Isolation and Characterization of a Novel Thermostable and Catalytically Efficient Laccase from *Peniophora* sp. Strain *UD4*

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Abstract

Enzymes are becoming an effective tool in industrial processes, from crude applications such as bioremediation to fine processes such as chirally selective biocatalysis. The ligninolytic enzymes have recently received considerable attention for industrial application due to both their broad substrate range and their ability to degrade the most recalcitrant natural polymer, lignin. This group of enzymes was therefore identified as the target group for this study.

Improved enzyme properties are constantly being sought to enhance the range of applications for enzymes. Biodiversity provides a wide variety of enzymes. Several researchers have concentrated on extremophiles as their primary source of superior enzymes, consequently neglecting temperate environments in their search for these enzymes. The relatively neglected fungal biodiversity of South Africa provided an opportunity to test the hypothesis that potentially important industrial enzymes with unusual properties could be isolated from mesophilic basidiomycetous fungi.

Subsequent screening of Eastern Cape biodiversity for thermostable ligninolytic enzymes from basidiomycetes resulted in the isolation of a novel laccase enzyme from a basidiomycetous species. This fungus was identified as *Peniophora* sp. UD4 by phylogenetic analysis of rDNA ITS sequences. Initial studies indicated a superior optimum temperature of 70°C and thermostability, indicated by no loss in activity at 60°C over nine hours. Further characterization of the laccase revealed a broader than usual substrate range through its unusual ability to oxidatively couple DMAB and MBTH. The laccase also exhibited a broad pH oxidation range for ABTS (pH 2 – 6.8), and a relatively high affinity (K_m = 0.0123 mM) and catalytic efficiency (63 252 $mM^{-1}s^{-1}$) for ABTS as a substrate. The laccase activity from *Peniophora* sp. UD4 was shown to be comprised of

three isozymes with a molecular weight of 62 kDa and pI's of 6.33, 6.45 and 6.50.

Investigation of the nutrient and physical factors affecting ligninolytic enzyme production and growth of *Peniophora* sp. UD4 indicated that the wild-type organism was unsuitable for large scale production of the thermostable laccase due to the low levels of laccase production.

The thermostable laccase was applied to defouling of ultrafiltration membranes, bioremediation of industrial waste streams, biocatalysis, and biosensor technology as potential applications. Application of the *Peniophora* sp. UD4 laccase to defouling of membranes used for ultrafiltration of brown water showed large flux recoveries of 31, 21 and 21% after the first three defouling recycles respectively, compared to 3% for the control without immobilized enzyme. The novel laccase showed potential for the bioremediation of industrial waste streams, the most successful being that of bleach plant effluent, where a reduction of 66% of the phenolic load was achieved. Application of the novel laccase to biocatalytic oxidation of ferulic acid and (±)-α-pinene showed higher product yield as compared to oxidation of these compounds by *Trametes versicolor* laccase in mediated and non-mediated systems. The major products of (±)-α-pinene oxidation were identified as verbenol and trans-sorberol. The *Peniophora* sp. UD4 laccase was successfully applied to biosensor technology, which benchmarked significantly better than *Trametes versicolor* laccase for the detection of 4-chlorophenol. The biosensor developed with laccase from *UD4* by covalent binding to a glassy carbon electrode exhibited the best combination of sensitivity and stability.

This thesis shows that a laccase with superior properties was obtained from a mesophilic South African basidiomycete. The catalytic properties displayed by the novel laccase from *Peniophora* sp. UD4 all contribute to the increased

industrial applicability of laccases, and may be the most industrially feasible enzyme of its class isolated to date.

One secret of success in life is for a man to be ready for his opportunity when it comes.

Benjamin Disraeli

It is the mark of an educated mind to be able to entertain a thought without accepting it.

Aristotle

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Table of Contents

Chapter 1 General Introduction

Chapter 2 Screening of Basidiomycetous Fungi for Thermostable Ligninolytic Enzymes

Chapter 3 Purification and Partial Characterization of a Thermostable Laccase From An Unidentified Basidiomycete

Chapter 4 Identification and Phylogenetic Analysis of the Basidiomycetous Fungus Producing a Thermostable Laccase

Chapter 5 Production of Laccase and Manganese Peroxidase Under Various Culture Conditions Aimed at Improving the yield of a Thermostable Laccase from *UD4*

Chapter 6 Applications of the Thermostable Laccase

Chapter 7 General Conclusions

Appendices

References

List of Abbreviations

2-D	Two dimension
ABTS	2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt
ACH-cinnamic acid	α-Cyano-4-hydroxycinnamic acid
ACN	Acetonitrile
ASR	Ancestral state reconstruction
ATCC	American type culture collection
BPE	Bleach plant effluent
CBS	Centraalbureau voor Schimmelcultures
Da	Dalton
DMAB	3-Dimethylaminobenzoic acid
DNP	2,4-dinitrophenylhydrazine
EDTA	Ethylene diamine tetra-acetic acid
EIA	Enzyme linked immunoassay
EPR	Electron paramagnetic resonance
ET	Electron transfer
FC	Folin Ciocalteu
GC-MS-EI	Gas chromatography coupled with electrospray ionization mass spectrometry
GCE	Glassy carbon electrode
H_2O_2	Hydrogen peroxide
HAT	Hydrogen atom transfer
HBT	Hydroxybenzotriazole
HPI	N-hydroxypthalimide
HPLC	High performance liquid chromatography
IEF	Isoelectric focusing
ITS	Internal transcribed spacer
kDa	Kilodalton

Lac	Laccase
LiP	Lignin Peroxidase
LMS	Laccase mediated system
MALDI-TOF MS	Matrix assisted laser desorption ionization time of flight mass spectrometry
MBTH	3-Methyl-2-benzothiazolinone hydrazone
Med	Mediator
MiP	Manganese Independent peroxidase
MnP	Manganese peroxidase
MW	Molecular weight
N_2	Nitrogen gas
OMW	Olive mill wastewater
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PSD	Post-source decay
QSAR	Quantitative structure-activity relationship
rDNA	Ribosomal DNA
SDS	Sodium dodecyl sulfate
SE	Sasol effluent
SGL	Strip gas liquor
Taq Pol	*Thermus aquaticus* DNA polymerase
TEMED	N,N,N',N'-Tetramethylethylenediamine
TEMPO	2,2,6,6-tetramethylpiperidin-1-yloxy
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
Tris	2-Amino-2-(hydroxymethyl)-1,3-proanediol
UV	Ultraviolet
VP	Versatile peroxdase
WDE	Wine distillery effluent
WDI	Wine distillery influent

List of Schemes, Figures and Tables

Schemes

Figures

Tables

Chapter 1
General Introduction

1.1 Applications and Market for Industrial Enzymes

Enzymes are finding a broad applications base in industrial processes owing to the wide range of chemical reactions they can catalyze, relatively clean technology, and their chiral or regiospecific selectivity. This specific activity of enzymes is considered one of their major advantages over chemical synthesis. Alternatively, non-specific enzymes have also developed feasible technologies owing to their wide substrate range; laccase is an example of such an enzyme.

A market survey for industrial enzymes predicted an exponential growth in the sale of industrial enzymes as developing technologies were becoming realized (Magnani, 1998. A conservative prediction for the sale of industrial enzymes estimated an increase of $ 0.3 billion, from $ 1.5 billion in 1997 to $ 1.8 billion in 2002 (Magnani, 1998). More specifically, market sectors with potential laccase applications that were predicted to show the highest increases in sales were: detergents (from $ 705 million to $ 833.1 million); textiles ($ 161 million to $ 182.7 million); pulp and paper production (from $ 97.6 million to $ 136 million); and chemical synthesis ($ 59.2 million to $ 67.6 million) in the same period (Magnani, 1998). This market was subsequently realized, and the market for industrial enzymes was estimated to be approximately $ 2 billion in 2004 (Thakore, 2004).

1.2 Applicability of Enzymes to Industry

Considerable emphasis has been placed on developing environmentally benign or “green” technologies to replace existing technologies, including the treatment of industrial wastes (Bermek *et al*., 2002). A major component of the green technology revolution is the use of enzymes, which are finding increasing applications in the food, materials and chemical industries.

Enzymes are loosely defined as biological catalysts. Several important differentiations may be drawn between enzymes and their chemical counterparts:

(i) The reaction rates of enzymes often exceed those of chemical catalysts (Call and Mücke, 1997),

(ii) Enzyme catalyzed reactions transpire under milder reaction conditions. However, although many enzymes are active in organic solvents and relatively high temperatures, many optimal reaction conditions may be detrimental to the enzymes (Call and Mücke, 1997),

(iii) Enzymes display a high substrate and reaction specificity, and often enzymes are able to recognize and catalyze reactions of a few substrates (Call and Mücke, 1997). Reaction specificity is often important since the production of chemically pure isomers may be achieved. Several enzyme classes do however exhibit a broad substrate range, and this class includes the ligninolytic enzymes (such as laccase) but these reactions are often non-specific in terms of enantioselectivity but in general they do retain regioselectivity,

(iv) Tailoring of enzymes to perform specific reactions is possible through a thorough understanding of the protein structure, often achieved by computer modeling, followed by genetic engineering techniques such as site directed mutagenesis or gene shuffling,

(v) Although chemicals are often a less expensive alternative to the use of enzymes, this disadvantage may be offset by the reduced cost of effluent treatment afforded by biocatalytic reactions. These treatment

costs are becoming more costly due to the increasingly stringent laws regarding the toxicity of industrial effluent release.

1.3 Market size and Applications of Laccase

1.3.1 Bioremediation

1.3.1.1 Organisms and Enzymes

The advantages and disadvantages of the preferential use of organisms or enzymes for bioremediation have been well documented. The major advantage of enzymes over microbes is that they are largely unaffected by toxic components found within the effluents. The major advantages of organisms often includes are considered to be a reduction in the cost of treatment, since the effluent may act as a microbial growth medium.

Although in certain instances the use of enzymes is inevitable, methods exist to enable organisms to withstand adverse environments that contain higher toxin concentrations, these include immobilization of the cells, long-term cultivation under selective pressure of the xenobiotics, and genetic manipulations (Leontievsky *et al*., 2002). An example of conditioning of a fungus by long-term cultivation under selective pressure may be seen in the white-rot fungus *Panus tigrinus*, which was successfully adapted to live in increasing concentrations of chlorophenols (Leontievsky *et al*., 2002).

1.3.1.2 Bioremediation of Xenobiotics

Removal of hazardous phenolic compounds from effluents is a major problem currently experienced by many chemical industries including paper, textile, coal conversion, petroleum refining as well as many other sources (Atlow *et al*.,

1984). Furthermore, many of these phenolics are considered phytotoxic (Casa *et al*., 2003), antimicrobial, mutagenic and carcinogenic (Zilly *et al*., 2002). Current methods for the removal of these phenols from wastewater include solvent extraction, microbial degradation, adsorption and chemical oxidation (Buchanan and Nicell, 1997). The requirement for a reduction in treatment costs and the development of an energy efficient process for the oxidation and removal of these phenols has been noted by Aktaş and Tanyolaç (2003). Considerable emphasis has recently been placed on enzymatic treatment by phenoloxidases (family of oxidoreductive enzymes, which includes laccases) for the removal of aromatic compounds via polymerization (Aktaş and Tanyolaç 2003; Collins *et al*., 1996; Kang *et al*., 2002). Many research groups consider phenoloxidase enzymes attractive for industrial application due to their lack of hazardous side effects during oxidation of xenobiotics (Fernández-Sánchez *et al*., 2002). The secondary activity has also recently been shown to be effective at removing endocrine disruptors such as oestrogens from effluent streams (Suzuki *et al*., 2003). Increasingly stringent requirements imposed on the release of industrial wastewaters caused by an increasing concern of the effect on the environment, have prompted research into the field of bioremediation of waste streams polluted with several toxic phenolic compounds, and also into the activity of laccase against chromophores found in these streams (Gianfreda *et al.*, 1999).

Bollag *et al*. (2003) demonstrated the oxidative transformation of chlorophenols to polymers by laccase. These halogenated organic chemicals often pollute soil and groundwater and are usually produced by paper, herbicide and fungicide manufacturers (Bollag *et al*., 2003; Kang *et al*., 2002). Dehalogenation usually occurs as a secondary reaction in the laccase-mediated oxidation of these phenols.

Polycyclic aromatic hydrocarbons are present in many industrial effluents, and may exhibit carcinogenic and mutagenic activities (Vandertol-Vanier *et al*., 2002). The ability of the polyphenoloxidase enzymes, laccases, to oxidize polcyclic

aromatic hydrocarbons was demonstrated using mediators (chemicals forming reactive intermediate compounds that may in turn act on other substrates through electron transfer reactions), the most effective of which was the natural mediator 4-hydroxybenzyl alcohol with laccase from *Trametes versicolor* (Johannes and Majcherczyk, 2000).

Laccases have been implicated in the formation of humic materials (Bollag and Bollag, 1990; Bollag and Loll, 1983; Gianfreda *et al*., 1999). This assumption is based on the activity of laccases towards phenols and the resultant formation of polymeric aggregates that tend to be less soluble and more stable than their monomers. It has shown a large potential in the field due to the non-specificity of the enzyme (Gianfreda *et al*., 1999). The ability of laccase to transform phenol and other aromatic compounds into insoluble polymers has spurred interest in the application of this technology to treat groundwater and wastewater (Gianfreda *et al*., 1998). Concern has been raised about the fate of the final polymerization product, since often dimers and trimers may be more toxic than their monomeric precursors. Fortunately, the release of smaller subunits from the phenolic complexes was shown to be slow enough to be handled by natural biodegradation processes (Bollag and Bollag, 1990; Bollag and Loll, 1983).

1.3.1.3 Dye Degradation and Bio-bleaching

Synthetic dyes are released into the environment in wastewater streams from textile and dye manufacturing industries. These dyes are considerably recalcitrant to biodegradation in wastewater treatment facilities, and pose a severe environmental risk since they may be mutagenic and/or carcinogenic (Zilly *et al*., 2002). Current methods for dye-decolourisation are chemically derived and include adsorption, chemical transformation, and incineration (Zilly *et al*., 2002). It has been suggested that enhanced microbial decolourisation may provide a less expensive and more environmentally acceptable alternative to chemical treatment (Selvam *et al*., 2003).

Dye degradation by laccases is a well-studied phenomenon, and the application of laccases for bio-bleaching of these industrial textile dyes, including triarylmethane, indigoid, azo and anthraquinonic dyes has been suggested (Abadulla *et al*., 2000; Campos *et al*., 2001; Claus *et al*., 2002; Martins *et al*., 2003; Zilly *et al*., 2002). Azo dyes constitute half of all synthetic dyes, and are available in many forms. Some of the most toxic are amino-substituted azo dyes, which are often mutagenic and carcinogenic (Selvam *et al*., 2003). Current processes to treat azo dye-wastewater produce large quantities of sludge and fail to degrade dye mixtures, providing degradative enzymes with a large potential market (Lorenzo *et al*., 2002). An advantage of using fungal oxidative mechanisms to degrade azo dyes over other microorganisms is that it is possible to avoid the formation of hazardous breakdown products such as anilines formed by the reductive cleavage of azo dyes (Martins *et al*., 2003).

The use of laccases for this purpose is however hindered by the slow reaction rates attained. The feasibility of using a laccase-mediated system (LMS) over laccase catalysis may provide a feasible solution to this problem (Call and Mücke, 1997).

1.3.1.4 Pulp and Paper

Pulp bleaching is currently achieved by treating pulps with chlorine-based chemicals. This results in the formation of chlorinated aliphatic and aromatic compounds that could be acutely toxic, mutagenic and carcinogenic (Bermek *et al*., 2002; Monteiro and Carvalho, 1998). In recent years there have been intensive studies performed to develop enzymatic, environmentally benign, bleaching technologies (Bermek *et al*., 2002; Crestini and Argyropoulos, 1998). The use of laccase-mediated systems has shown potential for the bio-bleaching of pulp, but the feasibility of its use is hindered by the lack of an inexpensive mediator (Bermek *et al*., 2002).

The bioremediatory role of laccases in the pulp and paper industry is hindered by the alkalinity of the effluent. Thus several researchers have spent considerable effort in identifying laccases that could be suitable for this type of remediation. The laccase from *Coriolopsis gallica* has been implicated in the decolourisation of alkaline effluents such as the effluent from the pulp and paper industry (Calvo *et al*., 1998). Laccases have also been shown to be applicable to the bioremediation of pulp and paper industry waste by effecting direct dechlorination (Taspinar and Kolankaya, 1998) and the removal of chorophenols and chlorolignins from bleach effluents (Milstein *et al.*, 1988).

Other uses of laccases for the pulp and paper industry include reduction of the kappa number of pulp (Bajpai, 1999) and an improvement in the paper making properties of pulp (Wong *et al*., 2000).

1.3.1.5 Olive Mill Wastewater

Considerable effort has been directed to the bioremediation of olive mill wastewater (OMW), and therefore has been selected as a model effluent for discussing bioremediation of industrial effluent. OMW is a by-product of the olive oil extraction process, and is of concern especially in the Mediterranean area where the most recent estimate of 30 million m^3 of OMW is produced annually (Casa *et al.*, 2003). OMW is characterized by a dark colour due to the presence of polymerized pigment (Blánquez *et al.*, 2002; Ruiz *et al.*, 2002). The high organic load, intense colour, and presence of suspension solids qualify OMW as a hazardous pollutant waste (Blánquez *et al.*, 2002).

The release of OMW is governed by stringent standards that aim to protect the aquatic environment (Casa *et al.*, 2003). An alternative to releasing OMW into aquatic systems is to spread the effluent on land, but Casa *et al.* (2003) demonstrated that the phenols in the OMW displayed phytotoxic effects. They

also demonstrated that these effects could be overcome by oxidation of the phenols in the OMW with fungal laccases. OMW also displayed antibacterial activity, making traditional biological methods ineffective for treatment of OMW (Ruiz *et al.*, 2002). Laccases are generally stable in the presence of OMW toxic components, which indicated the potential of laccases for OMW detoxification (Ruiz *et al.*, 2002). This indicated that OMW could likely be used as a growth substrate for *Phanerochaete flavido-alba* (Ruiz *et al.*, 2002).

1.3.2 Diagnostics

1.3.2.1 Immunoassay

Laccases are finding application as the conjugate enzymes in enzyme linked immunoassays (EIA) due to both their high catalytic constants and the use of air oxygen as the co-substrate (Yaropolov *et al*., 1994). Several factors making laccases suitable alternatives to peroxidases, such as horse-radish peroxidases, have been identified. A major advantage of laccases for EIA is the use of dioxygen rather than peroxide as the co-substrate, and thus there is less inactivation of the conjugate enzyme laccase by oxidation (Aktaş *et al*., 2000; Buchanan and Nicell, 1997; Wu *et al*., 1999), and no formation of unproductive enzyme-substrate complexes (Yaropolov *et al*., 1994). Other advantages over peroxidases include a lower sensitivity to the content of variable valence metallic ions in the medium (Yaropolov *et al*., 1994). Since the substrates are the same for both enzymes, it is possible to substitute laccase for peroxidase and still use the same equipment and reagents in the immunoassay (Yaropolov *et al*., 1994). The advantages of oxygen as a co-substrate include the abundance of oxygen in the reaction, whereas hydrogen peroxide can only be added in low concentrations, since excess may lead to oxidation of the enzyme. Another disadvantage of low concentrations of the co-substrate is that it leads to the formation of unproductive enzyme-substrate complexes (Yaropolov *et al*., 1994). A constant supply of hydrogen peroxide may be achieved by the addition of

glucose and glucose oxidase, but the addition of these increases the cost of the process.

1.3.2.2 Biosensors and Biofuel Cells

The use of laccase in biosensor technology is mainly attributed to its broad substrate range allowing for the detection of a broad range of phenolics (Kuznetsov *et al*., 2001), this does however disallow the detection of specific constituents. Biosensors that utilize laccase include an electrode that may be used for the detection of phenols, such as catechols in tea (Ghindilis *et al*., 1992), phenolic compounds in wine, and lignins and phenols in wastewaters (Yaropolov *et al*., 1994). Novel biosensors have been developed using beneficial properties of laccase, such as the potentiometric imunosensor for the detection of antigens (Yaropolov *et al*., 1994). Laccase has displayed a significant potential for its use in biofuel cells (Tayhas *et al*., 1999). The major reason for this interest is the use of oxygen as a substrate, which is converted into water. The obvious advantage of this is the potential use in nanotechnology for medical applications in living animals, since oxygen may be scavenged from the bloodstream, while the byproduct (water) is benign. The drawback of using laccase in this technology is its inability to reduce oxygen at the physiological pH of blood, a technical hurdle that must be overcome.

1.3.3 Organic synthesis through Biocatalysis

Organic synthesis of chemicals suffers from several drawbacks, including the high cost of chemicals, cumbersome multi-step processes and toxicity of reagents (Yaropolov *et al*., 1994). Enzymatic polymerization has drawn considerable attention recently since it is capable of generating polymers that are impossible to produce through conventional chemical synthesis (Aktaş and Tanyolaç, 2003).

The application of laccase in organic synthesis has arisen due to its broad substrate range, and the conversion of substrates to unstable free (cation) radicals that may undergo further non-enzymatic reactions such as polymerization or hydration. This may also be achieved vicariously using an LMS as mentioned above. The choice of mediator has also been known to affect the final product of organic synthesis (d'Acunzo *et al*., 2002). Alternative mediators may yield different final products when using the same precursors. For example, the mediator 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO) selectively catalyzes the oxidation of hydroxymethyl groups to aldehyde groups in the presence of laccase, whereas N-hydroxypthalimide (HPI) may be used for ring-opening and phenol coupling (d'Acunzo *et al*., 2002).

Several other enzymes are capable of producing a similar secondary reaction, including the peroxidases. However, the use of peroxidases is subject to several disadvantages as discussed above (see 1.3.2.1 Immunoassay above). The use of peroxidases does however have the advantage allowing for the oxidation of compounds with higher redox potentials.

Examples of the potential application of laccases for organic synthesis include the oxidative coupling of katarantine and vindoline to vinblastine. Vinblastine is an important anti-cancer drug, especially useful in the treatment of leukemia. Vinblastine is a natural product that may be extracted from the plant *Catharanthus roseus*. The compound is however only produced in small quantities in the plant, whereas the precursors, namely katarantine and vindoline at much higher concentrations, and thus are relatively inexpensive to obtain and purify. A method of synthesis has been realized through the use of laccase to perform this coupling, with preliminary yields reaching 40 % conversion of the precursors to vinblastine (Yaropolov *et al*., 1994). Laccase coupling has also resulted in the production of several other novel compounds that exhibit beneficial properties, e.g. antibiotic properties (Pilz *et al*., 2001).

The polymerization property of laccase has been applied to catechol monomers for the production of polycatechol (Aktaş and Tanyolaç, 2003). Polycatechol is considered a valuable redox polymer, two important applications of which include chromatographic resins, and the formation of thin films for biosensors. Current methods for the production of polycatechol use soybean peroxidase or horseradish peroxidase, which suffer from the aforementioned disadvantages of peroxidases (see 1.3.2.1 *Immunoassay* above) (Aktaş *et al*., 2000). Inert phenolic polymers, for example poly(1-napthol) polymers, may also be produced by laccase-catalyzed reactions (Aktaş *et al*., 2001). These polymers have application in wood composites, fibre bonding, laminates, foundry resins, abrasives, friction and molding materials, coatings and adhesives (Aktaş *et al*., 2001; Dodrick *et al*., 1987).

Catechins are the condensed structural units of tannins, which are considered important anti-oxidants found in herbs, vegetables and teas. Catechins ability to scavenge free radicals makes them important in preventing cancer, inflammatory and cardiovascular diseases. Oxidation of catechin by laccase has yielded products with enhanced anti-oxidant capability (Hosny and Rosazza, 2002).

1.3.4 Food and Beverage Industry

The beverage industry is also set to be a benefactor of laccase. Laccase may prevent undesirable changes such as discoloration, clouding, haze or flavour changes in beer, fruit juices and wine improving their shelf life by removing phenols such as coumaric acids, flavans and anthocyanins (Gianfreda *et al*., 1999; Giovanelli and Ravasinin, 1993).

1.4 Factors affecting Laccase Application in Industry

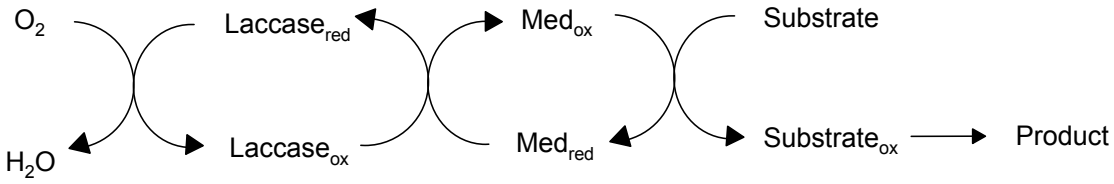
Laccases exhibit a broad natural substrate range, which is a major reason for the attractiveness of laccases to biotechnological applications (Nyanhongo *et al*., 2002). Even more interesting however is the application of laccase activity to a broader substrate range through the secondary activity of the free (cation) radical formed by oxidation of its substrates. The substrates that result in this type of activity towards other compounds are termed mediators. The industrial applicability of laccase may therefore be extended by the use of a laccase-mediator system.

Laccases are often considered the most important of the ligninolytic oxidative enzymes (Vasdev and Kuhad, 1994), and are capable of oxidizing phenolic and non-phenolic subunits of lignin model compounds. The broad substrate range of laccases that is derived from their non-specific formation of a free radical from a suitable substrate, and the use of air oxygen as a second substrate, make laccases attractive candidates for industrial application. Gianfreda *et al*., (1999) noted that the use of laccase in industrial operations is hampered by several drawbacks. The use of laccases is still expensive, even that of crude extract, and there is no easily available source of the enzyme. The characteristic catalytic efficiency, affinity and stability of laccases also have to be improved significantly before the enzymes can be used for bioremediation (Gianfreda *et al*., 1999). The most important factors affecting the applicability of laccases to industrial processes are outlined below.

1.4.1 Mediators

The oxidative action of laccases may be extended to uncharacteristic substrates by the addition of low molecular weight chemicals, which are termed mediators (scheme 1) (Fernández-Sánchez *et al*., 2002; Johannes and Majcherczyk, 2000). These mediators improve the applicability of laccases for bioremediatory and

biotechnological applications. Several naturally occurring mediators that are often produced by fungi have been identified, these include phenol, aniline, 4-hydroxybenzoic acid and 4-hydroxybenzyl alcohol (Johannes and Majcherczyk, 2000). Several artificial mediators have also been identified, these include 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1-hydroxybenzotriazole (HBT). Although the addition of mediators may broaden the applicability of laccase, there are two major drawbacks hindering their use, they are expensive and are often toxic (Bermek *et al*., 2002; Johannes and Majcherczyk, 2000).

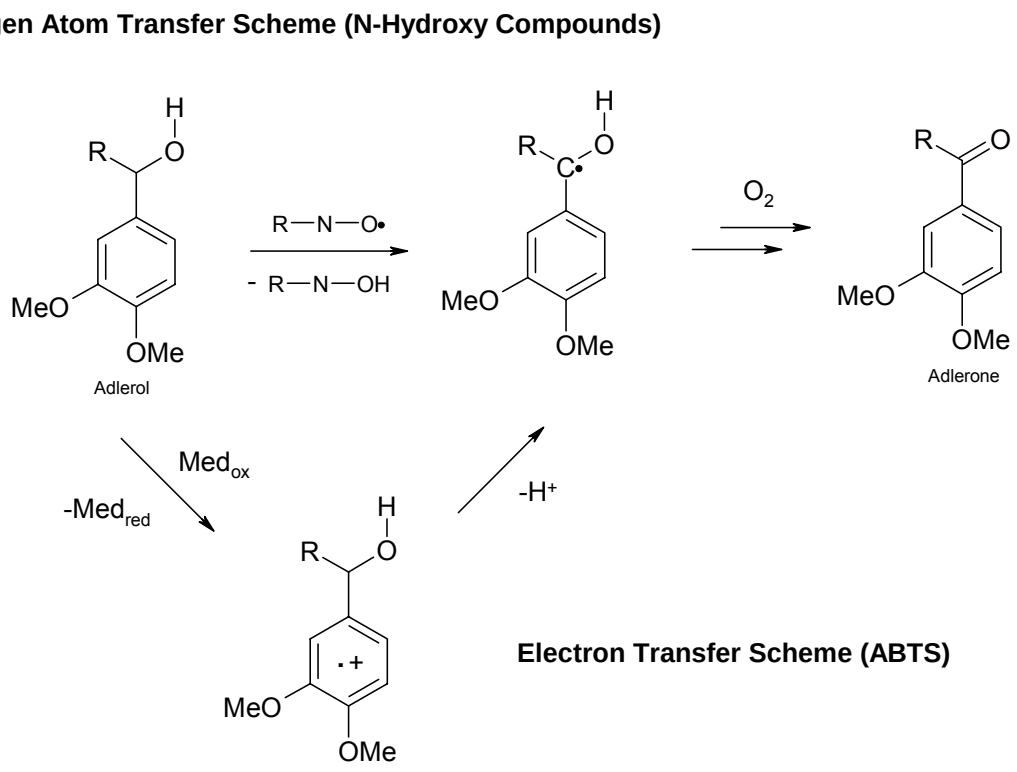


Scheme 1.1: Reaction mechanism of laccase indicating the role of mediators in the oxidation of non-usual substrates (Fabbrini *et al*., 2002)

As mentioned above the activity of laccase may be indirectly applied to a larger substrate range through the addition of a recognized substrate, which may indirectly oxidize other compounds, termed secondary substrates, which cannot enter the active site of the enzyme through steric hindrance, or that exhibit a redox potential higher than that possessed by the enzyme alone. The most commonly used mediators of laccase activity for industrial applications are ABTS [2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] and HBT [1-hydroxybenzotriazole] (Johannes and Majcherczyk, 2000). Currently over 200 mediators have been identified. The choice of mediator is also an important consideration when attempting to apply LMS to a specific technology since the mediator may affect the final product, by forming part of the final product, or preferentially performing a specific oxidation based on its effective redox potential or structure (d'Acunzo *et al*., 2002; Johannes and Majcherczyk, 2000).

Due to the extra expense associated with the addition of a mediator to realize an application, a differentiation should be drawn between the use of laccase and LMS for the industrial application of laccases. The activity of laccase may be applied to natural laccase substrates that may in turn act as mediators. Therefore the removal of phenolics from industrial waste may be achieved without the addition of a mediator if there is a sufficient quantity of natural substrate present in the effluent.

The addition of mediators for application studies is however commonplace. Two reaction mechanisms have been proposed for various mediators in LMS (Scheme 2), while the reaction mechanism of several others have not yet been elucidated (Fabbrini *et al*., 2002).



Scheme 1.2: Diagram indicating the difference between two proposed mechanisms for the oxidation of non-usual substrates by LMS, an electron transfer system (ABTS) and a hydrogen atom transfer mechanism (N-hydroxy compounds) (Barecca *et al*., 2003).

1.4.2 Stability of Laccases

Stability in enzymes is defined as resilience under adverse physiological conditions such as pH, temperature, and by counteracting inhibition by various other sources (Gianfreda *et al*., 1999). Thermostability of enzymes is an attractive feature for their biotechnological application (Berka *et al*., 1997). Although laccases may be viewed as moderately thermostable, considerable emphasis has been directed to the isolation of increasingly thermostable varieties.

1.4.3 Solvent Tolerance of Laccases

Several potential applications require that the processes take place in an organic solvent, or mixture of organic solvent and water, particularly in the field of organic synthesis where the precursors are generally organic compounds. An example where laccases requiring improved solvent tolerance would be beneficial is the potential oxidation of organosulfur compounds in petroleum, which helps reduce viscosity and improves the general quality of the petroleum (Van Hamme *et al*., 2003).

1.4.4 Kinetic Properties

Even with technologies such as recombinant production of enzymes, a severe hindrance to the use of these enzymes in industry is considered the relative expense of enzymes when compared to traditional synthetic chemical technologies. This disadvantage may be overcome by the isolation of novel laccases with improved kinetic properties, thereby reducing the quantity of enzyme required to perform a particular reaction.

1.5 Methods for Improving Laccase Activity

Due to the large laccase applications base, there is a potentially large market for laccases displaying enhanced characteristics. Bio-prospecting of laccases may yield alternative sources, capable of overcoming the shortfalls of currently available laccases.

1.5.1 Bioprospecting

Bioprospecting of wild isolates for laccases with improved properties may yield laccases with the desired qualities mentioned above. Characterization of these laccases can help develop an understanding of the important structural features involved in bestowing these enhanced attributes.

1.5.2 Protein Engineering

Crystallographic structure determination aids in the fundamental understanding of tertiary protein structure and identification of important residues. Recent crystal structures of laccases as determined by Bertrand *et al*. (2002) and Ducros *et al*. (1998) will assist researchers in designing new laccases with better substrate specificity/affinity, catalytic efficiency, and/or stability. The work by Bertrand *et al*. (2002) identified two particularly important amino acid residues, a histidine that coordinates the copper acting as the first electron acceptor, and an aspartate that is conserved among fungal laccases interacting with the amino group of 2,5-xylidine (the complexed substrate for this crystallographic study). Several amino acid residues that make hydrophobic interactions with the aromatic ring of the substrate were also identified (Bertrand *et al*., 2002). These important residues may then be altered through site directed mutagenesis in an attempt to improve the properties of laccase, such as specific activity and optimal pH. Positive results have been obtained using site directed mutagenesis of potentially important laccase amino acids (Xu *et al*., 1998).

1.5.3 Chemical Modification

The catalytic activity of laccase from *Coriolopsis gallica* was improved by treatment of the enzyme with polyethylene glycol (PEG: 5000 MW) (Vandertol-Vanier *et al*., 2002). The chemical modification of this laccase resulted a 1300 fold improvement in the catalytic efficiency for syringaldazine oxidation and a 1000 fold improvement in oxidation of polycyclic aromatic hydrocarbons.

1.5.4 Immobilization

Immobilization is a proven method of enhancing the stability of enzymes in unfavourable conditions (Ahn *et al*., 2002). The use of a natural support medium such as clay or soil is desirable since it poses no environmental risk, and is therefore beneficial for terrestrial bioremediation (Ahn *et al*., 2002). The benefits of immobilization may however be offset by the increased cost and loss of enzyme activity during immobilization (Ahn *et al*., 2002).

1.6 Laccase and Ligninolytic Enzymes

Lignin is the second most abundant polymer on earth, second only to cellulose, and is the most abundant aromatic material (Leonowicz *et al*., 1999), it may also be considered among the most recalcitrant natural products produced in the biosphere, and thus bioconversion of lignin is among the most important processes occurring in the carbon cycle (Rogalski *et al*., 1991). Primarily fungi of the class basidiomycota, ascomycetes and certain bacteria conduct the breakdown of lignin. Phenoloxidases, which include manganese peroxidase (Mn(II):hydrogen-peroxide oxidoreductase; EC 1.11.1.13), lignin peroxidase (Diarylpropane:oxygen, hydrogen-peroxide oxidoreductase; EC 1.11.1.14) and laccase (Benzenediol:oxygen oxidoreductase; EC 1.10.3.2) form an integral part of the biodegradation of lignin. The molecular size of these enzymes prevents

them from penetrating the lignin, leading to the assumption that lignin is oxidized indirectly by low molecular weight compounds (mediators), which may be found in the soil or produced by the fungus during growth (Evans *et al*., 1994).

1.6.1 Laccases

1.6.1.1 Classification and Description

Yoshida first discovered laccases in 1883 after an observation that latex from the Japanese lacquer tree (*Rhus vernicifera*) hardened in the presence of air (Call and Mücke, 1997; Gianfreda *et al*., 1999). Laccases are multinuclear copper-containing glycoproteins that belong to the family of enzymes known as oxidases, more specifically "blue" oxidases (Yaropolov *et al*., 1994), and polyphenol oxidases (Gianfreda *et al*., 1999). Laccases are one of six enzyme classes capable of reducing dioxygen to water, five of which belong to the multicopper oxidase family (the only enzyme not in this class being cytochrome-c oxidase, a heme/copper containing enzyme). Laccase is a polyphenol oxidase, indicative of the fact that laccases can oxidize a phenolic substrate that in turn can initiate a polymerization reaction (Gianfreda *et al*., 1999). Laccases from various sources vary greatly with respect to their degree of glycosylation, molecular weight and kinetic properties (Yaropolov *et al*., 1994).

1.6.1.2 Laccase Isozymes

A single organism may also possess several laccase isozymes (or isoforms) that may differ in their amino acid sequence and display different kinetic properties towards standard laccase substrates. Four different laccase isozymes have been detected in the basidiomycete *Rhizoctonia solani* (Wahleithner *et al*., 1996) and the ascomycete *Fusarium proliferatum* (Kwon and Anderson, 2001). Fungi may produce several isozymes of laccase that differ from one another with respect to both the degree of glycosylation and type of carbohydrate residues. An example

of this is *Trametes versicolor*, that was found to produce five isozymes differing only in carbohydrate content (Bertrand *et al*., 2002). The carbohydrate content of laccases may constitute 10 to 45 % of the molecular mass of the protein (Yaropolov *et al*., 1994), and in fungal laccases this usually constitutes between 15 and 20 % of the molecular mass (Thurston, 1994).

1.6.1.3 Laccases as Metallo-enzymes

Laccases have been shown to contain four copper(II) atoms per molecule that are essential for its catalytic activity (Ragusa *et al*., 2002). These four copper(II) atoms can be classified into three groups, type-1, type-2, and type-3 pair (Gianfreda *et al*., 1999, Ragusa *et al*., 2002) and are defined in terms of their spectroscopic properties and their electronic potential as determined by and their electron paramagnetic resonance (EPR) pattern (Gianfreda *et al*., 1999; Ragusa *et al*., 2002). Type-1 and type-2 copper atoms display strong electronic absorption, and have well defined EPR spectra, while the type-3 pair of copper(II) atoms are strongly coupled and are EPR silent (Ragusa *et al*., 2002), which may be activated by strong anion binding (Gianfreda *et al*., 1999). Laccases generally exhibit two absorption peaks when subjected to a UV-Vis wavelength scan, a strong absorbance is visible at 600 nm and is associated with the type-1 copper, while a shoulder at 330 nm is indicative of the type-3 pair of copper atoms. The occurrences of laccases that do not display this characteristic spectrum have been reported. A “white” laccase was said to be isolated from *Pleurotus ostreatus* (Palmieri *et al*., 1997), while Leontievsky *et al*. (1997) reported the presence of “yellow” laccases. The loss of the absorption peak at 600 nm of the “white” laccase was attributed to the presence of only a single copper atom in the metal cluster, the other three atoms being replaced by two zinc and one iron atom (Palmieri *et al*., 1997). Leontievsky *et al*. (1997) attributed the loss of this peak in the case of “yellow” laccases to copper atoms being present in their reduced state.

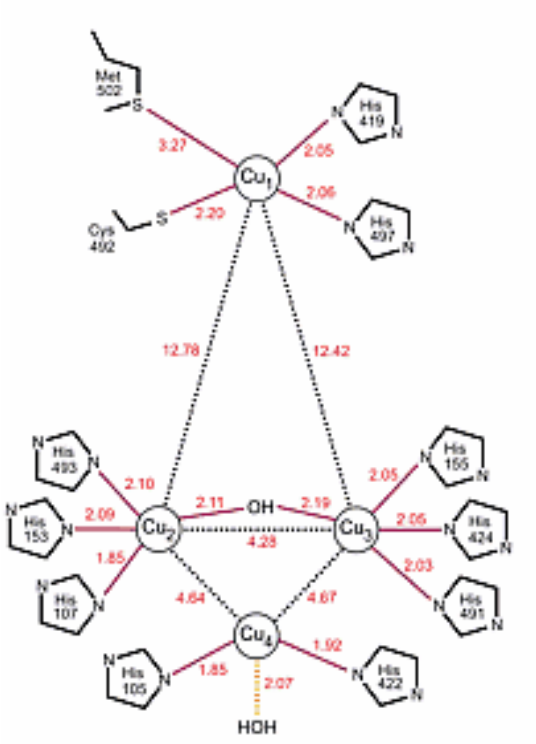


Figure 1.1: Illustration of the active site of *Bacillus subtilis* CotA laccase showing the relative orientation of the copper atoms as determined by x-ray chrystallography (reproduced from Enguita *et al.*, 2003).

1.6.1.4 Reaction Mechanism

The reaction mechanism may be described as the reduction of molecular oxygen, by various organic compounds, to water without the step of hydrogen peroxide formation (Yaropolov *et al*., 1994). Laccases exhibit a high affinity for oxygen as their electron acceptor, however they display a low affinity for their reducing substrates (Fernández-Sánchez *et al*., 2002). Laccases catalyze the oxidation of mono- and polyphenolic substrates and aromatic amines by the removal of a hydrogen atom from their hydroxyl group or removal of a single electron, to form a free radical. The radicals are susceptible to further oxidation by laccase or they may undergo non-enzymatic reactions such as hydration or polymerization (Yaropolov *et al*., 1994).

Although the exact mechanism of laccase activity has not been elucidated (Burke and Cairney, 2002), it is believed to comprise three major steps;

(i) type-1 copper reduction by the reducing substrate,

(ii) internal electron transfer from type 1 copper to type 2 and type 3 copper trinuclear cluster,

(iii) molecular oxygen reduction to water at type-2 and type-3 copper atoms (Gianfreda *et al*., 1999).

The type 2 and 3 copper atoms are thought to be associated in a trinuclear cluster (Gianfreda *et al*., 1999; Figure 1.1) at the site where the electrons from the substrate (transferred to the type-1 copper) are transferred to the oxygen molecule (Yaropolov *et al*., 1994). The fundamental understanding of the catalytic mechanism of laccase has recently received considerable attention and several researchers are currently investigating the interaction of laccase with its substrates through crystallography and x-ray diffraction studies (Antorini *et al*., 2002; Bertrand *et al*., 2002; Ducros *et al*., 1998; Piontek *et at.*, 2002). The four metal ions have been shown to be essential for optimal activity of laccase (Ducros *et al*., 1998).

1.6.1.5 Inhibitors of Laccase Activity

Anions such as the halides, azide, cyanide and hydroxide bind to the type 2 and 3 copper atoms of laccases, which disrupts the electron transfer system, resulting in enzyme inhibition (Gianfreda *et al*., 1999; Malkin *et al*., 1968). The inhibition by hydroxide generally prevents catalysis of substrates at alkaline pH (Xu, 1997). The inhibition of activity by hydroxide prevents autoxidation at alkaline pH, with a resultant increase in stability at alkaline pH (Xu, 1996). The inhibition by halides varies according to the laccase isozyme, and therefore likely related to the size of the channel of the trinuclear cluster (where oxygen binds) (Xu, 1996). Other types of inhibitors include certain metal ions (e.g. Hg^{2+}), fatty acids, sulfhydral reagents, hydroxyglycine, kojic acid, and cationic quaternary ammonium detergents (Gianfreda *et al*., 1999). These compounds may affect the laccase by chelating the copper(II) atoms, modification of amino acid residues or they may elicit a conformational change in the glycoprotein.

1.6.2 Other Ligninolytic Enzymes, Manganese Peroxidases (MnP) and Lignin Peroxidases (LiP)

Manganese peroxidases, a common peroxidase among white-rot fungi (Eggert *et al*., 1996; Hatakka, 1994), require the co-substrates hydrogen peroxide and Mn^{2+} for enzyme activity. It has been proposed that this enzyme uses a similar method for the degradation of lignin as laccase, but instead of using a phenolic substrate to oxidize lignin, it oxidizes Mn^{2+} to Mn^{3+}, which in turn oxidizes lignin (Wariishi *et al*., 1989). The presence of similar peroxidases have been reported (Keharia and Madamwar, 2002; Lobarzewski, 1981) that do not require Mn^{2+} as a co-substrate, differentiated from laccase only because they still require hydrogen peroxide for activity. These enzymes have been termed manganese-independent peroxidases (MiP).

Lignin peroxidases (LiP) are characterized by their ability to oxidize high redox potential substrates using hydrogen peroxide as their co-substrate (Martínez, 2002). LiP is a heme-containing glycoprotein differentiated from the other ligninolytic enzymes by its ability to oxidize veratryl alcohol as a substrate (Linko, 1992).

The defining characteristic has recently become obscured due to the definition of another ligninolytic enzyme termed versatile peroxidase (VP) from the genera *Pleurotus* and *Bjerkandera*, which are capable of oxidizing veratryl alcohol and Mn^{2+} to Mn^{3+}, LiP and MnP substrates respectively (Heinfling *et al*., 1998; Martínez, 2002).

1.7 Biological Function and Distribution of Laccases

The major source of laccase appears to be the ligninolytic fungal class known as basidiomycota, although it is not limited to these fungi. Certain plants such as *Rhus vernicifera* have also been shown to produce laccase. Several non-ligninolytic fungi, and a few bacteria have demonstrated laccase activity (Gianfreda *et al.*, 1999), which has led to increased speculation about the function of laccases in nature, rather than limitation to lignin degradation or protection against toxic phenolic compounds.

1.7.1 Fungi

The biological function of laccases in fungi remains unclear, but several biological functions have been proposed with a good scientific basis. The activity of laccases have been demonstrated in lignin decomposition and more specifically the polymerization of lignin oxidation products (Yaropolov *et al*., 1994), but this function is characteristic of the ligninolytic fungi and is not applicable to plants where the function remains more obscure (Gianfreda *et al.*, 1999). The role of laccases in lignin degradation has been demonstrated by laccase$^-$ mutants of *Pycnoporus cinnabarinus* as being greatly reduced in their ability to degrade lignin (Eggert *et al.*, 1997). The initial step in laccase-mediated lignin degradation is an oxidative reaction with the loss of one electron from the phenolic hydroxyl groups of lignin to produce phenoxy-radicals, which in turn may cause cleavage of lignin side chains, specifically alkyl chains (Gianfreda *et al.*, 1999). The overall metabolic mechanism by which lignin depolymerization by enzymes is achieved is not clearly defined, but seems to involve synchronized effects of other enzymes which interact to create an equilibrium between polymerization (by laccases) and lignin depolymerization; indeed, there is evidence indicating laccases are involved in both polymerization and depolymerization activities (Gianfreda *et al.*, 1999).

Laccases have been identified as possibly having an important role in fruit-body formation in fungi (Gianfreda *et al*., 1999). It has been observed that laccase activity increased during the formation of fruiting bodies, which indicates a possible function in their evolution (Gianfreda *et al*., 1999). This is not the only example of growth stimulation by laccases running parallel with growth improvement, laccase synthesis was observed to be an principal factor in growth and development of rhizomorphs in *Armillaria mellea*, further evidence of its importance in rhizomorph formation was decreased growth with the addition of laccase inhibitors (Gianfreda *et al*. 1999).

Laccase has also been implicated in virulence of the fungus *Botyritis cinerea*. The fungus was unable to attack its host plant when laccase was inhibited in the growing culture by the addition of a suppressor that prevented the production of laccase (Gianfreda *et al*., 1999). Similar results were obtained for *Heterobasidion annosum* (Johannsson *et al*., 1999) where it was noted that laccase was proportional to the aggressiveness of the root pathogen. Laccase has also been proposed to be involved in sclerotization in insects (Barrett, 1987), melanin biosynthesis (Dijkstra and Walker, 1991), and the formation of humus (Stevenson, 1994).

1.7.2 Plants

The presence of laccase in *Rhus vernicifera* is mentioned above. A proposed function of laccase in plants is the polymerization of monolignols into dimers and trimers during lignification (Bertrand *et al*., 2002). A possible role of laccase in the defense functioning of plants has been proposed (Mayer and Staples, 2002) since it was found in resin ducts of *Anacardiaceae*.

1.7.3 Bacteria

The majority of laccases occur in fungi, but several laccase-like enzymes have been noted in bacteria (Claus, 2003). Many bacteria have been shown to contain genes strongly resembling laccase genes, whose gene products are mainly involved in cell pigmentation and metal oxidation (Alexandre and Zhulin, 2000). The diversity of laccase in prokaryotes has recently been shown to include a thermostable laccase in the form of a coat protein in *Bacillus subtilis*, spore CotA protein (Hullo *et al*., 2001).

1.8 Production of Laccases from Basidiomycetes

The industrial importance of laccases has led to the need to enhance the laccase-producing ability of fungi (Lorenzo *et al.*, 2002). The production of ligninolytic enzymes by fungi is highly regulated by nutrients (Ben Hamman *et al.*, 1999; Miura *et al.*, 1997), making medium optimisation and investigation into alternative nutrient sources a useful tool for improving production of ligninolytic enzymes from fungi.

Previous findings indicate that for most fungi industrial scale production of laccase may not be gained from production in the host plant or fungal producer (Gianfreda *et al.*, 1999). The enzyme is produced in relatively low quantities from the host organism, and improvements using inducers is often not satisfactory alternative, since these inducers are generally toxic, and expensive (Gianfreda *et al.*, 1999). These factors have contributed to continuous emphasis being placed on alternative production strategies such as cloning and heterologous expression of laccase genes. The highest recorded natural production of laccase is likely from *Pycnoporus cinnabarinus*, a well-characterized over producer of laccase (Herpoël *et al*., 2000). Recently, 1 to 1.5 g/l of laccase was produced from the monokaryotic strain ss3, using ethanol as a low cost inducer (Lomascolo *et al.*, 2003).

1.8.1 Medium Optimisation

The ligninolytic production pattern of these enzymes has been shown to be species dependent, and even strain dependent for *Trametes versicolor* (Pickard *et al.*, 1999; Rogalski *et al.*, 1991), and thus medium optimisation provides a useful tool for an improvement in ligninolytic enzyme production. Several parameters for the improvement in production of laccases from white-rot fungi have been noted, and these have been linked to factors involved with the production of the enzyme. Nutrient starvation, either nitrogen or carbon, has been mentioned as a possible reason for the production of extracellular lignin degrading enzymes (Collins and Dobson, 1997; van der Woude *et al.*, 1993), and in several instances, limitation of one or more of these major nutrient components has been shown to improve production of lignin degrading extracellular enzymes. The oxygen availability is also an important consideration in the production of extracellular enzymes, since it is thought that oxygen excess enhances lignin degradation through production of ligninolytic enzymes (Dosoretz *et al.*, 1990). Oxygen rich environments have also provided improvements in the yield of lignin peroxidase (Rothschild *et al.*, 1995). It has been demonstrated that the type of ligninolytic enzymes produced by fungi can be directly controlled using medium components.

Increasing the production of lignin modifying enzymes may be achieved by modifying the source of carbon and nitrogen in the medium (Gayazov and Rodakiewicz-Nowak, 1996). Alterations in the source of carbon and nitrogen have provided significant improvements in the extracellular ligninolytic enzyme production experienced by several researchers. The use of malt extract as the carbon and nitrogen source in *Cyathus bulleri* resulted in a much higher yield of laccase than in mineral medium (Vasdev and Kuhad, 1994). Bran flakes have successfully been used as a carbon source, and provided a 794 fold increase in laccase production in *Trametes versicolor* UAMH 8272 and a 3190 fold

improvement in laccase production in *Coriolopsis gallica* UAMH 8260 (Pickard *et al.*, 1999).

A potential solution to the relative expense of enzymes for industrial application may be to decrease their production costs by investigating less expensive substrates, as opposed to a chemically defined medium (Berka *et al.*, 1997; Yaver *et al.*, 1999; Papinutti *et al.*, 2003). Investigation into the use of agricultural wastes such as cotton stalk extract (Ardon *et al.*, 1996; Castillo *et al.*, 1997), corn straw extracts (Crestini *et al.*, 1996) and potato waste (Trojanowski *et al.*, 1995) have yielded significant quantities of laccase.

Industrial effluents such as sugar cane baggase (Perumal and Kalaichelvan, 1996) and olive oil mill wastewater (Sanjust *et al.*, 1991) have also been investigated as potential nutrient sources for the production laccase. This strategy of using industrial effluents provides not only a less-expensive growth medium, but has the added benefit of allowing for bioremediation of the effluent.

1.8.2 Inducers

An important distinction may be drawn between laccases, they may either be inducible or constitutively expressed (Gianfreda *et al.*, 1999, Yaver *et al.*, 1996; Bollag and Leonowicz, 1984). The constitutive, or non-inducible group, do not react readily to dissolved compounds that exhibit properties similar to their substrates, or no inducer producing significant improvements in their yield has as yet been isolated. Single inducers may not elicit the desired response of laccase production, and a complex mixture of inducers may be required (Marbach *et al.*, 1985).

Several compounds may elicit a positive response on laccase production, these compounds known as inducers include the metal ions copper (Baldrian and Gabriel, 2002; Dittmer *et al.*, 1997) or cadmium (Baldrian and Gabriel, 2002),

cyclohexamide (Grotewold *et al.*, 1989) low molecular weight aromatic or organic acids, such as veratric acid (Collins and Dobson, 1997) and ferulic acid (Leonowicz and Trojanowski, 1975), as well as other phenolic or aromatic compounds, such as 2,5-xylidine (Bollag and Leonowicz, 1984) and veratryl alcohol (Pickard *et al.*, 1999; Rogalski *et al.*, 1991). These may form an integral prosthetic group within the enzyme, such as copper ions (Collins and Dobson, 1997), or natural substrates such as aromatic/phenolic compounds and lignin derivatives such as veratryl alcohol and 2,5-xylidine (Rogalski *et al.*, 1991). There is evidence, in *Trametes versicolor*, that these compounds cause an increase in mRNA levels, but only copper was involved in increasing laccase mRNA translation (Collins and Dobson, 1997). The exact action of inducers is however unknown. It has been demonstrated that fungi may possess several isozymes of laccase encoded by several laccase genes, and these may be differentially regulated (Collins and Dobson, 1997). It has further been suggested that the action of certain inducers may be as a direct result of their toxicity to the fungus, and the capability of laccase to polymerize and detoxify them (Collins and Dobson, 1997; Fenice *et al.*, 2003; Thurston, 1994). The use of inducers does however suffer from several disadvantages including their toxicity and the extra expense associated with the addition of an inducer.

It has been suggested that the addition of veratryl alcohol may not elicit an inductive effect, rather it may act as a protective agent against inactivation by hydrogen peroxide produced endogenously by the fungus (Dosoretz *et al.*, 1990), thereby indirectly eliciting a higher enzyme production.

1.8.3 Surfactants

The addition of detergents, e.g. Tween 20 or 80, has resulted in higher yields of ligninolytic enzymes in certain fungi. There is evidence that these detergents result in higher permeability of oxygen and extracellular enzyme transport through the cell membranes of fungi (Leštan *et al.*, 1994; Rothschild *et al.*, 1995).

Effective induction of laccase from *Pleurotus floridae* with anionic and cationic surfactants has been demonstrated (Dombrovskaya and Kostyshin, 1996).

1.8.4 Heterologous Expression

Recent advances in the field of genetic engineering have allowed the development of efficient expression vectors for the production of functional laccase. Common problems associated with heterologous expression of fungal enzymes are incorrect folding and inefficient codon usage of expression organisms, resulting in non-functional or low yields of enzyme. The incorrect substitution of carbohydrate residues during glycosylation of proteins, which is due to preferential utilization of specific carbohydrates by the expression organism, may pose an additional problem to heterologous expression. These problems are being overcome by using more advanced organisms as expression vectors whose codon usage and molecular folding apparatus are suitable for correct expression of these proteins. The most commonly used organisms include *Pichia pastoris* (Hong *et al.*, 2002; O'Callaghan *et al.*, 2002), *Aspergillus oryzae* (Berka *et al.*, 1997) and *Aspergillus niger* (Record *et al*., 2002).

1.8.5 Strain Improvement

A natural approach to increasing the ligninolytic enzyme production in basidiomycetes is through the genetic crossing of monokaryotic strains derived from spores, and screening of the resultant dikaryotic strains for improved production of enzymes. Using this methodology Eichlerová and Homolka (1999) increased the production of laccase ten-fold in *Pleurotus ostreatus*.

Increased production of laccase may be achieved through the mutation of wild-type fungal strains, followed by selection for the improved character. The use of a mutation-selection strategy was demonstrated successfully by Dhawan *et al.* (2003), and achieved a 6-fold increase in the production of laccase from *Cyathus*

bulleri after mutation with ethidium bromide. The major disadvantage of employing this strain improvement technique is the development of undesirable side effects, such as pleiotropism (Eichlerová and Homolka, 1999).

1.9 Research Hypothesis

South African biodiversity is an understudied source of potentially important industrial enzymes with unusual properties. Laccase with beneficial properties may be obtained from South African biodiversity.

1.10 Objectives

- Isolate and screen South African basidiomycetes for thermostable laccases
- Purify and characterize the thermostable laccases
- Characterize the organism producing these enzymes by identifying the basidiomycete and enhance the production by altering the medium
- Develop applications, and evaluate current applications using the thermostable laccase.

Chapter 2
Screening of Basidiomycetous Fungi for Thermostable Ligninolytic Enzymes

2.1 Introduction

Thermostability of enzymes is an attractive feature for their biotechnological application (Berka *et al*., 1997; Pilz *et al*., 2003), especially so for laccase due to its extensive and developing applications base. Isolation of these enzymes through bioprospecting does not only offer a superior alternative for current industrial applications, but may enable the realization of technologies where laccase may only be partially applicable owing to instability. The use of a thermostable laccase is especially applicable to processes that require a high temperature environment so that faster reaction rates may be achieved, or mechanical agitation, since thermostability is directly proportional to mechanical stability (Pilz *et al*., 2003).

Recently Ragusa *et al*. (2002), using *Rigidoporus lignosus* laccase, demonstrated that the high thermostability of laccase could be directly attributed to the metal content of laccase, as well as the presence of a compact protein structure thought to be composed of β-sheets. They also identified a part of the tertiary structure that is particularly sensitive to high temperatures, and suggested that this would likely be the reason for loss in activity of laccase above its average optimum temperature of 50°C.

Inadequate experimentation may lead to the misidentification of a laccase for other phenol oxidizing enzymes such as manganese peroxidase (Mayer and Staples, 2002). The use of substrates such as syringaldazine and ABTS should be used with caution since these substrates are catalyzed by both laccase and

manganese peroxidase (Mayer and Staples, 2002). Thus it is important to differentiate between these enzymes by means of their co-substrates, or the use of more specific assay substrates such as 3-dimethylaminobenzoic acid (DMAB) and 3-methyl-2-benzothiazolinone hydrazone (MBTH), the oxidative coupling of which is classified as a manganese peroxidase specific reaction due to its higher oxidative capability (Castillo *et al*., 1994).

This chapter describes the isolation and screening of South African basidiomycetes for the presence of the thermostable ligninolytic enzymes laccase, manganese-dependent and -independent peroxidase after a market for these enzymes was identified.

2.2 Objectives

- To isolate a thermostable laccase from South African biodiversity that may help realize industrial applications of laccase technology.

2.3 Methods and Materials

2.3.1 Chemicals

The assay substrates 3-dimethylaminobenzoic acid (DMAB), 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were obtained from Sigma. Guaiacol and bovine liver catalase were also obtained from Sigma. Ethylene diamine tetra-acetic acid (EDTA) and hydrogen peroxide (H_2O_2: 30 %) were obtained from Merck. Benomyl (50 %) was obtained from Efekto.

2.3.2 Isolation

Basidiomycetous carpophores were isolated from the Eastern Cape Province of South Africa, specifically Hogsback and Sam Knott Nature Reserve. Hogsback is a forested mountainous region of the Eastern Cape, whereas Sam Knott Nature Reserve is situated in a flatter, more arid region of the Eastern Cape.

Carpophores were picked and stored in cardboard boxes. The samples were tentatively identified using a field guide (van der Westhuizen and Eicker, 1994). The samples were then sprayed with 70 % ethanol, and thereafter contained in petri-dishes for a minimum of 5 minutes for surface sterilization. A sample of the interior flesh of the fruiting body was excised and placed on basidiomycete selective media, containing benomyl, chloramphenicol, guaiacol, and malt extract (de Jong *et al*., 1992), wherein hemp stem wood was substituted with yeast extract (0.1 %).

2.3.3 Culturing and Harvesting Techniques

Samples were incubated at room temperature (± 24°C) to ensure growth of culturable basidiomycetes. Two plugs were removed from these plates and subcultured at opposite ends of a petri-plate containing the selective medium. This subculturing was performed until a monoculture had been attained for all culturable basidiomycetes.

Four 5 mm diameter plugs from the monoculture plates were inoculated into 10 ml Tien and Kirk medium in 100 ml flasks (in hexaplate), and incubated as static culture for 2 weeks to ensure stationary phase had been reached. The flasks were flushed with pure oxygen once daily for 5 minutes at a flow rate of 100 ml a minute (Tien and Kirk, 1988; Kaal *et al*., 1995).

Contents of the replicate flasks were then pooled, and centrifuged at 10 000 rpm for 10 minutes using a Beckman JA-14 rotor to remove the mycelial mat. The supernatant was dialyzed against 2 litres of 10 mM Na-acetate buffer pH 6, four changes for three hours each (Tien and Kirk, 1988).

Following dialysis, the supernatant was freeze-dried without cryoprotectants, and a sample of freeze-dried extract was resuspended and used for the thermostability, pH and temperature profile assays.

2.3.4 Screening Optimum Temperature Assays

Optimum temperature profiles were performed using a Shimadzu spectrophotometer (UV 160 A) with waterbath attached to a water-jacketed cuvette holder for temperature control. The waterbath and Shimadzu spectrophotometer were equilibrated to assay temperature for 10 minutes prior to assaying. Samples, reagents and quartz cuvette used for assay, were equilibrated to assay temperature by placing them in the waterbath at the relevant temperature for 1 minute.

Initial optimum temperature assays were performed using ABTS as the substrate (Gold and Glenn, 1988) and resulting activity was measured at 420 nm with an extinction coefficient of 36 000 $M^{-1}cm^{-1}$ (refer to Table 2.1, A). The reagents contained Mn^{2+} ions (20 mM, in the form of manganous sulphate) and hydrogen peroxide (H_2O_2) (0.05 mM). This assay detected manganese oxidizing peroxidase (MnP), manganese independent peroxidase (MiP) and laccase, and was thus used as a screening assay for all the aforementioned enzymes.

2.3.5 Assays and Characterization of Enzyme Type

Once a thermostable extract was identified, differential enzyme assays were performed to determine whether the isolated thermostable enzyme was a laccase, MnP or MiP. These assays are described in Table 2.1 below, and referenced in the text where appropriate.

After the isolation of the enzyme/s with high temperature optima, the assay was adjusted to distinguish between MiP, MnP and laccase. General ligninolytic activity was determined with ABTS as the substrate with H_2O_2 (0.05 mM) and Mn^{2+} ions (20 mM) (Experiment A, Table 2.1), while MiP and laccase activity were determined by the addition of H_2O_2 exluding the addition of Mn^{2+} ions and the addition of ethylene diamine tetra-acetic acid (EDTA) to remove residual Mn^{2+} ions from the reagents (Experiment B, Table 2.1). Furthermore laccase specific activity was determined by the exclusion of H_2O_2 and the addition of bovine liver catalase (EC 1.11.1.6, Hydrogen-peroxide:hydrogen-peroxide oxidoreductase) at a concentration of 200 Units/l to remove residual H_2O_2 from the reagents (Experiment C, Table 2.1) (Kaal *et al*., 1995).

Further confirmation of the designated laccase was achieved by another version of the ABTS assay. This assay excluded Mn^{2+} ions, H_2O_2 and bovine liver catalase, and the addition of EDTA (Experiment D, Table 2.1), in equimolar concentration to Mn^{2+} ions concentration (20 mM) used for MnP assays. The inhibitory effect of EDTA on laccase was investigated, by assaying the laccase with high optimum temperature in the presence and absence of EDTA (20 mM).

To confirm that the enzyme in question was indeed laccase, a negative control assay was performed, namely the oxidative coupling of DMAB and MBTH in the presence of Mn^{2+} and H_2O_2. This assay was included to further overcome the problem of overlap of MnP, MiP and laccase activity using ABTS as the

substrate, since this assay is characterized as being manganese peroxidase specific (Castillo *et al*., 1994). A control assay was performed by omitting Mn^{2+} and H_2O_2 in the reagents, and the addition of EDTA and catalase, which would ensure the specificity of the reaction for MiP and MnP.

One enzyme unit was defined as the amount of enzyme required to convert 1 µmol of substate to product in 1 minute, under the assay condition used.

Table 2.1: Experimental strategy to define the activities present in the fungal culture supernatants.

Experiment	Detects	Mn^{2+}	H_20_2	Catalase	EDTA	Substrate
A	MnP, Lac, MiP	Y	Y	N	N	ABTS
B	MiP, Lac	N	Y	N	Y	ABTS
C	Lac	N	N	Y	N	ABTS
D	Lac	N	N	N	Y	ABTS

2.3.6 Thermostability

Thermostability was determined by incubation of the resuspended extract in a waterbath equilibrated to 60°C, and activity assayed at regular intervals, using the EDTA included version of the laccase assay with ABTS as the substrate (Experiment D, Table 2.1). The culture was re-isolated from its carpophore, cultured, and re-grown in liquid media under the same conditions, and assayed again for optimum temperature and thermostability, so as to ensure the candidate fungal isolate was indeed producing the thermostable enzyme, and not a contaminant.

2.4 Results

2.4.1 Results for Mycological Isolation Procedures and Temperature Optimum

Twenty-two samples were isolated from the field, and cultured as mentioned above in methods. Nineteen of these samples were culturable on the solid selective media, and of these nine isolates produced laccase, manganese peroxidase or manganese independent peroxidase in static liquid culture medium under the conditions mentioned above. These results are summarized in Table 2.2.

2.4.2 Enzyme Characterization

All organisms isolated were initially tested for thermostable laccase, manganese peroxidase and manganese independent peroxidase simultaneously using the methods mentioned above (Experiment A, Table 2.1). This was possible because of the non-specificity of the ABTS assay containing Mn^{2+} ions and H_2O_2, which detected activity of all three enzyme classes. The data showing the total ABTS oxidizing activity of isolates at different temperatures is shown in Figure 2.1. From Figure 2.1, it was observed that most species, except for *Trametes versicolor* and UD4 exhibited a biphasic profile (dual optima) curve of activity versus temperature. The temperature optima and nature of curve are summarized in Table 2.2. From Figure 2.1 and Table 2.2, the activity profile of UD4 looked most promising, and was therefore subjected to further observation.

Table 2.2: Summary of Results obtained for isolation and culture conditions from the field as well as the optimum temperature of the enzyme/s obtained as well as the nature of the graph obtained for the optimum temperature profile.

Species	Culturable	ABTS Oxidizing Activity	Biphasic Profile	Temperature Optima (°C)	Max Activity (Units/l at temperature optimum)
Phellinus rimosus	Yes	Yes	Yes	52	21.2 ± 1.0
Phellinus gilvus	Yes	Yes	Yes	56	23.4 ± 0.6
Phellinus spp.	Yes	Yes	Yes	53	27.9 ± 1.9
Stereum ostrea	Yes	No	-	-	-
Crepidotus spp.	Yes	Yes	Yes	57	10.3 ± 0.2
Ganoderma lucidum	Yes	Yes	Yes	53	22.0 ± 1.4
Coriolopsis polyzona	Yes	Yes	Yes	52	5.1 ± 0.3
Pycnoporus sanguineus	Yes	No	-	-	-
Schizophyllum commune	Yes	No	-	-	-
Trametes versicolor	Yes	Yes	No	59	140.9 ± 3.5
Trametes cingulata	Yes	Yes	Yes	53	17.7 ± 2.0
Gymnopilus junonius	No	-	-	-	-
Lenzites betulina	Yes	No	-	-	-
Coprinus plicatilis	No	-	-	-	-
UD1	Yes	No	-	-	-
UD2	Yes	No	-	-	-
UD3	Yes	No	-	-	-
UD4	Yes	Yes	No	70	21.8 ± 0.4
UD5	Yes	No	-	-	-
UD6	No	-	-	-	-
UD7	Yes	No	-	-	-
UD8	Yes	No	-	-	-

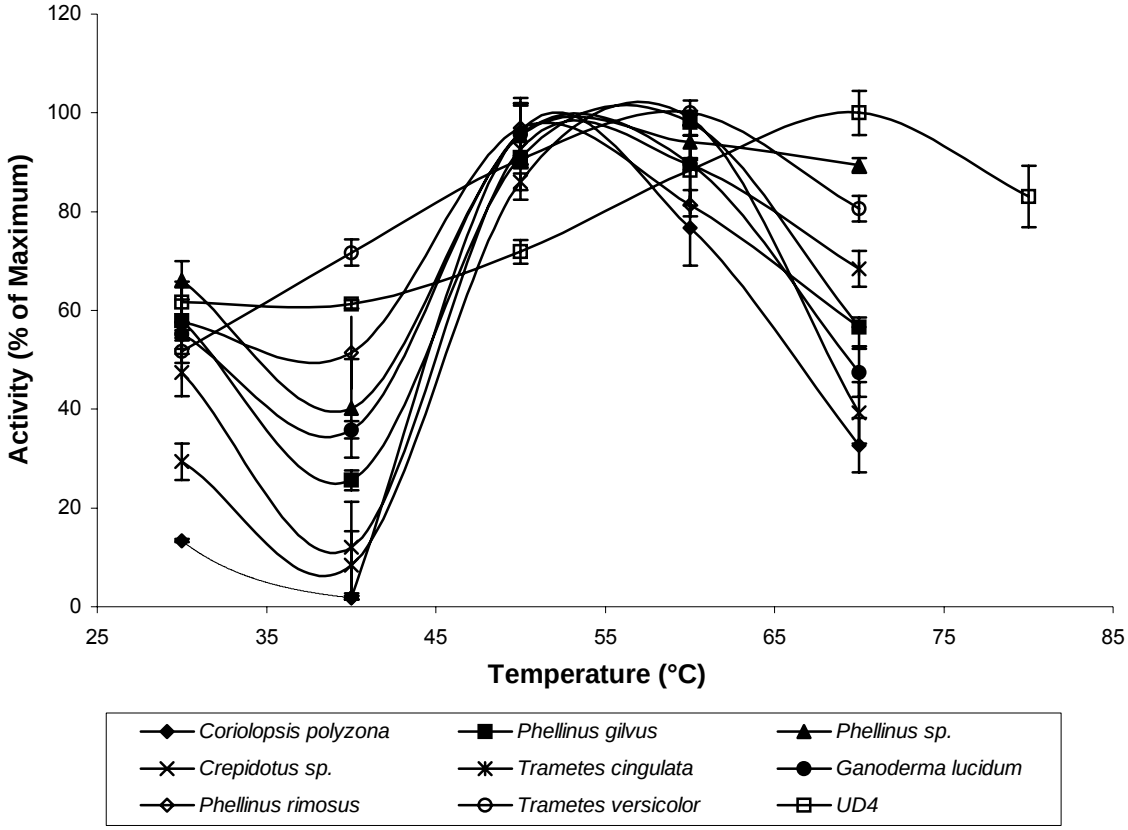


Figure 2.1: Indicates the temperature profiles for all organisms producing measurable quantities of enzyme in the culture conditions used. Most of profiles are biphasic (dual optima), except for the profiles obtained from *Trametes versicolor* and UD4. These two profiles indicate a broad range of activity, and only a single temperature optimum. Values indicated are means of triplicate assays at these temperatures, and error bars are indicative of standard deviations of these values.

The assays to determine the nature of the enzymes produced by UD4, which would show activity in the initial optimum temperature profile, yielded approximately the same activity for all three types of assay procedures used. Since enzyme assay A (Table 2.1) detects MnP, MiP and Laccase activity, and enzyme assay B (Table 2.1) detects MiP and laccase activity, and assay C

(Table 2.1) detects laccase activity only, these results can be used to calculate the relative activities for each three enzymes. The calculations and results are outlined in Table 2.3 below. The results indicate that the only enzyme produced by UD4, under the culture conditions described above, was laccase.

Table 2.3: Determination of the contribution of the enzyme laccase by means of deduction.

Enzyme Assay Used	Specificity of Assay (Enzymes showing oxidative activity)	Activity (Units/L)
A	MnP, MiP, Laccase	45.55 ± 2.65
B	MiP, Laccase	46.12 ± 1.56
C	Laccase	45.41 ± 2.41

The use of EDTA was investigated as an alternative to the use of catalase to ensure laccase specificity at high temperatures, such as the temperature optimum profiles. It was thought that at these high temperatures the selective agent, catalase, could denature and be unable to remove residual H_2O_2 from the reagents. This was investigated as an alternative method for the assay of the laccase. It was therefore necessary to determine the possible inhibitory effect of EDTA on laccase, since EDTA is a well-known inhibitor of metalloenzymes. If no inhibitory effect was noted, then EDTA could be employed to ensure specificity of further assays for laccase. The results for this experiment are outlined in Table 2.4.

Table 2.4: Results obtained for determination of inhibitory effect of EDTA on laccase isolated. The difference between the results obtained for these experiments indicates no inhibitory effect of EDTA on the thermostable laccase produced by UD4.

Enzyme Assay Used (Table 1)	Activity (% of Maximum)
C	96.52 ± 2.81
D	95.34 ± 2.69

Despite the use of controls including catalase and EDTA, still further differentiation between laccase, and MnP was sought by using other, more specific, substrates. Therefore another MnP and MiP specific assay technique was employed, the oxidative coupling of DMAB and MBTH (Castillo *et al*., 1994). These substrates were used in conjunction with H_2O_2 and Mn^{2+}, as well as the negative control reaction without the addition of these requirements for MnP and MiP activity, using catalase and EDTA in the reaction. The results indicated that there was no difference in the enzyme activity obtained for either version of the enzyme assay used. This is unusual since it indicates that the enzyme is a laccase with DMAB and MBTH oxidative coupling activity.

Once the nature of the enzyme was ascertained, it was subjected to a thermostability assay at 60°C, incubating two enzyme preparations, UD4 laccase and *Trametes versicolor* laccase. UD4 laccase exhibited an unusually high stability in comparison to that of *T.versicolor* laccase (the control). The results are indicated in Figure 2.2 below. The half-life of *T.versicolor* laccase is 9 hours at 60°C whereas laccase from UD4 did not lose significant activity at this temperature over the 9-hour incubation period.

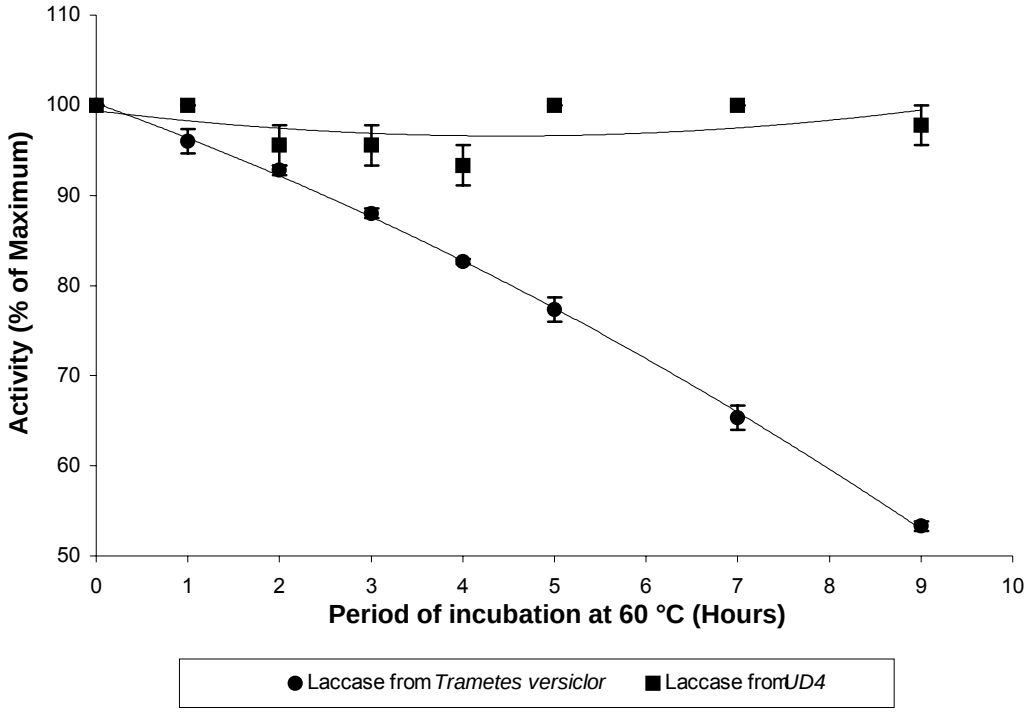


Figure 2.2: Thermostability assay for the 2 enzymes yielding the highest optimum temperature. Laccase from *Trametes versicolor* loses half its activity after approximately 9 hours incubation, while UD4 laccase remains stable, and retains 100 % of its activity after 9 hours incubation at 60°C.

2.5 Discussion

Initial temperature profile assays were performed on culture supernatants using a non-specific ABTS assay that detected manganese peroxidase, manganese independent peroxidase and laccase activity, as a simple screening method for selecting a thermotolerant laccase or manganese peroxidase.

It is unusual that *Pycnoporus sanguineus* did not produce any ABTS oxidizing enzymes under the culture conditions used, since it is characterized as an efficient producer of laccase (Herpoël *et al*, 2000). This may be attributed to

the culture conditions used, especially the length of incubation. Many basidiomycetes produce these enzymes after two or three days of growth, and these may have denatured or turned over during the two weeks of incubation. This methodology, used for the production of extracellular enzymes, could be improved by constant monitoring of the production of these enzymes of interest, rather than establishing a set incubation time.

Considerable effort was subsequently expended to determine whether the enzyme of interest from UD4 was a laccase, manganese peroxidase or manganese independent peroxidase. One of the difficulties in distinguishing between the enzymes is that they can all oxidize ABTS, but only laccase can catalyze the reaction in the absence of Mn^{2+} and H_2O_2. It was therefore important to remove all traces of Mn^{2+} and H_2O_2 for the laccase assay using ABTS as a substrate. Catalase effectively removes H_2O_2, and chelating agents can be used to remove Mn^{2+}. EDTA, a good chelating agent, has however been proposed as a possible inhibitor of laccase activity, but in most cases has not proven to be an efficient inhibitor of laccase (Shin and Lee, 2000; Palmieri, 1997). The results obtained for the EDTA inhibition study indicated that EDTA did not significantly affect the activity of the thermostable laccase, and thus could be used to improve the specificity of the assays used, as well as allow specificity at higher temperatures where catalase may become denatured, and thus incapable of removing H_2O_2.

Temperature optima obtained for all (excluding UD4) of the enzymes tested correspond well with literature values available. The first peaks fall below 40°C, this is most likely attributable to manganese peroxidase activity. The values reported in the available literature indicate that manganese peroxidase has an optimal activity of between 23 and 40°C (Forrester *et al*., 1990; Paszczynski *et al*., 1986; Wariishi *et al*., 1992; Aitken and Irvine, 1989). The second, and most predominant temperature optimum present in the temperature profiles, falls

within a range of 52 – 59°C. This corresponds well with the laccase optimum temperature values reported for *Botrytis cinerea,* i.e. 2 isozymes of 55 and 60°C (Zouari *et al.*, 1987), laccase from *Fomes fomentarius*, which has an optimum temperature of 52°C (Rogalski *et al*., 1991), and *Chaetomium thermophilium* laccase, which has an optimum temperature of between 50 and 60°C (Chefetz *et al*., 1998).

Trametes versicolor and UD4 appear to have only one optimum temperature present in their temperature profiles, whereas the other isolates appear to have two. Since only laccase was present in these two samples tested this gives credibility to the assumption that one temperature optimum represents MnP activity and the other laccase activity. Both *Trametes versicolor* and UD4 exhibit a high level of laccase activity at low temperature values (40°C), where the other species show minimal activity, which indicates that the other laccases may be inoperable or exhibit minimal activity at these lower temperatures. This result indicates a beneficial property for the UD4 laccase, since it may be used in lower temperature operations with significant activity, whereas larger quantities of the other laccases would be necessary at lower temperatures to yield equivalent catalytic rates.

The assay results for confirmation of the ligninolytic enzymes present, that showed high temperature stability, indicated that there was no significant amount of MiP and MnP present in the freeze-dried culture filtrate being investigated, and thus the thermostable enzyme was deduced to be laccase. This was later confirmed by the addition of EDTA and catalase to the reaction, which ensured absolute specificity for laccase. The assay technique used for thermostability at 60°C used reagents containing both EDTA and catalase, so as to ensure correlation of the data for laccase from *Trametes versicolor* and UD4.

The thermostability of the enzyme may provide applications in processes that require mechanical agitation, or in the treatment of heated effluents. There are also indications of the presence of a wider than normal substrate range, since the laccase catalyzes the oxidative coupling of DMAB and MBTH in the absence of Mn^{2+} and H_2O_2. This is astonishing since this assay technique has thus far been shown to be specific for manganese peroxidase (Castillo *et al*., 1994).

2.6 Conclusion

Twenty-five mycological carpophores yielded only twenty culturable isolates, of which only nine produced extracellular ligninolytic enzymes after cultivation in Tien and Kirk medium. The biphasic optimum temperature profiles obtained may be explained by the simultaneous presence of the two most common ABTS oxidizing enzymes produced by basidiomycetes, namely laccase and MnP.

A thermostable laccase was successfully identified (in the fungus UD4), which has an optimum temperature of approximately 70°C, and retains 100 % of its activity at 60°C during a 9-hour incubation (Jordaan and Leukes, 2003).

The laccase in question is capable of catalyzing the oxidative coupling of MBTH and DMAB, which has previously been thought to be indicative of MnP activity, MnP was proven to be absent during the enzyme assays employing ABTS as the substrate. This indicates the enzyme in question may exhibit a broader substrate range than typical laccases, and requires further investigation.

Chapter 3
Purification and Partial Characterization of a Thermostable Laccase From An Unidentified Basidiomycete

3.1 Introduction

After identification of a thermostable laccase from the basidiomycete UD4, a comparative study of the catalytic constants was performed to compare with that of alternative laccase sources. High catalytic efficiency would indicate an increased relevance for biotechnological application (Yaropolov *et al.,* 1994; Call and Mücke, 1997).

The most readily studied substrates are those that are most industrially applicable due to their activity. The use of ABTS as a mediator is the most extensively studied since ABTS is functionally the most useful of the mediators of laccase activity due to its two oxidative states, allowing for a broad range of secondary reactions to occur. Other commonly characterized substrates include syringic acid and guaiacol. The oxidation of guaiacol is of interest to researchers since it is a monomeric lignin model compound (Rittstieg *et al*., 2003), and therefore of importance as a model compound for the fundamental understanding of the biodegradation of lignin. It is also of biotechnological importance since it is used in the synthesis of flavour and fragrance compounds (Overhage *et al*., 2002). Syringic acid and guaiacol are also well-recognized substrates for determining phenoloxidase activity. It was also thought important to kinetically characterize the oxidative coupling of DMAB and MBTH since this reaction may be of biotechnological importance to the application of the UD4 laccase, since it is indicative that UD4 laccase is capable of performing reactions requiring a higher than usual redox potential for enzymes of its class.

The purification of a cell free laccase is an essential step for the determination of accurate kinetic parameters due to the possible presence of compounds from the host fungus that may act as natural mediators (Johannes and Majcherczyk, 2000), or the presence of similar enzymes that may exhibit significantly different reaction kinetics.

The most commonly used method for laccase purification is salt elution from an anion-exchange resin, probably due to the higher stability of laccase at neutral to slightly alkaline pH, as well as the pI of laccases (around 4.5). Isozyme purification may be achieved by using quaternary ammonium (QA) anion exchange resins rather than diethylaminoethyl (DEAE), using a shallow salt gradient to achieve effective separation of similar proteins (isozymes). Another commonly used method for isozyme purification is isoelectric focusing, since the various isozymes often exhibit significant differences in their pI.

The methods and results of attempting to get laccase amino acid sequences are delineated in this chapter.

3.2 Objectives

- To purify the thermostable laccase for characterization of its kinetic properties
- To compare the kinetic properties of the thermostable laccase to currently available sources of the enzyme.
- To obtain peptide sequences for derivation of laccase gene specific sequences.

3.3 Methods and Materials

3.3.1 Chemicals

Toyopearl® Super Q 650 M quaternary ammonium anion exchange resin was purchased from TosohBiosep. Molecular weight markers XIV were purchased from Roche. Unless otherwise stated A Mini-PROTEAN 3 Electrophoresis Cell (BioRad) was used for polyacrylamide gel electrophoresis. Immobiline DryStrips pH 3-10 (24 cm, non-linear), IPG buffer pH 3-10, Ettan CAF MALDI sequencing kit, ammonium persulfate, N,N,N',N'-tetramethylethylenediamine (TEMED) and bromophenol blue were obtained from Amersham Biosciences. Sodium thiosulfate, agarose, acetic acid, CHAPS, formaldehyde, sodium bicarbonate, DL-dithiothreitol, iodoacetamide, acrylamide/bis solution (29:1), glycine and sodium dodecyl sulfate (SDS) were obtained from Sigma-Aldrich. 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris), methanol and glacial acetic acid were obtained from Merck.

3.3.2 Organism, Maintenance and Culture Conditions

The production organism used was an unidentified fungal species, tentatively named UD4, which was found to produce a thermostable laccase. Cultures were maintained on agar slants containing malt extract agar (2 %) and 0.06 g/l Benomyl (Efekto) to suppress bacterial and ascomycetous contaminants. Laccase was produced using two culturing methods. The organism was grown in shallow, stationary, liquid culture conditions with Tien and Kirk chemically defined medium. Liquid cultures consisted of 100 ml of growth medium in 1 L flasks. These were inoculated with 5 x 10 mm^2 agar plugs from the growing edge of a fungal colony on malt extract agar. Liquid cultures were centrifuged at 10000 g for 10 minutes and the supernatant dialyzed.

3.3.3 Laccase Assays

Laccase assays were performed, in most cases, by using 0.1 mM 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as the substrate in 100 mM succinate-lactate buffer, pH 4.5. The absorbance of the solution was measured at 420 nm (Gold and Glenn, 1988). The pH profiles of the enzyme were performed using universal buffer containing 50 mM phosphate (di-sodium hydrogen orthophosphate), 33 mM citrate (citric acid) and 50.7 mM borate (boric acid), adjusted to pH values ranging from 2 to 8 (Perrin and Dempsey, 1974). The pH values were measured again after addition of the substrates for accurate plots of pH profiles for laccase activity. The Michaelis-Menten coefficient (K_m) values for the different substrates were determined in 50 mM tartrate buffer (Fukushima and Kirk, 1995) and 100 mM succinate-lactate buffer. The pH of the buffer was adjusted to the optimum pH for oxidation of the substrate studied. The K_i values for inhibitors were determined in 100 mM succinate-lactate buffer, pH 4.5 with ABTS as the substrate (Tien and Kirk, 1988). One unit of enzyme was defined as the amount of enzyme required to convert 1 µmol of substrate to product in 1 minute under the assay conditions employed. All assays were performed in hexaplate using a PowerWave Microtitre Plate Reader (BioTek), and kinetic assays were performed at 25°C. Data is represented as means ± standard deviation.

3.3.4 Enzyme Preparation

Liquid cultures (800 ml) were harvested and pooled after 14 days, this was then centrifuged at 3 800 X g using a JA 14 rotor (Beckman) for 5 minutes (to remove mycelial mass) and freeze-dried as a concentration step. This freeze-dried extract was purified as described and used for enzyme characterization.

The lyophilized extract from the liquid culturing was resuspended in the minimal amount of water and dialyzed against 20 mM Tris Buffer (pH 7.2), containing 50

mM NaCl (equilibration buffer), using SnakeSkin® (Pierce) pleated dialysis tubing (10 kDa MW cutoff). The resulting solution was applied to a Super Q 650 M (TosohBiosep) anion exchange resin column pre-equilibrated with equilibration buffer, with column dimensions of 30 cm X 2 cm. Bound protein was eluted with a 400 ml linear salt gradient from 50 mM to 400 mM NaCl in 20 mM Tris buffer (pH 7.2) at a flow rate of 100 ml/h, collecting 3 ml fractions. Fractions were assayed for protein by monitoring ultraviolet absorption at 280 nm, and laccase activity was determined for each fraction using ABTS as the substrate (0.1mM) in 100 mM succinate-lactate buffer pH 4.5 (Tien and Kirk, 1988). Fractions containing laccase activity were pooled and dialyzed against 10 mM Tris Buffer (pH 7.2). The dialyzed eluate was then lyophilized and stored at –20°C. The lyophilized extracts were then pooled and re-dissolved in a minimum amount of 20 mM Tris buffer (pH 7.2), containing 120 mM NaCl. The solution was dialyzed against the same buffer, and re-applied to the anion exchange column, pre-equilibrated with 20 mM Tris buffer containing 120 mM NaCl (pH 7.2), and eluted with a 400 ml gradient from 120 mM to 280 mM, at a flow rate of 100 ml/h. Fractions were collected and monitored as mentioned above. Laccase-containing fractions were collected, pooled and dialyzed against 10 mM Tris Buffer (pH 7.2). This solution was then lyophilized and stored at –20°C.

3.3.5 Laccase Characterization

3.3.5.1 Molecular Weight Determination

The lyophilized samples were dissolved in a minimum amount of distilled water, and subjected to denaturing and non-denaturing PAGE on 10 % acrylamide gels. SDS-PAGE was performed to determine sample purity and to determine the approximate mass of the thermostable laccase. The approximate molecular mass of the laccase was determined by calibration against broad range molecular weight markers (BioRad), which contained the proteins myosin (200 kDa), ß-galactosidase (116.3 kDa), phosphorylase B (97.4 kDa), bovine serum

albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa). SDS-PAGE and non-denaturing PAGE revealed the presence of 2 proteins. Non-denaturing PAGE was performed to ascertain which protein correlated to laccase activity. The non-denaturing gel with duplicate sets of samples was bisected and half was stained with Coomassie Brilliant Blue R-250, the other half was stained with guaiacol to determine which band correlated to laccase activity.

3.3.5.2 pI Determination

pI's for UD4 laccase isozymes were determined using an Ettan IPGPhor™ iso-electric focusing (IEF) unit (Amersham Biosciences) on a 13 cm pH 4-7 linear Immobiline Strip (Amersham Biosciences).

3.3.5.3 Kinetic Constants

K_m values were determined using Lineweaver-Burk plots for the individual substrates guaiacol (0.05 to 1 mM), syringic acid (0.01 to 1 mM) ABTS (0.005 to 0.1 mM), and the combined substrates 3-dimethylaminobenzoic acid (DMAB) and 3-methyl-2-benzothiazolinone hydrazone (MBTH) (0.3 to 1 mM for DMAB and to 0.021 to 0.07 mM: ratio of 1:0.07 DMAB:MBTH). As mentioned in Chapter 2, DMAB and MBTH oxidative coupling activity is typically a manganese peroxidase specific reaction (Castillo *et al.,* 1994). The k_{cat} for each substrate was also determined and the k_{cat}/K_m calculated. The wavelengths for measuring laccase activity with the abovementioned substrates were determined spectrophotometrically by allowing a reaction with laccase to proceed to completion, performing a spectral scan, and selecting suitable λ_{max} values. The λ_{max} values obtained were 420 nm for ABTS, 450 nm for guaiacol, 365 nm for syringic acid, and 590 nm for DMAB and MBTH as the substrates.

3.3.5.4 Inhibitor Constants

The effect of various potential inhibitors was investigated using ABTS as the substrate and determined by primary and secondary Lineweaver-Burk plots of the data. Ethylene diamine tetra-acetic acid (EDTA) was investigated at concentrations up to 20 mM, thioglycolic acid (mercapto-acetic acid) was investigated as a potential inhibitor, up to a concentration of 0.5 mM, sodium azide was investigated up to 0.05 mM, and hydroxylamine up to a concentration of 4 mM. K_i determination was performed on compounds that showed significant inhibition. For K_i determination ABTS concentrations were varied from 0.005 to 0.05 mM.

3.3.6 Peptide Fingerprinting and Sequencing

3.3.6.1 2D PAGE

Partially purified protein was subjected to non-denaturing 2-D PAGE. The first dimension, or IEF was performed on an Ettan IPGPhor™ (Amersham Biosciences) using a 24 cm broad range non-linear (pH 3-10) Immobiline Strip (Amersham Biosciences). Rehydration buffer was modified from the 2-D Electrophoresis Handbook (Amersham Biosciences), and consisted of bromophenol blue (0.01%, from a stock of 1 % in 50 mM Tris base), glycerol (20%), CHAPS (2%) and 2.5 µl of IPG buffer (Amersham Pharmacia). Sample was loaded onto the immobiline strip using the rehydration loading protocol from the 2-D Electrophoresis Handbook (Amersham Biosciences). The second dimension non-denaturing gel electrophoresis was performed on a Protean™ II XI (Bio-Rad) with a 12% acrylamide gel run at 200V for 5 hours. The electrophoresis unit was fitted with a cooling waterbath and maintained at 4°C. To accommodate the use of the Protean™ II XI for the second dimension PAGE, strips were cut to 16 cm from the pH 10 end.

3.3.6.2 Activity Staining

Laccase activity stain was performed with 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS - Fluka) as the substrate, using agarose to localize the ABTS oxidation by laccase. Non-denaturing gels were allowed to stand in 100 ml of succinate-lactate buffer (100 mM, pH 4.5) for 10 minutes to allow for pH adjustment. The acrylamide gel was removed from the buffer and placed on a clean surface. Substrate (1ml of 10 mM ABTS) was spread evenly over the surface of the gel and allowed to stand for 2 minutes. This was then covered with warm (40°C) 0.5 % agarose in succinate-lactate buffer (100 mM, pH 4.5). Laccase activity spots were indicated by the development of a green colour, and were extracted from the gel using a 2 mm cork-borer. Acrylamide fragments were placed in destaining solution containing 20 % methanol and 20 % glacial acetic acid in water. This destaining solution also served to localize the protein spot in the acrylamide fragment.

3.3.6.3 MALDI-TOF MS Analysis

All experiments were performed using a Bruker Autoflex MALDI-TOF MS (Bruker, Bremen, Germany). The Bruker Autoflex is equipped with a N_2 laser and a variable voltage reflectron. The parameter settings were optimized for analysis of peptides in both reflector and post-source decay (PSD) mode. The samples for MALDI analysis were prepared by the dried-droplet method whereby equal volumes of the tryptic digest or derivatized sample were mixed with a solution of 10 mg/mL α-cyano-4-hydroxycinnamic acid in 0.1% TFA/ 50%ACN. About 1 μL of the resulting solution was deposited on polished stainless steel sample plate. The instrument calibration was performed externally using seven peptides ranging from m/z 1046.51 to 3147.47 Da.

3.3.6.4 Tryptic Digest Fingerprinting

In-gel digestion of proteins was performed using sequencing grade modified trypsin (Promega) and peptide extraction from the acrylamide gel was performed as per the manufacturer's protocol. Digested laccase isozymes were desalted using C_{18} ZipTips (μZT, Millipore Corporation) following the manufacturers protocol. Digested samples were dried in a α-cyano-4hydroxycinnamic acid (ACH-cinnamic acid) matrix and analyzed using a Bruker Autoflex MALDI-TOF MS as described above (3.3.6.3).

3.3.6.5 Derivatization of Peptides

Peptides were immobilized on solid-phase supports (μZT), and derivatized using the Ettan CAF MALDI Sequencing Kit (Amersham Biosciences) following the manufacturers protocol. Derivatized peptides were then dried in a vacuum centrifuge and reconstituted in 2 μl of ACH-cinnamic acid matrix solution. Derivatized sample was loaded onto the polished stainless steel target and analyzed using a Bruker Autoflex MALDI-TOF MS as described above (3.3.6.3).

3.4 Results

3.4.1 Laccase Purification

The purification of laccase resulted in a relatively low yield of 12 % (unit/unit) of the original 4023 units of enzyme. This was probably due to selecting fractions containing 50 % or greater activity when compared to the fraction containing the highest number of units after anion exchange chromatography. A large loss was also experienced during lyophilization without cryoprotectant, which may have affected the structure of the protein resulting in a loss of protein or protein aggregation.

Table 3.1: Purification table of thermostable laccase from UD4.

Purification Step	Total Protein (mg)	Enzyme Activity (Units)	Specific Activity (Units/mg)	Yield (%)	Fold Purification
Dialyzed Culture Filtrate (Crude Enzyme)	862.24	4023	5	100	1.00
Super Q Anion Exchange Chromatography	69.45	2291	33	57	7.07
Freeze Drying and Dialysis	58.74	1176	20	29	4.29
Super Q Anion Exchange Re-chromatography (SG)	9.03	473	52	12	11.23

SG – shallow gradient (120 mM to 280 mM)

3.4.2 Molecular Weight Determination

The molecular weight of the thermostable laccase was determined by comparison with the molecular weight standards mentioned above. The molecular weight of the protein was calculated to be approximately 62 kDa, a co-purified protein of 38 kDa was also visualized.

3.4.3 pI Determination

Three isozymes were identified and labeled ‘L’, ‘M’ and ‘R’. pI’s for these three isozymes were calculated as 6.33, 6.45 and 6.50 respectively. Coomassie staining of the IEF strip was used to determine the relative abundance of the three isozymes. The concentration of the M isozyme far exceeded those of the L and R isozymes.

3.4.4 pH Optima

The pH profiles for the various substrates are shown in Figure 3.1. DMAB and MBTH oxidative coupling reaction had an optimal pH similar to that of guaiacol at pH 6. The optimal pH for syringic acid oxidation was 4.5, and 4 for ABTS. The enzyme exhibited a comparatively broader pH profile with ABTS than the other substrates tested.

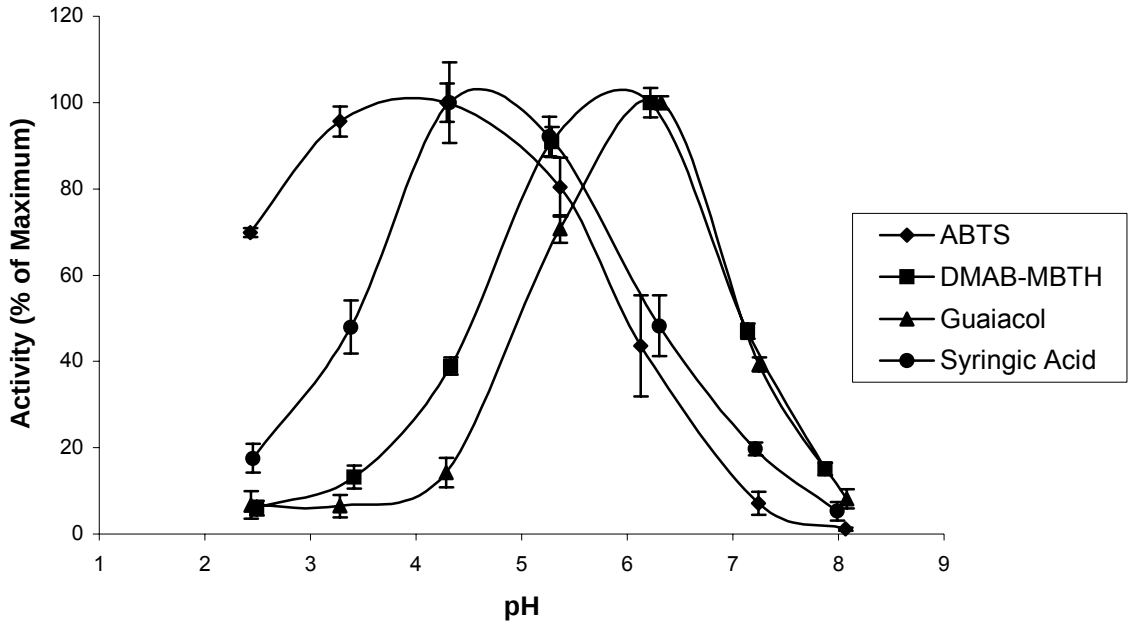


Figure 3.1: pH Profiles for the thermostable laccase with various substrates. Error bars indicate mean ± standard deviation of triplicate samples.

3.4.5 Determination of Kinetic Parameters

As mentioned in the methods, the laccase was characterized in terms of its Michaelis Menten constant (K_m), turnover number (k_{cat}) and specificity constant (k_{cat}/ K_m) with various substrates (see Table 3.2). Inhibitor constants (K_i) were determined for inhibitors that were found to exhibit significant inhibition of the thermostable laccase. Figures 3.2, 3.3 and 3.4 show the Lineweaver-Burk plots for the determination of apparent K_i values with different concentrations of inhibitor. Figures 3.5, 3.6 and 3.7 indicate the secondary plots for the determination of K_i for sodium azide, hydroxylamine and thioglycolic acid respectively.

Table 3.2: Kinetic properties obtained for the thermostable laccase from UD4 at various pH values. Data was obtained from Lineweaver-Burk plots, where x intercept = $-1/K_m$, and y intercept = $1/V_{max}$. The turnover number (k_{cat}) and specificity constant for each substrate were also determined.

Substrate	Optimum pH	pH Range*	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	Buffering System
ABTS	4	2 – 6.8	0.0123	778	63300	Succinate-Lactate
DMAB - MBTH	6	3.8 – 7.8	6.90	368	53	Tartrate
Syringic Acid	4.5	2.5 – 7.2	0.026	10.1	388	Tatrate
Guaiacol	6	4.5 – 7.8	0.251	42.1	168	Succinate-Lactate

* - The range end-points correspond to pH values at 20 % of maximum activity.

Hydroxylamine, sodium azide and thioglycolic acid, exhibited significant inhibition of the thermostable laccase, while EDTA did not inhibit the laccase up to a concentration of 20 mM. For thioglycolic acid, the initial velocity (u_0) was preceded by a lengthy lag phase. The K_i for thioglycolic acid was calculated from velocity values obtained after the lag phase. The Lineweaver-Burk profiles observed for thioglycolic acid as an inhibitor (Fig. 4) exhibited competitive mixed inhibition. The experiment was repeated, and the same data was obtained.

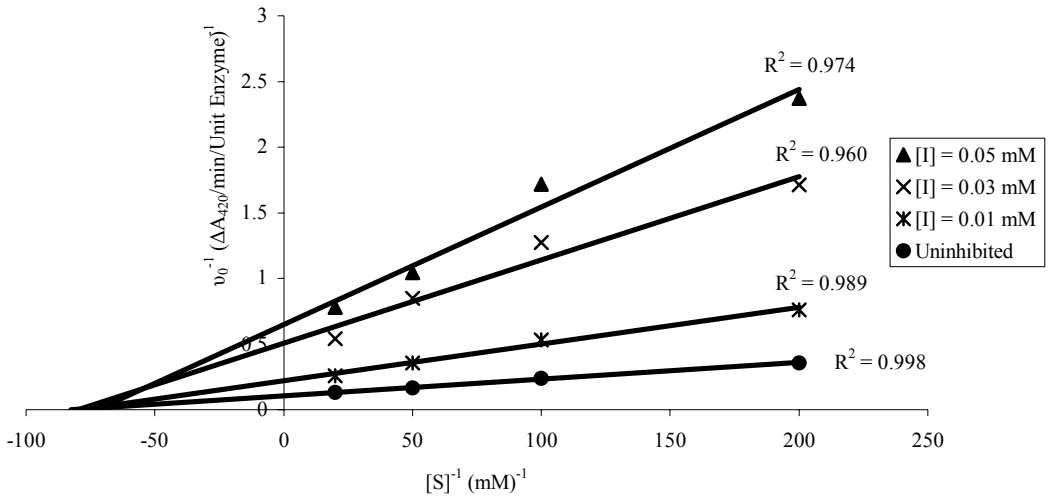


Figure 3.2: Lineweaver-Burk plots for different concentrations of sodium azide used with different concentrations of ABTS, in the presence of the thermostable laccase from UD4. This graph indicates non-competitive inhibition of the laccase with sodium azide.

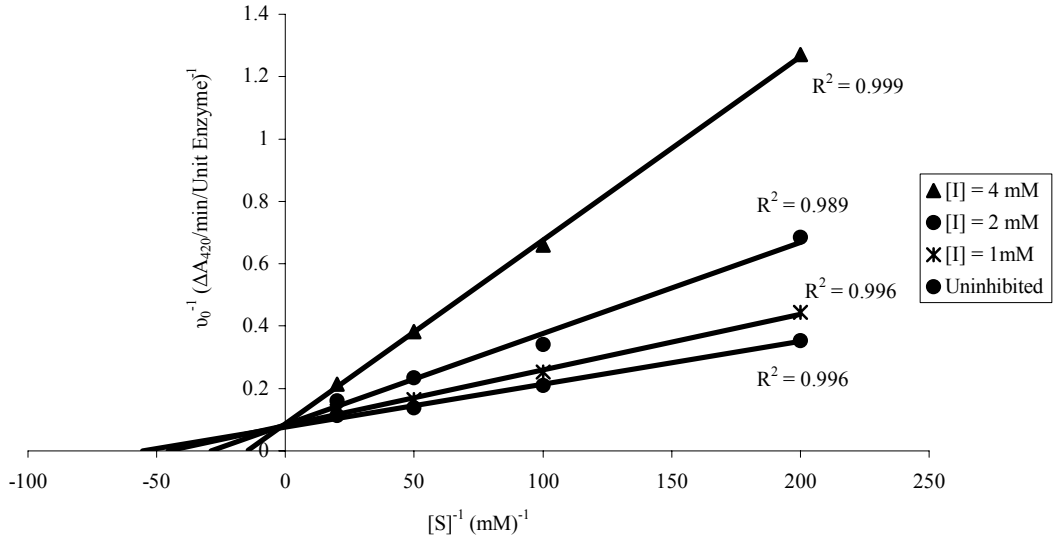


Figure 3.3: Lineweaver-Burk plots for different concentrations of hydroxylamine with different concentrations of ABTS, in the presence of laccase. This graph indicates competitive inhibition of the thermostable laccase with hydroxylamine.

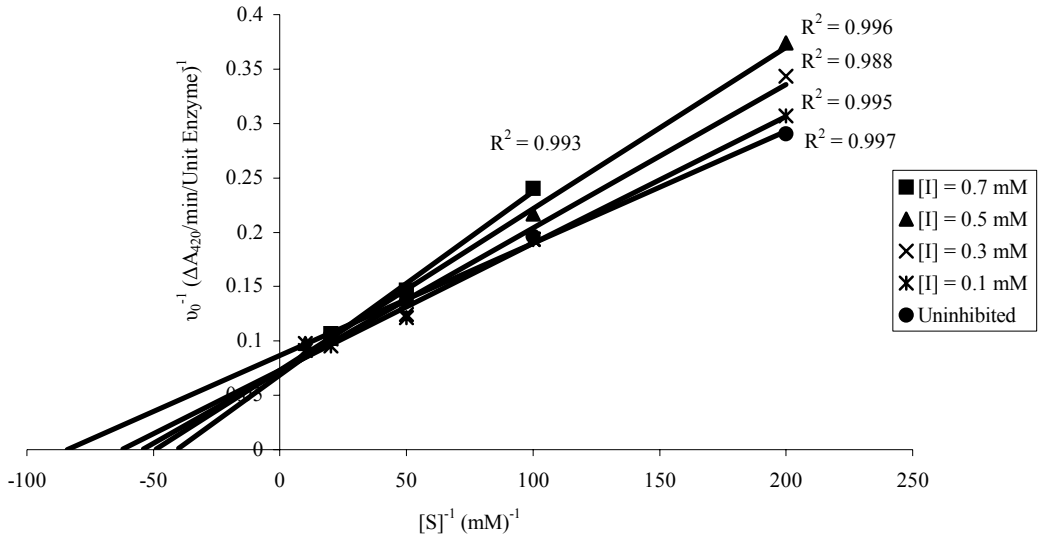


Figure 3.4: Lineweaver-Burk plots for the study of inhibition of laccase by thioglycolic acid. The graph indicates that thioglycolic acid exhibits mixed competitive inhibition of the thermostable laccase. The initial velocity was determined after the initial lag phase.

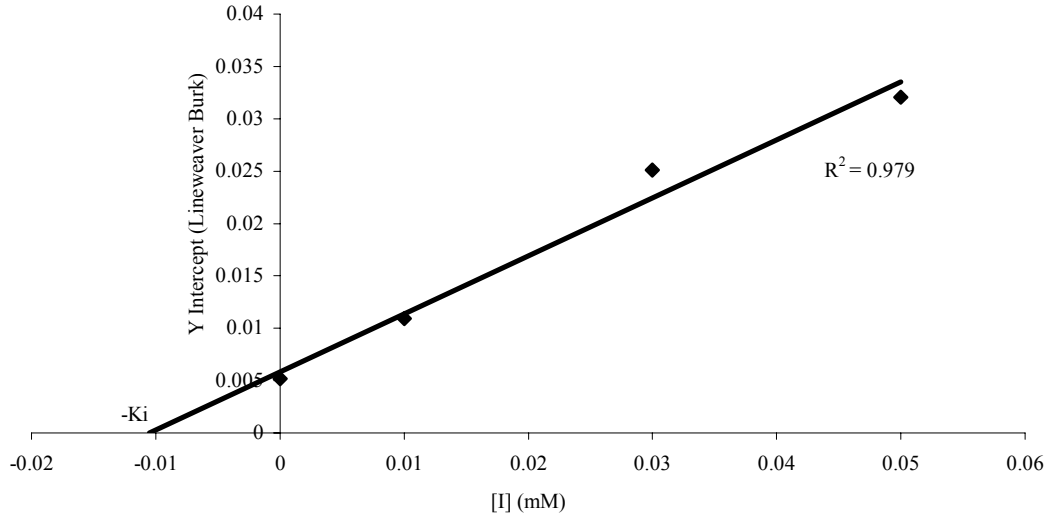


Figure 3.5: Secondary plot for the determination of K_i for sodium azide, where x-intercept is equal to $-K_i$.

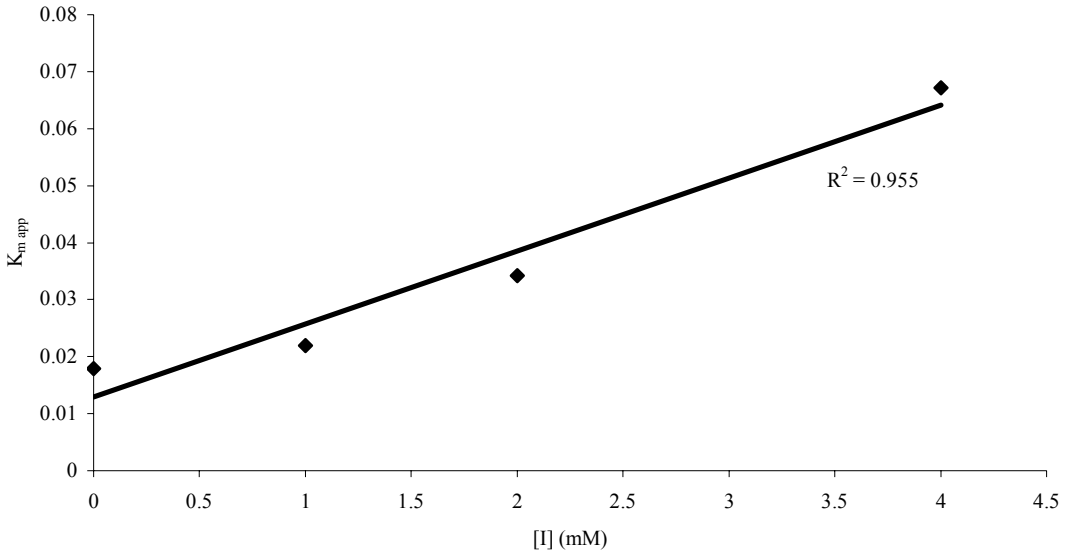


Figure 3.6: Secondary plot for the determination of K_i for hydroxylamine. The K_i can be calculated from the slope, which is equal to K_m/K_i, and the y-intercept is equal to the K_m for ABTS as the substrate.

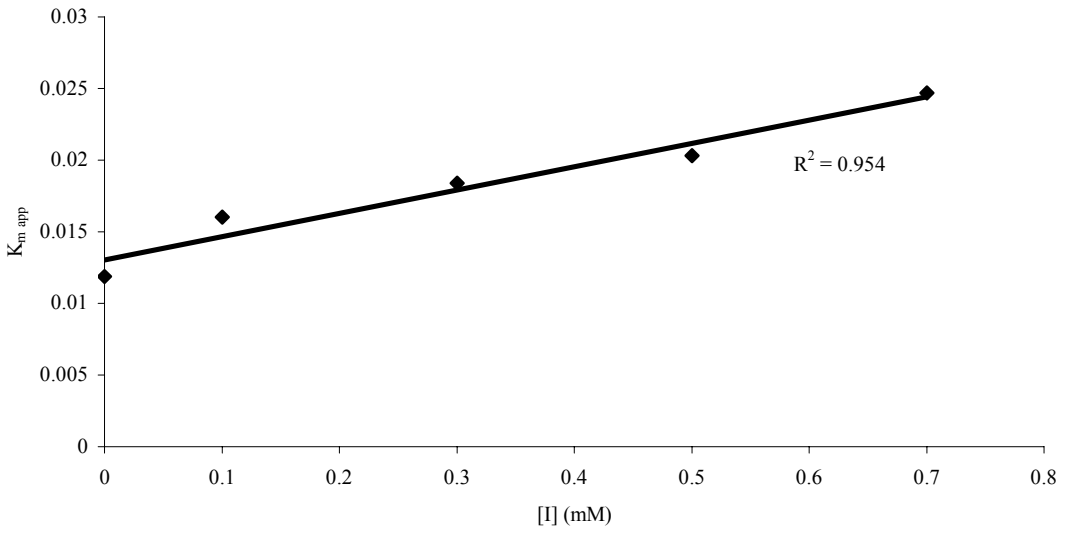


Figure 3.7: Secondary plot for laccase inhibition by thioglycolic acid with ABTS as the substrate, used for the determination of the K_i using the slope (K_m/K_i) and the y-intercept (K_m).

3.4.6 Peptide Fingerprinting and Sequencing

3.4.6.1 2D PAGE

The three spots exhibiting laccase activity were excised from the non-denaturing 2D PAGE and labeled ‘L’, ‘M’ and ‘R’ (Figure 3.8 and 3.9).

Figure 3.8: Activity stained non-denaturing 2D PAGE of partially purified laccase.

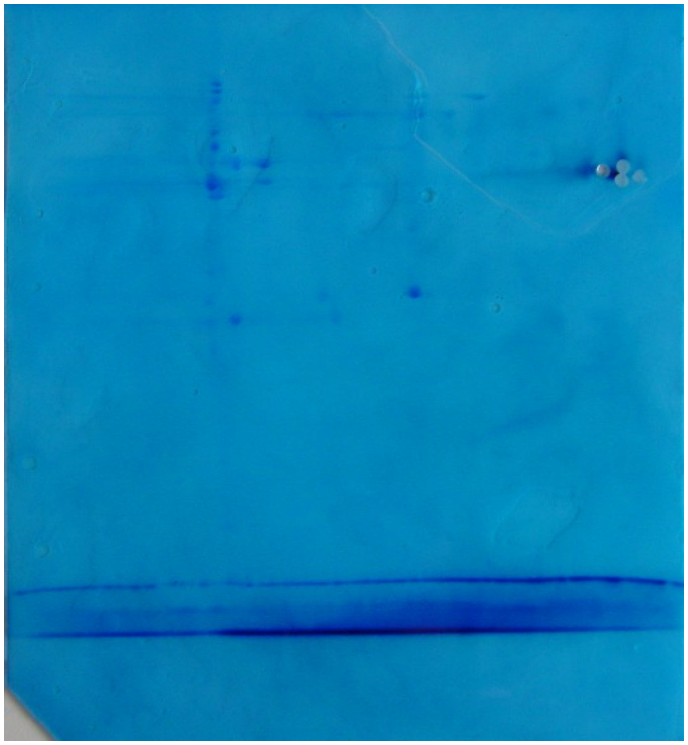


Figure 3.9: Coomassie stained non-denaturing 2D PAGE after excision of 3 laccase activity spots.

3.4.6.2 Tryptic Digestion and Derivatization

Each spot was in turn digested and derivatized using the Ettan CAF MALDI sequencing kit. The mass fingerprint spectra of the three laccase isozymes are shown in Figures 3.10-3.12. The spectrum of the derivatized spot 'L' is shown in Figure 3.13. However quality spectra of derivatized protein spots designated 'M' and 'R' could not be obtained.

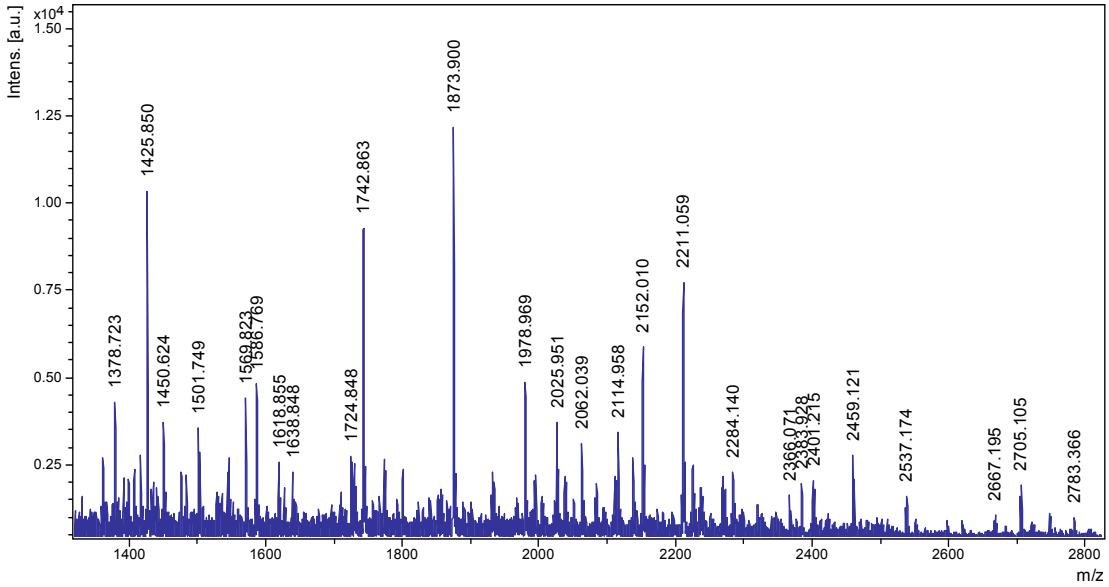


Figure 3.10: Left protein spot 'L' peptide fingerprint partial spectrum.

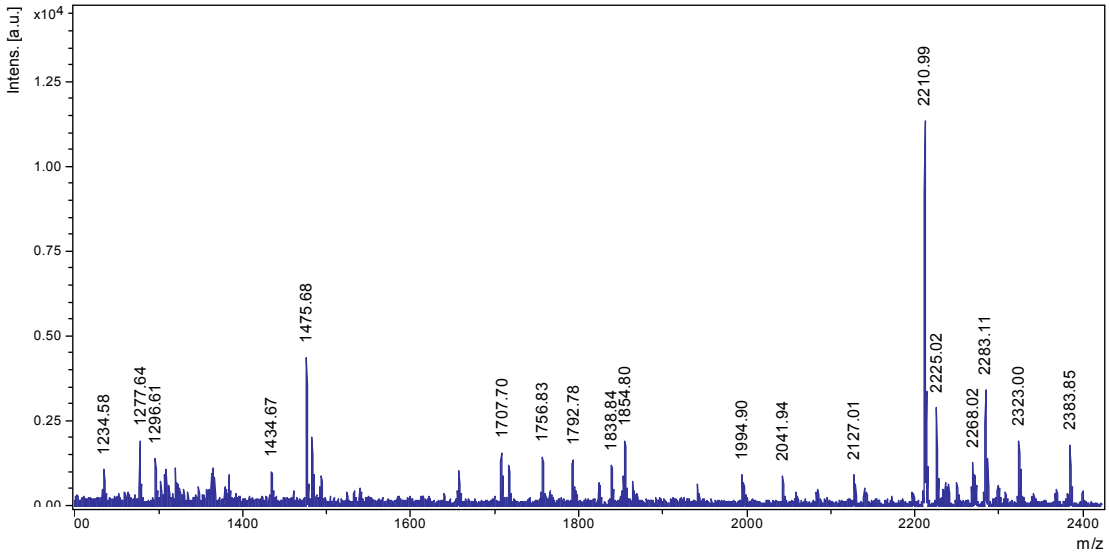


Figure 3.11: Middle protein spot 'M' peptide fingerprint partial spectrum.

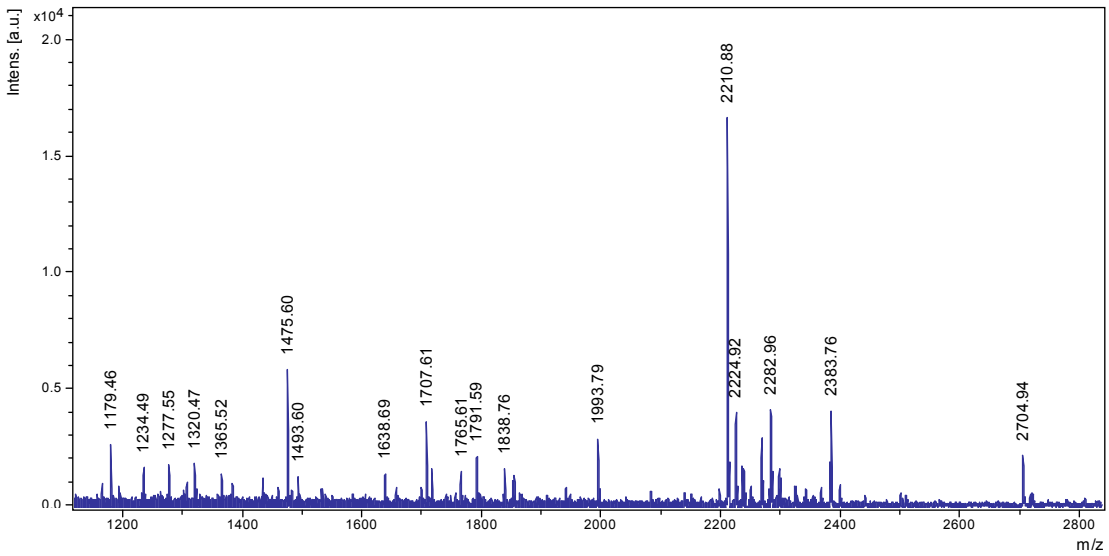


Figure 3.12: Right protein spot 'R' peptide fingerprint partial spectrum.

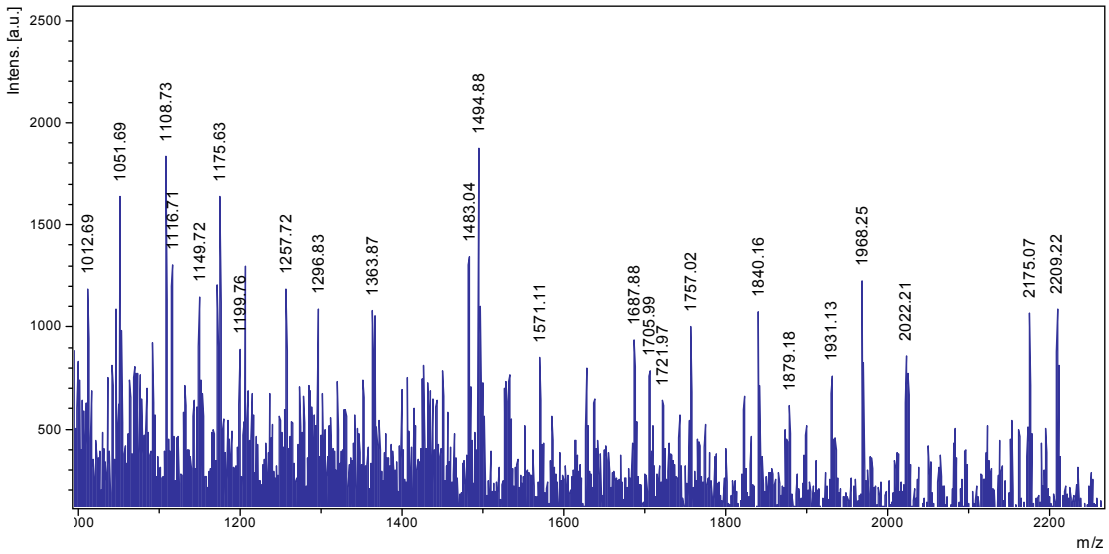


Figure 3.13: Derivatized tryptic peptides for left protein spot 'L'.

Post source decay experiment of peptide with MW 1285.75 Da for the left protein digest was carried out yielding the spectra shown in Figure 3.14. When attempting to readout the peptide amino acid sequence of fragmented derivatized MW 1285.75 Da peptide it was realised that the amino acid sequence resulted from a mixture of two different peptides. This may occur when attempting to carry out a PSD experiment on peptides with a similar mass range. However, it was

possible to derive two sequences from this PSD spectrum. The sequences obtained were DW(L/I)(Q/K)EGR and TGRT(F/Mo)(F/Mo)G ('Mo' refers to oxidized methionine).

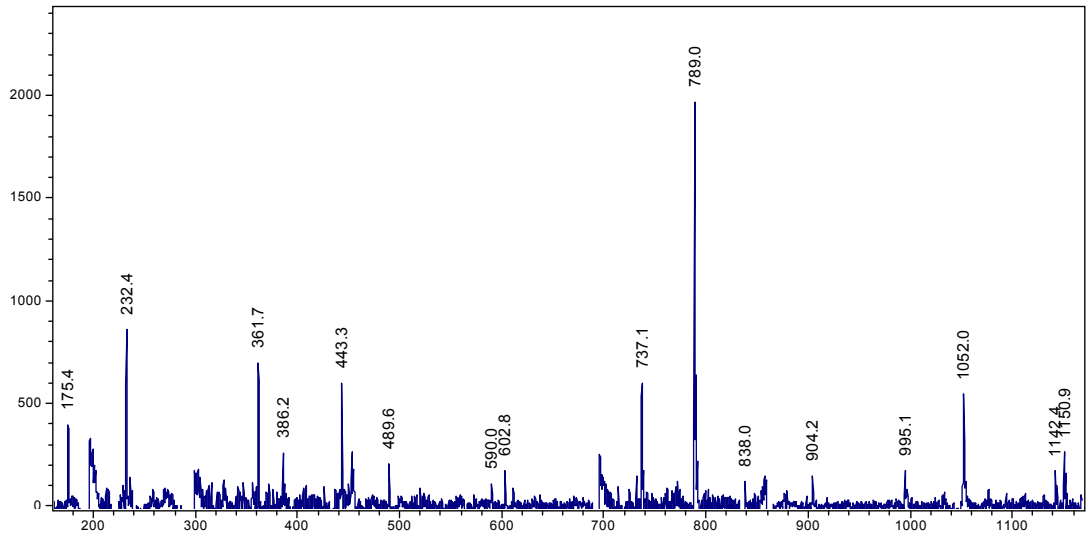


Figure 3.14: PSD spectra of derivatized 'L' protein tryptic peptide with MW 1285.75 Da.

An attempt was made to obtain amino acid sequences from derivatized 'M' and 'R' peptides. Good quality signals were however not forthcoming with these derivatized tryptic peptides and therefore no sequences could be derived. Further experiments are currently being performed to improve mass fingerprint and derivatizion signals so that further sequences may be elucidated from peptides of all three protein spots.

3.5 Discussion

It is commonly observed that optimum pH values vary by substrate for laccases, and may vary with isozymes of laccase produced by a single organism (Fukushima and Kirk, 1995). Thus, it was not unusual to note differences in the optimum pH values obtained for the oxidation of the four different substrates

tested. Laccase oxidation of ABTS exhibits a broad pH range of activity (from pH 2 to 6.8), and since this compound is used as a mediator for laccase activity (Bajpai, 1999; Crestini and Argyropoulos, 1998) with phenolic compounds, a broad pH range enhances the industrial applicability of the thermostable enzyme.

Only laccase activity, and no manganese peroxidase (MnP) activity, was detected in the Tien and Kirk defined medium after growth of UD4 without veratryl alcohol, used for the growth of the fungus and production of extracellular ligninolytic enzymes. This aided in the simplification of the purification techniques and characterization of the thermostable laccase.

Molecular weight as well as pI values of the laccase isozymes from UD4 are well within the range of other basidiomycetous laccases. The laccase suite of UD4 consists of three isozymes of 62 kDa molecular mass with pI values of 6.33, 6.45 and 6.5.

K_m values were determined in 50 mM tartrate buffer (Fukushima and Kirk, 1995) and 100 mM succinate-lactate buffer (a buffer commonly used for assaying laccase and manganese peroxidase activity) to determine any variability in kinetic parameters for the thermostable laccase, due to variation in buffer. An interesting observation made was that the kinetic parameters were improved in succinate-lactate buffer compared to tartrate buffer with ABTS as the substrate. However, the opposite was found with DMAB-MBTH or syringic acid as the substrate/s (refer to Table 3.2) indicating that this is not the general rule. The K_m, k_{cat} and specificity constants for guaiacol as a substrate were found to be comparable to those of isozymes L1 and L2 from *Ceriporiopsis subvermispora* (Fukushima and Kirk, 1995). Published values are reported in Table 3.3. The K_m values for ABTS as a substrate for the thermostable laccase were 1.6 fold lower than the lowest reported for laccase (L2 from *Ceriporiopsis subvermispora*, see Table 3.3) (Fukushima and Kirk, 1995). With syringic acid as a substrate the thermostable laccase exhibited a 3.8 fold lower K_m value than the lowest

published values (L2 from *Ceriporiopsis subvermispora*, see Table 3.3). The turnover number (k_{cat}) for the thermostable laccase is exceeded only by "white" laccase (POXA1) from *Pleurotus ostreatus* with ABTS as the substrate (Palmieri *et al.,* 1997). However, the specificity constant for the thermostable laccase was found to be in the same range as that of POXA1, with ABTS as the substrate, whereas no other laccase isozymes mentioned in Table 3.3 have a specificity constant in the same order of magnitude of laccase isozymes from *Pleurotus ostreatus* (POXA1) and UD4 (thermostable laccase). The thermostable laccase was found to be capable of catalyzing the manganese peroxidase-specific oxidative coupling of MBTH and DMAB (Jordaan and Leukes, 2003), with a K_m of 7 mM, k_{cat} of 368 s^{-1}, and a specificity constant of 53 $mM^{-1}s^{-1}$. This is unique among the laccase isozymes reported. The affinity of the thermostable laccase for the substrates (K_m) ABTS, syringic acid and guaiacol are indicated in Table 3.3, and are all considerably lower than reported values for other laccase iso-enzymes.

Table 3.3: Kinetic properties of other published laccase isozymes from fungal sources

Source of Laccase	Substrate	Optimum pH	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	Reference
UD4	ABTS	4	**0.0123**	**778**	**63252**	This work
UD4	Guaiacol	6	**0.251**	42.1	168	This work
UD4	**DMAB MBTH**	6	**7.964**	**368**	**53**	This work
UD4	Syringic Acid	4.5	**0.026**	10.1	388	This work
Rigidoporus lignosus B	ABTS	3	0.08	535	6700	Bonomo *et al.,* 1998
Rigidoporus lignosus D	ABTS	3	0.049	578	11800	Bonomo *et al.,* 1998
Panaeolus sphinctrinus	ABTS	3	0.0324	/	/	Heinzkill *et al.,* 1998
Panaeolus papilionaceus	ABTS	3	0.0506	/	/	Heinzkill *et al.,* 1998
Coprinus friesii	ABTS	5	0.0414	/	/	Heinzkill *et al.,* 1998
Polyporus pinsitus	ABTS	3	0.0223	/	/	Heinzkill *et al.,* 1998
Ceriporiopsis subvermispora L1	ABTS	3	0.03	101	3400	Fukushima and Kirk, 1995
Ceriporiopsis subvermispora L1	Guaiacol	3	1.6	52	33	Fukushima and Kirk, 1995
Ceriporiopsis subvermispora L1	Syringic Acid	3	0.13	31	240	Fukushima and Kirk, 1995
Ceriporiopsis subvermispora L2	ABTS	3	**0.02**	96	4800	Fukushima and Kirk, 1995
Ceriporiopsis subvermispora L2	Guaiacol	5	**0.44**	66	**150**	Fukushima and Kirk, 1995
Ceriporiopsis subvermispora L2	Syringic Acid	4	**0.1**	78	780	Fukushima and Kirk, 1995
Polyporus pinsitus *	ABTS	/	0.058	45	776	Xu *et al.,* 1996
Rhizoctonia solani *	ABTS	/	0.052	42	808	Xu *et al.,* 1996
Myceliophthora thermophila *	ABTS	/	0.096	7.3	76	Xu *et al.,* 1996
Scytalidium thermophilium *	ABTS	/	0.089	0.75	8	Xu *et al.,* 1996
Rhus vernicifera *	ABTS	/	0.039	0.45	12	Xu *et al.,* 1996
Chaetomium thermophilium	ABTS	/	0.19	/	/	Chefetz *et al.,* 1998
Chaetomium thermophilium	Guaiacol	/	0.4	/	/	Chefetz *et al.,* 1998
Pleurotus ostreatus POXA1	ABTS	3	**0.09**	**5833**	**64815**	Palmieri *et al.,* 1997
Pleurotus ostreatus POXA1	Guaiacol	NA	**NA**	**NA**	**NA**	Palmieri *et al.,* 1997
Pleurotus ostreatus POXA2	ABTS	3	0.12	/	/	Palmieri *et al.,* 1997
Pleurotus ostreatus POXA2	Guaiacol	6	3.1	/	/	Palmieri *et al.,* 1997
Pleurotus ostreatus POXC	ABTS	3	0.28	266	950	Palmieri *et al.,* 1997
Pleurotus ostreatus POXC	Guaiacol	6	1.2	2.5	2.1	Palmieri *et al.,* 1997
Pycnoporus cinnabarinus	Guaiacol	4	0.75	/	/	Eggert *et al.,* 1995
Trametes hirsutus	Guaiacol	/	0.75	/	/	Eggert *et al.,* 1995
Trametes sp. Strain AH 28-2	ABTS	/	0.025	692	27700	Xiao *et al*., 2003
Trametes sp. Strain AH 28-2	Guaiacol	/	0.42	69	164	Xiao *et al*., 2003
Trametes pubescens	ABTS	3	0.014	690	48000	Galhaup *et al*., 2002
Trametes pubescens	Guaiacol	/	0.36	180	500	Galhaup *et al*., 2002

* - The authors have not mentioned or determined the optimum pH for the laccase before determining the kinetic parameters, they used pH 5.3, and thus may have led to the low values obtained for the K_m k_{cat} and turnover number for the respective laccases

NA - Laccase was determined to be inactive with the substrate

/ - Kinetic parameters were not determined by the researchers

The laccase isozyme POXA1 from *Pleurotus ostreatus* has approximately the same specificity constant as the laccase obtained from UD4 as mentioned above, with ABTS as the substrate, but does not catalyze the oxidation of guaiacol and DMAB-MBTH, indicating a narrower substrate range than UD4 laccase. POXA1 is also thermostable, with an optimum temperature of between 45 and 65°C (Palmieri *et al.,* 1997), which is lower than that of the laccase from UD4 that showed an optimal activity at 70°C. Laccase from UD4 exhibits no loss in activity over a nine hour incubation at 60°C while POXA1 laccase from *Pleurotus ostreatus* exhibited a half-life ($t_{1/2}$) of three hours and twenty minutes at the same temperature (Palmieri *et al.,* 1997).

The POXA1 laccase from *Pleurotus ostreatus* was defined as a “white” laccase (Palmieri *et al.,* 1997), as it does not have the blue colour as determined by a visible wavelength scan, due to the presence of a single copper atom within its structure, and the other 3 copper atoms of “blue” laccases replaced by 2 zinc atoms and 1 iron atom. The authors are conducting further research to determine the nature of the metallic centers in the thermostable laccase from UD4.

Inhibition studies revealed that EDTA was not an efficient inhibitor of the thermostable laccase, which is consistent with findings by other researchers working with other laccase isozymes (Fukushima and Kirk, 1995; Heinzkill *et al.,* 1998). Sodium azide was strongly inhibitory with a calculated K_i of 0.011 mM (Figure 3.5). Sodium azide is an inhibitor of metallo-enzymes (Heinzkill *et al.,* 1998) and demonstrated pure non-competitive inhibition (Figure 3.2) with the thermostable laccase. This observation is not consistent with the findings of Heinzkill *et al.* (1998) who noted competitive inhibition with sodium azide for laccase from four species of fungi, and an average K_i of approximately 1 µM. The K_i obtained for the UD4 thermostable laccase was in the region of 10 µM, indicating that the presence of the sodium azide is less inhibitory to the thermostable laccase than laccase isozymes from these other sources.

Hydroxylamine exhibited competitive inhibition (Figure 3.3) with laccase, and a K_i of 1.01 mM was calculated (Figure 3.6). Fukushima and Kirk (1995) observed that hydroxylamine did not significantly inhibit laccase isozymes L1 and L2 from *Ceriporiopsis subvermispora*. Thioglycolic acid exhibited some inhibition after an initial lag phase was observed. The lag phase was thought to be due to the oxygen chelating activity of the thioglycolic acid, and since oxygen is the final electron acceptor for the laccase-catalyzed reaction, the reaction pathway could not be completed until the thioglycolic acid was oxygen saturated. This observation was substantiated by another observation: only freshly prepared thioglycolic acid caused the lag phase before initial activity. Thioglycolic acid exhibited mixed competitive inhibition (Figure 3.4) with a K_i of 0.802 mM (Figure 3.7).

Sequence data was successfully derived from protein spot 'L' which will aid in designing primers for extracting the laccase gene sequences from UD4. Further experimentation is currently underway to obtain additional peptide sequences from the three isozymes. N-terminal HPLC sequencing of the three laccase isozymes is also currently underway to provide further laccase specific sequences to aid in designing primers for heterologous expression. Full gene sequences will also be used for phylogenetic analysis with other known laccase sequences.

3.6 Conclusions

The purified thermostable laccase has unique catalytic properties when compared to other sources of the enzyme. The high catalytic efficiency of the enzyme is primarily due to the high affinity of the laccase for its substrates, the converse is true for *Pleurotus ostreatus* POXA1. Determining the structural components involved in this high affinity may prove invaluable in quantitative structure activity relationships of laccases and help identify residues involved in

substrate binding. The high catalytic efficiency of the laccase from UD4 as well as its thermostability makes it a suitable candidate for industrial application.

Identifying the host organism will ascertain pre-discovery and novelty of the thermostable laccase from this basidiomycete, and may also help in the elucidation of factors controlling the production of the thermostable laccase from UD4.

Chapter 4
Identification and Phylogenetic Analysis of the Basidiomycetous Fungus Producing a Thermostable Laccase

4.1 Introduction

Taxonomic Identification of the fungus producing the thermostable laccase was seen as an important component of the research, since this would reveal related species that may produce alternative thermostable varieties of laccases. Another benefit of identification could be increased understanding of physiological aspects and medium components required for optimal production of laccase from the host organism. Moreover phylogenetic analysis is an essential component of demonstrating novelty of the production organism for patenting purposes, and to determine any prior isolation of a similar thermostable laccase from a similar host.

Molecular characterization may currently be seen as the preferred method of species identification, and several genera have undergone reclassification based specifically on DNA sequence analysis rather than their structural or breeding similarities (Lim and Jung, 1998, Ko and Jung, 1999). Species identification based primarily on morphological characteristics is an experienced-based science (Taylor *et al.*, 2000) and therefore not readily available to many researchers. Fungal taxonomy is currently undergoing a revolution, since molecular data is discrediting many relationships previously based on biological and morphological characteristics. These include the colour of the basidiocarp, rot type (brown or white) and hyphal systems (Ko and Jung, 1999). Characteristics of the basidiocarp have also become a limited method of classifying fungal genera and families. This is due in part to the fact that several fungal classifications, such as the Boletales, exhibit plesiomorphic forms that

include poroid, gilled and resupinate forms, and other relatives of gilled mushrooms are non-gilled Hymenomycetes (Hibbett *et al.,* 1997). An example of phylogenetic analysis supporting morphologically diverse clades may be seen with *Lentinus tigrinus* (a gilled mushroom), which is phylogenetically associated with the Polyporaceae (bracket fungi) (Hibbett *et al.,* 1997).

The strain, tentatively named *UD4*, was isolated as an effused-reflexed basidiocarp, indicating its possible relationship with the basidiomycete families Coriolaceae and Polyporaceae. The fungus was not identifiable from the field guide available on the excursion (van der Westhuizen and Eicker, 1994), and further morphological characterization was inconclusive. Other noteworthy characteristics of the fungus were that it belonged to the group of fungi that preferentially attack the lignin component of wood resulting in “white-rot”, as opposed to “brown-rot” fungi, which preferentially attack the cellulose component of wood resulting in the brownish appearance of decomposing wood, the lignin. The basidiomycete was isolated from wood treated with creosote, indicating a possible application in the bioremediation of wastewaters containing poly-aromatic hydrocarbons that are present in high quantities in creosote (Barr and Aust, 1994).

Due to increased species resolution of the internal transcribed spacer (ITS) sequence analysis, and the availability of the largest database for sequence comparison, this region was selected as the best candidate to identify and phylogenetically analyse UD4 as described in this chapter.

4.2 Objectives

- Identification of the basidiomycetous fungus *UD4*, using molecular taxonomy techniques, to aid in ascertaining the the novelty of the thermostable enzyme

- To aid in determining any previous isolation and characterization of the host organism that may simplify further research on the organism.

4.3 Methods and Materials

4.3.1 Chemicals

Benomyl (Benlate®, 50 %) was purchased from Efekto. Forward primers GM1 and ITS3, and reverse primers GM2 and ITS4 were synthesized by WhiteHead Scientific. Taq Pol and dNTP's used in this research were purchased from TaKaRa.

4.3.2 Organisms and Maintenance

Isolate *UD4* was maintained on malt extract agar (Biolab, Merck) (2 %) slants containing 0.06 g/l benomyl (50 %). The control organism *Peniophora aurantiaca* (Centraalbureau voor Schimmelcultures, Utrecht, Accession number CBS 396.50) was maintained on malt extract agar (Biolab, Merck) (2 %) slants. Slants were stored at 4 °C.

4.3.3 DNA Isolation, PCR and Sequencing

DNA was isolated from *UD4* and *Peniophora aurantiaca* using a DNeasy™ Tissue Mini Kit (Qiagen), following the manufacturers modified protocol for yeast. Chromosomal and PCR DNA concentrations were quantified using a GeneQuant DNA spectrophotometer (Pharmacia). The primers used for PCR of the ITS1 region were GM1 (5' - TGTACACACCGCCCGTC – 3') and GM2 (5' – CTGCGTTCTTCATCGAT – 3') (Brookman *et al.,* 2000). PCR of the ITS2 region was performed using the universal primers ITS3 (5' – GCATCGATGAAGAACGCAGC - 3') and ITS4 (5' -

TCCTCCGCTTATTGATATGC - 3') (Fujita *et al.,* 2001). PCR was performed using reagent concentrations stipulated by the manufacturers (TaKaRa). The PCR protocol was modified from Brookman *et al*. (2000). The temperature and times used for PCR were as follows: initial denaturation at 94 °C for 3 minutes, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 45 °C for 1 minute and extension at 72 °C for 1 ½ minutes. The PCR product was checked for purity and approximate size on a 2 % agarose gel, using 100 base pair ladder Molecular Weight Marker XIV (Roche Molecular Biochemicals). Amplified DNA was purified using a High Pure PCR Product Purification Kit (Roche Molecular Biochemicals). Cycle sequencing was performed in 20 µl reaction volumes using ABI PRISM® BigDye™ Terminator v 3.0 Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) with a GeneAmp PCR System 9700 Thermal Cycler. BigDye™ terminated sequences were purified using a DNA Clean and Concentrator Kit™ -5 (Zymo) according to the manufacturers protocol. Sequencing of the resultant PCR fragments was performed in the forward and reverse direction, and was performed in duplicate by the Rhodes University DNA Sequencing Facility using an ABI PRISM® 3100 Genetic Analyzer (PE Applied Biosystems).

4.3.4 Sequences for alignment

A nucleotide Blast search (http://www.ncbi.nlm.nih.gov/blast) was performed for the sequenced DNA from *UD4*, and the twenty most similar meaningful sequences were downloaded for further analysis. Sequences of interest from other basidiomycetous genera were manually downloaded from GenBank.

4.3.5 Analysis of DNA sequences

Reverse sequences were complemented, and aligned to forward sequences to determine any mismatches between the two sequenced strands. Sequences were aligned using ClustalX version 1.81 (Thompson and Jeanmougin, 2000).

The ITS1 region was defined as the region between the 3' end of the 18S ribosomal coding region (defined as the sequence 5'- TCCGTAGGTGAA - 3') and the 5' end of the 5.8S ribosomal coding region (defined as the sequence 5' - GGCTCTCGCATC - 3'). The ITS2 region was defined as the region between the 5' end of the 5.8S ribosomal coding region (defined as the sequence 5' - ACTTACAAGCTC - 3') and the 3' end of the 28S ribosomal coding region (defined as the sequence 5' - TGACCTCAAATC - 3'). All sequences were aligned and edited manually to correspond to the region from the 18S to 5.8S, and 5.8S to 28S, as defined above before using ClustalX alignment. Gaps were removed from all sequences and edited sequences were realigned using ClustalX. The alignments were manually verified and edited (Appendix 2A and 2B). Manual editing was performed using BioEdit version 5.0.6 (Hall, 2001).

4.3.6 Phylogenetic analysis

Aligned sequences were subsequently used to produce guide trees using the ClustalX neighbour joining method, correcting for multiple substitutions and treating gaps as missing data. A group of 5 ascomycetes were used to define the outgroup (Zhang *et al.,* 1997), two *Aspergillus niger* strains (GenBank accession numbers AF455542 and AJ280006), *Penicillium commune* (AF455544), *Penicillium chrysogenum* (AF034451) and *Penicillium roseopurpureum* (AF455492). The strength of internal branches of the resultant phylogenetic trees were statistically tested by using bootstrap analysis (Felsenstein, 1985) with 500 replicates, and significant bootstrap percentages over 50 % are indicated in Figure 4.1. PAML© (Yang, 1997) was used for ancestral state reconstruction of closely related sequences of the Lachnocadiaceae in an attempt to ascertain the relationship of the morphologically diverse fungi with the resupinate and anamorphous fungi within this clade (Figure 4.2).

4.4 Results

4.4.1 Blast Results

The Blast search performed against GenBank (http://www.ncbi.nih.gov/genbank/) revealed a large number of similar ITS1 sequences, the most similar being *Peniophora aurantiaca*. To confirm the validity of the methods used, a control organism was ordered (*Peniophora aurantiaca,* CBS 396.50). Chromosomal DNA was extracted and the ITS1 sequence was amplified using the same methods followed for *UD4*. The blast results, followed by alignment showed 100 % similarity between an available GenBank entry for *Peniophora aurantiaca* (accession number AF210819), giving credibility to the methods used and the results obtained for *UD4*.

Blast searches revealed a strong ITS1 and ITS2 similarity between *UD4* and *Amanita tenuifolia* (GenBank Accession number AF085492), which prompted the incorporation of various other *Amanita* species into the phylogenetic study to determine any possible relationship between the Amanitaceae and the Lachnocladiaceae.

Another unusual occurrence was observed for the ITS2 blast results, indicating another relationship between *UD4* and *Phlebia serialis*. Therefore this sequence was included in the ITS2 tree, however no ITS1 sequence data was available and therefore *Phlebia serialis* could not be included in the ITS1 tree. The other *Phlebia* species were however still included in the ITS2 tree.

4.4.2 Phylogenetic Analysis

The phylogenetic tree for the ITS1 region of the rDNA (Figure 4.1) included several bracket fungal species from the Polyporaceae and Corioloaceae to determine any evolutionary relationship between *UD4* and these fungi. Figure 4.1

indicates that phylogenetically, *UD4* falls within a clade constructed mainly of Lachnocladiaceae, however *Entomocorticium*, which is currently classified within the Coriolaceae, is also placed within this clade. *Entomocorticium* species are classified taxonomically due to their association with the gut of insects. Another noteworthy observation is that *Peniophora* species are characterized by being resupinate (effused) in nature, whereas *UD4* was isolated from an effused-reflexed basidiocarp.

The tree constructed from the ITS2 sequence data indicates a similar positioning within the clade consisting of *Peniophora* and *Entomocorticium* species. The ITS1 tree contains the sequence for *Peniophora aurantiaca* CBS 396.50, which is not present in the ITS2 tree since no ITS2 sequencing was performed on this fungus. There is no sequence data available for the ITS2 region of *Phlebia serialis* (AF141629) rDNA and therefore this species has been excluded from the ITS1 tree.

Ancestral state reconstruction (ASR) using PAML© was performed for a combination of both ITS regions and the result is indicated in Figure 4.3.

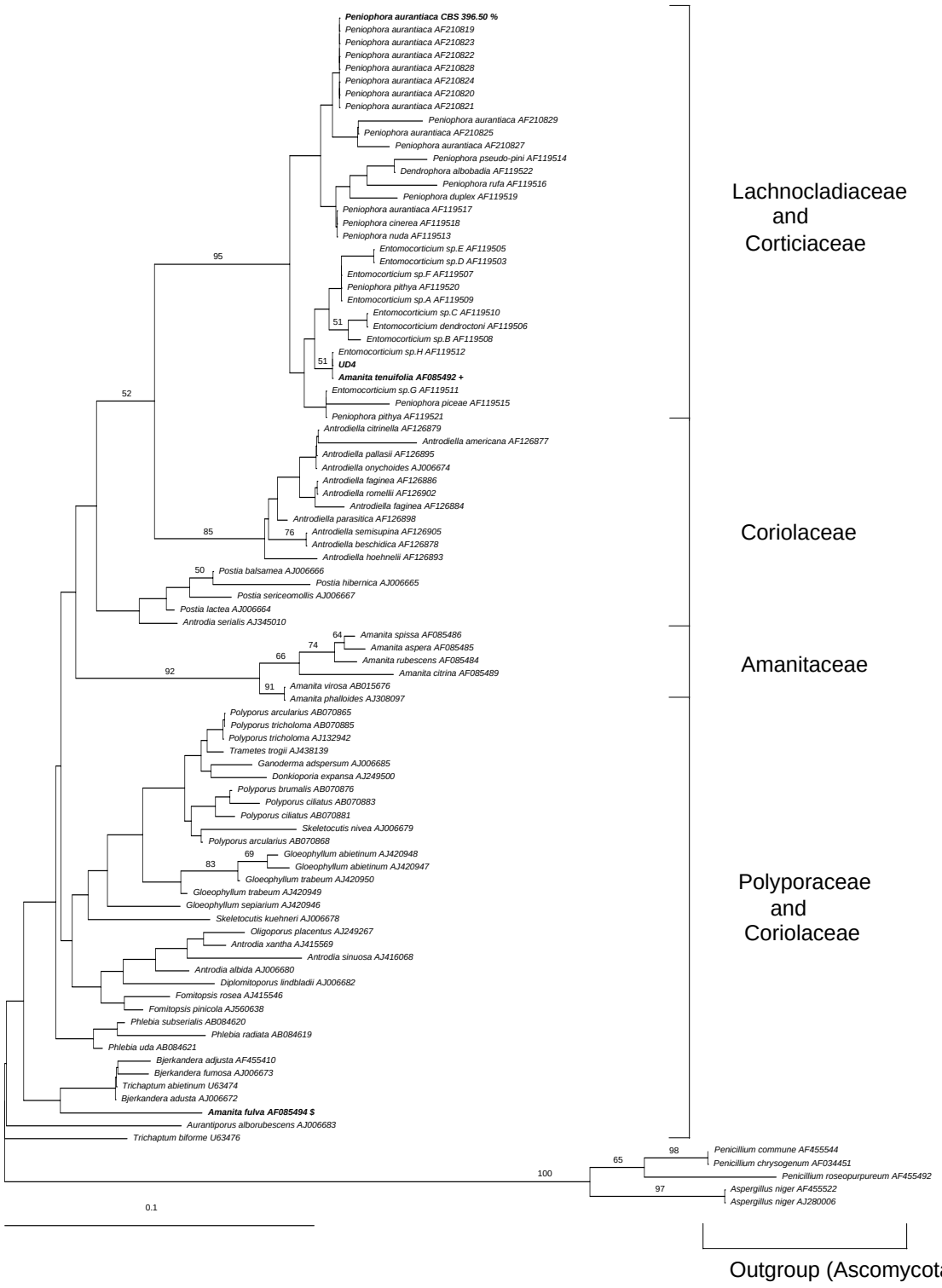


Figure 4.1: Phylogenetic tree for ITS1 using the neighbour joining algorithm of ClustalX (Thompson and Jeanmougin, 2000), indicating the placement of organisms of interest in bold. Bootstrap support of over 50 % is indicated on the internal branches. Names of species are followed by the relative GenBank accession numbers for the retrieval of sequences used.

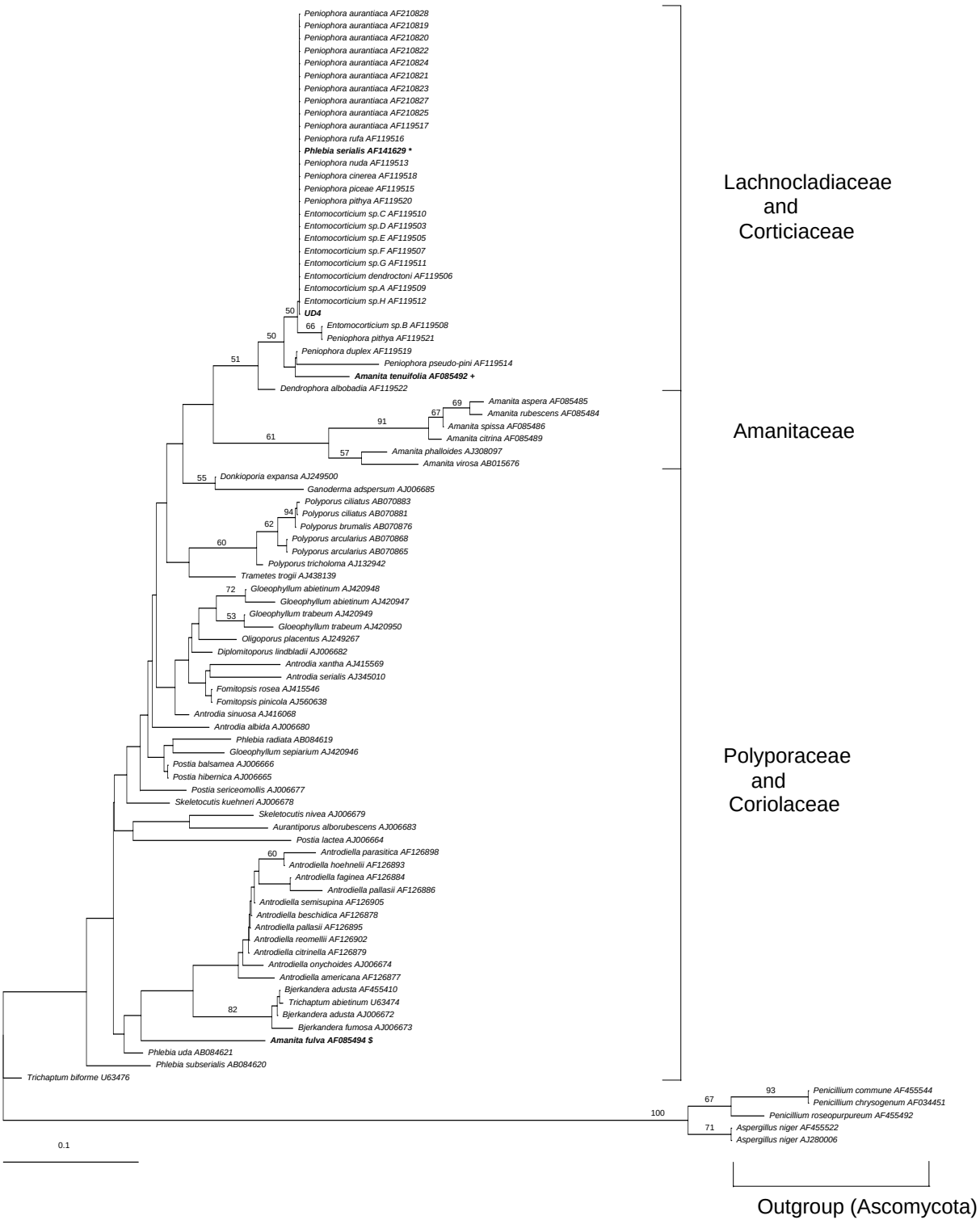


Figure 4.2: Phylogenetic tree for ITS2 ribosomal DNA using the neighbour joining algorithm of ClustalX (Thompson and Jeanmougin, 2000), indicating the placement of organisms of interest in bold. Bootstrap support of over 50 % is indicated on the internal branches. Names of species are followed by the relative GenBank accession numbers for the retrieval of sequences used.

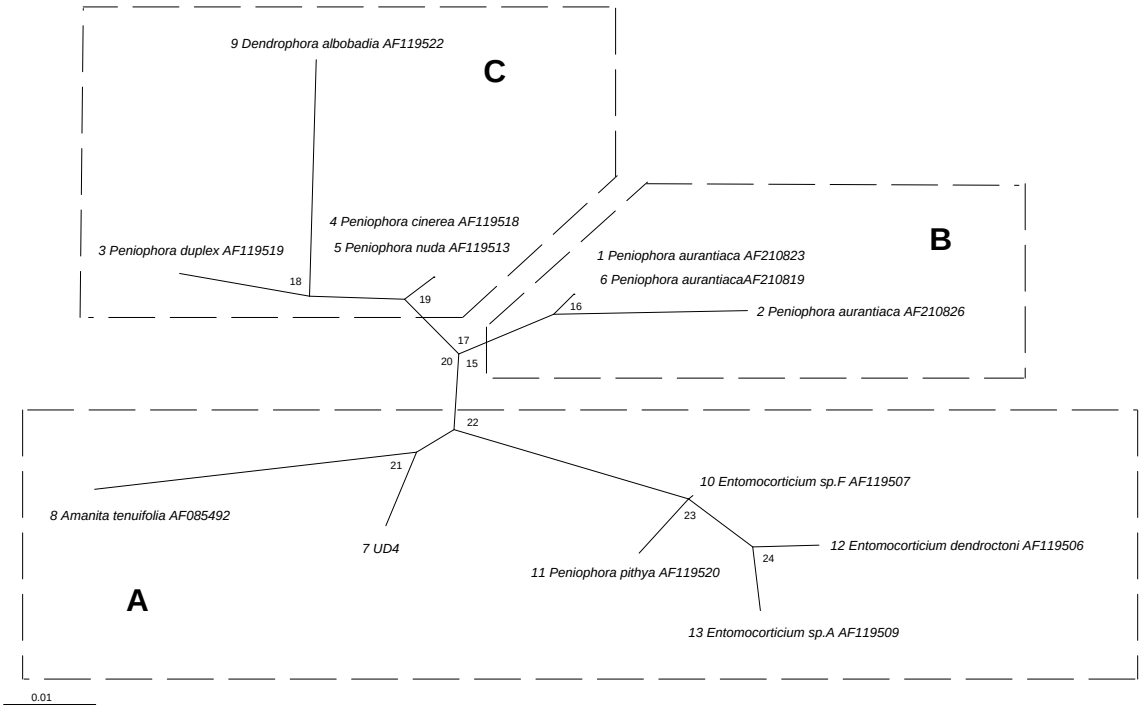


Figure 4.3: Radial tree indicating relationships between molecularly related species within the Lachnocladiaceae. Numbers 1 to 13 indicate original sequences, and numbers 15 to 24 indicate ancestrally reconstructed sequences. Groups A, B and C appear to be the three major groups derived from a common ancestor (node 15/17/20). *Amanita tenuifolia* and *UD4* form a structurally advanced grouping within the tree and the ancestral species indicated by node 21 is structurally advanced as determined by ASR.

4.5 Discussion

Initial analysis shows *UD4* to be a structurally advanced member of the Lachnocladiaceae, more specifically of the genus *Peniophora*. Further analysis also shows a similarity to the fungi associated with the gut of insects, namely *Entomocorticium* species. *UD4* was, as mentioned above, isolated from an effused-reflexed (bracket) fungus, but ITS sequence analysis revealed a strong relationship with the Lachnocladiaceae and Corticiaceae, which is statistically supported by a bootstrap analysis (50 % for ITS2 and 95 % for ITS1). This evidence strongly supports the classification of *UD4* within the genus *Peniophora*.

Zhang *et al*. (1997) mention that amplification from contaminating DNA is a potential problem when using PCR based techniques, and therefore a control *Peniophora aurantiaca* strain was purchased from CBS and subjected to the exact methods used for DNA extraction, PCR amplification, and identification as *UD4*. The CBS *Peniophora aurantiaca* strain partial 18S rDNA and ITS1 sequences were identical to alignable sequence data for *Peniophora aurantiaca* (Accession number AF210823). This gives credibility to the methods followed for the isolation and sequencing of ITS1 rDNA followed in this chapter.

The blast search revealed an unlikely similarity between the ITS regions of *UD4, Peniophora* species, and *Amanita tenuifolia*. Lim and Jung (1998) conducted a study on the phylogenetic relationships of several *Amanita* species using ITS sequencing techniques and concluded that *Amanita tenuifolia* (ATCC 26764) did not group within the three major clades within the Amanitaceae they defined. Furthermore, bootstrap analysis indicated a weak support for *Amanita tenuifolia* belonging in the family Amanitaceae (Lim and Jung, 1998). Several species from the three major clades they defined were used in Figure 4.1 and 4.2. It is however interesting to note that another species identified as *Amanita fulva* does not group with the Amanitaceae in the trees above, showing that the

Amanitaceae may exhibit a polyphyletic evolution. *Amanita tenuifolia* may require reclassification to within the Lachnocladiaceae or Corticiaceae as a morphologically advanced species. Alternatively, the purity or correct identification of this *Amanita tenuifolia* culture might require re-validation. *Phlebia serialis* is morphologically similar to species of *Peniophora,* which may have led to the incorrect identification of this species, or it may be indicative of the polyphyletic evolution of *Phlebia* species as mentioned above. Limited rDNA sequence data is available for other *Phlebia* species, therefore no conclusions can be drawn on the exact nature of the evolution followed by the genus *Phlebia*.

The enhanced resolution of the ITS1 over the ITS2 region can be seen in the trees above (Figure 4.1 and 4.2). The ITS1 region can clearly delineate between the *Peniophora* and *Entomocorticium* species, whereas ITS2 sequences exhibited a lower divergence and therefore no differentiation between these closely related species was possible. The length of the ITS2 sequence may be the limitation in using this sequence for species identification. The use of two regions for identification and phylogenetic analysis is however the preferred method (Taylor *et al*., 2000).

This study provides not only a phylogenetic placement and identification of *UD4* but also a strong link between *Peniophora* and *Entomocorticium* genera. This is the first evidence of an effused-reflexed fungus being grouped within the Lachnocladiaceae or Corticiaceae. This grouping provides further evidence for the need to carry out a reclassification of the fungi based on molecular phylogenetic analysis rather than morphological or biochemical similarities. With the strong molecular evidence supporting resupinate fungi as ancestral to more morphologically advanced fungi (Hibbett and Binder, 2003), and the concept that morphology is under stronger selective pressure than molecular divergence (Moncalvo *et al.,* 2000), the likely progression of *UD4* has been from differentiation of a *Peniophora* like resupinate ancestor. This relationship was confirmed by ancestral state reconstruction using members of the

Lachnocladiaceae. *UD4* and "*Amanita tenuifolia*" appear to form a single morphologically diverse grouping within this clade. Evolutionary influences such as the semi-arid African climate may explain the morphological divergence of *UD4* from its closest relatives. This is the first recorded isolation of a species exhibiting a close molecular relationship to *Peniophora* species, with morphologically incongruent characteristics.

4.6 Conclusions

Based on molecular data the isolated strain designated *UD4* is likely a *Peniophora* species, and we have conclusively shown that it has not been isolated before. There is however contradicting evidence complicating this identification, mainly the presence of an effused-reflexed basidiocarp, but as mentioned this may be explained by a recent evolutionary event in the *Peniophora* clade.

Chapter 5

Production of Laccase and Manganese Peroxidase Under Various Culture Conditions Aimed at Improving the Production of a Thermostable Laccase from *Peniophora* sp. UD4

5.1 Introduction

Production of ligninolytic enzymes in the wild-type organism is often advantageous owing to the use of parent host cell machinery, therefore avoiding incorrect folding or low yields due to alternative codon usage, as well as various other problems associated with heterologous expression (as outlined in Chapter 1). It was therefore deemed necessary to exhaust methods of production by the wild type organism. Accordingly, the effect of nutrient and growth conditions needed to be ascertained to determine the feasibility of wild-type organism production before progressing to genetic engineering and mutation strategies for improved production. Unfortunately, no information was available on ligninolytic enzyme production from any of the closely related species as identified in Chapter 4, and therefore the key factors affecting ligninolytic enzyme production in basidiomycetes were identified for evaluation of their potential for enhancing laccase production in *Peniophora* sp. UD4.

Optimization of production through strain improvement technologies was also considered of interest and a potentially feasible option for the purposes of this work. This methodology requires the production of a fruiting body from the host organism for the isolation of basidiospores that may then be screened for increased enzyme production and subjected to breeding strategies for enhanced production. The monokaryotic cultures may also exhibit enhanced production, such as *Pycnoporus cinnabarinus* ss3 that is capable of producing commercial quantities of laccase as high as 1.5 g/l media of laccase (Lomascolo *et al.,* 2003).

However, Induction of a carpophore from a basidiomycetous fungus is not trivial, and several factors have been identified as being involved in fruiting body formation in basidiomycetes. These factors have to be continually monitored and modified to ensure the correct conditions for fructification (Stamets, 1993).

Repeated attempts to find an identical carpophore at the same locality have been unsuccessful in this study. Attempts at inducing fructification on synthetic media have also failed. The lack of basidiospores therefore limited potential studies using strain improvement techniques.

It is well known that the production of ligninolytic enzymes is regulated by nutrients, and several conditions having a substantial effect on the ligninolytic enzyme production have been documented. These include the source of carbon and nitrogen in the medium, nutrient limitation, and environmental stress such as the presence of potentially toxic inducers (Gianfreda *et al*., 1999).

A frequently evaluated method for improvement in the yield of ligninolytic enzymes is the addition of inducers, but unless a dramatic improvement in enzyme production is achieved, the benefit of enhanced production may be outweighed by the cost of the inducers as well as the cost of post treatment and effluent disposal problems due to their toxicity. Therefore it is essential to take these factors into consideration when developing media for large-scale production, since the costs may outweigh the improvement in enzyme yield experienced. Any full medium development strategy for biotechnological application should consider cost implications and effluent disposal problems, since these can limit the commercialization of enzyme production.

The chemical nature, quantity added (Gianfreda *et al.,* 1999) and time of addition of an inducer (Shuttleworth *et al.,* 1986) may influence the production of laccase. The most common inducers include mainly phenolic compounds, which are structurally related to lignin or lignin derivatives. There are however many non-

phenolic compounds that have proven to be effective inducers of laccase production, such as copper, which is involved in electron transfer during oxidation reactions (Dittmer *et al.,* 1997; Baldrian and Gabriel, 2002).

Production of laccase has been shown to be sensitive to nitrogen concentration. Several fungi require the concentration of nitrogen to be in excess, while others produce only when induced by nitrogen starvation. *Lentinula edodes* (Buswell *et al.,* 1995) and *Phanerochaete chrysosporium* (Dittmer *et al.,* 1997) provide examples of improved laccase production in nitrogen sufficient media. A nitrogen deficient medium was however required for high production of laccase in *Pycnoporus sanguineus* (*cinnabarinus*) (Eggert *et al.,* 1996). The effect of the carbon source on the production of ligninolytic enzymes from fungi has been characterized, although not as widely as that of the effect of the nitrogen source.

This chapter characterizes the effect of nutrient conditions on the production of ligninolytic enzymes from *Peniophora* sp. UD4. Changes in growth conditions and nutrients are also characterized in terms of growth kinetics for these various medium sources.

5.2 Objectives

- To enhance the production of laccase from *Peniophora* sp. UD4 through an understanding of the nutrient and physical factors affecting growth and enzyme production
- Determine the effects of changes in nutrient and physical conditions on the ligninolytic enzyme production profile of *Peniophora* sp. UD4
- To determine the efficiency of wild-type production for large-scale production of the thermostable laccase.

5.3 Methods and Materials

5.3.1 Chemicals

The assay substrates used, 3-dimethylaminobenzoic acid (DMAB), 3-methyl-2-benzothiazolinone hydrazone (MBTH), and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were obtained from Sigma. Ethylene diamine tetra-acetic acid (EDTA), hydrogen peroxide (H_2O_2: 30 %) and dimethylsuccinic acid dimethyl ester were obtained from Merck. Benomyl (50 %) was obtained from Efekto. Orcinol, ferulic acid, and veratryl alcohol were purchased from Sigma.

5.3.2 Organism maintenance

Isolate *Peniophora* sp. UD4 was maintained on malt extract agar (Biolab, Merck) (2 %) slants. A number of slants were supplemented with 0.06 g/l benomyl (50 %). This was done to attempt to provide selective pressure for the fungus to enhance ligninolytic enzyme production, since it is thought that laccase is the primary enzyme involved in this detoxification pathway.

5.3.3 Monitoring of Growth Parameters

Mycelial growth was measured using the method developed by Langvad (1999) utilising optical density in microtitre plates, which were read on a PowerWave Microtitre Plate Reader (BioTek) at 630 nm, where 1 absorbance unit is equivalent to 4.2 mg/ml dry fungal biomass concentration (Langvad, 1999). Data is represented as means of octaplate samples ± standard deviation.

5.3.4 Calculation of Growth Parameters

The specific growth rate was determined as the slope of the natural logarithm of the exponential portion of the plotted growth curve. The best four points were used as determined by analyzing the best fit for the linear regression in Microsoft Excel. Lag phase was determined as the X intercept of two linear plots, the exponential portion of the curve (the four points exhibiting the best fit) and the linear regression for the initial lag phase.

5.3.5 Enzyme Production Monitoring

The production of manganese peroxidase (MnP) and laccase was followed in both static and agitated cultures, static state cultures consisted of 50 ml of Tien and Kirk medium in 500 ml Erlenmeyer flasks. Flasks were inoculated with four 5 mm^2 plugs of mycelia, cut from the growing edge of *Peniophora* sp. UD4 cultivated on malt extract agar (2 % agar). Shaking cultures consisted of 200 ml of media in 500 ml flasks, also inoculated with plugs. In both examples samples (1 ml) were extracted daily from 3 flasks and pooled, this culture media was then assayed for ligninolytic enzymes as mentioned below.

5.3.6 Enzyme Assays

Enzyme assays for MnP and laccase were performed using ABTS as the substrate (Gold and Glenn, 1988). DMAB-MBTH oxidative coupling (Castillo *et al.,* 1994) was used for the determination of laccase and MnP when using distillery effluent as a potential medium, and activity was differentiated as described in Chapter 2. LiP activity was determined by the method of Tien and Kirk (1988) using veratryl alcohol as the substrate. One enzyme unit was defined as the amount of enzyme required to convert 1 µmol of substrate to product in 1 minute at 25 °C. All assays were performed in hexaplate using a PowerWave

Microtitre Plate Reader (BioTek). All enzymatic data represented graphically below are indicated as means ± standard deviation.

5.3.7 Media

Media consisted of modifications of Tien and Kirk medium (Tien and Kirk, 1988). All vitamins were filter sterilized and simple sugars, such as glucose, were autoclaved separately from other media components. All media contained basal III and trace element solutions as described by Tien and Kirk (1988) (Appendix 1).

5.3.8 Conditions Investigated

The physiological and nutrient conditions investigated for their effects on growth and enzyme production were as follows; pH of medium, temperature of incubation, effect of agitation, carbon and nitrogen source concentration, carbon and nitrogen source, vitamin supplementation, oxygen availability, action of known inducers, and the use of wine distillery effluent as an alternative medium.

5.3.8.1 Carbon and Nitrogen Source and Concentration

Medium composition was based on Tien and Kirk defined medium (Tien and Kirk, 1988), excluding the addition of veratryl alcohol, thiamin and dimethylsuccinic acid dimethyl ester as the buffer (pH 6.0). Modifications to the medium are described below in Table 1. High and low concentrations of carbon and organic nitrogen were considered to be 1 g/l, and 0.1 g/l respectively. High and low concentrations of inorganic nitrogen sources were considered to be 56 mM, and 5.6 mM respectively. Intermediate concentrations for these macro-nutrients was considered to be half the high concentrations used. Supplementation of ammonium tartrate (56 mM) with yeast extract (0.1 g/l) was also investigated. Since the carbon and nitrogen concentration are considered to be important

factors governing the production of extracellular ligninolytic enzymes, this experiment was performed first, and subsequent media were based on the carbon and nitrogen concentrations best suited for laccase production.

5.3.8.2 Wine Distillery Effluent

Alternative nutrient sources, such as nutrient rich effluents, may be used reduce the cost of enzyme production since they are inexpensive medium sources. Wine distillery effluent was noted as potentially having all the nutrients required for fungal growth (Leukes and Dekker, 2001). It was therefore chosen as a potential medium for the production of laccase from *Peniophora* sp. UD4. Due to the potentially high concentrations of soluble and solid nutrients, several dilutions of wine distillery effluent were used to establish the effect on growth and enzyme production. Although the less attractive use of wine distillery influent as the growth medium (higher cost) it was also assessed as a potential nutrient source since it was noted that distillation might affect the nutrient composition of the wine industry waste, such as the caramelization of simple sugars into more complex carbohydrates, which may have and effect on growth and/or enzyme production.

5.3.8.3 Optimum Incubation Temperature

Optimum temperature experiments were perfomed in shallow static state culture conditions using Tien and Kirk defined medium (Tien and Kirk, 1988) excluding veratryl alcohol, at 24, 28, and 37°C, three frequently used temperatures for the growth of fungi and the production of ligninolytic enzymes. One microtitre plate (Costar) was incubated at each temperature with 62 wells inoculated with *Peniophora* sp. UD4 and 24 blank wells, inoculated with water. Extracellular enzyme production at these temperatures was followed as mentioned above.

5.3.8.4 Optimum pH

The optimum pH for growth using shallow static state culture conditions was determined using Tien and Kirk defined medium (Tien and Kirk, 1988) excluding veratryl alcohol. Dimethylsuccinate buffer was replaced with nitrogen free universal buffer (Perrin and Dempsey, 1974) adjusted to the pH value of interest. Microtitre plates (Costar) were inoculated with 8 blanks per pH value tested and 24 sample wells inoculated with *Peniophora* sp. UD4. One plate was therefore used for three pH values. Enzyme production was monitored in cultures as mentioned above.

5.3.8.5 Vitamin Supplementation

The basal vitamin solution used during this experimental set was based on the vitamin solution used for the culturing of *Microbacterium* species (Atlas, 1993). The effect of each vitamin was determined individually and by the addition of a vitamin solution excluding the vitamin of interest. All vitamin supplementation experiments were conducted using modified Tien and Kirk medium as described above, under carbon limited (1 % glucose) and nitrogen rich (56 mM ammonium tartrate) conditions.

5.3.8.6 Agitation

The effect of agitation on growth was investigated using comparative shaking and static state culture conditions. Shaking cultures were agitated at two speeds, 100 and 200 rpm on a Labcon© orbital shaker. The action of detergents on enzyme production was also monitored in shaking cultures. Tween 20 (sorbitan polyoxyethylene monolaureate) or Tween 80 (sorbitan polyoxyethylene monooleate) were added to flasks at the rotation speeds mentioned above, as well as to controls of static state cultures at concentrations of 1 g/l, to determine the potential effect of each detergent on nutrient modification. Flasks were

cultivated in hexaplate at 24 °C. Samples were extracted daily from three flasks from each experimental set and assayed for laccase and MnP activity as mentioned above.

Solid substrate shaking cultures were also performed using a medium consisting of soybean meal (30 g/l), cotton-seed meal (10 g/l), glucose (1 g/l) and glycerol (1.58 %). The medium was agitated at 100 rpm.

5.3.8.7 Oxygen Supplementation

The oxygen requirement of the fungus for the production of extracellular enzymes was investigated using shallow static fermentations as described above. Several flasks were oxygenated with pure oxygen at a flow rate of 5 ml/min for 10 minutes daily, while other flasks were bunged with cotton-wool, allowing for diffusion of gases. The presence of detergents, which could improve oxygen uptake by *Peniophora* sp. UD4, was also investigated using the same technique. To this end, Tween 20 and 80 (1 g/l) were added to media and the cultures were subjected to pure oxygen sparging and diffusion as mentioned above.

5.3.8.8 Inducers

Ferulic acid, orcinol, and veratryl alcohol were investigated as potential inducers of ligninolytic enzyme production. Inducers were added to shallow static cultures at three-day intervals (day 3, 6 or 9 of incubation) during the time course of the experiment to a final concentration of 100 µM.

5.4 Results

No production of ligninolytic enzymes was observed in agitated culture conditions with Tien and Kirk medium, therefore shallow static state culture conditions were chosen for the determination of the effect of various other medium components and conditions as described below.

5.4.1 Calculation of Growth Kinetic Parameters

An example of the enzyme production profiles (Figure 5.1) and the growth curves (Figure 5.2) obtained are shown below, as well as an illustration of the methods used for calculation of the growth kinetic parameters (Figure 5.3).

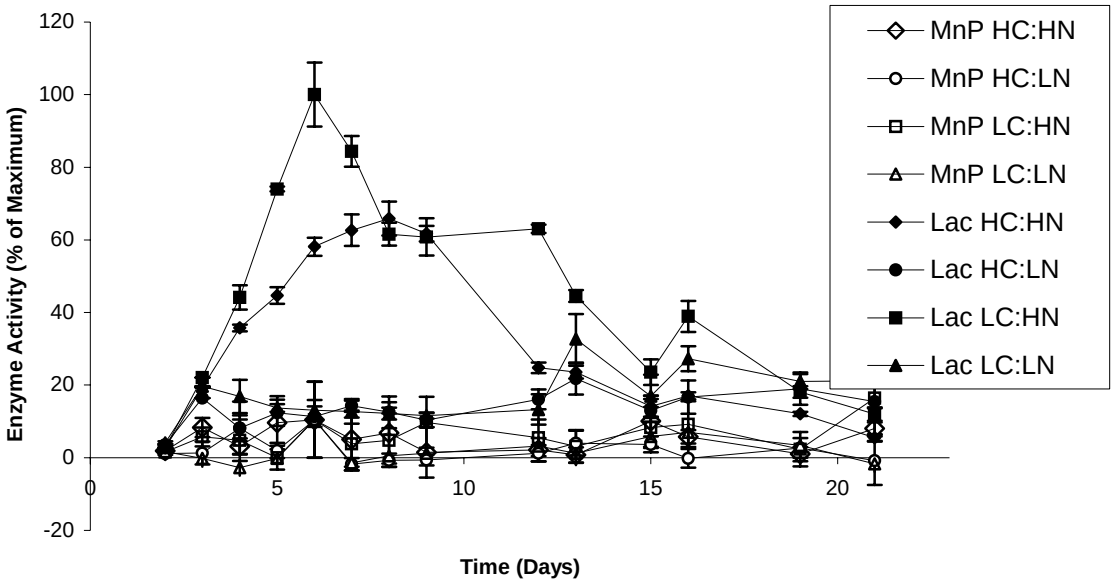


Figure 5.1: Enzyme production profile obtained for MnP and laccase obtained under different concentrations (L – low; H – High) of carbon (C) and nitrogen (N).

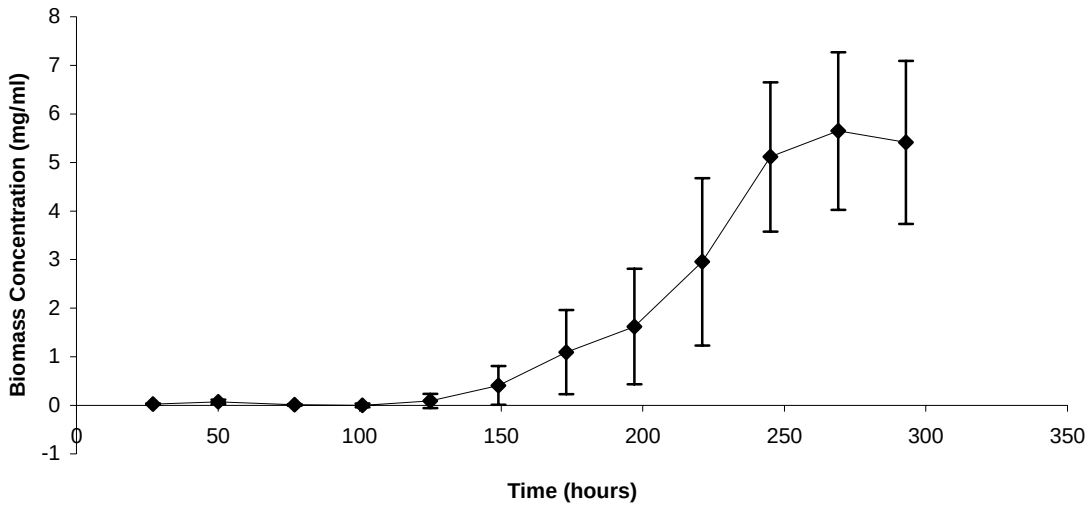


Figure 5.2: Example of a typical growth curve, this curve is representative of the sample containing all the vitamins in the vitamin supplementation experiments. Values are means ± standard deviation.

Lag phase was determined as illustrated in Figure 5.3 below, and the specific growth rate was determined as shown in Figure 5.4 below. For the sake of brevity no other calculations, growth curves or enzyme production profiles are illustrated in this thesis. Instead, the derived growth parameters have been summarized into Tables 5.1 to 5.6 and enzyme production data have been summarized into Figures 5.5 to 5.11 below.

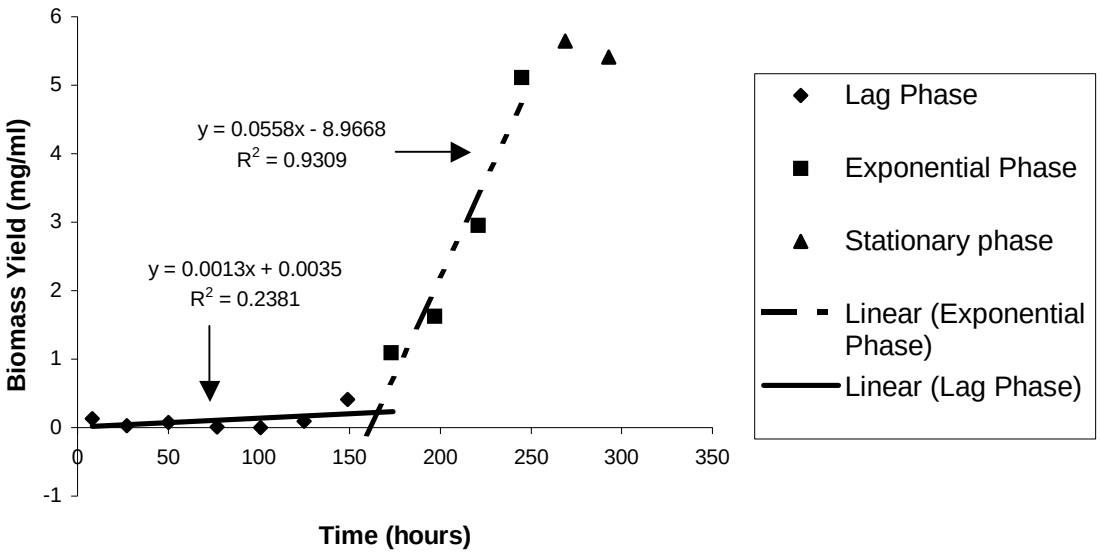


Figure 5.3: Example of a graph used for the determination of the lag phase, the x intercept of the two linear plots of the lag phase and exponential phase was used as the lag time for a sample. This graph is representative of the growth profile in Figure 5.2.

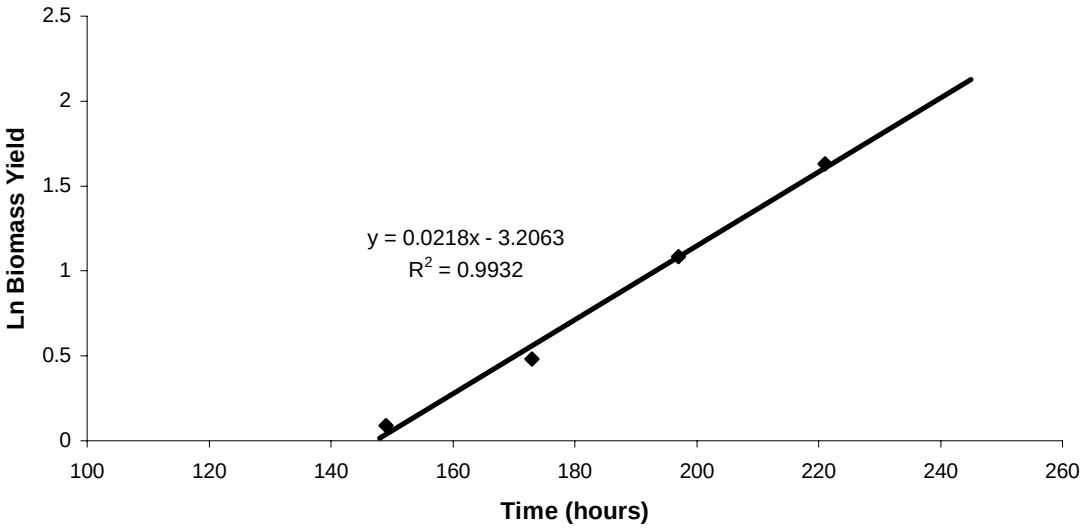


Figure 5.4: Example of graph used to calculate specific growth rate, where specific growth rate is represented by the slope of the linear plot, of the natural logarithm of biomass yield at each specific time measurement.

5.4.2 Carbon and Nitrogen Source and Concentration

Laccase production in *Peniophora* sp. UD4 appears to be strongly regulated by nitrogen source and concentration, while carbon limitation also appeared to be a definitive inducer of laccase production (Figure 5.5). Low concentrations of glucose result in a prolonged lag time before exponential growth but this is coupled with higher specific growth rates and results in similar biomass yields as low carbon source concentrations (Table 5.1). Organic nitrogen sources have a positive influence on the production of MnP (Table 5.2), and inorganic nitrogen such as ammonium tartrate enhances the production of laccase (Figure 5.6). Organic nitrogen sources have a positive influence on both the biomass yield and specific growth rate of *Peniophora* sp. UD4 (Table 5.2).

Table 5.1: Growth Parameters for *Peniophora* sp. UD4 cultured with liquid media with differing carbon to nitrogen ratios.

Ratios of Carbon and Nitrogen	Lag Time (hours)	Specfic Growth Rate (h^{-1})	Biomass Yield (mg/ml) ± Standard Deviation
HC:HN	140.4	0.0129	1.11 ± 0.21
HC:LN	149.1	0.0160	1.45 ± 0.20
LC:HN	156.6	0.0185	1.06 ± 0.40
LC:LN	184.5	0.0201	1.08 ± 0.21

L: Indicates low concentration
H: Indicates high concentration
C: Carbon in the form of Glucose
N: Nitrogen in the form of Ammonium Tartrate

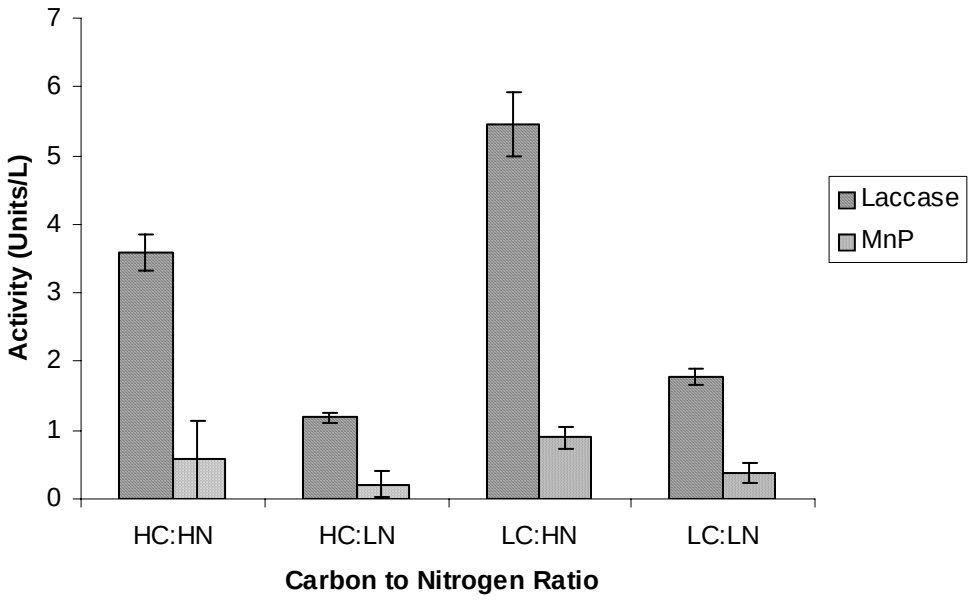


Figure 5.5: Enzyme production patterns for *Peniophora* sp. UD4 cultured with liquid media with differing carbon to nitrogen ratios. Values indicated are means ± standard deviation.

Table 5.2: Growth parameters observed when culturing *Peniophora* sp. UD4 on media containing different sources of carbon and nitrogen.

Alteration in Nutrient Composition	Lag Time (hours)	Specfic Growth Rate	Biomass Yield (mg/ml) ± Standard Deviation
Glucose Control	185.2	0.0208	1.02 ± 0.30
Fructose (C)	170.5	0.0193	1.42 ± 0.50
Dextrose (C)	180.5	0.0207	1.03 ± 0.24
Sucrose (C)	178.2	0.0241	1.48 ± 0.33
Mollasses (S)	180.2	0.0189	2.68 ± 0.58
Malt Extract (C)	95.4	0.0220	3.65 ± 0.92
Yeast Extract (S)	91.0	0.0200	6.08 ± 2.34
Peptone (N)	95.7	0.0402	9.76 ± 0.97
Yeast Extract (N)	166.7	0.0288	4.72 ± 1.89

C: Used Exclusively as an alteration of the Carbon Source
N: Used exclusively as an alteration of the Nitrogen Source
S: Supplementation of Medium containing Glucose and Ammonium Tartrate Medium

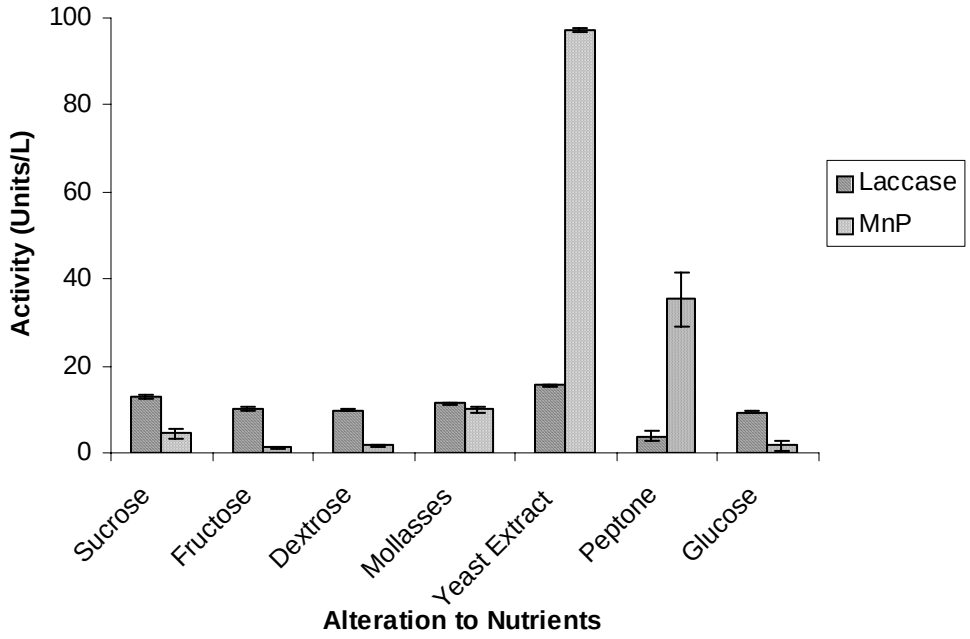


Figure 5.6: Enzyme production patterns for *Peniophora* sp. UD4 cultured on media containing alternative carbon and nitrogen sources and supplementation with nutrients. Values indicated are means ± standard deviation.

5.4.3 Wine Distillery Effluent

The resultant yields of MnP and laccase activity from *Peniophora* sp. UD4 cultivated on wine distillery effluent are indicated in Figure 5.7. The comparative biomass yields and specific growth rates are shown in Table 5.3.

What is not apparent in figure 5.7 is that laccase production is induced in wine distillery influent and effluent. Maximum laccase activity is achieved after the second day of growth in the influent and after the third day in the effluent. Activity appears in the early stages of growth on wine distillery effluent when compared to all other growth conditions investigated, which generally occurs after the seventh day of growth. MnP activity does not appear to be induced under these conditions and maximum yields are obtained after the fourth and tenth days in the influent and effluent respectively. This may be as a direct result of an inductive effect of high concentrations of specific low molecular weight acids

present after wine fermentations on the laccase. The ligninolytic enzyme production is highest at a 1 in 10 dilution of the distillery effluent (Figure 5.5).

Table 5.3: Growth Parameters, Laccase and MnP production patterns for *Peniophora* sp. UD4 cultured using wine distillery effluent at different concentrations as a medium.

Growth Medium	Lag Time (hours)	Specfic Growth Rate (h^{-1})	Biomass Yield (mg/ml) ± Standard Deviation
Influent (1/2)*	0	0.0115	0.34 ± 0.04
Influent (1/5)*	0	0.0172	0.30 ± 0.05
Influent (1/10)*	0	0.0092	0.31 ± 0.02
Influent (1/20)*	0	0.0105	0.34 ± 0.03
Effluent (1/2)$^{\$}$	0	0.0076	0.19 ± 0.06
Effluent (1/5)$^{\$}$	0	0.0060	0.34 ± 0.06
Effluent (1/10)$^{\$}$	0	0.0058	0.21 ± 0.02
Effluent (1/20)$^{\$}$	0	0.0077	0.22 ± 0.01

*: Influent refers to the liquid waste from the wine industry before distillation
$^{\$}$: Effluent refers to the liquid after distillation
( ): The dilution of influent or effluent with deionized water used for growth

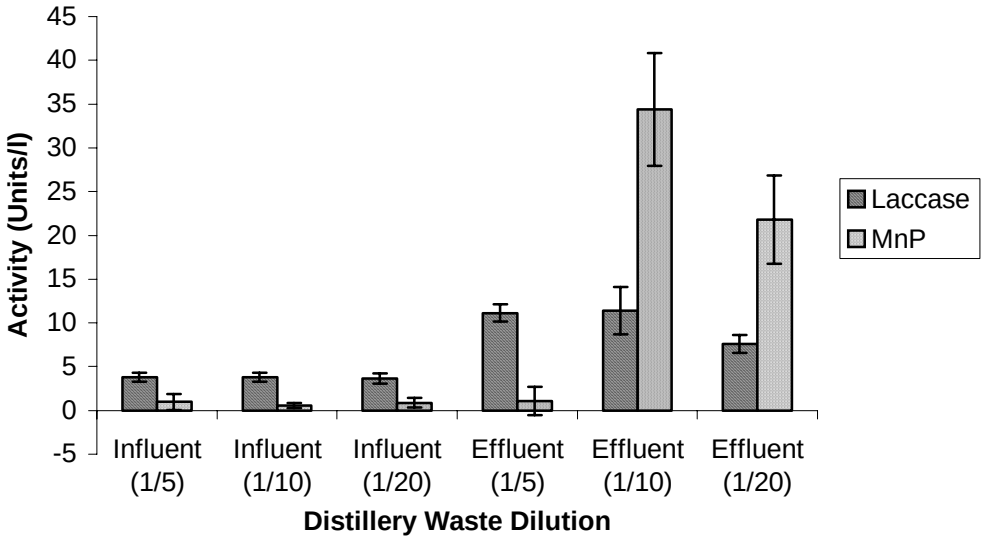


Figure 5.7: Enzyme Production patterns observed for laccase and MnP produced under various concentrations of wine distillery effluent. Values indicated are means ± standard deviation.

5.4.4 Optimum Incubation Temperature

Three temperatures were identified and chosen as test temperatures, namely 24, 28 and 37°C, three frequently used temperatures for fungal growth. The effect of these temperatures was investigated in terms of their influence on growth (Table 5.4) and their effect on ligninolytic enzyme production (Figure 5.8). At 37°C the fungus exhibited no growth, but enzyme activity was still detected, which remained constant over a period of 12 days during incubation. This laccase activity is possibly due to the release of ligninolytic enzymes stored in vacuoles in the mycelial cells (Barrasa *et al*., 1998) when incubated at this temperature. Mycelia from these flasks were harvested and subcultured on malt extract agar at 24°C. No growth was observed during this incubation indicating the fungus had died during incubation at 37°C.

Table 5.4: Effect of incubation temperature on growth kinetics and enzyme production of *Peniophora* sp. UD4 grown on Tien and Kirk medium.

Incubation Temperature (°C)	Lag Phase (hours)	Specific Growth Rate (h^{-1})	Biomass Yield (mg/ml) ± Standard Deviation
24	124.7	0.0147	5.66 ± 1.01
28	133.7	0.0120	4.22 ± 0.62
37	196.4	0.0067	0.15 ± 0.07

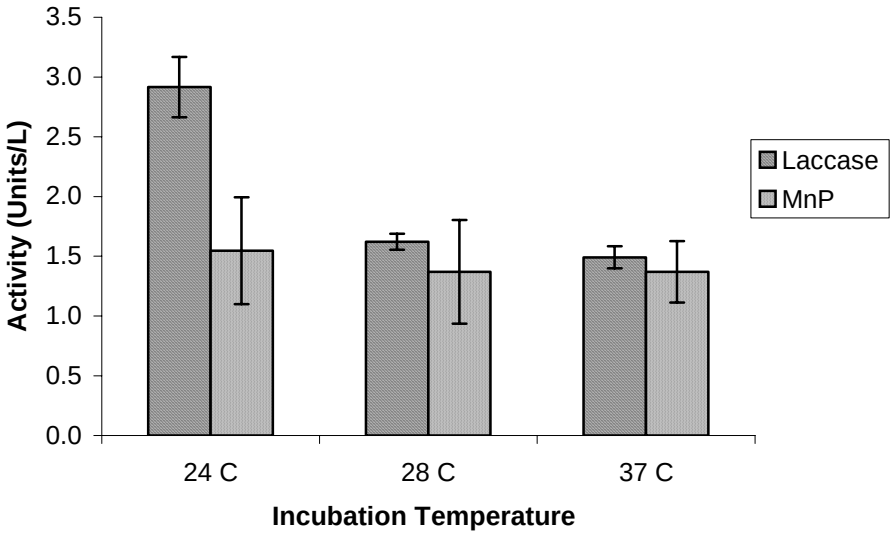


Figure 5.8: Maximum ligninolytic enzyme yields for *Peniophora* sp. UD4 incubated at different temperatures. Values are indicated as means ± standard deviation.

5.4.5 Optimum pH

The effect of pH on enzyme production from *Peniophora* sp. UD4 and the corresponding effects of pH on growth kinetics of the fungus were monitored and are represented in Figure 5.8 below. No growth was observed at pH values above 6.4 and below 3.5 (results not shown). Optimal biomass production and specific growth rate occur at slightly acidic pH of approximately 5.0 (Table 5.5). However, no enzyme activity was observed with a medium pH below 5.0. Maximal enzyme production occurs at pH 6.2 for those media that exhibit both growth and enzyme production. No growth was observed with media at pH 6.4 and above, but significant quantities of enzyme were recovered from these media (Figure 5.9).

Table 5.5: Growth characteristics for *Peniophora* sp. UD4 grown at various pH

pH of Medium	Lag Time (hours)	Specific Growth Rate (h^{-1})	Biomass Yield (mg/ml) ± Standard Deviation
4.1	119.7	0.0211	6.16 ± 0.96
4.6	122.8	0.0229	6.41 ± 0.70
5	132.4	0.0228	7.60 ± 0.93
5.4	133.6	0.0148	5.36 ± 0.82
5.7	151.7	0.0133	5.21 ± 0.74
6	135.7	0.0098	3.32 ± 0.62
6.4 *	69.2	0.0062	1.54 ± 0.40
5.4 (No Buffer)	122.4	0.0144	5.40 ± 0.93

*: Above pH 6.4 no growth was observed
All media contained vitamin supplementation
values.

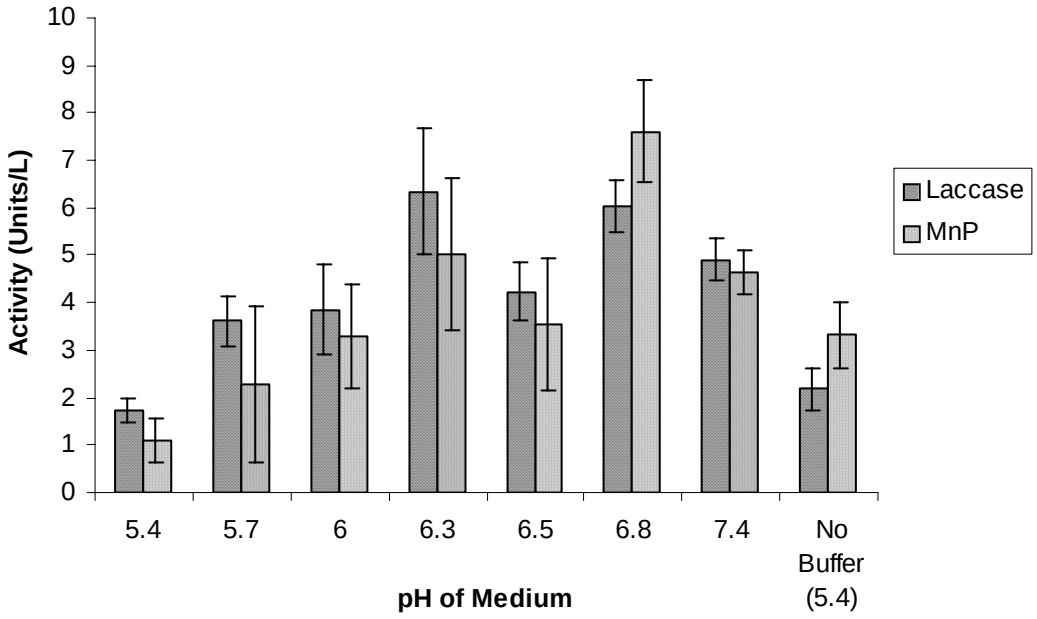


Figure 5.9: Ligninolytic enzyme production pattern for *Peniophora* sp. UD4 grown at various pH values. Yields are indicated as means ± standard deviation.

5.4.6 Vitamin Supplementation

The effect of individual vitamins as well as combinations of all vitamins excluding one from a vitamin stock were determined in an attempt to improve enzyme

production yields from *Peniophora* sp. UD4 and to investigate the potential use of vitamins to improve the biomass yield and specific growth rate from this organism (Table 5.6).

Table 5.6: Growth parameters determined for *Peniophora* sp. UD4 grown with vitamin supplemenation with a single vitamin and complex vitamin mixtures.

Vitamin Added	**Lag Time (hours)**	**Specfic Growth Rate (h^{-1})**	**Biomass Yield (mg/ml) ± Standard Deviation**
Nicotinic Acid	161.5	0.0182	0.78 ± 0.23
Aminobenzoic Acid	115.0	0.0081	2.41 ± 0.23
Riboflavin	142.2	0.0145	1.37 ± 0.41
Folic Acid	107.2	0.0354	1.50 ± 0.24
Thiamin	193.8	0.0231	2.07 ± 0.59
Ca Pantothenate	170.7	0.0202	2.07 ± 0.59
Pyridoxine	190.1	0.0259	0.89 ± 0.33
Biotin	119.1	0.0361	2.14 ± 0.87
Vitamin Excluded			
Nicotinic Acid	145.1	0.0262	5.03 ± 1.67
Aminobenzoic Acid	145.0	0.0258	6.10 ± 1.25
Riboflavin	137.8	0.0267	3.84 ± 1.58
Folic Acid	143.1	0.0207	4.15 ± 1.46
Thiamin	146.5	0.0243	5.70 ± 2.22
Ca Pantothenate	127.5	0.0240	3.92 ± 1.37
Pyridoxine	132.9	0.0304	4.17 ± 1.17
Biotin	141.6	0.0293	4.67 ± 1.67
Controls			
All Vitamins	164.7	0.0251	5.59 ± 2.24
No Vitamins	185.2	0.0208	1.02 ± 0.30

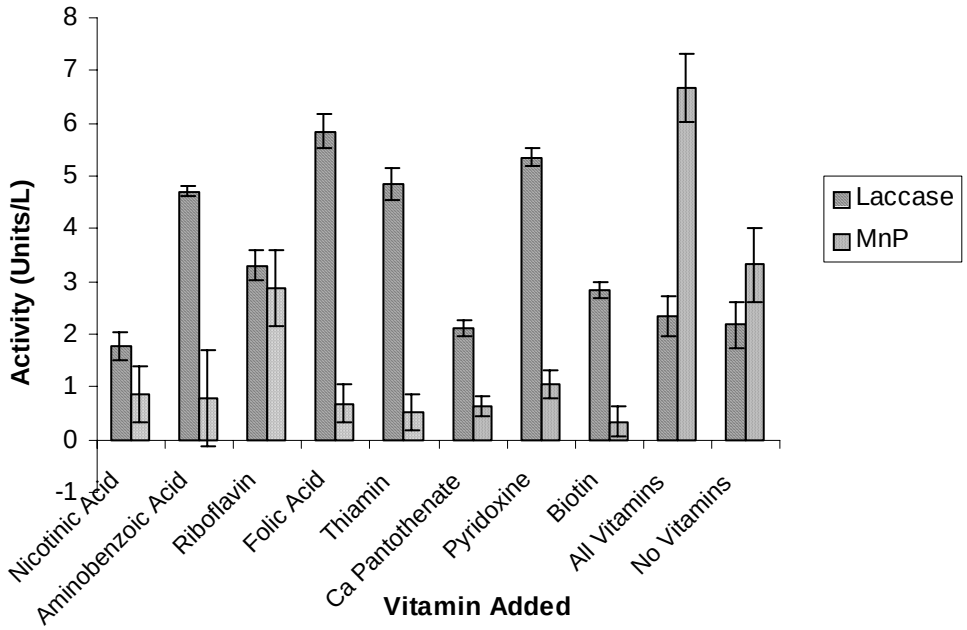


Figure 5.10: Enzyme production patterns of *Peniophora* sp. UD4 with media supplemented with a single vitamin. Enzyme yields are indicated as means ± standard deviation.

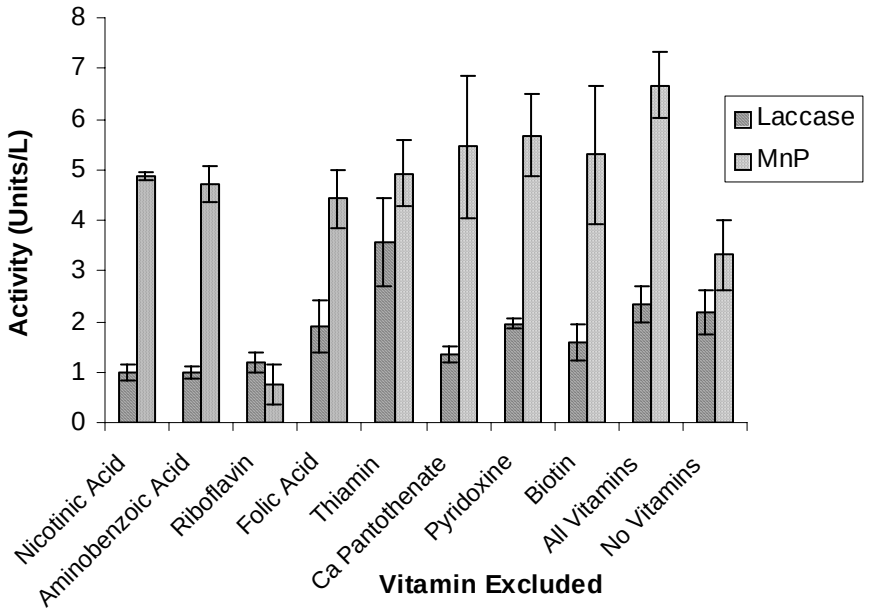


Figure 5.11: Enzyme production patterns of *Peniophora* sp. UD4 grown in media supplemented with vitamin mixtures excluding a single vitamin. Enzyme yields are indicated as means ± standard deviation.

The addition of biotin and folic acid enhance the specific growth rate of *Peniophora* sp. UD4 significantly, but still only offer a low biomass yield (Figure 5.7 A). Coupled with this high specific growth rate is a significant increase in the production of laccase with Folic acid that is not observed with Biotin supplementation at the concentration supplied (Figure 5.7 B). Pyridoxine supplementation provided the second highest laccase production but did not improve the biomass yield (Figure 5.7 A and B). The addition of all single vitamins resulted in the repression of manganese peroxidase (MnP) activity (Figure 5.7 B). Enzyme production with riboflavin supplemented medium results in a similar enzyme production pattern as the control medium without vitamin supplementation (Figure 5.7 B), however, the exclusion of riboflavin in a medium containing a complex mixture of vitamins results in repressed laccase and MnP activity (Figure 5.7 D). The addition of complex vitamin mixtures has the general effect of improving the biomass yield but appears to have no significant effect on the specific growth rate (Figure 5.7 C). The addition of nicotinic acid reduces the laccase yield from the medium. Another interesting phenomenon is that the additions of most single vitamins to the medium, with the exclusion of riboflavin, reduce the yield of MnP. This phenomenon is inversely true for the complex vitamin mixtures, where the exclusion of riboflavin results in a decrease in MnP yield, while all other complex vitamin mixtures increase the yield. It is also interesting to note that MnP and laccase appear to be independently regulated by vitamins.

5.4.7 Agitation

The production of laccase from *Peniophora* sp. UD4 was unaffected by the presence of detergents in both static and shaking culture conditions. *Peniophora* sp. UD4 did not produce laccase under agitated conditions in liquid Tien and Kirk medium (results not shown), however production of laccase and MnP were observed (results not shown) using agitated culture conditions with a solid

substrate medium containing cotton-seed and soybean meal indicating the possibility for production under agitated conditions.

5.4.8 Oxygen Supplementation

Increasing the oxygen content of the culture medium did not enhance the production of ligninolytic enzymes from the fungus *Peniophora* sp. UD4 (results not shown). The surfactants tested also failed to enhance the ligninolytic enzyme production of *Peniophora* sp. UD4. This indicates a relatively low oxygen demand for *Peniophora* sp. UD4 for the production of its ligninolytic enzymes.

5.4.9 Inducers

Laccase production appeared to be unaffected by the presence of ferulic acid, coumaric acid and veratryl alcohol as the inducer. Combinations of these inducers also failed to yield a positive response in laccase yield (data not shown). Production of laccase in the early stages of growth (day 2 and 3) in distillery effluent indicates that the laccase is however inducible. In future studies, a more substantial range of typical laccase inducers and mixtures of inducers (Marbach *et al.,* 1985) should be investigated to determine the nature of the induction observed in wine-distillery effluent. An investigation into the phenolic and low molecular weight acid content of wine-distillery effluent to determine which recognized inducers are present may be of interest to future research into induction of laccase in *Peniophora* sp. UD4.

5.5 Discussion

The use of distillery effluent as a medium proved to be beneficial for the production of MnP, rather than laccase from *Peniophora* sp. UD4. Liquid effluent prior to distillation (influent) from the wine industry was less efficient a medium

than the effluent after distillation. This indicates a change in the nutrient composition of the medium during distillation, and may perhaps be linked to the caramelization of simple sugars to complex carbohydrates or perhaps the lysis of yeast cells to release essential amino acids and carbohydrates. The initial laccase yields (on the second and third day of inoculation) are probably related to the inductive effect of the potentially toxic phenols present in the wine distillery influent and effluent. This is supported by the lowering of laccase yield in the 1/20 dilution of effluent. A greater inductive effect on laccase production is noted in the effluent, rather than the influent, which indicates the potential conversion of compounds to more inductive compounds during distillation of wine effluent. Maximum laccase yields are however reached a day earlier in the influent rather than the effluent, which may be due to the inductive effect of ethanol (Lomascolo *et al*., 2003). MnP yield is significantly higher than that of laccase activity in the effluent, which may be linked to the presence of yeast extracts from wine fermentations, which are released during distillation by yeast lysis. Increased laccase yields are obtained in the early stages of inoculation into effluent rather than influent, which may again be indicative of compositional changes in the phenolic content in the liquid during distillation. The increased yield of MnP in the wine effluent may be explained by the increased yield of MnP when yeast extract is used as the sole nitrogen source. Yeast lysis may occur during distillation, releasing yeast extracts for use as a nitrogen source. Organic nitrogen in the form of peptone or yeast extract enhances the growth and biomass yields of *Peniophora* sp. UD4, however only yeast extract improves the yield of MnP indicating the presence of inductive compounds in yeast extract.

Increased laccase production by nitrogen rich media with organic nitrogen, like *Peniophora* sp. UD4 with yeast extract, is supported by findings for other white-rot fungi such as *Lentinus edodes*, *Pleurotus* spp. (Kaal *et al.,* 1995) and *Ceriporiopsis subvermispora* (Ruttimann-Johnson *et al.,* 1993) for peptone containing medium, and *Pleurotus ostreatus* (Garzillo *et al.,* 1992) with medium supplemented with yeast extract. Higher growth rates on nitrogen rich media

containing organic nitrogen have also been observed for *Lentinus edodes* and *Pleurotus* spp. (Kaal *et al.,* 1995). *Phanerochaete chrysosporium* has also been shown to have a higher growth rate on an organic nitrogen source (Reid, 1983), but unlike the organisms mentioned above it produces higher yields of ligninolytic enzymes with a nitrogen deficient medium (Kaal *et al.,* 1995). A possible explanation for the increased production of ligninolytic enzymes when using organic nitrogen sources may be linked to the increased biomass observed (Kaal *et al.,* 1995), although in the present study the highest biomass yield was obtained with peptone rather than yeast extract while the highest MnP and laccase yield was achieved with yeast extract as the sole nitrogen source. The nature of the ligninolytic enzymes produced under various culture conditions is best illustrated using the example of *Phanerochaete chrysosporium* BKM-F1767 (Srinivasan *et al.,* 1995). Prior to the experimentation it was thought that *Phanerochaete chrysosporium* did not produce laccase (de Jong *et al.,* 1994; Hattaka, 1994; Thurston, 1994), but Srinivasan *et al.* (1995) demonstrated laccase production in a medium supplemented with cellulose.

The reduced production of laccase under agitated culture conditions has been observed in basidiomycetes (Nakamura *et al.,* 1999), although there are cases where agitation has provided an increase in laccase production, such as *Rhizoctonia praticola* (Shuttleworth *et al.,* 1986). The production of ligninolytic enzymes under bioreactor conditions has met limited success for two main reasons; under agitated culture conditions there is a loss in the production capability due to a change in the morphology of the fungus (Cui *et al.,* 1998; Venkatadri and Irvine, 1990; Nakamura *et al.,* 1999), or the agitation may lead to mechanical inactivation of the enzyme of interest (Venkatadri and Irvine, 1990). These two problems may be overcome by using low-shear bioreactors (Miura *et al.,* 1997; Nakamura *et al.,* 1999) such as those employing airlift or with immobilized fungus (Miura *et al.,* 1997; Nakamura *et al.,* 1999). The use of detergents such as Tween 20 and 80 have also provided positive results in overcoming these problems (Jager *et al.,* 1985) but are unsuitable for bioreactors

because of foaming. Since simple bioreactor configurations, such as airlift bioreactors, require a fair degree of agitation the effect of agitation on *Peniophora* sp. UD4 was investigated. Under agitated culture conditions *Peniophora* sp. UD4 produced little or no detectable laccase activity, even in the presence of detergents.

Production of laccase in *Peniophora* sp. UD4 appears to be under strong nitrogen and carbon regulation, with the highest yield of laccase being observed in a carbon limited and nitrogen rich environment. The presence of an organic nitrogen source such as yeast extract or peptone increases the ligninolytic enzyme production of *Peniophora* sp. UD4, and especially that of MnP. It appears that the production of MnP increases with the use of organic nitrogen sources such as peptone and yeast extract.

The DMAB-MBTH oxidative coupling reaction was used to determine the relative enzyme activities of MnP and laccase when using distillery effluent as a medium, since the use of ABTS was hindered due to its action as a mediator in the presence of other phenolic compounds. The use of post-distillation wine industry liquid waste for the production of ligninolytic enzymes proved to be more effective than pre-distillation waste. The reason for this is still unclear but may be due to the presence of ethanol in the pre-distillation liquid waste, or enhancing the availability of yeast extracts during distillation through yeast lysis at high temperatures.

The highest production of MnP and laccase was observed at pH 6.8. At this pH however the fungus did not exhibit any growth, this therefore may have been as a direct result of the release of MnP and laccase stored inside vacuoles (Barrasa *et al*., 1998) due to incorrect functioning of cellular components at this pH. The medium pH that produces the highest yields of laccase and MnP, as well as a growth rate is pH 6.2. This could be due to a balance between laccase production and low laccase degradation due to the laccase having a better

stability at neutral to slightly alkaline conditions, or it could be perhaps attributed to this pH being close to optimum physiological state of the fungus therefore improving protein folding.

The addition of vitamins has a pronounced effect on the production of ligninolytic enzymes from *Peniophora* sp. UD4. Four of the individual vitamins have a marked positive influence on the production of laccase from *Peniophora* sp. UD4, namely thiamin, aminobenzoic acid, folic acid and pyridoxine, which generally corresponds to suppression in the production of MnP. The addition of riboflavin does not exhibit the inhibitory effect on MnP production exhibited by the other single vitamins, and stimulates MnP expression. In the presence of a complex combination of vitamins there is a trend towards the production of MnP over that of laccase. The addition of nicotinic acid as well as the addition of most of the complex vitamin mixtures results in a decrease in laccase yield. The mechanisms of these phenomena require further investigation with *Peniophora* sp. UD4 and other fungi since they could significantly enhance our understanding of the ligninolytic system.

No LiP activity was observed in any of the abovementioned experiments, even when veratryl alcohol was tested for its inductive effect on the production of ligninolytic enzymes. Veratryl alcohol is the most commonly used LiP inducer, and since enzyme production was monitored in a diverse range of culture media, it is proposed that *Peniophora* sp. UD4 does not produce detectable levels of LiP, and that its chief lignin degrading enzymes are MnP and laccase. Several organisms are well known for being deficient in one or more of the three major classes of lignin modifying enzymes; *Ceriporiopsis subvermispora* is LiP deficient (Jensen *et al.,* 1996), and *Pycnoporus cinnabarinus* produces laccase as its sole lignin-modifying enzyme (Geng and Li, 2002; Herpoël *et al.,* 2000).

5.6 Conclusions

The objective of this study was to attempt to improve the laccase production of *Peniophora* sp. UD4 through alteration of medium components and culture conditions. Several enhancements in enzyme production and biomass yield were achieved, however no single component was identified that could drastically improve the enzyme yield to levels sufficient for large-scale production from the host organism.

A disappointing outcome of this research was the lack of enzyme production in shaking culture. Currently the most efficient method for fermentation of fungi is in agitated liquid culture conditions, although solid substrate fermentations are gaining understanding and momentum and therefore cannot be discounted.

It was shown that the nutrient composition of the medium plays a major role in the control of the ligninolytic enzyme production pattern. Of particular importance was the organic nitrogen sources appeared to favour MnP production. Laccase was produced in most of the medium compositions tested, although only small improvements in yield were noted. In general culture conditions such as pH and temperature affected both the ligninolytic enzymes produced.

The conclusion is thus that medium optimization is unlikely to generate laccase production suitable for use in large-scale fermentations. The use of inducers to enhance laccase production may yet offer improved yields since only a few inducers and mixtures of inducers were tested, but these inducers are generally expensive and therefore unsuitable for use in large-scale fermentations.

At this stage alternative production strategies such as mutation-selection and heterologous expression were considered. However, it was thought best to evaluate applications and establish an applications base for the novel laccase before these potentially arduous and expensive strategies were considered.

Chapter 6
Applications of the Thermostable Laccase

6.1 Introduction

The thermostable laccase from *Peniophora* sp. UD4 may have many potential applications in industrial processes due to its enhanced stability, broader than usual substrate range and high catalytic efficiency. Several advantages over applications using other sources of laccase were hypothesized, and are summarized in Table 6.1 below.

Table 6.1: Potential advantages of laccase from *Peniophora* sp. UD4 over other sources of the enzyme for current and developing technologies of laccase.

Technology	Advantages of Thermostable Laccase from *UD4*
Bioremediation	> Broader substrate range: Ability to recognize and oxidize or polymerize a broader range of pollutants. > Thermostability: Higher temperature bioremediation improves efficiency. > Improved catalytic efficiency: Cost saving since lower laccase requirement to bioremediate effluents.
Biosensors	> Broader substrate range: Increases the sensory capability to substrates not detectable by other laccase sources. > Thermostability: Longer life of biosensor; shelf life and life of working biosensor.
Immunoassay	> Thermostability: Longer shelf life of conjugate enzyme. > Improved catalytic efficiency: Reduction in cost owing to lower enzyme requirement and higher sensitivity. > Broad temperature profile: exhibits significant activity at lower temperatures improves sensitivity at lower temperatures.
Cosmetics *	> Thermostability: Improved shelf life of consumables. > Improved catalytic efficiency: Cost effective owing to lower enzyme requirement.
Organic Synthesis	> Broader substrate range: Development of novel oxidative products and biopolymers due to broader natural substrate range and mediators not recognized by other laccase sources > Improved catalytic efficiency: Less enzyme required to perform same oxidative function > Thermostability: Increased mechanical stability in agitated bioreactors

* - Hair dye, toothpaste etc.

The thermostable laccase was tested for its application in several technologies including biosensors, phenol reduction, defouling of membranes and organic synthesis. Applications were selected to demonstrate the potentially superior characteristics of the thermostable laccase from *Peniophora* sp. UD4 over other sources of laccase.

6.1.1 Defouling of Membranes

The application of the laccase in a system requiring high mechanical stability was investigated, since the attributed thermostability was thought to be a major attribute for enhancing the applicability of this enzyme to laccase based technologies. A potential application requiring a high degree of mechanical stability was identified in the “defouling on demand” strategy (Leukes *et al*., 2003), utilizing immobilized enzymes for defouling of ultafiltration membranes used for water purification. The lack of a suitable enzyme with high mechanical stability has hampered the realization of this technology, and thus provided a potential niche market for the thermostable laccase for this application.

Initial experiments performed in the field of membrane defouling used manganese peroxidase as the immobilized enzyme. Manganese peroxidase was later replaced with laccase as the immobilized enzyme as a superior alternative since the use of hydrogen peroxide and manganese ions to initiate defouling was considered undesirable. Potential damage of the ultrafiltration membrane due to the addition of hydrogen peroxide was noted as a major concern, as well as the addition of manganese ions that could further add to the problem of water pollution, negating the benefit of using this technology for water purification (Leukes *et al.*, 2003).

6.1.2 Bioremediation of Industrial Waste Streams

The potential bioremediation of high-strength phenolic industrial effluents required investigation for two major reasons: (i) the bioremediation of industrial effluents produced in South Africa offers a potential market for the thermostable laccase; (ii) relatively little information is available concerning the removal of potentially toxic phenolic components of these effluents. If significant removal of these toxic phenolic compounds can be achieved, the realization of further detoxification strategies by processes such as microbial degradation would be more attractive. The presence of phenols in the effluents is presently considered the limiting factor preventing the application of microbial breakdown of effluents by bacterial systems since many of these compounds exhibit antibacterial properties (Ruiz *et al.,* 2002)

6.1.3 Organic Synthesis through Biocatalysis

Organic synthesis may be considered an important application of laccase, since there is a trend towards the cleaner production of chemicals through the use of enzymes. The major disadvantage of using laccase however is the use of potentially toxic mediators to initiate the necessary oxidations (see chapter 1: 1.6.1). The oxidation of (±)-α-pinene and ferulic acid were investigated in the presence and absence of selected mediators.

Three mediators were selected for the indirect oxidation of ferulic acid through a laccase mediator system (LMS), namely 1-hydroxybenzotriazole (HBT), 2,2',6,6'-Tetramethylpiperidine free radical (TEMPO) and 2,2'-Azinobis-(3-ethylbenzylthiozoline-6)-sulphonic acid (ABTS). For the oxidation of (±)-α-pinene, a broader range of 5 mediators were selected, namely HBT, TEMPO, ABTS, 4-hydroxybenzoic acid (HBA) and N-hydroxyphthalimide (HPI).

6.1.4 Biosensors

Biosensors are becoming an increasingly important product for the fast and efficient determination of phenols and other toxic compounds in effluents and waste-streams, and may provide a sensitive method for field and online monitoring of phenolic pollutants. The thermostable laccase was applied to biosensor technology to determine whether the thermostable laccase was suitable for this application, since not all enzymes are suitable candidates for this technology. Furthermore, the laccase from *Peniophora* sp. UD4 was compared to laccase from *Trametes versicolor* for the sensitive detection of 4-chlorophenol and catechol using physical adsorption, biomolecule entrapment, covalent binding and crosslinking enzyme immobilization techniques.

6.2 Objectives

- To evalute the thermostable laccase for existing and proposed applications.

6.3 Methods

6.3.1 Enzyme Preparation

Unless otherwise stated, partially purified laccase was extracted by homogenization of solid substrate Tien and Kirk agar medium (Tien and Kirk medium supplemented with 2% agar) cultures of *Peniophora* sp. UD4. Twenty agar plates (approximately 400 ml of medium) were homogenized (Ultra Turrax) in 1 liter of water. This was then centrifuged at 15 000 X g for 5 minutes, and supernatant filtered through Whatman no. 1 filter paper. The filtrate was then subjected to a final round of centrifugation at 22 000 X g for 10 minutes to

remove any residual agar and mycelia. The supernatant was freeze-dried and a concentration step and subsequently purified according to the methods described in Chapter 3. The partially purified enzyme was freeze-dried and stored at –20°C, until required for applications testing.

6.3.2 Defouling of Membranes

Enzymatic defouling of polysulfone ultafiltration capillary membranes (supplied by Stellenbosch University) was measured under constant flux during the ultrafiltration of brown water. Two methodologies for enzymatic defouling were investigated, namely immobilization of the enzyme on the membranes, followed by purging with oxygen to remove enzyme bound residues, and periodic washing of membranes with a suspension of activated enzyme to remove membrane bound residues. The application of the thermostable laccase from *Peniophora* sp. UD4 to “defouling on demand” was performed by Mr. Clive Garcin under the supervision of Dr. Winston Leukes.

6.3.3 Bioremediation of Industrial Waste Streams

6.3.3.1 Phenol Assays

Phenol assays were performed using the Folin Ciocalteu reagent method. A standard curve was constructed using phenol as the standard.

6.3.3.2 Effluents

Effluents selected for phenol reduction were selected for their high concentration of phenolic constituents, these were strip gas liquor (SGL), Sasol effluent (SE), wine distillery influent (WDI) and effluent (WDE) (as differentiated in chapter 5) and bleach plant effluent (BPE). The specific compounds tested for removal were phenol, cresol and tannic acid.

6.3.3.3 Reaction mixtures

Tannic acid, phenol and cresol (500 mg/L), as well as effluents were adjusted to pH 4.5 using hydrochloric acid or sodium hydroxide for improved enzyme activity. A partially purified, peroxidase free, laccase preparation from the culture filtrate of *Peniophora* sp. UD4 (shallow static culture, refer to Chapter 3) in a ratio of 4 parts effluent: 1 part enzyme preparation. Two different quantities of enzyme were added to the samples, 0.5 Units (High) and 0.05 Units. Samples were incubated at room temperature (24°C) and 60°C for a period of 16 hours. Samples were prepared in quadruplicate and assayed individually for phenol concentration.

Another set of reaction mixtures for the effluents WDE, WDI, SE and SGL were prepared based on the high degree of phenolic content removal observed for BPE, since the possibility of these easily oxidizable substrates were considered as potential mediators for the laccase activity with the other effluents. The reaction mixture contained effluent : BPE : enzyme preparation : water in the ratio of 4:2:2:2.

Controls were performed for both temperatures where the enzyme preparation was replaced with water. The interference of protein with the FC reagent method for the determination of phenols was investigated and determined to be negligible.

6.3.4 Organic Synthesis through Biocatalysis

6.3.4.1 Chemicals

(±)-α-Pinene and ferulic acid and vanillin were purchased from Sigma-Aldrich. Laccase from *Trametes (Coriolus) versicolor* (1.5 U/mg) was purchased from Fluka. 2,2'-Azinobis-(3-ethylbenzylthiozoline sulphonic acid) (ABTS) was

purchased from Roche. All other mediators were purchased from Sigma-Aldrich. Absolute ethanol was purchased from SaarChem. Sulphuric acid was purchased from Merck.

6.3.4.2 Reaction Conditions

Enzymes were stable in the presence of dioxane (50%) as determined by ABTS oxidation and therefore reactions were performed in a dioxane water (1:1) system. Reactions were allowed to progress for 48 hours at 28 °C. Samples were extracted with 1 ml ethyl-acetate for further analysis. Extract (20 µl) was applied to the TLC plate. Reactions were performed at 28°C on a rotary shaker at 150 rpm. (±)-α-Pinene reaction mixtures consisted of 20 mM borate buffer (pH 5.5), 40 mM (±)-α-pinene, 0.006 U/ml laccase and 5 mM mediator in a final reaction volume of 4 ml. Ferulic acid oxidations were performed in reaction mixtures containing 20 mM borate (pH 5.5) or succinate-lactate buffer (pH 4.5), 40 mM ferulic acid, 0.006 U/ml laccase and 5 mM mediator in a final reaction volume of 4 ml. (±)-α-Pinene and ferulic acid were added from 10X stock solutions dissolved in ethanol.

6.3.4.3 Mediators

Mediators investigated for the oxidation of (±)-α-pinene were 2,2'-azinobis-(3-ethylbenzylthiozoline sulphonic acid) (ABTS), 1-hydroxybenzotriazole (HBT), 4-hydroxybenzoic acid (HBA), N-hydroxyphthalimide (HPI) and 2,2,6,6-tetramethylpiperidinooxy free radical (TEMPO).

6.3.4.4 Thin-layer Chromatography (TLC)

TLC was performed on Silica gel 60 F_{254} aluminium sheets (Merck). The chromatographic solvent for (±)-α-pinene consisted of hexane:diethyl ether (2:1), and for ferulic acid a toluene:acetic acid (20:1) chromatographic solvent was

used. TLC plates were viewed using ultraviolet light (CAMAG UV-Betrachter) and duplicate plates of (±)-α-pinene oxidation were stained using acidified vanillin (2% vanillin and 2% sulphuric acid in ethanol) (Figure 6.3: plates B and D) or 2,4-dinitrophenylhydrazine (DNP) (Figure 6.3: plates A and C below). Standards were applied as 15 µl samples containing 20 mM of the standard compound dissolved in absolute ethanol. (±)-α-Pinene was applied from a 1 M stock solution due to decreased resolution in acidified vanillin.

6.3.4.5 GC-MS-EI

Reactions of interest were further analyzed by GC-MS-EI. Gas chromatography was performed on a Hewlett Packard 5890 fitted with a 7673A auto sampler, flame ionization detector, and a Stabilwax-DA column (Restek). Oven parameters were an Initial oven temperature was 40°C with a final temperature of 240°C increased at a 15°C.min^{-1} increments. Data was analysed using Chemstation software (Hewlett Packard). Mass analysis was performed on a Finnigan TSQ 700 MS.

Table 6.2: Samples and controls for the oxidation of (±)-α-pinene by laccase. Labels are pertinent to Figure 6.3 below.

TLC Plate Label	Major Division	Minor Division	Mediator	Pinene	Laccase	Laccase Source
A & B	Samples	1	-	Yes	Yes	*Peniophora* sp. UD4
		2	-	Yes	Yes	T. versicolor
		3	ABTS	Yes	Yes	*Peniophora* sp. UD4
		4	ABTS	Yes	Yes	T. versicolor
	Controls	1	Yes	No	Yes	Mix
		2	No	No	Yes	Mix
		3	Yes	No	No	-
		4	No	Yes	No	-
C & D	Samples	1	HBT	Yes	Yes	*Peniophora* sp. UD4
		2	HPI	Yes	Yes	*Peniophora* sp. UD4
		3	TEMPO	Yes	Yes	*Peniophora* sp. UD4
		4	HBA	Yes	Yes	*Peniophora* sp. UD4
		5	HBT	Yes	Yes	T. versicolor
		6	HPI	Yes	Yes	T. versicolor
		7	TEMPO	Yes	Yes	T. versicolor
		8	HBA	Yes	Yes	T. versicolor
	Controls	1	HBT	Yes	No	-
		2	HPI	Yes	No	-
		3	TEMPO	Yes	No	-
		4	HBA	Yes	No	-
		5	HBT	No	Yes	Mix
		6	HPI	No	Yes	Mix
		7	TEMPO	No	Yes	Mix
		8	HBA	No	Yes	Mix

Table 6.3: Labels for standards run, applicable to Figure 6.3 below. Standards were based on potential reaction products for laccase oxidation as outlined by Niku-Paavola and Viikari (2000).

Plates	Number	Standard
A & B	1	Mix (2-5)
	2	Pinene
	3	Verbenone
	4	Carvone
	5	Carveol
C & D	1	Pinene
	2	Pinene oxide
	3	Verbenone
	4	Verbenol
	5	Carvone
	6	Carveol
	7	Mix (1-6)

6.3.5 Biosensors

The thermostable laccase was applied to biosensor technology for the detection of 4-chlorophenol and catechol as test compounds. The thermostable laccase from *Peniophora* sp. UD4 was applied to biosensor technology by Dr. Janice Limson and Mr Ron Fogel (Rhodes University). The thermostable laccase from *Peniophora* sp. UD4 and the comparative laccase from *Trametes versicolor* were adhered to the surface of a glassy carbon electrode through physical adsorption, entrapment and covalent binding techniques. Adherence of the laccases by physical adsorption to the surface of a glassy carbon electrode (GCE) was performed by dipping the electrode in a concentrated solution of water and laccase for five minutes, followed by air-drying. Entrapment of the laccases around the GCE was achieved using both resin-entrapment and dialysis tubing

entrapment. Covalent binding of the laccases was achieved by carbodiimide treatment of the GCE followed by dipping in an enzyme solution.

Following enzyme immobilization, the electrode was applied directly to a sample containing 4-chlorophenol or catechol. Enhanced detection of 4-chlorophenol and catechol was monitored by cyclic voltammetry in mediated (with ferrocyanide) and non-mediated systems.

6.4 Results

6.4.1 Defouling of Membranes

The use of laccase as a washing solution to remove the cake layer after ultrafiltration with brown water met with limited success and only small flux recoveries were observed. However, immobilization of the thermostable laccase to the polysulfone membrane through high-pressure dead end filtration, followed the brown water filtration, and washing with oxygenated water at 18, 43 and 54 hours resulted in large flux recoveries of 31 %, 21 % and 21 % respectively. These three washes resulted in increases to 87 %, 67 % and 67 % of the initial pure water flux respectively. The control without immobilized enzyme was subjected to oxygenation, which only resulted in a 3 % increase in flux, indicating that the flux recovery was directly attributable to the action of the immobilized enzyme rather than shear caused by the clean water (Leukes *et al.*, 2003).

6.4.2 Bioremediation of Industrial Waste Streams

The phenolic load reduction using two enzyme concentrations at two different temperatures are illustrated in Figures 6.1 and 6.2 below. Initial experiments indicated the BPE might contain a mediator due to the large phenol reduction exhibited when treated with laccase from *Peniophora* sp. UD4, and it was

therefore added to other effluents in an attempt to increase the phenol reduction in these effluents. Results for the application of a BPE mediated laccase treatment of the other industrial effluents, are tabulated below (Table 6.4).

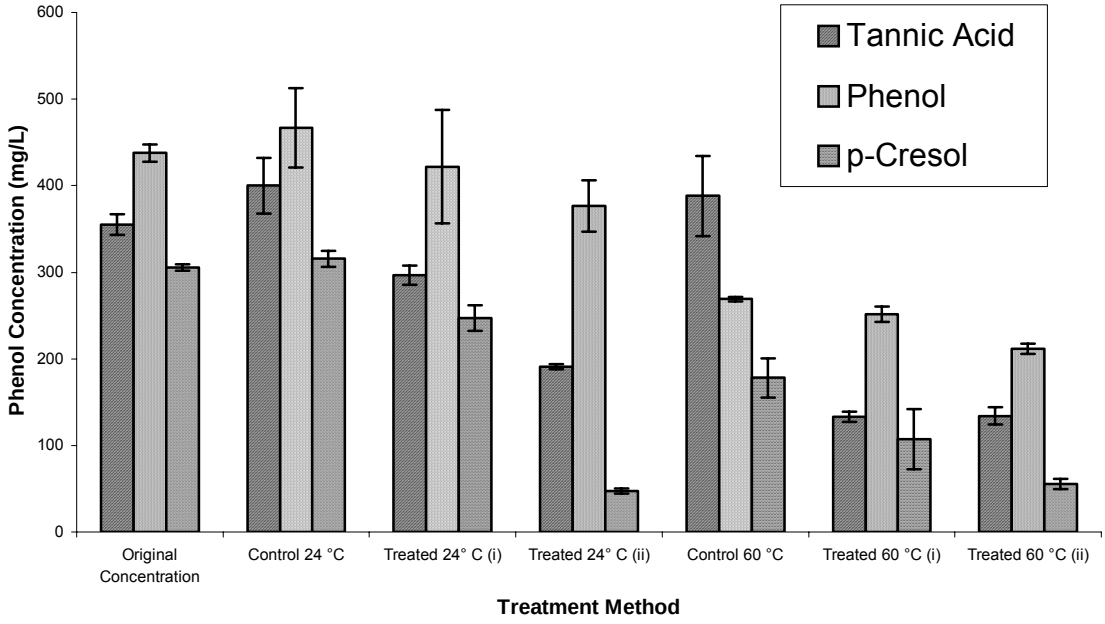


Figure 6.1: Results obtained for the oxidation of phenolic test compounds, as determined by assaying the total phenolic content of the reaction mixture. The samples containing low quantities of enzyme (0.05 Units/ml) are indicated by (i), and higher quantities of enzyme (0.5 Units/ml) are indicated by (ii).

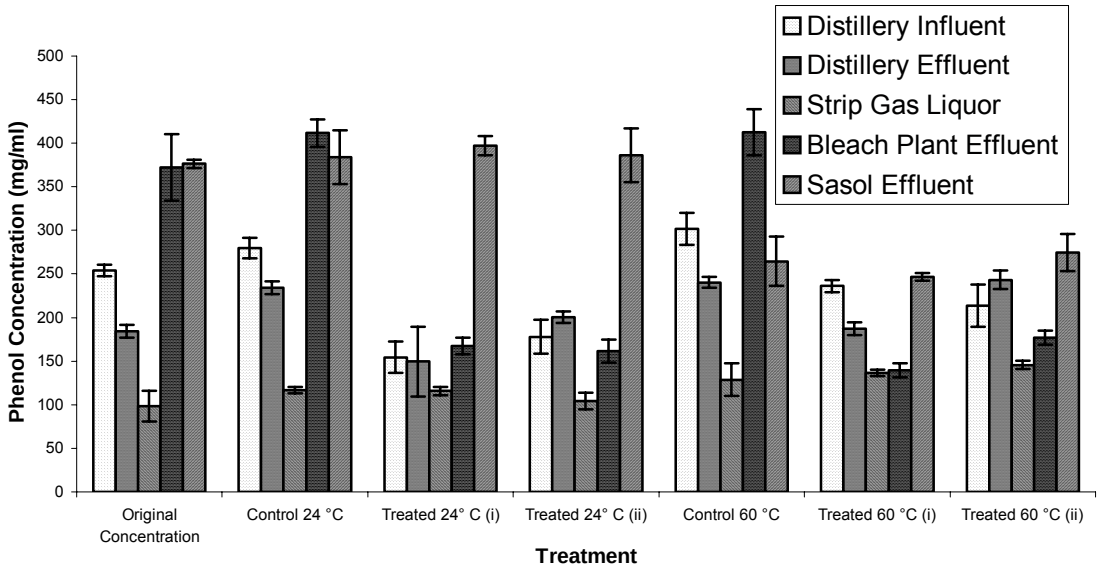


Figure 6.2: Graph illustrating the results obtained for removal of phenolic compounds from industrial effluents by the laccase from *Peniophora* sp. UD4. The samples containing low quantities of enzyme (0.05 Units/ml) are indicated by (i), and higher quantities of enzyme (0.5 Units/ml) are indicated by (ii).

Table 6.4: Comparative results obtained for the removal of phenolics in effluents with the addition of BPE.

	Without BPE			Containing BPE		
Sample	Phenolic Load Reduction (%)	Optimum Temperature for Removal (°C)	Enzyme Requirement*	Phenol Reduction (%)^	Optimum Temperature for Removal (°C)	Enzyme Requirement*
Distillery Influent	45	24	Low	41	24	Low
Distillery Effluent	36	24	Low	42	24	Low
Strip Gas Liquor	11	24	High	22	60	High
Bleach Plant Effluent	66	60	Low	-	-	-
Sasol Effluent	6	60	High	5	24	High

*: Low = 0.05 Units/ml; High = 0.5 Units/ml

^: The percentage reduction in phenol content is corrected for the removal of phenols from BPE. An increase in the percentage reduction is therefore an indication of the removal of the industrial effluents tested.

6.4.3 Organic Synthesis through Biocatalysis

6.4.3.1 (±)-α-Pinene Oxidation

Under the reaction conditions used laccase from *Peniophora* sp. UD4 was shown to catalyze the oxidation of (±)-α-pinene in the absence of a mediator, yielding a hydrophilic product with R_f = 0. This direct oxidation of pinene via laccase may be of importance since direct enzymatic oxidations are often chirally or enantiomerically selective. The same spot was observed with a greater intensity for the oxidation of (±)-α-pinene in the presence of *Peniophora* sp. UD4 laccase with HBT as a mediator.

It should also be mentioned that a spot with the same R_f value of pinene oxide was observed for the oxidation of (±)-α-pinene in the presence of all mediators, but more pronounced when using HBT as a mediator (Figure 6.3: C). This is not unusual since an epoxide has been proposed as an intermediate in the oxidation of other compounds by laccase, such as 1-(3,4-dimethoxyphenyl)-1-propene (Chen *et al.*, 2000).

DNP staining (Figure 6.3: A and C) revealed that none of the reaction products were aldehydes or ketones since this method is used for the derivitization of these compounds.

Another mentionable observation is that the product of catalysis with *Peniophora* sp. UD4 laccase and HBT (R_f = 0) is the only compound that exhibited fluorescence when subjected to both 254 and 366 nm wavelengths (Figure 6.3: C and D, green circles).

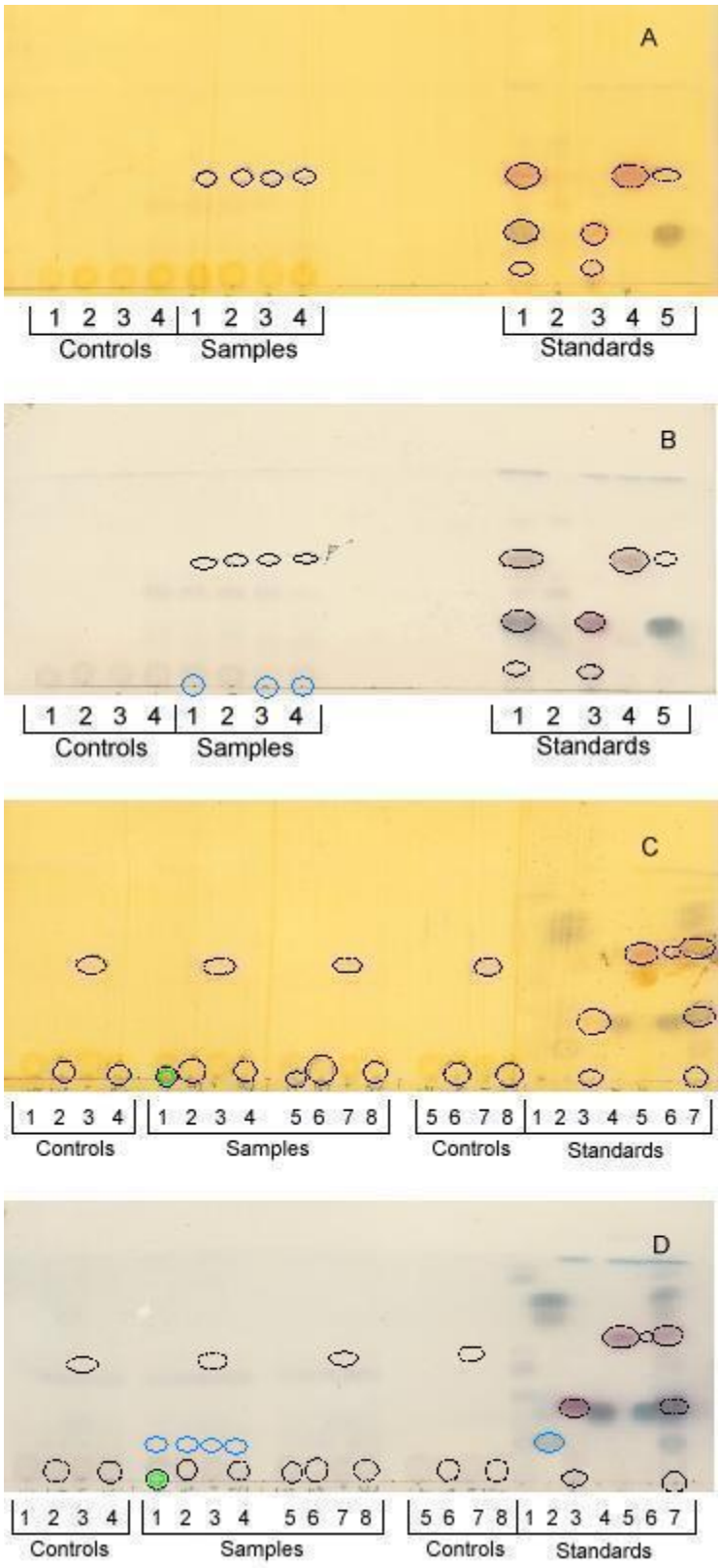


Figure 6.3: TLC results for the oxidation of pinene with laccase from *Peniophora* sp. UD4 and *Trametes versicolor*. Black circles indicate spots that luminesce when irradiated with UV light at 254 nm, green circles indicate spots that emit luminesce when irradiated under UV light at 366 nm. Blue circles indicate oxidation products that are not clearly visible due to loss of resolution upon editing of the TLC scans. Sample labels, control labels and standards are provided in Tables 6.2 and 6.3 above.

6.4.3.2 Ferulic Acid Oxidation

The oxidation of ferulic acid by laccase in the presence and absence of mediators was analyzed by TLC using the method mentioned above (results not shown). All samples were further analyzed by HPLC using a Luna C18 column with 5 mM KH_2PO_4 (pH 2.5) and acetonitrile mobile phase. Results of HPLC analysis are summarized in Table 6.4 below.

The oxidation of ferulic acid resulted in the production of several compounds including ethyl ferulate, which may have resulted from the oxidative coupling of ethanol (the solvent used to dissolve the ferulic acid) and ferulic acid. Two major compounds produced in almost every reaction condition tested, with retention times of 14.54 and 17.95 minutes may correspond to the two dimerization products of ferulic acid which have previously been identified (Carunchio *et al*., 2001), since they were the most abundant oxidation products in the mediatorless oxidation of ferulic acid by laccase from *Pyricularia oryzae*. The compound with a retention time of 17.95 minutes was the most abundant product.

Table 6.5: Summarized results obtained from HPLC analysis of reaction products from the oxidation of ferulic acid by laccase and LMS, major products are indicated in bold.

		Retention Time		**7.07**	**9.47**	**10.04**	**10.89**	**11.44**	**13.13**	**14.54**	**15.21**	**15.59**	**15.73**	**16.10**	**16.52**	**16.95**	**17.25**
		Compound if Identified			Vanillic Acid			Vanillin	Guaiacol								
Sample Name	Enzyme Source	Mediator	pH of Sample	Relative Quantity (Area Percent)													
FA NM 4	None	None	4.5				0.28										0.35
T NM 4	*Trametes versicolor*	None	4.5				0.25										0.32
P NM 4	*UD4*	None	4.5					0.42		**0.97**	0.76		**0.97**				0.37
FA NM 5	None	None	5.5					0.30									
T NM 5	*Trametes versicolor*	None	5.5	0.71		0.96	0.31	**1.25**		0.98	0.84		**1.47**			0.21	0.20
P NM 5	*UD4*	None	5.5					0.46		**1.63**	0.39		0.98	0.57	0.57		0.56
FA T 4	None	TEMPO	4.5		**1.03**			**1.96**		0.66	0.55		**1.38**				0.21
T T 4	*Trametes versicolor*	TEMPO	4.5		**1.41**			**1.85**		0.82	0.87		**1.46**				
P T 4	*UD4*	TEMPO	4.5		**1.49**			**1.93**		1.00	0.99		**1.70**				0.26
FA T 5	None	TEMPO	5.5		**0.94**	0.27		**1.88**	0.43	0.47	0.21	0.45	0.41				
T T 5	*Trametes versicolor*	TEMPO	5.5					**0.46**		0.88	0.16		0.40	0.27			0.22
P T 5	*UD4*	TEMPO	5.5		**1.24**	0.29		**1.63**		0.98	0.27	0.69	0.60	0.29			0.21
FA H 4	None	HBT	4.5		0.30											0.28	
T H 4	*Trametes versicolor*	HBT	4.5		0.38					0.57	0.45		0.45				0.22
P H 4	*UD4*	HBT	4.5		0.37					0.85	0.62		0.60				0.27
FA H 5	None	HBT	5.5					0.43		0.74	0.21		0.42				0.31
T H 5	*Trametes versicolor*	HBT	5.5					0.45		**1.44**	0.39		0.57	0.22			0.52
P H 5	*UD4*	HBT	5.5					0.56		**1.70**	0.41		0.69	0.22			0.59
FA A 4	None	ABTS	4.5					0.30									
T A 4	*Trametes versicolor*	ABTS	4.5					0.48		0.38	0.29		0.47				0.25
P A 4	*UD4*	ABTS	4.5					0.64		**1.07**	0.74		**1.22**	0.27			0.45
FA A 5	None	ABTS	5.5					0.54		1.14			0.50	0.39	0.21		0.34
T A 5	*Trametes versicolor*	ABTS	5.5				0.49	0.85		**1.79**	0.42		**1.09**	0.62	0.28		0.58
P A 5	*UD4*	ABTS	5.5				0.36	0.97		**1.76**	0.45		**1.30**	0.63	0.29		0.74
		Retention Time		**17.50**	**17.95**	**18.40**	**18.61**	**18.83**	**19.03**	**19.39**	**19.60**	**20.08**	**20.42**	**20.60**	**21.18**	**22.00**	**26.67**
		Compound if Identified					Vinyl Guaiacol				Ethyl Ferulate						
Sample Name	Enzyme Source	Mediator	pH of Sample														
FA NM 4	None	None	4.5			0.43											
T NM 4	*Trametes versicolor*	None	4.5			0.35											
P NM 4	*UD4*	None	4.5	0.47	**1.46**	0.72	0.57		0.21		0.71	0.27	0.71	0.54			
FA NM 5	None	None	5.5	0.41	0.40	0.27		0.20						0.14			
T NM 5	*Trametes versicolor*	None	5.5	0.53	0.92	0.59					0.48	0.27	0.42	0.30			
P NM 5	*UD4*	None	5.5	0.46	**4.03**	1.38			0.40		**1.52**	0.36	1.31	0.61	0.29	0.21	
FA T 4	None	TEMPO	4.5		**1.21**	0.68					0.48		0.26	0.23			0.37
T T 4	*Trametes versicolor*	TEMPO	4.5	0.45	**0.70**	0.68					0.44	0.22	0.22	0.39			0.36
P T 4	*UD4*	TEMPO	4.5	0.45	**1.12**	0.78					0.60	0.28	0.34	0.43			0.41
FA T 5	None	TEMPO	5.5	0.36	0.90	0.48					0.30						
T T 5	*Trametes versicolor*	TEMPO	5.5	0.49	**1.69**	0.72	0.20				0.54	0.20	0.57	0.41			
P T 5	*UD4*	TEMPO	5.5	0.49	**1.79**	0.70					0.55	0.32	0.32	0.40			
FA H 4	None	HBT	4.5				0.35										
T H 4	*Trametes versicolor*	HBT	4.5	0.42	0.44	0.28							0.30	0.30	0.22		
P H 4	*UD4*	HBT	4.5	0.38	0.53	0.32					0.21		0.42	0.39	0.31		
FA H 5	None	HBT	5.5	0.38	**1.06**	0.35	0.23				0.25		0.35				
T H 5	*Trametes versicolor*	HBT	5.5	0.37	**1.36**	0.45				0.23	0.42		0.52	0.31	0.43		
P H 5	*UD4*	HBT	5.5	0.40	**1.67**	0.53				0.31	0.51		0.58	0.42	0.55		
FA A 4	None	ABTS	4.5	0.41	0.41		0.44	0.21									
T A 4	*Trametes versicolor*	ABTS	4.5	0.47	0.92	0.38	0.53				0.30		0.22				
P A 4	*UD4*	ABTS	4.5	0.42	**2.20**	0.83			0.20		0.84		0.36	0.29			
FA A 5	None	ABTS	5.5	0.38	**2.20**	0.73					0.79		0.22				
T A 5	*Trametes versicolor*	ABTS	5.5	0.50	**3.99**	1.25			0.28		**1.44**		0.61				
P A 5	*UD4*	ABTS	5.5	0.44	**5.18**	1.64			0.30		**1.74**		0.72	0.40	0.21		

6.4.4 Biosensors

4-chlorophenol produced an irreversible oxidation wave at + 0.8V vs Ag/AgCl. This oxidation wave is natively observed at a blank glassy carbon electrode. 4-chlorophenol produces a wave at a glassy carbon electrode, but this wave was significantly enhanced in the presence of a laccase-modified electrode, indicating its potential application in the sensitive detection of phenolic compounds.

Peniophora sp. UD4 laccase provided an enhanced sensitivity when compared to *Trametes versicolor* laccase for the non-mediated detection of catechol and 4-chlorophenol except in the case of covalent modification of the laccase. For mediated systems, the physical adsorption of *Peniophora* sp. UD4 laccase to a GCE proved to be the most sensitive electrode modification. In contrast to non-mediated systems, covalent binding of *Peniophora* sp. UD4 laccase outperformed *Trametes versicolor* laccase providing the most stable and sensitive GCE modified electrode for the detection of 4-chlorophenol.

6.5 Discussion

6.5.1 Defouling of Membranes

Laccase from *Peniophora* sp. UD4 was shown to effective in defouling ultrafiltration membranes during brown river water purification. The best results were obtained with immobilized laccase, which showed rapid membrane defouling after flushing with oxygenated water, and a marked flux recovery was achieved. The use of enzymes for defouling of ultrafiltration membranes is highly advantageous over current defouling technologies that include potentially hazardous and expensive cleaning reagents. Ultrafiltration membranes with immobilized laccase could be used continuously for ten days before becoming blocked by suspended solids or enzyme washout occurred. The life expectancy of the ultrafiltration membranes with immobilized laccase may be improved by a pre-filtration step to remove suspended solids and/or a more permanent method of immobilization such as gluteraldehyde cross-linking.

6.5.2 Bioremediation of Industrial Waste Streams

Bleach plant effluent (BPE) treatment with the laccase from *Peniophora* sp. UD4 showed the highest reduction in phenolic load (66 %), but promising results were

also obtained for wine distillery influent (WDI) and effluent (WDE) (45 and 36 % respectively), indicating the potential for enzymatic oxidation of phenols present in these effluent streams. The percentage reduction in the phenolic load of BPE indicates the presence of easily oxidizable laccase substrates, which could perhaps act as mediators for the oxidation of other phenolic compounds in other waste streams. This prompted the experimental determination of a potential increase in the phenolic load reduction in other effluents by the addition of BPE. Promising results for the enhanced oxidation of phenolic compounds in SGL were obtained by a measured reduction in phenolic load from 11 % with laccase, to 22 % with laccase and BPE.

Two enzyme concentrations were used to determine the quantity of enzyme required for the treatment to take place in reasonable time (16 hours). The enzyme requirement for WDI, WDE and BPE was relatively low, probably due to the presence of potential mediators. The enzyme requirement for the limited reduction of phenolic compounds in the sasol effluent (SE) and strip gas liquor was relatively high, therefore limiting the application of laccase to these effluents without the addition of mediators. The problematic phenolic compounds in these effluents may require a higher redox potential for their oxidation, which may be achieved by the addition of mediators that exhibit two oxidation states (e.g. ABTS), however this technology is limited due to the current cost of these mediators. Due to this limitation, laccase mediated systems were not investigated for their potential in the detoxification of waste streams.

Laccase oxidizing ability was also tested against several single phenolic compounds. An 85 % reduction in the cresol content after treatment with laccase from *Peniophora* sp. UD4 was observed after 16 hours under mild reaction conditions (24°C). At 60°C a high reduction in cresol is observed (42 %) in the control reaction without laccase, and since a closed system was used for treatment a natural heat induced oxidation of cresol may account for this loss. The oxidation of tannic acid (66 % reduction achieved) is also promising. No heat

attributable loss in tannic acid was observed at 60°C. Phenol reduction was also observed in the presence of laccase, but to a lesser extent than the other two compounds tested. A heat-associated reduction of phenol was also observed (39 %). A further reduction of 19 % of the phenol content of the reaction was achieved with laccase in this study, indicating this recognized laccase substrate has a low oxidation rate at the enzyme concentrations used. Rates of reaction for tannic acid, cresol and phenol were 164, 135 and 57 $mg.Units^{-1}.hour^{-1}$ respectively, calculated for the samples at room temperature containing 0.05 Units enzyme for the total time interval of 16 hours.

6.5.3 Organic Synthesis through Biocatalysis

6.5.3.1 (±)-α-Pinene Oxidation

Mediators were selected for their stability as redox intermediates and efficiency of mediation towards non-phenolic secondary substrates. Another important consideration was the different pathways for the oxidation of secondary substrates, namely hydrogen atom transfer (HAT) in the case of HBA HBT and HPI, electron transfer (ET) in the case of ABTS, and the as yet unidentified mode of action in the case of TEMPO. The mediators also vary in their redox potentials required for direct laccase oxidation and therefore oxidation potentials for the catalysis of secondary substrates. Due to the ease of oxidation of ferulic acid, only three mediators were selected that displayed the three different methods of oxidation, ABTS (ET), HBT (HAT) and TEMPO (unknown mechanism).

This experiment was performed to determine the oxidative potential of laccase towards (±)-α-pinene, and no optimization has yet been performed. The results do however indicate that the oxidation of (±)-α-pinene is feasible using laccase from *Peniophora* sp. UD4 in the presence and absence of mediators. The most promising results were achieved using laccase from *Peniophora* sp. UD4 in the presence of ABTS or HBT as a mediator, which yielded hydrophilic products.

These reaction mixtures were subjected to GC-MS-EI for the identification of reaction products. Two oxidative products were identified from these samples, namely verbenol and trans-sorberol.

6.5.3.2 Ferulic Acid Oxidation

The oxidation of ferulic acid by the thermostable laccase and laccase from *T. versicolor* at various pH values yielded several reaction products, many of which were identified by HPLC using relevant standards, namely vanillin, ethyl ferulate and vinyl guaiacol in varying concentrations which were dependant on the source of laccase, pH and mediator present. The most promising results were obtained using laccase from *Peniophora* sp. UD4. Enhancement of product formation for laccase from *Peniophora* sp. UD4 may be attributed to enhanced stability in the presence of mediators that have been shown to inactivate laccase through indirect oxidation of functional amino acids in the enzyme.

6.5.4 Biosensors

The biosensor developed for measuring the concentration of 4-chlorophenol in a liquid was successful, yielding a measurable charge on the electrode. The biosensor was however not re-usable, probably due to the fouling of the electrode due to the secondary polymerizing activity of the laccase or the relatively slow turnover of 4-chlorophenol as a substrate. A better strategy for modifying the electrode such as an immobilization matrix is required to overcome the effects of fouling. Alternatively, this simple protocol could be effective for use in disposable electrodes for the detection of phenolic components of a liquid. The measurable change in charge at the laccase-modified electrode shows the potential for using the thermostable laccase for biosensor applications.

Laccase from *Peniophora* sp. UD4 outperformed laccase from *Trametes versicolor* in almost every instance in mediated and non-mediated systems for

the detection of 4-chlorophenol. The best GCE modified electrode in this study for the detection of 4-chlorophenol was obtained with covalent binding of *Peniophora* sp. UD4 laccase in a ferrocyanide mediated system, which provided the best combination of sensitivity and stability.

6.6 Conclusions

6.6.1 Defouling of Membranes

Initial enzyme defouling using the thermostable laccase also provided promising results with large flux recoveries being achieved without optimization and the use of relatively low concentrations of laccase. The membranes could be run for ten days before membrane blockage by suspended solids, or enzyme washout occurred. Further research is required to enhance the longevity of the membranes during brown water filtration, such as improved immobilization techniques, flow rate optimization and pre-filtering to remove large suspended solids.

6.6.2 Bioremediation of Industrial Waste Streams

Effluent that exhibited the highest reduction in phenolic load was bleach plant effluent, with wine distillery influent and effluent also exhibiting significant reduction in the phenolic load. However, the use of a laccase mediated system for the oxidation of phenolic compounds in these problematic effluents may provide more promising results, but owing to the expense and/or toxicity of currently available mediators this technology is not currently feasible. These results reinforce the need to screen for alternative mediators that can be used for this application.

6.6.3 Organic Synthesis through Biocatalysis

The thermostable laccase from *Peniophora* sp. UD4 performed significantly better in organic synthesis applications than commercially available laccase from *Trametes versicolor*. The significantly higher oxidation rates achieved for ferulic acid with laccase from *Peniophora* sp. UD4 over laccase from *Trametes versicolor* in almost all of the products detected may be directly attributable to enhanced stability of the laccase in the presence of mediators, or the enhanced catalytic efficiency of the thermostable laccase over other sources of the enzyme (Chapter 3). The relatively clean production of verbenol and trans-sorberol during (±)-α-pinene oxidation warrants further research since these compounds are of significant value.

6.6.4 Biosensors

The application of the novel laccase from *Peniophora* sp. UD4 to biosensors indicated that the laccase could generate voltage at an electrode, demonstrating its potential for use in this developing technology. Further research performed at Rhodes University to compare the thermostable laccase against *Trametes versicolor* laccase for the sensitive detection of 4-chlorophenol showed that laccase from *Peniophora* sp. UD4 was superior in most instances. The use of *Peniophora* sp. UD4 laccase also provided the most promising biosensor with covalent binding of the laccase to the GCE providing the best combination of stability and sensitivity. Further research in biosensor technology is currently underway to compare various other immobilization strategies for electrode modification and their effects on sensitivity and stability of electrodes.

6.6.5 General

The laccase applications tested during this research indicated the range of applications for the thermostable laccase is broad, and where applicable benchmarks significantly better than laccase from *Trametes versicolor.* These successes bode well for the application of the thermostable laccase from *Peniophora* sp. UD4 to current and developing technologies that require alternative sources of the enzyme exhibiting superior characteristics.

Chapter 7
General Conclusions

7.1 Introduction

At the beginning of the study the market opportunity for laccases with enhanced thermostability and increased catalytic efficiency was identified. A laccase with these properties would provide several benefits to the current technologies that incorporate laccase's catalytic properties (including its broad substrate range, use of air oxygen as the second substrate and the secondary activities exhibited by laccase substrates). Given South Africa's understudied biodiversity, the potential for the isolation of stable laccases was identified. No previous work on the bioprospecting of South African fungi for ligninolytic enzymes with enhanced properties has previously been undertaken and published.

A thermostable laccase would have several benefits, such as a prolonged shelf life of products containing the enzyme. It is likely that the thermostable laccase would also be more resistant to mechanical agitation, which would enhance its applicability in the use of bioreactors for organic synthesis or other applications requiring mechanical stability.

7.2 Screening for a Thermostable Laccase

This work has shown that bioprospecting of mesophilic fungi led to the discovery of a thermostable laccase from a *Peniophora* species. It is surprising that a wild isolate from a temperate environment would require the production of a thermostable laccase. The enzyme was only produced in small quantities, even after investigation into possible nutrient variations for increased production.

Therefore it is possible that the thermostability is a convenient alternative to production of the enzyme in large quantities, since the laccase may last longer in the environment, and thereby improve delignification and cellulose degradation, while limiting the amount of energy expended on enzyme production, enabling more energy to be directed to other metabolic activities.

7.3 Characterization of the Thermostable Laccase

The improved catalytic properties of the thermostable laccase, such as the high affinity of the laccase for its substrate and relatively high turnover numbers, especially for the oxidation of ABTS (Jordaan *et al.*, 2004), indicate that this enzyme may prove to be highly competitive for industrial application. This improved catalytic efficiency for ABTS as a substrate is a desired property in industrial laccases since ABTS is considered the most effective mediator for redox oxidations by laccase.

The specificity constant (k_{cat}/K_m), which takes into account both the specificity of the laccase for its substrate (K_m) as well as the catalytic efficiency (k_{cat}), is similar to the specificity constant of “white” laccase from *Pleurotus ostreatus* (Palmieri *et al.*, 1997). However, the two enzymes have different properties as *Pleurotus ostreatus* laccase (POXA1) has a high rate of conversion for its substrates, but a low affinity for them, whereas the laccase from *Peniophora* sp. UD4 has a high affinity for its substrates but a relatively low turnover number (compared with *P. ostreatus*, but higher than other fungal laccases). POXA1, the only similar laccase in terms of kinetic attributes does not possess the thermostability or substrate range of the laccase from *Peniophora* sp. UD4 (Jordaan *et al*., 2004). Owing to these improved catalytic properties, the broad pH range for the oxidation of ABTS, thermostability and flat optimum temperature curve (Jordaan and Leukes, 2003), the production of this thermostable laccase in sufficient yields may provide a valuable alternative enzyme source for industrial applications.

The ability of laccase to oxidize the manganese peroxidase specific oxidative coupling of DMAB and MBTH (Jordaan and Leukes, 2003) may be of considerable importance to applications of the thermostable laccase. The broader than usual substrate range, and the ability to oxidize substrates with a higher oxidative potential indicates the improved applicability in the treatment of phenol containing wastes, since phenols present in effluents that are not recognized by other laccases may be oxidized and in turn act as mediators for improved laccase activity.

Sequence information has been obtained from one of three isozymes produced by *Peniophora* sp. UD4. Optimization of derivitization methods is currently underway to provide further sequence information by MALDI-TOF MS. HPLC sequencing of N-terminal residues is also currently underway for all three isozymes.

7.4 Identification of UD4

UD4, the tentative name for the organism producing the thermostable laccase, was not identifiable from its morphological characteristics. Phylogenetic analysis indicates that UD4 groups closely with *Peniophora* species within the Lachnocladiaceae clade with strong statistical support (bootstrap analysis). However, this is not supported by morphological characteristics since UD4 has an effused-reflexed basidiocarp and all other fungi within this clade are characteristically effused. UD4 is the first species exhibiting both an effused-reflexed basidiocarp and strong phylogenetic relationship to *Peniophora* and *Entomocorticium* spp., and the ITS1 and ITS2 sequences are the first molecular data obtained for this novel species from Southern Africa.

While constructing UD4's phylogenetic tree it became apparent that the classification of *Amanita tenuifolia* should be reviewed (on the assumption that the culture held by ATCC is *Amanita tenuifolia*). The close molecular relationship between *Peniophora* species and *Entomocorticium* species requires further investigation since these species may be interchangeable, and may exhibit two different habitats, free or in association with the gut of insects. This work provides further evidence for the need to develop a new taxonomical approach involving the use of molecular data as a primary approach, followed by analysis of morphological divergence for further classification.

This work shows that UD4 is a morphologically advanced species that should be classed within the Lachnocladiaceae based on molecular similarity of the ITS1 and ITS2 sequences. Due to the close relationship of *Entomocorticium* and *Peniophora* spp. as illustrated in this work, including the nesting of several *Peniophora* species within the *Entomocorticium* clade, these two genera may be interrelated and exhibit two specific habitats. This relationship has to be validated but is not considered to be in the scope of this work.

Ancestral state reconstruction revealed that the two morphologically advanced species, UD4 and *Amanita tenuifolia* (ATCC 26764), are derived from the same ancestor, indicating the possible presence of an ancestor that that may recently have undergone hyper-evolution in response to an environmental stress. The *Amanita teunuifolia* strain held by the ATCC has to be taxonomically validated before a definite conclusion can be drawn.

Based on habitat and molecular data UD4 should likely be classed as a *Peniophora* species. This identification of UD4 remains debatable, due mainly to its morphological diversity. The conclusion of this work however is the novelty of the basidiomycete known as UD4 based on its unique ITS1 and ITS2 sequences and morphological diversity from organisms with similar sequence data.

7.5 Growth Conditions

Peniophora sp. UD4 is a mesophilic basidiomycete that exhibits optimum growth kinetics at approximately 25°C. Laccase and MnP production is limited in agitated liquid culture, but agitated solid substrate culture conditions yielded significant quantities of laccase and MnP. The nature of the nitrogen source differentiates between the production of laccase and MnP. Organic nitrogen sources, such as peptone and yeast extract, improve the production of manganese peroxidase significantly in comparison to the use of inorganic nitrogen sources such as ammonium tartrate and urea. Laccase production by *Peniophora* sp. UD4 is increased under carbon limiting conditions, indicating the production of laccase is enhanced by carbon depletion.

The individual vitamins pyridoxine, thiamine, folic acid and aminobenzoic acid elicited positive responses on laccase production while simultaneously suppressing the production of MnP. Complex mixtures of vitamins had the opposite effect by enhancing the production of MnP while suppressing the production of laccase, with the exception of riboflavin. Removing riboflavin from the complex mixture of vitamins suppressed the production of laccase and MnP. The ligninolytic system of *Peniophora* sp. UD4 is composed primarily of laccase and MnP. LiP was not detected in the diverse range of culture media used for the cultivation of *Peniophora* sp. UD4, indicating the high probability that *Peniophora* sp. UD4 is LiP deficient. This characteristic has been noted in other fungi (see chapter 5).

Laccase yields attained in this work are currently unsatisfactory to apply wild-type organism production to a large-scale production apparatus. The alternative production strategy investigated, involving the use of distillery effluent as a nutrient source, did stimulate the production of ligninolytic enzymes from *Peniophora* sp. UD4, but not to an extent that may be considered commercially feasible. Production on this effluent does however indicate the feasibility of using

wine distillery effluent as a potential medium source for the production of ligninolytic enzymes from other source organisms.

7.6 Applications

The application for the bioremediation of wine distillery influent and effluent, and bleach plant effluent (BPE) using laccase from *Peniophora* sp. UD4 demonstrated significant potential. BPE may be considered the most favourable effluent for enzymatic treatment using *Peniophora* sp. UD4 laccase, since the phenolic load could be reduced by 66 % in batch reactions. The oxidation of BPE phenolics was best achieved at a high temperature (60°C) using a relatively low concentration of enzyme. Addition of BPE to strip gas liquor (SGL) improved the phenolic reduction by 11 % for SGL without any optimization, indicating the potential for a mixed effluent treatment scheme for these effluents.

The application of the thermostable laccase to organic synthesis was successfully demonstrated by the oxidation of ferulic acid and (±)-α-pinene. The efficiency of oxidation was greater with the laccase from *Peniophora* sp. UD4 than the commercial laccase from *Trametes versicolor*.

The applications demonstrated in this thesis show a large potential application base for the thermostable laccase that may benefit from the enhanced properties of the laccase from *Peniophora* sp. UD4. Enhancement of product formation during organic synthesis over the commercially available laccase from *Trametes versicolor* is testament to the potential commercial value of the thermostable laccase.

The results from the immobilization of the thermostable laccase used for defouling of ultrafiltration membranes investigated showed promise for the realization of this technology. Thermostability of an enzyme is usually associated

with an increased degree of mechanical stability (Pilz *et al*., 2003). Therefore, this laccase could be a valuable resource for the defouling of membranes due to the large mechanical and shear forces experienced during ultrafiltration. The resultant use of air oxygen as a cleaning agent is also advantageous since it is relatively cleaner and less expensive than current cleaning reagents.

The thermostable laccase from *Peniophora* sp. UD4 was successfully used in a biosensor application for the detection of 4-chlorophenol. Promising results for the detection of 4-chlorophenol were obtained with *Peniophora* sp. UD4 laccase modified glassy carbon electrodes, with covalent binding of the laccase in a ferrocyanide mediated system providing the best combination of sensitivity and selectivity. Laccase from *Peniophora* sp. UD4 also benchmarked significantly better than laccase from *Trametes* versicolor with respect to sensitivity in almost every immobilization strategy in mediated and non-mediated systems, with the exception of covalent binding in a non-mediated system. This study provides promising results towards the development of effective laccase-based biosensors through the use of *Peniophora* sp. UD4 laccase.

7.7 Future Work

The taxonomical implication of molecular phylogeny is currently undergoing a revolution, and many arguments pertaining to other methods such as morphological and biochemical techniques for identification are currently being investigated. The method best suited to organisms, with the least bias, is the sequencing and phylogenetic analysis of the ITS region of fungi. The delimitation of species based on molecular data is currently a problem and standards are currently under investigation, which disallows the definition of what constitutes a specific genus, species or strain within an organism class. This is likely to change within the next couple of years as the database of rDNA sequences evolves and

these definitions are defined. The exact identification of the species will therefore be available once a clear delimitation and standard identification protocol have been established.

Molecular characterization and quantitative structure-activity relationship (QSAR) of the gene and the protein may aid in determining the factors involved in enhanced thermostability of laccases, as well as that of enhanced substrate binding as demonstrated by the K_m values obtained through characterization. This could be useful in guiding genetic improvements of laccases.

Due to the inefficiency of the wild-type organism (*Peniophora* sp. UD4) production of the thermostable laccase alternative production strategies have to be investigated. Production strategies, such as strain improvement and heterologous expression, will have to be investigated to provide larger yields of this thermostable laccase for industrial applications. To this end, laccase gene specific sequences are currently being established by MALDI-TOF MS and N-terminus HPLC sequencing.

The novelty of the organism and the isolation of the thermostable laccase as a novel product (the thermostable laccase) warrant further investigation into other potentially useful metabolites that may be produced from *Peniophora* sp. UD4. The characterization of other industrially important enzymes is a possible area of interest, including phytase (Lassen *et al.,* 2001), and compounds with antibiotic properties, known as peniophorins, that have previously been identified from *Peniophora* species (Gerber *et al.,* 1980).

7.8 Conclusion

In conclusion, this thesis demonstrates a high probability for the isolation of novel natural products from the untapped biodiversity of South Africa. Much research is concentrated on the isolation of thermostable enzymes from thermophilic organisms, and although the probability may be higher when focusing on thermophiles, other sources of valuable thermostable enzymes are available from mesophilic organisms. The novelty of the laccase from *Peniophora* sp. UD4 is not limited to its high thermostability, but includes a broader than usual substrate range and relatively high catalytic efficiency compared to other sources of the enzyme. These properties all contribute to the increased applicability of laccases in industry, potentially making this novel laccase the most industrially feasible enzyme of its class isolated to date.

Appendices

Appendix 1: Tien and Kirk Medium Composition

Appendix 2A: Alignment of 18s rDNA ITS1 sequences

Appendix 2B: Alignment of 18s rDNA ITS2 sequences

Appendix 1: Tien and Kirk Medium Composition

Tien and Kirk Medium

Basal III (per liter)

To 700 ml of Water add

KH2PO4	20 g
MgSO4	5 g
CaCl2	1 g
Trace Element Soln.	100 ml

Make up to 1 L

Trace Elements (per liter)

Dissolve 1.5 g Nitrilotriacetate in 700 ml water, adjust to pH 6.5 with KOH

Add in order:

MgSO4	3 g
MnSO4	0.5 g
NaCl	1.0 g
FeSO4·7H2O	0.1 g
CoCl2	0.1 g
ZnSO4·7H2O	0.1 g
CuSO4	0.1 g
AlK(SO4)2·12H2O	0.01 g
H3BO3	0.01 g
Na2MoO4	0.01 g

Make up to 1 L

Medium Composition (per liter)

Glucose (10%)	10 ml	(autoclaved separately)
Ammonium Tartrate	5 g	
Basal III	100 ml	
Trace Elements	60 ml	
Thiamin (0.1 g/L)	10 ml	
Dimethylsuccinate Buffer (pH 4.5)	100 ml	(100 mM stock)

Make up to 980 ml, add glucose when cool and filter sterilize the 10 ml Thiamin.

Appendix 2A: Alignment of 18s rDNA ITS1 sequences

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CLUSTAL X (1.81) multiple sequence alignment


Entomocorticium_sp.E_AF119505       TCCGTAGGTGAACCTGCGGAAGGATCATTTGCGAA-------GTTCGG--
Entomocorticium_sp.D_AF119503       TCCGTAGGTGAACCTGCGGAAGGATCATTTGCGAA-------GTTCGG--
Entomocorticium_sp.C_AF119510       TCCGTAGGTGAACCTGCGGAAGGATCATTTGCGAA-------GTTCGG--
Entomocorticium_sp.B_AF119508       TCCGTAGGTGAACCTGCGGAAGGATCATTTGCGAA-------GTTTGG--
Entomocorticium_sp.H_AF119512       TCCGTAGGTGAACCTGCGGAAGGATCATTTGCGAA-------GTCTGG--
Entomocorticium_sp.G_AF119512       TCCGTAGGTGAACCTGCGGAAGGATCATTTGCGAA-------GTTCGG--
Peniophora_rufa_AF119516            TCCGTAGGTGAACCTGCGGAAGGATCATTTGCGAA-------GTTCGG--
Peniophora_pithya_AF119521          TCCGTAGGTGAACCTGCGGAAGGATCATTTGCGAA-------GCTCGG--
Peniophora_piceae_AF119515          TCCGTAGGTGAACCTGCGGAAGGATCATTTGCGAA-------GTACGG--
Peniophora_pseudo-pini_AF119514     TCCGTAGGTGAACCTGCGGAAGGATCATTTGCGAA-------GTCCGG--
Peniophora_aurantiaca_AF119517      TCCGTAGGTGAACCTGCGGAAGGATCATTTGCGAA-------GCTCGG--
Peniophora_aurantiaca_AF210829      CCCGTAGGGGAACC-GCGG-AG--TC-TT---GAA-------G-TCGG--
Peniophora_aurantiaca_AF210824      TCCGTAGGTGAACCTGCGGAAGGATCATTTGCGAA-------GCCCGG--
Peniophora_aurantiaca_AF210820      TCCGTAGGTGAACCTGCGGAAGGATCATTAGCGAA-------GCTCGG--
Peniophora_aurantiaca_AF210821      TCCGTAGGTGAACCTGCGGAAGGATCATTAGCGAA-------GCTCGG--
Peniophora_aurantiaca_AF210822      TCCGTAGGTGAACCTGCGGAAGGATCATTAGCGAA-------GCTCGG--
Peniophora_aurantiaca_AF210825      TCCGTAGGTGAACCTGCGGAAGGATCATTAGCGAA-------GCTCGG--
Peniophora_aurantiaca_AF210827      TCCGTAGGTGAACCTGCGGAAGGATCATTAGCGAC-------GCTCGG--
Peniophora_aurantiaca_AF210828      TCCGTAGGTGAACCTGCGGAAGGATCATTAGCGAA-------GCTCGG--
Peniophora_aurantiaca_AF210823      TCCGTAGGTGAACCTGCGGAAGGA-CATT-GCGAA-------GCTCGG--
Peniophora_duplex_AF119519          TCCGTAGGTGAACCTGCGGAAGGATCATTAGCGAA-------GCTCGG--
Peniophora_cinerea_AF119518         TCCGTAGGTGAACCTGCGGAAGGATCATTAGCGAA-------GCTCGG--
Peniophora_nuda_AF119513            TCCGTAGGTGAACCTGCGGAAGGATCATTAGCGAA-------GCTCGG--
Peniophora_aurantiaca_AF210819      TCCGTAGGTGAACCTGCGGAAGGATCATTAGCGAA-------GCTCGG--
P_aurantiaca_CBS_396.50             TCCGTAGGTGAACCTGCGGAAGGATCATTAGCGAA-------GCTCGG--
UD4                                 TCCGTAGGTGAACCTGCGGAAGGATCATTAGCGAA-------GCTCGG--
Amanita_tenuifolia_AF085492         TCCGTAGGTGAACCTGCGGAAGGATCATTAGCGAA-------G--CGG--
Entomocorticium_spF_AF119507        TCCGTAGGTGAACCTGCGGAAGGATCATTAGCGAA-------GTTCGG--
Peniophora_pithya_AF119520          TCCGTAGGTGAACCTGCGGAAGGATCATTAGCGAA-------GTTCGG--
E._dendroctoni_AF119506             TCCGTAGGTGAACCTGCGGAAGGATCATTAGCGAA-------GTTCGG--
Entomocorticium_spA_AF119509        TCCGTAGGTGAACCTGCGGAAGGATCATTAGCGAA-------GTCTGG--
Dendrophora_albobadia_AF119522      TCCGTAGGTGAACCTGCGGAAGGATCATTAGCGAA-------GTTCGG--
An_citrinella_AF126879              TCCGTAGGTGAACCTGCGGAAGGATCATTAATGAATGA----ACTTGG--
Antrodiella_pallasii_AF126895       TCCGTAGGTGAACCTGCGGAAGGATCATTAATGAATGA----ACTTGG--
Antrodiella_faginea_AF126886        TCCGTAGGTGAACCTGCGGAAGGATCATTAATGAATGA----ACTTGG--
Antrodiella_romellii_AF126902       TCCGTAGGTGAACCTGCGGAAGGATCATTAATGAATGA----ACTTGG--
An_parasitica_AF126898              TCCGTAGGTGAACCTGCGGAAGGATCATTAATGAATGA----ACTTGG--
Antrodiella_faginea_AF126884        TCCGTAGGTGAACCTGCGGAAGGATCATTAATGATTNA----ACTTGG--
An_onychoides_AJ006674              TCCGTAGGTGAACCTGCGGAAGGATCATTAATGAATGA----ACTTGG--
An_semisupina_AF126905              TCCGTAGGTGAACCTGCGGAAGGATCATTAATGAATGA----ACTTGG--
An_beschidica_AF126878              TCCGTAGGTGAACCTGCGGAAGGATCATTAATGAATGA----ACTTGG--
Antrodiella_hoehnelii_AF126893      TCCGTAGGTGAACCTGCGGAAGGATCATTAATGAATGA----ACTTGG--
Antrodiella_americana_AF126877      TCCGTAGGTGAACCTGCGGAAGGATCATTAATGAATGA----ATTTGG--
Amanita_spissa_AF085486             TCCGTAGGTGAACCTGCGGAAGGATCATTACTGAACGA-----AATGG--
Amanita_aspera_AF085485             TCCGTAGGTGAACCTGCGGAAGGATCATTACTGAACGA-----AATGG--
Amanita_rubescens_AF085484          TCCGTAGGTGAACCTGCGGAAGGATCATTACTGAACGA-----AATGG--
Amanita_citrina_AF085489            TCCGTAGGTGAACCTGCGGAAGGATCATTACTGAAGAAC----AATGG--
Amanita_virosa_AB015676             TCCGTAGGTGAACCTGCGGAAGGATCATTACTGAAA-----TGAACCTT-
Amanita_phalloides_AJ308097         TCCGTAGGTGAACCTGCGGAAGGATCATTAATGAAA-----TGAACCTT-
Amanita_fulva_AF085494              TCCGTAGGTGAACCTGCGGAAGGATCATTATCGAGTTT--TGAAC-----
Au_alborubescens_AJ006683           TCCGTAGGTGAACCTGCGGAAGGATCATTAACGAGTTT--TGAAC-----
Trichaptum_abietinum_U63474         TCCGTAGGTGAACCTGCGGAAGGATCATTATCGAGTTT--TGAAC-----
Bjerkandera_adjusta_AF455410        TCCGTCGGTGAACCTGCGGA-GGATCATTATCGAGTTT--TGAAC-----
Bjerkandera_adusta_AJ006672         TCCGTAGGTGAACCTGCGGAAGGATCATTATCGAGTTT--TGAAC-----
Bjerkandera_fumosa_AJ006673         TCCGTAGGTGAACCTGCGGAAGGATCATTATCGAGTTT--TGAAC-----
Polyporus_brumalis_AB070876         TCCGTAGGTGAACCTGCGGAAGGA-CATTATCGAGHTC--TGAAA-----
Skeleto_kuehneri_AJ006678           TCCGTAGGTGAACCTGCGGAAGGATCATTATCGAGTTT--TTGAA-----
Gloeo_abietinum_AJ420948            TCCGTAGGTGAACCTGCGGAAGGATCATTAACGAGTAAA-CGAGGGA---
Gloeo_abietinum_AJ420947            TCCGTAGGTGAACCTGCGGAAGGATCATTAACGAGTAAA-CAGGGGA---
Gloeophyllum_trabeum_AJ420950       TCCGTAGGTGAACCTGCGGAAGGATCATTAACGAGTAAA-CAGGAGA---
Gloeophyllum_trabeum_AJ420949       TCCGTAGGTGAACCTGCGGAAGGATCATTATCGAGTATTTTGAAAGAG--
Gloeo_sepiarium_AJ420946            TCCGTAGGTGAACCTGCGGAAGGATCATTATCGAGTCT-TTGAAAGAA--
Skeletocutis_nivea_AJ006679         TCCGTAGGTGAACCTGCGGAAGGA-CATTATCGAGTTT--TGAAATGG--
Ganoderma_adspersum_AJ006685        TCCGTAGGTGAACCTGCGGAAGGATCATTATCGAGTTT--TG-ACTGG--
Donkioporia_expansa_AJ249500        TCCGTAGGTGAACCTGCGGAAGGATCATTATCGAGTTT--TGAAAGGG--
Trametes_trogii_AJ438139            TCCGTAGGTGAACCTGCGGAAGGATCATTATCGAGTTT--TGAAATGG--
Phlebia_subserialis_AB084620        TCCGTAGGTGAACCTGCGGAAGGATCATTAACGAGTTTT--GAAATGG--
Phlebia_uda_AB084621                TCCGTAGGTGAACCTGCGGAAGGA-CATTAACGAGTTTTT-GAAAGGG--
Phlebia_radiata_AB084619            TCCGTAGGTGAACCTGCGGAAGGATCATTATCGAGTTTT--GAAACGG--
Polyporus_arcularius_AB070865       TCCGTAGGTGAACCTGCGGAAGGA-CATTATCGAGHTC--TGAAACGG--
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Polyporus_ciliatus_AB070883         TCCGTAGGTGAACCTGCGGAAGGA-CATTATCGAGATC--TGAAACGG--
Polyporus_tricholoma_AJ132942       TCCGTAGGTGAACCTGCGGAAGGA-CATTATCGAGHTC--TGAAACGG--
Polyporus_tricholoma_AB070885       TCCGTAGGTGAACCTGCGGAAGGA-CATTATCGAGHTC--TGAAACGG--
Trichaptum_biforme_U63476           TCCGTAGGTGAACCTGCGGAAGGA-CATTATCGAGHTT--TGAAATAT--
Postia_balsamea_AJ006666            TCCGTAGGTGAACCTGCGGAAGGATCATTATTGAATTT-TTGAAGGAG--
Postia_sericeomollis_AJ006667       TCCGTAGGTGAACCTGCGGAAGGATCATTATTGAATCTTTTGAAGGAG--
Postia_hibernica_AJ006665           TCCGTAGGTGAACCTGCGGAAGGATCATTATTGAATCT-TTGAAGGAG--
Postia_lactea_AJ006664              TCCGTAGGTGAACCTGCGGAAGGATCATTACTGAATTT-TTGAAGGAG--
Di_lindbladii_AJ006682              TCCGTAGGTGAACCTGCGGAAGGATCATTATCGAATTT---GAAA-GG--
Oligoporus_placentus_AJ249267       TCCGTAGGTGAACCTGCGGAAGGATCATTAATGAATTT--TGAAAGGG--
Antrodia_xantha_AXA415569           TCCGTAGGTGAACCTGCGGAAGGATCATTAACGAATTT--TGAAAGGG--
Antrodia_serialis_AJ345010          TCCGTAGGTGAACCTGCGGAAGGATCATTAAAGAATTT--TGAAAGGG--
Antrodia_albida_AJ006680            TCCGTAGGTGAACCTGCGGAAGGATCATTATTGAATTT--TGAATGGG--
Antrodia_sinuosa_AJ416068           TCCGTAGGTGAACCTGCGGAAGGATCATTAATGAATTT--TGAAAGGG--
Fomitopsis_rosea_AJ415546           TCCGTAGGTGAACCTGCGGAAGGATCATTAATGAATTT--TGAAAGGG--
Fomitopsis_pinicola_AJ560638        TCCGTAGGTGAACCTGCGGAAGGATCATTAATGAATTC--TGAAAGGG--
Polyporus_arcularius_AB070868       TCCGTAGGTGAACCTGCGGAAGGA-CATTATCGAGATC--HGAAACGG--
Polyporus_ciliatus_AB070881         TCCGTAGGTGAACCTGCGGAAGGA-CATTATCGAGATC--HGACAHGG--
Aspergillus_niger_AF455522          TCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGC-------GGGTC
Aspergillus_niger_AJ280006          TCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGC-------GGGTC
Penicillium_commune_AF455544        TCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGA-------GGGCC
P_chrysogenum_AF034451              TCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGA-------GGGCC
P_roseopurpureum_AF455492           TCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGA-------GGGCC
                                     **** ** ***** ****  *   * **   **

Entomocorticium_sp.E_AF119505       -AATGC--GTGTT-CGGTACTG----ATGCTGCCCGG----CA----ACG
Entomocorticium_sp.D_AF119503       -AATGC--GTGTT-CGGTACTG----ATGCTGCCCGG----CA----ACG
Entomocorticium_sp.C_AF119510       -AATGC--GTGTC-CGGTACTG----ATGCTGCCCGG----CA----ACG
Entomocorticium_sp.B_AF119508       -AATGC--GTGTT-CGGTACTG----ATGCTGCCCGG----CA----ACG
Entomocorticium_sp.H_AF119512       -AATGC--GTGTT-CGGTATTG----ATGCTGCCCGG----CA----ACG
Entomocorticium_sp.G_AF119512       -AATGC--GCGTT-CGGTACTG----ATGCTGCC-GG----CA----ACG
Peniophora_rufa_AF119516            -AACTC--GCTTT-CGGTGCTG----ATGCTGCCCGG----CA----ACG
Peniophora_pithya_AF119521          -AACGTC-GCACT-CGGTACTG----ATGCTGCCCGG----CA----ACG
Peniophora_piceae_AF119515          -AACGC--GTGTG-AGG-ACTG----A-GCTGCCCAG----CG----ATG
Peniophora_pseudo-pini_AF119514     -TACGC--GCGTT-TGACACTG----A-GCTGCCCAG----CG----ATG
Peniophora_aurantiaca_AF119517      -AATGCT-GTGCTTCGGTGCTG----ATGCTGCCCAG----CA----ATG
Peniophora_aurantiaca_AF210829      -AATCC--GTGTT-CGGT-CTG----ATGCTGCCCAG----CA----ATG
Peniophora_aurantiaca_AF210824      -AATGC--GTGTT-CGGTACTG----ATGCTGCCCAG----CA----ATG
Peniophora_aurantiaca_AF210820      -AATGC--GTATT-CGGTACTG----ATGCTGCCCAG----CA----ATG
Peniophora_aurantiaca_AF210821      -AATGC--GTGTT-CGGTACTG----ATGCTGCCCAG----CA----ATG
Peniophora_aurantiaca_AF210822      -AATGC--GTGTT-CGGTACTG----ATGCTGCCCAG----CA----ATG
Peniophora_aurantiaca_AF210825      -AATGC--GTGTT-CGGT-CTG----ATGCTGCCCAG----CA----ATG
Peniophora_aurantiaca_AF210827      -AATGC--GTGTT-CGGT-CTG----ATGCTGCCCAG----CA----ATG
Peniophora_aurantiaca_AF210828      -AATGC--GCGTT-CGGT-CTG----ACGCTGCCCAG----CA----ATG
Peniophora_aurantiaca_AF210823      -AATGC--GTGTTGCGGTACTG----ATGCTGCCCAG----CA----ATG
Peniophora_duplex_AF119519          -AATGC--GTGTT-CGGTGCTG----ATGCTGCCCAG----CA----ATG
Peniophora_cinerea_AF119518         -AATGC--GTGTT-CGGT-CTG----ATGCTGCCCAG----CA----ATG
Peniophora_nuda_AF119513            -AATGC--GTGTT-CGGT-CTG----ATGCTGCCCAG----CA----ATG
Peniophora_aurantiaca_AF210819      -AATGC--GTGTT-CGGTACTG----ATGCTGCCCAG----CA----ATG
P_aurantiaca_CBS_396.50             -AATGC--GTGTT-CGGTACTG----ATGCTGCCCAG----CA----ATG
UD4                                 -AATGC--ATGTT-CGGTACTG----ATGCTGCCCAG----CA----ATG
Amanita_tenuifolia_AF085492         -AACGC--GCGTT-CGGTACTG----TAGCTGCCCAG----CA----ATG
Entomocorticium_spF_AF119507        -AATGC--GTGTT-CGGTACTG----ATGCTGCCCGG----CA----ACG
Peniophora_pithya_AF119520          -AATGC--GTGTT-CGGTACTG----ATGCTGCCCGG----CA----ACG
E._dendroctoni_AF119506             -AATGC--GTGTC-CGGTACTG----ATGCTGCCCGG----CA----ACG
Entomocorticium_spA_AF119509        -AATGC--GTGTT-CGGTATTG----ATGCTGCCCGG----CA----ACG
Dendrophora_albobadia_AF119522      -AATGC--ATGTT-CGATGTTG----ATGCTGCCCAG----CA----ATG
An_citrinella_AF126879              --GCAAA-GTTGTC----GCTG------GC--CTCAG----CA----ATG
Antrodiella_pallasii_AF126895       --GCAAA-GTTGTC----GCTG------GC--CTCAG----CA----ATG
Antrodiella_faginea_AF126886        --GCAAA-GTTGTC----GCTG------GC--CTCAG----CA----ATG
Antrodiella_romellii_AF126902       --GCAAA-GTTGTA----GCTG------GC--CTCAG----CA----ATG
An_parasitica_AF126898              --GCAAA-GTTGTC----GCTG------GC--CTCAG----CA----ATG
Antrodiella_faginea_AF126884        --GCAAA-GTTGTC----GCTG------GC--CTCAG----CA----ATG
An_onychoides_AJ006674              --GCAAA-GTTGTA----GCTG------GC--CTTAG----CA----ATG
An_semisupina_AF126905              --GCATA-GTTGTA----GCTG------GC--CTCAG----CA----ATG
An_beschidica_AF126878              --GCATA-GTTGTA----GCTG------GC--CTCAG----CA----ATG
Antrodiella_hoehnelii_AF126893      --GCGTG-GTTGTA----GCTG------GC--CTCAG----TG----ATG
Antrodiella_americana_AF126877      --ATGGA-GTTGTA----GCTG------GT--CTCAG----AA----ATG
Amanita_spissa_AF085486             --GTGGC-AA-------GGCTGT---A-GCTGGCT------CG----AAG
Amanita_aspera_AF085485             --GTGGC-AA-------GGCTGT---C-GCTGGCT------CA----AAT
Amanita_rubescens_AF085484          --GTGGC-AA-------GGCTGT---C-GCTGGCT------CT----AAT
Amanita_citrina_AF085489            --GTGGC-AA------AGGCTGT---T-GCTGGCT------TG----AGT
Amanita_virosa_AB015676             --GAGG-------------CTGT---T-GCTGGCC------CA----TCT
Amanita_phalloides_AJ308097         --GAGG-------------CTGT---C-GCTGGCCC-----CT----CTG
Amanita_fulva_AF085494              -GG--GTTGTA----------------GCTGGCCTC--TC------ACG
Au_alborubescens_AJ006683           AGG--GTTGTA----------------GGCTGGTTTTCTTAATG---GAG
Trichaptum_abietinum_U63474         -GG--GTTGT----------------CTGCTGGCT-----CG-----CAA
Bjerkandera_adjusta_AF455410        -GG--GTTGT----------------CTGCTGGCT-----CG-----CAA
Bjerkandera_adusta_AJ006672         -GG--GTTGT----------------CTGCTGGCT-----CG-----CAA
Bjerkandera_fumosa_AJ006673         -GG--GTTGT----------------CTGCTGGCTCG---C------GAA
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Polyporus_brumalis_AB070876         CGG--GHTGT-----------------AGCTGGCC----TT------CCG
Skeleto_kuehneri_AJ006678           -----GTGGGGG---------CTTTGATGCTGGCT----TT--------A
Gloeo_abietinum_AJ420948            ----G-TTGT-----------------AGCTGGC-----CG------C-A
Gloeo_abietinum_AJ420947            ----G-TTGT-----------------AGCTGGCCG---C---------A
Gloeophyllum_trabeum_AJ420950       ----G-TTGT-----------------AGCTGGCCG---C---------A
Gloeophyllum_trabeum_AJ420949       ----G-TTGT-----------------AGCTGGCCTTT-C---------G
Gloeo_sepiarium_AJ420946            ----G-TTGT-----------------AGCTGGCCTTT-C---------G
Skeletocutis_nivea_AJ006679         ----G-TTGT-----------------AGCTGGCCTT-CC---------G
Ganoderma_adspersum_AJ006685        ----G-TTGT-----------------AGCTGGCCTT-CC---------G
Donkioporia_expansa_AJ249500        ----G-TTGT-----------------TGCTGGCCTTTCC--------GG
Trametes_trogii_AJ438139            ----G-TTGT-----------------AGCTGGCTTCTCC--------GG
Phlebia_subserialis_AB084620        ----G-TTGT-----------------AGCTGGCCTTA---------CGA
Phlebia_uda_AB084621                ----G-TTGT-----------------AGCTGGCCAAAAA-------CAA
Phlebia_radiata_AB084619            ----G-TTGT-----------------AGCTGTCCTTC------GTAAGG
Polyporus_arcularius_AB070865       ----G-HTGH-----------------AGCTGGCCTTCC----------G
Polyporus_ciliatus_AB070883         ----G-HTGH-----------------AGCTGGCCTTCC----------G
Polyporus_tricholoma_AJ132942       ----G-HTGT-----------------AGCTGGCCTTCC----------G
Polyporus_tricholoma_AB070885       ----G-HTGT-----------------AGCTGGCCTTCC----------G
Trichaptum_biforme_U63476           ----G-GGGHT--------------GAAGCTGGCCTTAC----------G
Postia_balsamea_AJ006666            ------CTGTT----------------TGCTGGC-CTCTA-G-----C-G
Postia_sericeomollis_AJ006667       ------CTGTT----------------TGCTGGC-CCTTAAT-----CTT
Postia_hibernica_AJ006665           ------CTGTT----------------TGCTGGA-CTCTAA------GTG
Postia_lactea_AJ006664              ------CTGTT----------------TGCTGGCCCTGTC-------AAG
Di_lindbladii_AJ006682              ---GG-TTGT-----------------AGCTGGCCTTTCA--------CG
Oligoporus_placentus_AJ249267       ----G-TTGT-----------------AGCTGGCCTTTT--------AGG
Antrodia_xantha_AXA415569           ----G-TTGT-----------------AGCTGGCTTTTC--------ACG
Antrodia_serialis_AJ345010          ----G-TTGT-----------------AGCTGGC-------------C--
Antrodia_albida_AJ006680            ----G-TTGT-----------------AGCTGGCCTTTC--------AAC
Antrodia_sinuosa_AJ416068           ----G-TTGT-----------------AGCTGGTCTTTT--------CAG
Fomitopsis_rosea_AJ415546           ----G-TTGT-----------------TGCTGGCCTTTT---CGTTTGAG
Fomitopsis_pinicola_AJ560638        ----G-TTGT-----------------AGCTGGCYGTTT---CGTTTGAG
Polyporus_arcularius_AB070868       ----G-HTGH-----------------AGCTGGCCTT----------CCG
Polyporus_ciliatus_AB070881         ----G-HTGH-----------------AGCTGGCCTT----------ACC
Aspergillus_niger_AF455522          ------CT---------------------TTGGGCC---------CAACC
Aspergillus_niger_AJ280006          ------CT---------------------TTGGGCC---------CAACC
Penicillium_commune_AF455544        ------CT---------------------CTGGGTC---------CAACC
P_chrysogenum_AF034451              ------CT---------------------CTGGGTC---------CAACC
P_roseopurpureum_AF455492           ------CT---------------------CTGGGTC---------CAACC


Entomocorticium_sp.E_AF119505       GGA-----TGTGCTCG-----TCTGGA-TGCGTGTC-----CCTT-CTCT
Entomocorticium_sp.D_AF119503       GGA-----TGTGCTCG-----TCTGGA-TGCGTGTC-----CCTT-CTCT
Entomocorticium_sp.C_AF119510       GGA-----TGTGCTCG-----TCTGGA-TGCGTGTC-----CCTT-CTCT
Entomocorticium_sp.B_AF119508       GGA-----TGTGCTCG-----TCTGGA-TGCGTGTC-----CCTT-CTCT
Entomocorticium_sp.H_AF119512       GGA-----TGTGCTCG-----TCTGGA-TGCGTGTC-----CCTT-CTCT
Entomocorticium_sp.G_AF119512       GGA-----TGTGCTCG-----TCCGGA-TGCGTGTC-----CCTT-CTCT
Peniophora_rufa_AF119516            G-A-----CGTGCTCG-----TCCGGA-TTCGAGTC-----CCTT-CTCT
Peniophora_pithya_AF119521          GCA-----TGTGCTCG-----TCCGGA-TGCGTGTC-----CCTT-CCCT
Peniophora_piceae_AF119515          GGA-----CGTGCTCG-----CCCTCA-TGCGTGTC-----CCTT-CACT
Peniophora_pseudo-pini_AF119514     GGA-----TGTGCTCG-----TTCTCACTGCGTGTC-----CCTT-CTCT
Peniophora_aurantiaca_AF119517      GGA-----TGTGCTCG-----TCCGGA-TGCGTGTC-----CCTT-CTCT
Peniophora_aurantiaca_AF210829      GGA-----TGTGCTCG-----TCTGGA-TGCGTGTC-----CCTT-CTCT
Peniophora_aurantiaca_AF210824      GGA-----TGTGCTCG-----TCCGGA-TGCGTGTC-----CCTT-CTCT
Peniophora_aurantiaca_AF210820      GGA-----TGTGCTCG-----TCCGGA-TGCGTGTC-----CCTT-CTCT
Peniophora_aurantiaca_AF210821      GGA-----TGTGCTCG-----TCCGGA-TGCGTGTC-----CCTT-CTCT
Peniophora_aurantiaca_AF210822      GGA-----TGTGCTCG-----TCCGGA-TGCGTGTC-----CCTT-CTCT
Peniophora_aurantiaca_AF210825      GGA-----TGTGCTCG-----TCTGGA-TGCGTGTC-----CCTT-CTCT
Peniophora_aurantiaca_AF210827      GGA-----TGTGCTCG-----TCTGGA-TGCGTGTC-----CCTT-CTCT
Peniophora_aurantiaca_AF210828      GGA-----TGTGCTCG-----TCCGGA-TGCGTGTC-----CCTT-CTCT
Peniophora_aurantiaca_AF210823      GGA-----TGTGCTCG-----TCCGGA-TGCGTGTC-----CCTT-CTCT
Peniophora_duplex_AF119519          GGAC----TGTGCTCG-----CTCGGA-CGTGTGTC-----CCTT-CTCT
Peniophora_cinerea_AF119518         GGA-----TGTGCTCG-----GCCGGA-TGCGTGTC-----CCTT-CTCT
Peniophora_nuda_AF119513            GGA-----TGTGCTCG-----GCCGGA-TGCGTGTC-----CCTT-CTCT
Peniophora_aurantiaca_AF210819      GGA-----TGTGCTCG-----TCCGGA-TGCGTGTC-----CCTT-CTCT
P_aurantiaca_CBS_396.50             GGA-----TGTGCTCG-----TCCGGA-TGCGTGTC-----CCTT-CTCT
UD4                                 GGA-----TGTGCTCG-----TCTGGA-CGTGTGTC-----CCTT-CTCT
Amanita_tenuifolia_AF085492         GGA-----TGTGCTCG-----TCTGGA-TGCGTGTC-----CCTT-CTCT
Entomocorticium_spF_AF119507        GGA-----TGTGCTCG-----TCTGGA-TGCGTGTC-----CCTT-CTCT
Peniophora_pithya_AF119520          GGA-----TGTGCTCG-----TCTGGA-TGCGTGTC-----CCTT-CTCT
E._dendroctoni_AF119506             GGA-----TGTGCTCG-----TCTGGA-TGCGTGTC-----CCTT-CTCT
Entomocorticium_spA_AF119509        GGA-----TGTGCTCG-----TCTGGA-TGCGTGTC-----CCTT-CTCT
Dendrophora_albobadia_AF119522      GGA-----TGTGCTCG-----CTCGGA-TGTGTGTC-----CCTT-CTCT
An_citrinella_AF126879              GGGCA---TGTGCACA----CTTTGT-----TCA----T--CCAC-CCTT
Antrodiella_pallasii_AF126895       GGGCA---TGTGCACA----CTTTGT-----TCA----T--CCAC-CCTT
Antrodiella_faginea_AF126886        GGGCA---TGTGCACA----CTTTGT-----TCA----T--CCAC-CCTT
Antrodiella_romellii_AF126902       GGGCA---TGTGCACA----CTTTGT-----TCA----T--CCAC-C-TT
An_parasitica_AF126898              GGGCA---TGTGCACA----CTTTGT-----TCA----T--CCAC-CCTT
Antrodiella_faginea_AF126884        GGGCA---TGTGCACG----CTTTGT-----TCA----T--CCAC-CCTT
An_onychoides_AJ006674              GGGCA---TGTGCACA----CTTTGT-----TCA----T--CCAC-CCTT
An_semisupina_AF126905              AGGCA---TGTGCACA----CTGTGT-----TCA----T--CCAC-CCTT
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An_beschidica_AF126878              AGGCA---TGTGCACA----CTGTGT-----TCA----T--CCAC-CCTT
Antrodiella_hoehnelii_AF126893      GGGCA---TGTGCACA----CTGCGT-----TCAA---T--CCAC-CCTT
Antrodiella_americana_AF126877      CGGCA---TGTGCACA----CTTTGT-----TCA----T--TCAC-CCTT
Amanita_spissa_AF085486             GAGCA---TGTGCACG---TCTTTT-GCTGCTTG-----CTTC-ATTCTC
Amanita_aspera_AF085485             GAGCA---TGTGCACG---TCTTTT-GCTGCTTA-----CTTC-ATTCTC
Amanita_rubescens_AF085484          GAGCA---TGTGCACG---TCTTTT-GCTGCTTG-----CTTC-ATTCTC
Amanita_citrina_AF085489            GAGCA---TGTGCACG---CCTTTT-GTTGCTTA-----TTTC-ATTC--
Amanita_virosa_AB015676             GGGCA---TGTGCACG---TCTCTG-G-TCATTA-------CCAA-----
Amanita_phalloides_AJ308097         GGGCA---TGTGCACG---TCTCTG-G-TCATTA-------CCAA-----
Amanita_fulva_AF085494              AGGCA---TGTGCACG---CC--TG----GCTCAT------CCAC-TCTT
Au_alborubescens_AJ006683           AGACA---TGTGCACG---CCC-TG----CTCAAT------CCAC-TCTC
Trichaptum_abietinum_U63474         GGGCA---TGTGCACG---CC--TG-T-CT--CAT------CCAC-TCTC
Bjerkandera_adjusta_AF455410        GGGCA---TGTGCACG---CC--TG-T-CT--CAT------CCAC-TCTC
Bjerkandera_adusta_AJ006672         GGGCA---TGTGCACG---CC--TG-T-CT--CAT------CCAC-TCTC
Bjerkandera_fumosa_AJ006673         GAGCA---TGTGCACG---CCTGTC-T-T---CAT------CCA-CTCTC
Polyporus_brumalis_AB070876         AGGCA---TGHGCACG----CCCTGC-----TCA----T--CCA-CTCT-
Skeleto_kuehneri_AJ006678           TTGCA---TGTGCTCAGGCCCCCGC----TCCA--AT---CCA---CTC
Gloeo_abietinum_AJ420948            AGGCA---TGTGCACG----CTTTCT--TACTCAAT-----CCAAC-CTT
Gloeo_abietinum_AJ420947            AGGCA---TGTGCACG----CTCCCT---ACTCAAT-----CCCAC-CTT
Gloeophyllum_trabeum_AJ420950       AGGCA---TGTGCACG----CTCTCT---ACTCAAT-----CCAAC-CTT
Gloeophyllum_trabeum_AJ420949       GGGCAT--TGTGCACA----CCTTGC-----TCAATT----CCAACTCTT
Gloeo_sepiarium_AJ420946            GGGCA---TGTGCACG----CTTCGC-----TCAAAT----CCAACTCAT
Skeletocutis_nivea_AJ006679         AGGCA---TGTGCACGC---CC-TGC-----TCAT------CCAC-TCT-
Ganoderma_adspersum_AJ006685        AGGCA---CGTGCACG----CCCTGC-----TCAT------CCAC-TCT-
Donkioporia_expansa_AJ249500        AGGCA---TGTGCACG----CTCCGC-----TCATT-----CCAC-TCT-
Trametes_trogii_AJ438139            AGGCA---TGTGCACG----CCCTGC-----TCAT------CCAC-TCT-
Phlebia_subserialis_AB084620        AGGCA---TGTGCACA----CCCTG--CTCA-T--------CCAC-TCTC
Phlebia_uda_AB084621                AGGCA---TGTGCACA----CCCTGCT-CAATT--------CCAC-TCTC
Phlebia_radiata_AB084619            AGGT---ACGTGCACG----CCTTGCT-CA-T---------CCAC-TCTT
Polyporus_arcularius_AB070865       AGGCA---TGHGCACG----CCCTGC-----TCAT------CCAC-TCT-
Polyporus_ciliatus_AB070883         AGGCA---TGHGCACG----CCCTGC-----TCAT------CCAC-TCT-
Polyporus_tricholoma_AJ132942       AGGCA---TGYGCACG----CCCTGC-----TCAT------CCAC-TCT-
Polyporus_tricholoma_AB070885       AGGCA---TGTGCACG----CCCTGC-----TCAT------CCAC-TCT-
Trichaptum_biforme_U63476           AGGCA---TGHGCTCGC---CCCTGC-----TCAT------CCAC-TCT-
Postia_balsamea_AJ006666            GGGCA---TGTGCACG----CTCCGT-----TCAAAT----CCAA--CCT
Postia_sericeomollis_AJ006667       GGGCA---TGTGCACG----CTTCGT-----TCAAATT---CCAA-ACCC
Postia_hibernica_AJ006665           GGGCA---TGTGCACG----CTTCGT-----TCAAAAT---CCAA--CCT
Postia_lactea_AJ006664              GGGCA---TGTGCACG----CTCCGT-----TCAAAACTTTCCA--CCTT
Di_lindbladii_AJ006682              AGGCA---TGTGCACA----CCCTGC-----TCAT------CCA---CTT
Oligoporus_placentus_AJ249267       AGGCA---TGTGCACG----CCCTGC-----TCTT------CCAT-TCTT
Antrodia_xantha_AXA415569           AGGCA---TGTGCACA----CCCTGC-----TCAT------CCAT-TCT-
Antrodia_serialis_AJ345010          GGAAA---TGTGCACG----CCTCGA--TCATCAT------CCAT-CTCA
Antrodia_albida_AJ006680            GGGCA---TGTGCACG-----CTCGCT-T--CAAT------CCAC-TT--
Antrodia_sinuosa_AJ416068           AGACA---TGTGCACG----CCCTGC-----TCAT------CCAT-TCC-
Fomitopsis_rosea_AJ415546           AGGCATTTCGTGCACG----CCCTGA-----TCATTC---TCCAT-TTCA
Fomitopsis_pinicola_AJ560638        CGGCA---TGTGCACG----CCCTGA-----TCATTA---TCCAT-CTCA
Polyporus_arcularius_AB070868       AGGCA---TGHGCACG----CCCTGC-----TCAT------CCAC-TCT-
Polyporus_ciliatus_AB070881         AGGCA---TGHGCACG----CCCTGC-----TCAT------CCAC-TCT-
Aspergillus_niger_AF455522          TCCCA---T-----------CCGTGT-----CTATTGTA--CCCT-GTTG
Aspergillus_niger_AJ280006          TCCCA---T-----------CCGTGT-----CTATTGTA--CCCT-GTTG
Penicillium_commune_AF455544        TCCCA---C-----------CCGTGT-----TTATTTTA--CCTT-GTTG
P_chrysogenum_AF034451              TCCCA---C-----------CCGTGT-----TTATTTTA--CCTT-GTTG
P_roseopurpureum_AF455492           TCCCA---C-----------CCGTGT-----TT-CCGAA--CCTT-GTTG
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Entomocorticium_sp.E_AF119505       ATT-CCACCCCACTGTGAACC---------------AAGC-GT-------
Entomocorticium_sp.D_AF119503       ATT-CCACCCCACTGTGAACC---------------AAGC-GT-------
Entomocorticium_sp.C_AF119510       ATT-CCACCCCACTGTGAACC---------------ACGC-GT-------
Entomocorticium_sp.B_AF119508       ATT-CCACCCCATTGTGAACC---------------ACGC-AT-------
Entomocorticium_sp.H_AF119512       ATT-CCACCCCACTGTGAACC---------------AAGC-GT-------
Entomocorticium_sp.G_AF119512       ATT-CCACCCCACTGTGAACC---------------AAGC-GT-------
Peniophora_rufa_AF119516            AT--CCACCCCTTTGTGAACC---------------AAGC-GT-------
Peniophora_pithya_AF119521          ATC-CCACCCCACTGTGAACC---------------AAGC-GT-------
Peniophora_piceae_AF119515          AT--CCACCCCACTGTGAACC---------------AAGT-GT-------
Peniophora_pseudo-pini_AF119514     AT--CCACCCCATTGTGAACC---------------AAGT-GT-------
Peniophora_aurantiaca_AF119517      ATT-CCACCCCTTTGTGAACC---------------AAGT-GT-------
Peniophora_aurantiaca_AF210829      ATT-CCACCCCTTTGTGAACC---------------AAGT-GT-------
Peniophora_aurantiaca_AF210824      ATT-CCACCCCTTTGTGAACC---------------AAGT-GT-------
Peniophora_aurantiaca_AF210820      ATT-CCACCCCTTTGTGAACC---------------AAGT-GT-------
Peniophora_aurantiaca_AF210821      ATT-CCACCCCTTTGTGAACC---------------AAGT-GT-------
Peniophora_aurantiaca_AF210822      ATT-CCACCCCTTTGTGAACC---------------AAGT-GT-------
Peniophora_aurantiaca_AF210825      ATT-CCACCCCTTTGTGAACC---------------AAGT-GT-------
Peniophora_aurantiaca_AF210827      ATT-CCACCCCTTTGTGAACC---------------AAGT-GT-------
Peniophora_aurantiaca_AF210828      ATT-CCACCCCTTTGTGAACC---------------AAGT-GT-------
Peniophora_aurantiaca_AF210823      ATT-CCACCCCTTTGTGAACC---------------AAGT-GT-------
Peniophora_duplex_AF119519          ATT-CCACCCCATTGTGAACC---------------AAGT-GT-------
Peniophora_cinerea_AF119518         ATT-CCACCCCTTTGTGAACC---------------AAGT-GT-------
Peniophora_nuda_AF119513            ATT-CCACCCCTTTGTGAACC---------------AAGT-GT-------
Peniophora_aurantiaca_AF210819      ATT-CCACCCCTTTGTGAACC---------------AAGT-GT-------
P_aurantiaca_CBS_396.50             ATT-CCACCCCTTTGTGAACC---------------AAGT-GT-------
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UD4                              ATT-CCACCCCACTGTGAACC---------------AAGT-GT-------
Amanita_tenuifolia_AF085492      ATT-CCACCCCACTGTGAACC---------------AAGC-GT-------
Entomocorticium_spF_AF119507     ATT-CCACCCCACTGTGAACC---------------AAGC-GT-------
Peniophora_pithya_AF119520       ATT-CCACCCCACTGTGAACC---------------AAGC-GT-------
E._dendroctoni_AF119506          ATT-CCACCCCACTGTGAACC---------------ACGC-GT-------
Entomocorticium_spA_AF119509     ATT-CCACCCCACTGTGAACC---------------AAGC-AT-------
Dendrophora_albobadia_AF119522   AT--CCACCCCATTGTGAACC---------------AAGT-GT-------
An_citrinella_AF126879           -----CACACCTCTGTGCACT-------TCTC--ATGGGTC-GGG-----
Antrodiella_pallasii_AF126895    -----CACACCTCTGTGCACT-------TCTC--ATGGGTC-GGG-----
Antrodiella_faginea_AF126886     -----CACACCACTGTGCACT-------TCTC--ATGGGTC-GGG-----
Antrodiella_romellii_AF126902    -----CACACCACTGTGCACT-------TCTC--ATGGGTC-GGG-----
An_parasitica_AF126898           -----CACACCACTGTGCACT-------TCTC--ATGGGTT-GGG-----
Antrodiella_faginea_AF126884     -----CACACCACTGTGCACT-------TCTC--ATGGGTC-GGG-----
An_onychoides_AJ006674           -----CACACCTCTGTGCACT-------TCTC--ATGGGTTTGGG-----
An_semisupina_AF126905           -----CACACCTCTGTGCACT-------TCTC--ATGGGTTTGGG-----
An_beschidica_AF126878           -----CACACCTCTGTGCACT-------TCTC--ATGGGTTTGGG-----
Antrodiella_hoehnelii_AF126893   -----CACACCTCTGTGCACT-------TCTC--ATGGGTTCGGG-----
Antrodiella_americana_AF126877   -----CACACCTCTGTGCACT-------TCTC--ATGGGTC-GGG-----
Amanita_spissa_AF085486          T----TTTCCACCTGTGCACT--------CTTT-GTAGACACTCGGGAT-
Amanita_aspera_AF085485          T----TTTCCACCTGTGCACT--------CTTT-GTAAACACTTGGGAT-
Amanita_rubescens_AF085484       T----TTTCCACCTGTGCACT--------CTTT-GTAGACACTCGGGAT-
Amanita_citrina_AF085489         -ATC-TTTCCTCCTGTGCACG--------TTTT-GTAGACACTTGGGA--
Amanita_virosa_AB015676          ------TTCCACCTGTGCACA--------CACTTGTAGACACTTGGGA--
Amanita_phalloides_AJ308097      ------TTCCACCTGTGCACA----------CTTGTAGACACTTGGGA--
Amanita_fulva_AF085494           -------AACCTCTGTGCACT--------TA-T-GTAAGAGAAAAAA---
Au_alborubescens_AJ006683        ------AAACCCCTGTGCACT--------TACT-GTAGGTT---------
Trichaptum_abietinum_U63474      -------AACTTCTGTGCACT-------TTTC--ATAGGC----------
Bjerkandera_adjusta_AF455410     -------AACTTCTGTGCACT-------TTTC--ATAGGC----------
Bjerkandera_adusta_AJ006672      -------AACTTCTGTGCACT-------TTTC--ATAGGC----------
Bjerkandera_fumosa_AJ006673      -------AACTTCTGTGCACT-------TTTC--ATAGGC----------
Polyporus_brumalis_AB070876      --------ACACCTGTGCACT--------TAC-TGTAGGCTTCAGGAGCT
Skeleto_kuehneri_AJ006678        A-------ACCCCTGTGCACTATTCGGAAT-T-TGTGGGATT-AG-----
Gloeo_abietinum_AJ420948         --------ACACCTGTGCATC--------TAT-TGTAGGTCGGGTA----
Gloeo_abietinum_AJ420947         --------ACACCTGTGCATC--------TAT-TGTAGGTCGGGTGGCTT
Gloeophyllum_trabeum_AJ420950    --------ACACCTGTGCACC--------TAT-TGTAGGTCGAGTGGCT-
Gloeophyllum_trabeum_AJ420949    -------AACACCTGTGCACT--------CAT-TGTAGGTTGACTTTC--
Gloeo_sepiarium_AJ420946         T---TTCAACACCTGTGCACA--------TAT-TGTAGG-ATGGTTGAT-
Skeletocutis_nivea_AJ006679      --------ACACCTGTGCACT--------TAC-TGTAGGYTTCAGGAG--
Ganoderma_adspersum_AJ006685     --------ACACCTGTGCACT--------TAC-TGTGGGTTTACCGGTC-
Donkioporia_expansa_AJ249500     --------ACACCTGTGCACT--------TAC-TGTGGGTTT-CAGAC--
Trametes_trogii_AJ438139         --------ACACCTGTGCACT--------TAC-TGTGGGTATCGGGAG--
Phlebia_subserialis_AB084620     -AT-----ACCCCTGTGCACC---------TATTGTAGGCT-TGGTGG--
Phlebia_uda_AB084621             -A-----CACCCCTGTGCACT--------TAC-TGTAGGCTATGGTCG--
Phlebia_radiata_AB084619         -A-----AACCCCTGTGCACT--------TTT-TGTAGGCT-TGGTGG--
Polyporus_arcularius_AB070865    --------ACACCTGHGCACT--------TAC-TGHGGGHTWCAGGAG--
Polyporus_ciliatus_AB070883      --------ACACCTGHGCACT--------TAC-TGHGGGHTTCAGGAG--
Polyporus_tricholoma_AJ132942    --------ACACCTGTGCACT--------TAC-TGTGGGHTTCAGGAG--
Polyporus_tricholoma_AB070885    --------ACACCTGTGCACT--------TAC-TGTGGGTTTCAGGAG--
Trichaptum_biforme_U63476        --------ACACCTGTGCACT--------TAC-TGTAGGCTTCAGGAG--
Postia_balsamea_AJ006666         T----CATACCCCTGTGCACC------TTT---TGTAGG-GTCGT-----
Postia_sericeomollis_AJ006667    T----CATACACCTGTGCACC------TTT---TGTAGG-ACCG------
Postia_hibernica_AJ006665        TT---CATACCCCTGTGCACC------TTT---TGTAGG-GTCGT-----
Postia_lactea_AJ006664           -----CACACCCCTGTGCACC------TTC---TGTAGG-GTCGA-----
Di_lindbladii_AJ006682           --------ACACCTGTGCAC--------AT-T-TGTAGGTTAGAT--GC-
Oligoporus_placentus_AJ249267    --------ACCCCTGTGCAC--------ACTC-TGTAGGTCGGTTT----
Antrodia_xantha_AXA415569        --------ACACCTGTGCAC--------ATTC-TGTAGGTCGGTTT----
Antrodia_serialis_AJ345010       ---------CACCTGTGCAC--------ACAC-TGTAGGTCGGTTT----
Antrodia_albida_AJ006680         --------ACCCCTGTGCAC--------ACTT-TGTAGGTCGGCT-----
Antrodia_sinuosa_AJ416068        --------ACCCCTGTGCACA--------CTC-TGTAGGTCGGTTTT---
Fomitopsis_rosea_AJ415546        T-------ACACCTGTGCACA--------CAC-TGTAGGTCGGTTTGC--
Fomitopsis_pinicola_AJ560638     C-------ACACCTGTGCACA--------TAC-TGTAGGTCGGCTTTT--
Polyporus_arcularius_AB070868    --------ACACCTGHGCACT--------TAC-TGHAGGCTTC-------
Polyporus_ciliatus_AB070881      --------ACACCTGHGCACT--------TAC-TGTAGGHCT--------
Aspergillus_niger_AF455522       ---------CTTCGGCGGGCC-----CGCCGCTTGTCGGCCGCCG-----
Aspergillus_niger_AJ280006       ---------CTTCGGCGGGCC-----CGCCGCTTGTCGGCCGCCG-----
Penicillium_commune_AF455544     ---------CTTCGGCGGGCC-----CGCC-TTAACTGGCCGCCG-----
P_chrysogenum_AF034451           ---------CTTCGGCGGGCC-----CGCC-TTAACTGGCCGCCG-----
P_roseopurpureum_AF455492        ---------CTTCGGCGGGCC-----CGCC-TCA--CGGCCGCCG-----
                                          *    * *

Entomocorticium_sp.E_AF119505    --------GCGAG-----------------CCGAAGA-------------
Entomocorticium_sp.D_AF119503    --------GCGAG-----------------CCGAAGA-------------
Entomocorticium_sp.C_AF119510    --------GCGAG-----------------CCGAAGA-------------
Entomocorticium_sp.B_AF119508    --------GCGAG-----------------CCGAAGA-------------
Entomocorticium_sp.H_AF119512    --------GCGAG-----------------CCGAAGA-------------
Entomocorticium_sp.G_AF119512    --------GCGAG-----------------CCGAAGA-------------
Peniophora_rufa_AF119516         --------GCGAG-----------------CCGAAGA-------------
Peniophora_pithya_AF119521       --------GTGGA-----------------CCGATGA-------------
Peniophora_piceae_AF119515       --------G--AG-----------------CCGAAGA-------------
Peniophora_pseudo-pini_AF119514  --------GCGAG-----------------CCGAAGA-------------
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Peniophora_aurantiaca_AF119517      ---------CGAG-----------------CCGATGA-------------
Peniophora_aurantiaca_AF210829      --------GCGAG-----------------CCGATGA-------------
Peniophora_aurantiaca_AF210824      --------GCGAG-----------------CCGATGA-------------
Peniophora_aurantiaca_AF210820      --------GCGAG-----------------CCGATGA-------------
Peniophora_aurantiaca_AF210821      --------GCGAG-----------------CCGATGA-------------
Peniophora_aurantiaca_AF210822      --------GCGAG-----------------CCGATGA-------------
Peniophora_aurantiaca_AF210825      --------GCGAG-----------------CCGATGA-------------
Peniophora_aurantiaca_AF210827      --------GCGAG-----------------CCGATGA-------------
Peniophora_aurantiaca_AF210828      --------GCGAG-----------------CCGATGA-------------
Peniophora_aurantiaca_AF210823      --------GCGAG-----------------CCGATGA-------------
Peniophora_duplex_AF119519          --------GCGAG-----------------CCGAAGA-------------
Peniophora_cinerea_AF119518         --------GCGAG-----------------CCGAAGA-------------
Peniophora_nuda_AF119513            ---------CGAG-----------------CCGAAGA-------------
Peniophora_aurantiaca_AF210819      --------GCGAG-----------------CCGATGA-------------
P_aurantiaca_CBS_396.50             --------GCGAG-----------------CCGATGA-------------
UD4                                 --------GTGAG-----------------CCGAAGA-------------
Amanita_tenuifolia_AF085492         --------GTGAG-----------------CCGACGA-------------
Entomocorticium_spF_AF119507        --------GCGAG-----------------CCGAAGA-------------
Peniophora_pithya_AF119520          --------GCGAG-----------------CCGAAGA-------------
E._dendroctoni_AF119506             --------GCGAG-----------------CCGAAGA-------------
Entomocorticium_spA_AF119509        --------GCGAG-----------------CCGAAGA-------------
Dendrophora_albobadia_AF119522      --------GTGAG-----------------CCGAAGA-------------
An_citrinella_AF126879              --------TTGCG-----------------TCTGAAA-------------
Antrodiella_pallasii_AF126895       --------TTGCG-----------------TCTGAAA-------------
Antrodiella_faginea_AF126886        --------TTGCG-----------------TCTGAAA-------------
Antrodiella_romellii_AF126902       --------TTGCG-----------------TCTGAAA-------------
An_parasitica_AF126898              --------TTGCG-----------------TCTGAAA-------------
Antrodiella_faginea_AF126884        --------TTGCG-----------------TCTGAAA-------------
An_onychoides_AJ006674              --------TTGCG-----------------TCTGAAAA------------
An_semisupina_AF126905              --------TCAAG-----------------TCTGAAA-------------
An_beschidica_AF126878              --------TCAAG-----------------TCTGAAA-------------
Antrodiella_hoehnelii_AF126893      --------TTGCG-----------------TCTGAAA-------------
Antrodiella_americana_AF126877      --------CTGCG-----------------TCTGAAA-------------
Amanita_spissa_AF085486             --------GGGGGGAGA-GGTTGGCGTTGATTTGTTGAC-C-CCTCTCTT
Amanita_aspera_AF085485             --------GTGAGAGAG-GGTTGGCATTTATT-GTTGAA---CCTCTCTT
Amanita_rubescens_AF085484          --------GGGAGAGA--GGT-GGCATCGAAT-GTTAA-----CTCTCTT
Amanita_citrina_AF085489            --------ATGAGA----GGTTGGTTGTAATATAATGAT-TTGACCTCTT
Amanita_virosa_AB015676             --------ATGAGAGA-------C---------TTTG-AC-CAG-TCTCTT
Amanita_phalloides_AJ308097         --------ATGAG------AGACCTTGACCAGTC------------TCTT
Amanita_fulva_AF085494              -------ATGGTGGAAGCTTCCAGGATCTCGCGAGAGGT----C---T-T
Au_alborubescens_AJ006683           -------ATGGTGGGAT----AGCTGGCCCC--TGGGCC----------A
Trichaptum_abietinum_U63474         ----------CGGCTTGTGGGT-----CGTTCGCG-GC----TC-----
Bjerkandera_adjusta_AF455410        ----------CGGCTTGTGGGTG----CGTTCGCGCAC----TT-----
Bjerkandera_adusta_AJ006672         ----------CGGCTTGTGGGTG----CGTTCGCGCAC----TT-----
Bjerkandera_fumosa_AJ006673         ----------CGGCTTGTGGGTGC--GCGTTCGCGTGC----GT-----
Polyporus_brumalis_AB070876         T----CGA-AGCGAGGG----------HTTTAA----T-----CGCT--
Skeleto_kuehneri_AJ006678           -----CTTTTTTTCCT-------------AGAAAGGA-------------
Gloeo_abietinum_AJ420948            ------------GCCT--AAG---TCTTTGCG---GA---C---------
Gloeo_abietinum_AJ420947            ------------GGC-----------TCCATG------------------
Gloeophyllum_trabeum_AJ420950       ----------GGAC------------TTGTT------------------
Gloeophyllum_trabeum_AJ420949       --------GAGAGA------GTATTCTTTATGG--GGTA-CGC-------
Gloeo_sepiarium_AJ420946            ---------ACAAAC---------GAAGAACTTAGGT-T-CCTC------
Skeletocutis_nivea_AJ006679         --------CTTCACGGG-----------CTTTCATTA-------------
Ganoderma_adspersum_AJ006685        --------GCGAAACGG-------GCTCGTTT-ATT-------CGGGC--
Donkioporia_expansa_AJ249500        --------GTGGTGCGA--------GCC-TTT-A---------CCGG--T
Trametes_trogii_AJ438139            --------GTGTCGCG-------------TCG-----TT-TA--------
Phlebia_subserialis_AB084620        --------GA-AGACTG-GNGTCACA----ACCAG---------------
Phlebia_uda_AB084621                -GA-TAGCTGACTTCG---------GTTNG-------------------
Phlebia_radiata_AB084619            -GATAGGCTGACTTCG---------GTCGGC------------------
Polyporus_arcularius_AB070865       ----------CTTCGA---------AGCG-AGGG--HT-TAACCGCT--
Polyporus_ciliatus_AB070883         ----------CTTCGA---------AGCG-GGGG--CT-TAATCGCC--
Polyporus_tricholoma_AJ132942       ----------CTTCGA---------AGCGAGTGC--TT--AACCGCT--
Polyporus_tricholoma_AB070885       ----------CTTCGA---------AGCGAGGGT--TT--AATCGCT--
Trichaptum_biforme_U63476           --------CTTCTCGGG-----------CTTTCATTA-------------
Postia_balsamea_AJ006666            ----------------------------GGC-------------------
Postia_sericeomollis_AJ006667       -----------------------------GT-------------------
Postia_hibernica_AJ006665           ----------------------------GGC-------------------
Postia_lactea_AJ006664              --------------------------------------------------
Di_lindbladii_AJ006682              -GGACTGGGGACTCTG--------TGTT----------------------
Oligoporus_placentus_AJ249267       ----------GAGCGGCTCTCTCTAA--CGGGGG----------------
Antrodia_xantha_AXA415569           ----------GAATGGTTCTCTC-----AGGG----------------
Antrodia_serialis_AJ345010          ------GTG-GGGCGAGATCTT-CATTGATTT------------------
Antrodia_albida_AJ006680            -----CAAG-GGACAGATTCTTAAC-----GGGA----------------
Antrodia_sinuosa_AJ416068           ----------GAAGTGATTCCT-C-----ACGG----------------
Fomitopsis_rosea_AJ415546           ----------GAGGCGGAGCCTTC--ATTGGCGT----------------
Fomitopsis_pinicola_AJ560638        -----GATT-GGAGTGGGGTCTTCA----TCGACT--CTGCTTTTT----
Polyporus_arcularius_AB070868       ------AGGAGCTTCGA---------AGCGAGGGHTTA----ATCGCT--
Polyporus_ciliatus_AB070881         ---------------G----------GCGHGGGCT-TC----GGGCCTT
Aspergillus_niger_AF455522          --------GGGGGGCG------------CCTCTGC-------------
Aspergillus_niger_AJ280006          --------GGGGGGCG------------CCTCTGC-------------
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Penicillium_commune_AF455544        --------GGGGG----------------CTTACGC--------------
P_chrysogenum_AF034451              --------GGGGG----------------CTTACGC--------------
P_roseopurpureum_AF455492           --------GGGGG----------------CTTCCGC--------------


Entomocorticium_sp.E_AF119505       GAGA---TCGGAAG-------CTC-GTGCGCAA---CCCTTAAT------
Entomocorticium_sp.D_AF119503       GAGA---TCGGAAG-------CTC-GTGCGCAA---CCCTTAAT------
Entomocorticium_sp.C_AF119510       GAGA---TCGGAAG-------CTC-GTATGCAA---TCCTTAAT------
Entomocorticium_sp.B_AF119508       GAGA---TCGGAAG-------CT--GTATGCAA---TCCTTAAT------
Entomocorticium_sp.H_AF119512       GAGA---TCGGAAG-------CTC-GTATGCAA---TCCTTAAT------
Entomocorticium_sp.G_AF119512       GAGA---TCGGAAG-------CCC-GTATGCAA---CCCTTAAT------
Peniophora_rufa_AF119516            GAGA---TCGGAGG-------CTT-GCATGCGAA--CCCTTAAC------
Peniophora_pithya_AF119521          GAGA---TCGGAGG-------TCC-GCATGCAA---CCTTTAAC------
Peniophora_piceae_AF119515          GAGA---TCGGAGG-------CTC-GCATGCAA---CCCTTAAC------
Peniophora_pseudo-pini_AF119514     GAGA---TCGGAGG-------CTC-GCATGCAAA--CCCTTAAC------
Peniophora_aurantiaca_AF119517      GAGA---TCGGAGG-------CTC-GCATGCAAA--CCCTTAAC------
Peniophora_aurantiaca_AF210829      GAGA---TCGGAGG-------CTC-GCATGCAAA--CCCTTAAC------
Peniophora_aurantiaca_AF210824      GAGA---TCGGAGG-------CTC-GCATGCAAA--CCCTTAAC------
Peniophora_aurantiaca_AF210820      GAGA---TCGGAGG-------CTC-GCATGCAAA--CCCTTAAC------
Peniophora_aurantiaca_AF210821      GAGA---TCGGAGG-------CTC-GCATGCAAA--CCCTTAAC------
Peniophora_aurantiaca_AF210822      GAGA---TCGGAGG-------CTC-GCATGCAAA--CCCTTAAC------
Peniophora_aurantiaca_AF210825      GAGA---TCGGAGG-------CTC-GCATGCAAA--CCCTTAAC------
Peniophora_aurantiaca_AF210827      GAGA---TCGGAGG-------CTC-GCATGCAAA--CCCTTAAC------
Peniophora_aurantiaca_AF210828      GAGA---TCGGAGG-------CTC-GCATGCAAA--CCCTTAAC------
Peniophora_aurantiaca_AF210823      GAGA---TCGGAGG-------CTC-GCATGCAAA--CCCTTAAC------
Peniophora_duplex_AF119519          GAGA---TCGGAGG-------CTC-GCATGCAAA--CCCTTAAC------
Peniophora_cinerea_AF119518         GAGA---TCGGAGG-------CTC-GCATGCAA---CCCTTAAC------
Peniophora_nuda_AF119513            GAGA---TCGGAGG-------CTC-GCATGCAA---CCCTTAAC------
Peniophora_aurantiaca_AF210819      GAGA---TCGGAGG-------CTC-GCATGCAAA--CCCTTAAC------
P_aurantiaca_CBS_396.50             GAGA---TCGGAGG-------CTC-GCATGCAAA--CCCTTAAC------
UD4                                 GAGA---TCGGAAG-------CTC-GCATGCAA---CACTTAAC------
Amanita_tenuifolia_AF085492         GAGA---TCGGAGG-------CTT-GCATGCAA---CACTTAAC------
Entomocorticium_spF_AF119507        GAGA---TCGGAAG-------CCC-GCATGCAA---CCCTTAAT------
Peniophora_pithya_AF119520          GAGA---TCGGAAG-------CCC-GCATGCAA---CCCTTAAT------
E._dendroctoni_AF119506             GAGA---TCGGAAG-------CT--GTATGCAA---TCCTTAAT------
Entomocorticium_spA_AF119509        GAGA---TCGGAAG-------CT--GTATGCAA---TCCTTAAT------
Dendrophora_albobadia_AF119522      GAAA---TCGGACG-------CTCCGCATGCAAA--CCTTTAAC------
An_citrinella_AF126879              TATG---GCAAAGC------CCTTCTCATGT-----GTTTTAACC-----
Antrodiella_pallasii_AF126895       TATG---GCAAAGC------CCTTCTCATGT-----GTTTTAACC-----
Antrodiella_faginea_AF126886        TATG---GCAAAGC------CCTTCTCATGT-----GTTTTAACC-----
Antrodiella_romellii_AF126902       TATG---GCAAAGC------CCTTCTCATGT-----GTTTTA-CC-----
An_parasitica_AF126898              TATG---GCAAAGC------CCTTCTCATGT-----GTTTTAACC-----
Antrodiella_faginea_AF126884        TATG---GCAAAGC------CCTTCTCATGT-----GTTTTAACC-----
An_onychoides_AJ006674              TATG---ACAAAGCT-----CCTTCTCATGT-----GTTTTAACC-----
An_semisupina_AF126905              TATG---GCGAAGCC-C---CCTTCTCATGT-----GTTTTAAAC-----
An_beschidica_AF126878              TATG---GCGAAGCC-C---CCTTCTCATGT-----GTTTTAAAC-----
Antrodiella_hoehnelii_AF126893      TATG---GCAAAGC------CCTTCTCATGT-----GTTTTAACC-----
Antrodiella_americana_AF126877      TATG---GCGAGGC------ACCTCTCATGT-----GTTTATACC-----
Amanita_spissa_AF085486             GATA---TTGAAAAG-----TCTGGGTGTTTAT-GTATTTTTTT------
Amanita_aspera_AF085485             GATA---TTNAAAAA-G---TCTGGGTGTTTAT-GTATTTTTT-------
Amanita_rubescens_AF085484          GATA---TCGAAAG------TCTGG-TGTCTAT-GTGTTTTGC-------
Amanita_citrina_AF085489            GAGA---TCAG---------TCTGAGTGTCTATGGCATTTTTATAA----
Amanita_virosa_AB015676             GAGAGA-TTTCATA------TCTGGGTGTCTAT-GCCTTTTTATT-----
Amanita_phalloides_AJ308097         GAGAAG-TTGAAAA------TCTGGGTGTCTAT-GCCATTTTAT------
Amanita_fulva_AF085494              TGGTTGAACA--------AGCCGTTTTTCTTTCTTATGT-TTT-------
Au_alborubescens_AJ006683           GTATTGAA-----------------AGCCTTGCCTATGTTTC--------
Trichaptum_abietinum_U63474         --GTAGGN-----------------GTCGGGCTTATGCTTTA-------
Bjerkandera_adjusta_AF455410        --GTAGGT-----------------GTCGGGCTTATGCTTTA-------
Bjerkandera_adusta_AJ006672         --GTAGGT-----------------GTCGGGCTTATGCTTTA-------
Bjerkandera_fumosa_AJ006673         CTGTAG-------------------GTCTGGTTTATGCTTTA-------
Polyporus_brumalis_AB070876         ---CTCGCCGAGTTGT---------GACTGGGCCTACGTTTACC------
Skeleto_kuehneri_AJ006678           ----AG-CTAGTTT---T------GCCTTTT----CCGTTCCTTA--TAA
Gloeo_abietinum_AJ420948            --TCGGGCTGCT-------------CTT-CTT---ATG-TTTT-------
Gloeo_abietinum_AJ420947            --TCAGGCTGTC-------------CTTCCT----ATG-TCT--------
Gloeophyllum_trabeum_AJ420950       --CTGG-TCGCT-------------CTTCCT----ATGTTTY--------
Gloeophyllum_trabeum_AJ420949       --TTAAGTGGT--------------CTTCCT----ATGTTTTA-------
Gloeo_sepiarium_AJ420946            --TCGAGTTGTTGACGC--------CTTCCT----ATGTTTCATTT----
Skeletocutis_nivea_AJ006679         -----GTCCGGGTTGT---------AACTGGGCTTACGTTTACTACA---
Ganoderma_adspersum_AJ006685        TTGTGGAGCGC-------------ACTTGTTGCCTGCGTTT---------
Donkioporia_expansa_AJ249500        TCGTGAAGCG---------------TCTGT-GCCTGCGTTT---------
Trametes_trogii_AJ438139            CGGCGAGGCGTTAACCG---------TGCCT----ACGTTTTACT-----
Phlebia_subserialis_AB084620        -TCGG-----AAAGCCT---------TGCCT----ATGTTTTA-------
Phlebia_uda_AB084621                -TTTG-----AAAGCCT---------TGCTT----ATGTTATT-------
Phlebia_radiata_AB084619            -TTTG-----AATGCCT---------CGCCT----ATGTATTA-------
Polyporus_arcularius_AB070865       ---CTCGCCGAGHTGT---------TACTGGGCCTACGHTTATC------
Polyporus_ciliatus_AB070883         ---TTCGCCGAGHTGT---------TACTGGGCCTACGHTTACC------
Polyporus_tricholoma_AJ132942       ---CTCGCCGAGTTGT---------TACTGGGCCTACGTTTATC------
Polyporus_tricholoma_AB070885       ---CTCGCCGAGTTGT---------TACTGGGCCTACGTTTATC------
Trichaptum_biforme_U63476           -----GCTCGGGHTGT---------AACTGAGCTTACGHTTA-C------
Postia_balsamea_AJ006666            ---CGCGAGGCTG------------CGCT----CTATGTT--CA------
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Postia_sericeomollis_AJ006667       ---CGAAAGGCCG--------------TTC---CTATGTT--CATT----
Postia_hibernica_AJ006665           ---TGTGAGGCTG------------CGCT----CTATGTT--TATCA---
Postia_lactea_AJ006664              GGTCGAGAGGCCAA-----------CACC----CTATGTT---GT-----
Di_lindbladii_AJ006682              ------CTCGGTGCAAG---TCTGCCTATGTCT------T----------
Oligoporus_placentus_AJ249267       ----ATGCTGTTTGGC---------CTTCCT----ATGTTTT--------
Antrodia_xantha_AXA415569           ----GAGCTGTTTGGC---------CTTCCT----ATGTTTTT-------
Antrodia_serialis_AJ345010          ----TGCTTTGCGGGC---------CTTCCT----ATGTTTT--------
Antrodia_albida_AJ006680            ----TTTGTTTCCAGT---------CTTCCT----ATGTTTT--------
Antrodia_sinuosa_AJ416068           ----AGTCGCTCGGGC---------CTTCCT----ATGTTTTC-------
Fomitopsis_rosea_AJ415546           ----AGCTTTGTGGGC---------CTTCCT----ATGTTTT--------
Fomitopsis_pinicola_AJ560638        ----AG-TT-G-GGGC---------CTTCCT----ATGTTTT--------
Polyporus_arcularius_AB070868       ---CTCGCCGAGHTGH---------GACTGGGCCTACGHTT---------
Polyporus_ciliatus_AB070881         TACGGGCTTGAAGC-----------GTTCTGCCTGCCTATGH-------
Aspergillus_niger_AF455522          -----CCCCCGGGC-------CCGTGCCCGCCGGAGACCCCAA-------
Aspergillus_niger_AJ280006          -----CCCCCGGGC-------CCGTGCCCGCCGGAGACCCCAA-------
Penicillium_commune_AF455544        -----CCCC-GGGC-------CCGCGCCCGCCGAAGACACC---------
P_chrysogenum_AF034451              -----CCCC-GGGC-------CCGCGCCCGCCGAAGACACC---------
P_roseopurpureum_AF455492           -----CCCC-GGGC-------CCGCGCCCGCCGAAGACACC---------


Entomocorticium_sp.E_AF119505       ---------AT--ACCCC-AT----CATGTATCAG--AATGTACC-T---
Entomocorticium_sp.D_AF119503       ---------AT--ACCCC-AT----CATGTATCAG--AATGTACC-T---
Entomocorticium_sp.C_AF119510       ---------AT--ACCCC-AT----CATGTATCAG--AATGTACC-T---
Entomocorticium_sp.B_AF119508       ---------AT--ACCCC-AT----CATGTATCAG--AATGTACC-T---
Entomocorticium_sp.H_AF119512       ---------AT--ACCCC-AT----CATGTATCAG--AATGTACC-T---
Entomocorticium_sp.G_AF119512       ---------AT--ACCCC-AT----CATGTATCAG--AATGTACC-T---
Peniophora_rufa_AF119516            ---------AT--ACCCC-AAG--TCA-GTATCAG--AATGTAAC-T---
Peniophora_pithya_AF119521          -------ATAT--ACC-CCTA--CGAA-GTATC-AG-AATGTACC-T---
Peniophora_piceae_AF119515          ---------AT--ACCCC-AA--C-AA-GTATCAG--AATGTACC-T---
Peniophora_pseudo-pini_AF119514     ---------AT--ACCCC-AA--CGAA-GTATCAG--AATGTACC-T---
Peniophora_aurantiaca_AF119517      ---------AT--ACCCC-AA--TGAA-GTATCAG--AATGTACC-T---
Peniophora_aurantiaca_AF210829      ---------AT--ACCCC-CAACTGAA-GTATCAGT-AATGTACCCT---
Peniophora_aurantiaca_AF210824      ---------AT--ACCCC-AA--TGAA-GTATCAG--AATGTACC-T---
Peniophora_aurantiaca_AF210820      ---------AT--ACCCC-AA--TGAA-GTATCAG--AATGTACC-T---
Peniophora_aurantiaca_AF210821      ---------AT--ACCCC-AA--TGAA-GTATCAG--AATGTACC-T---
Peniophora_aurantiaca_AF210822      ---------AT--ACCCC-AA--TGAA-GTATCAG--AATGTACC-T---
Peniophora_aurantiaca_AF210825      ---------AT--ACCCC-AA--TGAA-GTATCAG--AATGTACC-T---
Peniophora_aurantiaca_AF210827      ---------AT--ACCCC-AA--TGAA-GTATCAG--AATGTACC-T---
Peniophora_aurantiaca_AF210828      ---------AT--ACCCC-GA--TGAA-GTATCAG--AATGTACC-T---
Peniophora_aurantiaca_AF210823      ---------AT--ACCCC-AA--TGAA-GTATCAGA-A-TGTACC-T---
Peniophora_duplex_AF119519          ---------AT--ACCCC-AA--TGAA-GTATCAGA-A-TGTACC-T---
Peniophora_cinerea_AF119518         ---------AT--ACCCC-AA--TGAA-GTATC-AGAA-TGTACC-T---
Peniophora_nuda_AF119513            ---------AT--ACCCC-AA--TGAA-GTATC-AGAA-TGTACC-T---
Peniophora_aurantiaca_AF210819      ---------AT--ACCCC-AA--TGAA-GTATC-AGAA-TGTACC-T---
P_aurantiaca_CBS_396.50             ---------AT--ACCCC-AA--TGAA-GTATC-AGAA-TGTACC-T---
UD4                                 ---------AT--ACCCC-AA--TGAA-GTATC-AGAA-TGTACC-T---
Amanita_tenuifolia_AF085492         ---------AT--ACCCC-AA--TGAA-GTATC-AGAA-TGTACC-T---
Entomocorticium_spF_AF119507        ---------AT--ACCCC-A---TCAT-GTATC-AGAA-TGTACC-T---
Peniophora_pithya_AF119520          ---------AT--ACCCC-A---TCAT-GTATC-AGAA-TGTACC-T---
E._dendroctoni_AF119506             ---------AT--ACCCC-A---TCAT-GTATC-AGAA-TGTACC-T---
Entomocorticium_spA_AF119509        ---------AT--ACCCC-A---TCAT-GTATC-AGAA-TGTACC-T---
Dendrophora_albobadia_AF119522      ---------AT--ACCCC-AA--TGAA-GTATC-AGAA-TGTACC-T---
An_citrinella_AF126879              ---------AC--ACACT-AT---ACAAGTTTT-AGAA-TGTAAC-AA--
Antrodiella_pallasii_AF126895       ---------AC--ACACT-AT---ACAAGTTTT-AGAA-TGTAAC-AA--
Antrodiella_faginea_AF126886        ---------AC--ACACT-AT---ACAAGTTTT-AGAA-TGTAAC-AA--
Antrodiella_romellii_AF126902       ---------AC--ACACT-A-----CAAGTTTT-AGAA-TGTAAC-AA--
An_parasitica_AF126898              ---------AC--ACACT-AT---ACAAGTTTT-AGAA-TGTAAC-AA--
Antrodiella_faginea_AF126884        ---------AC--ACACT-AT---ACAAGTTTT-AGAA-TGTAAC-AA--
An_onychoides_AJ006674              ---------AC--ACACT-AT---ACAAGTTTT-AGAA-TGTAAC-AA--
An_semisupina_AF126905              ---------AC--ACACT-AT---ACAAGTTTT-AGAA-TGTAAC-AA--
An_beschidica_AF126878              ---------AC--ACACT-AT---ACAAGTTTT-AGAA-TGTAAC-AA--
Antrodiella_hoehnelii_AF126893      ---------AC--ACACT-AT---ACAAGTTTA-AGAA-TGTAAC-AA--
Antrodiella_americana_AF126877      ---------AC--ACACT-TTG--AAAAGTTTT-AGAA-TGTAAC-AA--
Amanita_spissa_AF085486             ------GACAT--ACACA-GTT--GAATGTCTATAGAA-TGAAA--TG-T
Amanita_aspera_AF085485             ------GACAT--ACACG-GTC--GAATGTCTATAGAA-TGAAAT-TAAT
Amanita_rubescens_AF085484          -------ACAT--ACACAAGTT--AAATGTCTATAGAA-TGAAA--TG-T
Amanita_citrina_AF085489            ---------AC--AG-CG-ACT--GGACGTGTATAGAA-TGAGAT-TG-T
Amanita_virosa_AB015676             ---------AC--ACACTAGTT--GCATGTTTATAGAA-TGATGA-T---
Amanita_phalloides_AJ308097         --------TAA--ACACTAGTT--GCATGTTTATAGAA-TGATGA-T---
Amanita_fulva_AF085494              --ACT----AC-AAAC-GCTTCA-----GTTAT-AGAA-TGTC-A-AC--
Au_alborubescens_AJ006683           --ATT----AC-AAAC-GCTTCA-----GTCAA-AGAA-TGTAAC-AT--
Trichaptum_abietinum_U63474         ---CT----AC-AAAC-GATTCA-----GTTTT-AGAA-TGTCAT-AC--
Bjerkandera_adjusta_AF455410        ---TT----AC-AAAC-GATTCA-----GTTTT-AGAA-TGTCAT-AC--
Bjerkandera_adusta_AJ006672         ---TT----AC-AAAC-GATTCA-----GTTTT-AGAA-TGTCAT-AC--
Bjerkandera_fumosa_AJ006673         -TACT----AC-AAAC-GAATCA-----GTTTT-AGAA-TGTCAT-AC--
Polyporus_brumalis_AB070876         ---------AC-AAAC-ACTT---TAAAGTAAC-AGAA-TGTA-A-TC--
Skeleto_kuehneri_AJ006678           CAAT-----AC-AAAC--TCTTATTT-TATTT---GAA-TGTCAC-TTT-
Gloeo_abietinum_AJ420948            --AC-----AC-ATACACTT-GTA----GTCTC-AGAA-TGTAAT-CT--
Gloeo_abietinum_AJ420947            --AC-----AC-ATACACTT-GTA----GTCTC-AGAA-TGTAAT-CT--
Gloeophyllum_trabeum_AJ420950       --AY-----AC-ATACACTTTATA----GTTTC-AGAA-TGTAAA-CT--
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Gloeophyllum_trabeum_AJ420949       --AC-----AT-AC--ACTTTATA----GTTTC-AGAA-TGTAAA-CT--
Gloeo_sepiarium_AJ420946            --AC-----AT-AC--ACTT-GTA----GTCTC-AGAA-TGTAAT-CA--
Skeletocutis_nivea_AJ006679         ---------AC---AC---TCATTGTC-GTATCATGAA-TGTTTT-TT--
Ganoderma_adspersum_AJ006685        --ATC----AC-AAACTCCA-TAAA---GTATT-AGAA-TGTGTA-TT--
Donkioporia_expansa_AJ249500        --ATT----AC-AAACACTATTAAA---GTATT-AGAA-CGTGTA-TT--
Trametes_trogii_AJ438139            --AC---AAAC---G--ATTC------AGTATC-AGAA-TGTGTA-TT--
Phlebia_subserialis_AB084620        ACACA--A-AC-G----CTTC------AGT-TTTAGAA-TGTCACATTT-
Phlebia_uda_AB084621                --ACA--A-AC-G----CTTC------AGT-TAAAGAA-TGTCAT-ACC-
Phlebia_radiata_AB084619            AAACA--A-AC-G----CTTC------AGT-TT-AGAA-TGTAAC-CTT-
Polyporus_arcularius_AB070865       ---------AC--AAC--TCTT-A--AAGTATC-AGAA-TGTAAA-----
Polyporus_ciliatus_AB070883         ---------AC--AAC--TCTT-ATA-ARTAAC-AGAA-TGTTAA-T---
Polyporus_tricholoma_AJ132942       ---------AC-AAAC--TCTT-ATAAAGTATC-AGAA-TGTGTA-TT--
Polyporus_tricholoma_AB070885       ---------AC-AAACA-TC---ATAAAGHATC-AGAA-TGHGHA-TT--
Trichaptum_biforme_U63476           --------TACAAAACACTTTATTGTC-TTGTC--GAA-TGTATTAGCC-
Postia_balsamea_AJ006666            -------TCAT-AAACTCT-TCAGTA-TGTGT--AGAA-TGTTCA-T---
Postia_sericeomollis_AJ006667       --------TAT-AAACTCA-ACAGTA-TGTGT--AGAA-TGT-CA-A---
Postia_hibernica_AJ006665           --------TAT-AAACTCT-TCAGTA-TGTGC--AGAA-TGA-CA-C---
Postia_lactea_AJ006664              --------TAC-AAACCCT-GCTATG-T-TAT--AGAA-TGT-CA-T---
Di_lindbladii_AJ006682              -----TAC-AT--ACTCTGTTC----AGTCAATGA--A-TGTAAT-CT--
Oligoporus_placentus_AJ249267       --AT----AAC-AAACTCTGTAA-----TGTCATAGAA-TGTCAT-C---
Antrodia_xantha_AXA415569           --AT----TAT-ACACACTTTTA-----AGTCAAAGAA-TGTAAT-CT--
Antrodia_serialis_AJ345010          --AT---C-AC-AAA--CTACTA-----GTTTAAAGAA-TGTCTA-CT--
Antrodia_albida_AJ006680            --AT----TAT-AAAC-GTTTAA-----AGTCAAAGAA-TGTACT-C---
Antrodia_sinuosa_AJ416068           --AT----AC--AAACACTTTAA-----AGTCAAAGAA-TGTAAT-C---
Fomitopsis_rosea_AJ415546           --AT---C-AC-AAA--CTACT------GTTTAAAGAA-TGTCTAC-T--
Fomitopsis_pinicola_AJ560638        --AT---C-AC-ACA--CTACTTCA---GTTWAAAGAA-TGTCCTCTT--
Polyporus_arcularius_AB070868       ------ACCAC--AAC--TCTT-AAA--GTATC-AGAA-TGT-AA-AC--
Polyporus_ciliatus_AB070881         ---------ATCACTAACTCTT-ATAA-GHAAC-AGAA-TGHTAA-TC--
Aspergillus_niger_AF455522          --------CACGAACACTGTCT--GAAAGCGTGCAGTC-TGAGTT-----
Aspergillus_niger_AJ280006          --------CACGAACACTGTCT--GAAAGCGTGCAGTC-TGAGTT-----
Penicillium_commune_AF455544        --------CTCGAACTCTGTCT--GAAGAT-TGTAGTC-TGAGT------
P_chrysogenum_AF034451              --------CTCGAACTCTGTCT--GAAGAT-TGTAGTC-TGAGT------
P_roseopurpureum_AF455492           --------TGTGAACGCTGTCT--GAAG-T-TGCAGTC-TGAGA------
                                                                              *

Entomocorticium_sp.E_AF119505       ------TGCGT------------TTAACTCGC----ACAAATACAACTTT
Entomocorticium_sp.D_AF119503       ------TGCGT------------TTAACTCGC----ACAAATACAACTTT
Entomocorticium_sp.C_AF119510       ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Entomocorticium_sp.B_AF119508       ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Entomocorticium_sp.H_AF119512       ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Entomocorticium_sp.G_AF119512       ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Peniophora_rufa_AF119516            ------TGCGT------------T-AACTCGC----ACATATATAACTTT
Peniophora_pithya_AF119521          ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Peniophora_piceae_AF119515          ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Peniophora_pseudo-pini_AF119514     ------TGCGT------------T-AACTCGC----ACAAATATAACTTT
Peniophora_aurantiaca_AF119517      ------TGCGT------------T-AACTCGC----ATAAATACAACTTT
Peniophora_aurantiaca_AF210829      ------TGCGT------------T-A-CTCGC----ACAAATACAACTTT
Peniophora_aurantiaca_AF210824      ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Peniophora_aurantiaca_AF210820      ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Peniophora_aurantiaca_AF210821      ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Peniophora_aurantiaca_AF210822      ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Peniophora_aurantiaca_AF210825      ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Peniophora_aurantiaca_AF210827      ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Peniophora_aurantiaca_AF210828      ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Peniophora_aurantiaca_AF210823      ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Peniophora_duplex_AF119519          ------TGCGT------------T-GACTCGC----ACAAATACAACTTT
Peniophora_cinerea_AF119518         ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Peniophora_nuda_AF119513            ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Peniophora_aurantiaca_AF210819      ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
P_aurantiaca_CBS_396.50             ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
UD4                                 ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Amanita_tenuifolia_AF085492         ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Entomocorticium_spF_AF119507        ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Peniophora_pithya_AF119520          ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
E._dendroctoni_AF119506             ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Entomocorticium_spA_AF119509        ------TGCGT------------T-AACTCGC----ACAAATACAACTTT
Dendrophora_albobadia_AF119522      ------TGCGT------------T-AACTCGC----ACAAATATAACTTT
An_citrinella_AF126879              ---TCATGCAT------------C-AATGCAT----A-TAATACAACTTT
Antrodiella_pallasii_AF126895       ---TCATGCAT------------C-AATGCAT----A-TAATACAACTTT
Antrodiella_faginea_AF126886        ---TCATGCAT------------C-AATGCAT----A-TAATACAACTTT
Antrodiella_romellii_AF126902       ---TCATGCAT------------T-AATGCAT----A-TAATACAACTTT
An_parasitica_AF126898              ---TCATGCGT------------C-AATGCAT----A-TAATACAACTTT
Antrodiella_faginea_AF126884        ---TCATGCAT------------C-AATGCAT----A-TAATACAACTTT
An_onychoides_AJ006674              ---TCATGCAT------------T-AATGCATT---A-TAATACAACTTT
An_semisupina_AF126905              ---TCATGCGT------------C-AATGCAT----A-TAATACAACTTT
An_beschidica_AF126878              ---TCATGCGT------------C-AATGCAT----A-TAATACAACTTT
Antrodiella_hoehnelii_AF126893      ---TCATGCTT------------T-AATGCAT----A-TAATACAACTTT
Antrodiella_americana_AF126877      ---TCATGCAT------------C-AATGCAT----A-TAATACAACTTT
Amanita_spissa_AF085486             AGGCTTTTGTCA----GCCTTT--AAATGA-T----A-AAATACAACTTT
Amanita_aspera_AF085485             AGGCTCTTGTCA----GCCTTC--AAATGA-TC---A-AAATACAACTTT
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Amanita_rubescens_AF085484          AGGCTTTCGTCA----GCGTTT--AAATGA-T----A-AAATACAACTTT
Amanita_citrina_AF085489            AGGTTTTTAATAAAAAGCCGTT--AAATGAAT---AA-AGCTACAACTTT
Amanita_virosa_AB015676             ------TTGATTA------------AAT--AT----A-AAATACAACTTT
Amanita_phalloides_AJ308097         ------TTGATTA------------AAT--AT----A-AAGTACAACTTT
Amanita_fulva_AF085494              -----TGTGTA-----------T-AACACATTT----ATATACAACTTT
Au_alborubescens_AJ006683           -----TGGATT------------T-AATGGAATT----AAATACAACTTT
Trichaptum_abietinum_U63474         ----TTTGCTA------------T-AACGCAATT----ATATACAACTTT
Bjerkandera_adjusta_AF455410        ----TTTGCTA------------T-AACGCAATTT---ATATACAACTTT
Bjerkandera_adusta_AJ006672         ----TTTGCTA------------T-AACGCAATTT---ATATACAACTTT
Bjerkandera_fumosa_AJ006673         ----TTTGCTA------------T-AACGCAATT----ATATACAACTTT
Polyporus_brumalis_AB070876         -------GCGT-----------CT-AACGCATCT----ATATACAACTTT
Skeleto_kuehneri_AJ006678           -------GCGCTATT------AAT-AACGCATG-----AAATATAACTTT
Gloeo_abietinum_AJ420948            -------GCGTT-----------T-AACGCATCT----ATAAACAACTTT
Gloeo_abietinum_AJ420947            -------GCGTT-----------T-AACGCATCT----ATAAACAACTTT
Gloeophyllum_trabeum_AJ420950       -------GCGTA-----------T-AACGCATCT----ATAAACAACTTT
Gloeophyllum_trabeum_AJ420949       -------GCGTA-----------T-AACGCATCT----ATAAACAACTTT
Gloeo_sepiarium_AJ420946            -------GCGTA-----------T-AACGCATTA----TAAAACAACTTT
Skeletocutis_nivea_AJ006679         -----GCGCTTA---------AAT-AACGCA--TT---ATATATAACTTT
Ganoderma_adspersum_AJ006685        -------GCGA----------TGT-AACGCATCT----ATATACAACTTT
Donkioporia_expansa_AJ249500        -------GCGAA---------CAT-AACGCATTT----ATATACAACTTT
Trametes_trogii_AJ438139            -------GCGA----------TGT-AACGCATCT----ATATACAACTTT
Phlebia_subserialis_AB084620        -------GCG-T----------CT-AACGCATG-----AAATACAACTTT
Phlebia_uda_AB084621                -------GCG-T----------AT-AACGCATT-----AAATACAACTTT
Phlebia_radiata_AB084619            -------GCATA----------AT-AA-GCA-AAT---AAATACAACTTT
Polyporus_arcularius_AB070865       ------CGCGTC-----------T-AACGCATCT----ATATACAACTTT
Polyporus_ciliatus_AB070883         ------CGCGTG-----------T-AACGCATCT----ATATRCAACTTT
Polyporus_tricholoma_AJ132942       -------GCGAT----------GT-AACGCATCT----ATATACAACTTT
Polyporus_tricholoma_AB070885       -------GCGAT----------A--AACGCATCT----ATATACAACTTT
Trichaptum_biforme_U63476           -------TCTCG----------TTAGGCGAAACTT---AAATACAACTTT
Postia_balsamea_AJ006666            ------TGCGTG-----------T-AACGCATCT----TTATACAACTTT
Postia_sericeomollis_AJ006667       ------CGCGTG-----------T-AACGCATCT----TTATACAACTTT
Postia_hibernica_AJ006665           ------CGCGTG-----------T-AACGCATCT----TTATACAACTTT
Postia_lactea_AJ006664              ------TGCGTG-----------T-AACGCATTT----ATATACAACTTT
Di_lindbladii_AJ006682              -------GCGAT-----------AAACGCATTT----TAATACAACTTT
Oligoporus_placentus_AJ249267       -------GCGTA-----------T-AACGCATTA----TAATATAACTTT
Antrodia_xantha_AXA415569           -------GCGTC-----------T-AACGCATCT----TA-TACAACTTT
Antrodia_serialis_AJ345010          -------GCGTG-----------T-AACGCATTT----ATATACAACTTT
Antrodia_albida_AJ006680            -------GCGTC-----------T-AACGCATTA----TA-TACAACTTT
Antrodia_sinuosa_AJ416068           -------GCGTG-----------T-AACGCATT-----TAATACAACTTT
Fomitopsis_rosea_AJ415546           -------GCGTG-----------T-AACGCATTT----AAATACAACTTT
Fomitopsis_pinicola_AJ560638        -------GCGTC-----------T-AACGCATTT----AAATACAACTTT
Polyporus_arcularius_AB070868       -------GCGTC-----------T-AACGCATCT----ATATACAACTTT
Polyporus_ciliatus_AB070881         -------GCGHG-----------H-AACGCATCT----ATATACAACTTT
Aspergillus_niger_AF455522          -------GATTG-------------AATGCAATC----AGTTAAAACTTT
Aspergillus_niger_AJ280006          -------GATTG-------------AATGCAATC----AGTTAAAACTTT
Penicillium_commune_AF455544        -------GAAAA-------------TATA-AATT----ATTTAAAACTTT
P_chrysogenum_AF034451              -------GAAAA-------------TATA-AATT----ATTTAAAACTTT
P_roseopurpureum_AF455492           -------AACTA-------------GCTA-AATT----AGTTAAAACTTT
                                                                          ******

Entomocorticium_sp.E_AF119505       CAACAACGGATCTCTTGCGTCTCGCATC
Entomocorticium_sp.D_AF119503       CAACAACGGATCTCTTGCGTCTCGCATC
Entomocorticium_sp.C_AF119510       CAACAACGGATCTCTTGCGTCTCGCATC
Entomocorticium_sp.B_AF119508       CAACAACGGATCTCTTGCGTCTCGCATC
Entomocorticium_sp.H_AF119512       CAACAACGGATCTCTTGGCTCTCGCATC
Entomocorticium_sp.G_AF119512       CAACAACGGATCTCTTGCGTCTCGCATC
Peniophora_rufa_AF119516            CAACAACGGATCTCTTGCGTCTCGCATC
Peniophora_pithya_AF119521          CAACAACGGATCTCTTGCGTCTCGCATC
Peniophora_piceae_AF119515          CAACAACGGATCTCTTGCGTCTCGCATC
Peniophora_pseudo-pini_AF119514     CAACAACGGATCTCTTGCGTCTCGCATC
Peniophora_aurantiaca_AF119517      CAACAACGGATCTCTTGCGTCTCGCATC
Peniophora_aurantiaca_AF210829      CAACAACGGATCTCTTGGCTCTCGCATC
Peniophora_aurantiaca_AF210824      CAACAACGGATCTCTTGGCTCTCGCATC
Peniophora_aurantiaca_AF210820      CAACAACGGATCTCTTGGCTCTCGCATC
Peniophora_aurantiaca_AF210821      CAACAACGGATCTCTTGGCTCTCGCATC
Peniophora_aurantiaca_AF210822      CAACAACGGATCTCTTGGCTCTCGCATC
Peniophora_aurantiaca_AF210825      CAACAACGGATCTCTTGGCTCTCGCATC
Peniophora_aurantiaca_AF210827      CAACAACGGATCTCTTGGCTCTCGCATC
Peniophora_aurantiaca_AF210828      CAACAACGGATCTCTTGGCTCTCGCATC
Peniophora_aurantiaca_AF210823      CAACAACGGATCTCTTGGCTCTCGCATC
Peniophora_duplex_AF119519          CAACAACGGATCTCTTGCGTCTCGCATC
Peniophora_cinerea_AF119518         CAACAACGGATCTCTTGCGTCTCGCATC
Peniophora_nuda_AF119513            CAACAACGGATCTCTTGCGTCTCGCATC
Peniophora_aurantiaca_AF210819      CAACAACGGATCTCTTGGCTCTCGCATC
P_aurantiaca_CBS_396.50             CAACAACGGATCTCTTGGCTCTCGCATC
UD4                                 CAACAACGGATCTCTTGGCTCTCGCATC
Amanita_tenuifolia_AF085492         CAACAACGGATCTCTTGGCTCTCGCATC
Entomocorticium_spF_AF119507        CAACAACGGATCTCTTGCGTCTCGCATC
Peniophora_pithya_AF119520          CAACAACGGATCTCTTGCGTCTCGCATC
E._dendroctoni_AF119506             CAACAACGGATCTCTTGCGTCTCGCATC
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Entomocorticium_spA_AF119509        CAACAACGGATCTCTTGCGTCTCGCATC
Dendrophora_albobadia_AF119522      CAACAACGGATCTCTTGCGTCTCGCATC
An_citrinella_AF126879              CAGCAACGGATCTCTTGGCTCTCGCATC
Antrodiella_pallasii_AF126895       CAGCAACGGATCTCTTGGCTCTCGCATC
Antrodiella_faginea_AF126886        CAGCAACGGATCTCTTGGCTCTCGCATC
Antrodiella_romellii_AF126902       CAGCAACGGATCTCTTGGCTCTCGCATC
An_parasitica_AF126898              CAGCAACGGATCTCTTGGCTCTCGCATC
Antrodiella_faginea_AF126884        CAGCAACGGATCTCTTGGCTCTCGCATC
An_onychoides_AJ006674              CAGCAACGGATCTCTTGGCTCTCGCATC
An_semisupina_AF126905              CAGCAACGGATCTCTTGGCTCTCGCATC
An_beschidica_AF126878              CAGCAACGGATCTCTTGGCTCTCGCATC
Antrodiella_hoehnelii_AF126893      CAGCAACGGATCTCTTGGCTCTCGCATC
Antrodiella_americana_AF126877      CAGCAACGGATCTCTTGGCTCTCGCATC
Amanita_spissa_AF085486             CAACAACGGATCTCTTGGCTCTCGCATC
Amanita_aspera_AF085485             CAACAACGGATCTCTTGGCTCTCGCATC
Amanita_rubescens_AF085484          CAACAACGGATCTCTTGGCTCTCGCATC
Amanita_citrina_AF085489            CAACAACGGATCTCTTGGCTCTCGCATC
Amanita_virosa_AB015676             CAACAACGGATCTCTTGGCTCTCGCATC
Amanita_phalloides_AJ308097         CAACAACGGATCTCTTGGCTCTCGCATC
Amanita_fulva_AF085494              CAGCAACGGATCTCTTGGCTCTCGCATC
Au_alborubescens_AJ006683           TAGCAACGGATCTCTTGGCTCTCGCATC
Trichaptum_abietinum_U63474         CAGCAACGGATCTCTTG-CTCTCGCATC
Bjerkandera_adjusta_AF455410        CAGCAACGGATCTCTTGGCTCTCGCATC
Bjerkandera_adusta_AJ006672         CAGCAACGGATCTCTTGGCTCTCGCATC
Bjerkandera_fumosa_AJ006673         CAGCAACGGATCTCTTGGCTCTCGCATC
Polyporus_brumalis_AB070876         CAGCAACGGATCTCTTGGCTCTCGCATC
Skeleto_kuehneri_AJ006678           CAGCAACGGATCTCTTGGCTCTCGCATC
Gloeo_abietinum_AJ420948            CAACAACGGATCTCTTGGCTCTCGCATC
Gloeo_abietinum_AJ420947            CAACAACGGATCTCTTGGCTCTCGCATC
Gloeophyllum_trabeum_AJ420950       CAACAACGGATCTCTTGGCTCTCGCATC
Gloeophyllum_trabeum_AJ420949       CAACAACGGATCTCTTGGCTCTCGCATC
Gloeo_sepiarium_AJ420946            CAACAACGGATCTCTTGGCTCTCGCATC
Skeletocutis_nivea_AJ006679         TAGCAACGGATCTCTTGGCTCTCGCATC
Ganoderma_adspersum_AJ006685        CAGCAACGGATCTCTTGGCTCTCGCATC
Donkioporia_expansa_AJ249500        CAGCAACGGATCTCTTGGCTCTCGCATC
Trametes_trogii_AJ438139            CAGCAACGGATCTCTTGGCTCTCGCATC
Phlebia_subserialis_AB084620        CAGCAACGGATCTCTTGGCTCTCGCATC
Phlebia_uda_AB084621                CAACAACGGATCTCTTGGCTCTCGCATC
Phlebia_radiata_AB084619            CAGCAACGGATCTCTTGGCTCTCGCATC
Polyporus_arcularius_AB070865       CAGCAACGGATCTCTTGGCTCTCGCATC
Polyporus_ciliatus_AB070883         CAGCAACGGATCTCTTGGCTCTCGCATC
Polyporus_tricholoma_AJ132942       CAGCAACGGATCTCTTGGCTCTCGCATC
Polyporus_tricholoma_AB070885       CAGCAACGGATCTCTTGGCTCTCGCATC
Trichaptum_biforme_U63476           CAACAACGGATCTCTTGGCTCTCGCATC
Postia_balsamea_AJ006666            CAGCAACGGATCTCTTGGCTCTCGCATC
Postia_sericeomollis_AJ006667       CAGCAACGGATCTCTTGGCTCTCGCATC
Postia_hibernica_AJ006665           CAGCAACGGATCTCTTGGCTCTCGCATC
Postia_lactea_AJ006664              CAGCAACGGATCTCTTGGCTCTCGCATC
Di_lindbladii_AJ006682              CAGCAACGGATCTCTTGGCTCTCGCATC
Oligoporus_placentus_AJ249267       CAGCAACGGATCTCTTGGCTCTCGCATC
Antrodia_xantha_AXA415569           CAGCAACGGATCTCTTGGCTCTCGCATC
Antrodia_serialis_AJ345010          CAGCAACGGATCTCTTGGCTCTCGCATC
Antrodia_albida_AJ006680            CAGCAACGGATCTCTTGGCTCTCGCATC
Antrodia_sinuosa_AJ416068           CAGCAATGGATCTCTTGGCTCTCGCATC
Fomitopsis_rosea_AJ415546           CAGCAACGGATCTCTTGGCTCTCGCATC
Fomitopsis_pinicola_AJ560638        CAGCAACGGATCTCTTGGCTCTCGCATC
Polyporus_arcularius_AB070868       CAGCAACGGATCTCTTGGCTCTCGCATC
Polyporus_ciliatus_AB070881         CAGCAACGGATCTCTTGGCTCTCGCATC
Aspergillus_niger_AF455522          CAACAATGGATCTCTTGGTTCCGGCATC
Aspergillus_niger_AJ280006          CAACAATGGATCTCTTGGTTCCGGCATC
Penicillium_commune_AF455544        CAACAACGGATCTCTTGGTTCCGGCATC
P_chrysogenum_AF034451              CAACAACGGATCTCTTGGTTCCGGCATC
P_roseopurpureum_AF455492           CAACAACGGATCTCTTGGTTCCGGCATC
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Appendix 2B: Alignment of 18s rDNA ITS2 sequences

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CLUSTAL X (1.81) multiple sequence alignment


Amanita_aspera_AF085485              TTGG--ACA-------TTGGGAGTT---GCCGGC-CGCT----------GATAAAGT---
Amanita_rubescens_AF085484           TTGG--ACA-------TTGGGAGTT---GCCGGC-TGCT----------GATAAAGT---
Amanita_spissa_AF085486              TTGG--ACA-------TTGGGAGTT---GCCGGC-CGCT----------GATAAAGT---
Amanita_citrina_AF085489             TTGG--ACA-------TTGGGAGTT---GCTGGT-CACT----------GATAAAGT---
Amanita_phalloides_AJ308097          TGG---ATT------TTTGGGGGTT---GCAGGCT-GTTTC-------AAATAAAAAT--
Gloeophyllum_abietinum_AJ420948      -------TGGG----CTTGGA--TTT--GGGGGTTTGCT-----------GG-CTG-ACT
Gloeophyllum_abietinum_AJ420947      -------TGGG----CTTGGA--TTT--GGGGGTTTGCT-----------GG-CTG-ACT
Gloeophyllum_trabeum_AJ420949        -------TGGG----CTTGGA--CTT--GGAGGTATGCT-----------GG-CTTTACT
Gloeophyllum_sepiarium_AJ420946      -------TGGG----CTTGGA--CTT--GGAGGTATGCT-----------GG-CTG-ACT
Postia_balsamea_AJ006666             -------GG------CTTGGACTTGG---AGGCTTATG-CT----------TT-CTTAAC
Postia_hibernica_AJ006665            -------GGGAAG-GCTTGGACTTGG---AGGCTTGTG-CT----------TTCGTTTGA
Postia_lactea_AJ006664               -------TTGGA---CTTGGG--GGT---TTTGCTGGTGT-CT----------------
Gloeophyllum_trabeum_AJ420950        -------TGGG----CTTGGA--CTT--GGAGGTATGCT-----------GG-CTTTACT
Antrodia_albida_AJ006680             -------TTGGA---TTTGGA--GGC---CA---TTGCC-----------GGATTGTAAT
Skeletocutis_nivea_AJ006679          -------A-GCCC-TCTTC--ATCTT---TTTTGCAGG------------TTTAGTAAT
Skeletocutis_kuehneri_AJ006678       -------T-GGAT-TT--GGA--GGT---CTTTGCGGG------------TTTCACTGT
Postia_sericeomollis_AJ006677        -------T-GGAT-T---GGA--GGT---TTTTGCGGT------------TTT-----T
Trichaptum_biforme_U63476            -------T-GGAC-TT--GGA--GGT---TCG--T-GCT------------GGCTGC---
Diplomitoporus_lindbladii_AJ006682   --------TGGA---CTTGGGGGTCT----GC--CG--GCC----------TGTCCC---
Amanita_tenuifolia_AF085492          -------GGGA-----TGGGA--TTT--GGGAGCTTGCG-----------GGTCCCTGGC
UD4                                  -------GTGA---GCTGGGA--TTT--GGGAGCTTGCG-----------GGTCCCTGGC
Amanita_fulva_AF085494               AGCGGGCTTGGA---CTTGGAGGT-----TGTG-TC--GGC----------CCTTGTCG--
Amanita_virosa_AB015676              TGG---ATT------TTTGGGGGTTT--GCAGGCT-GTTTC-------AGATA-------
Entomocorticium_sp.H_AF119512        -------TTGG---GCTGGGA--TTT--GGGAGCTTGCG-----------GGTCCCTGGC
Entomocorticium_sp.A_AF119509        -------TTGG---ACTGGGA--TTT--GGGAGCTTGCG-----------GGTCCCTGGC
Entomocorticium_sp.B_AF119508        -------TTGG---GCTGGGA--TTT--GGGAGCTTGCG-----------GGTCCCTGGC
E._dendroctoni_AF119506              -------TTGG---GCTGGGA--TTT--GGGAGCTTGCG-----------GGTCCCTGGC
Entomocorticium_sp.G_AF119511        -------TTGG---GCTGGGA--TTT--GGGAGCTTGCG-----------GGTCCCTGGC
Entomocorticium_sp.F_AF119507        -------TTGG---GCTGGGA--TTT--GGGAGCTTGCG-----------GGTCCCTGGC
Entomocorticium_sp.E_AF119505        -------TTGG---GCTGGGA--TTT--GGGAGCTTGCG-----------GGTCCCTGGC
Entomocorticium_sp.D_AF119503        -------TTGG---GCTGGGA--TTT--GGGAGCTTGCG-----------GGTCCCTGGC
Entomocorticium_sp.C_AF119510        -------TTGG---GCTGGGA--TTT--GGGAGCTTGCG-----------GGTCCCTGGC
Peniophora_pithya_AF119520           -------TTGG---GCTGGGA--TTT--GGGAGCTTGCG-----------GGTCCCTGGC
Dendrophora_albobadia_AF119522       -------TTGG---GCTGGGA--TTT--GGGAGCTTGCA-----------GGTCCCTGGT
Peniophora_piceae_AF119515           -------TTGG---GCTGGGA--CCC--GGGAGCTTGCG-----------GGTCCCTGGC
Peniophora_cinerea_AF119518          -------TTGG---GCTGGGA--TTT--GGGAGCTTGCG-----------GGTCCCTGGC
Peniophora_nuda_AF119513             -------TTGG---GCTGGGA--TTT--GGGAGCTTGCG-----------GGTCCCTGGC
Peniophora_duplex_AF119519           -------TTGG---GCTGGGA--TTT--GGGAGCATGCG-----------GGTCCCTGGC
Peniophora_pseudo-pini_AF119514      -------TTGG---GCTGGGA--TTT--GGGAGCATGCG-----------GGTCCCTGGC
Phlebia_serialis_AF141629            -------TCGG---GCTGGGA--ATT--GGGAGCTTGCG-----------GGTCCCTGGT
Peniophora_rufa_AF119516             -------TCGG---GCTGGGA--ATT--GGGAGCTTGCG-----------GGTCCCTGGT
Peniophora_pithya_AF119521           -------TCGG---GCTGGGA--TTT--GGGAGCTTGCG-----------GGTCCCTGGC
Peniophora_aurantiaca_AF119517       -------TTGG---GCTGGGA--TTT--GGGAG-TTGCG-----------GGTCCCTGGC
Peniophora_aurantiaca_AF210825       -------TTGG---GCTGGGA--TTT--GGGAGC-TGCG-----------GGTCCCTGGC
Peniophora_aurantiaca_AF210827       -------TTGG---GCTGGGA--TTT--GGGAGC-TGCG-----------GGTCCCTGGC
Peniophora_aurantiaca_AF210823       -------TTGG---GCTGGGA--TTT--GGGAGC-TGCG-----------GGTCCCTGGC
Peniophora_aurantiaca_AF210821       -------TTGG---GCTGGGA--TTT--GGGAGC-TGCG-----------GGTCCCTGGC
Peniophora_aurantiaca_AF210824       -------TTGG---GCTGGGA--TTT--GGGAGC-TGCG-----------GGTCCCTGGC
Peniophora_aurantiaca_AF210822       -------TTGG---GCTGGGA--TTT--GGGAGC-TGCG-----------GGTCCCTGGC
Peniophora_aurantiaca_AF210820       -------TTGG---GCTGGGA--TTT--GGGAGC-TGCG-----------GGTCCCTGGC
Peniophora_aurantiaca_AF210819       -------TTGG---GCTGGGA--TTT--GGGAGC-TGCG-----------GGTCCCTGGC
Peniophora_aurantiaca_AF210828       -------TTGG---GCTGGGA--TTT--GGGAGC-TGCG-----------GGTCCCTGGC
Antrodia_xantha_AJ415569             -------TTGGA---------GGTTT----CTGCTGG------------CTC-TTCATT
Antrodia_sinuosa_AJ416068            -------TTGGA---------GGTTA----TTGCCGG------------CTT-GTCAT-
Antrodia_serialis_AJ345010           -------TTGGA---------GGT-A----TTGCTGG------------C-----CGTA
Oligoporus_placentus_AJ249267        -------TTGGA---TTTGGAGGTTT---AT-GCTGG------------CTT-G-TAAT
Donkioporia_expansa_AJ249500         -------TTGGA---CTTGGAGGCTT----GT--CGGC-----------TTT------A
Polyporus_ciliatus_AB070883          -------TTGGA---CTTGGAGGTTT---G---TCGG------------CTCAT-TAG-
Polyporus_ciliatus_AB070881          -------TTGGA---CTTGGAGGTTT---G---TCGG------------CTCAT-TAG-
Polyporus_brumalis_AB070876          -------TTGGA---CTTGGAGGCTT---G---CCGG------------CTC-T-TAG-
Fomitopsis_rosea_AJ415546            -------TTGGA---CTTGGAGG-TT--ATT-GCCGG-------------ACTTTAT-
Fomitopsis_pinicola_AJ560638         -------TTGGA---CTTGGAGGTTT---ATTGCC--------------GGTACCTGTG
Polyporus_arcularius_AB070868        -------TTGGA---CTTGGAGGCTT------GTCGG------------CTGCT-TAG-
Polyporus_arcularius_AB070865        -------TTGGA---CTTGGAGGCTT------GTCGG------------CT--C-TAG-
Ganoderma_adspersum_AJ006685         -------TTGGA---CTTGGAGGCTT----GT--CGGC-----------CTT----TAA
Trametes_trogii_AJ438139             -------TTGGA---CTTGGAAGGCT-----TGTCGGGCGA-CC------GTGAA-GGTC
Antrodiella_semisupina_AF126905      -------TTGGA---CTTGGAGGTTT---ATTGCTGGC-AT-TCAA---CTTGTTTGA--
Antrodiella_beschidica_AF126878      -------TTGGA---CTTGGAGGTTT---ATTGCTGGC-AT-TCAA---CTTGTTTGA--
Antrodiella_parasitica_AF126898      -------TTGGA---CTTGGAGGTTT---ATTGCTGGT-GT-TC------TCTTTTGA--
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Antrodiella_pallasii_AF126895        -------TTGGA---CTTGGAGGTTT---ATTGCTGGT-GT-TC------TCTTTTGA--
Antrodiella_faginea_AF126884         -------TTGGA---CTTGGAGGTTT---ATTGCTGGT-GT-TC------TCTTTTGA--
Antrodiella_pallasii_AF126886        -------TTGGA---CTTGGAGGTTT---ATTGCTGGT-GT-TC------TCTTTTGA--
Antrodiella_hoehnelii_AF126893       -------TTGGA---CTTGGAGGTTTT--ATTGCTGGC-GT-ACAAGCTTTTGTTTGA--
Antrodiella_reomellii_AF126902       -------TTGGA---CTTGGAGGT-----ATTGCCGGT-GT-TC------TCTTTTGA--
Antrodiella_citrinella_AF126879      -------TTGGA---CTTGGAGGTTT---ATTGCTGGT-GT-T------CTCTTTTGA--
Antrodiella_americana_AF126877       -------TTGGA---CTTGGAGGTATC--ACTGCTGGT-GT-TCA-------TTTTGA--
Bjerkandera_adusta_AF455410          -------TTGGA---CTTGGAGGT-----CGTGCCG---G-----------CTCTCG--
Trichaptum_abietinum_U63474          -------TTGGA---CTTGGAGGT-----CGTGTCG---G-----------CTCTCG--
Antrodiella_onchyoides_AJ006674      -------TTGGA---CTTGGAGGTTT---ATTGCTGGT-GT-TC-----TTTTTTTGA--
Bjerkandera_adusta_AJ006672          -------TTGGA---CTTGGAGGT-----CGTGCCG---GC----------TCTCG--
Bjerkandera_fumosa_AJ006673          -------TTGGA---CTTGGAGGT-----CGTGTCG---GC----------TCTCG--
Phlebia_radiata_AB084619             -------TTGGA---CTTGGAGGT-----CGTGTCG---GC----------GTTC---
Aurantiporus_alborubescens_AJ006683  -------TTGGA---TTTGGAGGT-----AGTGTTGGT--T----------TGCAA--
Phlebia_subserialis_AB084620         -------TTGGA---TGTGGAGGT-----CGTGTTGG-----------------CGC--
Phlebia_uda_AB084621                 -------GTGGA---CTTGGAGGT---C-TGTGTCG-GCT-------------CGCAA--
Penicillium_commune_AF455544         -------TTGTGTG--TTGGGCC-CC--GTCCTCCGATCCC-------------------
Penicillium_chrysogenum_AF034451     -------TTGTGTG--TTGGGCC-CC--GTCCTCCGATCCC-------------------
Aspergillus_niger_AF455522           -------TTGTGTG--TTGGGTCGCC--GTCCCCCTCTCCG-------------------
Aspergillus_niger_AJ280006           -------TTGTGTG--TTGGGTCGCC--GTCCCCCTCTCCG-------------------
Penicillium_roseopurpureum_AF455492  -------TTGTGTG--TTGGGCCCCC--GCCCCCCCGCGCTGG-----------------


Amanita_aspera_AF085485              ----GGTGGGCTCTTCTGA-AAAGCATTAGTTG----AGGAGCTTT-----GCA----C-
Amanita_rubescens_AF085484           ----GGTGGGCTCTTCTGA-AAAGCATTAGTTG----AGGAGCTTT-----GCA----C-
Amanita_spissa_AF085486              ----GGTGGGCTCTTCTGA-AAAGCATTAGTTG----AGGAGCTTT-----GCA----C-
Amanita_citrina_AF085489             ----GGCTGGCTCTTCTGA-AAAGCATTAGTTG----AGGAGCTTTT---TGTG----C-
Amanita_phalloides_AJ308097          ---AGCCTTGCTCTCTTTG-AATGTATTAGTGG--AGAAAAGCCA----TTGAA----C-
Gloeophyllum_abietinum_AJ420948      A-G-TC--GGCTCCCCTCTAAATGTATTAGCTG------AACCCGTT----GTGGA--TC
Gloeophyllum_abietinum_AJ420947      A-G-TC--GGCTCCCCTCTAAATGTATTAGCTG------AACTCGTT----GTGGA--TC
Gloeophyllum_trabeum_AJ420949        A-G-TC--GGCTCCTCTTTAAATGTATTAGCTG------GACCCGTT----ACGGA--TC
Gloeophyllum_sepiarium_AJ420946      A-G-TC--GGCTCCTCTCTAAATGCATTAGCTG------GACCTGTT----ATGGA--TC
Postia_balsamea_AJ006666             GGGATC-GG-CTCCTCTTG-AATGCATTAGCTT-----GAACCTATG----CTGTA--TC
Postia_hibernica_AJ006665            CCGATC-GG-CTCCTCTTG-AATGCATTAGCTT-----GAACCTATG----CTGTA--TC
Postia_lactea_AJ006664               -G--CC-AG-CTCCTCTTG-AATGCATTAGCTT-----GAACCTCTG----CTGTA--TC
Gloeophyllum_trabeum_AJ420950        A-G-TC--GGCTCCTCTTTAAATGTATTAGCTG------GACCCGTTA----CGGA--TC
Antrodia_albida_AJ006680             GAC-TC-GG-CTCCTCTTG-AATGCATTAGCTC-----GAATCTTTG----C-GGA--TC
Skeletocutis_nivea_AJ006679          AAA-TC-TG-CTCCTCTTG-AATGTATTAGCTG----GAAATCTTT-----GCTCA-GGT
Skeletocutis_kuehneri_AJ006678       AAA-TC-TG-CTCCTCTTG-AATACATTAGCTA----GAAACCTTT-----TGTTA--GG
Postia_sericeomollis_AJ006677        CAAAT--TG-CTCCTCTTG-AATGCATTAGCTG----GAA-CCTTT-----GGACC--GG
Trichaptum_biforme_U63476            AAAGTC-GG-CTCCTCTTG-AATGCATTAGCTT----GGA-CCTGT-----GCGCG--TT
Diplomitoporus_lindbladii_AJ006682   -------GG-CTCCCCTTG-AATGCATTAGCTC----GGA-CCCTT-----GCGGA--TC
Amanita_tenuifolia_AF085492          C-GATC--CGCTCTCCTTG-AATGAATTAGC-------GAAGCCCTT---TGCGG---CC
UD4                                  C-GATC--CGCTCTCCTTG-AATACATTAGC-------GAAGCCCTT----GCGG---CC
Amanita_fulva_AF085494               ---GTC-GA-CTCCTCTGA-AATGCATTAGCGT-----GAATCTTAC----GGA------
Amanita_virosa_AB015676              ----GC-TTGCTCTCCTTG-AATGTATTAGTGG-AGAAAGAGCC----ATTGA----AC-
Entomocorticium_sp.H_AF119512        C-GATC--CGCTCTCCTTG-AATACATTAGT-------GAAGCCCTT----GCGG---CC
Entomocorticium_sp.A_AF119509        C-GATC--CGCTCTCCTTG-AATACATTAGT-------GAAGCCCTT----GCGG---CC
Entomocorticium_sp.B_AF119508        C-GATC--CGCTCTCCTTG-AATACATTAGT-------GGAGCCCTT----GCGG---CC
E._dendroctoni_AF119506              C-GATC--CGCTCTCCTTG-AATACATTAGT-------GAAGCCCTT----GCGG---CC
Entomocorticium_sp.G_AF119511        C-GATC--CGCTCTCCTTG-AATACATTAGT-------GAAGCCCTT----GCGG---CC
Entomocorticium_sp.F_AF119507        C-GATC--CGCTCTCCTTG-AATACATTAGC-------GAAGCCCTT----GCGG---CC
Entomocorticium_sp.E_AF119505        C-GATC--CGCTCTCCTT-GAATACATTAGC-------GAAGCCCTT----GCGGC--C-
Entomocorticium_sp.D_AF119503        C-GATC--CGCTCTCCTT-GAATACATTAGC-------GAAGCCCTT----GCGGC--C-
Entomocorticium_sp.C_AF119510        C-GATC--CGCTCTCCTT-GAATACATTAGC-------GAAGCCCTT----GCGGC--C-
Peniophora_pithya_AF119520           C-GATC--CGCTCTCCTT-GAATACATTAGT-------GAAGCCCTT----GCGGC--C-
Dendrophora_albobadia_AF119522       T-GATC--CGCTCTCCTT-GAATACATTAGC-------GAAGCCCTTC---GCGGC--C-
Peniophora_piceae_AF119515           C-GATC--CGCTCTCCTT-GAATACATTAGC-------GAAGCCCTT----GCGGC--C-
Peniophora_cinerea_AF119518          C-GATC--CGCTCTCCTT-GAATACATTAGC-------GAAGCCCTT----GCGGC--C-
Peniophora_nuda_AF119513             C-GATC--CGCTCTCCTT-GAATACATTAGC-------GAAGCCCTT----GCGGC--C-
Peniophora_duplex_AF119519           G-GATC--CGCTCTCCTT-GAATGCATTAGC-------GAAGCCCTTT---GCGGC--C-
Peniophora_pseudo-pini_AF119514      T-GATC--CGCTCTCCTT-GAATGCATTAGC-------GAAGCCCTT----GCGGC--C-
Phlebia_serialis_AF141629            C-GATC--CGCTCTCCTT-GAATACATTAGC-------GAASCC-TT----GCGGC--C-
Peniophora_rufa_AF119516             C-GATC--CGCTCTCCTT-GAATACATTAGC-------GAAGCCCTT----GCGGC--C-
Peniophora_pithya_AF119521           C-GATC--CGCTCTCCTT-GAATACATTAGC-------AGAGCCCTC----GCGGC--C-
Peniophora_aurantiaca_AF119517       C-GATC--CGCTCTCCTT-GAATACATTAGC-------GAAGCCCTTT---GCGGC--C-
Peniophora_aurantiaca_AF210825       C-GATC--CGCTCTCCTT-GAATACATTAGC-------GAAGCCCTTT---GCGGC--C-
Peniophora_aurantiaca_AF210827       C-GATC--CGCTCTCCTT-GAATACATTAGC-------GAAGCCCTTT---GCGGC--C-
Peniophora_aurantiaca_AF210823       C-GATC--CGCTCTCCTT-GAATACATTAGC-------GAAGCCCTTT---GCGGC--C-
Peniophora_aurantiaca_AF210821       C-GATC--CGCTCTCCTT-GAATACATTAGC-------GAAGCCCTTT---GCGGC--C-
Peniophora_aurantiaca_AF210824       C-GATC--CGCTCTCCTT-GAATACATTAGC-------GAAGCCCTTT---GCGGC--C-
Peniophora_aurantiaca_AF210822       C-GATC--CGCTCTCCTT-GAATACATTAGC-------GAA-CCCT---TTGCGGC--C-
Peniophora_aurantiaca_AF210820       C-GATC--CGCTCTCCTT-GAATACATTAGC-------GAAGCCCT---TTGCGGC--C-
Peniophora_aurantiaca_AF210819       C-GATC--CGCTCTCCTT-GAATACATTAGC-------GAAGCCCT---TTGCGGC--C-
Peniophora_aurantiaca_AF210828       C-GATC--CGCTCTCCTT-GAATACATTAGC-------GAAGCCCT---TTGCGGC--C-
Antrodia_xantha_AJ415569             G-AATC--AGCTCCTCTT-GAATGCATTAGCTT-----GAA-CCTT----TGTGGAA-T-
Antrodia_sinuosa_AJ416068            G-AGTCT-GGCTCCTCTT-GAATTCATTAGCTT-----GAA-CCCT----TGTGGAT-C-
Antrodia_serialis_AJ345010           C-GGTC--GGCTCCTCTT-GAATTTATTAGCTC-----GAG-CCTT----TGTGGAT-C-
Oligoporus_placentus_AJ249267        G-AGTC--GGCTCCTCTT-GAATGCATTAGCTT-----GGA-CCTT----TGCGGAT-C-
Donkioporia_expansa_AJ249500         T-AGTC--GGCTCCTCTT-AAATGCATTAGCTT-----GAT-TCCT----TGCGGAT-C-
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Polyporus_ciliatus_AB070883          C-AGTC--GGCTCCTCTC-AAATGCATTARCTT-----GGTTCCT-----TGCGGAT-C-
Polyporus_ciliatus_AB070881          C-AGTC--GGCTCCTCTC-AAATGCATTAGCTT-----GGTTCCT-----TGCGGAT-C-
Polyporus_brumalis_AB070876          C-AGTC--GGCTCCTCTC-AAATGCATTAGCTT-----GGTTCCT-----TGCGGAT-C-
Fomitopsis_rosea_AJ415546            --TGTC--GGCTCCTCTT-GAATGCATTAGCTC-----GAG-CCTTT----GTGGAT-C-
Fomitopsis_pinicola_AJ560638         ---ATC--GGCTCCTCTT-GAATGCATTAGCTC-----GAA-CCTTT---TGTGGAT-C-
Polyporus_arcularius_AB070868        C-AGTC--GGCTCCTCTC-AAATGCATTAGCTT-----GGTTCCT-----TGCGGAT-C-
Polyporus_arcularius_AB070865        C-AGTC--GGCTCCTCTC-AAATGCATTAGCTT-----GGTTCCT-----TGCGGAT-C-
Ganoderma_adspersum_AJ006685         C-GGTC--GGCCCCTCTT-AAATGCATTAGCTT-----GATTTCCT----TGCGGAT-C-
Trametes_trogii_AJ438139             A-CATC--GACTCCTCTC-AAATGCATTAGCTT-----GATTCCT-----TGCGGAT-C-
Antrodiella_semisupina_AF126905      -ACGCG-GG-CTCCTCTG-AAATGCATTAGCTG-----GAATGTTA--CC-GAGC-----
Antrodiella_beschidica_AF126878      -ACGCG-GG-CTCCTCTG-AAATGCATTAGCTG-----GAATGTTA--CC-GAGC-----
Antrodiella_parasitica_AF126898      -ACGCG-GG-CTCCTCTG-AAATGCATTAGCTG-----GAATGTTA--CC-GAGC-----
Antrodiella_pallasii_AF126895        -ACGCG-GG-CTCCTCTG-AAATGCATTAGCTG-----GAATGTTA--CC-GAGC-----
Antrodiella_faginea_AF126884         -ACGCG-GG-CTCCTCTG-AAATGCATTAGCTG-----GAATGTTA--CC-GAGC-----
Antrodiella_pallasii_AF126886        -ACGCG-GG-CTCCTCTG-AAATGCATTAGCTG-----GAATGTTA--CC-GAGC-----
Antrodiella_hoehnelii_AF126893       -ACGCG-GG-CTCCTCTG-AAATGCATTAGCTG-----GAATGTTA--CC-GAGC-----
Antrodiella_reomellii_AF126902       -ACGCG-GG-CTCCTCTG-AAATGCATTAGCTG-----GAATGTTA--CC-GAGC-----
Antrodiella_citrinella_AF126879      -ACGCG-GG-CTCCTCTG-AAATGCATTAGCTG-----GAATGTTA--CT-GAGC-----
Antrodiella_americana_AF126877       -ACGCG-GG-CTCCTCTG-AAATGCATTAGCTG-----GAATTTTA--CT-GAGC-----
Bjerkandera_adusta_AF455410          -TAGTC-GG-CTCCTCTG-AAATGCATTAG-TGC----GAACGTTA--CC--GACC----
Trichaptum_abietinum_U63474          -TAGTC-GG-CTCCTCTG-AAATGCATTAG-TGC----GAANGTTA--CC--AGCC----
Antrodiella_onchyoides_AJ006674      -ACATT-GG-CTCCTCTG-AAATGCATTAGCTG-----GAATGTTA--CC-GAGC-----
Bjerkandera_adusta_AJ006672          -TAGTC-GG-CTCCTCTG-AAATGCATTAG-TGC----GAACGTTA--CC--AACC----
Bjerkandera_fumosa_AJ006673          -TAGTC-GA-CTCCTCTG-AAATGCATTAG-TGC----GAACGTTA--CC--AGCC----
Phlebia_radiata_AB084619             -GCGTC-GG-CTCCTCTG-AAATGCATTAG-CGT----GAATCTTA--CG--AATC----
Aurantiporus_alborubescens_AJ006683  -AAGTC-AA-CTCCTCTG-AAATGTATTAG-TGT----GAACCTTA--CG-GATC-----
Phlebia_subserialis_AB084620         -AAGTC-AA-CTCCTCTG-AAATGCATTAG-TGT----GAATCTTA--CG-GATC-----
Phlebia_uda_AB084621                 -GAGCG-GA-CTCCTCTT-AAATGCATTAG-CGT----GAACCTGA--CG-GATCG----
Penicillium_commune_AF455544         GGGGGACGGGCCC----G-AAAGGCA-GCGGCG-----GCACCGCGT----CCGGTC---
Penicillium_chrysogenum_AF034451     GGGGGACGGGCCC----G-AAAGGCA-GCGGCG-----GCACCGCGT----CCGGTC---
Aspergillus_niger_AF455522           GGGGGACGGGCCC----G-AAAGGCA-GCGGCG-----GCACCGCGT----CCGATC---
Aspergillus_niger_AJ280006           GGGGGACGGGCCC----G-AAAGGCA-GCGGCG-----GCACCGCGT----CCGATC---
Penicillium_roseopurpureum_AF455492  GGGGG-CGGGCCC----G-AAAGGCA-GCGGCG-----GCACCGCGT----CCGGTC---
                                                     * *       **   *

Amanita_aspera_AF085485              ---TCTATT-GGTGTGATAGA--CTATC-TATGCCAAGA--GACGC-TTCATGA------
Amanita_rubescens_AF085484           ---TCTATT-GGTG--ATAAA--CTATC-TATGCCAGGA--GAAGC-TTCATGA------
Amanita_spissa_AF085486              ---TCTATT-GGTGTGATAGA--CTATC-TATGCCAGGA--GATGC-TTCATGA------
Amanita_citrina_AF085489             ---TCTATT-GGTGTGATAGA--CTATC-TATGCCAGGA--GAAGCATTAATGA------
Amanita_phalloides_AJ308097          ---TCCATT-GGTGTGATAAAACCTATC-AATGCCAGGA-GC-AATATCACT--------
Gloeophyllum_abietinum_AJ420948      ---AGTACTCGGTGTGATAAT---TGTC-TACGCCGTGTCTGTGAAGCGCCTTA------
Gloeophyllum_abietinum_AJ420947      ---AGTACTCGGTGTGATAAT---TGTC-TACGCCGTGTCTGTGAAGCGCCTTA------
Gloeophyllum_trabeum_AJ420949        ---AGTACTCGGTGTGATAAT---TGTC-TACGCCGTGTCTGTGAAGTGCCTTA------
Gloeophyllum_sepiarium_AJ420946      ---AGTACTCGGTGTGATAAT---TGTC-TACGCCGTGTCTGTGAAGTGC-TTGA-----
Postia_balsamea_AJ006666             G--GCTGTTCGGTGTGATAAT---TGTC-TACGCCGTGGCTGTGAGGCCCGTTGA-----
Postia_hibernica_AJ006665            G--GCTGTTCGGTGTGATAAT---TGTC-TACGCCGTGGCTGTGAGGCTA--TA------
Postia_lactea_AJ006664               G--GCTTCAACGTG--ATAAT---TGTC-TACGTTGTGGCTGAGAGGCGCTCCGTCGT--
Gloeophyllum_trabeum_AJ420950        ---AGTACTCGGTGTGATAAT---TGTC-TACGCCGTGTCTGTGAAGTGCCTTA------
Antrodia_albida_AJ006680             A--GCTTTT-GGTGTGATAAT---TGTC-TACGCCGCTAACT-GAGATGCATGA------
Skeletocutis_nivea_AJ006679          CTAGCT--TCAGTGTGATA-A--TTGTC-TACGC--TGTAGCTGTCTAAGCA----C---
Skeletocutis_kuehneri_AJ006678       CTGGCT--TCAGTGTGATA-A--TTGTC-TGCGC--TGTAGTCGTCTA-GCA----C---
Postia_sericeomollis_AJ006677        ---CCT--TCGGTGTGATA-A--CTATC-TACGCCGTGGTC--ACCGG------------
Trichaptum_biforme_U63476            T--GCT--AGCGTGTAATACA--TTTTA-TTCACCACGGGCC-GTGTCAC----------
Diplomitoporus_lindbladii_AJ006682   A--GCTA-TCGGTGTGATA-A--TTGTC-TACGCCGTGGTTT-GTGATGCC--ACCTT--
Amanita_tenuifolia_AF085492          --------TTGGTGTGATA-G--TCATC-TACGCCTCGGCTTAGC-GAACCTAA------
UD4                                  --------TTGGTGTGATA-G--TCATC-TACGCCTCGGCTTAGC-GAACCTA-------
Amanita_fulva_AF085494               --TCGCCTTCAGTGTGATAATTATCTGCGC-TGCGGTGTT-GAAGTATCA-TG-------
Amanita_virosa_AB015676              ---TCCATT-GGTGTGATAAAATCTATC-AATGCCAGGA-GC-CATGTTAGTT-------
Entomocorticium_sp.H_AF119512        --------TTGGTGTGATA-G--TCATC-TACGCCTCGGTTTAGC-GAACTTA-------
Entomocorticium_sp.A_AF119509        --------TTGGTGTGATA-G--TCATC-TACGCCTCGGCTTAGC-GAACTTA-------
Entomocorticium_sp.B_AF119508        --------TTGGTGTGATA-G--TCATC-TACGCCTCGGCTTAGC-GAACTTA-------
E._dendroctoni_AF119506              --------TTGGTGTGATA-G--TCATC-TACGCCTCGGCTTAGC-GAACTTA-------
Entomocorticium_sp.G_AF119511        --------TTGGTGTGATA-G--TCATC-TACGCCTCGGCTTAGC-GAACATATG-----
Entomocorticium_sp.F_AF119507        --------TTGGTGTGATA-G--TCATC-TACGCCTCGGCTTAGC-GAACGTA-------
Entomocorticium_sp.E_AF119505        --------TTGGTGTGATA-G--TCATC-TACGCCTCGGCTTAGC-GAACATA-------
Entomocorticium_sp.D_AF119503        --------TTGGTGTGATA-G--TCATC-TACGCCTCGGCTTAGC-GAACATA-------
Entomocorticium_sp.C_AF119510        --------TTGGTGTGATA-G--TCATC-TACGCCTCGGCTTAGC-GAACATA-------
Peniophora_pithya_AF119520           --------TTGGTGTGATA-G--TCATC-TACGCCTCGGCTTAGC-GAACATA-------
Dendrophora_albobadia_AF119522       --------TTGGTGTGATA-A--TTATC-TGCGCCTTGGCTTAGC-GAACCTA-------
Peniophora_piceae_AF119515           --------TTGGTGTGATA-G--TCATC-TACGCCTCGGCTTAGC-GAACATA-------
Peniophora_cinerea_AF119518          --------TTGGTGTGATA-G--TCATC-TACGCCTCGGTTTAGC-GAACATA-------
Peniophora_nuda_AF119513             --------TTGGTGTGATA-G--TCATC-TACGCCTCGGTTTAGC-GAACATA-------
Peniophora_duplex_AF119519           --------TTGGTGTGATA-A--TCATC-TACGCCTCGGTTTAGC-AAACTTA-------
Peniophora_pseudo-pini_AF119514      --------TTTGTGTGATA-A--TCATC-TACGCCTCGGTTTAGC-GAACATG-------
Phlebia_serialis_AF141629            --------TTGGTGTGATA-G--TCATC-TACGCCTCGGCTTAGC-GAACCCA-------
Peniophora_rufa_AF119516             --------TTGGTGTGATA-G--TCATC-TACGCCTCGGTTTAGC-GAACCCA-------
Peniophora_pithya_AF119521           --------TTGGTGTGATA-A--TCATC-TACGCCTCGGTTTAGC-GCATGAA-------
Peniophora_aurantiaca_AF119517       --------TTGGTGTGATA-G--TCATC-TACGCCTCGGCTTAGC-GAATCAA-------
Peniophora_aurantiaca_AF210825       --------TTGGTGTGATA-G--TCATC-TACGCCTCGGTTTAGC-GAATCAA-------
Peniophora_aurantiaca_AF210827       --------TTGGTGTGATA-G--TCATC-TACGCCTCGGTTTAGC-GAATCAA-------
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Peniophora_aurantiaca_AF210823      --------TTGGTGTGATA-G--TCATC-TACGCCTCGGTTTAGC-GAATCAA-------
Peniophora_aurantiaca_AF210821      --------TTGGTGTGATA-G--TCATC-TACGCCTCGGTTTAGC-GAATCAA-------
Peniophora_aurantiaca_AF210824      --------TTGGTGTGATA-G--TCATC-TACGCCTCGGTTTAGC-GAATCAA-------
Peniophora_aurantiaca_AF210822      --------TTGGTGTGATA-G--TCATC-TACGCCTCGGTTTAGC-GAATCAA-------
Peniophora_aurantiaca_AF210820      --------TTGGTGTGATA-G--TCATC-TACGCCTCGGTTTAGC-GAATCAA-------
Peniophora_aurantiaca_AF210819      --------TTGGTGTGATA-G--TCATC-TACGCCTCGGTTTAGC-GAATCAA-------
Peniophora_aurantiaca_AF210828      --------TTGGTGTGATA-G--TCATC-TACGCCTCGGTTTAGC-GAATAAA-------
Antrodia_xantha_AJ415569            --GGCTTATCGGTGTGATAA---TTGTC-TACGCCGTTGGCT-GT-GAAGCTCATACACT
Antrodia_sinuosa_AJ416068           --GGCTACTCGGTGTGATAA---TTGTC-TACGCCGTGTGCT-GT-GAAGCTTTCAAACA
Antrodia_serialis_AJ345010          --AGCT--TCGGTGTGATA-A--TTGTC-TACGCCGTT--CT-GT-GAAGCA---TAACT
Oligoporus_placentus_AJ249267       --AGCTTTTCGGTGTGATAA---TTGTC-TACGCCGTGGGCT-GT-GATGCTGTTTAACA
Donkioporia_expansa_AJ249500        --GGCTC-TCGGTGTGATA-A--TTGTC-TACGCCGCGA-CC-GT-GAAGC---------
Polyporus_ciliatus_AB070883         --GGCTC-ACGGTGTGATA-A--TTGTC-TACGCCGCGACCG-TT-GAAGC---------
Polyporus_ciliatus_AB070881         --GGCTC-ACGGTGTGATA-A--TTGTC-TACGCCGCGACCG-TT-GAAGC---------
Polyporus_brumalis_AB070876         --GGCTC-ACGGTGTGATA-A--TTGTC-TACGCCGCGACCG-TT-GAAGC---------
Fomitopsis_rosea_AJ415546           --AGC--TTCGGTGTGATA-A--TTGTC-TACGCCGTT--CT-GT-GAAGCATACATAA-
Fomitopsis_pinicola_AJ560638        --AGCTTATCGGTGTGATAAA--ATGTC-TACGCCGTTAC-T-GT-GAAGCATATTAT--
Polyporus_arcularius_AB070868       --GGCTC-ACGGTGTGATA-A--TTATC-TGCGCCGCGACCG-TT-GAAGC---------
Polyporus_arcularius_AB070865       --GGCTC-ACGGTGTGATA-A--TTATC-TGCGCCGCGACCG-TT-GAAGC---------
Ganoderma_adspersum_AJ006685        --GGCTG-TCGGTGTGATA----ATGTC-TACTCCGCGA-CC-GT-GAAGC---------
Trametes_trogii_AJ438139            --GGCTC-TCGGTGTGATA-A--TTGTC-TACGCCGTGA-CC-GT-GAAGC---------
Antrodiella_semisupina_AF126905     ---ATGATTCAATGTGATA-A--TTGTC-TACGT--TGCTT-CAACTTGGT--ATTAA--
Antrodiella_beschidica_AF126878     ---ATGATTCAATGTGATA-A--TTGTC-TACGT--TGCTT-CAACTTGGT--ATTAA--
Antrodiella_parasitica_AF126898     ---ATGATTCAATGTGATA-A--TTGTC-TATGT--TGCTT-CAACTTGGT--ATTAA--
Antrodiella_pallasii_AF126895       ---ATGATTCAATGTGATA-A--TTGTC-TACGT--TGCTT-CAACTTGGT--ATTAA--
Antrodiella_faginea_AF126884        ---ATGATTCAATGTGATA-A--TTGTC-TACAT--TGCTT-CAACTTGGT--ATTAA--
Antrodiella_pallasii_AF126886       ---ATGATTCAACGTGATA-A--TTGTC-TACAT--TGCTT-CAACTTGGT--ATTAA--
Antrodiella_hoehnelii_AF126893      ---ATGATTCAATGTGATA-A--TTGTC-TACGT--TGCTT-CAACTTGGT--ATTAA--
Antrodiella_reomellii_AF126902      ---ATGATTCAATGTGATA-A--TTGTC-TACGT--TGCTT-CAACTTGGT--ATTAA--
Antrodiella_citrinella_AF126879     ---ATGATTCAATGTGATA-A--TTGTC-TACGT--TGTTT-CAACTTGGT--GTTAG--
Antrodiella_americana_AF126877      ---ATGATTCAATGTGATA-A--TTGTC-TACGT--TGTCT-CAACTTGGT--ATTAAA-
Bjerkandera_adusta_AF455410         ---GC--TTCAGCGTGATA-A--TTATC-TGCGT--TGCTGTG--GAGGGT--ATTC---
Trichaptum_abietinum_U63474         ---GC--TTCAGCNNNATA-A--TTATC-TGCGT--TGCTGCG--GAGGGT--ATTC---
Antrodiella_onchyoides_AJ006674     ---ATGATTCAATGTGATA-A--TTGTC-TACGT--TGCTT-CATCTTTGT--ATTAA--
Bjerkandera_adusta_AJ006672         ---GC--TTCAGCGTGATA-A--TTATC-TGCGT--TGCTGTG--GAGGGT--ATTC---
Bjerkandera_fumosa_AJ006673         ---GC--TTCAGCGTGATA-A--TTATC-TGCGT--TGCTGTG--GAGGGT--ATGC---
Phlebia_radiata_AB084619            ---GCC-CTCGGTGTGATA-A---TTTCT-ACGCCGTAGACGTGCAGT--ATA-------
Aurantiporus_alborubescens_AJ006683 ---ACC-TTCAGTGTGATA-A--TTGTC-TACACTGTGGTGTTGAAGT--ATCT------
Phlebia_subserialis_AB084620        ---GCC-TCCAGTGTGATA-A--GTATC-TACGCTGTGGTGTTGAAGT--ATAT------
Phlebia_uda_AB084621                -----CCTTCAGTGTGATA-A--TTTTC-TGCGCTGTGGTGT-GAAGTC-AATT------
Penicillium_commune_AF455544        --CTCGAGCGTATGGGGC-----TTTGT---CACC-CGCTCT------------------
Penicillium_chrysogenum_AF034451    --CTCGAGCGTATGGGGC-----TTTGT---CACC-CGCTCT------------------
Aspergillus_niger_AF455522          --CTCGAGCGTATGGGGC-----TTTGT---CACA-TGCTCT------------------
Aspergillus_niger_AJ280006          --CTCGAGCGTATGGGGC-----TTTGT---CACA-TGCTCT------------------
Penicillium_roseopurpureum_AF455492 --CTCGAGCGTATGGGGC-----TTCGT---CACC-CGCTCTT-----------------


Amanita_aspera_AF085485             --------------TCCC-TCTGCTGTC---TAACTGCC-TTTATCG-------------
Amanita_rubescens_AF085484          --------------TCC--TCTGCTATC---TAACTGC---TTATC--------------
Amanita_spissa_AF085486             --------------TCC--TCTGCCATC---TAACTGTC-TTTATT-------------
Amanita_citrina_AF085489            -------------AGCC--TCTGCTGTC---TAACAGTC-GTAATT-------------
Amanita_phalloides_AJ308097         --------------TCTC-TCTGCTGTC---TAACTGTG-ACTGTCTGTATAAATTTATA
Gloeophyllum_abietinum_AJ420948     --------------GTTG--AT-TGGCTTCT-AATCGTCTTC------------------
Gloeophyllum_abietinum_AJ420947     --------------GTGG--AT-TGGCTTCT-AATCGTCTTC------------------
Gloeophyllum_trabeum_AJ420949       --------------GTGG--AT-CAGCTGCT-AATCGTCTTC------------------
Gloeophyllum_sepiarium_AJ420946     --------------ATGG--AC-TAGCTTCT-AACCGTCTTC------------------
Postia_balsamea_AJ006666            ----AAGACGGTG-GTAGG-AT-CAGCTTCT-AACCGTCCCTG---------------C
Postia_hibernica_AJ006665           ----AAA-----TTGTAGG-AT-CAGCTTCG-AACCGTCCCTC----------------
Postia_lactea_AJ006664              ---------------AG--GTT-TGGCTTCT-AACGGTCTCTTTGAT-------------
Gloeophyllum_trabeum_AJ420950       --------------GTGG--AT-CAGCTGCT-AATCGTCTTC------------------
Antrodia_albida_AJ006680            --------------ATGGG-AT-CGGCTTAC-AATCGTCCTTGAA---------------
Skeletocutis_nivea_AJ006679         -------ATGTAT-G-GA--ATTCCGCTTCT-AGTTGTCTTTTAAAGA------------
Skeletocutis_kuehneri_AJ006678      -------TTGTAT-A-AAGGATTTTGCTTCTTAATCGTCTTTT----------------
Postia_sericeomollis_AJ006677       ---------GTGT-C-GAGGAT-CTGCTTAT-AATTGTCTCC------------------
Trichaptum_biforme_U63476           --------TAT-T--AG--GGT-CTGCTTCT-AATCGTCCTTAC----------------
Diplomitoporus_lindbladii_AJ006682  ---TCAGGTGT-T--GAGGGAT-CGGCTTCT-AATTGTCCCCCC----------------
Amanita_tenuifolia_AF085492         ---------------TGGGAGT-CG-CTTCT-AACAGTCTCGCA----------------
UD4                                 ---------------TGGGAAT-CG-CTTCC-AACCGTCTCGCA----------------
Amanita_fulva_AF085494              -------------GTGTT-CATGCTTC----GAACCGTCTCCTGCCG--------AGACA
Amanita_virosa_AB015676             -----C--------TCTC---TGCTGTC---TAACCGCT-AACAGTTGTCTGTA---AAA
Entomocorticium_sp.H_AF119512       --------------ATGGGACT-CG-CTTCC-AACCGTCTTGCA----------------
Entomocorticium_sp.A_AF119509       --------------ATGGGACT-CG-CTTCC-AACCGTCTCGCA----------------
Entomocorticium_sp.B_AF119508       --------------CCGGGACT-CG-CTTCC-AACCGTCTTGCA----------------
E._dendroctoni_AF119506             --------------CCGGGACT-TG-CTTCC-AACCGTCTCGCA----------------
Entomocorticium_sp.G_AF119511       -----------TA-CCGGGACT-CG-CTTCC-AACCGTCTCGCA----------------
Entomocorticium_sp.F_AF119507       --------------CCGGGACT-CG-CTTCC-AACCGTCTCGCA----------------
Entomocorticium_sp.E_AF119505       --------------TGGGGACT-CG-CTTCC-AACCGTCTCGCA----------------
Entomocorticium_sp.D_AF119503       --------------TGGGGACT-CG-CTTCC-AACCGTCTCGCA----------------
Entomocorticium_sp.C_AF119510       --------------CGGGGACT-TG-CTTCC-AACCGTCTCGCA----------------
Peniophora_pithya_AF119520          --------------CGGGGACT-CG-CTTCC-AACCGTCTCGCA----------------
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Dendrophora_albobadia_AF119522      --------------TAGGGACT-TG-CTTCC-AACCGTCTCGCA----------------
Peniophora_piceae_AF119515          ---------------TGGGAAT-CG-CTTCC-AACCGTCTCGCG----------------
Peniophora_cinerea_AF119518         ---------------CGGGAAT-CG-CTTCC-AACCGTCTCGCA----------------
Peniophora_nuda_AF119513            ---------------CGGGAAT-CG-CTTCC-AACCGTCTCGCA----------------
Peniophora_duplex_AF119519          ---------------CGGGAAT-CG-CTACC-AACCGTCTCGCA----------------
Peniophora_pseudo-pini_AF119514     ---------------AGGGAAT-CG-CTTAC-AGCCGTCTCGCA----------------
Phlebia_serialis_AF141629           ---------------CGGACGT-CG-CTTCC-AACCGTCTCGCA----------------
Peniophora_rufa_AF119516            ---------------CGGACAT-CG-CTTCC-AACCGTCTCGCA----------------
Peniophora_pithya_AF119521          ---------------CGGGCAT-TG-CTTCC-AACCGTCTCGCA----------------
Peniophora_aurantiaca_AF119517      --------------TGGGGAAT-TG-CTTCC-AACCGTCTCGCA----------------
Peniophora_aurantiaca_AF210825      --------------TGGGGAAT-CG-CTTCC-AACCGTCTCGCA----------------
Peniophora_aurantiaca_AF210827      --------------TGGGGAAT-CG-CTTCC-AACCGTCTCGCA----------------
Peniophora_aurantiaca_AF210823      --------------TGGGGAAT-TG-CTTCC-AACCGTCTCGCA----------------
Peniophora_aurantiaca_AF210821      --------------TGGGGAAT-TG-CTTCC-AACCGTCTCGCA----------------
Peniophora_aurantiaca_AF210824      --------------TGGGGAAT-TG-CTTCC-AACCGTCTCGCA----------------
Peniophora_aurantiaca_AF210822      --------------TGGGGAAT-TG-CTT-CCAACCGTCTCGCA----------------
Peniophora_aurantiaca_AF210820      --------------TGGGGAAT-TG-CTT-CCAACCGTCTCGCA----------------
Peniophora_aurantiaca_AF210819      --------------TGGGGAAT-TG-CTT-CCAACCGTCTCGCA----------------
Peniophora_aurantiaca_AF210828      ---------------CGGGGAT-TG-CTT-CCAACCGTCTTGCA----------------
Antrodia_xantha_AJ415569            TCGGTG--TGAGG-------AT-CAGCTT-CTAATGGTCCTTCA----------------
Antrodia_sinuosa_AJ416068           CTTTGT--TGTGTT-GAGGGAT-TTGCTT-CTAATCGTCCTTCA----------------
Antrodia_serialis_AJ345010          ATG-------GGA-------CT-CGGCTT-CCAATAGTCCTTCA----------------
Oligoporus_placentus_AJ249267       TGCTTCGGCGTGTT--GGGGTT-CTGCTT-CTAATGGTCCCTTT----------------
Donkioporia_expansa_AJ249500        --GTTT----TG--------GC-GAGCTT-CTAACCGTCTCTTA----------------
Polyporus_ciliatus_AB070883         --GTTT--AATG--------GC-CAGCTT-CTAATCGTCTCTTG----------------
Polyporus_ciliatus_AB070881         --GTTTT-AATG--------GC-CAGCTT-CTAATCGTCTCTTG----------------
Polyporus_brumalis_AB070876         --GTTT--AATG--------GC-CAGCTT-CTAATCGTCTCTTG----------------
Fomitopsis_rosea_AJ415546           --------TGGGT-------TT-CGGCTTCC-AATCGTCCTTTG----------------
Fomitopsis_pinicola_AJ560638        --------TCGG--------------CTT-CCAATCGTCCTTCA----------------
Polyporus_arcularius_AB070868       --GTTT--AATG--------GC-CAGCTT-CTAATCGTCTCTTG----------------
Polyporus_arcularius_AB070865       --GTTT--AATG--------GC-CAGCTT-CTAATCGTCTCTTG----------------
Ganoderma_adspersum_AJ006685        --GTTT-----G--------GC-AAGCTT-CTAACCGTCTCGTT----------------
Trametes_trogii_AJ438139            --GTTT----TG--------GC-AAGCTT-CTAACCGTCTCTAA----------------
Antrodiella_semisupina_AF126905     -------TTCTGT--------TTCAGCTT-CTAACCGTCCCCTT----------------
Antrodiella_beschidica_AF126878     -------TTCTGT--------TTCAGCTT-CTAACCGTCCC-TT----------------
Antrodiella_parasitica_AF126898     -------TTCTGT--------TTCAGCTT-CAAACTGTCC---T----------------
Antrodiella_pallasii_AF126895       -------TTCTGT--------TTCAGCTT-CAAACCGTCC---T----------------
Antrodiella_faginea_AF126884        -------TTCTGT--------TTCAGCTT-CAAACCGTCCC--T----------------
Antrodiella_pallasii_AF126886       -------TTCTGT--------TTCAGCTT-CAAACCGTCCC--T----------------
Antrodiella_hoehnelii_AF126893      -------TTCTGT--------TTCAGCTT-CGAACTGTCCT--T----------------
Antrodiella_reomellii_AF126902      -------TTCTGT--------TTCAGCTT-CTAACCGTCCG------------------
Antrodiella_citrinella_AF126879     -------TTCTGT--------TTCAGCTT-CAAACCGTCCTT-----------------
Antrodiella_americana_AF126877      -------ATTTGT--------TTCAGCTT-CTAAACGTCTT------------------
Bjerkandera_adusta_AF455410         -------TAGTGT--------TCACGCTT-CTAACCGTC--------------------
Trichaptum_abietinum_U63474         -------TAGTGT--------TCGCGCTT-CTAACCGTCTT------------------
Antrodiella_onchyoides_AJ006674     -------TTCTGT--------TTCAGCTTTCTAATCGTCTT------------------
Bjerkandera_adusta_AJ006672         -------TAGTGT--------TCACGCTT-CTAACCGTCTT------------------
Bjerkandera_fumosa_AJ006673         -------TAGTGT--------TCGCGCTT-CTAACCGTCTT------------------
Phlebia_radiata_AB084619            -------TAGTGT--------TCGTGCTT-ACAACCGTCTG------------------
Aurantiporus_alborubescens_AJ006683 -------TAGTGT--------TTGTGCTT-CTAACTGTCTGT-----------------
Phlebia_subserialis_AB084620        -------AGGCAT--------TCATGCTT-CTAATCGTCTCTTC---------------
Phlebia_uda_AB084621                --------AGTGT--------TCGAGCTT-ACAACCGTCCGC----------------
Penicillium_commune_AF455544        -----GTAGGC-C----CGGCCGGCGCTTGCCGATCAACCC----------------
Penicillium_chrysogenum_AF034451    -----GTAGGC-C----CGGCCGGCGCTTGCCGATCAACCC----------------
Aspergillus_niger_AF455522          -----GTAGGA-T----TGGCCGGCGCCTGCCGACGTTTTC----------------
Aspergillus_niger_AJ280006          -----GTAGGA-T----TGGCCGGCGCCTGCCGACGTTTTC----------------
Penicillium_roseopurpureum_AF455492 -----GTAGGC-C----CGGCCGGCGCCAGCCGACCCCCCT----------------


Amanita_aspera_AF085485             ------AG-ACAAT--ATGATA-------A-CT--TGACCTCAAATC
Amanita_rubescens_AF085484          ------AG-ACAAT--ATGATA-------AACT--TGACCTCAAATC
Amanita_spissa_AF085486             ------GG-ACAAT--ATGATA-------AACT--TGACCTCAAATC
Amanita_citrina_AF085489            ------GG-ACAATT-ATGATA-------AACT--TGACCTCAAATC
Amanita_phalloides_AJ308097         TGGATGGGGACAACT--TGACC-------AACTT--GACCTCAAATC
Gloeophyllum_abietinum_AJ420948     ------GG-ACAATC-AT----TGAC---ATTTT--GACCTCAAATC
Gloeophyllum_abietinum_AJ420947     ------GG-ACAATC-AT----TGAC---ATTTT--GACCTCAAATC
Gloeophyllum_trabeum_AJ420949       ------GG-ACAATT-------TGAC---ATTTT--GACCTCAAATC
Gloeophyllum_sepiarium_AJ420946     ------GG-ACAACT-AT----TGAC---ATTTT--GACCTCAAATC
Postia_balsamea_AJ006666            GAACGGGACAACATCTTA--ATTGAC----CTCT--GACCTCAAATC
Postia_hibernica_AJ006665           ----GCGACAATATCTTC--ATTGAC----CTCT--GACCTCAAATC
Postia_lactea_AJ006664              ------GAGACATACAC---CTTTGA-C---CTCT-GGCCTCAAATC
Gloeophyllum_trabeum_AJ420950       ------GG-ACAGTT-------TGAC---ATT-T-TGACCTCAAATC
Antrodia_albida_AJ006680            ------GG-ACAATTTAT--TATGAC----TTCT--GACCTCAAATC
Skeletocutis_nivea_AJ006679         CAGATTGTAATGATC---TTTTTGAC---ATCT---GACCTCAAATC
Skeletocutis_kuehneri_AJ006678      ---CTTAAGACAATC-----T-TGAC---ATCT---GACCTCAAATC
Postia_sericeomollis_AJ006677       -------GGACAAACTTTTCTGAC-----ATCT---GACCTCAAATC
Trichaptum_biforme_U63476           -----CGG-ACAATAATAAACTTTAT-G--ACTCT-GACCTCAAATC
Diplomitoporus_lindbladii_AJ006682  -----CGGGACAATTAA---CTTTTT-A--AATCT-GACCTCAAATC
Amanita_tenuifolia_AF085492         -----AGGGACAAC-ACA--TACC-----AACT--TGACCTCAAATC
UD4                                 -----AGAGACAACGACT--TACC-----AACT--TGACCTCAAATC
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Amanita_fulva_AF085494              ATCTCCG--ACAATC--------------------TGAGCTCAAATC
Amanita_virosa_AB015676             AAAA-TG-GACAACT--TGACC-------AACT--TGACCTCAAATC
Entomocorticium_sp.H_AF119512       -----AGAGACAACTAC---TACC-----AACT---GACCTCAAATC
Entomocorticium_sp.A_AF119509       -----AGAGACAACTAC---TACC-----AACT---GACCTCAAATC
Entomocorticium_sp.B_AF119508       -----AGAGACAACTAC---TACC-----AACT---GACCTCAAATC
E._dendroctoni_AF119506             -----AGAGACAACTAC---TACC-----AACT---GACCTCAAATC
Entomocorticium_sp.G_AF119511       -----AGAGACAACTAC---TACC-----AACT---GACCTCAAATC
Entomocorticium_sp.F_AF119507       -----AGAGACAACTAC---TACC-----AACT---GACCTCAAATC
Entomocorticium_sp.E_AF119505       -----AGAGACAACTAC---TACC-----AACT---GACCTCAAATC
Entomocorticium_sp.D_AF119503       -----AGAGACAACTAC---TACC-----AACT---GACCTCAAATC
Entomocorticium_sp.C_AF119510       -----AGAGACAACTAC---TACC-----AACT---GACCTCAAATC
Peniophora_pithya_AF119520          -----AGAGACAATCAC---TTCA-----A-CT---GACCTCAAATC
Dendrophora_albobadia_AF119522      -----AGAGACAATCAC---TTCA-----AACT---GACCTCAAATC
Peniophora_piceae_AF119515          -----AGAGACAACTAC---TACG-----AACT---GACCTCAAATC
Peniophora_cinerea_AF119518         -----AGAGACAATCAC---TTCA-----AACT---GACCTCAAATC
Peniophora_nuda_AF119513            -----AGAGACAATCAC---TTCA-----AACT---GACCTCAAATC
Peniophora_duplex_AF119519          -----AGAGACAATCAC---TTCA-----AACT---GACCTCAAATC
Peniophora_pseudo-pini_AF119514     -----AGAGACATTGAC---T-CC-----AACT---GACCTCAAATC
Phlebia_serialis_AF141629           -----AGAGACAACGACC--TACC-----AACT---GACCTCAAATC
Peniophora_rufa_AF119516            -----AGAGACAATCAC---TACC-----AACT---GACCTCAAATC
Peniophora_pithya_AF119521          -----AGGGACAACGAC---TACC-----AACT---GACCTCAAATC
Peniophora_aurantiaca_AF119517      -----AGAGACAACGAC---TT-------AACT---GACCTCAAATC
Peniophora_aurantiaca_AF210825      -----AGGGACAACGAC---TTTT-----AACT--TGACCTCAAATC
Peniophora_aurantiaca_AF210827      -----AGGGACAACGAC---TTTT-----AACT--TGACCTCAAATC
Peniophora_aurantiaca_AF210823      -----AGAGACAACGAC---TTAT-----AACT--TGACCTCAAATC
Peniophora_aurantiaca_AF210821      -----AGAGACAACGAC---TTAT-----AACT--TGACCTCAAATC
Peniophora_aurantiaca_AF210824      -----AGAGACAACGAC---TTAT-----AACT--TGACCTCAAATC
Peniophora_aurantiaca_AF210822      -----AGAGACAACGAC---TTAT-----AACT--TGACCTCAAATC
Peniophora_aurantiaca_AF210820      -----AGAGACAACGAC---TTAT-----AACT--TGACCTCAAATC
Peniophora_aurantiaca_AF210819      -----AGAGACAACGAC---TTAT-----AACT--TGACCTCAAATC
Peniophora_aurantiaca_AF210828      -----AGAGATAACGAC---TTTT-----AACT--TGACCTCAAATC
Antrodia_xantha_AJ415569            -----ATGGACAAT------CTTTG----ACCTC-TGGCCTCAAATC
Antrodia_sinuosa_AJ416068           -----GTGGACAAT------TTTTG----ACCTC-TGACCTCAAATC
Antrodia_serialis_AJ345010          -----CTGGACAATA--T--CTTTG----ACCTT-TGACCTCAAATC
Oligoporus_placentus_AJ249267       GG---TGGGACAACTT-A--CTTTG----ACCTC-TGACCTCAAATC
Donkioporia_expansa_AJ249500        -----CGAGACAATTA-----CTTG----ACATC-TGACCTCAAATC
Polyporus_ciliatus_AB070883         -----CGAGACAGCAT-T--CATCG----AACTC-TGACCTCAAATC
Polyporus_ciliatus_AB070881         -----CGAGACAGCAT-T--CATCG----AACTC-TGACCTCAAATC
Polyporus_brumalis_AB070876         -----CGAGACAGCAT-T--CATCG----AACTC-TGACCTCAAATC
Fomitopsis_rosea_AJ415546           -----CGGGACAATACAC---TATG----ACCTT-TGACCTCAAATC
Fomitopsis_pinicola_AJ560638        -----CGGGACAATAAC----TTTG----ACCTT-TGACCTCAAATC
Polyporus_arcularius_AB070868       -----CGAGACAGCTT-T--CATCG----AACTC-TGACCTCAAATC
Polyporus_arcularius_AB070865       -----CGAGACAGCTT-T--CATCG----AACTC-TGACCTCAAATC
Ganoderma_adspersum_AJ006685        AC---AGAGACAGCT-----TTATG----ACCTC-TGACCTCAAATC
Trametes_trogii_AJ438139            -----CGAGACAGCTT-A--CTTTG----ACCTC-TGACCTCAAATC
Antrodiella_semisupina_AF126905     --TGCGGG-ACAA-----TATCTTG-AACATCT---GACCTCAAATC
Antrodiella_beschidica_AF126878     --TGCGGG-ACAA-----TATCTTG-AACATCT---GACCTCAAATC
Antrodiella_parasitica_AF126898     --TGCGG--ACAA-----TATCTTG-AACATCT---GACCTCAAATC
Antrodiella_pallasii_AF126895       --TGCGG--ACAA-----TATCTTG-AACATCT---GACCTCAAATC
Antrodiella_faginea_AF126884        --TGCGG--ACAA-----TATCTTG-AACATCT---GACCTCAAATC
Antrodiella_pallasii_AF126886       --TGCGG--ACAA-----TATCTTG-AACATCT---GACCTCAAATC
Antrodiella_hoehnelii_AF126893      --CACCGG-ACAA-----TACTTTGTAACATCT---GACCTCAAATC
Antrodiella_reomellii_AF126902      --CAAGG--ACAA-----TATCTTG-AACATCT---GACCTCAAATC
Antrodiella_citrinella_AF126879     --TGCGG--ACAA-----TATCTTG-AACATCT---GACCTCAAATC
Antrodiella_americana_AF126877      --CACAG--ACAA-----TATCT-G-AACATCT---GACCTCAAATC
Bjerkandera_adusta_AF455410         --TTCGG--ACAAA----TTTCT-G--AACTCT---GAGCTCAAATC
Trichaptum_abietinum_U63474         ----CGG--ACAA---A-TTTCT-G--AACTCT---GAGCTCAAATC
Antrodiella_onchyoides_AJ006674     --TACAG--ACAA-----TATCTTG-AACATCT---GACCTCAAATC
Bjerkandera_adusta_AJ006672         ----CGG--ACAA---A-TTTCT-G--AACTCT---GAGCTCAAATC
Bjerkandera_fumosa_AJ006673         ----CGG--ACAAC--A-TTTCT-A--AACTCT---GAGCTCAAATC
Phlebia_radiata_AB084619            -----CGAAG--ACAAC--ATCATTG-ACAATCT--GACCTCAAATC
Aurantiporus_alborubescens_AJ006683 -----ATAGAC-A-AG---TTACTTG-ACAATCT--GACCTCAAATC
Phlebia_subserialis_AB084620        ----ATTGAG--ACAA---TTACTTG-ACAATCT--GACCTCAAATC
Phlebia_uda_AB084621                ------AAGG--ACAA---TTACTTG-ACAATCT--GACCTCAAATC
Penicillium_commune_AF455544        ----------AAAT--TTTTATCC-----AGGTT--GACCTCGGATC
Penicillium_chrysogenum_AF034451    ----------AAAT--TTTTATCC-----AGGTT--GACCTCGGATC
Aspergillus_niger_AF455522          ----------CAACCATTCTTTCC-----AGGTT--GACCTCGGATC
Aspergillus_niger_AJ280006          ----------CAACCATTCTTTCC-----AGGTT--GACCTCGGATC
Penicillium_roseopurpureum_AF455492 ----------CAATCTATTTTTTC-----AGGTT--GACCTCGGATC
                                                                      *  ***  ***
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