures compared to the controls). The exception, isolate HR160, was included for comparative purposes because it is a representative of one of three *T. versicolor* cultures shown to mineralise PCP in soil in previous work at HRERM (Walter, Boul *et al.* 2004). Isolate HR112 was selected because of its vigour in producing laccase in the presence of PCP after 14 days (see Figure 2.5) and enzyme production appeared to be correlated to formation of a surface mat. Isolate HR197 was one of the slowest growing white-rot fungi. Although it was a good PCP degrader, it did not produce laccase over the 42 day PCP degradation experiment. Therefore, it was of interest to determine whether LiP and/or MnP were produced by this isolate. HR240 was selected as a representative of the *P. sacrata* isolates. Although it was not the best PCP degrader of the three *P. sacrata* isolates tested, it produced the most laccase over the 42 day period of these three isolates (see Figure 2.4a). Isolate HR577 was selected as a representative of the four isolates that degraded all of the PCP over the 42 day incubation period.

The three isolates selected for the second (PCP-amended) 15 day time course experiment were HR112, HR240 and HR577. These three isolates were selected as representatives of isolates that produced either a small amount of laccase (HR577) or quite a high amount of laccase (HR240) over the 42 day PCP degradation experiment, and an isolate that produced more laccase after formation of a surface mycelial mat (HR112).

Survival of Isolates Grown in Standard Liquid Medium Amended with PCP

For the three isolates grown in shallow liquid medium amended with PCP no fungal growth was observed over the 15 day time course experiment with two exceptions. The two exceptions, both from isolate HR577, were two single replicate bottles representing two different days (day 12 and day 15). Fungal growth in these two bottles was greatly reduced compared to fungal growth in shallow cultures grown without PCP amendment. This is in contrast to the fungal growth observed in submerged cultures where SCS was used as the inoculum. In the earlier 42 day experiment all cultures survived in the PCP-amended submerged cultures.

The observation that fungi did not survive in shallow cultures amended with PCP for the 15 day time course experiment is similar to the observations of Mileski, Bumpus *et al.* (1988). These researchers studied PCP degradation by *P. chrysosporium* in shallow cultures. The addition of PCP to cultures at concentrations above 4 mg L^{-1} was toxic to fungal spores of *P. chrysosporium*. However, PCP toxicity could be overcome if fungi were pre-grown for six days prior to addition of PCP to the liquid medium.

It is likely that the reason for fungal survival and growth in submerged cultures in the 42 day experiment was due to the use of isolates grown on SCS rather than isolates grown on agar as the inoculum.

Peroxidase Activity in Isolates Grown in PCP-Free and PCP-Amended Standard Liquid Medium

Peroxidases and laccases are part of the lignin degrading system in white-rot fungi (see Chapter 1). Fungi produce one or more of each of these types of ligninolytic enzymes. Five of the isolates selected for the PCP-free study produced laccase *in vitro* while the sixth isolate did not. It was of interest, therefore, to examine whether white-rot fungi produced LiP and MnP *in vitro*.

No LiP or MnP activity was observed over the 15 days of the experiment[33], either in PCP-free or PCP-amended liquid medium.

In this study the white-rot isolate HR197 was found to be one of the best isolates for removing PCP. However it did not produce any (detectable) LiP, MnP or laccase over the 15 day time-course experiment, confirming observations made in an earlier study (Walter, Guthrie *et al.* 2003). Thus, this isolate does not produce any extracellular ligninolytic enzymes or may produce the enzymes under different growth conditions. Alternately, it is possible other enzymes are produced by this isolate for lignin degradation. The studies in the literature report that white-rot fungi can also produce other types of peroxidases such as VP (see de Jong, Field and de Bont 1992; Miyata, Iwahori and Fujita 1998; Camarero, Sarkar *et al.* 1999; Davila-Vazquez, Tinoco *et al.* 2005 and the review in Chapter 1), and it is possible that HR197 may produce this type of peroxidase or a hitherto undiscovered ligninolytic enzyme. Because this fungus was very slow growing, no further experiments were carried out with this isolate.

Laccase Activity in Isolates Grown in PCP-Free Standard Liquid Medium

The results for laccase activity in PCP-free shallow cultures over the 15 day time course are shown in Figure 2.7. As mentioned above, HR197 did not produce any detectable levels of laccase. In contrast, all of the other isolates produced laccase in a cyclic manner over the 15 days (Figure 2.7).

[33] In a separate trial, both assay systems were shown to work using purified LiP and MnP from *P. chrysosporium*.

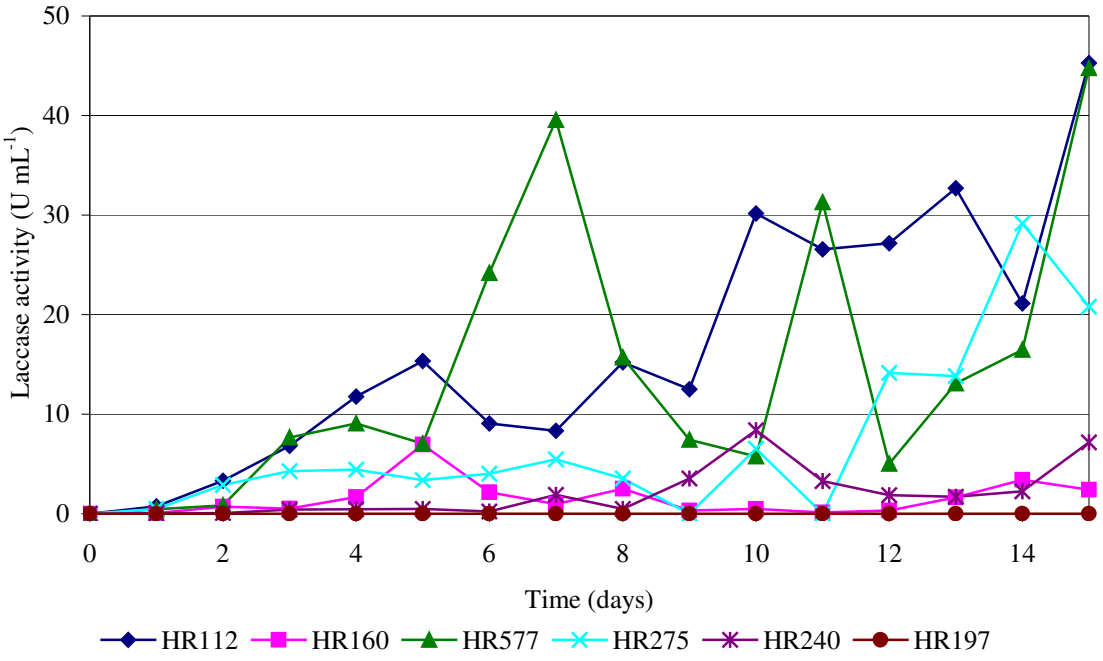


Figure 2.7: Laccase activity over 15 days for selected white-rot fungi. Fungi were grown in 200 mL medical flat bottles at 25ºC in the dark. Each data point on the graph represents the average of three replicate bottles for each isolate per day. HR112: unknown basidiomycete sp., HR160: *T. versicolor*, HR577: *Trametes* sp., HR275: *T. versicolor*, HR240: *P. sacrata*, and HR197: unknown basidiomycete sp. Laccase activities were determined with ABTS as the substrate (Wolfenden and Willson 1982). One unit of laccase activity was defined as the amount of enzyme required to oxidise 1 µmol of ABTS per minute at 25ºC.

The patterns of laccase activity seen here are not unusual amongst white-rot fungi. For example, with the white-rot *Pleurotus sajor-caju* only one peak of laccase activity was observed (on day 6) over a 22 day time course (Soden and Dobson 2001). In contrast, *T. versicolor* displayed two peaks of laccase activity over a 15 day time course (Collins and Dobson 1997a), and *Trametes cingulata, Pycnoporus sanguineus*, *Datronica concentrica*, *Irpex* spp., *Trametes elegans* and *Lentinus velutinus* manifested several peaks of activity over a 21 day period depending on the medium used to grow the fungi (Tekere, Zvauya and Read 2001).

Laccase Activity of Isolates Grown in Standard Liquid Medium Amended with PCP

Figure 2.8 shows the laccase activity over 15 days for isolates HR112, HR240 and HR577 grown in shallow cultures amended with PCP. As expected, no laccase activity was detected in liquid medium from cultures where no fungal growth was observed.

As mentioned earlier, two single replicates of isolate HR577, from day 12 and day 15, were able to overcome the toxicity of PCP as evidenced by the observation of fungal growth. The fungal growth in these two bottles was severely reduced compared to growth in the equivalent PCP-free replicates. However, the laccase activity observed in these two bottles from PCP-amended shallow cultures was much higher than the equivalent bottles containing PCP-free liquid medium. This suggests that laccase production in isolate HR577 can be induced by addition of PCP.

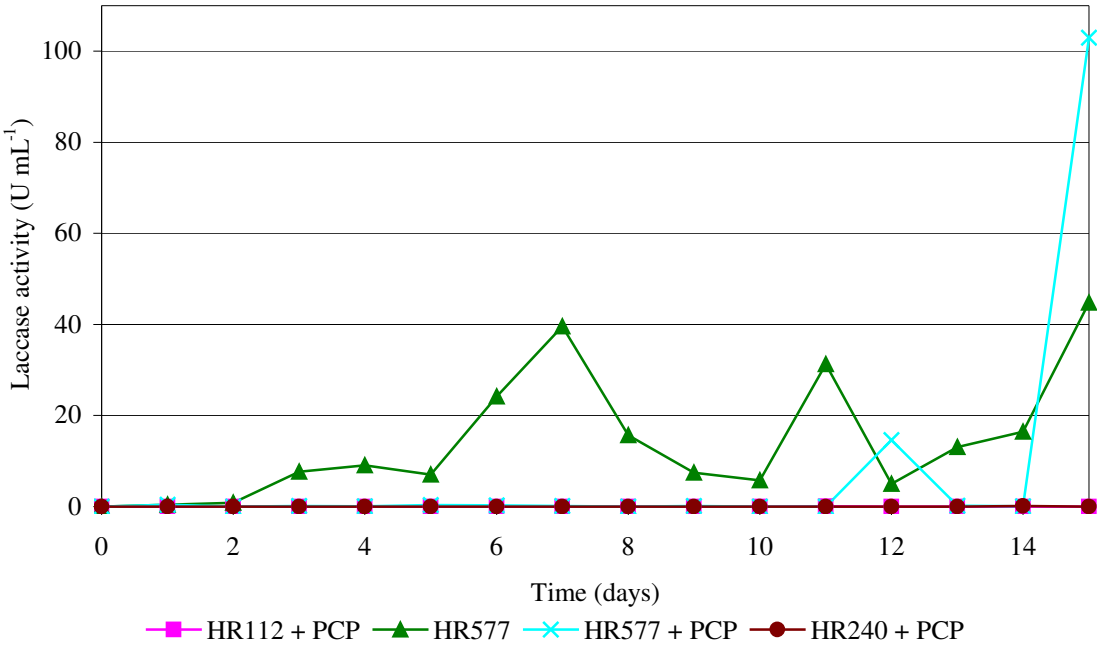


Figure 2.8: Laccase activity over 15 days for selected white-rot fungi. Fungi were grown in 200 mL medical flat bottles at 25ºC in the dark. These isolates were grown in standard liquid medium amended with 50 mg L^{-1} PCP. Each data point on the graph represents the average of three replicate bottles for each isolate per day. HR160: *T. versicolor*, HR577: *Trametes* sp., and HR240: *P. sacrata*. The results for HR577 from Figure 2.7 are presented in this graph as a reference. Laccase activities were determined with ABTS as the substrate (Wolfenden and Willson 1982). One unit of laccase activity was defined as the amount of enzyme required to oxidise 1 µmol of ABTS per minute at 25ºC.

Summary

None of the six isolates produced LiP or MnP over the 15 day (PCP-free) time course. Addition of PCP to cultures of three isolates grown in standard liquid medium using agar plugs as inoculum resulted in complete inhibition of fungal growth in all but two bottles of isolate HR577. For the two bottles of HR577 that grew in the presence of PCP the laccase activity was much higher than the laccase activity in the equivalent PCP-free cultures.

2.6 SELECTION OF AN ISOLATE FOR THE REMAINING EXPERIMENTS REPORTED IN THIS THESIS

This part describes the criteria used to identify a suitable white-rot isolate for further studies. Several selection criteria were used in this selection process (Table 2.3). The fungus chosen needed to be a unique native white-rot fungus that is not a proprietary isolate, tolerant to high concentrations of PCP with the ability to degrade PCP *in vitro*. It also needed to grow at a range of different temperatures and produce inducible ligninolytic enzymes.

Table 2.3: Criteria used to select the white-rot isolate for further studies

Criteria	White-rot isolate code									
	112	**122**	**131**	**154**	**160**	**226**	**235**	**240**	**275**	**577**
Tolerant to 200 mg L^{-1} PCP in agar[a]	√	√	√	√	√	√	√	√	√	√
Tolerant to 10,000 mg L^{-1} creosote in agar[a]	√	√	√	√	√	√	√	√	√	√
Able to grow at 0-40ºC[a]	√	√	√	√	√	X	X	X	√	√
Growth on 200 mg L^{-1} PCP in agar >50% than growth on nil control[a]	X	X	X	X	X	X	X	X	√	√
Good PCP degrader (95-100% PCP removal, see Table 2.4)[b]	√	√	X	X	X	√	√	√	√	√
Produces inducible ligninolytic enzymes	X	ND	ND	ND	ND	ND	ND	X	ND	√
Unique native isolate	√	√	X	X	X	X	X	X	X	√
Not a proprietary isolate[a]	√	√	√	√	√	X	X	X	X	√

ND = Not determined [a] See Table 2.1 [b] See Table 2.2

Trametes sp. HR577 was chosen for the remaining studies presented in this thesis as it fulfilled all of the selection criteria outlined above. The remainder of this chapter describes preliminary studies on the induction of laccase in this organism.

2.7 PRELIMINARY STUDY INTO INDUCTION OF LACCASE IN *TRAMETES* SP. HR577

This section outlines experiments carried out to determine if laccases in HR577 could be induced by addition of PCP and copper to the fungal medium.

Numerous fungal media components have been shown to affect production of laccase *in vitro* (Fåhraeus 1962; Buswell, Cai and Chang 1995; Collins and Dobson 1997a; Lee, Jung *et al.* 1999; Galhaup and Haltrich 2001; Klonowska, Le Petit and Tron 2001; Soden and Dobson 2001; Baldrian and Gabriel 2002; Mougin, Kollmann and Jolivalt 2002; Alves da Cunha, Barbosa *et al.* 2003). Here, the induction of laccase with PCP and copper was of interest.

PCP was selected because it appeared to induce laccase production in the experiment described earlier for some cultures of *Trametes* sp. HR577. Copper was chosen for this study, as laccase isozymes require four copper molecules per monomeric unit[34]. For this reason, it is also the metal that is most commonly studied for induction of laccase in white-rot fungi (Collins and Dobson 1997a; Palmieri, Giardina *et al.* 2000; Galhaup and Haltrich 2001; Klonowska, Le Petit and Tron 2001; Soden and Dobson 2001; Baldrian and Gabriel 2002; Galhaup, Goller *et al.* 2002; Levin, Forchiassin and Ramos 2002; Malhotra, Sharma and Capalash 2004).

[34] An exception is the so-called 'white' laccase isozyme produced by *P. ostreatus*. This isozyme uses one copper atom, two zinc atoms, and one iron atom for catalysis (Palmieri, Giardina *et al.* 1997).

Addition of PCP to One, Three and Five Day Old Fungal Cultures

Typically, PCP is toxic to fungal cultures. However, this toxicity can be overcome if fungi are allowed to grow for several days prior to addition of PCP to culture medium (Mileski, Bumpus *et al.* 1988). In the present study, an initial experiment was conducted in which PCP was added to 1, 3 and 5 day old cultures to determine an appropriate time to add PCP to induce laccase production. The results from this experiment are presented in Table 2.4.

Table 2.4: Relative laccase activity detected 24 and 48 hours after addition of PCP. Data reported is the average of three replicate cultures. Cultures were grown in 250 mL flasks for one, three or five days prior to addition of PCP. Laccase activity in the control cultures was measured on days 2-7 (e.g. the for the cultures where PCP was added on day 1, laccase activity was measured on days 2 and 3 in both PCP containing and control cultures). Laccase activity in control cultures for each day was ascribed a value of 100 and activity in all other treatments was given relative to the appropriate control culture.

Day PCP added	%Laccase activity relative to the control: 24 hours after addition of PCP	48 hours after addition of PCP
Day 1	40	110
Day 3	90	130
Day 5	100	280

Table 2.4 shows that laccase activity decreased in cultures after 24 hours if PCP was added on day 1 or day 3 but stayed the same if added on day 5. For all treatments the laccase activity detected after 48 hours was higher than that of control cultures. The highest increase in laccase production after 48 hours was observed when PCP was added to five day old cultures. Thus, in the remaining experiments reported in this chapter, PCP was added to five day old cultures.

The Effect of Copper, PCP or Both on Laccase Production

In order to study the effect of copper (**Cu**), PCP (**PCP**) or both compounds (**PCP/Cu**) on laccase production, fungal cultures of *Trametes* sp. HR577 were grown in standard liquid medium in shallow cultures. **Cu**, **PCP** or **PCP/Cu** was added to five day old cultures and the laccase activity measured after a further 48 hour incubation.

The effect of **Cu**, **PCP** or **PCP/Cu** on laccase specific activity is shown in Figure 2.9. All of these treatments led to increased laccase production on day 7 relative to the (day 7) control cultures (2- 6- and 13-fold for **PCP**, **Cu** and **PCP/Cu**, respectively).

The greatest effect on laccase production was from addition of a combination of both copper and PCP to five day old cultures.

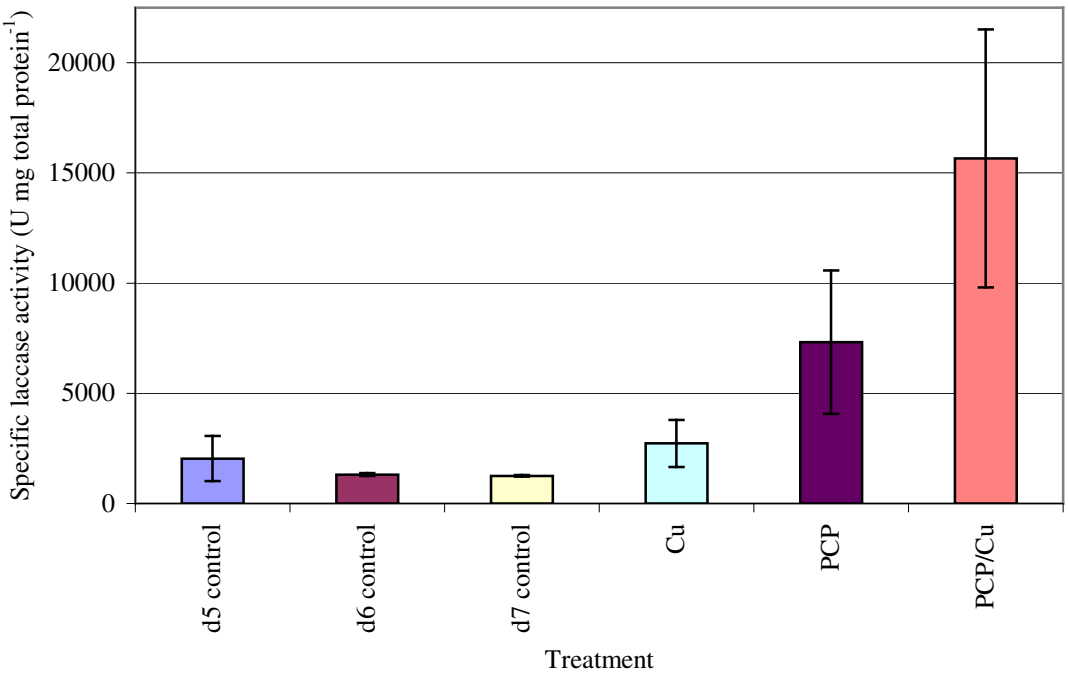


Figure 2.9: The effect of Cu, PCP or PCP/Cu on laccase production in *Trametes* sp. HR577. Each data point is the average of triplicate cultures and each error bar represents the standard deviation of these cultures. Cultures were grown in 15 mL of fungal medium for five, six or seven days. This experiment was repeated several times. The results presented here are from a representative experiment. Laccase activities were determined with ABTS as the substrate (Wolfenden and Willson 1982). One unit of laccase activity was defined as the amount of enzyme required to oxidise 1 µmol of ABTS per minute at 25ºC. Total protein was determined using the Bio-Rad Protein Assay (Bio-Rad), with BSA as standard.

These findings are similar to results reported by Collins and Dobson (1997a). These researchers studied the effect of copper, XYL (Figure 2.10) and a combination of both on laccase production in *T. versicolor*. In Collins and Dobson's study all three treatments gave increased laccase production relative to control cultures.

This effect was also shown to occur at the level of gene transcription. When copper and XYL were simultaneously present as *lcc* inducers, they acted synergistically to activate *lcc* transcription at a rate faster than that for either separately (Collins and Dobson 1997a).

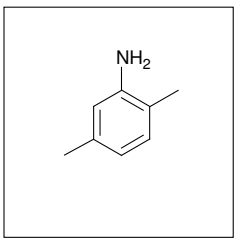


Figure 2.10: XYL

The Effect of Copper, PCP or Both on m*RNA Expression*

In this part of the study *m*RNA was extracted from HR577 cultures previously induced with PCP, copper or both PCP and copper. A preliminary RT-PCR of extracted RNA was carried out using primers designed to amplify a highly conserved region of the laccase gene[35]. The *m*RNA levels of *lcc* in *Trametes* sp. HR577 under different conditions are shown in Figure 2.11. These results indicate that the levels of gene transcription observed were similar to the levels of expressed laccase shown in Figure 2.9. However, a housekeeping gene needs to be used to validate that the levels observed were due to differences in *m*RNA transcript levels.

[35] The PCR primers used for this experiment are the same primers used later in Chapter 3 to obtain three partial laccase gene sequences from gDNA from *Trametes* sp. HR577

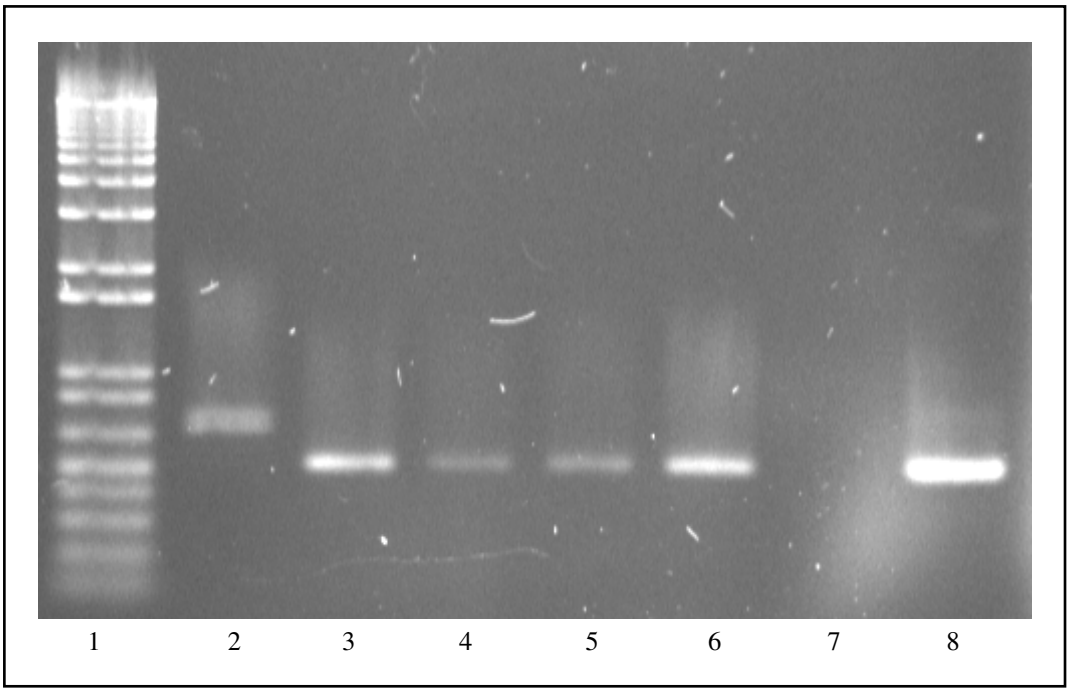


Figure 2.11: Agarose gel (1%) of *lcc* mRNA levels in *Trametes* sp. HR577. Lane 1: markers; Lane 2: negative control (gDNA); Lane 3: day 5 control; Lane 4: day 6 control; Lane 5: day 7 control; Lane 6: day 7 Cu; Lane 7: day 7 PCP and Lane 8: day 7 Cu/PCP. Cultures were grown for 5, 6 or 7 days at 25ºC in the dark. Fungal mycelium from three replicate cultures was combined and RNA was extracted (Gromoff, Treier and Beck 1989). RT-PCR experiments were carried out using 1 ng of total RNA (estimated by UV spectroscopy, see Chapter 7 for further experimental details). Lane 7 is blank because the RNA extracted from fungal mycelium for this treatment had degraded prior to the RT-PCR reaction being carried out.

Summary

The results from these preliminary experiments suggest that addition of copper, PCP or a combination of copper and PCP to five day old cultures results in increased laccase production in the white-rot fungus *Trametes* sp. HR577. This increase in laccase production appeared to be accompanied by an increase in laccase gene expression.

2.8 CONCLUSION AND FUTURE WORK

White-rot fungi from the HRCC were screened for temperature tolerance and sensitivity to PCP and creosote in agar. PCP and creosote sensitivity on agar and tolerance to a

range of temperatures provided a quick high throughput method for screening of white-rot fungi for bioremediation potential.

Of the total pool of 367 isolates in this collection, twenty isolates were selected for further screening based on their tolerance to 200 mg L^{-1} PCP in agar. Although PCP tolerance on agar did not correlate to PCP degradation *in vitro*, all isolates tolerant to 200 mg L^{-1} significantly reduced PCP in the liquid fraction compared to the PCP control.

Half of the isolates produced laccase over the six week experiment. Production of laccase in these isolates after 14 days appeared to be linked to formation of a mycelial mat on the liquid surface. However, formation of a surface mat on the liquid surface did not correlate to PCP degradation.

Six isolates were further screened for the presence of LiP, MnP and laccase in standard liquid medium inoculated from agar plates over a shorter time course (15 days). None of the isolates produced LiP or MnP over the 15 day period. For three of the isolates (HR160, HR226 and HR577) the 15 day time course experiment was also carried out with PCP added to the medium. PCP completely inhibited fungal growth in all but two of the replicate flasks from *Trametes* sp. HR577. Laccase activity was elevated in the two replicates that survived incubation with PCP compared to control cultures despite severely inhibited biomass growth in these cultures. Further experiments using this isolate indicated that both PCP and copper appeared to induce laccase production in this organism.

The white-rot fungus *Trametes* sp. HR577 is a NZ, non-proprietary isolate previously not part of the HRCC. It was selected for the remaining experiments described in this thesis based on its growth over a range of different temperatures (0-40ºC), tolerance to PCP in agar (200 mg L^{-1}), superior degradation of PCP *in vitro*, and production of high quantities of laccase in the presence of PCP.

Preliminary experiments with *Trametes* sp. HR577 suggested that addition of copper, PCP or a combination of copper and PCP to five day old cultures resulted in increased laccase production. Further studies into the induction of laccase production by PCP and copper also need to be carried out to see if laccase expression is induced at the level of gene transcription.

CHAPTER 3: IDENTIFICATION AND ENZYMATIC PROFILING STUDIES OF *TRAMETES* SP. HR577

3.1 INTRODUCTION

The HortResearch white-rot isolate HR577 was selected for the remaining studies reported in this thesis based in its high tolerance to PCP and degradation of PCP *in vitro* (see Chapter 2). It has been proposed that ligninolytic enzymes produced by white-rot fungi may be involved in degradation of PCP by white-rot fungi. Of the three ligninolytic enzymes, isolate HR577 produced laccase but not LiP or MnP *in vitro* in liquid culture[36]. Furthermore, addition of PCP to cultures appeared to induce laccase production in this organism (Chapter 2). This chapter describes the morphological and genetic techniques used to try to identify this white-rot fungus. It also compares the partial laccase gene sequences from white-rot isolate HR577 to laccase sequences from other white-rot fungi and discusses how these laccase sequences fit into classifications proposed by other research groups.

3.2 IDENTIFICATION OF WHITE-ROT ISOLATE HR577

Identification of White-Rot Fungi Using Morphological Features

White-rot fungi belong to the phylum Basidiomycetes and subphylum Hymenomycetes (Chapter 1). Nobles (1965) created a multiple-choice key for the identification of the Hymenomycetes that compared features of the mycelium including types of septation of hyphae, occurrence of special structures and accessory spores, colour of hyphae and mycelial mats, colour changes in agar, rates of growth, and interfertillity phenomena.

A plate of white-rot isolate HR577 on malt extract agar was sent to the NZ FRI for identification using this key. The results from this are listed in Table 3.1. White-rot isolate HR577 fits the key code 2.3.8.32. This describes species that produce

[36] Very low levels of manganese peroxidase were produced when this isolate was grown on solid media (Ford 2006).

extracellular oxidase, have nodose clamps present at all septa, hyphae are differentiated to form fibre hyphae, and have no conidia or chlamydospores. This group includes fungi from the genera *Fomes*, *Trametes*, *Polyporus*, *Lenzites*, *Cerrena*, *Poria*, *Favolus*, *Pleurotus*, and *Aporpium*.

Table 3.1: Nobles' key code for white-rot isolate HR577 and *T. hirsuta*

Key Codes	Description	White-rot isolate HR577	*T. hirsuta*
1-2	Production of extracellular oxidase	2: Positive for extracellular oxidase	2: Positive for extracellular oxidase
3-6	Septation of hyphae	3: Thin-walled hyphae consistently nodose-septate	3: Thin-walled hyphae consistently nodose-septate
7-26[1]	Occurrence of special structures formed by differentiation of hyphae	8: Hyphae differentiated to form special structures	8: Hyphae differentiated to form special structures
32-35[2]	Occurrence of conidia, chlamydospores, and oidia	32: no conidia, chlamydospores, and oidia34: no chlamydospores	32: no conidia, chlamydospores, and oidia34: no chlamydospores
36-37	Colour of hyphae and mycelial mats	36: hyphae hyline and mats white or pale in colour	36: hyphae hyline and mats white or pale in colour
38-40	Colour changes in agar induced by growth of fungus ("reverse")	38: reverse unchanged in colour	38: reverse unchanged in colour
41-47	Rate of growth[3]	42: Plates covered in 2 weeks	42: Plates covered in 2 weeks 43: Plates covered in 3 weeks
48-49	Production of Fruit bodies in culture	Not produced in culture	(48)[4]: Fruit bodies regularly produced before the end of 6 weeks.
50-53	Odour of cultures	52: Odour suggesting an antiseptic, such as the idoform odour of *Polyporus balsameus*	Not listed in key code
54-56	Host relationships	54: Associated with decay in broad-leaved trees	54: Associated with decay in broad-leaved trees 55: Associated with decay in coniferous trees
57-60	Interfertility phenomena	Could not be determined	60: Heterothallic, showing the tetrapolar type of interfertility

[1] In Noble's papers there are sketches of the different structures formed by differentiation of hyphae. [2] Code numbers 27-31 are not in use. [3] Fungi were grown on malt agar containing: Difco bacto malt extract 12.5 g, Difco bacto-agar 20.0 g, distilled water 1,000 ml. [4] The enclosing of the symbol in parentheses (48) indicates that fruit body formation occurs in some isolates and not in others.

The descriptions given for the mycelial mat and other features of the vegetative culture for all of the species listed under Nobles' key code 2.3.8.32 were used to try to identify white-rot isolate HR577. The closest match using this key was to *Trametes hirsuta* (the code for this organism is also shown in Table 3.1). However, there was one difference between the description of *T. hirsuta* and white-rot isolate HR577 and one key code could not be tested with white-rot isolate HR577. The difference observed between *T. hirsuta* cultures and white-rot isolate HR577 was in the odour of the fungal cultures. There is no odour listed for *T. hirsuta* but white-rot isolate HR577 has a scent that suggests an antiseptic odour.

The key code that could not be tested for cultures in *Trametes* sp. HR577 related to the interfertility of the isolate. A mature fruiting body is required in order to test interfertility of a culture. Formation of a fruiting body occurs with some *T. hirsuta* isolates but not others. White-rot isolate HR577 did not form a mature fruiting body over the time it was at the FRI. While this does fit the description of *T. hirsuta*, without a fruiting body the final test on the interfertility of the culture cannot be carried out.

Nobles' paper states that the four isolates from the genus *Trametes* listed in her key (*T. hirsuta*, *T. pubescens*, *T. zonatus* and *T. versicolor*) can only be identified with certainty if "Fruit bodies are formed, from which single spore cultures can be isolated and paired with single spore isolates from fruit bodies collected in nature. Interfertillity, indicated by the formation of nodose-septate hyphae, is accepted as proof of conspecificity."

There are several photos of *T. hirsuta* fruiting bodies on the Internet[37]. The fruiting bodies of *T. hirsuta* are white to grey and often possess a brownish edge. Their top side is has rough, rigid hair, hence the name hirsuta (from the Latin word meaning hairy, shaggy, rough or unadorned[38]).

A mature fruiting body was not available to send to FRI for identification of white-rot isolate HR577. However, some photos of the fruiting bodies had been taken prior to its

[37] The following links are but a few of the examples of *T. hirsuta* pictures available on the internet:
http://www.hlasek.com/trametes_hirsuta_a8145.html
http://perso.orange.fr/champi.fc/aphyllos/images/Trametes_hirsuta.jpg
http://www.nahuby.sk/sk/sources/obrazok_detail.php?id=15027
http://www.nic.funet.fi/pub/sci/bio/life/fungi/gasteromycetes/polyporales/coriolaceae/trametes/hirsuta-1.jpg
http://botit.botany.wisc.edu/images/332/Basidiomycota/Hymenomycetes/Polyporaceae/Trametes/T.hirsuta_tjv.html
[38] http://www.archives.nd.edu/cgi-bin/lookup.pl?stem=hirsutus&ending=

inclusion in the HortResearch culture collection (Figure 3.1). Although the fruiting bodies of white-rot isolate HR577 shown in Figure 3.1 are white in colour, fitting the colour range given for *T. hirsuta*, they do not have any rough rigid hair typical of *T. hirsuta* fruiting bodies. This suggests that isolate HR577 is not a *T. hirsuta* isolate.

Four partial laccase sequences from white-rot isolate HR577 show some similarity to those of *T. hirsuta* (see later for a comparison of laccase sequences from both organisms). However, they are much closer in sequence identity to laccase gene sequences from other *Trametes* sp. (*T. versicolor* and *T. villosa*). The production of mature fruiting bodies from white-rot fungi for a definitive taxonomic identification is non-trivial and outside of the scope of this PhD project.

Figure 3.1 Fruiting bodies of *Trametes* sp. HR577 growing on a birch log

Identification of White-Rot Fungi Using Gene Sequences

An alternative method of fungal classification is by using gene sequences. The most common method involves amplifying ribosomal rRNA genes (see chapter 1).

Genomic DNA was purified from *Trametes* sp. HR577 using the methods outlined by Collins and Dobson (1995a, see Chapter 7). Two sets of primers were used to try to identify white-rot isolate HR577. The first set of primers (EF4fwd and F5rev) amplified a short segment of the 18S rRNA gene (van Elsas, Duarte *et al.* 2000). The gDNA PCR product from this reaction was a single band of the expected size (530 bp) and was cloned and sequenced.

The resulting sequence was entered into a BLAST (Basic Local Alignment Search Tool) search on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/) to search for matching or closely related sequences. At the time the sequence was obtained it matched only one sequence in the gene library, that of a brown-rot fungus, *Wolfiporia cocos* (100% sequence identity at the nucleotide level). As with *T. hirsuta*, the fruiting bodies of this fungus did not resemble that of white-rot isolate sp. HR577. In addition, other research conducted by the HRERM group indicated that white-rot isolate HR577 produced white-rot rather than brown-rot when grown on wood (Walter, Guthrie *et al.* 2003).

When the 18S rRNA sequence was re-entered into a sequence similarity search two years after it was first obtained, over 100 matches were found. Included in this match were several *Trametes* spp. sequences (also 100% sequence identity at the nucleotide level) and other basidiomycetes (both brown- and white-rot fungi). Presumably, with such a range of matching species, this particular part of the 18S rRNA gene is unsuitable for identification of Polypores as it does not contain enough variation to distinguish between species. These primers, therefore, confirmed that white-rot isolate HR577 was a basidiomycete but could not be used to identify the isolate further.

The other set of primers used to try to identify white-rot isolate HR577 were for the internal transcribed spacer region (ITS, De Koker, Nakasone *et al.* 2003). Primers that amplify the ITS region have been shown to have a higher degree of variation than other

genic regions of rDNA[39]. Therefore, the sequences obtained using these primers were anticipated to give a more suitable sequence for identification of white-rot isolate HR577. The nucleotide sequence obtained using these primers was entered into a similarity search on the NCBI website (using the BLASTN search engine[40]). Of the 100 matches producing significant alignment, 59 of the sequences had an E value of 0 (scores between 658-747 bits). Of the 59 sequences with an E value of 0, 77% of the sequences were from the genus *Trametes*. Based on this information and the previous Nobles' code information, this white-rot fungus was assigned the name *Trametes* sp. HR577, which has been used for this isolate throughout this thesis.

3.3 PARTIAL GENOMIC DNA LACCASE SEQUENCES FROM *TRAMETES* SP. HR577

The primary objective in purifying genomic DNA from *Trametes* sp. HR577 was to study whether laccase isozymes were produced by this organism. To this end, PCR primers based on highly conserved copper binding regions of previously cloned basidiomycete laccase genes (Collins and Dobson 1997a) were used to produce a PCR product giving a single band at about 690 bp[41] on an agarose gel. This band was excised, purified and cloned. Three clones were sequenced and assigned the names *lac1*, *lac2* and *lac3*[42].

The sequence identity between the three nucleotide sequences is given in Table 3.2. Nucleotide sequences *lac1* and *lac3* had high sequence homology (91%) to each other but poor homology to the *lac2* sequence (65 and 64% for *lac1* and *lac3*, respectively).

[39] http://www.biology.duke.edu/fungi/mycolab/primers.htm
[40] http://www.ncbi.nlm.nih.gov/BLAST/
[41] The sequences obtained from this PCR product represent only a small portion of the laccase gene.
[42] For clarity and quick recognition, several of the laccase sequences mentioned in this chapter have been colour coded both in the text and the figures.

Table 3.2: Percent identity of *lac1*, *lac2* and *lac3* from *Trametes* sp. HR577

Fungal laccase[a]	% Identity[b]		
	lac1	***lac2***	***lac3***
lac1		65	91
lac2			64
lac3			

[b]Calculated using ClustalW (http://www.ebi.ac.uk/clustalw/, Chenna, Sugawara *et al.* 2003)

Confirmation of the Identity of Trametes *sp. HR577 Using Partial gDNA Laccase Gene Sequences*

Since *Trametes* sp. HR577 was identified as belonging to the *Trametes* genus, it follows that the laccase sequences should cluster with those of closely related *Trametes* laccase genes. As with the ITS sequences obtained earlier, sequence similarity searches of the laccases in the NCBI GenBank database (using the BLASTN search on the NCBI website) nucleotide sequences were performed with *lac1*, *lac2* and *lac3*.

The three nucleotide sequences showed significant sequence alignments with laccase genes from *T. versicolor* and *T. villosa*. *Trametes* sp. HR577 *lac1* had the highest score for alignment with *T. villosa lcc2* (GI[43]:1100245, score 1081 bits, E value 0) and *T. versicolor lccI* (GI:1174244, score 1048 bits, E value 0). *Trametes* sp. HR577 *lac2* had the highest score for alignment with *T. villosa lcc4* (GI: 1322078, score 983 bits, E value 0) and *T. versicolor lcc1* (GI:2598856, score 920 bits, E value 0). *Trametes* sp. HR577 *lac3* had the highest score for alignment with *T. versicolor lccI* (GI:1174244, score 985 bits, E value 0) and *T. villosa lcc2* (GI:1100245, score 763 bits, E value 0).

The sequence identity of *lac1*, *lac2* and *lac3* to nucleotide sequences from *T. versicolor* and *T. villosa* laccases is given in Table 3.3. The sequence identity of *lac1*, *lac2* and *lac3* to nucleotide sequences from *T. hirsuta* laccases are also shown in this table. This table shows that the sequence identities at the nucleotide level was between 46-98% (60-94%, 60-98% and 46-69% for *T. villosa*, *T. versicolor* and *T. hirsuta*, respectively) and amino acid sequence identity ranged from 64-98% (72-98%, 71-98% and 67-85% for *T. villosa*, *T. versicolor* and *T. hirsuta*, respectively). These results confirm that white-rot isolate HR577 belongs to the genus *Trametes*.

[43] GI = GenInfo Identifier. The GI numbers here are the ones assigned by the NCBI as opposed to those used by other databases.

Table 3.3: Sequence identity of *lac1*, *lac2* and *lac3* from *Trametes* sp. HR577 to laccase nucleotide sequences for *T. versicolor* and *T. villosa*. Sequence identity was calculated using ClustalW (Chenna, Sugawara *et al.* 2003)

Organism	Isozyme	GI	Nucelotide % Sequence ID (Amino acid % ID)			Reference
			lac1	*lac2*	*lac3*	
T. villosa	*lcc2*	1100245	94 (98)	68 (72)	92 (98)	Yaver, Xu *et al.* 1996
T. villosa	*lcc3*	1321615	62 (68)	62 (64)	61 (68)	Yaver and Golightly 1996
T. villosa	*lcc4*	1322078	61 (72)	94 (97)	60 (72)	Yaver and Golightly 1996
T. villosa	*lcc5*	1322080	65 (75)	69 (79)	65 (74)	Yaver and Golightly 1996
T. villosa	*lcc1*	1100243	66 (80)	67 (72)	65 (80)	Yaver, Xu *et al.* 1996
T. versicolor	*lccI*	1174244	93 (98)	67 (72)	98 (98)	Unpublished
T. versicolor	*lccIII*	19848919	67 (79)	68 (71)	66 (80)	Unpublished
C. versicolor	*CVL3*	2388516	65 (79)	69 (71)	65 (80)	Unpublished
C. versicolor	*CVLG1*	2598856	63 (75)	67 (79)	64 (74)	Mikuni and Morohoshi 1997
T. versicolor	*lcc1*	886718	62 (72)	92 (97)	60 (72)	Jönsson, Sjöström *et al.* 1995
T. hirsuta		167464	67 (85)	69 (72)	67 (85)	Kojima, Tsukuda *et al.* 1990
T. hirsuta		167466	67 (85)	70 (72)	67 (85)	Kojima, Tsukuda *et al.* 1990
T. hirsuta		46578390	44 (77)	46 (67)	46 (78)	Unpublished

Laccase Protein Sequences for lac1, lac2 *and* lac3 *from* Trametes *sp. HR577*

In order to further characterise the partial laccase sequences from *Trametes* sp. HR577, the three partial laccase nucleotide gene sequences were translated in three open-reading frames (ORF) using a web page on the NCBI website[44].

Intron and exon positions were deduced by comparison of these sequences with *lcc2* from *T. villosa* (GI:1100245) as *lac1* and *lac3* showed a high degree of sequence similarity (94 and 92%, respectively at the nucleotide level, both were 98% identical to *lcc2* at the amino acid level, see Figure 3.3 above) with this sequence[45]. The alignment of the *Trametes* sp. HR577 sequences with *lcc2* from *T. villosa* is given in Figure 3.2. At the amino acid level *lac1* and *lac3* share high sequence identity to the *T. villosa lcc2* sequence (both are 98% identical to *T. villosa lcc2*) but *lac2* only shares 72% identity.

[44] http://www.ncbi.nlm.nih.gov/gorf/gorf.html

[45] One of the *T. versicolor* sequences listed in Table 3.3 showed higher sequence homology to the *lac1* and *lac3* nucleotide sequences. However, the sequence was not from a published paper and the nomenclature for *T. versicolor* laccase isozymes tends to be inconsistent between publications (Necochea, Valderrama, *et al.* 2005). In contrast, the sequence used for comparison was published in one of two papers by the same research group (Yaver and Golightly 1996; Yaver, Xu *et al.* 1996). The nomenclature used for the five *T. villosa* laccase sequences was also straightforward. In addition, their publications also included purification and characterisation of two expressed laccase isozymes from *T. villosa*, allowing comparison with purified laccases from *Trametes* sp. HR577 described in chapter 4.

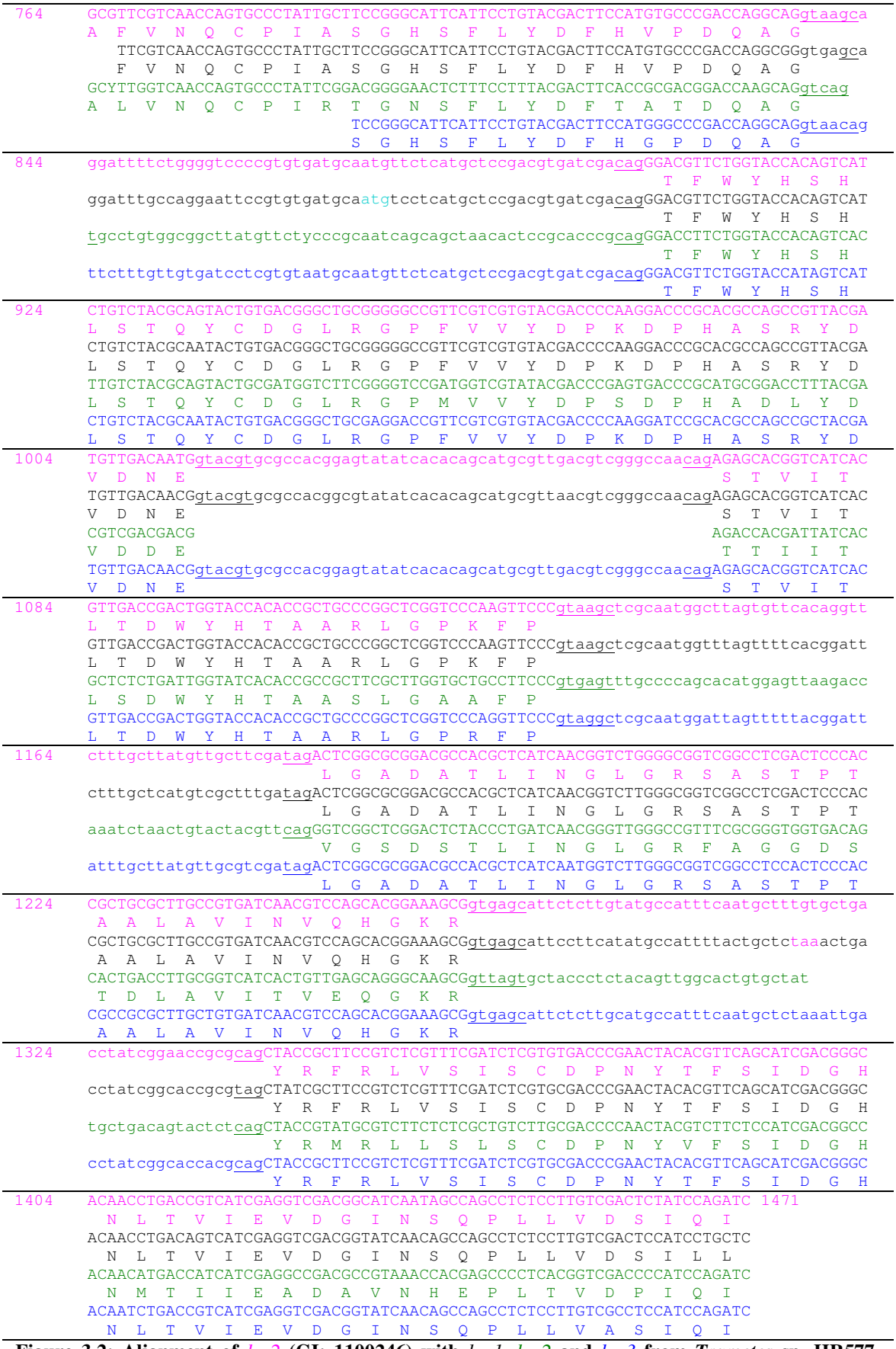


Figure 3.2: Alignment of *lcc2* (GI: 1100246) with *lac1*, *lac2* and *lac3* from *Trametes* sp. HR577. Exons and introns are in capital and lower case letters, respectively. No attempt was made to align the introns from *lac2* since the introns lengths were different. For the full sequence see Chapter 3 Appendix.

The gene structure for *lac1* and *lac3* from *Trametes* sp. HR577 is closer to *lcc2* from *T. villosa* than *lac2* from *Trametes* sp. HR577. This is reflected in the length and number of introns (Figures 3.2 and 3.3). For the *lac1* and *lac3* sequences there are four introns while *lac2* only has three introns. Only the third *lac2* intron is the same length as the equivalent intron in the other two nucleotide sequences but it only shares 29% sequence identity to the other introns. The 5' splice sites of some of the introns from *lac1*, *lac2* and *lac3* do not strictly follow the consensus sequence (GTANGT, Gurr, Unkles and Kinghorn 1987). All but one of the changes occur at either position 3 or position 6 of the consensus sequence. The most common substitution is a C at position 6. This has also been observed for all of the *T. villosa lcc* genes reported by Yaver *et al.* (Yaver and Golightly 1996; Yaver, Xu *et al.* 1996).

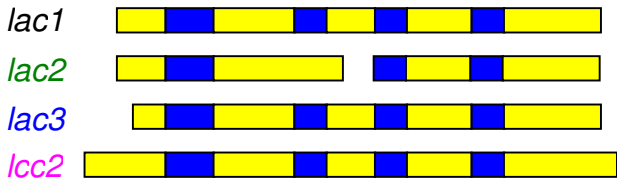


Figure 3.3: The intron/exon structure of the partial laccase genes from *Trametes* sp. HR577 in comparison to part of the *lcc2* gene from *T. villosa*. The exons are indicated by yellow boxes and the introns by blue boxes.

While the *lac2* nucleotide sequence from *Trametes* sp. HR577 did not show high homology to the other two partial laccase sequences from this organism, it showed a very high degree of sequence similarity to *lcc4* from *T. villosa* (GI:1322078, 93% and 97% identity at the nucleotide and amino acid level, respectively, see Table 3.3). An alignment of the *T. villosa lcc4* nucleotide sequence with the *Trametes* sp. HR577 *lac2* nucleotide is given in Figure 3.4. The introns in these two sequences are the same length and share high sequence identity.

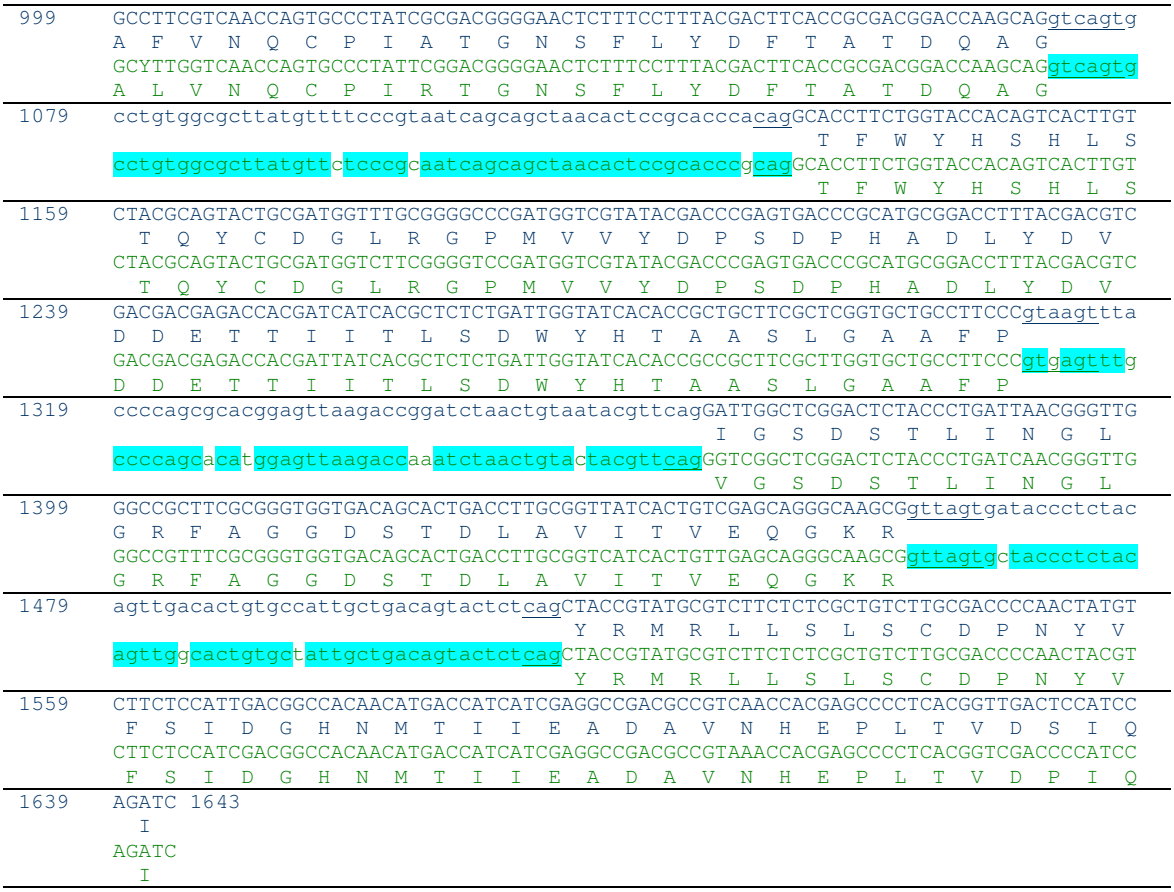


Figure 3.4: Alignment of *lcc4* from *T. villosa* (GI: 1322078, Yaver, Xu *et al.* 1996) with *lac2* from *Trametes* sp. HR577. Exons are in capital letters and introns are in lower case letters. Introns from *lac2* that are the same as those in *lcc4* are highlighted in turquoise. For clarity, only the portion of *lcc4* that overlaps with the *lac2* partial HR577 sequence is shown here. For the full sequence see the Appendix for Chapter 3.

As has just been illustrated for the *Trametes* sp. HR577 and *T. villosa* isozyme sequences, intron length and position can vary considerably within a species. Hoegger, Navarro-González *et al.* (2004) studied the introns in the laccase gene family in *Coprinus cinereus* (a white-rot fungus from the order Agaricales). They sequenced eight non-allelic laccase genes and analysed these for the position and length of introns. Only four introns were conserved over all eight laccase genes while the other twelve introns were found in one to six of the genes. Sequence identity of introns within a gene varied considerably between 6-63%. In the case of one intron (position 4) sequence identity was only 14-33%. They proposed that "evolutionary force conserved the intron positions but not their nucleotide sequences".

3.4 PARTIAL COMPLEMENTARY LACCASE GENE SEQUENCE FROM *TRAMETES* SP. HR577

In order to further study laccase from *Trametes* sp. HR577, degenerate primers were designed to amplify a larger portion of a laccase gene from cDNA. The primer design was based on an alignment of the *T. villosa lcc2* (GI:1100246) and *T. versicolor lccI* (GI:1174245) laccase amino acid sequences. A back translation of the peptide secretion signal and C-terminus portions of the sequence alignments using a *T. villosa* codon frequency table was used to design primers to amplify a longer laccase gene sequence from *Trametes* sp. HR577.

A PCR was carried out over several annealing temperatures (50-60ºC) and a single band (1.6 kb) at 53ºC was observed and subsequently purified, sequenced and designated *lac4*. Although this sequence was longer than the three gDNA laccase sequences *lac1*, *lac2* and *lac3* from *Trametes* sp. HR577, it still did not encode a full laccase gene. This was due to the primer design, which was designed for the beginning of the secretion peptide and the end of the C-terminus.

Comparison of the Amino Acid Sequences of Laccases from Trametes *sp. HR577*

The sequence identity of the four partial laccase amino acid sequences from *Trametes* sp. HR577 is listed in Table 3.4.

Table 3.4: Percent identity of *lac1*, *lac2*, *lac3* and *lac4* amino acid sequences from *Trametes* sp. HR577

Fungal laccase[a]	% Identity[b]			
	lac1	***lac2***	***lac3***	***lac4***
lac1		70	96	98
lac2			71	71
lac3				97
lac4				

[b]Calculated using ClustalW (http://www.ebi.ac.uk/clustalw/, Chenna, Sugawara *et al.* 2003)

Three of the four laccase sequences had very high sequence homology to each other (96-98%) while the other sequence was quite different from the other three (only 70-71% sequence homology at the amino acid level). This points to the presence of at least

two laccase isozymes in this organism. This is consistent with the elution profiles from an anion exchange of crude proteins from *Trametes* sp. HR577 (see Chapter 4, Figure 4.4), which showed two major peaks of laccase activity.

The first laccase active peak from the anion exchange column was eluted in the flow through. It was further purified by hydrophobic interaction chromatography and appeared to resolve into three peaks of laccase activity (Figure 3.5). While the fractionation is unoptimised, it does suggest that a total of four laccase isozymes could be expressed in cultures of *Trametes* sp. HR577. While the three highly homologous laccase sequences *lac1*, *lac3* and *lac4* from *Trametes* sp. HR577 may simply arise from allelic variation or errors from the PCR reactions; the results from the hydrophobic column suggest that these three sequences may represent three different closely related isozymes.

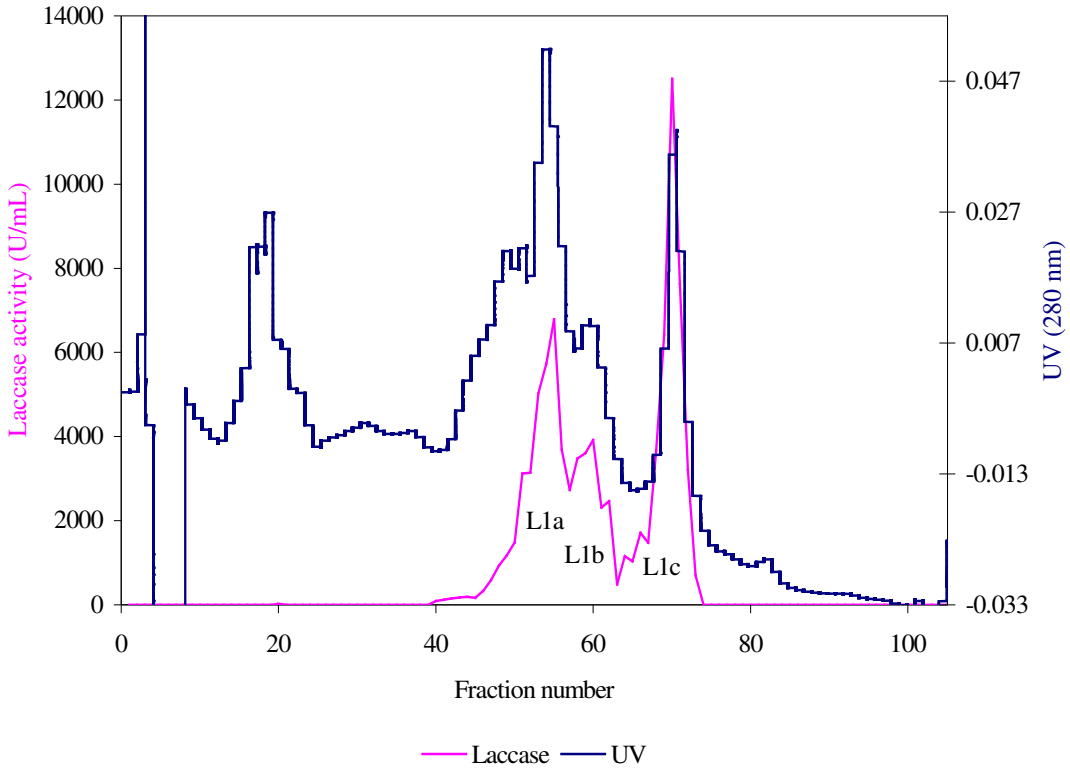


Figure 3.5 Elution profiles of laccases isozymes on Source Phe Column. For experimental details see Chapter 7.

Comparison of lac4 *from* Trametes *sp. HR577 With Other* Trametes *spp. Laccase Sequences*

The laccase nucleotide sequence for *lac4* was translated and the protein sequence (506 amino acids) was loaded into an NCBI similarity search (http://www.ncbi.nlm.nih.gov/BLAST/). All of the most closely matched sequences to *lac4* (with a score above 850 bits, E value = 0) were from the genus *Trametes*. The laccase protein encoded by *lac4* is highly homologous to laccase protein sequences from nine other *Trametes* spp. proteins with identities ranging from 77.6 to 99.8% (Table 3.5).

Table 3.5: Percent identity of *lac4* from *Trametes* sp. HR577 predicted amino acid sequence to sequences of to the nine closest sequences in the NCBI database. For an alignment of these sequences see the Appendix for Chapter 3.

Fungal laccase[a]	% Identity[b]									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1		95.4	94.8	95.0	94.8	93.6	93.4	86.2	86.2	77.6
2			98.6	98.8	98.6	97.4	97.0	88.4	88.4	80.2
3				99.0	98.8	98.4	98.0	87.2	87.2	79.4
4					99.8	97.8	97.4	87.8	87.8	79.6
5						97.6	97.2	87.8	87.8	79.6
6							96.8	86.4	86.4	78.2
7								86.2	86.2	78.2
8									99.8	80.6
9										80.4
10										

[a]Key	GI	Genus	species	Definition	Locus
1	-	*Trametes*	sp. HR577	*lac4*	-
2	23200086	*Trametes*	*versicolor*	TvL, Chain A	1GYC_A
3	56786630	*Trametes*	*versicolor*	Laccase 1	AAW29420
4	1172163	*Trametes*	*versicolor*	Laccase I	AAC49828
5	2833233	*Trametes*	*versicolor*	Laccase 2 precursor	Q12718
6	63147344	*Trametes*	*versicolor*	Laccase2	BAD98306
7	15617227	*Trametes*	*versicolor*	Laccase 1	AAL00887
8	33334369	*Trametes*	sp. I-62	laccase	AAQ12268
9	33334367	*Trametes*	sp. I-62	laccase	AAQ12267
10	2842752	*Trametes*	*villosa*	Laccase 1 precursor	Q99044

[b]Calculated by the Clustal method (Thompson, Higgins and Gibson 1994)

As mentioned above, the most closely related sequences to *lac4* were all from the genus *Trametes*. This appears to provide further confirmation that this isolate belongs to the genus *Trametes*.

The use of fungal laccase sequence similarity to try to classify white-rot fungi has been reported by Mansur, Suárez *et al.* (1997). They used a comparison of laccase gene

sequences to confirm the identity of a white-rot isolate that could not be conclusively identified using morphological features. Comparison of laccase gene sequences suggested a phylogenetic proximity between this fungus and fungi from the genus *Trametes*. Therefore, they designated it as *Trametes* sp. I-62.

A phylogenetic analysis of protein sequences from both plant and fungal laccases with sequences from other closely related multicopper oxidase protein sequences has been reported in the literature (Hoegger, Kilaru *et al.* 2006). The analysis suggests that fungal laccases cluster by taxonomic group. However, if only basidiomycete fungal laccases are included in the phylogenetic analysis, not all of the sequences cluster strictly by taxonomic group. It was therefore proposed that laccase protein sequences in basidiomycetes are clustered by biological function rather than by taxonomic group (Hoegger, Kilaru *et al.* 2006).

Therefore, the laccase sequences from *Trametes* sp. HR577 can not be used as the only criteria to prove that this isolate is definitely part of the genus *Trametes*. However, these results suggest that laccases from this organism have a similar biological function to laccases from other isolates in the *Trametes* genus.

3.5 FEATURES OF LACCASE GENES

Laccase genes of *Trametes* spp. generally encode a protein of about 519 amino acids and have a signal sequence of around 20 amino acids. All laccase sequences have regions of highly conserved amino acids. These regions are involved in the binding of copper and maintenance of the enzyme tertiary structure. An alignment of the partial amino acid sequences of the laccase sequences from *Trametes* sp. HR577 is given in Figure 3.6. A full length laccase isozyme gene from *T. versicolor* (*TvL*, GI: 23200086, Piontek, Antorini and Choinowski 2002) was also included in the alignment in Figure 3.6 for comparison. The *T. versicolor* sequence was from a crystal structure and therefore, does not contain a secretion signal peptide. Although none of the four sequences from *Trametes* sp. HR577 are for a full length laccase gene, these sequences illustrate several of the common features of fungal laccases.

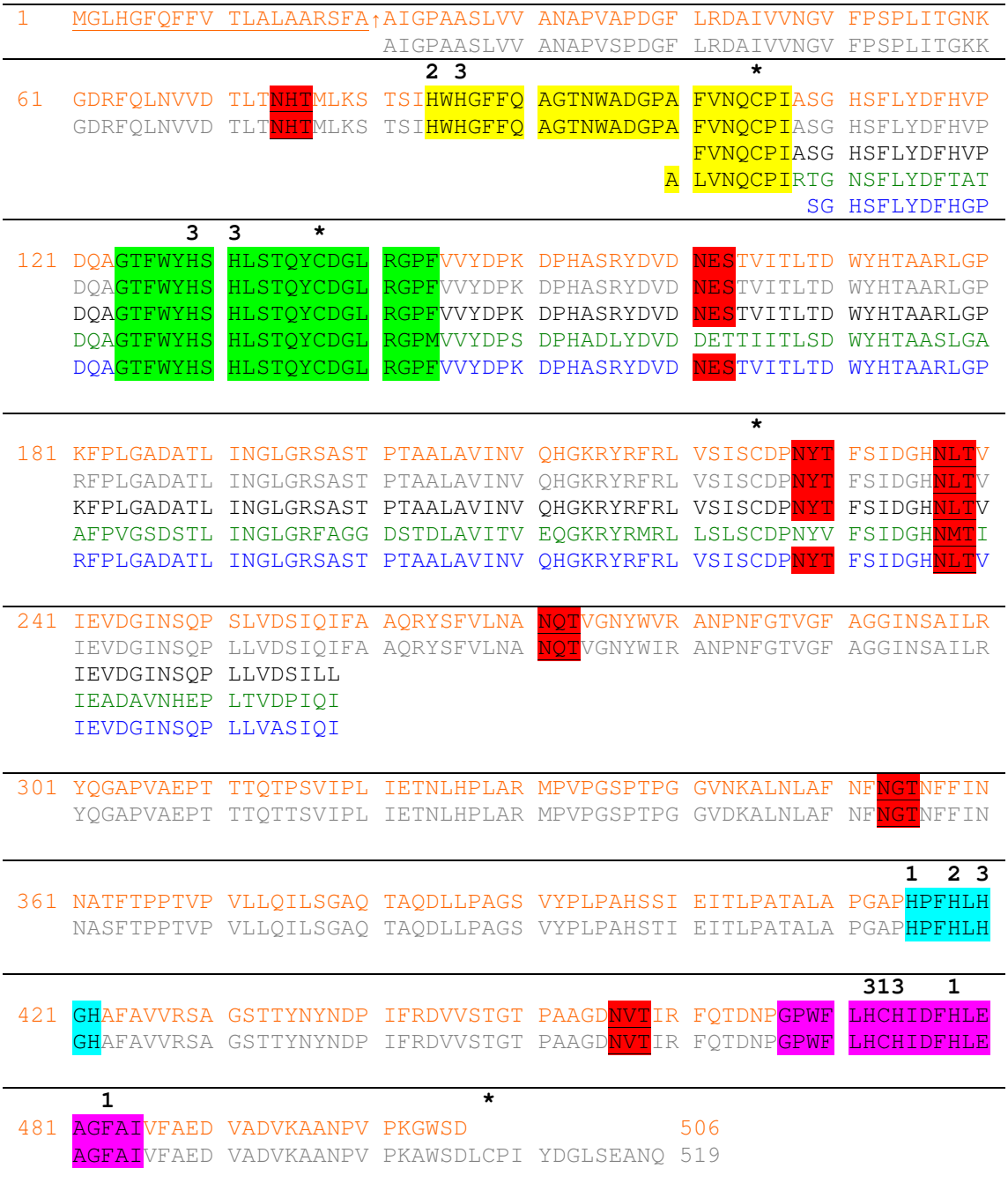


Figure 3.6: Alignment of *Lac1*, *Lac2*, *Lac3* and *Lac4* amino acid sequences from *Trametes* sp. HR577.
Key:
Lac4 (506 amino acids) *Lac1* (171 amino acids) *Lac2* (172 amino acids) *Lac3* (163 amino acids) *T. versicolor TvL*, 1GYC_A, GI: 23200086 (519 amino acids). The start of the N-terminal sequence of the secreted protein is indicated with ↑. The signal peptide is underlined in the *lac4* sequence. Conserved Cys residues involved in disulfide bonds are marked with an asterisk. Amino acids that coordinate to copper atoms are indicated by a number that refers to the type of copper atom bound. Laccase signature sequences are highlighted as follows: L1, L2, L3, L4 (Kumar, Phale *et al.* 2003). Putative *N*-glycosylation sites are highlighted in red. Putative *N*-glycosylation sites that are underlined indicate those that correspond to sites found to be glycosylated in the *TvL* crystal structure (Piontek, Antorini and Choinowski 2002)

Conserved Fungal Laccase Sequences

Ten His residues and five Cys residues are completely conserved over all laccase sequences. The His residues and one of the Cys residues are involved in binding four copper ions while the remaining four Cys residues form disulfide bonds. As expected for the partial sequence lengths, the partial *lac4* sequence contains all of the conserved His residues and four of the Cys residues (three of the Cys residues that form disulfide bonds and the Cys involved in copper binding) while the shorter sequences have 2-3 of the Cys residues (*lac3* has 2 and *lac1* and *lac2* have 3 Cys residues of the four that form disulfide bonds) and two of the type 3 copper binding His residues. The full sequence from *T. versicolor* has all of the conserved residues.

Kumar, Phale *et al.* (2003) analysed a large number of plant and fungal laccase sequences. They identified a set of four ungapped sequence regions (which they labelled L1-L4) that could be used to identify laccases and distinguish them from other multicopper oxidases. These four regions contained all of the copper binding residues. For the L2 sequence (which contains 21 residues) only the first eight residues are also conserved in plant laccases. Therefore this sequence is specific for fungal laccases. All four of these signature sequences are found in the *lac4* sequence and have been highlighted in Figure 3.6. The *lac3* sequence is only long enough to show the fungal specific L2 signature sequence. However, the *lac1* and *lac2* are slightly longer and contain both the L2 signature sequence and also part of the L1 signature sequence (see Figure 3.6).

Putative N-*Glycosylation Sites*

The putative *N*-glycosylation sites for *lac1-4* have also been highlighted in Figure 3.6 (see Chapter 4 for a discussion on glycosylation of purified laccases). Only one putative *N*-glycosylation site was found in all four sequences. The crystal structures of two *T. versicolor* laccase isozymes have been solved (Bertrand, Jolivalt *et al.* 2002, Piontek, Antorini and Choinowski 2002). The laccase amino acid sequence published by Piontek, Antorini and Choinowski (2002, GI:23200086) closely matched that of *lac4* from *Trametes* sp. HR577 (99% sequence identity, see Table 3.5 and Figure 3.6). Seven putative *N*-glycosylation sites were found in that sequence, but only five were

shown to be glycosylated in the crystal structure. The second of these glycosylated sites is the one that corresponds with the only putative site observed in all four partial laccase sequences from *Trametes* sp. HR577.

T1 Copper Ligation

The T1 copper redox potential is important for determining the range of substrates that can be oxidised by different fungal laccases (see Chapter 1). Fungal laccases have been classified into two groups based on the T1 copper redox potential. Laccases with a T1 E° of 0.400-0.550 V are classed as 'low' while those with a T1 E° between 0.700-0.800 V are classed as 'high'.

Several groups have attempted to predict whether fungal laccases are 'high' or 'low' based on comparison of 'high' and 'low' laccase gene sequences. Some of the amino acids that were proposed to play a role in determination of the T1 redox potential have been shown to be unimportant in determining the T1 redox potential (Xu, Berka *et al.* 1998; Xu, Palmer *et al.* 1999; Palmer, Szilagyi *et al.* 2003; Madzak, Mimmi *et al.* 2006). The role of other amino acid sequences in determining T1 redox potentials have yet to be confirmed or disproved (Piontek, Antorini and Choinowski 2002).

One of the current theories to predict whether a laccase isozyme will have a 'high' or 'low' T1 redox potential has been presented by Piontek, Antorini and Choinowski (2002). These researchers proposed that the reason for the high redox potential in a *T. versicolor* laccase isozyme is due to a hydrogen bond formed between Ser113 and Glu160. This bond causes an elongation of the bond from one of the His residues (His458) to the T1 copper. This is thought to result in an increase in the redox potential at this site. Comparison of the laccase sequence from *Trametes* sp. HR577 *lac4* with the sequence given for *T. versicolor* shows that *lac4* has both of the residues required to form the hydrogen bond. Therefore, *lac4* may also be a laccase isozyme with a high T1 redox potential.

While the theory proposed by Piontek, Antorini and Choinowski (2002) has yet to be completely substantiated, there is other data in the literature that agrees with their findings. The crystal structure of another high redox potential laccase, *Rigidoporus*

lignosus has been elucidated (Garavaglia, Cambria *et al.* 2004). The His-T1 copper bond length for this laccase is also long (2.20 Å) and there is a strong hydrogen bond between Ser and Glu at the equivalent structural positions to Ser113 and Glu160 in *T. versicolor*. This may provide confirmation that these amino acids are important for determining the T1 copper redox potential of fungal laccases. However, more research is needed to establish if these amino acids are important.

3.6 CLASSIFICATION OF THE *TRAMETES* SP. HR577 LACCASE SEQUENCES

Many attempts have been made to devise classification systems for laccases from differences between laccases both within a species and between different species (Eggert, LaFayette *et al.* 1998; Yaver and Golightly 1996; Yaver, Xu *et al.* 1996; Necochea, Valderrama *et al.* 2005). This section describes some of these laccase classification methods and how *lac1*, *lac2*, *lac3* and *lac4* from *Trametes* sp. HR577 may fit into the various classification systems.

Classification of Laccases Based on the Ligation of the T1 Copper

One method proposed for classification of plant and fungal laccases is based on the T1 copper axial ligand as this was originally proposed to account for differences in the redox potentials of plant and fungal laccases. Eggert, LaFayette *et al.* (1998) suggested that laccases should be divided into three classes based on the axial T1 copper ligand. The classes proposed were class 1 (Met, all plant laccases), class 2 (Leu, generally 'low' redox laccases) and class 3 (Phe, generally 'high' redox laccases).

Of the four partial laccase gene sequences reported in this thesis, only the last sequence, *lac4*, included the region containing the T1 copper ligands. Based on the classification system proposed by Eggert, LaFayette *et al.* (1998), *lac4* would be classified as a class 3 laccase (the class corresponding to fungal laccases with 'high' redox potentials). While this would appear to be confirmation that *lac4* is a 'high' redox laccase, it has been

shown that for fungal laccases Phe and Leu as axial T1 copper ligand have an equivalent effect on the T1 copper redox potential (see Chapter 1).

Classification of Laccases Based on Intron/Exon Structure

Yaver *et al.* (Yaver and Golightly 1996; Yaver, Xu *et al.* 1996) cloned five laccase genes from the white-rot fungus *T. villosa*. They analysed the genes and suggested that the laccase genes of *T. villosa* could be classified into two subfamilies based on the intron/exon structure. Two of the five *T. villosa* laccase genes reported by Yaver *et al.* (*lcc2* and *lcc4*) were used to deduce the intron/exon positions in the three partial laccase nucleotide sequences reported in Figures 3.4 and 3.6 due to high sequence homology of these sequences. The *lcc2* and *lcc4* sequences from *T. villosa* were classified in different subfamilies (Yaver and Golightly 1996). As *Trametes* sp. HR577 laccases *lac1*, *lac3* and *lac4* share high sequence identity with *lcc2* from *T. villosa* while *lac2* from HR577 shares the highest similarity to *lcc4* from *T. villosa*, these isozymes could be divided into similar subfamilies.

Classification of Laccases Based on Phylogenetic Analysis

Necochea, Valderrama *et al.* (2005) noted that the current laccase gene nomenclature for *T. versicolor*, which is based on isoelectric point (pI) prediction, is ambiguous. They carried out a phylogenetic analysis of ten *T. versicolor* laccase genes (plus a *P. ostreatus* laccase gene, and a *Neurosporra crassa* laccase gene as 'outgroups') and proposed a new system of classification for *T. versicolor* laccases. Their system divides the *T. versicolor* laccases into four groups (α, β, δ, γ). The sequence identity within each subgroup was at least 97% while identity between subgroups was approximately 70%. They suggested that the differences within subgroups might arise from allelic variation.

In order to see whether this model could be used to classify other *Trametes* sp. laccases, their phylogenetic tree was reconstructed using ClustalX[46] and TreeView[47], and the four laccase sequences from *Trametes* sp. HR577 were included in the analysis (Figure 3.7). *Lac1*, *lac3* and *lac4* all clustered with the α-subgroup while the most diverse sequence (that of *lac2*) was found clustered with the δ-subgroup.

While this would suggest that *lac1*, *lac2*, *lac3* and *lac4* from *Trametes* sp. HR577 could be classified using this system, this model needs to be further tested, as there may be some problems associated with this approach. Firstly, the original alignment used by Necochea, Valderrama *et al.* (2005) was only meant to classify *T. versicolor* laccase sequences and contained only 12 sequences: 10 from *T. versicolor* and 2 outliers (*P. ostreatus* and *N. crassa*). There are many other laccase sequences available in the GenBank library from other *Trametes* spp (*T. villosa, T. hirsuta, T. pubescens, Trametes* sp. C. 30, *Trametes* sp. I-62, *Trametes* sp. AH28-2 and *Trametes* sp. 420).

If the other sequences from *T. versicolor* plus the *T. hirsuta* and *T. pubescens* are included in the alignment then the model still holds with four distinct branches. However, addition of the *T. villosa* sequences lead to the inclusion of a new branch (designated ε, Figure 3.8). This new branch was much closer to the ascomycete outlier laccase sequence (*N. crassa*) than the basidiomycete outlier (*P. ostreatus*) sequence. The model broke down completely if *Trametes* sp. C30, *Trametes* sp. I-62, *Trametes* sp. AH28-2 or *Trametes* sp. 420 were also included.

[46] The ClustalX program is described by Thompson, Gibson, *et al.* (1997).
[47] The TreeView program used to generate Figures 3.9 and 3.10 was downloaded from http://taxonomy.zoology.gla.ac.uk/rod/rod.html and was written by Roderic D M Page, 2001

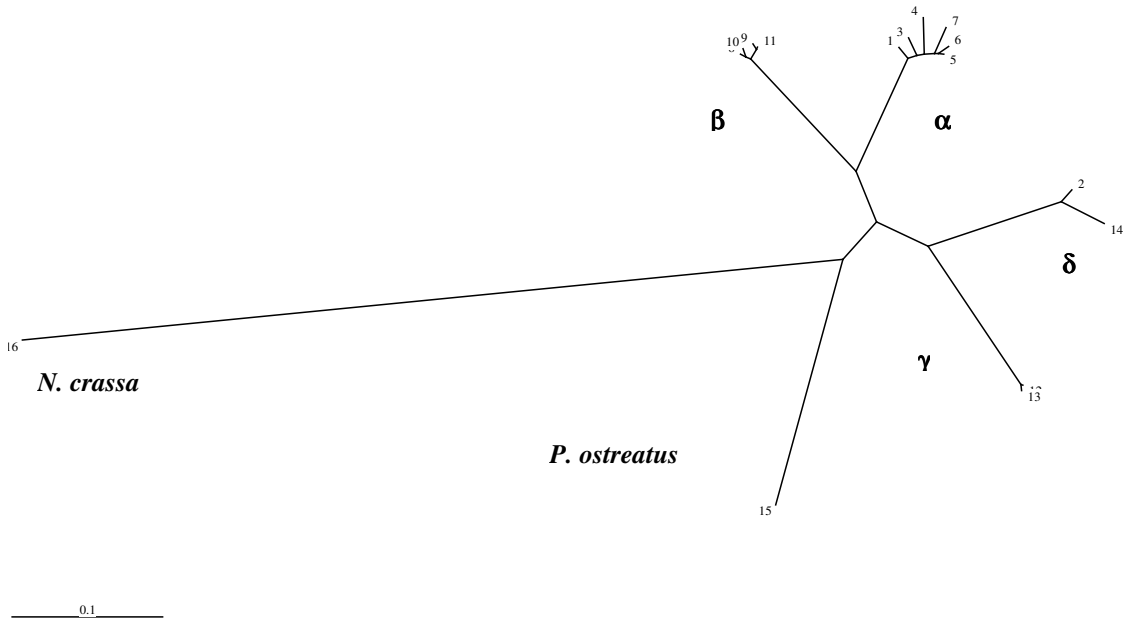


Figure 3.7: TreeView picture of *T. versicolor* laccases with *Trametes* sp. HR577 laccases

Key	Accession no./name	Organism	Reference	Sub-group
1	*Lac1*	*Trametes* sp. HR577	Current study	α
2	*Lac2*	*Trametes* sp. HR577	Current study	δ
3	*Lac3*	*Trametes* sp. HR577	Current study	α
4	*Lac4*	*Trametes* sp. HR577	Current study	α
5	AAC49828	*T. versicolor*	Ong, Pollock and Smith 1997	α
6	1GYC	*T. versicolor*	Johannes and Majcherczyk 2000	α
7	AAL00887	*T. versicolor*	O'Callaghan, O'Brien *et al.* 2002	α
8	AAL07440	*T. versicolor*	Bertrand, Jolivalt *et al.* 2002	β
9	CAA77015	*T. versicolor*	Jönsson, and Nyman, Unpublished (Submitted to NCBI: 27 August 1998)	β
10	BAA22153	*T. versicolor*	Mikuni and Morohoshi 1997	β
11	AAL93622	*T. versicolor*	Pfaller, and Friesenegger, Unpublished (Submitted to NCBI: 21 February 2002)	β
12	BAA23284	*T. versicolor*	Mikuni and Morohoshi 1997	γ
13	AAC49829	*T. versicolor*	Ong, Pollock and Smith 1997	γ
14	CAA59161	*T. versicolor*	Jönsson, Sjöström *et al.* 1995	δ
15	Q12739	*P. ostreatus*	Giardina, Aurilia *et al.* 1996	Out-group
16	P10574	*N. crassa*	Germann, Müller *et al.* 19881	Out-group

[1] NB: This sequence was replaced on 1 December 2006 by gi: 118572665 (P06811). It is included here as it is the sequence that was used by Necochea, Valderrama *et al.* (2005)

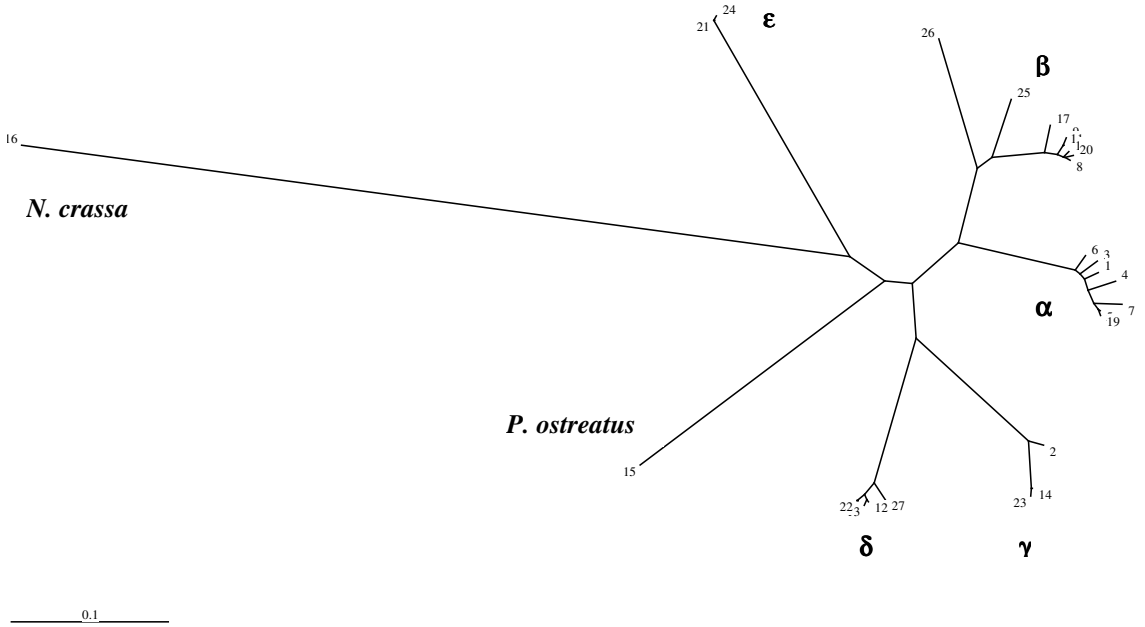


Figure 3.8: TreeView of Necochea, Valderrama *et al.* (2005) with *Trametes* sp. HR577, *T. hirsuta*, *T. pubescens* and *T. villosa* laccases

Key	Accession no./name	Organism	Reference	Sub-group
1	*Lac1*	*Trametes* sp. HR577	Current study	α
2	*Lac2*	*Trametes* sp. HR577	Current study	δ
3	*Lac3*	*Trametes* sp. HR577	Current study	α
4	*Lac4*	*Trametes* sp. HR577	Current study	α
5	AAC49828	*T. versicolor*	Ong, Pollock and Smith 1997	α
6	1GYC	*T. versicolor*	Johannes and Majcherczyk 2000	α
7	AAL00887	*T. versicolor*	O'Callaghan, O'Brien *et al.* 2002	α
8	AAL07440	*T. versicolor*	Bertrand, Jolivalt *et al.* 2002	β
9	CAA77015	*T. versicolor*	Jönsson, and Nyman, Unpublished (Submitted to NCBI: 27 August 1998)	β
10	BAA22153	*T. versicolor*	Mikuni and Morohoshi 1997	β
11	AAL93622	*T. versicolor*	Pfaller, and Friesenegger, Unpublished (Submitted to NCBI: 21 February 2002)	β
12	BAA23284	*T. versicolor*	Mikuni and Morohoshi 1997	γ
13	AAC49829	*T. versicolor*	Ong, Pollock and Smith 1997	γ
14	CAA59161	*T. versicolor*	Jönsson, Sjöström *et al.* 1995	δ
15	Q12739	*P. ostreatus*	Giardina, Aurilia *et al.* 1996	Out-group
16	P10574	*N. crassa*	Germann, Müller *et al.* 19881	Out-group
17	AAM18407	*T. pubescens*	Galhaup, Goller *et al.* 2002	β
18	Q99056	*T. villosa*	Yaver and Golightly 1996	γ
19	Q99046	*T. villosa*	Yaver, Xu *et al.* 1996	α
20	Q99044	*T. villosa*	Yaver, Xu *et al.* 1996	β
21	JC5355	*T. villosa*	Yaver and Golightly 1996	ε
22	AAB47735	*T. villosa*	Yaver and Golightly 1996	γ
23	AAB47734	*T. villosa*	Yaver and Golightly 1996	δ
24	AAB47733	*T. villosa*	Yaver and Golightly 1996	ε
25	Q02497	*T. hirsuta*	Kojima, Tsukuda *et al.* 1990	β
26	AAL89554	*T. hirsuta*	Rebrikov, Unpublished (Submitted to NCBI: 27 April 2004)	β
27	AAM18408	*T. pubescens*	Galhaup, Goller *et al.* 2002	γ

[1] NB: This sequence was replaced on December 1 2006 by gi: 118572665 (P06811). It is included here as it is the sequence that had been used by Necochea, Valderrama *et al.* (2005)

3.7 CONCLUSION AND FUTURE WORK

The white-rot isolate HR577 has been identified as belonging to the genus *Trametes* using morphological features and ITS gene sequences. Four partial putative laccase isozymes were sequenced from gDNA or cDNA extracted from this organism. These four sequences showed very high sequence homology (up to 99% identity at the amino acid level) to laccase genes from other *Trametes* spp., especially those of *T. versicolor* and *T. hirsuta*. The four partial laccase sequences represent at least two different laccase genes. Three of the laccases sequences show very high sequence identity to each other. This may indicate that these represent different alleles rather than different laccase genes. However, expressed laccases purified and reported in Chapter 4 indicate that these three sequences may represent three different laccase genes. The four partial laccase sequences from *Trametes* sp. HR577 share many of the common features of fungal laccases.

Further work is needed to identify *Trametes* sp. HR577 to the species level. This could be achieved either by production of a mature fruiting body or by using different universal primers designed to amplify a larger portion of the internal transcribed spacer region. More work is also needed to obtain full length laccase gene sequences from *Trametes* sp. HR577. These could be used to confirm whether the four partial laccase sequences described in this chapter represent four different laccase genes or simply indicate the presence of two laccase genes with different alleles. These may also show the presence of other laccase isozymes. Design of primers for specific laccase genes in this organism would allow further studies into the induction of laccase by PCP and copper to be carried out. These primers could be used in Real-Time RT-PCR to determine whether each laccase isozyme produced by *Trametes* sp. HR577 is induced or constitutive. Primers designed to amplify a housekeeping gene such as the β-tubulin gene would also be required for real-time RT-PCR to ensure that any increase observed was due to an increase in laccase gene transcription.

CHAPTER 4: PURIFICATION AND CHARACTERISATION OF LACCASE FROM *TRAMETES* SP. HR577

4.1 INTRODUCTION

Laccase is an enzyme produced by white-rot fungi that has potential for use in a variety of different applications (see Chapter 1). This PhD project is part of a larger study to examine ways to clean up soil contaminated with PCP. Laccases have been implicated in the degradation of PCP but there is still controversy over what role, if any, laccases play in PCP degradation. Laccase isozymes from white-rot fungi can have different biological properties (Mansur, Arias *et al.* 2003). It was of interest therefore, to purify and characterise the laccase isozymes from *Trametes* sp. HR577 to determine what role they might potentially play in PCP degradation in the environment (see Chapter 5).

The white-rot isolate *Trametes* sp. HR577 has at least two different laccase genes (see Chapter 3). These laccases genes share high sequence homology to laccase genes from other *Trametes* species. This chapter describes the purification and characterisation of laccase isozymes from strain HR577. To the best of the author's knowledge, this study represents the first ever purification of laccases from a NZ white-rot fungus. The chapter compares some of the properties of these enzymes to other white-rot laccase isozymes, especially those from other *Trametes* species. For each section of work presented here, the literature background that affects the experimental set up and the literature that relates to the results are discussed together.

4.2 OPTIMISATION OF LACCASE PRODUCTION

It has been reported in the literature that there are many different media components that can affect laccase production *in vitro*. These include the type of carbon and nitrogen source and concentration (Collins and Dobson 1997a; Tavares, Coelho *et al.* 2005), copper concentration (Palmieri, Giardina *et al.* 2000; Galhaup and Haltrich 2001; Baldrian and Gabriel 2002; Levin, Forchiassin and Ramos 2002), manganese concentration (Collins and Dobson 1997a; Soden and Dobson 2001), and the addition of

laccase inducers (Fåhraeus and Reinhammar 1967; Bollag and Leonowicz 1984; Yaver, Xu *et al.* 1996; Mougin, Kollmann and Jolivalt 2002).

Since the laccase genes from *Trametes* sp. HR577 share high sequence homology to laccases from other related *Trametes* spp. (see Chapter 3), it would be reasonable to expect that laccases produced by this fungus would be affected in a similar way by nutrient and environmental conditions. A full study of the many factors that affect the production of laccase isozymes is beyond the scope of this study. Here, only a few key conditions known to affect laccase production were tested.

Enzyme Expression in Cultures Grown on SCS

Growth on solid substrate was evaluated using SCS as described by Leštan *et al.* (Leštan, Leštan *et al.* 1996). The SCS was submerged in one of three solutions: standard liquid medium (based on Johansson and Nyman 1993), acetate buffer (pH 5) or distilled water (Table 4.1). The laccase activity after 7 days was compared to that of cultures inoculated with *Trametes* sp. HR577 grown on malt extract agar. Fungi grown on SCS in distilled water or acetate buffer produced much less laccase than the control cultures. In contrast, growth of *Trametes* sp. HR577 on SCS in standard liquid medium was almost double that of the controls.

Table 4.1: Enzyme Expression in Cultures Grown on SCS. Cultures were grown in standard liquid medium based on Johansson and Nyman (1993, see Chapter 7) or sterile distilled water or acetate buffer pH 5. Control flasks were inoculated with agar plugs from 7 day old cultures grown on malt extract agar. All other flasks were inoculated with cultures grown on SCS. SCS = Starch, Cornmeal, Sawdust and is based on the method of Leštan, Leštan *et al.* (1996). All experimental treatments were carried out in duplicate and are reported as a percentage relative to control cultures grown in standard liquid medium. Laccase activity was measured by oxidation of ABTS at 25ºC using the method of Wolfenden and Willson (1982).

Treatment	Relative laccase activity (%)
SCS in standard liquid medium	194
SCS in distilled water	27
SCS in acetate buffer, pH 5	11

The results of this study show that enzyme expression was most efficient in cultures grown on SCS in standard liquid medium. Similar observations have been made by other workers. For white-rot to grow in PCP contaminated soil (especially unsterilised soil), nutrient amendment is often required (Lamar and Dietrich 1990; Lamar, Larsen and Kirk 1990; Boyle 1995; Walter, Boul *et al.* 2004). One of the easiest ways to provide nutrients is by growing fungi on solid substrates such as wood chips. Several research groups have studied the effect of various solid substrates and lignin-model compounds on fungal growth and ligninolytic enzyme production (Scheel, Höfer *et al.* 2000; Ullah, Kadhim *et al.* 2000; de la Rubia, Ruiz *et al.* 2002; Farnet, Criquet *et al.* 2004; Terrón, González *et al.* 2004; Dong, Zhang *et al.* 2005; Meza, Lomascolo *et al.* 2005; Prasad, Mohan *et al.* 2005). Many of the compounds tested have been shown to enhance production of extracellular ligninolytic enzymes. As wood is the natural substrate for ligninolytic enzymes from white-rot fungi, it is not surprising that the use of lignin-containing or lignin-model compounds influences the production of peroxidases and laccase.

Leontievsky *et al.* (Leontievsky, Myasoedova *et al.* 1990, Leontievsky, 1997 #208) purified 'yellow' laccases from white-rot fungi such as *P. tigrinus*, *T. versicolor*, *Ph. radiata*[48] and *A. bisporus* cultured on wheat straw. These laccases were able to oxidise non-phenolic lignin model compounds in the absence of synthetic mediator compounds (see Chapter 1). It is possible that laccase purified from cultures of *Trametes* sp. HR577 grown in submerged culture on SCS in the present study might also be classed as 'yellow' laccases based upon their similarity to the laccases of Leontievsky's study. However, while this particular SCS formulation gave an increase

[48] *Ph. radiata* refers to *Phlebia radiata*, a white-rot fungus as opposed to *P. radiata* (*Pinus radiata*, pine)

in laccase production relative to the control cultures it is not necessarily the best formulation to use for laccase production.

Ford (2006) carried out experiments for the optimisation of soil colonisation by NZ white-rot fungi in parallel with these experiments. He found that the best formulation for soil colonisation was a Monterey pine-kibbled rye-calcium carbonate formulation. Those results were not available at the time of the current purification experiments, and use of SCS requires an extra week of incubation prior to enzyme purification. In addition, enzyme purification is a time consuming and laborious task, one that is made even more so when purification of the extracellular secreted fungal enzyme has to be extracted from a complex medium like a soil or wood slurry. Thus, it was decided to use a simple liquid medium to reduce the amount of labour required to purify laccase from *Trametes* sp. HR577 cultures.

Effect of Copper on Laccase Expression in Culture

Laccases require four copper atoms per monomeric subunit for catalysis. Increasing the copper concentration in fungal media generally leads to increased laccase production (see Chapter 2, Galhaup and Haltrich 2001; Klonowska, Le Petit and Tron 2001; Klonowska, Gaudin *et al.* 2002; Saparrat, Guillén *et al.* 2002; Arana-Cuenca, Roda *et al.* 2004). However, if the copper concentration is too high then it can have an adverse effect on fungal biomass growth (Gralla, Thiele *et al.* 1991; Cervantes and Gutierrez-Corona 1994; De Groot and Woodward 1999; Epstein and Bassein 2001). Therefore, the appropriate amount of copper to maximise laccase production without adverse effects on biomass growth in *Trametes* sp. HR577 was assessed (see Table 4.2).

Table 4.2: Effect of Copper on Laccase Expression in Culture. Cultures were grown in standard liquid medium containing 0-500 μM added copper (based on Johansson and Nyman 1993, see Chapter 7). All flasks were inoculated with agar plugs from 7 day old cultures grown on malt extract agar. All experimental treatments were carried out in duplicate and are reported as a percentage relative to control cultures grown in standard liquid medium (standard liquid medium contained 4 μM Cu). Laccase activity was measured by oxidation of ABTS at 25ºC using the method of Wolfenden and Willson (1982).

Copper (μM)	Relative laccase activity (%)	Fungal Biomass (mg)
0	15	79 ± 8
4 (Control)	100	70 ± 11
50	73	69 ± 2
100	147	76 ± 1
200	148	54 ± 35
300	187	39 ± 6
400	59	21 ± 1
500	87	23 ± 2

The levels of copper selected for testing (0-500 μM) were based on copper concentrations reported to enhance laccase production (Galhaup and Haltrich 2001; Klonowska, Le Petit and Tron 2001; Klonowska, Gaudin *et al.* 2002; Saparrat, Guillén *et al.* 2002; Arana-Cuenca, Roda *et al.* 2004) or inhibit fungal growth (Gralla, Thiele *et al.* 1991; Cervantes and Gutierrez-Corona 1994; De Groot and Woodward 1999; Epstein and Bassein 2001).

Although the treatment containing 300 μM of copper had the highest laccase activity (Table 4.2), the fungal growth in these flasks was inhibited. Figure 4.1 shows fungal growth in copper containing cultures on day 7. Fungal growth in the presence of 0-100 μM copper was similar, giving dry biomass yields between 69 and 79 mg (Table 4.2). When copper levels reached 200 μM there was a slight decrease in fungal growth relative to control cultures. Therefore 100 μM of copper was used in the final fungal growth medium.

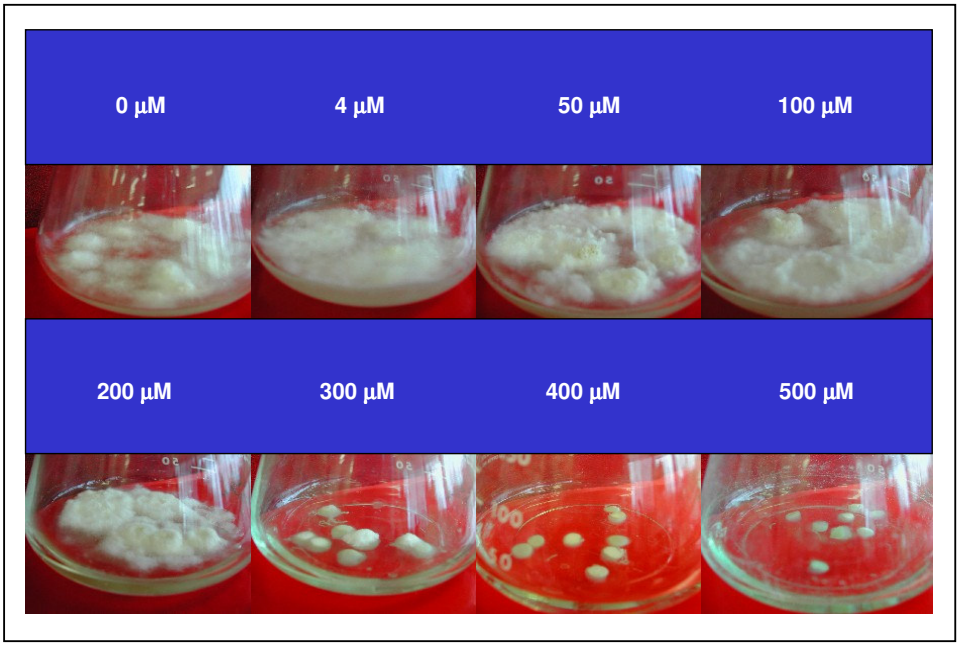


Figure 4.1: The effect of copper concentration on fungal growth. These photos show the fungal growth on day 7 in cultures that differ in the amount of copper added to the liquid medium. Each treatment was carried out in duplicate. Biomass data for the average of duplicate cultures is given in the Table 4.2 above.

The results of this study compare well with levels of copper added to other white-rot fungi to enhance laccase production. Collins and Dobson (1997a) tested the effect of six different copper concentrations (ranging from 0-400 µM) on laccase production and gene transcription in *T. versicolor*. Laccase production and gene expression were found to increase with increasing copper concentration. Collins and Dobson did not report whether any of the levels of copper added had any effect on biomass production for this white-rot fungus. Similarly, Galhaup and Haltrich (2001) studied the effect of a range of copper concentrations on laccase production by the white-rot fungus *T. pubescens*. Copper (0.1-2 mM) was added to cultures on either day 0, 4 or 8 and the effect on laccase activity was measured by assaying with ABTS. The optimal amount of copper to enhance laccase production in this white-rot was 1.5-2.0 mM. This effect was greatest for these copper concentrations if the copper was added to cultures on day 4. This is similar to observations for addition of copper to cultures of *Trametes* sp. HR577 on day 5 (see Chapter 2). Galhaup *et al.* observed there was a negligible effect on fungal biomass growth at lower copper concentrations (0-1 mM). However, if 1.5-2.0 mM copper was added to cultures of *T. pubescens* on day 0, a negative effect on fungal biomass growth was observed.

Effect of Manganese on Laccase Expression in Culture

The literature reports that addition of manganese to fungal cultures can enhance production of laccase (Scheel, Höfer *et al.* 2000; Soden and Dobson 2001). In this study, manganese concentrations between 0-300 μM were used to determine an appropriate level of manganese for laccase production. The levels of manganese tested were based on reported manganese levels used by other researchers. No differences in fungal biomass growth were observed between treatments. The best manganese concentration in terms of laccase production was 100 μM (Table 4.3).

Table 4.3: Effect of Manganese on Laccase Expression in Culture. Cultures were grown in standard liquid medium containing 0-300 μM added manganese (based on Johansson and Nyman 1993, see Chapter 7). All flasks were inoculated with agar plugs from 7 day old cultures grown on malt extract agar. All experimental treatments were carried out in duplicate and are reported as a percentage relative to control cultures grown in standard liquid medium. Laccase activity was measured by oxidation of ABTS at 25ºC using the method of Wolfenden and Willson (1982).

Manganese (μM)	**Relative laccase activity (%)**
0	68
2 (Control)	100
50	64
100	108
200	65
300	41

Figure 4.2 shows an SDS-PAGE of the extracellular proteins secreted into the culture medium when the fungus was grown with different amounts of manganese. The results show that as manganese concentration increased the concentration of two extracellular proteins of 45-50 kDa size also increased in the culture medium. The intensity of the bands for these two proteins increased with increasing manganese concentration to the point where their concentration was much higher than that of laccase (66 kDa).

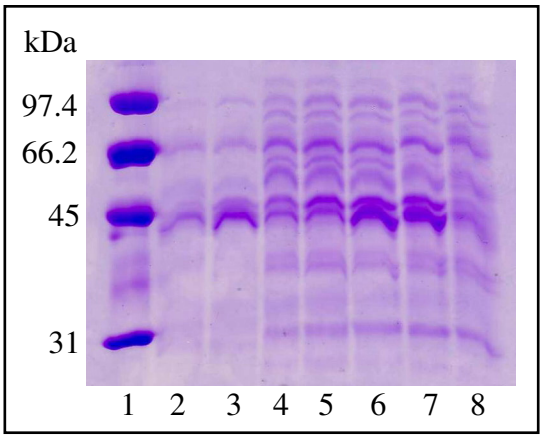


Figure 4.2: The effect of manganese concentration on extracellular fungal proteins. This gel shows the extracellular proteins produced by *Trametes* sp. HR577 with different amounts of manganese added to the fungal growth medium. Lane 1: molecular weight markers; Lane 2: control (no Mn); Lane 3: 2 µM Mn; Lane 4: 25 µM Mn; Lane 5: 50 µM Mn; Lane 6: 100 µM Mn; Lane 7: 200 µM Mn; Lane 8: 300 µM Mn.

The observation made in this study that two proteins of 45-50 kDa are expressed with manganese is consistent with the production of manganese peroxidases (MnP, typically molecular weight 45 kDa). Ford (2006) previously detected trace amounts of MnP in cultures of *Trametes* sp. HR577 grown on solid substrates. However, this needs to be validated by further studies. For the enzyme purification work described later in this chapter, manganese was omitted from the culture medium to minimise the expression of other extraneous non-laccase proteins that are expressed.

4.3 LACCASE PURIFICATION

Several isolation methods have been used to purify fungal laccase. They usually consist of four to five steps: initially the culture medium is concentrated by means of ultrafiltration or precipitation procedures, followed by two or three protein fractionation steps using ion exchange, gel filtration, or affinity chromatography (Gianfreda, Xu and Bollag 1999).

Many white-rot fungi produce large quantities of extracellular glycans. These can interfere with the laccase purification process. Roy-Arcand and Archibald (1991) noted that column purification of laccase from *T. versicolor* was difficult because of varying quantities of extracellular glycans produced by this fungus. This problem has also been

seen for *P. cinnabarinus* (Eggert, Temp and Eriksson 1996). The common method to overcome this problem is by precipitation of the protein with ammonium sulphate, acetone or ethanol.

Optimisation of Ammonium Sulphate Precipitation Step

Ammonium sulphate precipitation was chosen as the first step as it has been used widely in the purification of laccase isozymes from other *Trametes* sp. (Malmström, Fåhraeus and Mosbach 1958; Mosbach 1963; Fåhraeus and Reinhammar 1967; Roy-Arcand and Archibald 1991; Moldes, Lorenzo and Sanromán 2004).

To optimise this step, 50 mL volumes of crude filtrate were subjected to precipitation with different amounts of ammonium sulphate (25, 30, 40, 50, 75, 95% w/v). The precipitate was separated from the culture fluid by decantation after centrifugation and dialysed to remove ammonium sulphate. The laccase activity in the decanted liquid and the dialysed protein mixtures was measured and the ratios of laccase in each fraction are given in Table 4.4. For addition of 25% ammonium sulphate the majority of laccase activity was detected in the decanted liquid while at 75 and 95 % ammonium sulphate, the majority of laccase was found in the dialysed protein mixture. Therefore 75% ammonium sulphate (w/v) was used with a 25% (w/v) cutoff.

Table 4.4: Table of laccase activity for different ammonium sulphate fractionations. For details see the text. Laccase was measured as described in Table 4.1.

% $(NH_4)_2SO_4$	Ratio of laccase activity Filtrate:	Pellet
0	1:	2
25	20:	1
30	1:	11
40	1:	34
50	1:	28
75	1:	167
95	1:	243

Isolation of Two Laccases from Trametes sp. HR577

Twelve day old fungal cultures of *Trametes* sp. HR577 were filtered and solid ammonium sulphate (using cutoffs of 25% w/v and 75% w/v) was used to precipitate proteins in the culture filtrate. Ammonium sulphate precipitated crude laccase was dialysed against 10 mM phosphate buffer (pH 7) and loaded onto an FPLC anion exchange column (Source Q). Proteins bound to the column were eluted with a stepwise sodium chloride gradient (from 0-1M NaCl, see Chapter 7). Figure 4.3 shows that two peaks of laccase activity were eluted from the anion exchange column. These active peaks were labelled L1 and L2 based on their elution order from the column. Fractions with laccase activity were pooled separately, concentrated and dialysed with 10 mM phosphate buffer (pH 7).

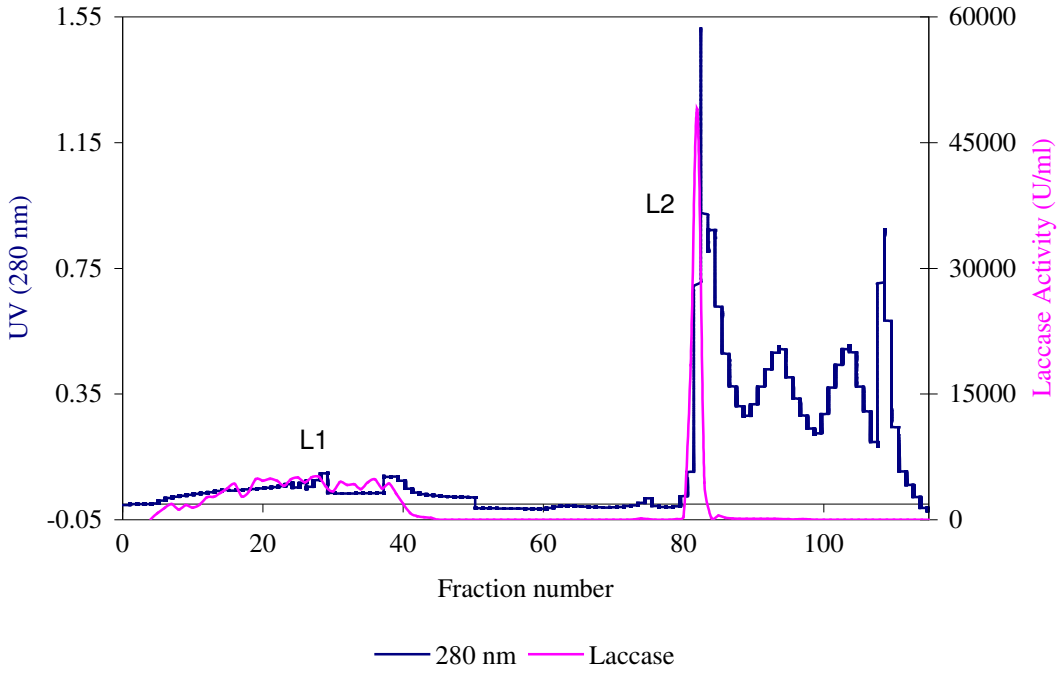


Figure 4.3: Elution Profiles of laccase isozymes on Source Q Column. The Source Q column was equilibrated in phosphate buffer (pH 7) and the dialysed, concentrated protein mixture from the ammonium sulphate step was loaded onto the column as described in the text. Laccase activity was measured by oxidation of ABTS using the method of Wolfenden and Willson (1982). One unit of laccase was defined as the amount of enzyme required to oxidise 1 µmol of ABTS per minute at 25ºC.

Further Purification of the L1 Laccase Active Fraction from Anion Exchange

Protein eluting in the L1 peak did not bind to the Source Q anion exchange column and came off in the flow through before the salt gradient was applied. This active fraction was subjected to hydrophobic interaction chromatography on a Source Phe column. The proteins bound to the column were eluted with a linear ammonium sulphate gradient (15-0% w/v, 1.1 mol L^{-1}). Figure 4.4 shows that the protein from the L1 fraction resolved into three peaks of laccase activity. These three peaks were pooled separately and designated L1a, L1b and L1c based on their order of elution from the Source Phe column. The L1a and L1b peaks overlapped slightly so it was decided not to further purify these two active fractions. The L1c peak did not overlap with the two other laccase active peaks. As most purified laccases were stored in sodium acetate buffer (the buffer used in laccase assays), purified L1c was dialysed against 10 mM sodium acetate buffer (pH 5), concentrated, flash frozen in liquid nitrogen and stored at -70ºC.

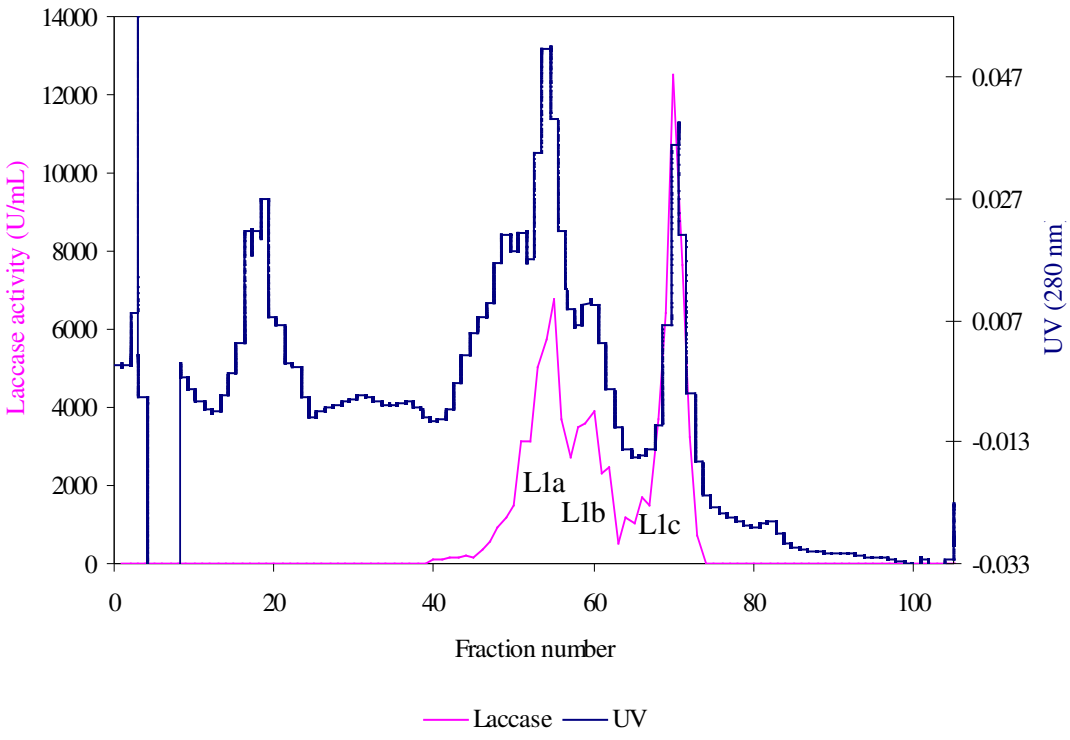


Figure 4.4 Elution profile of L1 on Source Phe Column. The Source Phe column was equilibrated in phosphate buffer (pH 7, 15% w/v ammonium sulphate) and the dialysed, concentrated protein mixture from the anion exchange step was loaded onto the column as described in the text. Laccase activity was measured as described in Figure 4.3.

Further Purification of the L2 Laccase Active Fraction from Anion Exchange

The active fraction that bound to the Source Q column and was eluted with NaCl, L2, was further purified on a hydrophobic column (phosphate, pH 7, Figure 4.5a) followed by a second anion exchange step on a Source Q column (acetate, pH 5, Figure 4.5b). Figure 4.5a shows that the proteins present in the L2 peak from the first anion exchange step were fractionated into four peaks. Laccase activity was associated only with the last peak eluting off the column. Figure 4.5b shows that the proteins that contained laccase activity in Figure 4.5a were further fractionated into two main peaks during the second anion exchange step. The earlier eluting protein peak contained laccase activity. The active fractions (32-41 in Figure 4.5b) were pooled, dialysed against 10 mM sodium acetate (pH 5), concentrated, flash frozen in liquid nitrogen and stored at -70ºC.

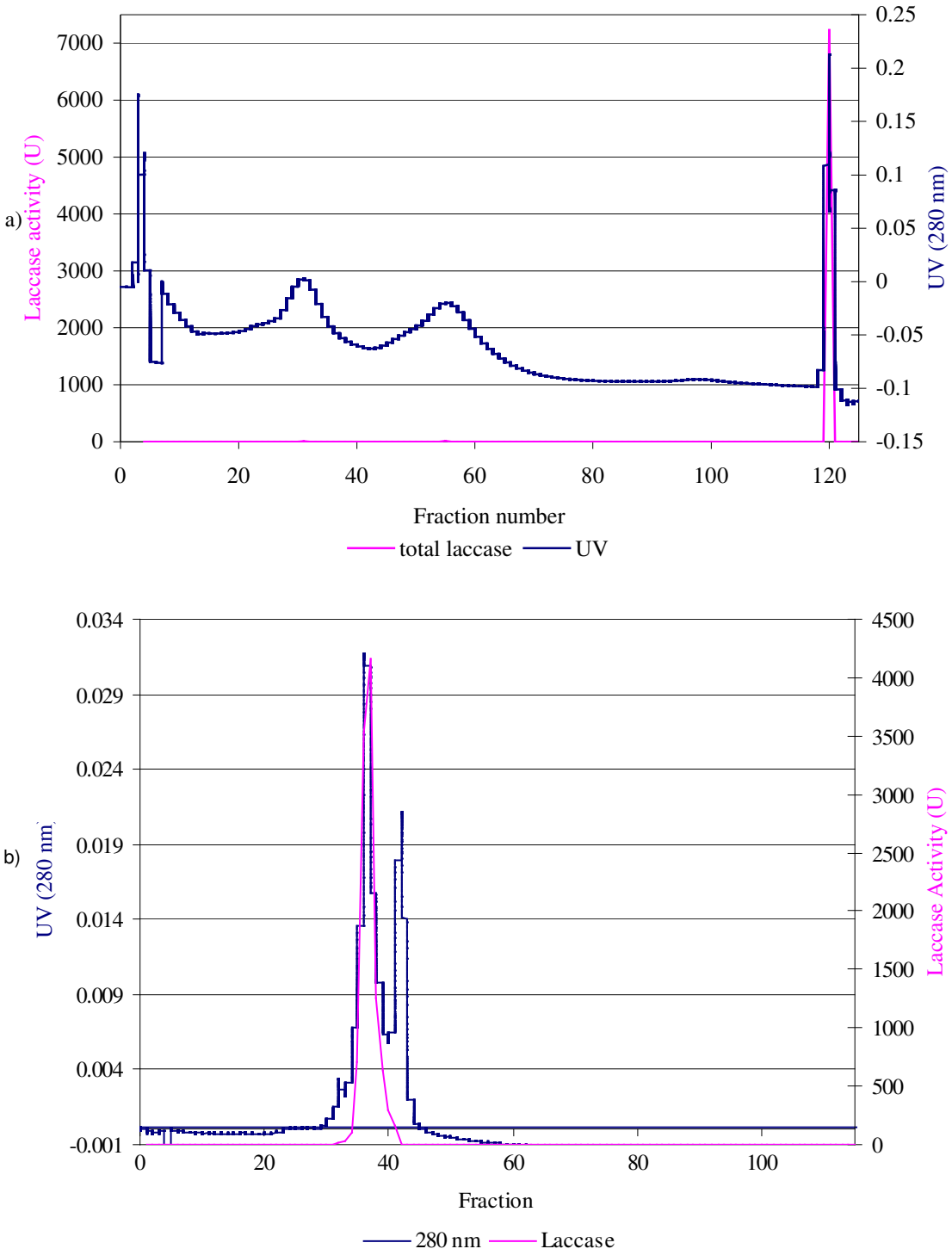


Figure 4.5: Elution of L2 from a) Source Phe column (pH 7) and b) Source Q column (pH 5). The Source Phe column was equilibrated in phosphate buffer (pH 7, 15% w/v ammonium sulphate) and the dialysed, concentrated protein mixture from the first anion exchange step was loaded onto the column as described in the text. The Source Q column was equilibrated in acetate buffer (pH 5) and the dialysed, concentrated protein mixture from the hydrophobic interaction chromatography step was loaded onto the column as described in the text. Laccase activity was measured as described in Figure 4.3.

Figure 4.5a shows that a single peak of laccase activity eluted from the Source Phe column at pH 7. The same enzyme eluted as a single peak of laccase activity on the

Source Q column at pH 5 (Figure 4.5b). Unlike L1, the L2 peak was not further fractionated into additional peaks of laccase activity on the Source Phe column.

Efficacy of Laccase Purification

Table 4.5 presents the summary of a typical purification of laccase isozymes from *Trametes* sp. HR577. At the end of the process, L1c and L2 were purified 12.6- and 5.3-fold, respectively with yields of 2.8 and 12.6%, respectively.

Table 4.5: Summary of the purification of extracellular laccase isozymes L1c and L2 from *Trametes* sp. HR577.

	Purification Step	Total Volume (ml)	Laccase (U)[b]	Protein (mg)	Specific Activity (U mg^{-1})	Yield (%)	Purification (Fold)
	Culture filtrate	900	470,000	18	26,000	100	1.0
	$(NH_4)_2SO_4$ precipitation	90	370,000	12	32,000	79	1.2
Laccase 1c	Source Q	3.7	150,000	1	103,000	31	4.0
	Source Phe	0.02	13,000	0.04	328,000	3	12.6
Laccase 2	Source Q	6.2	100,000	3	32,000	22	1.2
	Source Phe	5	59,000	1	63,000	13	2.4
	Source Q[a]	0.017	59,000	0.4	137,000	13	5.3

[a] Acetate buffer pH 5 was used for this FPLC step. For all the other FPLC steps the buffer was a phosphate buffer, pH 7. [b] One unit of laccase was defined as the amount of enzyme required to oxidise 1 µmol of ABTS per minute at 25ºC.

In this study ammonium sulphate precipitation was confirmed as a useful first step to remove cell debris and extraneous proteins in the culture medium to obtain a crude extract for further purification. Anion exchange on Source Q provides immediate access to the L2 laccase activity and L1 did not bind to the Source Q at pH 7. L1 could be further fractionated on Source Phe. L1 appears to contain three laccase activities. In future studies with *Trametes* sp. HR577 L1 could be purified using size exclusion chromatography. This might provide a way of resolving all the activities present in L1. It may also provide a further means of purifying the enzymes to homogeneity for characterisation studies.

The efficacy of purification compares well with some purification procedures (14.5 fold in Shin and Lee 2000) and not so well with others (for example 33.8 fold in Xiao, Tu *et al.* 2003).

Table 4.5 shows that the yields were low compared to yields reported for other purified *Trametes* sp. laccases. Some of the higher yields reported in the literature are due to XYL induction of laccases in the culture before the enzyme was purified (Malmström, Fåhraeus and Mosbach 1958; Mosbach 1963; Shin and Lee 2000; Xiao, Tu *et al.* 2003). In future studies it could be worth purifying enzymes from cultures where laccase has been induced by an aromatic compound such as XYL.

Presence of at Least Two Laccases

In the discussion that follows the terms 'purified enzyme L1c' and 'purified enzyme L2' refer to pooled fractions from the Source Q or Source Phe column as listed in Table 4.4. Figure 4.6 shows that these are semi-pure enzyme preparations that are not homogeneous in composition. The major proteins in these fractions are characterised in the section that follows as the putative laccase protein. These activities are referred to as isozymes because they catalyse the same reaction but probably have different properties as shown by different salt concentrations required for them to elute from the anion exchange and hydrophobic columns.

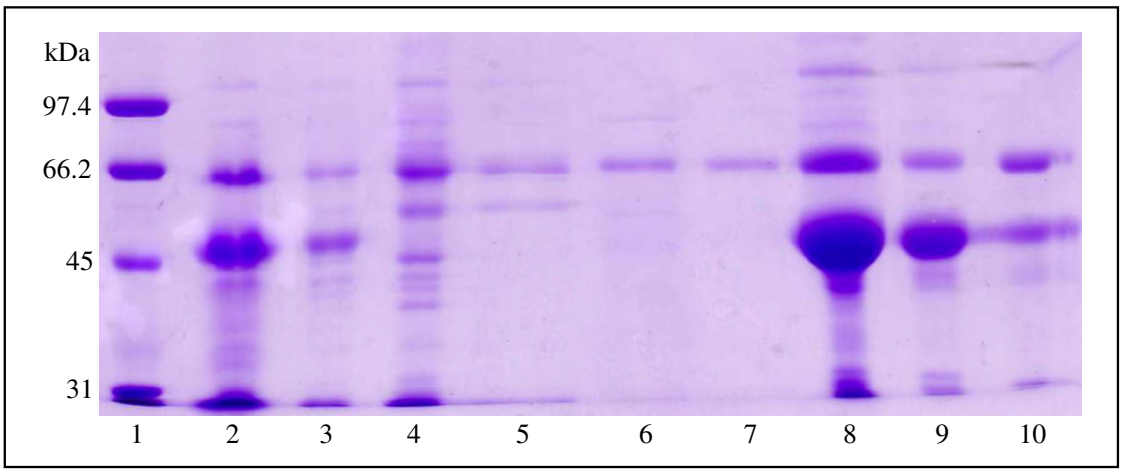


Figure 4.6: SDS-PAGE of the laccase purification steps. Lanes: 1, Molecular mass markers; 2, crude enzyme filtrate (11 µg); 3, dialysed protein after ammonium sulphate precipitation and dialysis (5 µg); 4, partially purified laccase 1 after Source Q column (pH 7, 5 µg); 5, partially purified laccase 1a after Source Phe column (1 µg); 6, partially purified laccase 1b after Source Phe column (1 µg); 7, purified laccase 1c after Source Phe column (1 µg); 8, partially purified laccase 2 after Source Q column (pH 7, 42 µg); 9, partially purified laccase 2 after Source Q column (pH 5, 20 µg); 10, purified laccase 2 after Source Phe column (12 µg). The electrophoresis was performed on a 12% gel and stained with Coomassie.

Four partial laccase gene sequences from *Trametes* sp. HR577 are reported in Chapter 3 (*lac1*, *lac2*, *lac3* and *lac4*[49]). Three of these showed high homology to each other (96-98% sequence identity at the amino acid level) whereas the fourth sequence showed much lower sequence homology (70-71% sequence identity at the amino acid level). This suggests that *Trametes* sp. HR577 has at least two distinct laccase genes. This was confirmed by anion exchange, which showed two distinct peaks of laccase activity (see Figure 4.3). The first of these peaks of activity did not bind to the column at pH 7. After further purification by hydrophobic interaction chromatography this peak resolved into three distinct peaks of laccase activity (Figure 4.4). Thus, the three putative laccases described in Chapter 3 that had high sequence homology to each other may be indicative of three distinct laccase isozymes. It is possible they may correspond to the three peaks of laccase activity observed on the hydrophobic column. The three peaks of laccase activity observed on the hydrophobic column for the L1 peak of activity may simply arise from differences in the glycosylation of the first laccase isozyme. More work is needed to clarify whether these three are separate isozymes or simply the same isozyme with a different glycosylation pattern.

Putative Isoelectric Points

Binding of the two purified extracellular laccases L1c and L2 from *Trametes* sp. HR577 to the anion exchange column suggests that the laccase isozymes have quite different isoelectric points. Since L1c eluted from the column in the flow through prior to applying a NaCl gradient, the isoelectric point of the protein is predicted to be above pH 7. Conversely, since L2 was retained on the anion exchange column when it was equilibrated in both phosphate buffer (pH 7) and in acetate buffer (pH 5), the isoelectric point of this protein is predicted to be below pH 5.

The production of multiple laccase isozymes with different properties from other *Trametes* species has also been observed (Yaver, Xu *et al.* 1996; Antorini, Herpoël-Gimbert *et al.* 2002). Most fungal laccase isozymes have acidic isoelectric points between pH 3-5 (Shin and Lee 2000). However, a few laccase isozymes with higher isoelectric points (pI 6.7-7.4) have been isolated (Palmieri, Giardina *et al.* 1997; Shin

[49] The colour and nomenclature used for laccase sequences reported in this chapter has been kept the same as that used in Chapter 3 for easy recognition.

and Lee 2000; Garzillo, Colao *et al.* 2001; Nagai, Kawata *et al.* 2003; Almansa, Kandelbauer *et al.* 2004). Two of the laccase isozymes with high isoelectric points (pI 7 and 7.4) are from *T. hirsuta*, a white-rot shown to have similar morphological features to *Trametes* sp. HR577 (see Chapter 3).

Purified laccases from *T. versicolor* and *T. villosa* have similar elution patterns on anion exchange to the laccases from *Trametes* sp. HR577 (Yaver, Xu *et al.* 1996; Antorini, Herpoël-Gimbert *et al.* 2002). Antorini, Herpoël-Gimbert *et al.* (2002) described the purification of three laccase isozymes from *T. versicolor* for crystal trials. The first laccase isozyme eluted from an anion exchange column prior to applying the NaCl gradient. This laccase isozyme was the only *T. versicolor* isozyme that they isolated that produced suitable crystals for X-ray diffraction. They also published the crystal structure of this laccase isozyme with a resolution of 1.9Å (Piontek, Antorini and Choinowski 2002). The laccase sequence for this isozyme showed high sequence homology to the partial laccase sequence *lac4* from *Trametes* sp. HR577 (95.4% identical at the amino acid level).

Similarly, Yaver, Xu *et al.* (1996) purified two laccase isozymes expressed by *T. villosa*. The first of these isozymes (form 1 laccase) was not retained on an anion exchange column, while the other isozyme (form 3 laccase) was retained on the column until the NaCl gradient was applied. The publication by Yaver, Xu *et al.* (1996) included the laccase gDNA sequences to the two isozymes that they purified: *lcc1* and *lcc2* (encoding laccase form 1 and form 3, respectively). The second of these isozymes showed very high sequence homology to *lac1* and *lac3* from *Trametes* sp. HR577 (94 and 92% identity at the nucleotide level, respectively, both were 98% identical to *lcc2* at the amino acid level) and was used to deduce the intron and exon positions in these two partial laccase sequences.

The isoelectric point values given for the *T. versicolor* and *T. villosa* laccase isozymes that eluted in the anion exchange column flow through were both 3.5 (Yaver, Xu *et al.* 1996; Piontek, Antorini and Choinowski 2002). These isoelectric point values are not what would be predicted for these isozymes based on their elution from the anion exchange column (predicted to be above pH 7 and 7.7 for *T. versicolor* and *T. villosa* flow through laccases, respectively). In contrast, the second laccase isozyme purified from *T. villosa* cultures (form 3 laccase) had a isoelectric point within the predicted

range (pI 5.5-6, below the pH of the buffer used for anion exchange). The reason for the isoelectric point values for the flow through laccase isozymes from these fungi have not been discussed by the authors of either paper.

A preliminary isoelectric focussing (IEF) gel of isozymes L1c and L2 from *Trametes* sp. HR577 indicated that the isoelectric point values for these two laccases fit the isoelectric point predictions based on the elution pattern observed on the anion exchange column. Homogeneously purified preparations are required to determine the exact isoelectric point values for these isozymes.

4.4 PROPERTIES OF PURIFIED LACCASES

There were limited quantities of the purified isozymes available for characterisation and PCP degradation studies. Therefore, not all of the studies reported in this chapter could be carried out with both of the laccase isozymes.

Characterisation of the Isolated Laccases

Both of the laccase isozymes from *Trametes* sp. HR577 were pale blue in colour after purification and concentration (Figure 4.7). This is typical of purified laccases, which are part of a group of multicopper oxidases known as 'blue' copper oxidases (Messerschmidt and Huber 1990). The type 1 (T1) copper of fungal laccases is coordinated to the sulphur atom of a Cys residue. The copper-sulphur bond has a charge transfer at about 600 nm giving laccase isozymes a blue appearance (see Chapter 1).

Figure 4.7: Photo of purified laccase isozyme L2 from *Trametes* sp. HR577. Purified L1c laccase is not shown. The blue colour shown in this photo is typical of the colour of laccase isozymes L1c and L2 purified from *Trametes* sp. HR577 from several different purification experiments.

Estimate of Laccase Molecular Mass

The molecular masses of L1c and L2 were both 66 kDa as determined by SDS-PAGE (see Chapter 7). This is consistent with the molecular masses reported for related laccase isozymes from other *Trametes* spp., which are typically 60-78 kDa per monomeric unit (Bourbonnais, Paice *et al.* 1995; Yaver, Xu *et al.* 1996; Leontievsky, Vares *et al.* 1997; Lee and Shin 1999; Shin and Lee 2000; Garzillo, Colao *et al.* 2001; Xiao, Tu *et al.* 2003; Almansa, Kandelbauer *et al.* 2004; Xiao, Chen *et al.* 2004a)[50].

Degree of Glycosylation

Peptide: *N*-glycosidase F (PNGase F) is an amidase enzyme that hydrolyses a range of common oligosaccharides from proteins (Tarentino, Gómez and Plummer 1985). This enzyme cleaves an asparagine linked oligosaccharide to generate a carbohydrate-free peptide and an intact oligosaccharide. During the hydrolysis the asparagine is converted to an aspartate residue with the concomitant production of ammonia (Figure 4.8).

[50] Fungal laccases from white-rot fungi generally have molecular weights between 50-80 kDa per laccase monomer (Muñoz, Guillén, *et al.* 1997).

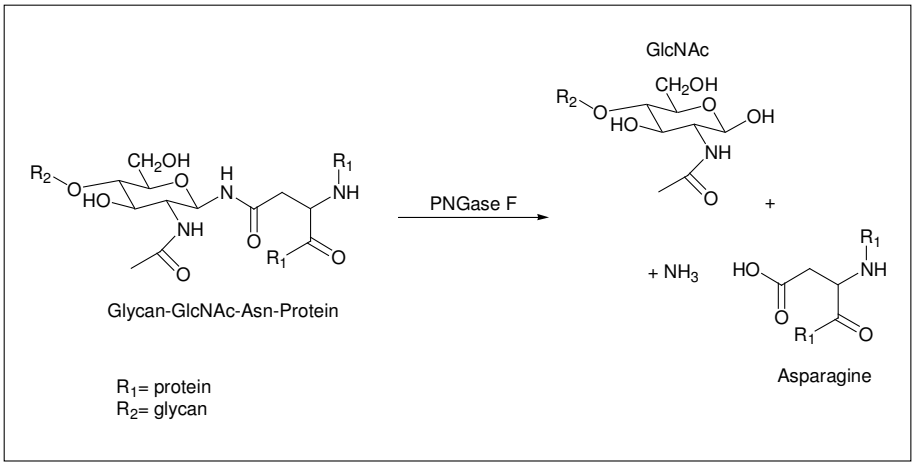


Figure 4.8: Deglycosylation of a protein with PNGase F.

Overnight incubation of isozyme L1c from *Trametes* sp. HR577 with PNGase F did not lead to cleavage of *N*-linked carbohydrates. However, if the isozyme was boiled prior to incubation with PNGase F then the carbohydrate content was estimated to be 19% by SDS-PAGE (Figure 4.9). This level of glycosylation is comparable to fungal laccases from other *Trametes* sp. (Mosbach 1963; Yaver, Xu *et al.* 1996; Koroljova-Skorobogat'ko, Stepanova *et al.* 1998; Dong and Zhang 2004).

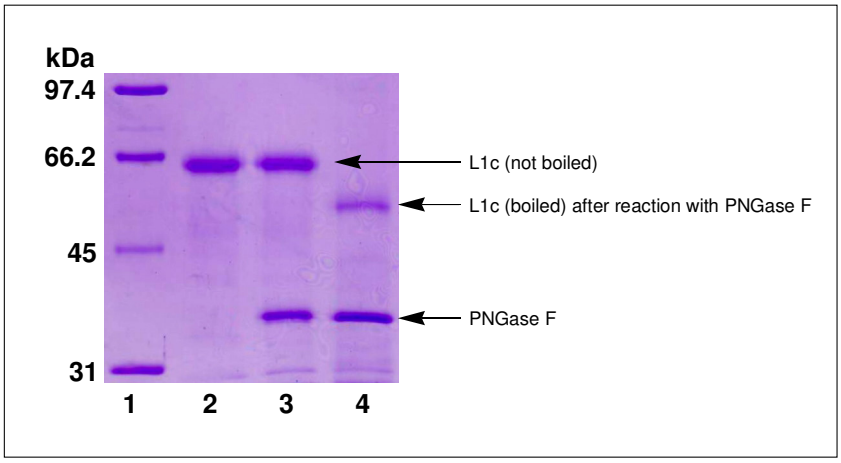


Figure 4.9: Deglycosylation of L1c from *Trametes* sp. HR577. Lane 1: markers; Lane 2: L1c; Lane 3: L1c (not boiled) + PNGase; Lane 4: L1c (boiled) + PNGase. Laccase isozyme L2 was not tested. Molecular weight was estimated from a plot of the log(molecular weight) versus the distance travelled for each of the protein bands.

The requirement to boil the enzyme prior to deglycosylation suggests that the isozyme is folded in such a way as to not expose the sites required for attack by PNGase F. One possibility is that the isozyme is multimeric rather than being monomeric. To test this proposition, laccases L1c and L2 were run on a native gel either in the native state or pre-boiled in SDS buffer. The band corresponding to the native form of L1c and L2 did

not occur at the same place in the native gel as the boiled (monomeric) form. This implies that both are probably multimeric proteins. Generally extracellular laccases purified from white-rot fungi are monomeric (Marques de Souza and Peralta 2003), although there are a few examples of multimeric laccases. Homodimeric extracellular laccases have been purified from *T. villosa* (Yaver, Xu *et al.* 1996), *Pleurotus pulmonarius* (Marques de Souza and Peralta 2003), *Phellinus ribis* (Min, Kim *et al.* 2001) and *A. bisporus* (Wood 1980).

pH Optimum[51]

The pH optima for the activity of the laccase isozymes L1c and L2 from *Trametes* sp. HR577 using ABTS as the substrate are given in Figure 4.10. The pH optimum for L2 was pH 2. This is typical for fungal laccases, which generally have low pH optima (commonly between pH 2 and pH 4) when ABTS is the substrate (Palmieri, Giardina *et al.* 1997; Lee and Shin 1999; Shin and Lee 2000; Garzillo, Colao *et al.* 2001; Baldrian 2004).

In the buffers tested, the pH that gave the highest enzyme activity with L1c was pH 1.5 (Figure 4.10). This is unusual among fungal laccases. The only other reported pH optimum below pH 2 is for the laccase from *Daedalea quercina* where the pH optimum is somewhere between pH 1.8 and 2.0 (Baldrian 2004).

[51] In both of the pH optimisation studies described here and the substrate specificity studies described below, an artificial substrate is being used for assaying laccase activity. This substrate bears little resemblance to the unknown real/natural substrate for the enzyme. Therefore, the relevance of the data to natural situations is open to question. The studies have been completed to get comparative data on the enzymes to compare to the laccases described in the literature for other *Trametes* sp.

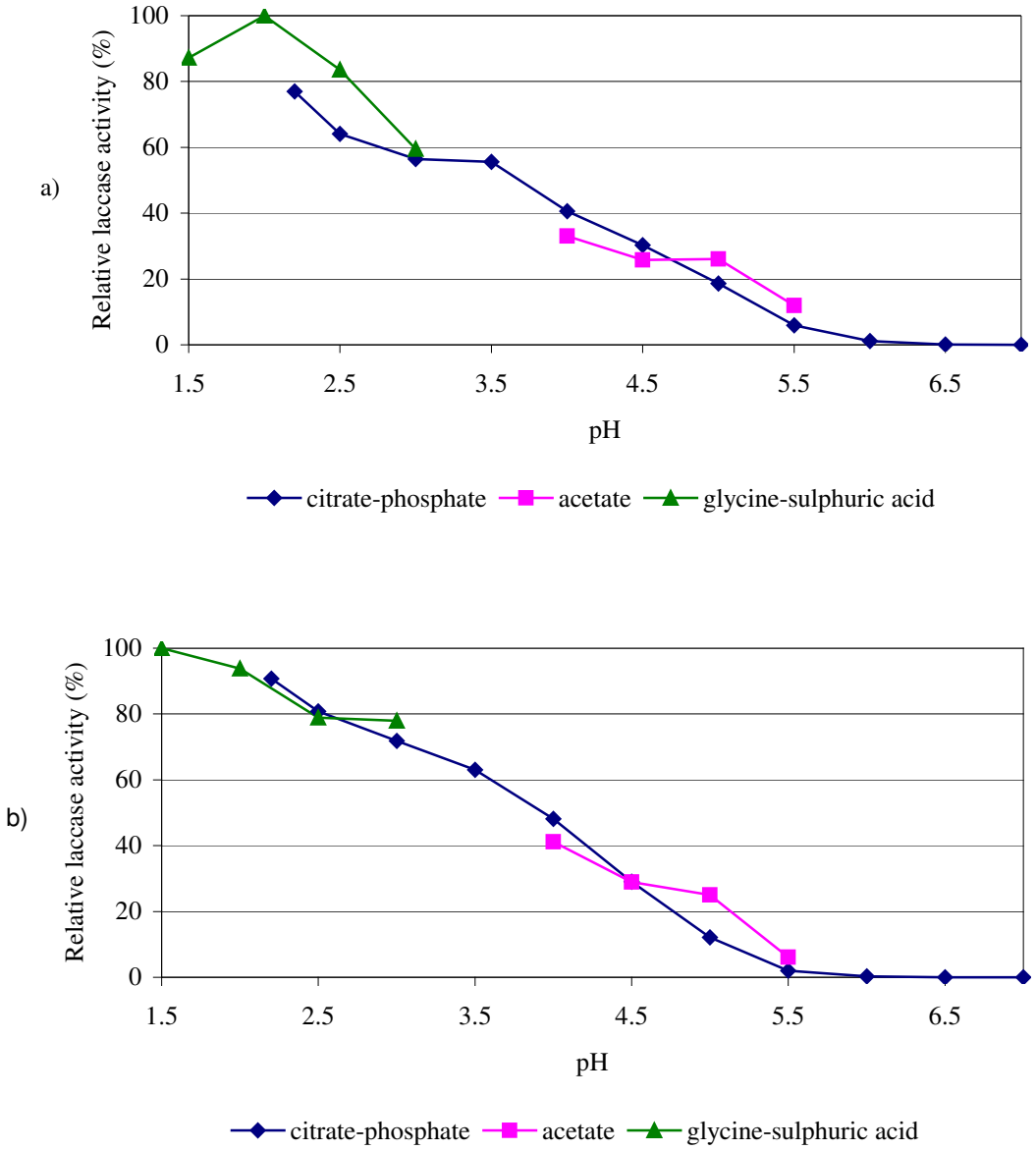


Figure 4.10: pH profiles of laccase isozymes a) L2 and b) L1c. The pH optimum for the activity of laccase isozymes from *Trametes* sp. HR577 was evaluated using ABTS as the substrate in 100 mM citrate-phosphate (pH 2.2-7), 100 mM glycine-sulphuric acid (pH 1.5-3) or 100 mM sodium acetate (pH 4-5.5) buffer at 25ºC. All reactions were initiated by addition of enzyme. No activity was seen in the absence of enzyme. A value of 100 was ascribed to the highest laccase activity and the other activities were expressed as a percentage of this value.

Lignin is the natural substrate for laccase (see Chapter 1) but, as it is a complex molecule, alternative substrates such as ABTS or DMP are often used to assay for laccase. The amount of laccase activity detected with these substrates is pH dependant (Xu 1997). Therefore, it is important to determine the pH optimum of each laccase isozyme with the assay substrate.

Substrate Specificity of L1c and L2

The kinetics constants of L1c and L2 from *Trametes* sp. HR577 using ABTS as the substrate are listed in (Table 4.6).

Table 4.6: Kinetic constants for L1c and L2 at different pH's. The pH's selected were chosen based on the standard ABTS assay pH (pH 5) and the pH optimum for each isozyme with ABTS determined in the previous section (pH 1.5 and 2, for L1c and L2, respectively).

Enzyme	pH	V_{max} (µmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	K_M (µM)	K_M/k_{cat} (µM s x 10^{-3})
L1c	5	3.3	3600	27	7.4
L1c	1.5	3.5	3800	62	16.3
L2	5	8.8	9700	30	3.1
L2	2	41.1	45000	23	0.5

The L1c and L2 isozymes had different V_{max} and k_{cat} values. While the V_{max} and k_{cat} for L1c did not appear to be affected by pH, those from L2 were greatly affected by pH.

Although the isozymes from *Trametes* sp. HR577 are only semi-pure enzyme preparations that are not homogeneous in composition, L1c and L2 had similar K_M and k_{cat} values to other fungal laccases (Shin and Lee 2000; Garzillo, Colao *et al.* 2001; Xiao, Tu *et al.* 2003; Chen, Ge *et al.* 2004a). The relatively high K_M values given for laccases using ABTS as the substrate reflects the broad substrate range of these enzymes compared to other multicopper oxidases.

Generally, most multicopper oxidases are very stereospecific for their natural substrates. However, because the natural laccase substrate (lignin) is a complex polymer containing a mixture of *R*- and *S*-enantiomeric forms, laccases are much less stereo- and regio-specific for lignin than most other multicopper oxidases are for their natural substrates (Solomon, Sundaram and Machonkin 1996).

Temperature for Optimum Activity

It is common for laccase isozymes from the same organism to have different temperature profiles. Most *Trametes* laccase isozymes purified to date have temperature optima between 45-55ºC (Lee and Shin 1999; Shin and Lee 2000; Tinoco, Pickard and Vazquez-Duhalt 2001; Xiao, Tu *et al.* 2003; Almansa, Kandelbauer *et al.*

2004; Xiao, Chen *et al.* 2004a). Therefore, it was of interest to determine the working temperature range of L1c and L2 from *Trametes* sp. HR577.

The temperature profiles for the laccase activity associated with L1c and L2 are shown in Figure 4.11. The results show that L2 is less active at lower temperatures than L1c. However, it has an optimum temperature of 70-75ºC while L1c has an optimum temperature of 65ºC (Figure 4.11).

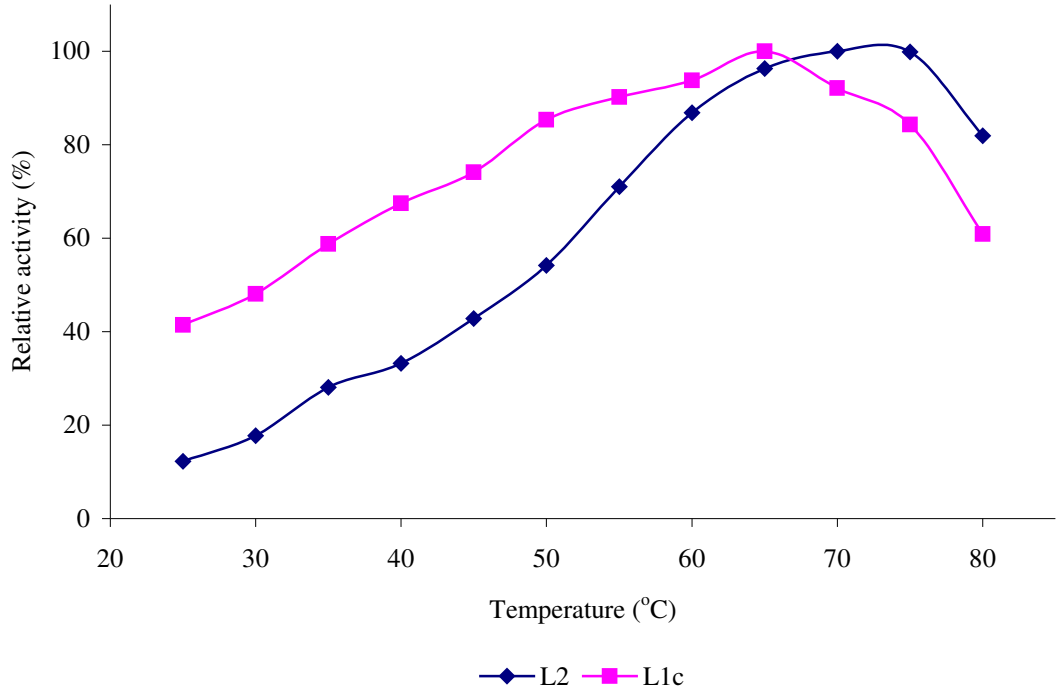


Figure 4.11: The effect of temperature on laccase activity of *Trametes* HR577 isozymes. The temperature optimum for the activity of the laccase isozymes from *Trametes* sp. HR577 was evaluated between 25-80ºC using ABTS as the substrate in 100 mM sodium acetate (pH 5) buffer at 25ºC. A value of 100 was ascribed to the highest laccase activity for each isozyme and the other activities were expressed as a percentage of this value.

The temperature optima of L1c and L2 from *Trametes* sp. HR577 are relatively high (65, and 70-75ºC, respectively) compared to other *Trametes* laccase isozymes (45-55ºC). However, such elevated temperature optima have been observed in laccase isozymes purified from a range of other white-rot fungi such as *D. quercina* (Baldrian 2004), *T. giganteum* (Wang and Ng 2004c) and *Marasmius quercophilus* (Dedeyan, Klonowska *et al.* 2000).

Enzyme Stability at 50 and 70 Degrees

While L1c and L2 from *Trametes* sp. HR577 have relatively high temperature optima, their stability at these elevated temperatures is quite low. The stability of L2 at 50 and 70ºC is shown in Figure 4.12. L2 has a half life of around 13 minutes at 70ºC. In contrast, the half life at 50ºC is about 3 hours (Figure 4.12) and the isozyme is nearly completely inactivated after 5 hours incubation.

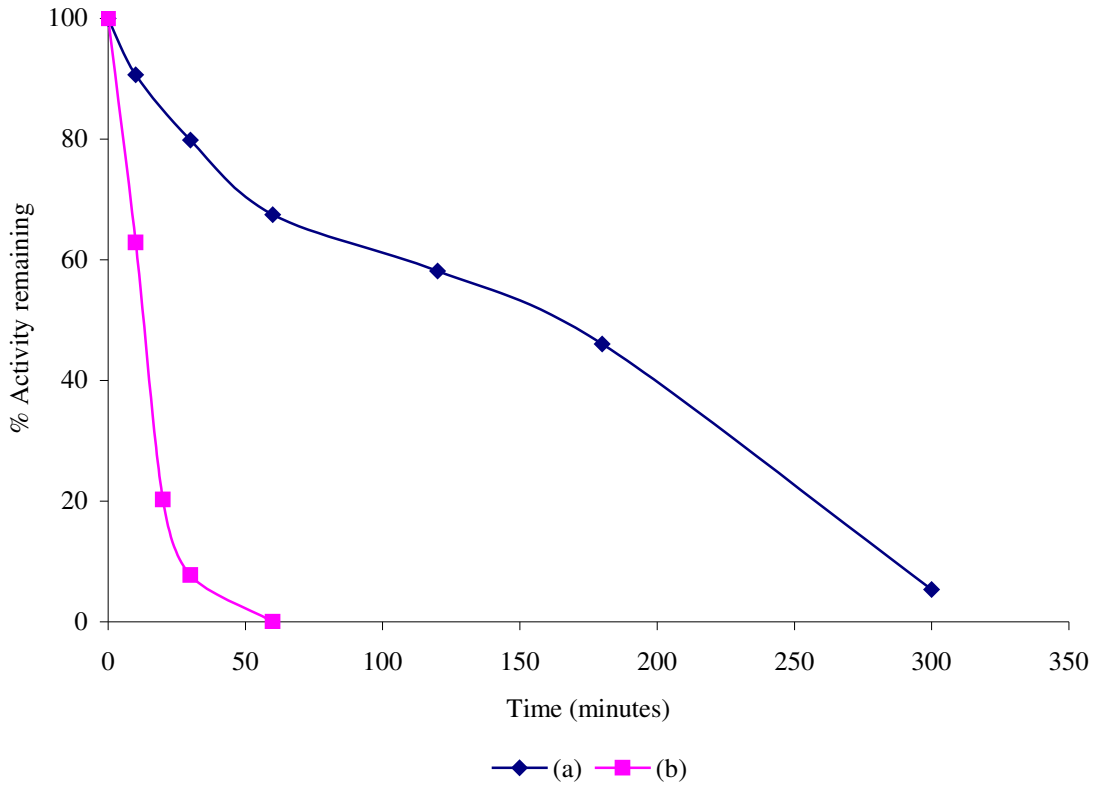


Figure 4.12: Thermal stability of L2 at a) 50 and b) 70ºC. L2 was incubated at either 50ºC or 70ºC for 10-60 or 10-300 minutes, respectively, cooled on ice and the laccase activity assayed at 25ºC. A value of 100 was ascribed to the highest laccase activity for each isozyme and the other activities were expressed as a percentage of this value.

Thermophilic ascomycete fungi such as *M. thermophila* and *Scytalidium thermophilum* have been shown to be activated by pre-incubation at high temperature. This phenomenon has also been observed in other ascomycete fungi such as *Melanocarpus albomyces* and in white-rot fungi such as basidiomycetes PM1 and *P. ribis* (Coll, Fernández-Abalos *et al.* 1993; Xu, Shin *et al.* 1996; Min, Kim *et al.* 2001; Kiiskinen, Viikari and Kruus 2002). These fungi all have high temperature optima of 60-80ºC, which are similar to the temperature optima for L1c and L2 from *Trametes* sp. HR577.

Unlike the laccases from these fungi, isozymes L1c and L2 from *Trametes* sp. HR577 were not activated by pre-incubation at 50 and 70ºC.

The Effect of Ethanol on Laccase Activity

White-rot fungi can degrade a variety of xenobiotics. Many of these xenobiotics are insoluble or sparingly soluble in water and hence have low bioavailability. Some researchers have found that they can improve fungal degradation of xenobiotics by including an organic, water miscible solvent into the test conditions. Ethanol is the most commonly used solvent to increase bioavailability of xenobiotics. However, solvents such as ethanol have been shown to have a negative effect on laccase activity. Therefore, before adding solvent to laccase reactions to increase xenobiotic solubility it is important to determine the effect of the solvent on the laccase activity.

In later studies the PCP degradation products from reactions with laccase isozymes will be examined (Chapter 5). For these studies, it was anticipated that ethanol would be required to solubilise the PCP for the enzymatic reaction.

The effect of ethanol on laccases laccase isozymes L1c and L2 was determined using ABTS as the substrate in the presence of increasing amounts of ethanol (0-50%). Figure 4.13 shows that a linear decrease in enzyme activity with increasing ethanol concentration occurred for both enzyme preparations.

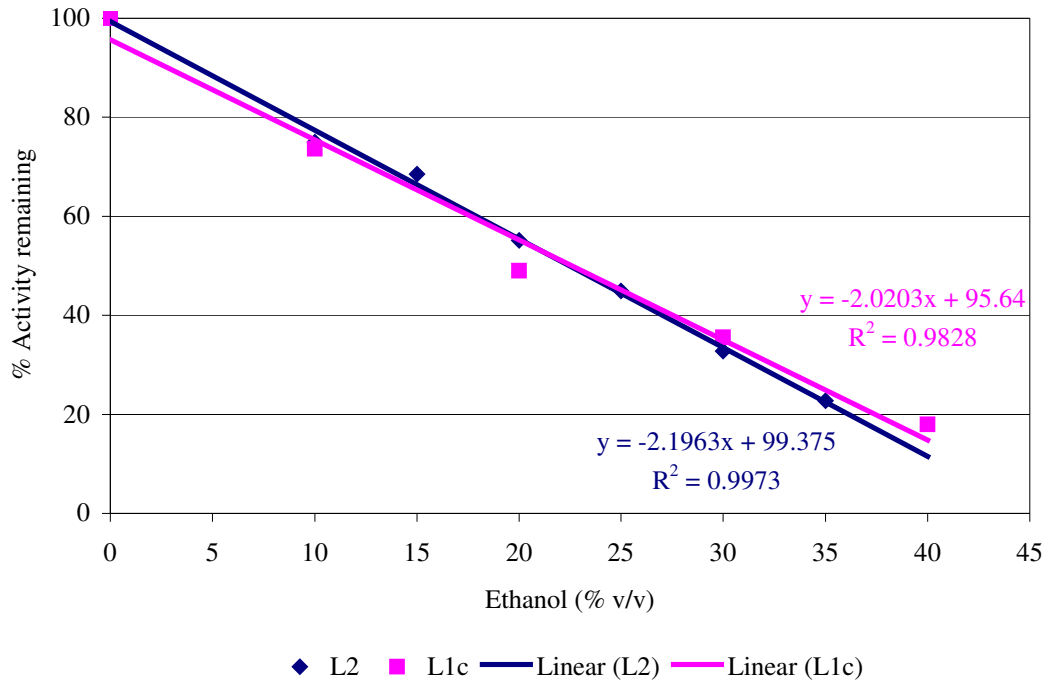


Figure 4.13: The effect of ethanol on laccase activity of L1c and L2. Laccase activity was measured as previously described (see Figure 4.1 legend) except that 0-40% ethanol was used in the assay. Assays were initiated by addition of laccase. A value of 100 was ascribed to the highest laccase activity for each isozyme and the other activities were expressed as a percentage of this value.

These results agree with the trends observed by other researchers. (Ullah, Bedford and Evans 2000) studied the reactions of *T. versicolor* laccase isozymes with PCP. Ethanol was used to increase the solubility of PCP in solution. This caused a linear decrease in laccase activity. At a concentration of 33% ethanol the remaining laccase activity was only about 50% compared to reactions in the absence of ethanol. However, the laccase isozymes were still able to react with PCP.

For laccase isozymes from *Trametes* sp. HR577, a linear decrease in enzyme activity with increasing ethanol concentration was also observed (see Figure 4.13). However, on addition of 30% ethanol to the enzyme assay, L1c and L2 retained less than 30% activity compared to the nil control.[52]

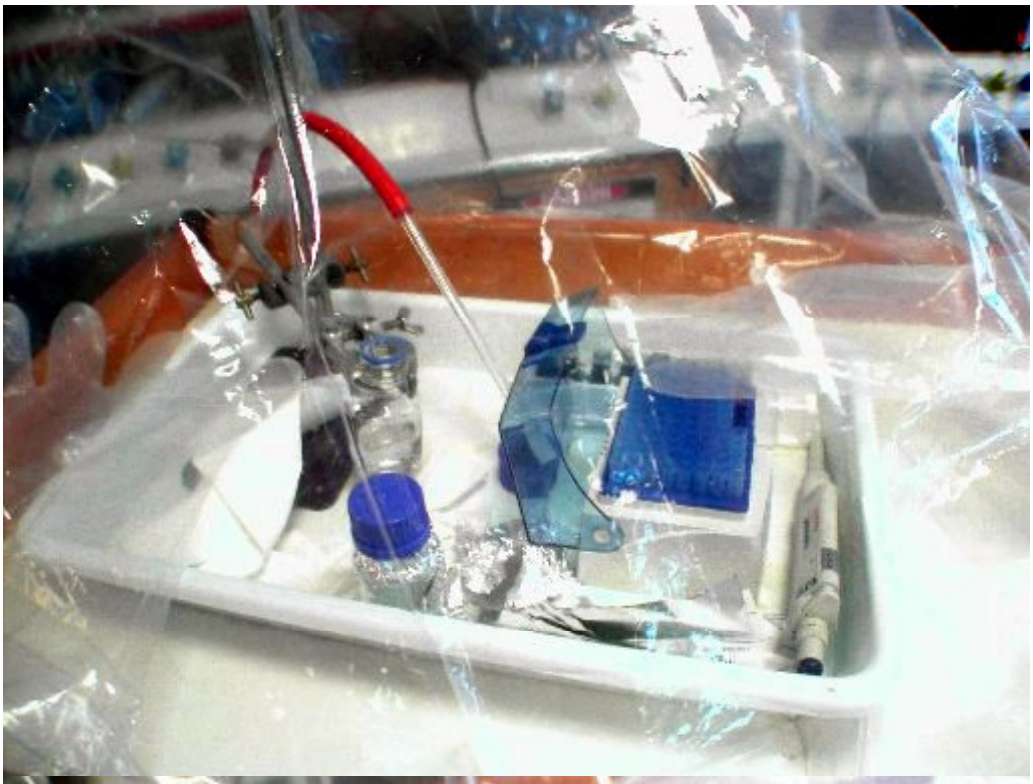
[52] Other researchers have also studied the effect of ethanol on enzyme activity. Tome, Nicolas and Drapron, (1978) studied the effect of several different solvents (methanol, ethanol, ethylene glycol, propylene glycol, diethylene glycol and glycerol) on the activity of a mushroom polyphenol oxidase (tyrosinase). At low solvent concentrations only a slight effect on enzyme activity was observed. At intermediate levels of solvent the reaction levels depended on the water to solvent ratio while at higher levels of solvent almost complete enzyme inhibition was observed. Rodakiewicz-Nowak, Haiber et al (1999) studied the effect of 0-8 M ethanol on the oxidation of DMP by laccases from *T. versicolor* and *P. tigrinus*. Their research suggests that *T. versicolor* laccase is denatured at ethanol concentrations above 12.6%.

Dependence on Oxygen

As mentioned previously (see Chapter 1), laccases catalyse four one-electron oxidation substrate reactions with the concomitant four-electron reduction of dioxygen to water. Each laccase requires four copper ions per monomeric enzyme unit. The first of these coppers (T1) is involved in binding of a reducing substrate such as XYL (Bertrand, Jolivalt *et al.* 2002). The other three copper atoms (one T2 and two T3 coppers) form a trinuclear copper cluster. The trinuclear copper cluster is the site of dioxygen binding and reduction of the oxygen to water (Piontek, Antorini and Choinowski 2002).

The dependence of laccase from *Trametes* sp. HR577 on dissolved oxygen for catalysis was tested. Dissolved oxygen was removed from the system. This was achieved by bubbling nitrogen through the laccase assay mixture (containing 10 mM ABTS and 100 mM sodium acetate at pH 5) in the absence of laccase for one hour under an inert nitrogen atmosphere (Figure 4.14). Cuvettes were capped with a rubber septum prior to removal from the inert atmosphere to prevent reoxygenation of the mixture. Although the laccase used for the assays was not deoxygenated prior to reaction with ABTS, the volume of laccase used was negligible compared to the overall assay volume (2.5 μL in 1 mL total volume). In some treatments, oxygen was added back into the liquid by bubbling for two hours with air.

(a)



(b)

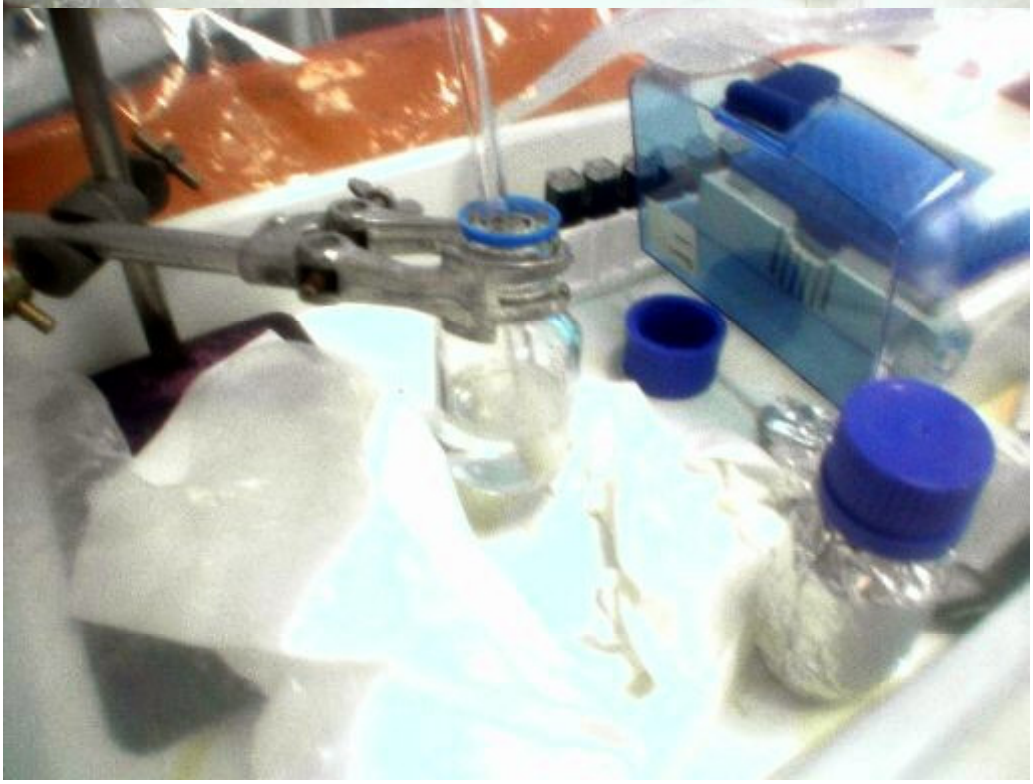


Figure 4.14: Inert Atmosphere set up: a) Glove bag containing everything required for bubbling N_2 into assay solution in an inert atmosphere; b) Close up of the assay solution with N_2 bubbling through it.

Table 4.7 shows that in the absence of dissolved oxygen, the laccase activity detected in cuvettes was only 9% of the oxidised (control) assay. However, if compressed air was bubbled through the deoxygenated solution in the cuvette for 2 hours prior to addition of laccase, 92% of the enzyme activity could be restored. This illustrates the dependence of this enzyme on the presence of oxygen for catalytic activity.

Table 4.7: The effect of O_2 on laccase activity for isozyme L1c. Nitrogen gas was pumped through a glove bag overnight. N_2 gas was then bubbled through the assay solution for 1 hour to remove dissolved oxygen. Laccase activity was measured using ABTS as the substrate as previously described (Figure 4.1). A value of 100 was ascribed to the highest laccase activity for each isozyme and the other activities were expressed as a percentage of this value.

Treatment	**% Laccase Activity**
Control	100
Bubbling with N_2 for 1 hour	9
Bubbling with N_2 for 1 hour followed by bubbling with air for 2 hours	92

4.5 CONCLUSION AND FUTURE WORK

The studies described above represent the first ever attempt at purifying laccase isozymes from a NZ white-rot fungus.

Fungal enzyme expression was studied prior to the purification attempt. Enzyme expression was best in cultures grown in the complex SCS media. Simple medium was chosen for growing the cultures so as to make the purification of secreted enzyme easier. Both manganese and copper at 100 μM were found to increase laccase expression, but the expression did not reach the levels seen when cultures were grown in SCS medium. The presence of manganese in the simple medium also increased the expression of two other extracellular secreted proteins of 45-50 kDa size in the culture medium.

Two laccase isozymes (L1 and L2) were isolated from *Trametes* sp. HR577 culture media after ammonium sulphate precipitation and anion exchange fractionation. Laccase L1 was revealed to contain three activities (L1a, L1b and L1c) when further fractionated by hydrophobic interaction chromatography. Because L1a and L1b were not fully resolved, only L1c and L2 were taken further for characterisation studies. The

enzyme preparations used in the characterisation studies were approximately 12.6 and 5.3 fold purified (for L1c and L2, respectively) by the three isolation procedures that were used.

Both laccase L1c and L2 were shown to be 66 kDa in molecular size. The putative isoelectric points are expected to be >7.5 for L1c and <5.0 for L2. L1c was shown to be a glycoprotein with approximately 19% *N*-linked glycosylation. The pH of optimum activity for was found to be around pH 2 for L2 and pH 1.5 for L1c.

These isozymes are semi-pure enzyme preparations that are not homogeneous in composition. Despite being semi-pure, the two isozymes displayed similar properties to laccase isozymes from other white-rot fungi. The molecular weight and degree of glycosylation of the two *Trametes* sp. HR577 isozymes is similar to those found in other *Trametes* laccases. Both isozymes appeared to be multimeric. While most white-rot laccases are monomeric, multimeric laccases have been found in the closely related white-rot *T. villosa*. The temperature optima for isozymes L1c and L2 were high (65 and 70-75ºC, respectively) compared to other *Trametes* laccases (45-55ºC). However, the isozymes were not stable for very long at 50 and 70ºC. The optimum pH with ABTS as the substrate for L1c and L2 were both acidic, which is similar to other *Trametes* laccases. Addition of ethanol resulted in a proportional decrease in laccase activity for both laccase isozymes. Laccase activity was severely inhibited in the absence of dissolved oxygen in solution. However, activity could be restored if air was bubbled back into the solution.

Further optimisation of the laccase purification method needs to be carried out to obtain homogeneous enzyme preparations. Gel filtration of purified laccase isozymes could be used to confirm whether the purified isozymes are monomeric or multimeric. Tryptic digests and sequencing of the resulting peptide fragments could be carried out. This could be used to determine whether the three peaks of laccase activity observed when the L1 active fraction was subjected to hydrophobic interaction chromatography are due to separate laccase isozymes or simply arise due to differences in post-translational modifications of the laccase isozyme. These peptide sequences could also be used to determine which of the partial gene sequences described in Chapter 3, if any, encode the expressed laccases.

CHAPTER 5: DEGRADATION OF PCP BY *TRAMETES* SP. HR577

5.1 INTRODUCTION

The review of the literature in Chapter 1 shows that for complete bioremediation PCP needs to be eventually mineralised to CO_2 and water. The mineralisation process can be expected to include PCP breakdown and oxidation reactions using both the intracellular and extracellular enzymes systems of the chosen white-rot fungus.

As mentioned in Chapter 1, the two most extensively studied white rot fungi are *P. chrysosporium* and *T. versicolor*. The degradation pathway of PCP by *P. chrysosporium* has been elucidated (Reddy and Gold 2000). The roles of LiP and MnP in this pathway have been suggested to be only in oxidation of PCP to 2,3,5,6-tetrachloro-2,5-cyclohexadiene-1,4-dione (TCHD, also called *p*-chloranil, Figure 5.1). For *T. versicolor* some studies have been published suggesting that laccase also carries out this reaction but that other metabolites are also produced. The white rot isolate chosen for this project, *Trametes* sp. HR577, produces only laccase in liquid cultures although very low levels of MnP have been detected when grown on solid media (Ford 2006).

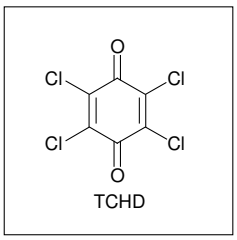


Figure 5.1: TCHD = 2,3,5,6-tetrachloro-2,5-cyclohexadiene-1,4-dione

This chapter describes studies that aimed to determine whether the pattern of degradation of PCP by *Trametes* sp. HR577 was similar to that already described for overseas white-rot fungi. The chapter begins by describing the pre-growth conditions required to overcome PCP toxicity to immature fungal cultures. It then describes and discusses PCP degradation by whole fungal cultures and laccase isozymes from the white-rot fungus *Trametes* sp. HR577.

There are many different factors that affect PCP degradation *in vitro*. These include whether whole cultures or purified enzymes are used, duration of incubation with PCP, the amount of PCP present, and the presence or absence of cofactors (e.g. ABTS). A fixed number of variables were chosen for study, largely because of the limited time, restricted availability of fungal culturing facilities and time-bounded access to GC and HPLC equipment at the time the studies were undertaken. In this chapter the results and discussion will be presented separately.

5.2 PRE-GROWTH OF *TRAMETES* SP. HR577 PRIOR TO ADDITION OF PCP

The studies published in the literature show that addition of PCP to fungal growth media prior to fungal inoculation can severely retard or completely inhibit fungal growth (Mileski, Bumpus *et al.* 1988; Alleman, Logan and Gilbertson 1992; Leontievsky, Myasoedova *et al.* 2000). Pre-growth of cultures to form a confluent mycelial mat can be used to circumvent this problem (Mileski, Bumpus *et al.* 1988).

In this study five day old cultures were used for the PCP degradation studies. For *Trametes* sp. HR577 five days is generally long enough for production of a confluent mycelial mat (Chapter 2, Figure 5.2 a), especially if the flasks used for fungal growth are sealed loosely with cotton wool and foil. This allows sufficient air exchange for fungal growth. However, after PCP is added to cultures, flasks need to be sealed using Teflon-lined lids to prevent loss of volatilised PCP metabolites.

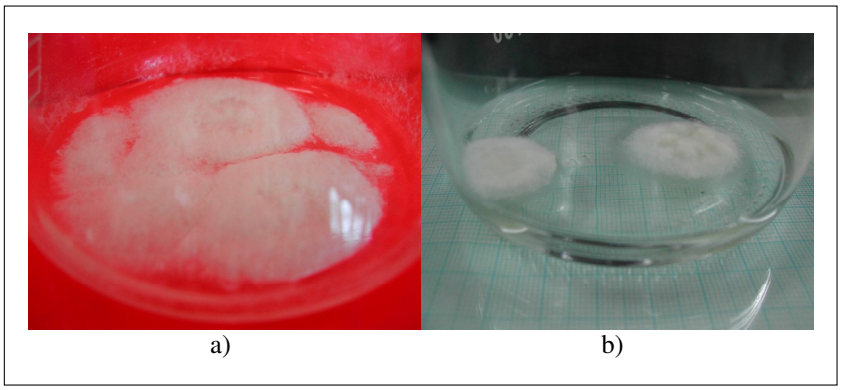


Figure 5.2: Fungal growth after 5 days in cultures sealed with a) cotton wool b) Teflon-lined lids

For the degradation studies described below, *Trametes* sp. HR577 cultures were grown for five days prior to addition of PCP (50 mg L^{-1}) in cultures with Teflon-lined lids. For the first five days the culture flasks were loosely capped to allow air exchange. Biomass growth in flasks and bottles loosely capped with Teflon-lined lids was reduced compared to bottles from previous experiments sealed with cotton wool (Figure 5.2 a and b). This suggests that the Teflon-lined lids limited air exchange even when the lids were loosely capped. Despite this, *Trametes* sp. HR577 was still able to degrade up to 76% of PCP in these experiments (see below for a discussion of these results).

5.3 PCP DEGRADATION BY WHOLE CULTURES OF *TRAMETES* SP. HR577

PCP Disappearance Quantified by HPLC

As mentioned above, PCP was spiked into five day old cultures of *Trametes* sp. HR577 in liquid culture to investigate PCP degradation by whole fungal cultures. After a further 72 hours whole cultures were extracted and residual PCP was quantified by HPLC using a C8 reverse-phase hydrophobic column (see Chapter 7).

Whole fungal cultures removed 76% of PCP (8 ± 2 mg L^{-1} residual PCP compared to 32 ± 2 mg L^{-1} residual PCP in control cultures) from liquid medium relative to the control cultures (Table 5.1). This equates to a rate of removal of PCP of 12.6 mg L^{-1} day^{-1}. Addition of crude or purified laccase isozymes[53] (100 U mL^{-1}) did not increase PCP disappearance from cultures. The results are given in Table 5.1.

[53] The term 'purified laccase' used in this chapter refers to laccase isozymes L1c and L2 from *Trametes* sp. HR577. Purification of these isozymes is described in Chapter 4. The terms 'crude laccase' and 'crude L1a-c' refer to a crude enzyme preparation of putative laccase isozymes L1a, L1b and L1c partially purified from cultures of *Trametes* sp. HR577 by ammonium sulphate precipitation, dialysis and passage through a Source Q anion exchange column (see Chapter 4).

Table 5.1: Residual PCP after incubation with *Trametes* sp. HR577 for 72 hours.

Treatment[a]	PCP remaining (mg L^{-1})	(% of control)
No fungus control	32 ± 2	100
Autoclaved whole culture control	24 ± 8	75
Whole culture	8 ± 2	24
Whole culture + 100 U mL^{-1} crude L1a-c[b]	8 ± 1	24
Whole culture + 100 U mL^{-1} L1c[b]	8 ± 1	24
Whole culture + 100 U mL^{-1} L2[b]	9 ± 1	28
No PCP control	0	0

[a]Culture conditions: all treatments contained freshly prepared culture medium and, with the exception of the "No fungus control" cultures, were inoculated with *Trametes* sp. HR577. All flasks or Schott bottles were incubated at 25ºC in the dark for five days. On day 5 the 'Autoclaved whole culture control' flasks were autoclaved and then PCP was added to all treatments except the "No PCP control" flasks. Laccase isozymes (crude L1a-c, purified L1c and purified L2) were added to some treatments. After addition of PCP all flasks and Schott Bottles were sealed to prevent loss of volatilised PCP metabolites. Residual PCP was measured after a further 72 hours incubation and quantified by HPLC. All treatments were carried out in triplicate. [b]One unit of laccase activity was defined as the amount of enzyme required to oxidise 1 μmol of ABTS per minute at 25ºC.

Quantification of Residual PCP by GC

The results above showed that 76% of the PCP was removed from whole fungal cultures over 72 hours. In these studies, the HPLC used for quantification of residual PCP was equipped with a UV detector that was set to measure only residual PCP. This method of analysis did not allow adequate quantification of PCP metabolites. For this reason, analysis of culture extracts was also carried out by GC.

GC was looked at as an alternative method of analysis. The GC was equipped with an electron capture detector (ECD) to quantify a range of different chlorophenol compounds. This allowed analysis of cultures for chlorinated PCP metabolites. The cultures were grown in an identical manner to those described above. The results for residual PCP quantified by GC are shown in Figure 5.3.

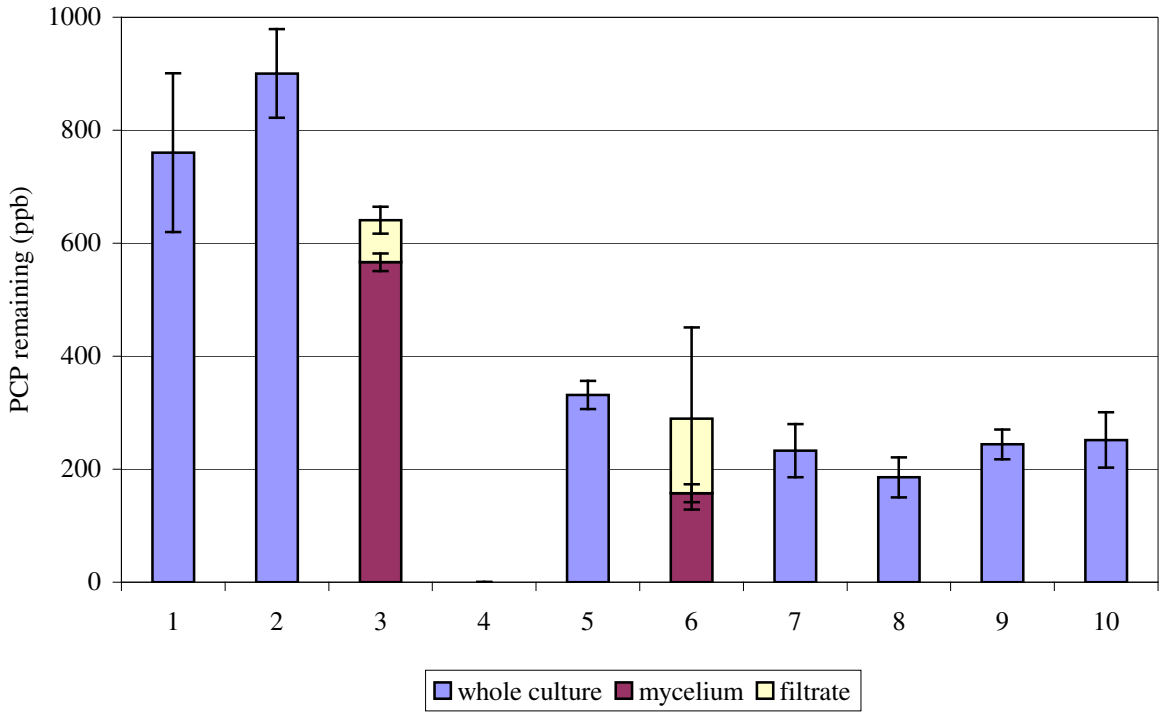


Figure 5.3: Comparison of PCP disappearance from whole cultures. Culture extracts were acetylated prior to quantification by GC. External standard calibration was used to quantify the acetylated chlorophenols (PCP, 2,3,4,5- TeCP, 2,3,4,6- TeCP, 2,3,5,6- TeCP, 2,3,4- TCP, 2,3,6-TCP, 2,4,5- TCP, 2,3,5- TCP and 2,4,6- TCP). The calibration standards were used to prepare calibration curves and quantify target compounds. Each data point is the average of triplicate cultures and each error bar represents the standard deviation of these cultures.

Key	Treatment
1	No fungus control
2	Autoclaved whole culture control
3	Autoclaved whole culture control[a]
4	No PCP control
5	Whole culture
6	Whole culture[a]
7	Whole culture plus autoclaved enzyme control[b]
8	Whole culture plus crude laccase preparation L1a-c[b]
9	Whole culture plus L1c[b]
10	Whole culture plus L2[b]

[a]Fungal biomass was separated from culture fluid by filtration and the fungal mycelium and culture filtrate were extracted, acetylated and analysed individually to give a measure of the amount of PCP removed from solution by adsorption to fungal mycelium. [b]The term 'purified laccase' used in this chapter refers to laccase isozymes L1c and L2 from *Trametes* sp. HR577. Purification of these isozymes is described in Chapter 4. The terms 'crude laccase' and 'crude L1a-c' refer to a mixture of putative laccase isozymes L1a, L1b and L1c partially purified from cultures of *Trametes* sp. HR577 by ammonium sulphate precipitation, dialysis and passage through a Source Q anion exchange column (see Chapter 4). One unit of laccase activity was defined as the amount of enzyme required to oxidise 1 µmol of ABTS per minute at 25ºC. The term 'autoclaved enzyme' refers to purified isozymes that were autoclaved prior to addition to fungal cultures.

The results presented in Figure 5.3 show that live fungal cultures removed approximately 60% of PCP relative to the control cultures. This equates to a removal rate of 10 mg L^{-1} day^{-1}, and compares favourably with the removal rate found by HPLC analysis.

For one set of live culture flasks (treatment 6, Figure 5.3) and one set of autoclaved culture flasks (treatment 3, Figure 5.3) the cultures were filtered prior to PCP extraction, acetylation and GC analysis. The fungal mycelium and culture filtrate were extracted, acetylated and analysed separately for these two treatments. For all other cultures, residual PCP and PCP metabolites were extracted from the fungal mycelium and culture filtrate together. While these results show that PCP adsorption to autoclaved cultures was quite high (566 ± 16 ppb, 75%[54]), significant amounts (157 ± 16 ppb, 21%[48]) also bound to live mycelium. Given that only 17% PCP remained in the culture filtrate, these results suggest that between 60 to 76% of the PCP supplied to the medium may be being degraded by live fungal cultures. There appeared to be a slight increase in PCP removal from cultures when fungal laccase isozymes from *Trametes* sp. HR577 were added.

Identification of PCP Metabolites by GC

GC analysis was used to examine whole culture extracts for PCP degradation. Standards for GC analysis included a range of lower chlorophenols (tri- and tetra-chlorophenols) as well as PCP. In addition, four other potential PCP metabolites (*p*-chloranil, *o*-chloranil, 3,4,5,6-tetrachloro-1,2-dihydroxybenzene and 2,3,5,6-tetrachloro-1,4-dihydroxybenzene) were also acetylated to use as reference standards. The standards were selected based on the chlorophenol standards available in the laboratory and PCP metabolites reported in the literature (Konishi and Inoue 1972; Mileski, Bumpus *et al.* 1988; Ricotta, Unz and Bollag 1996; Reddy and Gold 2000). The retention times of the reference standards as observed in the GC analysis are given in Table 5.2.

[54] Of the total PCP recovered from control treatments.

Table 5.2: Chlorophenol standards used to quantify residual PCP and PCP metabolites by GC

Chlorophenol	Retention time (minutes)
2,4,6-TCP	7.07
2,3,5-TCP	7.63
2,4,5-TCP	7.72
2,3,6-TCP	7.79
2,3,4-TCP	8.40
2,6-dibromo-4-methyl phenol[a]	9.34
2,3,5,7-TeCP	9.53
2,3,4,6-TeCP	9.60
2,3,4,5-TeCP	10.51
PCP	12.53

[a]Internal standard

The retention times in Table 5.2 show that all the chlorophenol standards were well resolved in the GC separation used in this study.

Table 5.2 shows that pure PCP standard eluted as a single peak with a retention time of 12.53 minutes. Technical grade PCP (85-90% purity) was used for fungal culture experiments. The GC analysis shows that it also contained 2,3,4,6-TeCP as a contaminant. Because the technical PCP used for the degradation studies described in this thesis contains 2,3,4,6-TeCP as a contaminant, the degradation of both the contaminant and PCP was examined.

2,3,4,6-TeCP Degradation

The fungal culture extracts from the experiment described in Figure 5.3 were examined for 2,3,4,6-TeCP removal. Figure 5.4 shows a comparison of 2,3,4,6-TeCP levels as quantified by GC analysis after 72 hours incubation for each treatment.

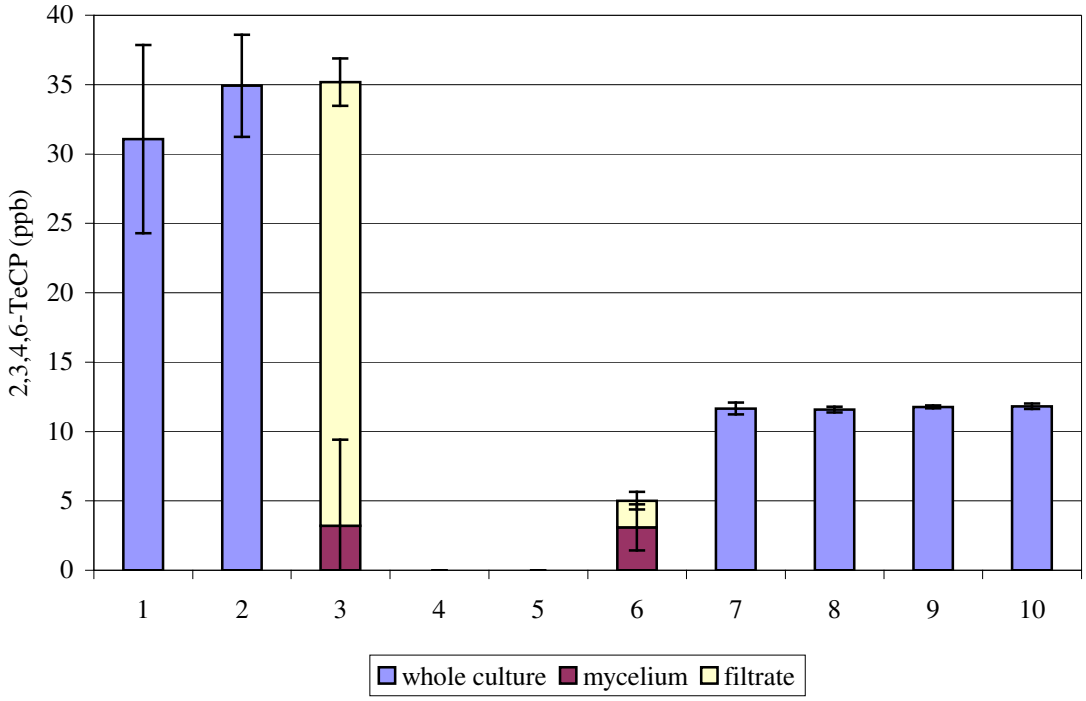


Figure 5.4: Comparison of 2,3,4,6-TeCP disappearance from whole cultures. Culture extracts were acetylated prior to quantification by GC. Each data point is the average of triplicate cultures and each error bar represents the standard deviation of these cultures.

Key	Treatment
1	No fungus control
2	Autoclaved whole culture control
3	Autoclaved whole culture control[a]
4	No PCP control
5	Whole culture
6	Whole culture[a]
7	Whole culture plus autoclaved[c] enzyme control[b]
8	Whole culture plus crude laccase isozymes L1a-c[b]
9	Whole culture plus L1c[b]
10	Whole culture plus L2[b]

[a]See footnote a in Figure 5.3. [b]See footnote b in Figure 5.3 [c]Purified enzyme was autoclaved prior to addition of 100 U mL^{-1} to whole fungal cultures.

As with the PCP results, all treatments containing live *Trametes* sp. HR577 significantly reduced the amount of 2,3,4,6-TeCP detected in cultures. For treatment 5 (whole fungal culture without any added laccase), no 2,3,4,6-TeCP was detected after 72 hours. In contrast, for treatment 6, which was identical to treatment 5 except that the mycelium and filtrate were analysed separately, a small amount of 2,3,4,6-TeCP was detected in both the fungal biomass and culture fluid after 72 hours. The total 2,3,4,6-TeCP recovered for the latter treatment amounted to about 5 ppb, which is close to the lower detection limit of the GC analysis in the method used. Therefore, it is possible that treatment 5 contains some residual 2,3,4,6-TeCP but that the levels are below the

detection limits of the GC. In contrast to results for PCP, only about 10% of the 2,3,4,6-TeCP was adsorbed to autoclaved and live fungal mycelium.

PCP DEGRADATION

Only one PCP metabolite was detected by GC when the whole culture fluids in the experiments described above were examined for PCP degradation products. This compound had a retention time of 11.6 minutes (Figure 5.5). This peak did not correspond to the retention time of any of the chlorophenol standards or any of the potential metabolites tested. From its retention time, it would appear to be some sort of tetra-chlorinated phenol derivative. Further investigation into the identity of this compound is required.

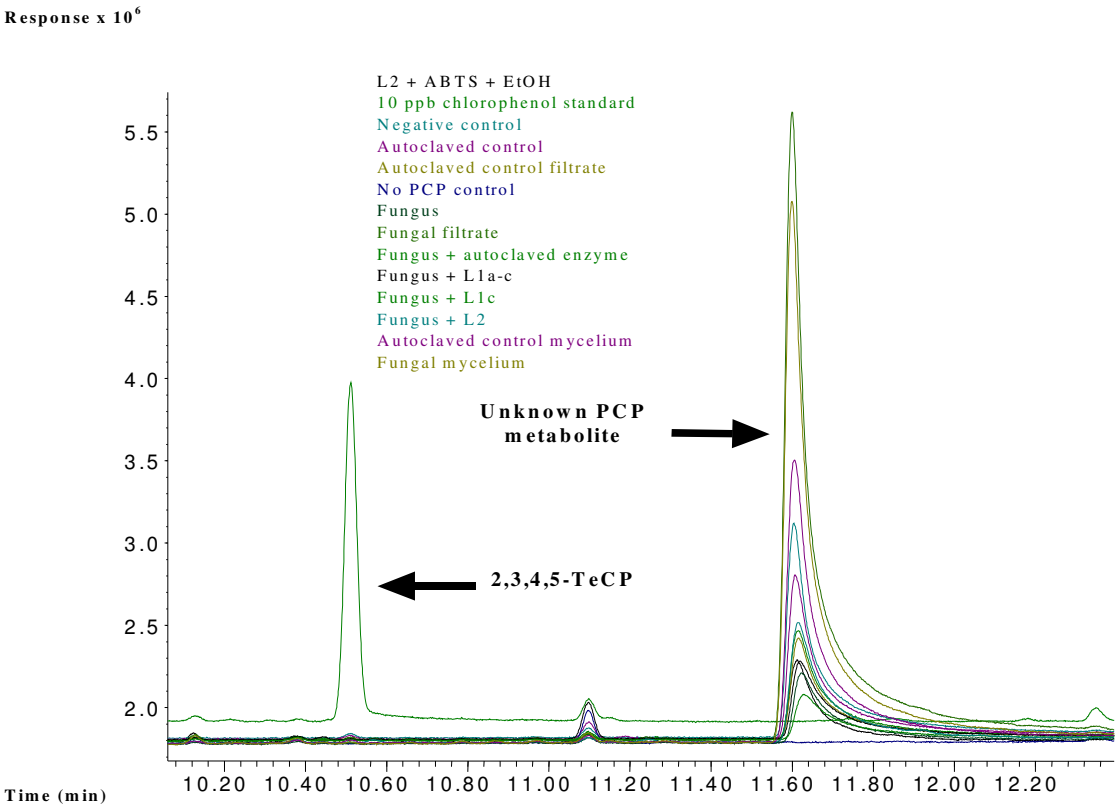


Figure 5.5: GC trace of treatments of whole cultures with PCP. This figure shows the unknown PCP metabolite (11.6 minutes) and 2,3,4,5-TeCP from the 10 ppb chlorophenol standard (10.51 minutes).

5.4 PCP DEGRADATION BY LACCASE ISOZYMES L1C AND L2 FROM *TRAMETES* SP. HR577

PCP Disappearance Quantified by HPLC

In order to examine the reactions of PCP with *Trametes* sp. HR577 laccase, the isolated isozymes need to be brought into contact with PCP as a substrate. Because PCP is sparingly soluble in water, some researchers have used ethanol to increase PCP solubility for enzyme degradation studies (Ullah, Bedford and Evans 2000). Given that xenobiotic degradation is sometimes aided by the presence of oxidation cofactors, other researchers have added ABTS to their enzyme incubation (Roy-Arcand and Archibald 1991; Collins, Kotterman *et al.* 1996). As a consequence, the effects of laccase, ethanol and ABTS on PCP degradation were examined here.

For the enzyme degradation studies PCP was added to tubes containing buffer and spiked with PCP (40 mg L^{-1}). For some incubations, 10% ethanol was added to increase availability of PCP without decreasing the laccase activity (see details on the effect of ethanol on laccase activity in Chapter 4). The culture tubes were then sealed with Teflon-lined lids and incubated for 72 hours. The results for PCP degradation by *Trametes* sp. HR577 laccase isozymes L1c and L2 are given in Tables 5.3 and 5.4, respectively.

Table 5.3: The effect of purified laccase on the percentage of PCP remaining. Reactions were carried out with 100 U mL^{-1} laccase and 40 mg L^{-1} PCP in sodium acetate buffer (1 ml, pH 5) for 72 hours at 25ºC. Residual PCP was quantified by HPLC.

Treatment	PCP mg L^{-1} (% of control)
No laccase control	33 ± 2 (100%)
No laccase + ABTS control	33 ± 1 (100 %)
No laccase + EtOH control	32 ± 2 (97%)
Autoclaved L1c control	33 ± 5 (100%)
No PCP control	0 (0%)
L1c	28 ± 4 (85%)
L1c + ABTS	22 ± 2 (67%)
L1c + ethanol	23 ± 11 (70%)
L1c + ABTS + ethanol	23 ± 2 (70%)

Table 5.4: The effect of purified laccase on the percentage of PCP remaining. Reactions were carried out with 100 U mL^{-1} laccase and 40 mg L^{-1} PCP in sodium acetate buffer (1 ml, pH 5) for 72 hours at 25ºC. Residual PCP was quantified by HPLC.

Treatment	PCP mg L^{-1} (% of control)
No laccase control	33 ± 2 (100%)
No laccase + ABTS control	33 ± 1 (100 %)
No laccase + EtOH control	32 ± 2 (97%)
Autoclaved L2 control	32 ± 3 (97%)
No PCP control	0 (0%)
L2	31 ± 1 (94%)
L2 + ethanol	29 ± 1 (88%)
L2 + ABTS	23 ± 5 (70%)
L2 + ABTS + ethanol	28 ± 1 (85%)

The results in Tables 5.3 and 5.4 show that on their own ABTS, ethanol and the autoclaved laccase isozymes L1c and L2 did not effect degradation of PCP. The data in the tables show that both *Trametes* sp. HR577 laccases L1c and L2 were able to remove PCP. The removal of PCP was better with the L1c preparation (15%) compared to L2 (6%).

PCP removal was improved when ABTS was added to the enzyme incubations. The extent of removal increased from 15% to 33% for L1c, and from 6% to 30% for L2. PCP removal was also improved when 10% ethanol was added to assist PCP solubility. The extent of PCP removal increased from 17% to 30% for L1c, and from 6% to 12% with L2. In both cases PCP disappearance was not as high as that observed from whole fungal culture incubations (76%, see Figure 5.3).

The data in Tables 5.3 and 5.4 also shows that for both enzymes PCP removal rates were not significantly improved slightly when both ethanol and ABTS were added together.

Identification of PCP Metabolites by GC

In this part of the study the metabolites produced during the degradation of PCP were compared to those from whole cultures. Selected culture extracts from the HPLC analysis [PCP controls (no enzyme, ABTS or ethanol), and selected L1c (L1c alone; L1c and ABTS; and L1c with ABTS and ethanol) and L2 (L2 alone; L2 and ethanol; and L2 with ABTS and ethanol)] were acetylated for GC analysis. Only three

compounds were detected by GC analysis. These were: residual PCP, residual 2,3,4,6-TeCP and an unidentified PCP metabolite with a retention time of 11.6 minutes.

Figure 5.6 shows an overlay of an expansion of the GC traces of the products derived from the enzyme incubations between 11.4 and 13 minutes. Figure 5.6 includes a trace of the 10 ppb chlorophenol standard mixture. The figure shows that the unknown PCP metabolite and residual PCP peaks resolve differently in the column separation and that the control cultures and 10 ppb chlorophenol standard traces do not have the unidentified PCP metabolite peak that elutes at 11.6 minutes.

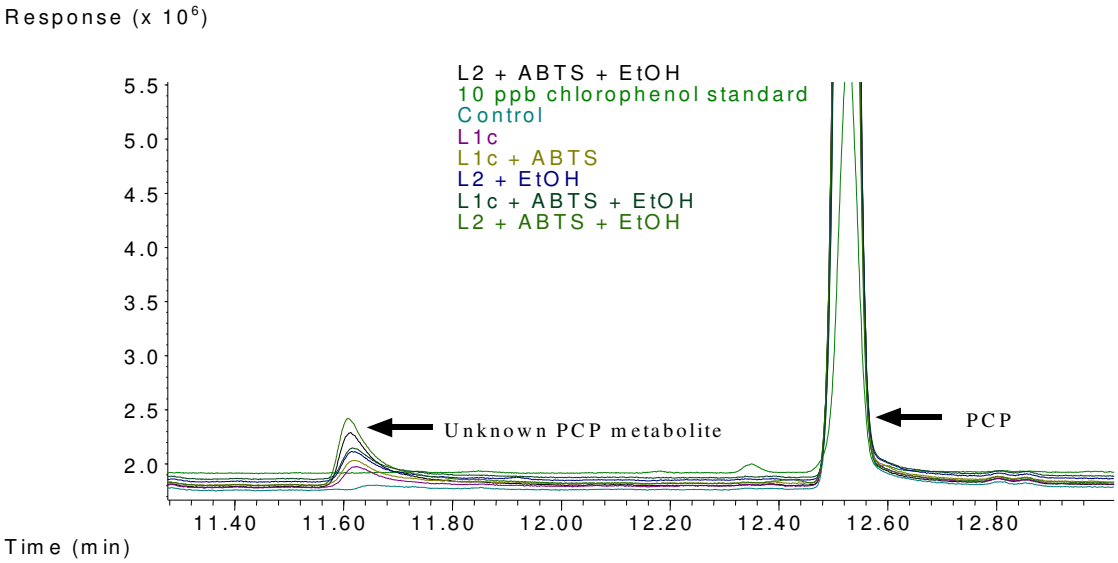


Figure 5.6: GC trace of treatments of purified laccase with PCP showing PCP and the unknown PCP metabolite.

Summary of Key Findings

The results in this study show that *Trametes* sp. HR577 cultures exhibit good capability for PCP degradation when cultures are pre-grown for five days prior to addition of PCP. Autoclaved whole cultures bind up to 75% of the PCP provided in the medium and live cultures bind up to 21% of the PCP provided in the medium. HPLC and GC analyses revealed that between 60 and 76% of the PCP was removed by live whole cultures. The technical grade PCP used for degradation studies showed that a contaminant (2,3,4,6-TeCP) was present. Whole cultures degraded 75-100% of the contaminant and only 10% was found to bind to the fungal mycelium.

Both laccase isozymes purified from *Trametes* sp. HR577 (L1c and L2) were able to degrade PCP (6-15% degradation over 72 hours). PCP removal rates improved significantly when ABTS was provided as a mediator and when 10% ethanol was included to assist PCP solubility.

Only one PCP degradation product was identified in the whole culture and purified enzyme incubations. The degradation product has not been identified and elutes from the GC column with a retention time of 11.6 minutes.

5.5 DISCUSSION

In the studies reported in Chapter 2 it was shown that submerged cultures of *Trametes* sp. HR577 removed all the PCP present in the culture medium over a six week period (see Table 2.3). In the present study, between 60 to 76% of the PCP was shown to be degraded by shallow cultures of *Trametes* sp. HR577 over a considerably shorter (72 hour) period (see Table 5.1 and Figure 5.3). These results indicate that the white-rot fungus *Trametes* sp. HR577 does remove PCP from its environment qualifying it a white-rot fungus with potential for bioremediation of PCP contaminated sites.

PCP degradation from shallow fungal cultures has been observed for other white-rot fungi. The extent of biodegradation depends on the amount of PCP added, the white-rot species used and the culture conditions. For example, the white-rot *P. chrysosporium* was shown to degrade 97% of 1.1 mg L^{-1} PCP added to 20 mL fungal cultures over a 28 hour period (Mileski, Bumpus, *et al*., 1988). Similarly, *T. versicolor* and *P. chrysosporium* were able to remove 89-100% of 5-40 mg L^{-1} PCP added to 50 mL fungal cultures over six days (Alleman, Logan and Gilbertson 1992). In contrast, no degradation was observed in cultures of three *Coprinus* sp. after a 5 day incubation period in 100 mL cultures containing 4 mg L^{-1} PCP (Guiraud, Steiman, *et al*. 1999).

The investigation above shows that the PCP used for the degradation studies reported in this chapter contained the contaminant 2,3,4,6-TeCP. About 10% of the contaminant adsorbed to *Trametes* sp. HR577 mycelium both in live and autoclaved cultures. In

contrast, 21% (live cultures) and 75% (autoclaved cultures) of the PCP added to HR577 cultures adsorbed to the fungal mycelium. The differences in xenobiotic binding observed here are consistent with those reported in the literature (Denizli, Cihangir *et al.* 2005).

The PCP adsorption observations are consistent with literature reports that show that the adsorption behaviour of white-rot fungi differs depending on fungal species, amount of PCP added to cultures and length of incubation period (Logan, Alleman *et al.* 1994; Sedarati, Keshavarz *et al.* 2003).

The results presented in Figure 5.3 show that only 24% of PCP was recovered from cultures incubated with live fungal cultures of *Trametes* sp. HR577. Of the PCP recovered approximately half was found in the mycelium and the rest in the culture filtrate. In contrast, autoclaved cultures of *Trametes* sp. HR577 were shown to bind 75% of the PCP provided. The above sets of results demonstrate that PCP disappearance from live fungal cultures is due to both degradation and mycelial binding, and not purely due to adsorption to the fungal mycelium.

When the metabolites resulting from the degradation of PCP were analysed none of the expected metabolites, namely *o*-chloranil and *p*-chloranil (TCHD, Konishi and Inoue 1972; Ricotta, Unz and Bollag 1996; Ullah, Bedford and Evans 2000), were detected. The only metabolite detected was an unidentified compound eluting as a single peak with a retention time of 11.6 minutes in the GC analysis. The identity of this peak needs to be ascertained. It is possible that it is a tetrachlorophenol derivative of PCP given that it eluted from the GC column between the tetrachlorophenol standards and the PCP peak.

Both the laccases purified from *Trametes* sp. HR577 were able to degrade PCP (Tables 5.3 and 5.4). Degradation was increased when PCP solubility was improved with ethanol addition or when the mediator compound ABTS was included. These results are consistent with the reports in the literature that show that addition of these cofactors improve the transformation rate of xenobiotics *in vitro* (Roy-Arcand and Archibald 1991; Field, Boelsma *et al.* 1995; Collins, Kotterman *et al.* 1996; Field, Vledder *et al.* 1996; Ullah, Bedford and Evans 2000).

The same PCP metabolite was observed in the GC traces for PCP incubated with two purified fungal laccases (L1c and L2) from *Trametes* sp. HR577 (Tables 5.3 and 5.4). These results suggest that laccase may play a role in PCP degradation by *Trametes* sp. HR577 since the same unidentified metabolite product was found for both whole culture and purified laccase incubations with PCP.

PCP degradation with whole fungal cultures was much higher than the amount of degradation observed for reactions of PCP with purified laccase. There are several possible reasons for this. It is possible that the two other laccases from *Trametes* sp. HR577 described in Chapter 4 (not purified or tested for PCP degradation here) or other hitherto unidentified laccase isozymes from *Trametes* sp. HR577 may be involved in addition to the enzymes tested in this study. Another reason could be that the amount of laccase added to culture tubes was not sufficient or representative of the level secreted by whole *Trametes* sp. HR577 cells to degrade PCP[55]. Additionally, or alternately, the lower activity detected may be due to the absence of essential cofactors that may have been lost in the process of purifying the two enzymes (Collins, Kotterman *et al.* 1996), or the effect of synergies that derive from the activity in concert of all of the enzymes working together (Makkar, Tsuneda *et al.* 2001).

In the present study, no experiments were carried out to test whether the laccase isozymes worked synergistically with each other or with other components from the extracellular fluid. Addition of the laccase mediator compound ABTS resulted in increased PCP degradation with laccases L1c and L2. This indicates that laccase mediator compounds produced by *Trametes* sp. HR577 may (also be involved in PCP degradation by laccase isozymes.

[55] The levels of laccase secreted by whole fungal cultures used for the PCP degradation experiments described in this chapter could not be quantified as the facility where the experiments were carried out did not have access to a UV-visible spectrophotometer. Laccase activity was determined for all other fungal culture experiments carried out for this thesis. However, since biomass growth was reduced in cultures grown in flasks with Teflon-lined lids (used only in the current set of experiments), the amount of laccase activity measured on day 5 for other experiments cannot be used to estimate the amount of laccase activity in the present experiments.

5.6 CONCLUSION AND FUTURE WORK

Whole cultures of *Trametes* sp. HR577 removed 60-76% of PCP from solution over 72 hours. About 21% of residual PCP was found associated with fungal mycelium indicating that PCP removal was due to degradation rather than simply adsorption to biomass. Purified laccase isozymes L1c and L2 removed much less PCP from solution than whole cultures (6-15%). Increased PCP removal was observed when either ethanol or ABTS was added to laccase cultures, suggesting that cofactors are required for PCP degradation by laccases from this white-rot fungus. These cofactors are very likely to be missing from purified laccase preparations due to the purification process and these are probably responsible for the reduced PCP degradation observed with laccase cultures relative to whole cultures. *Trametes* sp. HR577 has therefore been shown to be a good PCP degrader with the potential for use in bioremediation of PCP-contaminated sites. However, more research is required to identify the PCP metabolite(s) produced during degradation by this organism. Further work is also required to identify laccase cofactors and to purify other laccase isozymes and other enzymes involved in PCP degradation by *Trametes* sp. HR577.

CHAPTER 6: GENERAL DISCUSSION

6.1 GENERAL DISCUSSION

White-rot fungi have the potential to degrade PCP in soil. However, during degradation more toxic or persistent metabolites can be produced (McGrath and Singleton 2000). Therefore, it is important to screen isolates, not only for PCP disappearance, but to identify the PCP metabolites. The white-rot fungus *Trametes* sp. HR577 was selected for further studies from mass screening experiments conducted with 261 native white-rot fungi (see Chapter 2). This isolate grew on PCP- and creosote-amended agar and at all the temperatures tested (0-40ºC). These qualities mean that HR577 has more chance of surviving in PCP contaminated soil.

Trametes sp. HR577 produced laccase in both submerged and shallow cultures (see Chapter 2). Addition of PCP, copper or both PCP and copper to five day old shallow cultures resulted in increased laccase production. An apparent increase in *lcc* gene transcription levels was also observed when mRNA was extracted and amplified using laccase primers.

The four partial laccase sequences obtained from PCR products using laccase primers and amplified from cDNA and gDNA indicate that *Trametes* sp. HR577 has multiple laccase genes (see Chapter 3). Three of the partial laccase sequences showed very high sequence homology to each other but bear much less resemblance to the fourth sequence. Other laccase genes may also be present in the *Trametes* sp. HR577 genome. Further research is required to determine whether the three highly similar partial laccase sequences represent three separate genes or are due to allelic variation.

The longest partial laccase gene, from *Trametes* sp. HR577 cDNA, contained conserved amino acids found in sequences from laccases with high redox potentials. This suggests that the protein encoded by this laccase gene may also be a 'high' redox laccase. At this stage it is unknown whether the laccase isozymes from *Trametes* sp. HR577 have high T1 redox potentials. Laccases with high T1 redox potentials have a greater substrate range than 'low' redox potential laccases. Therefore, these laccases are likely to be useful for degrading a range of different xenobiotics. The redox potentials for L1c and

L2 could be determined by cyclic voltammetry using the method outlined by (Xu, Shin *et al.* 1996)

Two laccase isozymes, L1c and L2, were purified from *Trametes* sp. HR577 by ammonium sulphate precipitation, anion exchange and hydrophobic interaction chromatography (see Chapter 4). This represents the first report of purification of laccases from a NZ white-rot fungus.

Two other putative laccases, L1a and L1b, were semi-purified from fungal cultures. These two partially purified laccases eluted in the column flow through on the anion exchange column along with the L1c isozyme, but were observed as separate peaks of laccase activity on a hydrophobic column. Further development of the purification process, perhaps by using a different column or adjusting the column conditions may improve resolution of these three laccases.

The three peaks of laccase activity observed on the hydrophobic column are likely to be due to the presence of multiple laccase isozymes, although they may simply arise from differences in post-translational modification such as the degree of glycosylation. N-terminal sequencing of these laccases may give an indication of whether or not they represent different isozymes. These studies confirm that multiple isozymes are produced by *Trametes* sp. HR577. Other laccase isozymes may also be produced under different growth conditions. It is unclear whether the four putative laccase isozymes described above are all induced by addition of PCP or whether some of the isozymes are constitutive.

The increase in laccase production in shallow cultures after addition of PCP to fungal medium indicates that induced laccases may be involved in the PCP degradation pathway by *Trametes* sp. HR577. The purified laccase isozymes L1c and L2 degraded between 6 and 15% of PCP supplied in acetate buffer over a 72 hour period. A single PCP metabolite with a retention time of 11.6 minutes was observed in GC traces. This metabolite was also the only PCP metabolite detected during PCP degradation by whole fungal cultures of *Trametes* sp. HR577. PCP degradation with whole cultures was much higher than the rates of degradation observed with purified enzymes (between 60 and 76% degradation over 72 hours).

There are several possible reasons for this difference in PCP degradation by whole cultures compared to degradation by purified enzymes. Firstly, it has been shown that many white-rot fungi produce multiple laccase isozymes under various different growth conditions (Klonowska, Le Petit and Tron 2001). The reason for this multiplicity of isozymes is unclear. It is possible that the different isozymes work synergistically. Secondly, although many laccase isozymes degrade a variety of compounds without addition of cofactors, faster reactions can result with the addition of laccase mediator compounds such as ABTS (Collins, Kotterman *et al.* 1996). Addition of ethanol or ABTS to reactions with L1c or L2 increased PCP degradation over 72 hours (see Chapter 5). This suggests that laccase cofactors are required for effective PCP degradation by fungal laccases. The natural laccase cofactors produced by *Trametes* sp. HR577 were very likely to have been lost during the ammonium sulphate precipitation step during the laccase purification process. Collins, Kotterman *et al.* (1996) did not use ammonium sulphate precipitation to purify *T. versicolor* laccases but instead used ultrafiltration as the first purification step. They kept the ultrafiltrate and demonstrated that the level of anthracene oxidation was increased if ultrafiltrate was added to crude enzyme preparations. They suggested that the natural laccase mediator compounds produced by white-rot fungi are less than 10 kDa and heat stable.

Submerged cultures of *Trametes* sp. HR577 grown on SCS degraded all of the PCP supplied in the fungal medium over a six week period. Laccase production in submerged cultures of *Trametes* sp. HR577 was observed to be very low over the six week period (see Chapter 2). In contrast, laccase production in shallow cultures was increased when PCP was added to five day old cultures. Up to 76% of the PCP supplied in the fungal medium was degraded over 72 hours. Approximately half of the recovered PCP from whole cultures was found associated with the fungal mycelium while the other half was recovered from the culture filtrate (see Chapter 5).

Differences in laccase production observed between shallow and submerged cultures are likely to be due to the timing of when the PCP was added to fungal cultures and the type of inoculum used (SCS versus agar plugs). In submerged cultures PCP was added at the time that the cultures were inoculated. However, for shallow cultures, PCP was not added until day 5 after the fungus had established a mycelial mat on the liquid surface. Further experiments are required to determine whether the differences observed were due to the type of inoculum used or the timing of PCP addition.

Addition of purified laccase isozymes to whole cultures did not appear to enhance PCP degradation. However, since PCP degradation was low in laccase isozyme experiments and about 50% of the residual PCP from whole cultures was found associated with the fungal mycelium, it is probable that the effect of adding purified laccase isozymes was masked by the PCP adsorption effect.

PCP degradation occurred in cultures with high laccase production (shallow cultures) and low laccase production (submerged cultures) and some adsorption of PCP occurred. These results suggest that a mixture of both intracellular and extracellular enzyme processes are involved in the PCP degradation pathway by *Trametes* sp. HR577. However, more work is required to determine the exact role of laccase in PCP degradation and to establish the metabolites produced during this PCP degradation. If all the gene sequences encoding laccases in the *Trametes* sp. HR577 are found, gene knockout experiments could be performed to determine whether laccases are required for PCP degradation by this organism.

Overseas, the white-rot fungi *P. chrysosporium* and *T. versicolor* have been widely studied for bioremediation of PCP. Unlike *Trametes* sp. HR577, *P. chrysosporium* is a fast growing white-rot that produces numerous LiP and MnP isozymes under nutrient limited conditions (Farrell, Murtagh *et al.* 1989). The biotransformation of PCP in soils by these fungi leaves PCA, a persistent dead-end metabolite that is a more lipophillic species and can bioaccumulate (McGrath and Singleton 2000).

In contrast, it has been reported in the literature that the major ligninolytic enzyme produced by *T. versicolor* is laccase. At least three laccase isozymes are produced in cultures of this organism (Morohoshi, Fujita *et al.* 1987). This white-rot fungus has also been shown to produce LiP and MnP but only under certain conditions (Collins and Dobson 1995b). However, studies on PCP degradation by *T. versicolor* tend to focus exclusively on the role of laccase in the degradation pathway. Mineralisation of PCP in soil by *T. versicolor* occurs without accumulation of PCA (Tuomela, Lyytikäinen *et al.* 1999).

Trametes sp. HR577 belongs to the same genus as *T. versicolor*, and, as mentioned earlier, HR577 also produces multiple laccase isozymes (see chapters 3 and 4). While trace amounts of MnP have been detected in cultures of *Trametes* sp. HR577 grown on

solid media (Ford 2006), so far it has not been detected in liquid media. Whether MnP from *Trametes* sp. HR577 is involved in PCP degradation remains unknown.

The four partial laccase gene sequences from *Trametes* sp. HR577 share very high sequence homology (up to 98% at the nucleotide and amino acid level) to laccase gene sequences from *T. versicolor* and *T. villosa* (see Chapter 3). Laccase isozymes from *T. versicolor* and *T. villosa* have been purified and these isozymes have high T1 copper redox potentials (Xu, Palmer *et al.* 1999; Piontek, Antorini and Choinowski 2002). Therefore, it is possible laccase isozymes from *Trametes* sp. HR577 also have high T1 copper redox potentials. Comparison of full length gene sequences for laccases from *Trametes* sp. HR577 with those of *T. versicolor* and *T. villosa* laccase sequences may indicate whether this fungus produces 'high' redox laccases.

Purified laccases from *T. versicolor* react with PCP to form *p*-chloranil, *o*-chloranil or various polymeric metabolites depending on the culture conditions (Ricotta, Unz and Bollag 1996; Pallerla and Chambers 1998; Ullah, Bedford and Evans 2000; Sedarati, Keshavarz *et al.* 2003). It has been proposed that laccases do not play a role in the PCP degradation pathway by PCP (Ricotta, Unz and Bollag 1996). However, *T. versicolor* laccases appear to be able to catalyse the initial PCP detoxification step. Oxidative dechlorination by laccases from *T. versicolor* was first reported in 1963 (Lyr 1963) and has been demonstrated in several other studies over the years. In contrast, in these studies no evidence was found for the formation of *p*-chloranil, *o*-chloranil or polymeric products during PCP degradation by *Trametes* sp. HR577 (see Chapter 5). Similarly, none of those metabolites were observed during whole cultures PCP degradation by *Trametes* sp. HR577. More studies with *Trametes* sp. HR577 are needed to determine the exact PCP metabolites produced during degradation by whole cultures and purified isozymes. It is possible that dechlorination leads to the PCP metabolite with a retention time of 11.6 minutes observed in the GC chromatogram or to other products that bind more avidly to the fungal cells.

Experiments need to be undertaken to determine the PCP metabolites produced at different stages of the degradation pathway by both whole cultures and purified enzymes. Mineralisation of PCP to CO_2 could also be carried out by spiking cultures with ^{14}C-labelled PCP and collecting and analysing volatile fractions collected over a time-course experiment. These experiments could be used to compare the rate of PCP

mineralisation by *Trametes* sp. HR577 compared to other white-rot fungi. The results may also provide insight into the identity of PCP metabolites produced during the degradation process so that the PCP degradation pathway by *Trametes* sp. HR577 can be elucidated. This is required to ensure that the metabolites produced are not 'dead-end' metabolites or more toxic than the parent compound. The metabolite observed in the GC chromatogram with a retention time of 11.6 minutes needs to be identified. This could be achieved by using GC-MS.

Further purification of laccase isozymes L1a and L1b also needs to be carried out so the metabolites of PCP degradation by these two isozymes can be determined. Further development of the laccase purification process would also be useful. If an alternative step to ammonium sulphate precipitation such as ultrafiltration is used it might be possible to also purify and characterise some of the laccase cofactors that may be involved in the PCP degradation pathway.

Further work to obtain full length laccase gene sequences from both cDNA and gDNA is also required. It would be useful to also determine the sequences upstream from the laccase genes. The upstream region of laccase genes from other white-rot fungi has indicated the presence of laccase promoter elements such as putative xenobiotic resistance elements (XRE) and putative metal resistance elements (MRE, Soden and Dobson 2001). If laccase genes produced by *Trametes* sp. HR577 are inducible enzymes then XRE and/or MRE should be present upstream from the laccase gene coding regions.

There are several techniques reported in the literature for obtaining full length gene sequences. These include using amplified flanking region-PCR (AFR-PCR, Soden and Dobson 2003) and 3'-RACE protocol techniques (Colao, Garzillo *et al.* 2003).

Full length gene sequences could be cloned into vectors and transformed into yeast cells (e.g. *Pichia pastoris* or *Aspergillus niger*) to produce recombinant laccases. Purification and characterisation of recombinant laccases could be used to try to establish which laccase gene sequences correspond to the expressed wild type laccases that have already been purified from *Trametes* sp. HR577. They may also encode laccases that are not expressed under the conditions tested.

Overseas, it has been reported that immobilised laccases from *T. versicolor* have shown potential for xenobiotic degradation in bioreactors (Hublik and Schinner 2000; Jolivalt, Brenon *et al.* 2000; Leontievsky, Myasoedova *et al.* 2001). Production of recombinant laccase in yeast cells could be used to produce large quantities of laccase for bioremediation of contaminated waste water using similar processes.

A housekeeping gene for *Trametes* sp. HR577 needs to be found before more RT-PCR reactions can be carried out. Glyceraldehyde-3-phosphate (gdp) and β-tubulin have both been used as housekeeping genes for other white-rot fungi (Bogan, Schoenike *et al.* 1996; Soden and Dobson 2001; Stapleton and Dobson 2003). There are several white-rot gdp and β-tubulin sequences in the NCBI database. Alignments of these sequences would allow primers to be designed to amplify a highly conserved portion of these genes. Once a suitable housekeeping gene has been found, experiments can be carried out to determine whether laccase induction by PCP and copper is mediated at the level of gene transcription. In the past, RT-PCR reactions have been used to establish whether laccase expression is regulated at the level of gene transcription (Collins and Dobson 1997a; Soden and Dobson 2001). Given the problems that exist around the use of this technique to quantify levels of mRNA, Real Time RT-PCR would be the technique of choice for ensuring that levels of gene transcription observed are due to differences in gene transcription (Freeman, Walker *et al.* 1999).

The results reported in this thesis indicate that *Trametes* sp. HR577 has the potential to be an effective PCP degrader for cleanup of contaminated soils in NZ. More research is required to establish the PCP degradation pathway to ensure that no toxic or persistent metabolites are produced.

In this study, several other NZ white rot isolates were also effective PCP degraders (see Chapter 2). More research is needed to establish the PCP degradation pathway by these white-rot fungi both to determine whether ligninolytic enzymes play a role in this pathway and whether they are good choices for bioremediation. The white-rot isolate HR197, a slow growing isolate, is one such example of an effective PCP degrader. However, it did not produce LiP, MnP or laccase under the conditions tested, and, it is not yet known whether it produces ligninolytic enzymes for PCP degradation.

This study shows that the screening of new white-rot fungi may reveal new native organisms with a greater bioremediation potential than the ones so far described in the literature.

CHAPTER 7: EXPERIMENTAL

7.1 GENERAL EXPERIMENTAL

Culture Collection

All the cultures used for the studies described in this thesis are part of the HRCC. The HRCC culture collection is kept at HortResearch, Canterbury Science Centre, PO Box 51, Lincoln, NZ. New Zealand white-rot isolates in the HRCC were obtained from bioprospecting by HortResearch personnel as well as gifted from the Landcare Research, Auckland, and Forest Research, Rotorua, institutes. Not all isolates were identified. Two American isolates *P. chrysosporium* (ATCC 24725) and/or *P. sordida* (ATCC 90628) were included in the mass screening experiments (see Chapter 2) for control purposes.

Isolate Selection

Isolates used in the mass screening experiments described in Chapter 2 were randomly selected from the HRCC. The white-rot isolate *Trametes* sp. HR577 used for the majority of experiments described in this thesis was isolated from a birch log donated by John Daly and was found on a property in Christchurch. This culture was added to the HRCC just prior to commencement of the studies described in this thesis.

Culture Maintenance and Inoculum Production

All isolates were maintained as mycelial plugs in 7 mL bijou bottles (Samco Laboratories) of sterile distilled water at room temperature. Fungal inoculum was produced by transferring a mycelial plug, from the bijou bottle, onto a malt extract agar (MEA, Merck) plate. Plates were incubated in the dark at 25 for 7 or 14 days for the fast and slow growing isolates, respectively[56]. Culture plates then were stored at 4°C up to 4 weeks and used for further subculturing or as a source of inoculum in the various

[56] The white-rot isolate *Trametes* sp. HR577 was classified as a 'fast' growing isolate and, as such, culture plates were incubated for a period of seven days.

screening experiments. Fungal subcultures were taken from MEA plates for no more than 3 generations to avoid degeneration.

Culture Conditions

STANDARD CULTURE LIQUID MEDIUM FOR WHITE-ROT CULTURES

Unless otherwise stated, the liquid medium used for cultures contained, per litre, glucose (10 g), ammonium tartrate (1 g), potassium dihydrogen orthophosphate (1 g), sodium dihydrogen orthophosphate monohydrate (0.26 g), magnesium sulphate heptahydrate (0.5 g), and 2,2-dimethyl succinic acid (2.92 g). The medium was adjusted to pH 5.0 with potassium hydroxide, autoclaved and cooled before the addition of 1 mg thiamine (filter sterilised) and 10 mL of trace element solution (filter sterilised). The trace element solution contained, per 100 mL, iron sulphate heptahydrate (50 mg), manganese sulphate monohydrate (3.8 mg), zinc sulphate heptahydrate (5 mg), copper sulphate pentahydrate (10 mg), and calcium chloride dihydrate (66 mg).

CULTURE MEDIUM FOR ESCHERICHIA COLI *CULTURES*

All *E. coli* cultures used in this project were grown in Luria Broth (LB). The LB medium contained, per litre, 10 g tryptone, 5 g yeast extact and 10 g NaCl in Milli-Q water. Ten mL aliquots were dispensed into McCartney bottles, autoclaved and stored at room temperature until required. Sterile ampicillin (50 μL of a stock solution prepared as described below) was added prior to inoculation with colonies.

Ampicillin Solutions

Stock solutions of ampicillin (50 μg mL^{-1}) in Milli-Q water were filter sterilized (0.2 μM) and stored at -20ºC.

Enzyme Assays

LACCASE ACTIVITY

Unless otherwise stated, laccase activities were determined with ABTS as the substrate (Wolfenden and Willson 1982; Collins and Dobson 1997a) at 25ºC. ABTS assay mixtures contained 0.5 mM ABTS and 0.1 M sodium acetate (pH 5). Assays were initiated by addition of culture fluid or purified laccase. Oxidation of ABTS to $ABTS^{\cdot+}$ was monitored by determining the increase in A_{420} (ε=36,000 M^{-1} cm^{-1}). One unit of enzyme activity was defined as the amount of enzyme required to oxidise 1 µmol of ABTS per minute at 25ºC. Datum points in all cases are means for triplicate cultures. Absorbances were measured on either a Beckman 640 or a Varian Cary 50 or a Varian Cary 100 UV-visible spectrophotometer. Unless otherwise stated, assays of whole culture fluid were carried out in 1 mL disposable cuvettes. All assays with purified enzymes were carried out in 1 mL Quartz cuvettes.

LIP ACTIVITY

LiP activities were determined with veratryl alcohol (3,4-dimethoxyphenol) as the substrate (Tien and Kirk 1988). Assay mixtures contained 10 mM veratryl alcohol, 250 mM disodium tartrate (pH 3), 5 mM hydrogen peroxide (prepared fresh daily) and 500 µL aliquots of culture fluid in Quartz cuvettes. Oxidation of veratryl alcohol to veratryl aldehyde was monitored by determining the increase in A_{310} (ε=9,300 M^{-1} cm^{-1}). One unit of enzyme activity was defined as the amount of enzyme required to oxidise 1 µmol of veratryl alcohol per minute at 25ºC. Datum points in all cases are means for triplicate cultures. Absorbances were measured on a Beckman 640 UV-visible spectrophotometer.

MNP ACTIVITY

MnP activities were determined with manganese as the substrate (Wariishi, Valli and Gold 1992). Assay mixtures contained 10 mM manganese sulphate, 250 mM sodium

malonate (pH 4.5), 5 mM hydrogen peroxide (prepared fresh daily) and 700 µL aliquots of culture fluid in Quartz cuvettes. Oxidation of Mn^{2+} to Mn^{3+} was monitored by determining the increase in A_{270} (ε=6,500 M^{-1} cm^{-1}). One unit of enzyme activity was defined as the amount of enzyme required to oxidise 1 µmol of manganese per minute at 25ºC. Datum points in all cases are means for triplicate cultures. Absorbances were measured on a Beckman 640 UV-visible spectrophotometer.

Determination of Protein Concentration

Protein concentrations were determined by the method of Bradford (Bradford 1976), using BSA as a standard. Bradford reagent (Bio-Rad) was diluted 5-fold with Milli-Q water and filtered (0.2 µM). The BSA protein standards were made from dilution of a stock (Bio-Rad, 20 mg L^{-1}). The assays were performed by thoroughly mixing 100 µL of protein standard solution or sample with 1 mL of Bradford reagent. Absorbance readings (595 nm) were then taken of all the standards and samples using either a Cary 50 or Cary 100 UV-visible spectrophotometer. The concentration (mg mL^{-1}) was determined using the Cary UV-visible concentration program software (Varian).

Measurement of Dry Biomass

In cultures where biomass dry weight was measured, cultures were filtered through Miracloth and the fungal mycelium was transferred to a weighed glass petri dish. The mycelia was then dried at 50ºC until a constant weight was obtained. Unless otherwise stated, dry biomass weights are reported as the average of duplicate cultures.

DNA Extractions

Total DNA was prepared by a modification of the method of Collins and Dobson (1997b). Unless otherwise stated, triplicate analyses, representing the extraction of three identically grown cultures, were undertaken. Mycelia were separated from culture fluid by filtering through Miracloth (Calbiochem), quick frozen in liquid nitrogen, and

ground to a powder with a mortar and pestle. 10 mL of lysis buffer [50 mM EDTA, 50 mM Tris-HCl (pH 8) and 3% sodium dodecyl sulphate] was added, and the mixture was shaken gently by inversion. The mixture was then incubated at 65ºC for 30 minutes. During this incubation it was mixed gently every 10 minutes. After the incubation, the mixture was centrifuged for 5 minutes at 5,000 rpm and then the supernatant transferred to a fresh tube. Phenol-chloroform-isoamyl alcohol[57] (10 mL) was added and the solution mixed by inversion then centrifuged for 30 minutes at 13,000 rpm. The aqueous layer was transferred to a fresh tube and 3M sodium acetate (1 mL, pH 5.5) and ice cold isopropanol were added (5.5 mL) and mixed by inversion. The mixture was centrifuged for a further 15 minutes at 13,000 rpm. The supernatant was decanted and the pellet washed with ice cold 75% ethanol (10 mL), and centrifuged briefly and the supernatant removed. The pellet was then dried for 5-10 minutes at 50ºC. 500 µL of sterile distilled water was then added and the pellet resuspended at 4ºC overnight. RNase (10 µL) was then added to remove any contaminating RNA and the DNA was incubated at 37ºC for 1 hour followed by another incubation at 65ºC for 20 minutes. The extracted DNA was then stored at 4ºC until required. Total DNA was quantitated spectrophotometrically using the ratio of A260/A280.

RNA Extractions

Total RNA was prepared by a modification of the method of Gromoff, Treier and Beck (1989). Mycelia from seven day old triplicate cultures were separated from culture fluid by filtering through Miracloth (Calbiochem), quick frozen in liquid nitrogen, and ground to a powder with a mortar and pestle. Lysis buffer [0.6 M NaCl, 10 mM EDTA, 100 mM Tris-Cl (pH 8), 4% sodium dodecyl sulphate] (0.5 mL), and phenol-chloroform-isoamyl alcohol (25:24:1 v/v 0.5 mL) was added, and the mixture was shaken vigorously for 20 minutes and then centrifuged for 10 minutes at 14,000 rpm. After a further extraction step with phenol-chloroform-isoamyl alcohol (0.5 mL), lithium chloride (8 M, 0.75 mL) was added, and the mixture vortexed and incubated overnight at 4^{o}C. RNA was pelleted by centrifugation for 15 minutes at 14,000 rpm and resuspended in DEPC-treated water. The resuspended RNA was precipitated with sodium acetate (pH 5.5, 0.3 mL) and 99% ethanol (0.75 mL), washed (70% ethanol,

[57] From Sigma, 25:24:1 v/v

0.5-1 mL), and resuspended in DEPC-treated water (50 μL). Residual contaminating DNA was removed by digestion with DNase I (Invitrogen) according to the manufacturer's protocol. Total RNA was quantitated spectrophotometrically using the ratio of A260/A280.

Reverse Transcriptase (RT) Experiments

Total RNA was used as the template to generate first-strand cDNA using a SuperScript First-Strand Synthesis Kit according to the manufacturer's protocols (Invitrogen). Briefly, reaction mixtures contained 1 ng of total RNA, 200 ng of random hexamer primers and 0.5 mM (each) deoxynucleoside triphosphates (dNTPs). Reaction volumes were adjusted to 10 μL with DEPC treated water. Samples were incubated at 65°C for 5 minutes and incubated on ice for 1 minute. Then 2 μL of 10x RT buffer, 4 μL of $MgCl_2$ (25 mM), 2 μL DTT (0.1 m) and 1 μL of RNaseOUT Recombinant Ribonuclease Inhibitor were added to the reaction mixture. The reaction mixtures were incubated for 25°C for 2 minutes before addition of 50 U of SuperScript™ II RT to each tube. Reaction mixtures were then incubated a further 10 minutes at 25°C. The reaction tubes were incubated at 42°C for 50 minutes. The reactions were terminated at 70°C for 15 minutes and then chilled on ice.

Agarose Gel Electrophoresis

DNA fragments were separated on the basis of size using agarose gel electrophoresis. Agarose gels (1 % w/v) were prepared by adding 0.5 g agarose to 50 mL 1X TAE buffer (40 mM Tris.HCl, 20 mM acetic acid and 2 mM EDTA at pH 8.0) and heating until dissolved. All gels were run using a Sub-Cell® GT Agarose Gel electrophoresis system (Bio-Rad) in 1X TAE buffer. DNA samples were premixed with 6x loading buffer (0.2% w/v bromophenol blue in 50% v/v glycerol) and then loaded into the wells. Electrophoresis was performed at 80 V until the dye front had migrated to the other end of the gel. The DNA on the gels was visualized by staining the gel in ethidium bromide (~0.5 μg mL^{-1}), followed by exposure to ultraviolet light (302 nm). Pictures of the gels

were taken using an Alpha Imager gel documentation system (Alpha Innotech Corporation, USA).

Quantification and Size Determination of DNA Fragments

The concentration of DNA samples was estimated by comparison with DNA samples of known concentration run alongside on an agarose gel. The approximate size of a DNA band was estimated by comparing its migration through the gel against that of DNA standards with known size (1 Kb Plus DNA ladder, Invitrogen), which were run alongside the DNA sample of interest.

Digestion with EcoRI

5 µL of each plasmid was digested with 1 µL *EcoRI* (Roche) in a total volume of 21 µl in 1x SuRE/cut Buffer H (50 mM Tris-HCL, 100 mM NaCl, 10 mM $MgCl_2$, 100 µM DTT, pH 7.5, Roche) at 37ºC for ~2 hours.

DNA Sequencing

SEQUENCING OF GDNA

DNA sequencing services for gDNA PCR products were provided by Lark Technology Inc (UK). Sequences were determined by the dideoxy chain-termination method (Sanger, Nicklen and Coulson 1977) using the Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase, FS (Applied Biosystems), on a GeneAmp PCR system 2400 (Perkin Elmer), and run on an automated DNA sequencer (model 373 stretch, Applied Biosystems). The sequence data were assembled and processed using the Chromaslite software package.

SEQUENCING OF CDNA

DNA sequencing services for cDNA PCR products were provided by the Massey University Allan Wilson Centre for Molecular Evolution and Ecology Genome Service. DNA sequencing was carried out on either an ABI Prism 377-64 sequencer or an ABI Prism 3730 capillary sequencer, using BIGDYE labelled dideoxy chain termination chemistries (Applied Biosystems). The sequence data were assembled and processed using the Chromaslite software package.

Nucleotide and Amino Acid Sequence Alignments

Nucleotide sequences from gDNA were copied and pasted into the NCBI ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and translated into three open reading frames. Intron and exon positions of *lac1*, *lac2* and *lac3* from *Trametes* sp. HR577 were deduced by comparison of these sequences with the nucleotide and amino acid sequences for *lcc2* and *lcc4* from *T. villosa* (nucleotide sequences: GI:1100245, and GI:1322078, respectively, amino acid sequences: GI: 1100246 and GI: 1322079, respectively, (Yaver, Xu *et al.* 1996). Nucleotide and amino acid sequences shown in Figures 3.4, 3.6 and 3.8 were aligned manually in Microsoft Word. Sequence identities were calculated using ClustalW (http://www.ebi.ac.uk/clustalw/, Chenna, Sugawara *et al.* 2003).

Centrifugation

Centrifugation in this project was performed in one of three centrifuges: a SORVALL Evolution RC centrifuge, a SORVAL Heraeus multifuge® 1S-R or a MiniSpin® centrifuge (Eppendorf).

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method of Laemmli (1970) with a 4% (w/v) stacking gel and a 12%

(w/v) separating gel, using a Mini Protean III cell (Bio-Rad). All samples were prepared in SDS loading buffer and boiled for one minute before loading into wells. Low range SDS-PAGE molecular weight standards (Bio-Rad) were used. A constant voltage of 260 V was applied across the electrodes until the dye front reached the bottom of the separating gel. After electrophoresis, gels were stained for protein using Coomassie Blue. The staining solution consisted of 1 g L^{-1} Coomassie Brilliant Blue R 250 (Park Scientific) in 50% (v/v) methanol, 10% acetic acid (v/v) in water for approximately 20 minutes. The gel was then destained in identical solution without the dye. Molecular weight was estimated from a plot of the $\log_{(\text{molecular weight})}$ versus the distance travelled for each of the protein bands.

Fast Protein Liquid Chromatography (FPLC)

FPLC was carried out using a Bio-Rad Biologic protein chromatography system. Separations were conducted either at 4ºC or room temperature for anion exchange and hydrophobic interaction chromatography, respectively. All buffers and solvents for FPLC were filtered using a 0.2 μM filter (Millipore).

Chemicals for PCP Extraction and Analysis

All solvents and reagent chemicals were high purity (Mallinckrodt Nanograde, Merck-BDH analytical grade). Ultrapure water was prepared by distillation followed by a Milli-Q-system (Millipore, Milford, MA, USA). A certified standard of PCP (99% purity, Dr Ehrenstorfer GmbH, Augsburg, Germany) was used to prepare calibration standards for HPLC analysis.

HPLC Analysis

Liquid chromatographic separation and detection of PCP were performed with a Shimadzu LC-10A liquid chromatography system and Shimadzu SPD-10AV UV-Visible detector at 280 nm. Chromatography was performed at 35^{o}C with an isocratic

methanol/water/acetic acid mobile phase (81/18/1% by volume) and 5 μm, 150 x 4.5-mm-i.d. Luna C_8 reverse phase column (Phenomenex NZ Ltd). The column flow rate was 1.0 mL min^{-1} and injection volume 10 μl. Six PCP calibration standards (50, 20, 10, 5, 2.5, 1 μg mL^{-1}) were analysed with each batch of samples and the samples quantified against the constructed calibration curve.

GC Analysis

Acetylated sample extracts were analysed using an Agilent 6890N Network GC system with microcell electron-capture detector (ECD), programmed temperature vaporiser injector and 7683B series autoinjector. The injector temperature was programmed to start at 90°C where it was held for 0.1 minute, then increased at 200°C min^{-1} to 250°C where it was held for 23.7 minutes. 1 μL of sample extract and chlorophenol calibration standards were injected by autosampler using a splitless time of 1 minute after which the injector was purged at 200 mL min^{-1} with helium. Chromatographic separation was achieved using a 25 m x 0.20 mm I.D. Agilent Ultra-2 glass capillary column, coated with 0.33 μm 1% phenyl-dimethylsiloxane phase (Agilent, NZ). Helium carrier gas was supplied at 23 psi. The GC oven was programmed at 90°C (1 min), increased to 160°C at 20°C min^{-1}, followed by 5°C min^{-1} to 220°C, and increased at 50°C to 300°C (6.5 minute hold). Helium carrier gas was maintained at a head pressure of 23 psi. The ECD was maintained at 325°C and nitrogen was used as make-up gas.

Agilent Environmental Chemstation was used to acquire and analyse the data. External standard calibration was used to quantify the acetylated chlorophenols (PCP, 2,3,4,5-TeCP, 2,3,4,6- TeCP, 2,3,5,6- TeCP, 2,3,4- TCP, 2,3,6-TCP, 2,4,5- TCP, 2,3,5- TCP and 2,4,6- TCP) and the corresponding anisoles (2,3,5,6-tetrachloro- 2,5 - cyclohexadiene- 1,4-dione, 3,4,5,6- tetrachloro- 3,5- cyclohexadiene-1,2- dione, 3,4,5,6-tetrachloro- 1,2- dihydroxybenzene and 2,3,5,6- tetrachloro- 1,4-dihydroxybenzene). The calibration standards were used to prepare calibration curves and quantify target compounds.

Photographs

Unless otherwise stated, all photographs that appear in this thesis were taken by the author using a Nikon Coolpics 885 digital camera.

7.2 CHAPTER 2 EXPERIMENTAL

Mass Screening Experiments

ORGANISMS AND INOCULUM PREPARATION

All of the white-rot fungi chosen for the initial screening experiments were randomly selected from the 367 cultures held in the HRCC. The culture maintenance and inoculum production was identical to that described in the General Experimental section.

TEMPERATURE TOLERANCE

Isolates were studied for their temperature tolerance to determine their upper and lower temperature limits for growth. MEA plates were centrally inoculated with an 8 mm mycelial plug (mycelial side down) and incubated at 0, 5, 20, 25, 30, 35 and 40°C. There were two replicate plates for each isolate at each temperature. Mycelial growth diameter (mm) was measured in two perpendicular directions after three and seven days of incubation. If little or no visible growth was observed after the seven days incubation, isolates were further incubated at 25°C for an additional seven days. Isolates that did not resume growth were assessed as dead.

PCP RESISTANCE

Tests to determine resistance to PCP were carried out on agar plates amended with PCP (99%, Aldrich). PCP was dissolved in acetone and diluted with autoclaved MEA at 50-

55°C to give a range of PCP concentrations (0, 10, 20, 30, 40, 50 and 200 mg L^{-1} PCP). Agar was dispensed (20 ml) into vented sterile plastic petri dishes (85 mm diameter). The volume of acetone added (5 ml L^{-1} agar) was constant for all concentrations. The nil-control consisted of straight MEA plates (nil PCP and nil acetone). Acetone-control refers to the 0 mg L^{-1} PCP plus acetone carrier solvent. Plates were allowed to dry (lids closed) for one to two days at room temperature in the fume hood prior to inoculation with two 6 mm plugs of inoculum (mycelial side down) positioned on opposite margins of the plate. Mycelial growth was measured from the inner edge of the plug to the centre of the plate after three, seven and fourteen days incubation at 25°C in the dark. There were two replicate plates for each isolate at each concentration.

One hundred and sixty three native isolates were tested for PCP tolerance against the complete range of PCP concentrations (including acetone-control). An additional 88 isolates were screened for PCP tolerance at the 50 mg L^{-1} PCP concentration in comparison to the nil-control. All isolates showing growth at the 50 mg L^{-1} PCP level were also tested for PCP tolerance at 200 mg L^{-1}.

CREOSOTE RESISTANCE

Resistance to creosote was tested in a similar manner to resistance to PCP. Creosote was added neat to autoclaved MEA at 50-55ºC to give a range of creosote concentrations (500, 1,000, 5,000, 10,000 and 100,000 mg L^{-1} creosote). Plates were inoculated with one 8 mm plug of inoculum (mycelial side down) positioned in the centre of the plate. Mycelial growth was measured in two perpendicular directions after seven days incubation at 25ºC in the dark. There were two replicate plates for each isolate at each concentration.

PCP Degradation and Laccase Activity in Submerged Cultures

ORGANISMS AND INOCULUM PREPARATION

Twenty white-rot isolates from the HRCC were used for this experiment. The isolates used were: *A. biemmis* (HR145), *O. australis* (HR345), *P. sacrata* (HR226, HR235 and HR240), *S. faciatum* (HR348), *Trametes* sp. (HR192, HR196, HR197 and HR577), *T. versicolor* (HR131, HR154, HR160, HR275, HR277 and HR445) and five unknown basidiomycete species (HR112, HR122, HR152, and HR588).

Isolates were grown on a fungal growth substrate (SCS) consisting of fresh *P. radiata* sawdust cornmeal starch mixture (Lestan *et al.* 1996). SCS was adjusted to 50% gravimetric water content, weighed (20 g) into vented glass petri dishes (85 mm diameter) and sterilised by autoclaving at 121°C and 103.5 kPa for 1 hour over 2 successive days. The sterile SCS was then centrally inoculated with an 8 mm mycelial plug from a 7 day old white-rot isolate grown on MEA. SCS inoculated cultures were incubated for 3-4 weeks at 20 or 25°C in the dark and used for inoculum in PCP degradation experiments.

CULTURE CONDITIONS

Five grams (fresh weight) of colonised SCS inoculum was introduced into 100 mL of standard liquid medium (see General Experimental) containing 50 mg L^{-1} PCP (dissolved in acetone) in a 250 mL Erlenmeyer flask. Duplicate flasks were inoculated for each isolate. Flasks were incubated statically at room temperature (20-25°C) in the dark.

During incubation laccase activity was monitored at regular intervals. Laccase activities were determined with DMP (de Jong, Cazemier *et al.* 1994) as the substrate. DMP assay mixtures contained 10 mM DMP, 50 mM malonate buffer (pH 4.5) and 300-600 µL aliquots of culture fluid. Oxidation of DMP was monitored by determining the increase in A_{450} (ε=8550 M^{-1} cm^{-1}). One unit of laccase activity was defined as the amount of enzyme required to oxidise 1 µmol of DMP per minute at 25ºC. Datum points in all cases are means for duplicate cultures.

After 42 days the mycelium was filtered under vacuum through filter paper (Whatman No. 1) and the filtrate analysed for PCP by HPLC. An uninoculated PCP control and a fungus control (HR131) were analysed for residual PCP. The latter consisted of liquid medium and fungal inoculum without PCP.

PCP EXTRACTION AND ANALYSIS OF LIQUID CULTURES

Measured aliquots of filtered liquid culture samples (50-90 ml) were extracted with 100 mL acetone/hexane (2:3 v/v) by shaking for 60 minutes on a flat bed shaker (300 cycles/minute). After shaking, 150 mL of distilled water was added to separate the hexane phase containing extracted PCP and 0.25 mL was transferred to a 2 mL glass HPLC vial. Twenty microlitres of iso-butanol was added as keeper solvent and hexane was removed under a gentle stream of nitrogen gas at room temperature. Then 1.6 mL methanol/water (80:20 v/v) was added to each vial and the samples were stored under refrigeration until analysis by HPLC. PCP recovery during sample workup was calculated from control samples spiked with a known amount of PCP and was typically 90 to 100%. The amount of PCP, measured in a corresponding batch of samples, was corrected by the appropriate recovery factor. HPLC analysis was carried out as previously described (see General Experimental).

Ligninolytic Activities in Shallow Culture Medium in the Presence and Absence of PCP

ORGANISMS AND INOCULUM PREPARATION

The isolates chosen for the 15 day PCP-free time course experiment were HR112, HR122, HR160, HR197, HR240 and HR577. The isolates chosen for the 15 day PCP-amended time course experiment were HR112, HR240 and HR577. All isolates were from the HRCC and were maintained as described in the General Experimental section.

CULTURE CONDITIONS: 15 DAY TIMECOURSE IN PCP-FREE STANDARD LIQUID MEDIUM

The culture medium used for these experiments was the standard liquid medium described in the General Experimental section. Two agar plugs (6 mm diameter) from the outer circumference of a fungal colony growing on a MEA plate (7-28 days) were used as the inoculum. The fungi were grown in 15 mL stationary cultures in 200 mL medical flat bottles (BDH) in the dark. The incubation temperatures were 26°C for isolates HR112, HR122, HR160, HR197 and HR577 and room temperature for isolate HR240. Bottles were loosely capped to allow passive aeration.

Cultures were examined daily for the presence of laccase, LiP and MnP using standard assays for ligninolytic enzymes (see General Experimental). Datum points in all cases are means for triplicate cultures for each day of the experiment[58].

CULTURE CONDITIONS: 15 DAY TIMECOURSE IN PCP-AMENDED STANDARD LIQUID MEDIUM

The culture medium used for these experiments was the standard liquid medium described in the General Experimental section except that 50 mg L^{-1} PCP dissolved in acetone was added to cultures at the time of inoculation. Two agar plugs (6 mm diameter) from the outer circumference of a fungal colony growing on a MEA plate (7-28 days) were used as the inoculum. The fungi were grown in 15 mL stationary cultures in 200 mL medical flat bottles (BDH) in the dark. The incubation temperatures were 26°C for isolates HR112, HR122, HR160, HR197 and HR577 and room temperature for isolate HR240. Bottles were loosely capped to allow passive aeration. Datum points in all cases are means for triplicate cultures for each day of the experiment[59].

[58] i.e. a total of 45 bottles were inoculated for each isolate.
[59] i.e. a total of 45 bottles were inoculated for each isolate.

Preliminary Study into Induction of Laccase in Trametes sp. HR577

ORGANISM AND INOCULUM PRODUCTION

Isolate *Trametes* sp. HR577 used in these experiments was from the HRCC and was maintained as previously described (see General Experimental).

The culture medium used for these experiments was the standard liquid medium described in the General Experimental section. Two agar plugs (6 mm diameter) from the outer circumference of a fungal colony growing on a MEA plate (7 days) were used as the inoculum. The fungi were grown in 15 mL stationary cultures in 250 mL Erlenmeyer flasks at 25ºC in the dark. Flasks were loosely sealed with cotton wool and aluminium foil to allow passive aeration. Datum points in all cases are means for triplicate cultures.

CULTURE CONDITIONS: ADDITION OF PCP TO 1, 3 AND 5 DAY OLD FUNGAL CULTURES

Cultures of *Trametes* sp. HR577 were grown for 1, 3 or 5 days prior to addition of PCP (50 mg L^{-1}) to fungal liquid medium. Laccase activity was measured daily in all cultures over a seven day period using ABTS as the substrate (see General Experimental). Control cultures were grown in the same way as the other cultures without addition of PCP. The laccase activity reported is in U mL^{-1} of culture medium where 1 unit of enzyme activity was defined as the amount of enzyme required to oxidise 1 μmol of ABTS per minute at 25ºC.

CULTURE CONDITIONS: THE EFFECT OF COPPER, PCP OR BOTH ON LACCASE PRODUCTION

Fungal cultures of *Trametes* sp. HR577 were grown in standard liquid medium in shallow (15 mL) cultures for five days at 25ºC in the dark. $CuSO_4$ (300 μM), PCP (50 mg L^{-1}) or PCP and $CuSO_4$ (50 mg L^{-1} and 300 μM for PCP and Cu, respectively) were added to five day old cultures and the laccase activity and total protein (as

described in the General Experimental section) were measured after a further 48 hour incubation.

CULTURE CONDITIONS: THE EFFECT OF COPPER, PCP OR BOTH ON LACCASE MRNA LEVELS

Fungal cultures of *Trametes* sp. HR577 were grown under identical conditions to those described above. Laccase activity was monitored on days 5-7 for all treatments. On day 5 (control cultures only), day 6 (control cultures only) and day 7 (control, Cu, PCP and PCP/Cu cultures), RNA was extracted from mycelia from triplicate cultures as previously described (see General Experimental). Total RNA (1 ng) from each treatment was used to generate first-strand cDNA in RT reactions with random hexamer primers using a SuperScript™ First-Strand Synthesis kit (Invitrogen) according to the manufacturer's protocols. The resulting cDNA was amplified using forward (5'-ATTGGCACGGCTTCTTCC-3') and reverse (5'-GATCTGGATGGAGTCGAC-3') PCR primers based on conserved regions between previously described *lac* sequences from *Trametes* species and closely related fungi (Collins and Dobson 1997a). The PCR reaction mixture consisted of ~7 ng of genomic DNA, 2 ng μL^{-1} of both primers, 200 μM of each of the deoxyribonucleotide triphosphates (dNTPs), 1x polymerase buffer, 1.25 units of *Taq* DNA polymerase in a total reaction volume of 50 μL. The thermo-cycling program consisted of an initial denaturation at 95ºC for 1 minute, followed by 30 cycles of primer annealing at 55ºC for 1 minute with extension at 72ºC for 1 minute. After 30 cycles a final extension at 72ºC was carried out for 10 minutes. PCR products were analysed by agarose gel electrophoresis (see General Experimental). PCR reactions were carried out using an Eppendorf Thermal cycler.

7.3 CHAPTER 3 EXPERIMENTAL

Organism and Inoculum Preparation

Trametes sp. HR577 from the HRCC was used for all the experiments described in this chapter. The culture maintenance and inoculum production was identical to that described in the General Experimental section.

Culture Conditions

Triplicate cultures of *Trametes* sp. HR577 were grown in 15 mL of standard liquid medium in 250 mL Erlenmeyer flasks or 250 mL medical flat bottles. Two agar plugs (6 mm diameter) from the outer circumference of a fungal colony growing on a MEA plate (7 days) were used as the inoculum. Cultures were grown at 25°C in the dark for seven days. Culture flasks and bottles were loosely capped to allow adequate aeration.

DNA and RNA Extractions

DNA and RNA extractions were carried out as previously described (see General Experimental).

Reverse Transcriptase Experiments

RT experiments were carried out as previously described (see General Experimental) except that cDNA prepared to obtain the longer laccase gene sequence used Oligo d(T) primer instead of random hexamer primer. Reactions were carried out using an Eppendorf Thermal cycler.

PCR Primers

The primers used for the four different PCR reactions described in Chapter 3 are listed in Table 7.1.

Table 7.1: Primers used to amplify gene sequences from *Trametes* sp. HR577

Primer name	Primer sequence	Annealing temperature (ºC)
lcc (5′)[a]	ATTGGCACGGCTTCTTCC	55
lcc (3′)[a]	GATCTGGATGGAGTCGAC	55
Tlcc (5′)[b]	CGGCGCCCATATGGGKTTRCAGCGYTTCAGYTTYTTYGT	53
Tlcc (3′)[b]	ACGGGATCCATTACTGGTTAGCCTCRCTYAANCCRTCRTA	53
EF4fwd (5′)[c]	GGAAGGGRTGTATTTATTAG	48
F5rev (3′)[c]	GTAAAAGTCCTGGTTCCCC	48
ITS5 (5′)[d]	TCCTCCGCTTATTGATATGC	57
ITS4 (3′)[d]	GGAAGTAAAAGTCGTAACAAGG	57

Key to symbols: R=G+A, Y=C+T, N=A+C+G+T, K=G+T
[a]Collins and Dobson 1997a. [b]This thesis. [c]van Elsas, Duarte *et al.*, 2000. [d]De Koker, Nakasone *et al.* 2003

Primer design for *Tlcc* was based on an alignment of the amino acid sequences for two *T. versicolor* and *T. villosa* laccase genes (*T. villosa lcc2* and *T. versicolor lccI*, GI numbers 1100246 and 1174245, respectively). A back translation of the peptide secretion signal and C-terminus portions of the sequence alignments using a *T. villosa* codon frequency table was used to design primers to amplify a longer laccase gene sequence from *Trametes* sp. HR577.

Amplification of 18s rRNA Genes by PCR

gDNA purified from *Trametes* sp. HR577 was amplified using *Taq* DNA polymerase (New England BioLabs). The primers used were: *forward* 5'-GGAAGGGRTGTATTTATTAG (*EF4fwd*) and *reverse* 5'-GTAAAAGTCCTGGTTCCCC (*F5rev*). The PCR reaction mixture consisted of ~7 ng of genomic DNA, 2.5 ng μL^{-1} of both primers, 200 µM of each of the deoxyribonucleotide triphosphates (dNTPs), 1x polymerase buffer, 1.25 units of *Taq* DNA polymerase in a total reaction volume of 40 µL. The thermo-cycling program consisted of an initial denaturation at 95ºC for 1 minute, followed by 30 cycles of primer annealing at 48ºC for 1 minute with extension at 72ºC for 1 minute. After 30 cycles a final extension at 72ºC was carried out for 10 minutes. Amplification was performed in a PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, MA, USA).

Amplification of the ITS Region by PCR

cDNA purified from *Trametes* sp. HR577 was amplified using *Taq* DNA polymerase (New England BioLabs). The primers used were: *forward* 5'-TCCTCCGCTTATTGATATGC (*ITS5*) and *reverse* 5'-GGAAGTAAAAGTCGTAACAAGG (*ITS4*). The PCR reaction mixture consisted of ~7 ng of genomic DNA, 2 ng μL^{-1} of both primers, 200 μM of each of the deoxyribonucleotide triphosphates (dNTPs), 1x polymerase buffer, 0.5 units of *Taq* DNA polymerase in a total reaction volume of 50 μL. The thermo-cycling program consisted of an initial denaturation at 95ºC for 1 minute, followed by 30 cycles of primer annealing at 57ºC for 1 minute with extension at 72ºC for 1 minute. After 30 cycles a final extension at 72ºC was carried out for 10 minutes. Amplification was performed in an Eppendorf Thermal cycler.

Amplification of the Three Partial Laccase Genes from gDNA by PCR

gDNA purified from *Trametes* sp. HR577 was amplified using *Taq* DNA polymerase (New England BioLabs). The primers used were: *forward* 5'-ATTGGCACGGCTTCTTCC (*lccf*) and *reverse* 5'-GATCTGGATGGAGTCGAC (*lccr*). The PCR reaction mixture consisted of ~7 ng of genomic DNA, 2 ng μL^{-1} of both primers, 200 μM of each of the deoxyribonucleotide triphosphates (dNTPs), 1x polymerase buffer, 1.25 units of *Taq* DNA polymerase in a total reaction volume of 50 μL. The thermo-cycling program consisted of an initial denaturation at 95ºC for 1 minute, followed by 30 cycles of primer annealing at 55ºC for 1 minute with extension at 72ºC for 1 minute. After 30 cycles a final extension at 72ºC was carried out for 10 minutes. Amplification was performed in a PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, MA, USA).

The PCR the product was run on an agarose gel and the band was excised and the DNA purified using a QIAEX II Agarose gel extraction kit. The extracted PCR product was cloned into the pCR®2.1-Topo® vector and transformed into chemically competent *E. coli* cells (One Shot®, Invitrogen) using a TOPO TA cloning® kit (Invitrogen) according to the manufacturers protocols. The cells were plated onto LB plates containing X-Gal (50 μL of 300 μL per 500 mL), ampicillin (500 μL of a 50 $\mu g\ mL^{-1}$

solution) and IPTG (500 µL per 500 mL stock). The plates were incubated overnight at 37ºC. Ten white colonies were picked from plates and cultured in LB medium containing ampicillin (50 µL of a 50 µg mL^{-1} solution) overnight at 37ºC on a shaker. Plasmids were analysed by digestion with *Eco*RI (see protocol for digestion with *Eco*RI in the General Experimental Section) and agarose gel electrophoresis. Three plasmids were isolated using a spin plasmid kit (QIAprep Spin Plasmid Kit) and sent to Lark Technologies Inc for sequencing.

Amplification of Partial Laccase Gene from cDNA by PCR

cDNA purified from *Trametes* sp. HR577 was amplified using *Taq* DNA polymerase (New England BioLabs). The primers used were: *forward* 5'-CGGCGCCCATATGGGKTTRCAGCGYTTCAGYTTYTTYGT[60] (*Tlccf*) and *reverse* 5'-ACGGGATCCATTACTGGTTAGCCTCRCTYAANCCRTCRTA (*Tlccr*). The PCR reaction mixture consisted of ~7 ng of genomic DNA, 2 ng μL^{-1} of both primers, 200 µM of each of the deoxyribonucleotide triphosphates (dNTPs), 1x polymerase buffer, 2 units of *Taq* DNA polymerase in a total reaction volume of 50 µL. The thermo-cycling program consisted of an initial denaturation at 95ºC for 1 minute, followed by 30 cycles of primer annealing at 53ºC for 1 minute with extension at 72ºC for 1 minute. After 30 cycles a final extension at 72ºC was carried out for 10 minutes. Amplification was performed in an Eppendorf Thermal cycler.

7.4 CHAPTER 4 EXPERIMENTAL

Organism and Inoculum Production

Trametes sp. HR577 from the HRCC was used for all the experiments described in this chapter. The culture maintenance and inoculum production was identical to that described in the General Experimental section.

[60] Key to symbols: R=G+A, Y=C+T, N=A+C+G+T, K=G+T

Optimisation of Laccase Production

ENZYME EXPRESSION IN CULTURES GROWN ON SCS

Trametes sp. HR577 was grown on a fungal growth substrate (SCS) consisting of fresh *Pinus radiata* sawdust cornmeal starch mixture (Lestan *et al.* 1996). SCS was adjusted to 50% gravimetric water content, weighed (20 g) into vented glass petri dishes (85 mm diameter) and sterilised by autoclaving at 121°C and 103.5 kPa for 1 hour over 2 successive days. The sterile SCS was then centrally inoculated with an 8 mm mycelial plug from a 7 day old white-rot isolate grown on MEA. SCS inoculated cultures were incubated for 7 days at 20 or 25°C in the dark.

One gram (fresh weight) of colonised SCS inoculum was introduced into 15 mL of either standard liquid medium (see General Experimental), or sterile distilled water, or sodium acetate buffer (10 mM, pH 5) in a 250 mL Erlenmeyer flask. Control flasks were inoculated with 10 agar plugs (6 mm diameter) from the outer circumference of a fungal colony growing on a MEA plate (7 days). All results are for duplicate cultures. Flasks were incubated statically at room temperature (20-25°C) in the dark.

EFFECT OF COPPER ON LACCASE EXPRESSION IN CULTURE

Duplicate fungal cultures of *Trametes* sp. HR577 were grown in standard liquid medium (see General Experimental). A range of filter sterilised copper sulphate heptahydrate solutions (0, 4, 25, 50, 100, 200, 300, 400 and 500 μM) were added to cultures prior to inoculation. Eight agar plugs (6 mm diameter) from the outer circumference of a fungal colony growing on a MEA plate (7 days) were used as the inoculum. The fungi were grown in 15 mL stationary cultures in 250 mL Erlenmeyer flasks at 25°C in the dark.

EFFECT OF MANGANESE ON LACCASE EXPRESSION IN CULTURE

Duplicate fungal cultures of *Trametes* sp. HR577 were grown in standard liquid medium (see General Experimental). A range of filter sterilised manganese sulphate

monohydrate solutions (0, 2, 25, 50, 100, 200 and 300 μM) were added to cultures prior to inoculation. Eight agar plugs (6 mm diameter) from the outer circumference of a fungal colony growing on a MEA plate (7 days) were used as the inoculum. The fungi were grown in 15 mL stationary cultures in 250 mL Erlenmeyer flasks at 25°C in the dark.

Laccase Purification

Optimisation of Ammonium Sulphate Precipitation Step

The medium used for liquid culture contained, per litre, glucose (10 g), ammonium tartrate (1 g), potassium dihydrogen orthophosphate (1 g), sodium dihydrogen orthophosphate monohydrate (0.26 g), magnesium sulphate heptahydrate (0.5 g), and 2,2-dimethyl succinic acid (2.92 g). The medium was adjusted to pH 5.0 with potassium hydroxide, autoclaved and cooled before the addition of 1 mg thiamine (filter sterilised) and 10 mL trace elements solution (filter sterilised), which contained, per 100 mL, iron sulphate heptahydrate (50 mg), copper sulphate pentahydrate (100 mg), zinc sulphate heptahydrate (5 mg), and calcium chloride dihydrate (66 mg). A fungal culture (500 mL) was grown in a 5 L Erlenmeyer flask. Inoculum consisted of a whole MEA plate (7 day). On day 12 the culture was filtered through cheesecloth to separate mycelium from the filtrate.

50 mL aliquots of culture filtrate were subjected to ammonium sulphate precipitation using a range of different amounts of solid ammonium sulphate (0%, 25%, 30%, 40%, 50%, 75% and 95% ammonium sulphate w/v). The precipitate was separated from the culture fluid by decantation after centrifugation and dialysed to remove ammonium sulphate. The laccase activity in the decanted liquid and the dialysed protein mixtures was measured using ABTS as the substrate (see General Experimental). Total protein in the decanted liquid and the dialysed protein mixtures was measured using the Bio-Rad Protein Assay (see General Experimental).

ISOLATION OF TWO LACCASES FROM TRAMETES SP. HR577

The medium used for liquid cultures contained, per litre, glucose (10 g), ammonium tartrate (1 g), potassium dihydrogen orthophosphate (1 g), sodium dihydrogen orthophosphate monohydrate (0.26 g), magnesium sulphate heptahydrate (0.5 g), and 2,2-dimethyl succinic acid (2.92 g). The medium was adjusted to pH 5.0 with potassium hydroxide, autoclaved and cooled before the addition of 1 mg thiamine (filter sterilised) and 10 mL trace element solution (filter sterilised). The trace element solution contained, per 100 mL, iron sulphate heptahydrate (50 mg), copper sulphate pentahydrate (100 mg), zinc sulphate heptahydrate (5 mg), and calcium chloride dihydrate (66 mg). Cultures (500 mL) were grown in 5 L Erlenmeyer flasks. Inoculum consisted of a whole MEA plate (7 day). On day 12 cultures were filtered through cheesecloth to separate mycelium from the filtrate. Duplicate flasks were used to produce crude filtrate. The filtrate from duplicate cultures was combined for purification of laccase isozymes.

Ammonium sulphate (25% w/v) was added to the filtrate and the liquid was stirred overnight at 4ºC, centrifuged and the supernatant decanted from the insoluble fraction. Ammonium sulphate (50% w/v) was added to bring the total ammonium sulphate concentration up to 75% (w/v) and the liquid was stirred overnight at 4°C, centrifuged and the liquid decanted. The pellet was redissolved in 10 mM phosphate buffer (pH 7), dialysed overnight at 4°C and concentrated before filtering for anion exchange chromatography.

The crude extract was applied to a Source Q column (GE Healthcare), which had been equilibrated with 10 mM potassium phosphate buffer, pH 7.0, and was eluted with a stepwise sodium chloride gradient, which increased from 0 to 1.0 M. Two major peaks of laccase activity were observed and pooled separately. The first major peak of laccase activity came off the column in the flow through. These two laccases were named Laccase 1 and Laccase 2 based on their order of elution from the Source Q column. Both were concentrated by ultrafiltration (20 mL Vivaspins, 10,000 MW cutoff, NZMS) and dialysed against 10 mM phosphate, pH 7.0.

FURTHER PURIFICATION OF THE L1 LACCASE ACTIVE FRACTION FROM ANION EXCHANGE

Ammonium sulphate was added to the crude L1 laccase preparation to a final concentration of 15% (w/v), and it was further purified by chromatography on a Source Phe (GE Healthcare) column. Elution was with a linear ammonium sulphate gradient, which decreased from 15% to 5% (w/v). Three peaks of laccase activity were observed on the Source Phe column. These three peaks were pooled separately and named laccase L1a, L1b and L1c according to their order of elution from the Source Phe column. Laccases L1a, L1b and L1c were concentrated, dialysed against 10 mM sodium acetate (pH 5.0), snap frozen with liquid nitrogen and stored at -80ºC.

FURTHER PURIFICATION OF THE L2 LACCASE ACTIVE FRACTION FROM ANION EXCHANGE

Ammonium sulphate was added to the L2 pooled fractions to a final concentration of 15% (w/v), and was further purified by chromatography on a Source Phe (GE Healthcare) column. Elution was with a linear ammonium sulphate gradient, which decreased from 15% to 5% (w/v). L2 was further purified by a second anion exchange on a Source Q column equilibrated in 10 mM sodium acetate buffer, pH 5.0 and eluted with a linear gradient (0-1 M NaCl). The purified laccase isozyme L2 was concentrated, dialysed against 10 mM sodium acetate (pH 5.0), snap frozen with liquid nitrogen and stored at -80ºC.

Properties of Purified Laccases

ESTIMATE OF LACCASE MOLECULAR MASS AND DEGREE OF GLYCOSYLATION

The molecular mass of laccase isozymes L1c and L2 was estimated by SDS-PAGE from a plot of the $\log_{(\text{molecular weight})}$ versus the distance travelled for each of the protein bands.

N-linked carbohydrates in the purified laccase L1c were determined by treatment with PNGase F (donated by Trevor Loo, IMBS, Massey University). Purified L1c (1 µL of a

0.2 mg mL^{-1} solution in 10 mM acetate buffer, pH 5) was boiled for 5 minutes, and then incubated with PNGase F (2 µL of a 0.02 µg mL^{-1} solution in 10 mM phosphate buffer, pH 7) in 10 mM phosphate buffer (11 µL, pH 7) at room temperature for 16 hours. After this treatment, the molecular mass of the protein was estimated by SDS-PAGE as described above.

pH optimum

All laccase assays to determine the pH optima of L1c and L2 were carried out at 25ºC in 100 mM either citrate-sodium phosphate buffer (pH 2.2-7), glycine-sulphuric acid (pH 1-2.5) or sodium acetate (pH 4-5.5). Initial velocity was measured in 1 mL Quartz cuvettes with 1 cm path lengths on a Varian Cary 100 UV-visible spectrophotometer. Reactions were initiated by adding laccase. Laccase activities were determined with ABTS (0.5 mM) as the substrate. The wavelength used for determining catalytic reaction velocities was 420 nm for ABTS (ε=36,000 M^{-1} cm^{-1}). Datum points in all cases are means for triplicate cultures. A value of 100 was ascribed to the highest laccase activity and all other activities were expressed as a percentage of this value.

Substrate specificity of L1c and L2

All laccase catalytic assays were carried out at 25ºC. Assays to determine kinetic constants were carried out for both isozymes in 100 mM acetate buffer (pH 5). In addition, the catalytic assays were repeated using glycine-sulphuric acid buffer (pH 1.5 and pH 2 for L1c and L2, respectively). Initial velocity was measured in 1 mL Quartz cuvettes with 1 cm path lengths on a Varian Cary 100 UV-visible spectrophotometer. Reactions were initiated by adding laccase. Initial rates were calculated from the linear portion of the progress curve. The wavelength used for determining catalytic reaction velocities was 420 nm for ABTS (ε=36,000 M^{-1} cm^{-1}). The kinetic data obtained was fitted to a hyperbola by means of the Michaelis-Menten equation. The best values were determined by linear least-squares regression analysis. One unit of enzyme activity was defined as the amount of enzyme required to oxidise 1 µmol of ABTS per minute at 25ºC. Datum points in all cases are means for triplicate cultures.

TEMPERATURE FOR OPTIMUM ACTIVITY

The optimum temperature was determined between 25-80ºC in 100 mM acetate buffer (pH 5). All laccase assays were carried out in 100 mM acetate buffer (pH 5) with ABTS as the substrate (ε=36,000 M^{-1} cm^{-1} at 420 nm). Initial velocity was measured in 1 mL Quartz cuvettes with 1 cm path lengths on a Varian Cary 100 UV-visible spectrophotometer. Reactions were initiated by adding laccase. Datum points in all cases are means for triplicate cultures. A value of 100 was ascribed to the highest laccase activity and all other activities were expressed as a percentage of this value.

ENZYME STABILITY AT 50 AND 70 DEGREES

Thermal stability was investigated by incubating the enzyme at 50 or 70ºC for different times (0, 10, 30, 60, 120, 180 and 300 minutes at 50ºC and 0, 10, 20, 30, 60 minutes at 70ºC). Immediately afterwards, the enzyme was immersed in an ice bath and then residual activity was measured at 25ºC as previously described (see General Experimental). One unit of enzyme activity was defined as the amount of enzyme required to oxidise 1 µmol of ABTS per minute at 25ºC. Datum points in all cases are means for triplicate cultures. A value of 100 was ascribed to the highest laccase activity and all other activities were expressed as a percentage of this value.

THE EFFECT OF ETHANOL ON LACCASE ACTIVITY

Ethanol concentrations of 0-50% v/v were used to examine the effect of ethanol on laccase activity. Reactions were carried out in 100 mM sodium acetate buffer (pH 5) at 25ºC with ABTS as the substrate (ε=36,000 M^{-1} cm^{-1} at 420 nm). Initial velocity was measured in 1 mL Quartz cuvettes with 1 cm path lengths on a Varian Cary 100 UV-visible spectrophotometer. Reactions were initiated by adding laccase. Datum points in all cases are means for triplicate cultures. A value of 100 was ascribed to the highest laccase activity and all other activities were expressed as a percentage of this value.

DEPENDENCE ON OXYGEN

Nitrogen gas was bubbled through a solution of 1 mM ABTS in 10 mM sodium acetate buffer (pH 5.0) in a glove bag under an atmosphere of nitrogen for 1 hour at room temperature. An aliquot of this mixture was added to several cuvettes that were sealed with septa prior to removal from the nitrogen atmosphere to keep the mixtures under nitrogen. A control solution of ABTS in 10 mM sodium acetate buffer (pH 5.0) was kept at room temperature outside the glove bag. A duplicate set of cuvettes that had been subjected to nitrogen treatment had air bubbled through for two hours to restore some of the dissolved oxygen.

Reactions to examine the effect of oxygen on laccase activity were carried out in 100 mM sodium acetate buffer (pH 5) at 25ºC with ABTS as the substrate (ε=36,000 M^{-1} cm^{-1} at 420 nm). Initial velocity was measured in 1 mL Quartz cuvettes with 1 cm path lengths on a Varian Cary 100 UV-visible spectrophotometer. Reactions were initiated by adding laccase. Datum points in all cases are means for triplicate cultures. A value of 100 was ascribed to the highest laccase activity and all other activities were expressed as a percentage of this value.

7.5 CHAPTER 5 EXPERIMENTAL

Organism and Inoculum Production

Trametes sp. HR577 from the HRCC was used for all the experiments described in this section. The culture maintenance and inoculum production was identical to that described in the General Experimental section.

PCP Degradation by Whole Cultures of Trametes *sp. HR577*

CULTURE CONDITIONS

Triplicate cultures of *Trametes* sp. HR577 were grown in 15 mL of standard liquid medium in 250 mL Erlenmeyer flasks or 500 mL Schott bottles with Teflon-lined lids. Two agar plugs (6 mm diameter) from the outer circumference of a fungal colony growing on a MEA plate (7 days) were used as the inoculum. Cultures were grown at 25°C in the dark for five days. Culture flasks and bottles were loosely capped over the first five days of the experiment. PCP control cultures were not inoculated with *Trametes* sp. HR577. On day 5, PCP (50 mg L^{-1} dissolved in methanol 2.5% v/v) was added to all cultures except for the 'no PCP' control cultures. Laccase isozymes (100 U mL^{-1} of either purified L1c, purified L2 or crude L1a-c[61]) were also added to some flasks. To prevent loss of PCP or PCP degradation products through volatilisation, the lids were sealed after addition of PCP and purified laccase isozymes. All experiments were carried out in triplicate. Cultures were incubated at 25°C in the dark for a further 72 hours and then extracted as described below.

EXTRACTION OF WHOLE CULTURES FOR HPLC ANALYSIS

The contents of the liquid culture samples (15 mL) were extracted with 100 mL acetone/hexane (2:3 v/v) and 1 mL concentrated orthophosphoric acid by shaking for 60 minutes on a flat bed shaker (300 rpm). After shaking, 150 mL of distilled water was added to separate the hexane phase containing extracted PCP and 0.15 mL was transferred to a 20 mL scintillation vial. Twenty microlitres of iso-butanol was added as keeper solvent and hexane was removed under a gentle stream of nitrogen gas at room temperature. Then 1 mL methanol/water (80:20 v/v) was added to each vial. The vials were vortexed and the contents transferred to a GC vial and stored under refrigeration until analysed by HPLC. PCP recovery during sample workup was calculated from control samples spiked with a known amount of PCP and was typically 90 to 100%.

[61] The term 'purified laccase' used in this section refers to laccase isozymes L1c and L2 from *Trametes* sp. HR577. Purification of these isozymes is described in the Chapter 4 experimental section. The terms 'crude laccase' and 'crude L1a-c' refer to a mixture of laccase isozymes L1a, L1b and L1c partially purified from cultures of *Trametes* sp. HR577 by ammonium sulphate precipitation, dialysis and passage through a Source Q anion exchange column (see Chapter 4 experimental section).

The amount of PCP measured in a corresponding batch of samples was corrected by the appropriate recovery factor. The HPLC set up used to analyse culture extracts is outlined in the General Experimental section.

ACETYLATION OF CULTURE EXTRACTS FOR GC ANALYSIS

Sample extracts and control extracts in hexane (0.050 mL) were added to 15 mL screw cap test tubes containing 0.1 M sodium hydroxide (5 mL), firmly capped (with Teflon-lined lids) and partitioned by shaking at 300 rpm on a flatbed shaker for 30 minutes. Acetic anhydride (0.5 mL) was added and the tubes were shaken for a further 10 minutes at 300 rpm. A predetermined volume of heptane[62] was added to tubes and they were further shaken at 300 rpm for 1 hour followed by centrifugation at 2,000 rpm for 5 minutes. The top heptane layer was transferred to a 2 mL autosampler vial for GC analysis.

PCP Degradation by Laccase Isozymes L1c and L2 from **Trametes** *sp. HR577*

CULTURE CONDITIONS

Extracellular laccase isozymes from *Trametes* sp. HR577 were isolated from growth media as described previously (see Chapter 4 Experimental). Laccase reaction mixtures (1 mL) consisted of enzyme (100 U mL^{-1})[63], PCP (25 mg L^{-1}, in methanol 2.5% v/v) in 10 mM sodium acetate (pH 5.0). All reactions were carried out in 10 mL screw cap glass culture tubes sealed with Teflon lined lids. Reactions were carried out at 25°C, statically in the dark for 72 hours. Controls for all experiments included autoclaved enzyme and no enzyme. All reactions were carried out in triplicate.

[62] The volume of heptane depended on HPLC results: if the PCP remaining in cultures was 0-10 μg/mL, 2 mL was added. If the PCP remaining was 30-40 μg/mL PCP, 10 mL heptane was added.
[63] One unit of enzyme activity was defined as the amount of enzyme required to oxidise 1 μmol of ABTS per minute.

PCP EXTRACTION FOR HPLC ANALYSIS

The contents of the culture tubes were extracted by adding methanol (9 mL) and shaking for 30 minutes on a flat bed shaker (350 rpm). The tubes were then centrifuged (7 minutes at 1,000 rpm) and an aliquot removed for HPLC analysis. Samples were stored under refrigeration until analysis by HPLC. PCP recovery during sample workup was calculated from control samples spiked with a known amount of PCP and was typically 90 to 100%. The amount of PCP, measured in a corresponding batch of samples, was corrected by the appropriate recovery factor. HPLC analysis was carried out as previously described (see General Experimental).

ACETYLATION OF LACCASE CULTURE EXTRACTS FOR GC ANALYSIS

Extracted PCP culture from laccase experiments in 9:1 methanol/water (2 mL), concentrated orthophosphoric acid (0.15 mL), and hexane (2 mL) were added to a 15 mL screw cap test tube and shaken vigorously for 30 minutes (300 rpm on a flatbed shaker) and then centrifuged (2,000 rpm for 5 minutes) to extract chlorophenols into the hexane layer. Sample extracts and control extracts in hexane (0.050 mL) were added to 15 mL screw cap test tubes containing 0.1 M sodium hydroxide (5 mL), firmly capped and partitioned by shaking at 300 rpm on a flatbed shaker for 30 minutes. Acetic anhydride (0.5 mL) was added and the tubes were shaken for a further 10 minutes at 300 rpm. Heptane (2 mL, 1 mL for chlorophenol controls) was added to tubes and they were further shaken at 300 rpm for 1 hour followed by centrifugation at 2,000 rpm for 5 minutes. The top heptane layer was transferred to a 2 mL autosampler vial for GC analysis. GC analysis was carried out as previously described (see General Experimental).

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CHAPTER 2 APPENDIX

WHITE-ROT ISOLATE DATABASE TABLES

These tables contain the information that will used to select isolates suited to different contaminated sites. Only the research carried out for inclusion in this thesis is reported here. To simplify the database for this appendix, the database has been split into three different tables. The first table contains information on temperature tolerance of white-rot isolates at various temperatures (0-40ºC). The second table lists the growth of isolates on PCP-amended agar (50 and 200 mg L^{-1}) in agar. The final table give the results for studies carried out to determine the sensitivity of fungal cultures to 10,000 mg L^{-1} creosote in agar.

Because some of the information on isolates is commercially sensitive, all isolates have been given a number that does not match their culture collection number.

Table 1: Temperature data for fungal isolates. The ranking system used in this table was used to select fungi that were able to grow well over a range of different temperatures. Fungal cultures were grown at several different temperatures (0-40ºC) for seven days and fungal growth was measured in two directions. All plates were then incubated at 25ºC for a further seven days and the fungal growth was again measured. The rankings given for recovery at 0, 35 and 40ºC indicate the following:

0 Isolate does not grow at this temperature and does not resume growth after seven days at 25ºC

1 Isolate does not grow at this temperature but has some growth once it has been move to the 25ºC incubator for seven days.

2 Fungus grows at this temperature

3 Fungus grows as fast or faster at this temperature than at 25ºC

Code no.	Average growth at 25°C (7d)	Best growth temperature	Growth /recovery at 0°C	Growth /recovery at 35°C	Growth /recovery at 40°C
1	77	25	1	3	3
2	77	25	2	3	3
3	47	25	1	3	3
4	45	25 or 30	1	3	0
5	77	40	1	3	3
6	38	25	1	3	3
7	ND	ND	ND	ND	ND
8	18	25	1	3	0
9	ND	ND	ND	ND	ND
10	77	30	1	3	3
11	38	25	1	3	3
12	77	25	1	3	3
13	13	25	1	1	1
14	77	30	1	3	3
15	47	25	1	3	3
16	ND	ND	ND	ND	ND
17	ND	ND	ND	ND	ND
18	46	25	1	3	3
19	ND	ND	ND	ND	ND
20	ND	ND	ND	ND	ND
21	77	30	1	3	3
22	18	25	1	2	0
23	46	25	1	3	0
24	46	25 or 30	1	3	3
25	48	25	1	3	0
26	46	25	1	3	0
27	ND	ND	ND	ND	ND
28	44	25 or 30	1	3	3
29	11	20	1	0	0
30	77	40	1	3	3
31	ND	ND	ND	ND	ND
32	ND	ND	ND	ND	ND
33	ND	ND	ND	ND	ND
34	ND	ND	ND	ND	ND
35	17	25	2	3	0
36	13	20	1	0	0
37	59	35	1	3	3
38	ND	ND	ND	ND	ND
39	ND	ND	ND	ND	ND
40	48	25	1	3	3

Code no.	Average growth at 25°C (7d)	Best growth temperature	Growth /recovery at 0°C	Growth /recovery at 35°C	Growth /recovery at 40°C
41	77	25 or 30	1	3	3
42	39	25	1	3	3
43	72	25	1	3	3
44	77	30	1	3	3
45	39	25	1	3	0
46	46	25 or 30	1	3	3
47	42	25	1	3	0
48	45	25 or 30	1	3	3
49	29	25	1	3	0
50	77	25 or 30	2	3	3
51	71	25 or 30	2	3	3
52	69	25	1	3	3
53	77	25	1	3	0
54	ND	ND	ND	ND	ND
55	ND	ND	ND	ND	ND
56	31	25	1	3	1
57	38	25 or 30	1	3	1
58	47	25	1	3	3
59	77	40	1	3	3
60	ND	ND	ND	ND	ND
61	ND	ND	ND	ND	ND
62	41	25	1	3	0
63	43	25	1	3	1
64	ND	ND	ND	ND	ND
65	ND	ND	ND	ND	ND
66	ND	ND	ND	ND	ND
67	ND	ND	ND	ND	ND
68	ND	ND	ND	ND	ND
69	19	25	1	3	0
70	ND	ND	ND	ND	ND
71	17	25	1	3	0
72	ND	ND	ND	ND	ND
73	77	25 or 30	1	3	3
74	30	25 or 30	1	3	3
75	48	25	1	3	3
76	ND	ND	ND	ND	ND
77	19	25	2	3	0
78	ND	ND	ND	ND	ND
79	ND	ND	ND	ND	ND
80	ND	ND	ND	ND	ND

Code no.	Average growth at 25°C (7d)	Best growth temperature	Growth /recovery at 0°C	Growth /recovery at 35°C	Growth /recovery at 40°C
81	ND	ND	ND	ND	ND
82	18	25	1	0	0
83	ND	ND	ND	ND	ND
84	ND	ND	ND	ND	ND
85	77	40	1	3	3
86	18	25	1	0	0
87	16	25	1	0	0
88	16	25	1	0	0
89	ND	ND	ND	ND	ND
90	18	25	1	2	0
91	21	25	1	3	0
92	17	20	1	3	0
93	17	25	1	3	0
94	18	25	1	3	0
95	18	25	1	3	0
96	17	25	1	3	0
97	17	25	1	3	0
98	77	40	1	3	3
99	46	25	1	3	0
100	77	35	1	3	3
101	45	25	1	3	2
102	77	25	1	3	2
103	18	25	1	3	0
104	9	20 or 25	1	3	0
105	ND	ND	ND	ND	ND
106	ND	ND	ND	ND	0
107	46	25	1	3	0
108	46	25 or 30	1	3	0
109	77	25	1	3	0
110	45	25	1	3	0
111	63	25 or 30	1	3	0
112	46	25	1	3	3
113	48	25 or 30	1	3	3
114	11	20	1	0	0
115	42	20	1	0	0
116	66	25	1	0	0
117	61	25	1	0	0
118	37	20	1	0	0
119	49	20	1	0	0
120	30	20	1	0	0

Code no.	Average growth at 25°C (7d)	Best growth temperature	Growth /recovery at 0°C	Growth /recovery at 35°C	Growth /recovery at 40°C
121	43	20	1	0	0
122	45	20	1	0	0
123	54	25	1	0	0
124	77	20	1	0	0
125	38	20	1	0	0
126	39	20	1	0	0
127	35	20	1	0	0
128	65	25	1	0	0
129	44	20	1	0	0
130	43	20	1	0	0
131	19	25	1	0	0
132	27	20	1	0	0
133	43	25	1	0	0
134	39	20	1	0	0
135	ND	ND	ND	ND	ND
136	8	20	1	0	0
137	47	25	1	3	3
138	49	20	1	0	0
139	25	20	1	0	0
140	46	20	1	0	0
141	48	20	1	0	0
142	59	25 or 30	1	3	3
143	ND	ND	ND	ND	ND
144	16	25	1	2	2
145	58	25 or 30	1	3	0
146	46	25	1	3	0
147	77	40	1	3	3
148	62	30	1	3	0
149	61	25 or 30	1	3	3
150	47	25	1	3	3
151	47	25	1	3	3
152	77	25 or 30	1	3	3
153	13	20	1	3	0
154	ND	ND	ND	ND	ND
155	45	25 or 30	1	3	0
156	ND	ND	ND	ND	ND
157	46	25	1	3	1
158	ND	ND	ND	ND	ND
159	ND	ND	ND	ND	ND
160	44	25	1	3	0

Code no.	Average growth at 25°C (7d)	Best growth temperature	Growth /recovery at 0°C	Growth /recovery at 35°C	Growth /recovery at 40°C
161	18	25	1	3	0
162	49	25	1	3	3
163	ND	ND	ND	ND	ND
164	ND	ND	ND	ND	ND
165	77	30	1	3	3
166	41	25 or 30	1	3	3
167	16	25	1	0	0
168	ND	ND	ND	ND	ND
169	ND	ND	ND	ND	ND
170	ND	ND	ND	ND	ND
171	ND	ND	ND	ND	ND
172	9	20	1	0	0
173	7	ND	ND	ND	ND
174	ND	ND	ND	ND	ND
175	19	25	1	3	0
176	42	25 or 30	1	3	3
177	ND	ND	ND	ND	ND
178	ND	ND	ND	ND	ND
179	ND	ND	ND	ND	ND
180	46	25 or 30	1	3	0
181	49	25	1	3	3
182	77	30	1	3	3
183	77	25	0	3	3
184	ND	ND	ND	ND	ND
185	20	ND	ND	ND	ND
186	48	25 or 30	1	3	3
187	49	25	1	3	3
188	49	25	1	3	3
189	ND	ND	ND	ND	ND
190	6	ND	ND	ND	ND
191	ND	ND	ND	ND	ND
192	ND	ND	ND	ND	ND
193	39	25 or 30	1	3	3
194	46	25	1	3	3
195	48	25	1	3	3
196	42	ND	ND	ND	ND
197	ND	ND	ND	ND	ND
198	ND	ND	ND	ND	ND
199	49	25	1	3	3
200	47	25	1	3	3

Code no.	Average growth at 25°C (7d)	Best growth temperature	Growth /recovery at 0°C	Growth /recovery at 35°C	Growth /recovery at 40°C
201	48	25	1	3	3
202	46	25 or 30	1	3	3
203	48	25	1	3	3
204	ND	ND	ND	ND	ND
205	48	25	1	3	3
206	46	25	1	3	3
207	47	25	1	3	3
208	ND	ND	ND	ND	ND
209	47	25	1	3	3
210	48	25	1	3	3
211	47	25	1	3	3
212	49	25	1	3	3
213	ND	ND	ND	ND	ND
214	48	25	1	3	3
215	ND	ND	ND	ND	ND
216	ND	ND	ND	ND	ND
217	ND	ND	ND	ND	ND
218	ND	ND	ND	ND	ND
219	47	25	1	3	3
220	ND	ND	ND	ND	ND
221	ND	ND	ND	ND	ND
222	47	25	1	3	3
223	47	25	1	3	3
224	46	25	1	3	3
225	38	25	1	3	3
226	ND	ND	ND	ND	ND
227	ND	ND	ND	ND	ND
228	49	25	1	3	3
229	ND	ND	ND	ND	ND
230	44	25	1	3	1
231	47	25	1	3	3
232	47	25	1	3	3
233	ND	ND	ND	ND	ND
234	ND	ND	ND	ND	ND
235	21	25	1	0	0
236	ND	ND	ND	ND	ND
237	47	25	1	3	3
238	14	25	1	2	0
239	ND	ND	ND	ND	ND
240	47	25	1	3	3

Code no.	Average growth at 25°C (7d)	Best growth temperature	Growth /recovery at 0°C	Growth /recovery at 35°C	Growth /recovery at 40°C
241	ND	ND	ND	ND	ND
242	ND	ND	ND	ND	ND
243	77	25	1	3	3
244	20	25	1	0	0
245	77	25	1	3	3
246	72	25	1	3	3
247	72	25	1	2	3
248	ND	ND	ND	ND	ND
249	ND	ND	ND	ND	ND
250	ND	ND	ND	ND	ND
251	ND	ND	ND	ND	ND
252	ND	ND	ND	ND	ND
253	77	40	1	3	3
254	ND	ND	ND	ND	ND
255	ND	ND	ND	ND	ND
256	ND	ND	ND	ND	ND
257	ND	ND	ND	ND	ND
258	ND	ND	ND	ND	ND
259	ND	ND	ND	ND	ND
260	ND	ND	ND	ND	ND
261	ND	ND	ND	ND	ND
262	ND	ND	ND	ND	ND
263	52	25	1	0	3
264	ND	ND	ND	ND	ND
265	ND	ND	ND	ND	ND
266	ND	ND	ND	ND	ND
267	ND	ND	ND	ND	ND
268	ND	ND	ND	ND	ND
269	ND	ND	ND	ND	ND
270	ND	ND	ND	ND	ND
271	11	20	1	0	0
272	ND	ND	ND	ND	ND
273	ND	ND	ND	ND	ND
274	ND	ND	ND	ND	ND
275	ND	ND	ND	ND	ND
276	ND	ND	ND	ND	ND
277	ND	ND	ND	ND	ND
278	ND	ND	ND	ND	ND
279	ND	ND	ND	ND	ND
280	ND	ND	ND	ND	ND

Code no.	Average growth at 25°C (7d)	Best growth temperature	Growth /recovery at 0°C	Growth /recovery at 35°C	Growth /recovery at 40°C
281	ND	ND	ND	ND	ND
282	ND	ND	ND	ND	ND
283	ND	ND	ND	ND	ND
284	ND	ND	ND	ND	ND
285	ND	ND	ND	ND	ND
286	ND	ND	ND	ND	ND
287	ND	ND	ND	ND	ND
288	ND	ND	ND	ND	ND
289	ND	ND	ND	ND	ND
290	ND	ND	ND	ND	ND
291	ND	ND	ND	ND	ND
292	ND	ND	ND	ND	ND
293	ND	ND	ND	ND	ND
294	48	25	1	3	3
295	ND	ND	ND	ND	ND
296	ND	ND	ND	ND	ND
297	ND	ND	ND	ND	ND
298	ND	ND	ND	ND	ND
299	ND	ND	ND	ND	ND
300	ND	ND	ND	ND	ND
301	ND	ND	ND	ND	ND
302	ND	ND	ND	ND	ND
303	ND	ND	ND	ND	ND
304	ND	ND	ND	ND	ND
305	ND	ND	ND	ND	ND
306	ND	ND	ND	ND	ND
307	ND	ND	ND	ND	ND
308	ND	ND	ND	ND	ND
309	ND	ND	ND	ND	ND
310	ND	ND	ND	ND	ND
311	ND	ND	ND	ND	ND
312	ND	ND	ND	ND	ND
313	37	25 or 30	1	3	3
314	ND	ND	ND	ND	ND
315	ND	ND	ND	ND	ND
316	ND	ND	ND	ND	ND
317	ND	ND	ND	ND	ND
318	ND	ND	ND	ND	ND
319	ND	ND	ND	ND	ND
320	ND	ND	ND	ND	ND

Code no.	Average growth at 25°C (7d)	Best growth temperature	Growth /recovery at 0°C	Growth /recovery at 35°C	Growth /recovery at 40°C
321	ND	ND	ND	ND	ND
322	ND	ND	ND	ND	ND
323	ND	ND	ND	ND	ND
324	ND	ND	ND	ND	ND
325	ND	ND	ND	ND	ND
326	ND	ND	ND	ND	ND
327	ND	ND	ND	ND	ND
328	ND	ND	ND	ND	ND
329	ND	ND	ND	ND	ND
330	ND	ND	ND	ND	ND
331	ND	ND	ND	ND	ND
332	ND	ND	ND	ND	ND
333	ND	ND	ND	ND	ND
334	ND	ND	ND	ND	ND
335	16	25	1	0	0
336	ND	ND	ND	ND	ND
337	ND	ND	ND	ND	ND
338	ND	ND	ND	ND	ND
339	ND	ND	ND	ND	ND
340	ND	ND	ND	ND	ND
341	72	ND	ND	ND	ND
342	ND	ND	ND	ND	ND
343	66	ND	ND	ND	ND
344	ND	ND	ND	ND	ND
345	ND	ND	ND	ND	ND
346	ND	ND	ND	ND	ND
347	ND	ND	ND	ND	ND
348	ND	ND	ND	ND	ND
349	ND	ND	ND	ND	ND
350	ND	ND	ND	ND	ND
351	ND	ND	ND	ND	ND
352	ND	ND	ND	ND	ND
353	ND	ND	ND	ND	ND
354	ND	ND	ND	ND	ND
355	ND	ND	ND	ND	ND
356	ND	ND	ND	ND	ND
357	ND	ND	ND	ND	ND
358	ND	ND	ND	ND	ND
359	77	25 or 30	1	3	3
360	ND	ND	ND	ND	ND

Code no.	Average growth at 25°C (7d)	Best growth temperature	Growth /recovery at 0°C	Growth /recovery at 35°C	Growth /recovery at 40°C
361	ND	ND	ND	ND	ND
362	77	25	1	3	0
363	ND	ND	ND	ND	ND
364	ND	ND	ND	ND	ND
365	ND	ND	ND	ND	ND
366	ND	ND	ND	ND	ND
367	ND	ND	ND	ND	ND
368	ND	ND	ND	ND	ND
369	ND	ND	ND	ND	ND
370	ND	ND	ND	ND	ND
371	ND	ND	ND	ND	ND
372	ND	ND	ND	ND	ND
373	ND	ND	ND	ND	ND
374	49	25	1	3	2
375	48	25	1	3	3
376	ND	ND	ND	ND	ND
377	ND	ND	ND	ND	ND
378	ND	ND	ND	ND	ND
379	ND	ND	ND	ND	ND
380	ND	ND	ND	ND	ND
381	ND	ND	ND	ND	ND
382	ND	ND	ND	ND	ND
383	ND	ND	ND	ND	ND
384	ND	ND	ND	ND	ND
385	ND	ND	ND	ND	ND
386	ND	ND	ND	ND	ND
387	ND	ND	ND	ND	ND
388	ND	ND	ND	ND	ND
389	ND	ND	ND	ND	ND
390	48	25	1	3	3
391	ND	ND	ND	ND	ND
392	ND	ND	ND	ND	ND
393	47	25	1	3	3
394	ND	ND	ND	ND	ND
395	39	25 or 30	1	3	3
396	ND	ND	ND	ND	ND
397	ND	ND	ND	ND	ND
398	ND	ND	ND	ND	ND
399	ND	ND	ND	ND	ND
400	ND	ND	ND	ND	ND

Code no.	Average growth at 25°C (7d)	Best growth temperature	Growth /recovery at 0°C	Growth /recovery at 35°C	Growth /recovery at 40°C
401	ND	ND	ND	ND	ND
402	ND	ND	ND	ND	ND
403	ND	ND	ND	ND	ND
404	ND	ND	ND	ND	ND
405	ND	ND	ND	ND	ND
406	ND	ND	ND	ND	ND
407	ND	ND	ND	ND	ND
408	ND	ND	ND	ND	ND
409	ND	ND	ND	ND	ND
410	ND	ND	ND	ND	ND
411	ND	ND	ND	ND	ND
412	ND	ND	ND	ND	ND
413	ND	ND	ND	ND	ND
414	ND	ND	ND	ND	ND
415	ND	ND	ND	ND	ND
416	ND	ND	ND	ND	ND
417	ND	ND	ND	ND	ND
418	ND	ND	ND	ND	ND
419	ND	ND	ND	ND	ND
420	ND	ND	ND	ND	ND
421	ND	ND	ND	ND	ND
422	ND	ND	ND	ND	ND
423	ND	ND	ND	ND	ND
424	ND	ND	ND	ND	ND
425	ND	ND	ND	ND	ND
426	ND	ND	ND	ND	ND
427	ND	ND	ND	ND	ND
428	ND	ND	ND	ND	ND
429	ND	ND	ND	ND	ND
430	ND	ND	ND	ND	ND
431	ND	ND	ND	ND	ND
432	ND	ND	ND	ND	ND
433	ND	ND	ND	ND	ND
434	ND	ND	ND	ND	ND
435	ND	ND	ND	ND	ND
436	ND	ND	ND	ND	ND
437	ND	ND	ND	ND	ND
438	ND	ND	ND	ND	ND
439	ND	ND	ND	ND	ND
440	ND	ND	ND	ND	ND

Code no.	Average growth at 25°C (7d)	Best growth temperature	Growth /recovery at 0°C	Growth /recovery at 35°C	Growth /recovery at 40°C
441	ND	ND	ND	ND	ND
442	ND	ND	ND	ND	ND
443	ND	ND	ND	ND	ND
444	ND	ND	ND	ND	ND
445	ND	ND	ND	ND	ND
446	ND	ND	ND	ND	ND
447	ND	ND	ND	ND	ND
448	ND	ND	ND	ND	ND
449	ND	ND	ND	ND	ND
450	ND	ND	ND	ND	ND
451	ND	ND	ND	ND	ND
452	ND	ND	ND	ND	ND
453	ND	ND	ND	ND	ND
454	ND	ND	ND	ND	ND
455	ND	ND	ND	ND	ND
456	ND	ND	ND	ND	ND
457	ND	ND	ND	ND	ND
458	30	25 or 30	1	3	3
459	77	25	1	3	1
460	77	25	1	3	3
461	57	20	1	3	2
462	51	35	1	3	3
463	44	25	1	3	0
464	77	30	1	3	3
465	39	30	1	3	3
466	58	25	1	2	0
467	51	30	1	3	3
468	22	25	1	2	0
469	77	25 or 30	1	3	3
470	77	25 or 30	1	3	3
471	77	25 or 30	1	3	3
472	77	40	1	3	3
473	77	25 or 30	1	3	3
474	77	25 or 30	1	3	3
475	65	25 or 30	1	3	3
476	77	30	1	3	3
477	77				
478	21	25	1	2	0
479	36	25	1	2	0
480	ND	35	1	2	3
481	ND	ND	ND	ND	ND
482	ND	ND	ND	ND	ND

Table 2: PCP sensitivity of fungal isolates. Fungal cultures were grown at 25ºC for fourteen days on PCP amended agar (either 0, 50 or 200 mg L^{-1} PCP in agar) and growth was measured in two directions. Growth of cultures on 50 mg L^{-1} PCP in agar is listed as both the actual growth (mm) and % growth relative to the control cultures. Resistance to 200 mg L^{-1} PCP amended-agar is listed as yes, no or ND (not determined). Actual growth for cultures that were able to tolerate 200 mg L^{-1} PCP in agar and were selected for further studies are listed in Table 2.1 (see Chapter 2).

Code no.	Growth on 50 mg L^{-1} PCP-amended agar	% Growth 50 mg L^{-1} compared to control	Culture grows on 200 mg L^{-1} PCP-amended agar
1	9	23	No
2	15	39	Yes
3	0	0	ND
4	19	52	No
5	2	4	No
6	17	47	No
7	ND	ND	ND
8	0	0	ND
9	ND	ND	ND
10	0	0	ND
11	0	0	ND
12	9	25	Yes
13	0	0	ND
14	0	0	ND
15	0	0	ND
16	ND	ND	ND
17	ND	ND	ND
18	0	0	ND
19	ND	ND	ND
20	ND	ND	ND
21	37	100	Yes
22	0	0	ND
23	24	64	No
24	26	70	No
25	12	31	No
26	ND	ND	ND
27	ND	ND	ND
28	12	33	No
29	0	0	ND
30	0	0	ND
31	ND	ND	ND
32	ND	ND	ND
33	ND	ND	ND
34	ND	ND	ND
35	4	21	Yes
36	0	0	ND
37	0	0	ND
38	ND	ND	ND
39	ND	ND	ND
40	0	0	ND

Code no.	Growth on 50 mg L^{-1} PCP-amended agar	% Growth 50 mg L^{-1} compared to control	Culture grows on 200 mg L^{-1} PCP-amended agar
41	0	0	ND
42	2	5	Yes
43	0	0	ND
44	0	0	Yes
45	ND	ND	ND
46	0	0	ND
47	12	31	No
48	0	0	ND
49	0	0	ND
50	19	50	Yes
51	0	0	ND
52	0	0	ND
53	0	0	ND
54	ND	ND	ND
55	ND	ND	ND
56	0	0	ND
57	0	0	ND
58	0	0	ND
59	0	0	ND
60	ND	ND	ND
61	ND	ND	ND
62	0	0	ND
63	0	0	ND
64	ND	ND	ND
65	ND	ND	ND
66	ND	ND	ND
67	ND	ND	ND
68	ND	ND	ND
69	0	0	ND
70	ND	ND	ND
71	0	0	ND
72	ND	ND	ND
73	0	0	ND
74	0	0	ND
75	0	0	ND
76	ND	ND	ND
77	0	0	ND
78	ND	ND	ND
79	ND	ND	ND
80	ND	ND	ND

Code no.	Growth on 50 mg L^{-1} PCP-amended agar	% Growth 50 mg L^{-1} compared to control	Culture grows on 200 mg L^{-1} PCP-amended agar
81	ND	ND	ND
82	11	60	Yes
83	ND	ND	ND
84	ND	ND	ND
85	0	0	ND
86	10	51	Yes
87	11	63	Yes
88	0	0	ND
89	ND	ND	ND
90	5	24	No
91	0	0	ND
92	0	0	ND
93	0	0	ND
94	0	0	ND
95	0	0	ND
96	0	0	ND
97	0	0	ND
98	0	0	ND
99	0	0	ND
100	0	0	ND
101	0	0	ND
102	0	0	ND
103	0	0	ND
104	1	19	No
105	0	0	ND
106	2	15	No
107	0	0	ND
108	0	0	ND
109	0	0	ND
110	0	0	ND
111	0	0	ND
112	0	0	ND
113	21	56	No
114	0	0	ND
115	0	0	ND
116	10	28	Yes
117	0	0	ND
118	20	53	No
119	8	22	No
120	0	0	ND

Code no.	Growth on 50 mg L^{-1} PCP-amended agar	% Growth 50 mg L^{-1} compared to control	Culture grows on 200 mg L^{-1} PCP-amended agar
121	4	10	No
122	0	0	ND
123	0	0	ND
124	0	0	ND
125	2	5	Yes
126	0	0	ND
127	18	49	No
128	0	0	ND
129	0	0	ND
130	10	26	Yes
131	9	49	Yes
132	0	0	ND
133	0	0	ND
134	13	35	No
135	ND	ND	ND
136	0	1	No
137	0	0	ND
138	5	14	No
139	11	28	No
140	16	44	No
141	13	34	No
142	0	0	ND
143	ND	ND	ND
144	0	0	ND
145	0	0	ND
146	0	0	ND
147	0	0	ND
148	0	0	ND
149	0	0	ND
150	18	49	No
151	0	0	ND
152	0	0	ND
153	2	14	No
154	ND	ND	ND
155	0	0	ND
156	ND	ND	ND
157	0	0	ND
158	ND	ND	ND
159	ND	ND	ND
160	0	0	ND

Code no.	Growth on 50 mg L^{-1} PCP-amended agar	% Growth 50 mg L^{-1} compared to control	Culture grows on 200 mg L^{-1} PCP-amended agar
161	0	0	ND
162	0	0	ND
163	ND	ND	ND
164	ND	ND	ND
165	29	78	Yes
166	8	20	No
167	8	47	Yes
168	ND	ND	ND
169	ND	ND	ND
170	ND	ND	ND
171	ND	ND	ND
172	0	0	ND
173	0	0	ND
174	ND	ND	ND
175	0	0	ND
176	4	11	No
177	ND	ND	ND
178	ND	ND	ND
179	ND	ND	ND
180	1	27	No
181	0	0	ND
182	0	0	ND
183	37	100	No
184	ND	ND	ND
185	ND	ND	ND
186	12	31	No
187	22	58	No
188	15	41	No
189	ND	ND	ND
190	ND	ND	ND
191	ND	ND	ND
192	ND	ND	ND
193	17	47	No
194	0	0	ND
195	5	14	No
196	ND	ND	ND
197	ND	ND	ND
198	ND	ND	ND
199	0	0	ND
200	17	45	No

Code no.	Growth on 50 mg L^{-1} PCP-amended agar	% Growth 50 mg L^{-1} compared to control	Culture grows on 200 mg L^{-1} PCP-amended agar
201	13	34	No
202	0	0	ND
203	23	63	No
204	ND	ND	ND
205	0	0	ND
206	21	56	Yes
207	21	57	No
208	ND	ND	ND
209	0	0	ND
210	19	42	No
211	26	69	No
212	24	65	No
213	ND	ND	ND
214	30	80	No
215	ND	ND	ND
216	ND	ND	ND
217	ND	ND	ND
218	ND	ND	ND
219	19	50	No
220	ND	ND	ND
221	ND	ND	ND
222	16	44	No
223	19	50	No
224	23	61	No
225	0	0	ND
226	ND	ND	ND
227	ND	ND	ND
228	0	0	ND
229	ND	ND	ND
230	25	67	No
231	17	45	No
232	0	0	ND
233	ND	ND	ND
234	ND	ND	ND
235	9	51	Yes
236	ND	ND	ND
237	23	61	No
238	10	57	Yes
239	ND	ND	ND
240	20	54	No

Code no.	Growth on 50 mg L^{-1} PCP-amended agar	% Growth 50 mg L^{-1} compared to control	Culture grows on 200 mg L^{-1} PCP-amended agar
241	ND	ND	ND
242	ND	ND	ND
243	0	0	ND
244	11	63	No
245	16	44	No
246	0	0	ND
247	0	0	ND
248	ND	ND	ND
249	0	0	ND
250	3	7	No
251	ND	ND	ND
252	ND	ND	ND
253	0	0	ND
254	ND	ND	ND
255	ND	ND	ND
256	ND	ND	ND
257	ND	ND	ND
258	ND	ND	ND
259	ND	ND	ND
260	ND	ND	ND
261	ND	ND	ND
262	ND	ND	ND
263	4	9	No
264	ND	ND	ND
265	ND	ND	ND
266	ND	ND	ND
267	ND	ND	ND
268	ND	ND	ND
269	ND	ND	ND
270	ND	ND	ND
271	0	0	ND
272	ND	ND	ND
273	ND	ND	ND
274	ND	ND	ND
275	ND	ND	ND
276	ND	ND	ND
277	ND	ND	ND
278	ND	ND	ND
279	ND	ND	ND
280	ND	ND	ND

Code no.	Growth on 50 mg L^{-1} PCP-amended agar	% Growth 50 mg L^{-1} compared to control	Culture grows on 200 mg L^{-1} PCP-amended agar
281	ND	ND	ND
282	ND	ND	ND
283	ND	ND	ND
284	ND	ND	ND
285	ND	ND	ND
286	ND	ND	ND
287	ND	ND	ND
288	ND	ND	ND
289	ND	ND	ND
290	ND	ND	ND
291	ND	ND	ND
292	ND	ND	ND
293	ND	ND	ND
294	22	59	No
295	ND	ND	ND
296	ND	ND	ND
297	ND	ND	ND
298	ND	ND	ND
299	ND	ND	ND
300	ND	ND	ND
301	ND	ND	ND
302	ND	ND	ND
303	ND	ND	ND
304	ND	ND	ND
305	ND	ND	ND
306	ND	ND	ND
307	ND	ND	ND
308	ND	ND	ND
309	ND	ND	ND
310	ND	ND	ND
311	ND	ND	ND
312	ND	ND	ND
313	18	49	No
314	ND	ND	ND
315	ND	ND	ND
316	ND	ND	ND
317	ND	ND	ND
318	ND	ND	ND
319	ND	ND	ND
320	ND	ND	ND

Code no.	Growth on 50 mg L^{-1} PCP-amended agar	% Growth 50 mg L^{-1} compared to control	Culture grows on 200 mg L^{-1} PCP-amended agar
321	ND	ND	ND
322	ND	ND	ND
323	ND	ND	ND
324	ND	ND	ND
325	ND	ND	ND
326	ND	ND	ND
327	ND	ND	ND
328	ND	ND	ND
329	ND	ND	ND
330	ND	ND	ND
331	ND	ND	ND
332	ND	ND	ND
333	ND	ND	ND
334	ND	ND	ND
335	8	49	Yes
336	ND	ND	ND
337	ND	ND	ND
338	ND	ND	ND
339	ND	ND	ND
340	ND	ND	ND
341	ND	ND	ND
342	ND	ND	ND
343	ND	ND	ND
344	ND	ND	ND
345	ND	ND	ND
346	ND	ND	ND
347	ND	ND	ND
348	ND	ND	ND
349	ND	ND	ND
350	ND	ND	ND
351	ND	ND	ND
352	ND	ND	ND
353	ND	ND	ND
354	ND	ND	ND
355	ND	ND	ND
356	ND	ND	ND
357	ND	ND	ND
358	ND	ND	ND
359	0	0	ND
360	ND	ND	ND

Code no.	Growth on 50 mg L^{-1} PCP-amended agar	% Growth 50 mg L^{-1} compared to control	Culture grows on 200 mg L^{-1} PCP-amended agar
361	ND	ND	ND
362	0	0	ND
363	ND	ND	ND
364	ND	ND	ND
365	ND	ND	ND
366	ND	ND	ND
367	ND	ND	ND
368	ND	ND	ND
369	ND	ND	ND
370	ND	ND	ND
371	ND	ND	ND
372	ND	ND	ND
373	ND	ND	ND
374	ND	ND	ND
375	ND	ND	ND
376	ND	ND	ND
377	ND	ND	ND
378	ND	ND	ND
379	ND	ND	ND
380	ND	ND	ND
381	ND	ND	ND
382	ND	ND	ND
383	ND	ND	ND
384	ND	ND	ND
385	ND	ND	ND
386	ND	ND	ND
387	ND	ND	ND
388	ND	ND	ND
389	ND	ND	ND
390	12	32	No
391	ND	ND	ND
392	ND	ND	ND
393	23	61	No
394	ND	ND	ND
395	16	42	No
396	ND	ND	ND
397	ND	ND	ND
398	ND	ND	ND
399	ND	ND	ND
400	ND	ND	ND

Code no.	Growth on 50 mg L^{-1} PCP-amended agar	% Growth 50 mg L^{-1} compared to control	Culture grows on 200 mg L^{-1} PCP-amended agar
401	ND	ND	ND
402	ND	ND	ND
403	ND	ND	ND
404	ND	ND	ND
405	ND	ND	ND
406	ND	ND	ND
407	ND	ND	ND
408	ND	ND	ND
409	ND	ND	ND
410	ND	ND	ND
411	ND	ND	ND
412	ND	ND	ND
413	ND	ND	ND
414	ND	ND	ND
415	ND	ND	ND
416	ND	ND	ND
417	ND	ND	ND
418	ND	ND	ND
419	ND	ND	ND
420	ND	ND	ND
421	ND	ND	ND
422	ND	ND	ND
423	ND	ND	ND
424	ND	ND	ND
425	ND	ND	ND
426	ND	ND	ND
427	ND	ND	ND
428	ND	ND	ND
429	ND	ND	ND
430	ND	ND	ND
431	ND	ND	ND
432	ND	ND	ND
433	ND	ND	ND
434	ND	ND	ND
435	ND	ND	ND
436	ND	ND	ND
437	ND	ND	ND
438	ND	ND	ND
439	ND	ND	ND
440	ND	ND	ND

Code no.	Growth on 50 mg L^{-1} PCP-amended agar	% Growth 50 mg L^{-1} compared to control	Culture grows on 200 mg L^{-1} PCP-amended agar
441	ND	ND	ND
442	ND	ND	ND
443	ND	ND	ND
444	ND	ND	ND
445	ND	ND	ND
446	ND	ND	ND
447	ND	ND	ND
448	ND	ND	ND
449	ND	ND	ND
450	ND	ND	ND
451	ND	ND	ND
452	ND	ND	ND
453	ND	ND	ND
454	ND	ND	ND
455	ND	ND	ND
456	ND	ND	ND
457	ND	ND	ND
458	0	0	ND
459	0	0	ND
460	0	0	ND
461	0	0	ND
462	0	0	ND
463	0	0	ND
464	0	0	ND
465	1	3	No
466	2	4	No
467	9	25	Yes
468	0	0	ND
469	0	0	ND
470	0	0	ND
471	ND	ND	ND
472	0	0	ND
473	3	7	No
474	ND	ND	ND
475	0	0	ND
476	7	18	No
477	ND	ND	ND
478	14	37	No
479	8	38	Yes
480	5	13	Yes
481	14	37	No
482	ND	ND	ND

Table 3: creosote sensitivity of fungal isolates. Fungal cultures were grown at 25ºC for fourteen days on creosote-amended agar (either 0, or 10,000 mg L^{-1} creosote in agar) and growth was measured in two directions. Growth of cultures on 10,000 mg L^{-1} creosote in agar is listed as both the actual growth (mm) and % growth relative to the control cultures.

Code no.	Growth on 10,000 mg L^{-1} creosote-amended agar	% Growth on 10,000 mg L^{-1} compared to control
1	30	37
2	79	100
3	15	29
4	0	0
5	0	0
6	20	43
7	ND	ND
8	ND	ND
9	ND	ND
10	77	97
11	0	0
12	79	100
13	0	0
14	79	100
15	41	83
16	ND	ND
17	ND	ND
18	44	57
19	ND	ND
20	ND	ND
21	79	100
22	0	0
23	44	59
24	48	65
25	4	4
26	0	0
27	ND	ND
28	43	62
29	0	0
30	38	48
31	ND	ND
32	ND	ND
33	ND	ND
34	ND	ND
35	0	0
36	0	0
37	10	14
38	ND	ND
39	ND	ND
40	ND	ND

Code no.	Growth on 10,000 mg L^{-1} creosote-amended agar	% Growth on 10,000 mg L^{-1} compared to control
41	32	43
42	ND	ND
43	60	75
44	77	97
45	ND	ND
46	42	78
47	35	44
48	27	39
49	ND	ND
50	72	91
51	57	72
52	51	64
53	24	30
54	ND	ND
55	ND	ND
56	0	0
57	0	0
58	42	62
59	0	0
60	ND	ND
61	ND	ND
62	0	0
63	0	0
64	ND	ND
65	ND	ND
66	ND	ND
67	ND	ND
68	ND	ND
69	ND	ND
70	ND	ND
71	0	0
72	ND	ND
73	76	96
74	0	0
75	37	57
76	ND	ND
77	0	0
78	ND	ND
79	ND	ND
80	ND	ND

Code no.	Growth on 10,000 mg L^{-1} creosote-amended agar	% Growth on 10,000 mg L^{-1} compared to control
81	ND	ND
82	0	0
83	ND	ND
84	ND	ND
85	0	0
86	0	0
87	0	0
88	0	0
89	ND	ND
90	0	0
91	ND	ND
92	ND	ND
93	ND	ND
94	ND	ND
95	ND	ND
96	ND	ND
97	48	61
98	ND	ND
99	47	69
100	ND	ND
101	41	62
102	36	45
103	ND	ND
104	ND	ND
105	ND	ND
106	ND	ND
107	18	27
108	42	63
109	ND	ND
110	38	57
111	ND	ND
112	41	60
113	46	58
114	19	23
115	ND	ND
116	32	40
117	ND	ND
118	51	64
119	ND	ND
120	ND	ND

Code no.	Growth on 10,000 mg L^{-1} creosote-amended agar	% Growth on 10,000 mg L^{-1} compared to control
121	41	51
122	32	40
123	37	47
124	ND	ND
125	34	43
126	18	22
127	45	56
128	ND	ND
129	40	50
130	37	47
131	0	0
132	29	37
133	56	70
134	34	42
135	ND	ND
136	ND	ND
137	ND	ND
138	32	41
139	32	41
140	ND	ND
141	37	46
142	ND	ND
143	ND	ND
144	ND	ND
145	ND	ND
146	ND	ND
147	0	0
148	ND	ND
149	7	8
150	29	59
151	ND	ND
152	ND	ND
153	ND	ND
154	ND	ND
155	42	61
156	ND	ND
157	44	59
158	ND	ND
159	ND	ND
160	41	63

Code no.	Growth on 10,000 mg L^{-1} creosote-amended agar	% Growth on 10,000 mg L^{-1} compared to control
161	ND	ND
162	37	69
163	ND	ND
164	ND	ND
165	74	94
166	29	59
167	0	0
168	ND	ND
169	ND	ND
170	ND	ND
171	ND	ND
172	ND	ND
173	ND	ND
174	ND	ND
175	0	0
176	20	41
177	48	72
178	ND	ND
179	ND	ND
180	ND	ND
181	ND	ND
182	73	92
183	62	78
184	ND	ND
185	ND	ND
186	19	31
187	11	18
188	5	8
189	ND	ND
190	ND	ND
191	ND	ND
192	ND	ND
193	25	52
194	43	64
195	32	63
196	ND	ND
197	ND	ND
198	ND	ND
199	ND	ND
200	ND	ND

Code no.	Growth on 10,000 mg L^{-1} creosote-amended agar	% Growth on 10,000 mg L^{-1} compared to control
201	39	74
202	ND	ND
203	ND	ND
204	ND	ND
205	23	42
206	0	0
207	17	33
208	ND	ND
209	34	64
210	ND	ND
211	ND	ND
212	32	63
213	ND	ND
214	35	68
215	ND	ND
216	ND	ND
217	ND	ND
218	ND	ND
219	31	59
220	ND	ND
221	ND	ND
222	33	61
223	23	42
224	43	83
225	36	84
226	ND	ND
227	ND	ND
228	5	6
229	ND	ND
230	45	85
231	34	61
232	ND	ND
233	ND	ND
234	ND	ND
235	0	0
236	ND	ND
237	ND	ND
238	0	0
239	ND	ND
240	42	78

Code no.	Growth on 10,000 mg L^{-1} creosote-amended agar	% Growth on 10,000 mg L^{-1} compared to control
241	ND	ND
242	ND	ND
243	77	104
244	0	0
245	ND	ND
246	ND	ND
247	60	76
248	ND	ND
249	ND	ND
250	ND	ND
251	ND	ND
252	ND	ND
253	0	0
254	ND	ND
255	ND	ND
256	ND	ND
257	ND	ND
258	ND	ND
259	ND	ND
260	ND	ND
261	ND	ND
262	ND	ND
263	0	0
264	ND	ND
265	ND	ND
266	ND	ND
267	ND	ND
268	ND	ND
269	ND	ND
270	ND	ND
271	0	0
272	ND	ND
273	ND	ND
274	ND	ND
275	ND	ND
276	ND	ND
277	ND	ND
278	ND	ND
279	ND	ND
280	ND	ND

Code no.	Growth on 10,000 mg L^{-1} creosote-amended agar	% Growth on 10,000 mg L^{-1} compared to control
281	ND	ND
282	ND	ND
283	ND	ND
284	ND	ND
285	ND	ND
286	ND	ND
287	ND	ND
288	ND	ND
289	ND	ND
290	ND	ND
291	ND	ND
292	ND	ND
293	ND	ND
294	15	25
295	ND	ND
296	ND	ND
297	ND	ND
298	ND	ND
299	ND	ND
300	ND	ND
301	ND	ND
302	ND	ND
303	ND	ND
304	ND	ND
305	ND	ND
306	ND	ND
307	ND	ND
308	ND	ND
309	ND	ND
310	ND	ND
311	ND	ND
312	ND	ND
313	ND	ND
314	ND	ND
315	ND	ND
316	ND	ND
317	ND	ND
318	ND	ND
319	ND	ND
320	ND	ND

Code no.	Growth on 10,000 mg L^{-1} creosote-amended agar	% Growth on 10,000 mg L^{-1} compared to control
321	ND	ND
322	ND	ND
323	ND	ND
324	ND	ND
325	ND	ND
326	ND	ND
327	ND	ND
328	ND	ND
329	ND	ND
330	ND	ND
331	ND	ND
332	ND	ND
333	ND	ND
334	ND	ND
335	0	0
336	ND	ND
337	ND	ND
338	ND	ND
339	ND	ND
340	ND	ND
341	ND	ND
342	ND	ND
343	ND	ND
344	ND	ND
345	ND	ND
346	ND	ND
347	ND	ND
348	ND	ND
349	ND	ND
350	ND	ND
351	ND	ND
352	ND	ND
353	ND	ND
354	ND	ND
355	ND	ND
356	ND	ND
357	ND	ND
358	ND	ND
359	22	28
360	ND	ND

Code no.	Growth on 10,000 mg L^{-1} creosote-amended agar	% Growth on 10,000 mg L^{-1} compared to control
361	ND	ND
362	0	0
363	ND	ND
364	ND	ND
365	ND	ND
366	ND	ND
367	ND	ND
368	ND	ND
369	ND	ND
370	ND	ND
371	ND	ND
372	ND	ND
373	ND	ND
374	ND	ND
375	15	26
376	ND	ND
377	ND	ND
378	ND	ND
379	ND	ND
380	ND	ND
381	ND	ND
382	ND	ND
383	ND	ND
384	ND	ND
385	ND	ND
386	ND	ND
387	ND	ND
388	ND	ND
389	ND	ND
390	14	23
391	ND	ND
392	ND	ND
393	ND	ND
394	ND	ND
395	ND	ND
396	ND	ND
397	ND	ND
398	ND	ND
399	ND	ND
400	ND	ND

Code no.	Growth on 10,000 mg L^{-1} creosote-amended agar	% Growth on 10,000 mg L^{-1} compared to control
401	ND	ND
402	ND	ND
403	ND	ND
404	ND	ND
405	ND	ND
406	ND	ND
407	ND	ND
408	ND	ND
409	ND	ND
410	ND	ND
411	ND	ND
412	ND	ND
413	ND	ND
414	ND	ND
415	ND	ND
416	ND	ND
417	ND	ND
418	ND	ND
419	ND	ND
420	ND	ND
421	ND	ND
422	ND	ND
423	ND	ND
424	ND	ND
425	ND	ND
426	ND	ND
427	ND	ND
428	ND	ND
429	ND	ND
430	ND	ND
431	ND	ND
432	ND	ND
433	ND	ND
434	ND	ND
435	ND	ND
436	ND	ND
437	ND	ND
438	ND	ND
439	ND	ND
440	ND	ND

Code no.	Growth on 10,000 mg L^{-1} creosote-amended agar	% Growth on 10,000 mg L^{-1} compared to control
441	ND	ND
442	ND	ND
443	ND	ND
444	ND	ND
445	ND	ND
446	ND	ND
447	ND	ND
448	ND	ND
449	ND	ND
450	ND	ND
451	ND	ND
452	ND	ND
453	ND	ND
454	ND	ND
455	ND	ND
456	ND	ND
457	ND	ND
458	0	0
459	40	50
460	67	84
461	38	48
462	ND	ND
463	0	0
464	37	47
465	ND	ND
466	0	0
467	77	97
468	ND	ND
469	0	0
470	0	0
471	ND	ND
472	44	59
473	0	0
474	ND	ND
475	0	0
476	77	97
477	54	68
478	ND	ND
479	0	0
480	ND	ND
481	ND	ND
482	0	0

CHAPTER 3 APPENDIX

Figure 1: Alignment of *lcc2* from *T. villosa* (GI:1100245) with *lac1*, *lac2* and *lac3* from *Trametes* sp. HR577. Introns are indicated in lower case letters while exons are indicated in capital letters. Portions of the *lac* primers used to amplify *lac1*, *lac2* and *lac3* from *Trametes* sp. HR577 are highlighted in bright green (Collins and Dobson 1997a). Introns from lac1 and *lac3* of *Trametes* sp. HR577 that are the same as those of *lcc2* from *T. villosa* are highlighted in turquoise.

Key:
lac1 *lac2* *lac3* *T. villosa lcc2* (Yaver, Xu et al. 1996, NCBI GI:1100245)

```
1     gcggcgcacaaaccgtgggagccaacacactcccgtccactctcacactggccagattcg

61    cgcgaccgccgcctttcaggcccaaacagatctggcaggtttcgatggcgcacgccgccg

121   tgcctgccggattcaattgtgcgccagtcgggcatccggatggctctaccagcgcggttg

181   actggaagagaacaccgaggtcatgcattctggccaagtgcggccaaaggaccgctcgct

241   ggtgcggatacttaaagggcggcgcggggaggcctgtctaccaagctcaagctcgccttg

301   ggttcccagtctccgccaccctcctcttcccccacacagtcgctccatagcaccgtcggc

361   gccATGGGTCTGCAGCGATTCAGCTTCTTCGTCACCCTCGCGCTCGTCGCTCGCTCTCTT
1        M  G  L  Q  R  F  S  F  F  V  T  L  A  L  V  A  R  S  L

421   GCAGCCATCGGGCCGGTGGCGAGCCTCGTCGTCGCGAACGCCCCCGTCTCGCCCGACGGC
20    A  A  I  G  P  V  A  S  L  V  V  A  N  A  P  V  S  P  D  G

481   TTCCTTCGGGATGCCATCGTGGTCAACGGCGTGGTCCCTTCCCCGCTCATCACCGGGAAG
41    F  L  R  D  A  I  V  V  N  G  V  V  P  S  P  L  I  T  G  K

541   AAGgtcggcgtgttcgtcgtcgtcctactcctttgctgacagcgatctacagGGAGACCGC
81    K                                                    G  D  R

602   TTCCAGCTCAACGTCGTCGACACCTTGACCAACCACAGCATGCTCAAGTCCACTAGTATC
85    F  Q  L  N  V  V  D  T  L  T  N  H  S  M  L  K  S  T  S  I

662   gtaagtgtgacgatccgaatgtgacatcaatcggggctaattaaccgcgcacagCACTGG
105                                                          H  W

722   CACGGCTTCTTCCAGGCAGGCACCAACTGGGCAGAAGGACCCGCGTTCGTCAACCAGTGC
107   H  G  F  F  Q  A  G  T  N  W  A  E  G  P  A  F  V  N  Q  C
                                                    TTCGTCAACCAGTGC
                                                    F  V  N  Q  C
                                                 GCYTTGGTCAACCAGTGC
                                                 A  L  V  N  Q  C

782   CCTATTGCTTCCGGGCATTCATTCCTGTACGACTTCCATGTGCCCGACCAGGCAGgtaagc
127   P  I  A  S  G  H  S  F  L  Y  D  F  H  V  P  D  Q  A
      CCTATTGCTTCCGGGCATTCATTCCTGTACGACTTCCATGTGCCCGACCAGGCGGgtgagc
      P  I  A  S  G  H  S  F  L  Y  D  F  H  V  P  D  Q  A
      CCTATTCGGACGGGGAACTCTTTCCTTTACGACTTCACCGCGACGGACCAAGCAGgtcagt
      P  I  R  T  G  N  S  F  L  Y  D  F  T  A  T  D  Q  A
               TCCGGGCATTCATTCCTGTACGACTTCCATGGGCCCGACCAGGCAGgtaaca
               S  G  H  S  F  L  Y  D  F  H  G  P  D  Q  A
```

```
843   aggattttctggggtccccgtgtgatgcaatgttctcatgctccgacgtgatcgacagGG
                                                                 G
      aggatttgccaggaattccgtgtgatgcaatgtcctcatgctccgacgtgatcgacagGG
                                                                 G
        gcctgtggcggcttatgttctycccgcaatcagcagctaacactccgcacccgcagGG
                                                                 G
      gttctttgttgtgatcctcgtgtaatgcaatgttctcatgctccgacgtgatcgacagGG
                                                                 G

903   ACGTTCTGGTACCACAGTCATCTGTCTACGCAGTACTGTGACGGGCTGCGGGGGCCGTTC
146   T  F  W  Y  H  S  H  L  S  T  Q  Y  C  D  G  L  R  G  P  F
      ACGTTCTGGTACCACAGTCATCTGTCTACGCAATACTGTGACGGGCTGCGGGGGCCGTTC
      T  F  W  Y  H  S  H  L  S  T  Q  Y  C  D  G  L  R  G  P  F
      ACCTTCTGGTACCACAGTCACTTGTCTACGCAGTACTGCGATGGTCTTCGGGGTCCGATG
      T  F  W  Y  H  S  H  L  S  T  Q  Y  C  D  G  L  R  G  P  M
      ACGTTCTGGTACCATAGTCATCTGTCTACGCAATACTGTGACGGGCTGCGAGGACCGTTC
      T  F  W  Y  H  S  H  L  S  T  Q  Y  C  D  G  L  R  G  P  F

963   GTCGTGTACGACCCCAAGGACCCGCACGCCAGCCGTTACGATGTTGACAATGgtacgtg
166   V  V  Y  D  P  K  D  P  H  A  S  R  Y  D  V  D  N
      GTCGTGTACGACCCCAAGGACCCGCACGCCAGCCGTTACGATGTTGACAACGgtacgtg
      V  V  Y  D  P  K  D  P  H  A  S  R  Y  D  V  D  N
      GTCGTATACGACCCGAGTGACCCGCATGCGGACCTTTACGACGTCGACGAC
      V  V  Y  D  P  S  D  P  H  A  D  L  Y  D  V  D  D
      GTCGTGTACGACCCCAAGGATCCGCACGCCAGCCGCTACGATGTTGACAACGgtacgtg
      V  V  Y  D  P  K  D  P  H  A  S  R  Y  D  V  D  N

1022  cgccacggagtatatcacacagcatgcgttgacgtcgggccaacagAGAGCACGGTCATC
183                                              E  S  T  V  I
      cgccacggcgtatatcacacagcatgcgttacgtcgggccaacagAGAGCACGGTCATC
                                                 E  S  T  V  I
                                                GAGACCACGATTATC
                                                E  T  T  I  I
      cgccacggagtatatcacacagcatgcgttgacgtcgggccaacagAGAGCACGGTCATC
                                                 E  S  T  V  I

1082  ACGTTGACCGACTGGTACCACACCGCTGCCCGGCTCGGTCCCAAGTTCCCgtaagctcgca
      T  L  T  D  W  Y  H  T  A  A  R  L  G  P  K  F  P
      ACGTTGACCGACTGGTACCACACCGCTGCCCGGCTCGGTCCCAAGTTCCCgtaagctcgca
      T  L  T  D  W  Y  H  T  A  A  R  L  G  P  K  F  P
      ACGCTCTCTGATTGGTATCACACCGCCGCTTCGCTTGGTGCTGCCTTCCCgtgagtttgcc
      T  L  S  D  W  Y  H  T  A  A  S  L  G  A  A  F  P
      ACGTTGACCGACTGGTACCACACCGCTGCCCGGCTCGGTCCCAGGTTCCCgtaggctcgca
      T  L  T  D  W  Y  H  T  A  A  R  L  G  P  R  F  P

1143  atggcttagtgttcacaggttctttgcttatgttgcttcgatagACTCGGCGCGGACGCC
                                                    L  G  A  D  A
      atggtttagtttcacggattctttgctcatgtcgctttgatagACTCGGCGCGGACGCC
                                                    L  G  A  D  A
      ccagcacatggagttaagaccaaatctaactgtactacgttcagGGTCGGCTCGGACTCT
                                                    V  G  S  D  S
      atggattagtttttacggatttatttgcttatgttgcgtcgatagACTCGGCGCGGACGCC
                                                    L  G  A  D  A
```


1203
ACGCTCATCAACGGTCTGGGGCGGTCGGCCTCGACTCCCACCGCTGCGCTTGCCGTGATC
T L I N G L G R S A S T P T A A L A V I
ACGCTCATCAACGGTCTTGGGCGGTCGGCCTCGACTCCCACCGCTGCGCTTGCCGTGATC
T L I N G L G R S A S T P T A A L A V I
ACCCTGATCAACGGGTTGGGCCGTTTCGCGGGTGGTGACAGCACTGACCTTGCGGTCATC
T L I N G L G R F A G G D S T D L A V I
ACGCTCATCAATGGTCTTGGGCGGTCGGCCTCCACTCCCACCGCCGCGCTTGCTGTGATC
T L I N G L G R S A S T P T A A L A V I
1263
AACGTCCAGCACGGAAAGCGgtgagcattctcttgtatgccatttcaatgctttgtgct
N V Q H G K R
AACGTCCAGCACGGAAAGCGgtgagcattcctcatatgccatttactgctctaaact
N V Q H G K R
ACTGTTGAGCAGGGCAAGCGgttagtgctaccctctacagttggcactgtgctat
T V E Q G K R
AACGTCCAGCACGGAAAGCGgtgagcattctcttgcatgccatttcaatgctctaaatt
N V Q H G K R
1322
gacctatcggaaccgcgcagCTACCGCTTCCGTCTCGTTTCGATCTCGTGTGACCCGAAC
Y R F R L V S I S C D P N
gacctatcggcaccgcgtagCTATCGCTTCCGTCTCGTTTCGATCTCGTGCGACCCGAAC
Y R F R L V S I S C D P N
tgctgacagtactctcagCTACCGTATGCGTCTTCTCTCGCTGTCTTGCGACCCCAAC
Y R M R L L S L S C D P N
gacctatcggcaccacgcagCTACCGCTTCCGTCTCGTTTCGATCTCGTGCGACCCGAAC
Y R F R L V S I S C D P N
1382
TACACGTTCAGCATCGACGGGCACAACCTGACCGTCATCGAGGTCGACGGCATCAATAGC
Y T F S I D G H N L T V I E V D G I N S
TACACGTTCAGCATCGACGGGCACAACCTGACAGTCATCGAGGTCGACGGTATCAACAGC
Y T F S I D G H N L T V I E V D G I N S
TACGTCTTCTCCATCGACGGCCACAACATGACCATCATCGAGGCCGACGCCGTAAACCAC
Y V F S I D G H N M T I I E A D A V N H
TACACGTTCAGCATCGACGGGCACAATCTGACCGTCATCGAGGTCGACGGTATCAACAGC
Y T F S I D G H N L T V I E V D G I N S
1442
CAGCCTCTCCTTCTCGACTCTATCCAGATCTTCGCCGCACAGCGCTACTCCTTCGTGgtaa
Q P L L V D S I Q I F A A Q R Y S F V
CAGCCTCTCCTTGTCGACTCCATCCTGCTC
Q P L L V D S I L L
GAGCCCCTCACGGTCGACCCCATCCAGATC
E P L T V D P I Q I
CAGCCTCTCCTTGTCGCCTCCATCCAGATC
Q P L L V A S I Q I
1503
gtcctggcttgtcgatgctccaaagtggcctcactcatatactttcgttagTTGAATGCG
L N A
1563
AATCAAACGGTGGGCAACTACTGGGTTCGTGCGAACCCGAACTTCGGAACGGTTGGGTTC
N Q T V G N Y W V R A N P N F G T V G F
1623
GCCGGGGGGATCAACTCCGCCATCTTGCGCTACCAGGGCGCACCGGTCGCCGAGCCTACC
A G G I N S A I L R Y Q G A P V A E P T
1683
ACGACCCAGACGCCGTCGGTGATCCCGCTCATCGAGACGAACTTGCACCCGCTCGCGCGC
T T Q T P S V I P L I E T N L H P L A R

```
1743  ATGCCAGTGgtatgtctctttttctgatcatctgagttgcccgttgttgaccgcattatg
        M  P  V

1801  tgttactatctagCCTGGCAGCCCGACACCCGGGGGCGTCGACAAGGCGCTCAACCTC
                    P  G  S  P  T  P  G  G  V  D  K  A  L  N  L

1861  GCGTTTAACTTCgtaagtatctctactacttaggctggaggctcgtcgctgatcatacggtg
        A  F  N  F

1923  cttcagAACGGCACCAACTTCTTCATCAACAACGCGACTTTCACGCCGCCGACCGTCCCG
            N  G  T  N  F  F  I  N  N  A  T  F  T  P  P  T  V  P

1983  GTACTCCTCCAGATTCTGAGCGGTGCGCAGACCGCACAAGACCTGCTCCCCGCAGGCTCT
        V  L  L  Q  I  L  S  G  A  Q  T  A  Q  D  L  L  P  A  G  S

2043  GTCTACCCGCTCCCGGCCCACTCCACCATCGAGATCACGCTGCCCGCGACCGCCTTGGCC
        V  Y  P  L  P  A  H  S  T  I  E  I  T  L  P  A  T  A  L  A

2103  CCGGGTGCACCGCACCCCTTCCACCTGCACGGTgtatgttcccctgccttcccttcttat
        P  G  A  P  H  P  F  H  L  H  G

2163  ccccgaaccagtgctcacgtccgtcccatctagCACGCCTTCGCGGTCGTTCGCAGCGCG
                                        H  A  F  A  V  V  R  S  A

2223  GGGAGCACCACGTATAACTACAACGACCCGATCTTCCGCGACGTCGTGAGCACGGGCACG
        G  S  T  T  Y  N  Y  N  D  P  I  F  R  D  V  V  S  T  G  T

2283  CCCGCCGCGGGCGACAACGTCACGATCCGCTTCCAGACGGACAACCCCGGGCCGTGGTTC
        P  A  A  G  D  N  V  T  I  R  F  Q  T  D  N  P  G  P  W  F

2343  CTCCACTGCCACATCGACTTCCACCTCGACGCAGGCTTCGCGATCGTGTTCGCAGAGGAC
        L  H  C  H  I  D  F  H  L  D  A  G  F  A  I  V  F  A  E  D

2403  GTTGCGGACGTGAAGGCGGCGAACCCGGTTCCGAAGGCGTGGTCGGACCTGTGCCCGATC
        V  A  D  V  K  A  A  N  P  V  P  K  A  W  S  D  L  C  P  I

2463  TACGACGGGCTGAGCGAGGCTAACCAgtgagcggagggcgtggtgttgagcgtaaagctc
        Y  D  G  L  S  E  A  N  Q

2523  gggcgtcgacctgggggggttgaaggtgttctgattgaaatggtctttgggtttatttgtt

2583  gttattctaactcggttctctacgcaaggaccgaggattgtataggatgaagtaactttc

2643  ctaatgtattatgatatcaattgacggaggcatggactgcgaagtgt          2689
```

Figure 2: Alignment of *lcc4* from *T. villosa* (GI:1322078) with *lac2* from *Trametes* sp. HR577. Introns are indicated in lower case letters while exons are indicated in capital letters. Portions of the *lac* primers used to amplify *lac2* from *Trametes* sp. HR577 are highlighted in bright green (Collins and Dobson 1997a). Introns from *lac3* of *Trametes* sp. HR577 that are the same as those of *lcc4* from *T. villosa* are highlighted in turquoise.

Key:

lac2 *T. villosa lcc4* (Yaver, Xu et al. 1996, NCBI GI:1100245)

```
1     gaattccgatcggcttgccctcattcctccatgttcccccgaccgagcgggcgcgtcaat

61    ggcccgtttgcgaacacatatgcaggataaacagtgcgaaatatcaatgtggcggcgaca

121   caacctcgccggccgacactcgacgctgttgatcatgatcatgtcttgtgagcattctat

181   acgcagccttggaaatctcaggcgaatttgtctgaattgcgctgggaggctggcagcgca

241   gatcggtgtgtcggtgcagtagccgacgcagcacctggcggaagccgacatctcgggtac

301   gacttgatctccgccagatcactgcggttccgccatcggccgcggggcccattctgtgtg

361   tgcgctgtagcactctgcattcaggctcaacgtatccatgctagaggaccgtccagctgt

421   tggcgcacgattcgcgcagaaagctgtacaggcagatataaggatgtccgtccgtcagag

481   actcgtcactcacaagcctcttttcctcttcgcctttccagcctcttccaacgcctgcca

541   tcgtcctcttagttcgctcgtccattctttctgcgtagttaatcATGGGCAGGTTCTCATCT
                                                    M  G  R  F  S  S

603   CTCTGCGCGCTCACCGCCGTCATCCACTCTTTTGGTCGTGTCTCCGCCGCTATCGGGCCT
      L  C  A  L  T  A  V  I  H  S  F  G  R  V  S  A  A  I  G  P

663   GTGACCGACCTCACCATCTCCAATGGGGACGTTTCTCCCGACGGCTTCACTCGTGCCGCA
      V  T  D  L  T  I  S  N  G  D  V  S  P  D  G  F  T  R  A  A

723   GTGCTTGCAAACGGCGTCTTCCCGGGTCCTCTTATCACGGGAAACAAGgtacgtggcatg
      V  L  A  N  G  V  F  P  G  P  L  I  T  G  N  K

783   cgttcagtctacaccctacaagccttctaactcttttaccacagggcgacaacttccaga

843   tcaatgttatcgacaacctctctaacgagacgatgttgaagtcgacctccatcgtatgtg

903   cttctactgcttcttagtcttggcaatggctcaaggtctcctccgcagcattggcacggc

963   TTCTTCCAGAAGGGTACTAACTGGGCTGATGGAGCTGCCTTCGTCAACCAGTGCCCTATC
      F  F  Q  K  G  T  N  W  A  D  G  A  A  F  V  N  Q  C  P  I
                                       GCYTTGGTCAACCAGTGCCCTATT
                                       A  L  V  N  Q  C  P  I

1023  GCGACGGGGAACTCTTTCCTTTACGACTTCACCGCGACGGACCAAGCAGgtcagtgcctg
      A  T  G  N  S  F  L  Y  D  F  T  A  T  D  Q  A
      CGGACGGGGAACTCTTTCCTTTACGACTTCACCGCGACGGACCAAGCAGgtcagtgcctg
      R  T  G  N  S  F  L  Y  D  F  T  A  T  D  Q  A

1083  tggcgcttatgttttcccgtaatcagcagctaacactccgcacccacagGCACCTTCTGG
                                                       G  T  F  W
      tggcgcttatgttctcccgcaatcagcagctaacactccgcacccgcagGCACCTTCTGG
                                                       G  T  F  W
```

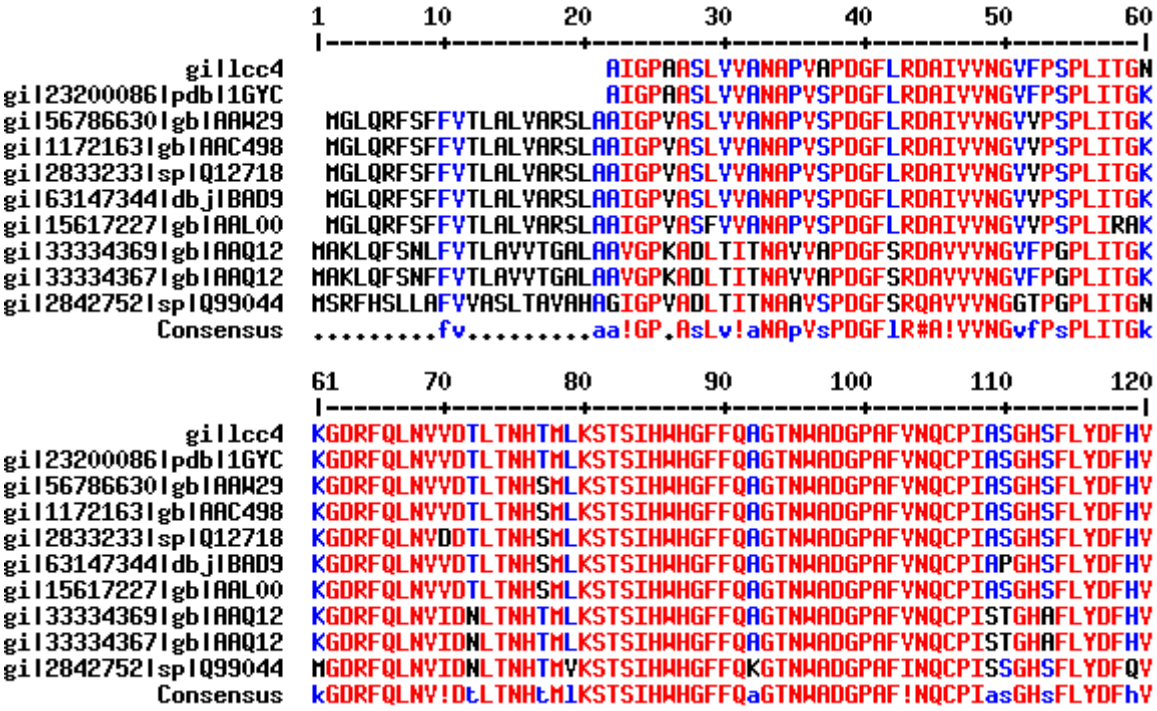
```
1143  TACCACAGTCACTTGTCTACGCAGTACTGCGATGGTTTGCGGGGCCCGATGGTCGTATAC
      Y  H  S  H  L  S  T  Q  Y  C  D  G  L  R  G  P  M  V  V  Y
      TACCACAGTCACTTGTCTACGCAGTACTGCGATGGTCTTCGGGGTCCGATGGTCGTATAC
      Y  H  S  H  L  S  T  Q  Y  C  D  G  L  R  G  P  M  V  V  Y

1203  GACCCGAGTGACCCGCATGCGGACCTTTACGACGTCGACGACGAGACCACGATCATCACG
      D  P  S  D  P  H  A  D  L  Y  D  V  D  D  E  T  T  I  I  T
      GACCCGAGTGACCCGCATGCGGACCTTTACGACGTCGACGACGAGACCACGATTATCACG
      D  P  S  D  P  H  A  D  L  Y  D  V  D  D  E  T  T  I  I  T

1263  CTCTCTGATTGGTATCACACCGCTGCTTCGCTCGGTGCTGCCTTCCCgtaagtttacc
      L  S  D  W  Y  H  T  A  A  S  L  G  A  A  F  P
      CTCTCTGATTGGTATCACACCGCCGCTTCGCTTGGTGCTGCCTTCCCgtgagtttgcc
      L  S  D  W  Y  H  T  A  A  S  L  G  A  A  F  P

1321  ccagcgcacggagttaagaccggatctaactgtaatacgttcagGATTGGCTCGGACTCT
                                                  I  G  S  D  S
      ccagcacatggagttaagaccaaatctaactgtactacgttcagGGTCGGCTCGGACTCT
                                                  V  G  S  D  S

1381  ACCCTGATTAACGGGTTGGGCCGCTTCGCGGGTGGTGACAGCACTGACCTTGCGGTTATC
      T  L  I  N  G  L  G  R  F  A  G  G  D  S  T  D  L  A  V  I
      ACCCTGATCAACGGGTTGGGCCGTTTCGCGGGTGGTGACAGCACTGACCTTGCGGTCATC
      T  L  I  N  G  L  G  R  F  A  G  G  D  S  T  D  L  A  V  I

1441  ACTGTCGAGCAGGGCAAGCGgttagtgataccctctacagttgacactgtgccattgctg
      T  V  E  Q  G  K  R
      ACTGTTGAGCAGGGCAAGCGgttagtgctaccctctacagttggcactgtgctattgctg
      T  V  E  Q  G  K  R

1501  acagtactctcagCTACCGTATGCGTCTTCTCTCGCTGTCTTGCGACCCCAACTATGTCTTC
                   Y  R  M  R  L  L  S  L  S  C  D  P  N  Y  V  F
      acagtactctcagCTACCGTATGCGTCTTCTCTCGCTGTCTTGCGACCCCAACTACGTCTTC
                   Y  R  M  R  L  L  S  L  S  C  D  P  N  Y  V  F

1563  TCCATTGACGGCCACAACATGACCATCATCGAGGCCGACGCCGTCAACCACGAGCCCCTC
      S  I  D  G  H  N  M  T  I  I  E  A  D  A  V  N  H  E  P  L
      TCCATCGACGGCCACAACATGACCATCATCGAGGCCGACGCCGTAAACCACGAGCCCCTC
      S  I  D  G  H  N  M  T  I  I  E  A  D  A  V  N  H  E  P  L

1623  ACGGTTGACTCCATCCAGATCTACGCCGGCCAACGTTACTCCTTCGTCgtacgtattccgg
      T  V  D  S  I  Q  I  Y  A  G  Q  R  Y  S  F  V
      ACGGTCGACCCCATCCAGATC
      T  V  D  P  I  Q  I

1683  aacagccatgatcacgccaagcccgatgctaacgcgcctaccctcagCTTACCGCTGAC
                                                     L  T  A  D

1742  CAGGACATCGACAACTACTTCATCCGTGCCCTGCCCAGCGCCGGTACCACCTCGTTCGAC
      Q  D  I  D  N  Y  F  I  R  A  L  P  S  A  G  T  T  S  F  D

1802  GGCGGCATCAACTCGGCTATCCTGCGCTACTCTGGTGCCTCCGAGGTTGACCCGACGACC
      G  G  I  N  S  A  I  L  R  Y  S  G  A  S  E  V  D  P  T  T

1862  ACGGAGACCACGAGCGTCCTCCCCCTCGACGAGGCGAACCTCGTGCCCCTTGACAGCCCC
      T  E  T  T  S  V  L  P  L  D  E  A  N  L  V  P  L  D  S  P
```

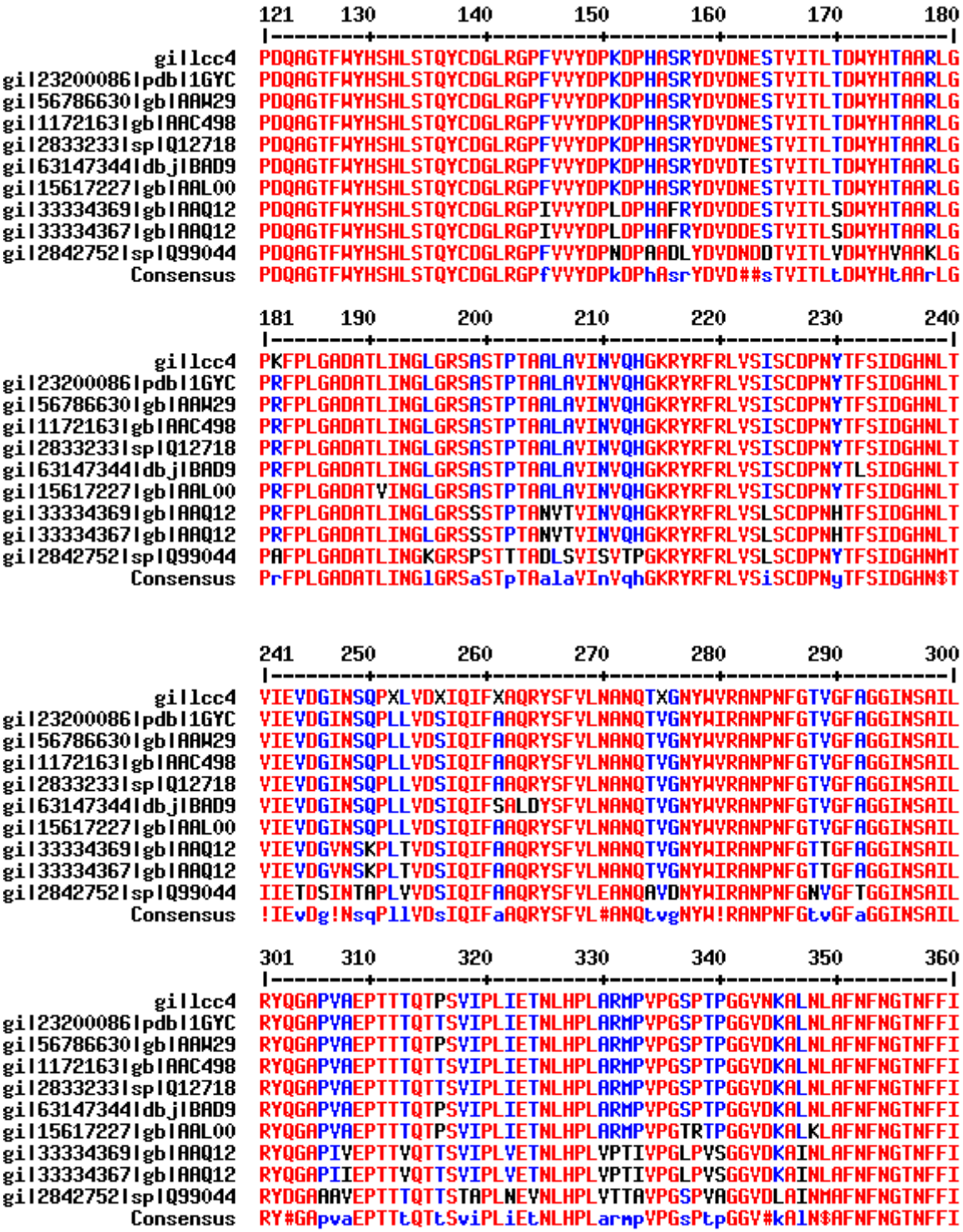
```
1922  GCTGCTgtacgtcgtattctgcgcttgcaaggatcgcacatactaacatgctcttgtag
      A  A

1981  CCCGGTGACCCCAACATTGGCGGTGTCGACTACGCGCTGAACTTGGACTTCAACTTCGAT
      P  G  D  P  N  I  G  G  V  D  Y  A  L  N  L  D  F  N  F  D

2041  GGCACCAACTTCTTCATCAACGACGTCTCCTTCGTGTCCCCCACGGTCCCTGTCCTCCTC
      G  T  N  F  F  I  N  D  V  S  F  V  S  P  T  V  P  V  L  L

2101  CAGATTCTTAGCGGCACCACCTCCGCGGCCGACCTTCTCCCCAGCGGTAGTCTCTTCGCG
      Q  I  L  S  G  T  T  S  A  A  D  L  L  P  S  G  S  L  F  A

2161  GTCCCGTCCAACTCGACGATCGAGATCTCGTTCCCCATCACCGCGACGAACGCTCCCGGC
      V  P  S  N  S  T  I  E  I  S  F  P  I  T  A  T  N  A  P  G

2221  GCGCCGCATCCCTTCCACTTGCACGGTgtacgtgtcccatctcatatgctacggagctcc
      A  P  H  P  F  H  L  H  G

2281  acgctgaccgccctatagCACACCTTCTCTATCGTTCGTACCGCCGGCAGCACGGATACG
                        H  T  F  S  I  V  R  T  A  G  S  T  D  T

2341  AACTTCGTCAACCCCGTCCGCCGCGACGTCGTGAACACCGGTACCGTCGGCGACAACGTC
      N  F  V  N  P  V  R  R  D  V  V  N  T  G  T  V  G  D  N  V

2401  ACCATCCGCTTCACGgtacgcagcactctcctaacattcccactgcgcgatcactgactcct
      T  I  R  F  T

2463  cgcccacagACTGACAACCCCGGCCCCTGGTTCCTCCACTGCCACATCGACTTCCACTTG
            T  D  N  P  G  P  W  F  L  H  C  H  I  D  F  H  L

2523  GAGGCCGGTTTCGCCATCGTCTTCAGCGAGGACACCGCCGACGTCTCGAACACGACCACG
      E  A  G  F  A  I  V  F  S  E  D  T  A  D  V  S  N  T  T  T

2583  CCCTCGAGTACgttgtgctcccgtgcccatctccgcgcgcctgactaacgagcacccctt
      P  S  S  T

2643  acagctgCTTGGGAAGATCTGTGCCCCACGTACAACGCTCTTGACTCATCCGACCTctaa
            A  W  E  D  L  C  P  T  Y  N  A  L  D  S  S  D  L

2703  tcggttcaaagggtcgctcgctaccttagtaggtagacttatgcaccggacattatctac

2763  aatggactttaatttgggttaacggccgttatacatacgcgcacgtagtataaaggttct

2823  ctggattggtcggacctacagactgcaattttcgtgacctatcaactgtatattgaagca

2883  cgacagtgaatggaaatagagaca 2906
```

Figure 3: Alignment of the predicted partial *lac4* amino acid sequence from *Trametes* sp. HR577 with closely related sequences from the NCBI database. This alignment was carried out by entering the sequences into the MultiAlign program on the INRA website (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html, Corpet 1988).

Key:

GI	Genus	species	Definition	Locus
-	*Trametes*	sp. HR577	*lac4*	-
23200086	*Trametes*	*versicolor*	TvL, Chain A	1GYC_A
56786630	*Trametes*	*versicolor*	Laccase 1	AAW29420
1172163	*Trametes*	*versicolor*	Laccase I	AAC49828
2833233	*Trametes*	*versicolor*	Laccase 2 precursor	Q12718
63147344	*Trametes*	*versicolor*	Laccase2	BAD98306
15617227	*Trametes*	*versicolor*	Laccase 1	AAL00887
33334369	*Trametes*	sp. I-62	laccase	AAQ12268
33334367	*Trametes*	sp. I-62	laccase	AAQ12267
2842752	*Trametes*	*villosa*	Laccase 1 precursor	Q99044

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gi|23200086|pdb|1GYC PDQAGTFWYHSHLSTQYCDGLRGPFVVYDPKDPHASRYDVDNESTVITLTDWYHTAARLG
gi|56786630|gb|AAW29 PDQAGTFWYHSHLSTQYCDGLRGPFVVYDPKDPHASRYDVDNESTVITLTDWYHTAARLG
gi|1172163|gb|AAC498 PDQAGTFWYHSHLSTQYCDGLRGPFVVYDPKDPHASRYDVDNESTVITLTDWYHTAARLG
gi|2833233|sp|Q12718 PDQAGTFWYHSHLSTQYCDGLRGPFVVYDPKDPHASRYDVDNESTVITLTDWYHTAARLG
gi|63147344|dbj|BAD9 PDQAGTFWYHSHLSTQYCDGLRGPFVVYDPKDPHASRYDVDTESTVITLTDWYHTAARLG
gi|15617227|gb|AAL00 PDQAGTFWYHSHLSTQYCDGLRGPFVVYDPKDPHASRYDVDNESTVITLTDWYHTAARLG
gi|33334369|gb|AAQ12 PDQAGTFWYHSHLSTQYCDGLRGPIVVYDPLDPHAFRYDVDDESTVITLSDWYHTAARLG
gi|33334367|gb|AAQ12 PDQAGTFWYHSHLSTQYCDGLRGPIVVYDPLDPHAFRYDVDDESTVITLSDWYHTAARLG
gi|2842752|sp|Q99044 PDQAGTFWYHSHLSTQYCDGLRGPFVVYDPNDPAADLYDVDNDDTVITLVDWYHVAAKLG
Consensus PDQAGTFWYHSHLSTQYCDGLRGPfVVYDPkDPhAsrYDVD##sTVITLtDWYHtAArLG
181 190 200 210 220 230 240
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gi|2833233|sp|Q12718 PRFPLGADATLINGLGRSASTPTAALAVINVQHGKRYRFRLVSISCDPNYTFSIDGHNLT
gi|63147344|dbj|BAD9 PRFPLGADATLINGLGRSASTPTAALAVINVQHGKRYRFRLVSISCDPNYTLSIDGHNLT
gi|15617227|gb|AAL00 PRFPLGADATVINGLGRSASTPTAALAVINVQHGKRYRFRLVSISCDPNYTFSIDGHNLT
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gi|33334367|gb|AAQ12 PRFPLGADATLINGLGRSSSTPTANVTVINVQHGKRYRFRLVSLSCDPNHTFSIDGHNLT
gi|2842752|sp|Q99044 PAFPLGADATLINGKGRSPSTTTADLSVISVTPGKRYRFRLVSLSCDPNYTFSIDGHNMT
Consensus PrFPLGADATLINGlGRSaSTpTAalaVInVqhGKRYRFRLVSiSCDPNyTFSIDGHN$T
241 250 260 270 280 290 300
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gi|23200086|pdb|1GYC VIEVDGINSQPLLVDSIQIFAAQRYSFVLNANQTVGNYWIRANPNFGTVGFAGGINSAIL
gi|56786630|gb|AAW29 VIEVDGINSQPLLVDSIQIFAAQRYSFVLNANQTVGNYWVRANPNFGTVGFAGGINSAIL
gi|1172163|gb|AAC498 VIEVDGINSQPLLVDSIQIFAAQRYSFVLNANQTVGNYWVRANPNFGTVGFAGGINSAIL
gi|2833233|sp|Q12718 VIEVDGINSQPLLVDSIQIFAAQRYSFVLNANQTVGNYWVRANPNFGTVGFAGGINSAIL
gi|63147344|dbj|BAD9 VIEVDGINSQPLLVDSIQIFSALDYSFVLNANQTVGNYWVRANPNFGTVGFAGGINSAIL
gi|15617227|gb|AAL00 VIEVDGINSQPLLVDSIQIFAAQRYSFVLNANQTVGNYWVRANPNFGTVGFAGGINSAIL
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Consensus !IEvDg!NsqPllVDsIQIFaAQRYSFVL#ANQtvgNYW!RANPNFGtvGFaGGINSAIL
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gi|23200086|pdb|1GYC RYQGAPVAEPTTTQTTSVIPLIETNLHPLARMPVPGSPTPGGVDKALNLAFNFNGTNFFI
gi|56786630|gb|AAW29 RYQGAPVAEPTTTQTPSVIPLIETNLHPLARMPVPGSPTPGGVDKALNLAFNFNGTNFFI
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gi|2833233|sp|Q12718 RYQGAPVAEPTTTQTTSVIPLIETNLHPLARMPVPGSPTPGGVDKALNLAFNFNGTNFFI
gi|63147344|dbj|BAD9 RYQGAPVAEPTTTQTPSVIPLIETNLHPLARMPVPGSPTPGGVDKALNLAFNFNGTNFFI
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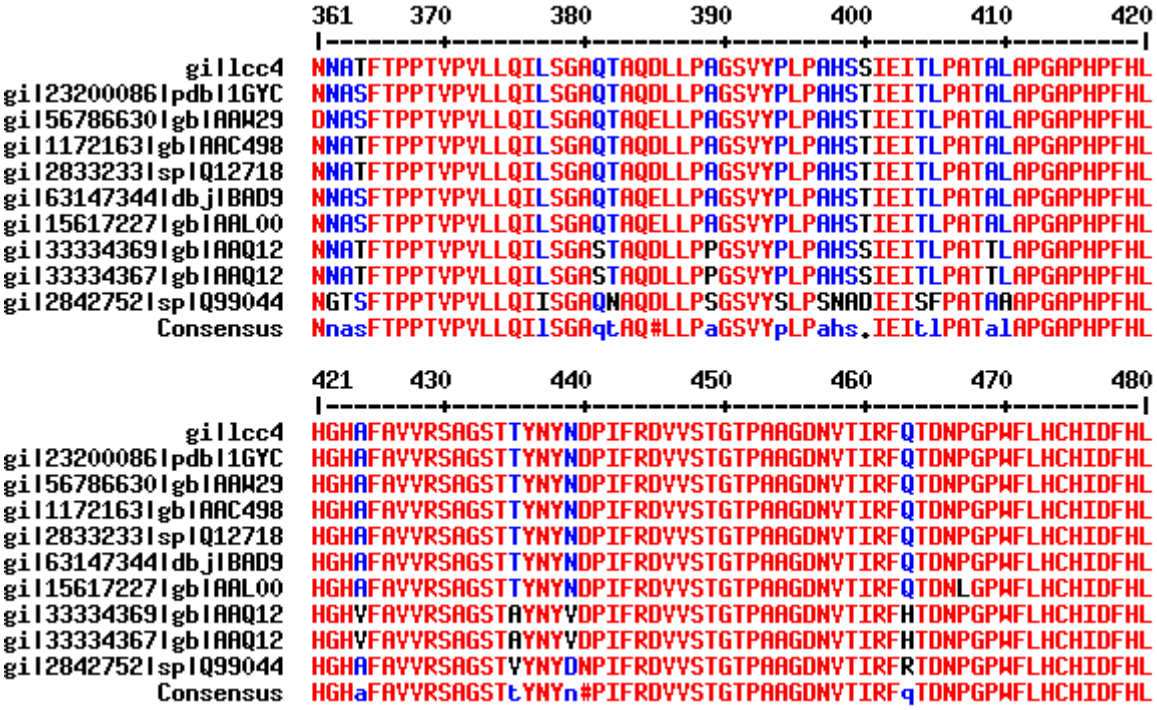
gi|2842752|sp|Q99044 RYDGAAAVEPTTTQTTSTAPLNEVNLHPLVTTAVPGSPVAGGVDLAINNAFNFNGTNFFI
Consensus RY#GApvaEPTTtQTtSviPLiEtNLHPLarmpVPGsPtpGGV#kAlN$AFNFNGTNFFI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gi|56786630|gb|AAW29 DNASFTPPTVPVLLQILSGAQTAQELLPAGSVYPLPAHSTIEITLPATALAPGAPHPFHL
gi|1172163|gb|AAC498 NNATFTPPTVPVLLQILSGAQTAQDLLPAGSVYPLPAHSTIEITLPATALAPGAPHPFHL
gi|2833233|sp|Q12718 NNATFTPPTVPVLLQILSGAQTAQDLLPAGSVYPLPAHSTIEITLPATALAPGAPHPFHL
gi|63147344|dbj|BAD9 NNASFTPPTVPVLLQILSGAQTAQELLPAGSVYPLPAHSTIEITLPATALAPGAPHPFHL
gi|15617227|gb|AAL00 NNASFTPPTVPVLLQILSGAQTAQELLPAGSVYPLPAHSTIEITLPATALAPGAPHPFHL
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Consensus NnasFTPPTVPVLLQIlSGAqtAQ#LLPaGSVYpLPahs.IEItlPATalAPGAPHPFHL
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Consensus HGHaFAVVRSAGSTtYNYn#PIFRDVVSTGTPAAGDNVTIRFqTDNPGPWFLHCHIDFHL
481 490 500 510 520
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gi|23200086|pdb|1GYC EAGFAIVFAEDVADVKAANPVPKAWSDLCPIYDGLSEANQ
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gi|2833233|sp|Q12718 DAGFAIVFAEDVADVKAANPVPKAWSDLCPIYDGLSEANQ
gi|63147344|dbj|BAD9 EAAFAIVFAEDVADVKAANPVPKAWSDLCPIYDGLSEANQ
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Consensus EAGFA!VFAED!aDVkaANpvpkawsdlcp.yd.l.e..q