The Laccase from *Micromonospora* sp. 044 30-1 as a Biocatalyst for Synthesis of Antioxidant Compounds

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THE LACCASE FROM *MICROMONOSPORA* SP. 044 30-1 AS A BIOCATALYST FOR SYNTHESIS OF ANTIOXIDANT COMPOUNDS

By

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Abstract

Laccases (EC 1.10.3.2) are blue multicopper oxidases that catalyse a single electron oxidation of various phenolic substrates with an associated four-electron reduction of dioxygen to water. The varied uses of laccase as a biocatalyst can be attributed to its ability to produce a free radical from a suitable substrate. Of importance to the pharmaceutical, chemical, and industrial sectors, are the laccase-catalysed reactions providing means for the synthesis of dimeric phenolics showing biological activity, including antioxidant activity. The objective of this research was to investigate the production of value-added compounds, with biological activity, *via* laccase-catalysed oxidation reactions.

Our laboratory has access to several unique and previously unexploited culture collections obtained from extreme environments spanning the globe. Potentially novel strains were screened for the ability to produce laccases: 14 environmental isolates, of which 2 strains were fungi, 7 were streptomycetes, and 5 were non-streptomycetes, representing the rare actinomycete genera *Gordonia*, *Rhodococcus*, *Mycobacterium*, *Amycolatopsis*, and *Micromonospora* were screened. This is the first report of laccase production in these species. A screening protocol, using criteria specifically suited to bioprocess development, was developed to investigate variables affecting the production of laccase by the native strains. Variables investigated included different types of media (nutritional variables), pH, temperature, incubation times, aeration and agitation, salt concentrations, and the effect of inducers on laccase production by the native strains.

Of the isolates investigated, actinomycete strain *Micromonospora* sp. 044 30-1 showed the greatest potential for the production of laccase. This strain may be novel and the role of laccase in this strain may be related to sporulation. Various growth requirements were investigated in order to optimise for maximal laccase production by strain 044 30-1. The optimal medium for laccase production was M172F medium, pH 5, supplemented with a high concentration of Cu^{2+} (8 mM), and 2.0% sodium chloride. Extracellular laccase production was higher than intracellular laccase production. The successful application of a *Micromonospora* strain in an airlift bioreactor specifically for the synthesis of laccase was demonstrated. The biocatalytic potential of the laccase from *Micromonospora* sp. 044 30-1 was investigated.

Laccases are responsible for the formation of radicals that can react non-enzymatically with each other to form dimers or oligomers linked by C-C or C-O bonds. This study reports on laccase-catalysed oxidative reactions involving, primarily, tyrosol, monoacetyltyrosol, and to a lesser extent, reactions with totarol, 3-hydroxyanthranilic acid, and 8-hydroxyquinoline. This study reports for the first time the isolation and structure determination of novel biocatalysis reaction products, specifically the dimeric products obtained through biocatalytic reaction of monoacetyltyrosol with *Trametes versicolor* laccase, and *Micromonospora* sp. 044 30-1 laccase.

The biocatalysis reaction products of the laccase-catalysed oxidation of tyrosol and monoacetylated tyrosol showed higher antioxidant activity than the parent compounds, as determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and low density lipoprotein (LDL) assays, showing that the dimeric derivatives of laccase-catalysed reactions with phenolic compounds have enhanced antioxidant capabilities.

The biocatalysis products were also evaluated as antimicrobials and showed antimicrobial activity against *E. coli*, a vancomycin resistant clinical strain of *Enterococcus faecium*, a clinically relevant strain of *Micrococcus*, and *Mycobacterium aurum*, a strain that displays a similar antibiotic susceptibility profile to *Mycobacterium tuberculosis*, the causative agent of the prevalent disease tuberculosis.

Abbreviations

3-HAA	3-hydroxyanthranillic acid
8-HQ	8-hydroxyquinoline
ALR	Air Lift Reactor
AO(s)	Antioxidant(s)
BM	Bennett's Agar
BMCO(s)	Blue multicopper oxidase(s)
CA	Cinnabarinic acid
COSY-NMR	Correlation spectroscopy NMR
Cu	Copper
DMP	Dimethoxyphenol
ESI-MS	Electrospray ionization-mass spectrometry
FA	Ferulic acid
ISP	International *Streptomyces* Project
LC-MS	Liquid chromatography-mass spectrometry
M172F	Modified m172F medium
MIC	Minimal inhibitory concentration
MCO	Multicopper oxidase
NMR	Nuclear magnetic resonance
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SCN	Starch-casein-nitrate
STR	Stirred Tank Reactor
T	Copper Type
TLC	Thin layer chromatography
TOF	Time of flight
WRF	White-rot fungi

Chapter 1
Review of the Literature

1.1 Introduction

Biocatalysis involves the utilization of natural catalysts, *i.e.* enzymes, to perform chemical transformations of organic compounds. Living, whole-cells producing enzyme, or isolated enzymes, are used in biocatalysis. The key advantage of biocatalysis is the specificity of the biocatalyst, which potentially results in high yields of a particular product (Burton, *et al.*, 2002). New biocatalytic processes are based on the availability of interesting, useful new enzymes, usually obtained by screening for microbes that carry out the desired target reaction (Schmid, *et al.*, 2001). Exploration of extreme environments can provide unique microbial culture collections that can be used in screening for suitable enzymes to perform a desired biocatalytic reaction (Schmid, *et al.*, 2001). These enzymes may then be used as biocatalysts in industrially relevant bioprocesses.

Laccases, the enzymes of interest in the current study, catalyse oxidation reactions, the products of which often possess useful biological activities. The majority of research previously reported on laccase has involved fungal enzymes. This review describes the common general characteristics of (not only fungal) laccases as well as the unique properties of these enzymes for biotechnological use, and provides discussion on the occurrence and significance of laccase in the natural environment. This study describes how fungal and actinomycete strains, derived from diverse environments, can be exploited as sources of potentially novel laccases to be used in biocatalysis reactions.

1.1.1 Biological novelty

New biocatalysis reactions can be based on the identification of desired products, after which a biocatalyst is then selected that converts available reactants (Schmid, *et al.*, 2001). The majority of enzymes currently being applied or developed for biocatalysis applications are microbial, and novel enzyme systems might be identified by focusing research on microbial strains that do not contain known systems (Burton, 2001). The structural characterization and engineering of these enzymes could provide more insight into enzymatic activation and may support the development of laccases for industrial applications (van Beilen and Funhoff, 2005).

1.2 Laccases: Blue multicopper oxidases with potential application as biocatalysts

Laccases (benzenediol:oxygen oxidoreductase or *p*-diphenol:dioxygen oxidoreductase; EC 1.10.3.2) are abundant in nature; plants and fungi, particularly **w**ood-**r**otting **f**ungi (WRF), are the best-known producers of laccases, but laccases also exist widely in bacteria. Laccases are **b**lue **m**ulti**c**opper **o**xidases (BMCO), belonging to the broad class of phenol oxidases. Other members of the BMCO group are plant ascorbate oxidase, mammalian plasma protein ceruplasmin, and **ph**enoxazinone

synthase (PHS) from the actinomycete *Streptomyces antibioticus* (Diamantidis, *et al.*, 2000). Laccases catalyse the oxidation of various phenolic substances, often to polymeric products, and these products frequently have useful applications. In the presence of redox mediators, the substrate range of laccases can be even wider. Of significance to the pharmaceutical, nutraceutical, and food industries, is the synthesis of dimeric and polymeric phenolics exhibiting biological activity, including antioxidant activity, which can be accomplished using laccases as biocatalysts. Most laccases have been isolated from fungal strains and are located extracellularly and/or intracellularly (at some stage of their development) in almost every fungus studied (Diamantidis, *et al.*, 2000). Generally, fungal laccases are described as copper-containing glycoproteins with varying carbohydrate content.

The physiological function of laccases, which can be secreted or may remain intracellular, is different in various microbes but they usually catalyse polymerisation or depolymerization reactions. It has been proposed that laccases are involved in the assembly of UV-resistant spores in bacterial species (Enguita, *et al.*, 2003). Often, substrates of interest cannot be oxidized directly by laccases, either because they are too large to fit into the enzyme active site or because they have a high redox potential. This limitation can be overcome by the addition of chemical mediators; these are compounds that act as intermediate substrates for the laccase, whose oxidized radicals are able to interact with the bulky or high redox-potential substrate targets (Canfora, *et al.*, 2008; Riva, 2006). A key characteristic to consider when using laccases is that a radical is the primary product of the reaction. It is generally considered that once formed, the radical product is released and is further transformed, depending on its own reactivity in the reaction medium (Tranchimand, *et al.*, 2006). Literature on the applications of laccases can be divided into two sets. In the first, laccases are used to oxidize a suitable chemical compound, which consequently acts as a mediator for the oxidation of a target substrate. The second set describes the direct oxidation of phenolic substrates to give dimers and oligomers. The main disadvantage of these biocatalytic reactions is the extensive polymerisation that may occur due to the radical mechanism of the oxidative process, producing a complex mixture of poly-phenolic oligomers (Nicotra, *et al.*, 2004).

1.3 Applications and uses of laccases

Currently, the catalytic properties of laccases are being exploited for a range of biotechnological applications, thus studies on laccase-producing organisms have been intensified (Niladevi and Prema, 2007). Laccases have attracted scientific attention due to their application in diverse industrial sectors such as the paper and pulp industry, removal of xenobiotics from waste streams, stabilization of fruit and vegetable juices, oxidation of phenolics in wine, denim washing, textile dye decolourisation, dye bleaching, lignin bleaching, and bleaching of cork for bottled wine (Mayer and Staples, 2002; Niladevi and Prema, 2007; Ergül, *et al.*, 2009). Most of the varied uses of laccase can be attributed to the ability of the enzyme to produce a free radical from a suitable substrate. The consequent secondary reactions are responsible for the versatility of laccases in producing many different products (Mayer and Staples, 2002).

Researchers have focused on the biochemical properties of laccases, on their applications in biotechnological and bioremediation processes, and their use in chemical reactions (Riva, 2006). The main limitation to the use of these 'green' enzymes (laccases use air and produce water as the only by-product) has been their limited availability (Nicotra, *et al.*, 2004). Increased knowledge of these enzymes will encourage laccase-based industrial bioprocesses in the future (Riva, 2006).

Oxidative biocatalytic systems can provide a means to controlled and predictable formation of polymers, a feat that remains challenging in organic synthetic chemistry, and thus laccases are receiving attention as an alternative to conventional synthetic chemical approaches (Burton, 2003). In addition to a wide substrate specificity, fungal laccases have other properties that make them useful for biotechnological application: Laccase does not require cofactors; its cosubstrate is oxygen, which is usually present in its environment; they are often produced extracellularly, making purification procedures easy; they are stable in the extracellular environment; and their inducible expression allows for their application in bioprocesses (Baldrian, 2006). The information available on the properties of fungal laccases can allow, to some extent, the selection of an enzyme suitable for a specific application (*e.g.* temperature-resistant or pH-stable).

1.4 Occurrence of laccases in fungi

Among fungal laccases, wide variability has been observed in the induction mechanism, degree of polymorphism, physico-chemical (molecular weight, isoelectric point, and carbohydrate glycosylation content; Table 1.1) and kinetic properties. Typical fungal laccases have acidic isoelectric points of around pH 4.0 (Solomon, *et al.*, 1996; Baldrian, 2006). In some fungal species, the addition of inducers to the culture medium results in the biosynthesis of new extracellular forms, and based on the literature, 2,5-xylidine seems to be the best laccase inducer (see, for example, Cordi, *et al.*, 2007; Bertrand, *et al.*, 2002; Galhaup, *et al.*, 2002; Eggert, *et al.*, 1996). Laccases produced by different fungi are distinctly different in terms of the number of isoforms, molecular weight, optimum pH, and specificity for the substrate. The redox potential of fungal laccases is independent of their species of origin and is in the range of 0.5 – 0.8V. Aeration requirements for laccase production are reported to vary from one fungal strain to another.

Table 1.1. Properties of fungal laccases (modified from Solomon, *et al.*, 1996).

Fungal Source:	Number of Subunits:	Molecular Weight (kDa):	Percent Carbohydrate:
Myceliophthora thermophila	2	160	14
Neurospora crassa	1	64 – 64.8	11 – 12
Phlebia radiata	1	64	11.8
Pleutrotus ostreatus	1	59 – 64	12.5
Podospora anserina			
I	4	390	20 – 23.7
II	1	70	22.7 – 24.9
III	1	80	22.7 – 23.1
Polyporus anisoporus	1	57.5	ND[a]
Polyporus pinsitus 1	2	126	7
Polyporus versicolor	1	64 – 65	10 – 12
Rhizoctonia solani 4	2	132	10
Scytalidium therophilum	1	75 – 80	ND

[a]ND, not determined.

Among taxonomic groups of fungi, laccases are generally produced by the WRF and related groups, *i.e.* species causing lignin degradation. The comparison of biochemical data reported for different fungal laccases, which would be important for biotechnological application, is problematic, as different experimental conditions have been used in different studies. There are only a few literature reports comparing laccase properties of enzymes from different sources and these comparisons are insufficient (Baldrian, 2006). Three possible functions have been attributed to fungal laccases: Pigment formation, lignin degradation, and detoxification (Solomon, *et al.*, 1996). Pigment formation is the most well-known role for certain fungal laccases. For example, laccase deficient mutants of *Aspergillus nidulans* produce yellow spores instead of the wild-type green spores (Solomon, *et al.*, 1996). Laccase is excreted by some fungi in order to remove potentially toxic phenols produced during lignin degradation.

Current knowledge concerning the structure and physico-chemical properties of fungal laccases has been based on the study of purified proteins, and >100 fungal laccases have been purified and characterized (Claus, 2003; Giardina, *et al.*, 2009). Based on literature, some general conclusions can be made about laccases, but it must be noted that most enzymes were purified from WRF and that other groups of fungi have been studied to a much lesser extent. The information on the molecular structure of laccases has mainly been derived from laccases isolated from higher fungi (Claus, 2003). The molecular weight of the monomer ranges in size from approximately 50 to 110 kDa. Laccases often occur as isoenzymes that oligomerize to form multimeric complexes. Like most fungal extracellular enzymes, laccases are glycoproteins. The extent of glycosylation ranges between 10% and 25% but laccases with a carbohydrate content >30% have been found (Claus, 2003; Solomon, *et*

al., 1996). In addition to the structural role, glycosylation can also protect the enzyme from proteolytic degradation. For the catalytic activity, a minimum of 4 copper atoms per active protein monomer is required. The copper atoms differ in their light absorbance and electron-paramagnetic behaviour, and three types of copper have been distinguished (Table 1.2). The three-dimensional structures of 5 fungal laccases and 1 bacterial laccase have been reported (Baldrian, 2006).

1.5 Copper centres of the laccase active site

Laccases are BMCOs, due to the presence of four copper ions coordinated at the active site of each enzyme monomer. These copper ions form one **T**ype **1** copper site, where the substrate is oxidised, one **T**ype **2** and two **T**ype **3** sites, which form the trinuclear oxygen-binding site (Solomon, *et al.*, 1996). The four Cu atoms have been classified according to their **e**lectron **p**aramagnetic **r**esonance (EPR) features: T1 or blue, T2 or normal (conferring no colour), and a T3 pair or coupled binuclear coppers that are antiferromagnetically (diamagnetically) coupled through a bridging ligand (EPR undetectable) (Table 1.2 and Figure 1.1). The T3 copper site is a pair of copper atoms that give a weak absorbance in the near UV range. Spectroscopy, combined with crystallography, has provided a detailed description of the active site of laccase. Magnetic circular dichroism and X-ray absorption spectroscopy of laccase have shown that the T2 and T3 centres combine to function as a trinuclear Cu cluster with respect to exogenous ligand interaction including reaction with dioxygen (Solomon, *et al.*, 1996). The T2 centre is 3-coordinate with two histidines and water as ligands, while the T3 coppers are each 4-coordinate having three histidines and bridging hydroxide ligands (Smith, *et al.*, 2006). The structural model of bridging between the T2 and T3 sites has provided insight into the catalytic reduction of oxygen to water. The T2 copper is required for the reduction of oxygen to water since bridging to the centre is involved in the stabilization of the peroxide intermediate (Solomon, *et al.*, 1996). Reduction of oxygen by laccase occurs in two 2-electron steps, the first of which is rate-determining (Durán, *et al.*, 2002). The function of the T1 site within the enzyme is long-range intramolecular electron transfer, shuttling electrons from the substrate to the trinuclear cluster (Solomon, *et al.*, 1996).

Table 1.2 Details of the copper atoms in fungal laccases (adapted from Claus, 2004).

Cu Type:	No. of Cu atoms/enzyme:	Characteristics:	Coordination with:	EPR signal and features:	Functions:
1	1	Blue Cu with absorbance at ~610 nm (oxidized state); redox potential of +785 mV	2 Histidines, 1 Cysteine, 1 Leucine	+ Paramagnetic	Substrate oxidation (one-electron step)
2	1	Affinity to azide, fluoride, and cyanide	Trinuclear cluster	+ Paramagnetic	Reoxidation of T1 Cu; stabilization of an H_2O_2 intermediate
3	2	Spin-coupled Cu pair; absorbance at ~330 nm (oxidized state)	8 Histidines	- Diamagnetic	O_2 reduction to water by enzyme oxidation (two two-electron steps)

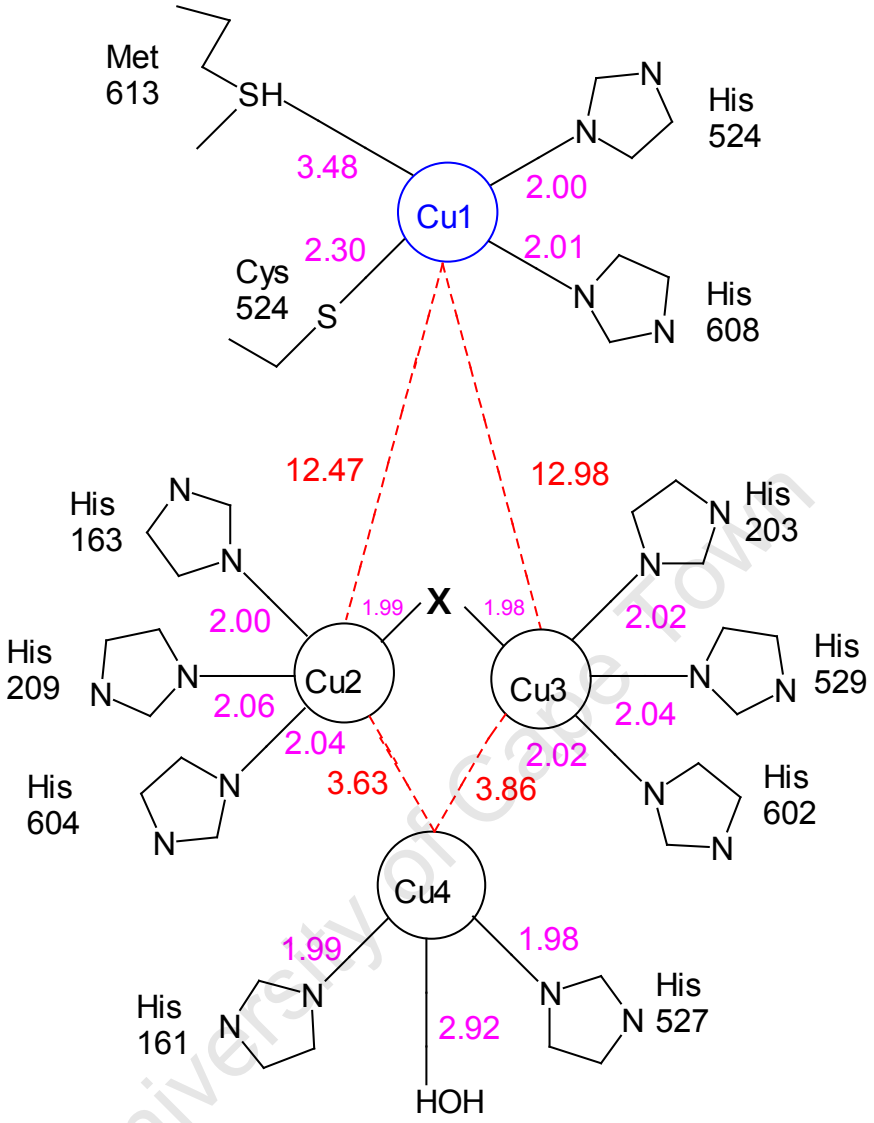


Fig. 1.1. Diagram of the arrangement of the four conserved copper atoms, the distances to the ligating atoms, and the distances between the copper atoms. X indicates an unidentified bridging ligand, *e.g.* OH. (Adapted from Smith, *et al.*, 2006.)

1.6 Reaction mechanism of laccase

The combination of four copper ions (Cu) in laccase allows coupling of four one-electron oxidations of substrate to the four-electron reduction of dioxygen to water. Laccase oxidizes the substrate molecule with the T1 Cu by four one-electron step-wise transfers (Figure 1.2A, B). The reoxidation of laccase is due to the T3 Cu pair, which transfers four electrons in two-electron steps to molecular oxygen, resulting in the reduction of oxygen to water. Thus, the oxidation of substrates by laccase creates reactive radicals that can undergo either oxidative coupling (in the case of monomers) or degradation (in the case of polymers) (Claus, 2003). The oxidation of monomers creates reactive radicals that can undergo non-enzymatic coupling reactions, *i.e.* polymerisation. Mediators, which are low-molecular-weight molecules, assist in the degradation of polymers: When the mediators are activated by laccase, they diffuse from the active site of the enzyme to susceptible components of the polymers, causing depolymerization reactions (Figure 1.2C).

⇒ Figure 1.2 contains images adapted with permission from Bertrand, *et al.* (2002), Claus (2003), Baldrian (2006), and Riva (2006).

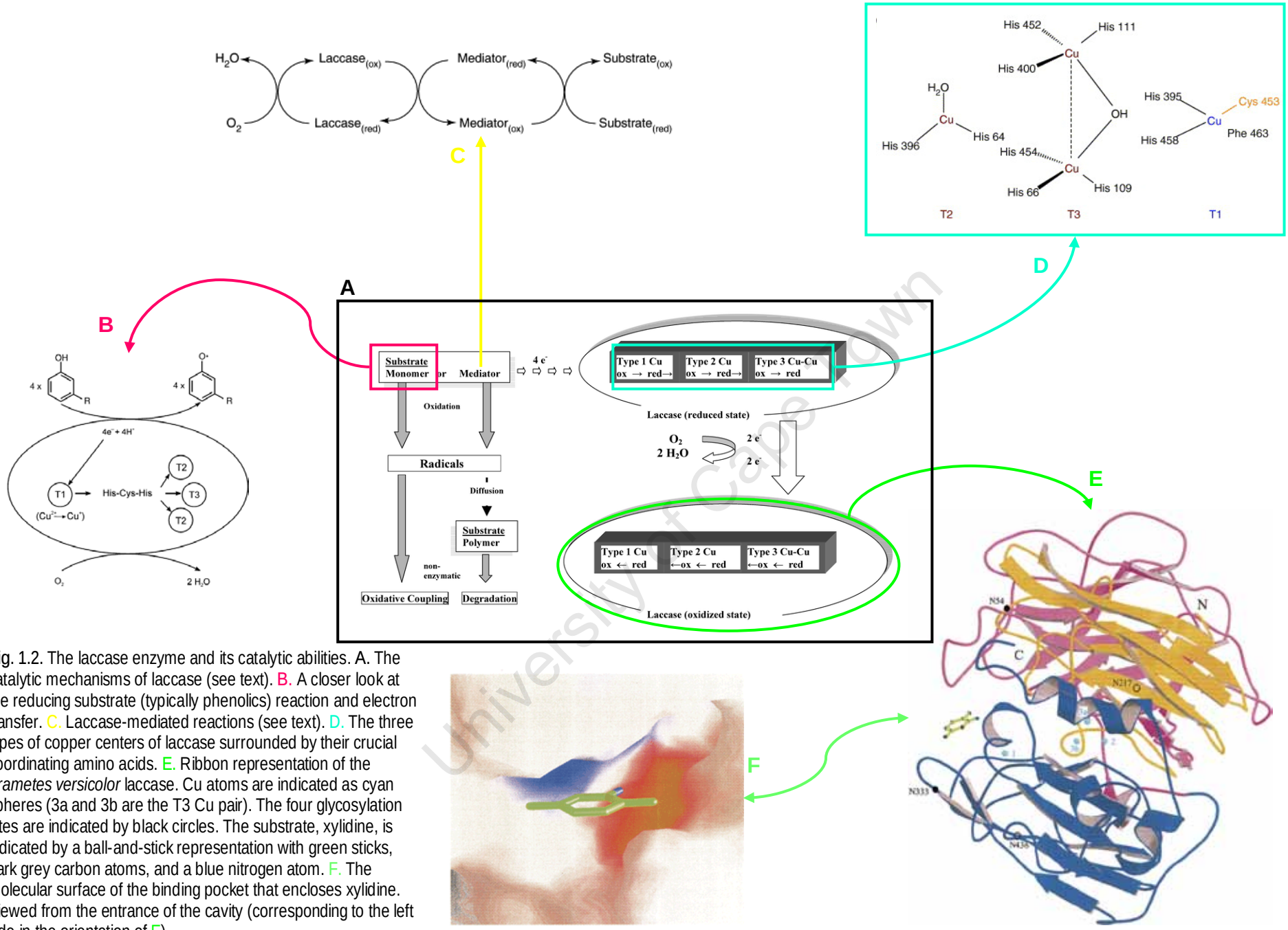


Fig. 1.2. The laccase enzyme and its catalytic abilities. A. The catalytic mechanisms of laccase (see text). B. A closer look at the reducing substrate (typically phenolics) reaction and electron transfer. C. Laccase-mediated reactions (see text). D. The three types of copper centers of laccase surrounded by their crucial coordinating amino acids. E. Ribbon representation of the *Trametes versicolor* laccase. Cu atoms are indicated as cyan spheres (3a and 3b are the T3 Cu pair). The four glycosylation sites are indicated by black circles. The substrate, xylidine, is indicated by a ball-and-stick representation with green sticks, dark grey carbon atoms, and a blue nitrogen atom. F. The molecular surface of the binding pocket that encloses xylidine. Viewed from the entrance of the cavity (corresponding to the left side in the orientation of E).

1.7 Redox potential and kinetics of laccases

The driving force for a redox reaction is expected to be proportional to the difference between the redox potentials of oxidant and reductant. For laccase-catalysed oxidations, an increase in the substrate redox potential (E^0) should therefore decrease the efficiency of the reaction (Zille, 2005). The oxidation of phenols depends on the redox potential difference (ΔE^0) between the phenolic compound and the T1 Cu, *i.e.* the higher the potential of the T1 Cu site, the higher the catalytic efficiency of laccase (Zille, 2005).

The E^0 of a phenol decreases when pH increases due to proton release, which implies enhanced oxidation rate. Laccase activity at higher pH is decreased by the binding of a hydroxide anion to the T2/T3 coppers, which interrupts the internal electron transfer from the T1 Cu to the T2/T3 Cu cluster (Baldrian, 2006). The rate of oxidation as well as the reaction products can differ with pH, as pH may affect ensuing non-enzymatic reactions of primary radicals catalysed by laccase (Baldrian, 2006).

The initial laccase-catalysed oxidation of phenol (aryl C-OH) compounds is similar to the oxidation of aryl (N-OH; phenol homolog) compounds in terms of the dependence of the initial rate on E^0 and pH (Xu, *et al.*, 2000). In general, the phenol is first oxidized to a highly unstable phenoxy radical (C-O$^{\bullet}$), which then gives up an additional electron (at a rate faster than that of the first electron transfer) to yield a stable but much less active quinone (Xu, *et al.*, 2000).

It has been reported that, despite the wide substrate range, the affinity for and the reaction rate of laccase-catalysed reactions are influenced by the nature of the substrate's substituents and by their reciprocal position on the aromatic ring (Xu, *et al.*, 2000; Zille, 2005). These factors could affect the degree of stabilization of the aryloxyradical generated by enzymatic action or aid its formation by increasing the electron density at the phenoxy group. It has been documented that phenolic structures bearing electron-donating substituents, such as methyl or methoxyl groups, are better laccase substrates than phenols substituted with electron-withdrawing functional groups such as the nitro or acetyl group (Xu, *et al.*, 2000; Rodakiewicz-Nowak, *et al.*, 2000; Zille, 2005; Baldrian, 2006).

1.8 Substrates for laccases

The full range of compounds that can be affected directly or indirectly by laccase activity is not known (Durán, *et al.*, 2002). Laccases oxidize polyphenols, methoxy-substituted phenols, aromatic diamines, and many other compounds but does not oxidize tyrosine as tyrosinases do (Baldrian, 2006). Oxidation of syringaldazine in combination with the inability to oxidize tyrosine has been accepted as confirmation of laccase activity. Syringaldazine is a substrate used exclusively for the detection of laccases (Baldrian, 2006). Laccases of various origins differ in their substrate specificities and several substrates should be tested to assess the activity of a putative laccase (McMahon, *et al.*, 2007).

1.8.1 Laccase activity tests (assays)

Activity of laccases *in vivo* or as purified isoenzymes is most commonly determined by spectrophotometric tests using phenolic substrates, and by monitoring the coloured oxidation products. The most common tests utilize guaiacol, 2,6-DMP, syringaldazine, or the electron-rich, non-phenolic substrate, ABTS. These substrates are specific for laccases, and because their oxidation products are intensively coloured, they are particularly useful for easy spectrophotometric determinations (Baldrian, 2006). 2,6-DMP is a naturally-occurring laccase substrate and has been used to detect laccases in bacteria but it is toxic (Claus, 2003).

1.8.2 Laccase mediators

Mediators are low-molecular-weight compounds that are activated by laccase and diffuse from the enzyme active site to initiate reactions such as depolymerisation reactions (Claus, 2003). Free radicals resulting from the laccase-catalysed oxidation of ABTS in the **l**accase-**m**ediator **s**ystem (LMS) oxidation process revealed that common laccase substrates, which form radicals, can also act as mediator compounds (Johannes and Majcherczyk, 2000). Many natural compounds, either produced by fungi or present during the degradation of lignocellulose substrates, are laccase mediators (Johannes and Majcherczyk, 2000). The first artificial mediator to be used in the LMS for pulp delignification was ABTS, in 1990 (Majcherczyk, *et al.*, 1999). Over 100 mediator compounds have been described but the most common are the azine ABTS and the triazole 1-hydroxybenzotriazole (HBT). ABTS is readily oxidized to the cation radical $ABTS^{+\bullet}$, and the concentration of the intensely coloured, green-blue cation radical correlates to laccase activity (Majcherczyk, *et al.*, 1999).

1.8.3 Inhibitors of laccase production

The characterization of laccases often includes the effect of potential inhibitor compounds, *i.e.* substances that selectively deactivate the laccase (Table 1.3). It is essential for biocatalysis reactions that the inhibitor substance does not react with the initial substrate or the final products, and it should not participate in any non-enzymatic reactions. Only sodium azide acts as a true laccase inhibitor and shows no significant interference with the enzyme activity tests (Johannes and Majcherczyk, 2000). By interfering with electron transfer in the copper centre of the enzyme, copper chelators of the copper centres also act as inhibitors (Johannes and Majcherczyk, 2000).

Table 1.3. List of inhibitors of laccase activity (from Johannes and Majcherczyk, 2000).

Inhibitor:	Molecular Weight (g/mol):
Sodium azide	65.01
Sodium fluoride	41.99
Diethyldithiocarbamic acid	720.68
Thioglycolic acid	92.11
Cysteine	121.15
EDTA	372.24

1.8.4 Laccase production and the effects of inducers

Many studies on the production of laccase or its optimisation have focused on the type, concentration, and time of addition of low-molecular-weight inducers, which are supplemented to the culture medium to stimulate laccase synthesis (Galhaup, *et al.*, 2002). The production of laccases can be enhanced by the addition of a range of compounds to fungal cultures, such as different aromatic compounds that are structurally related to lignin (Table 1.4). One of the key factors determining the induction capacity of such compounds is their chemical structure, and closely related aromatic compounds appear to have different effects on fungal laccase activity levels (Niladevi and Prema, 2007; Galhaup, *et al.*, 2002; Ryan, 2003).

Some inducers can show a distinct inhibition of fungal growth while others show a rapid inductive effect (Ryan, 2003). The presence of an individual aromatic compound may increase laccase titres without altering the isozyme pattern, induce new isoforms, or have no induction effect (Ryan, *et al.*, 2005). Induction of laccase synthesis could occur in response to toxic compounds produced during the degradation of the lignin component of lignocellulosic residues (which serve as natural growth inhibitors to fungi) or to antimicrobial agents secreted by microbial competitors (Niladevi and Prema, 2007).

Another effect that has been attributed to certain compounds, especially phenolic compounds, is to increase the long-term stability of laccase enzymatic activity in aqueous solutions. Compounds that cannot be oxidized can be effective in stabilizing the enzymes, as opposed to having an inductive effect, indicating a potentially important role for these compounds in practical applications (Terrón, *et al.*, 2004).

Table 1.4. List of inducers of laccase activity documented in the literature.

Inducer:	Molecular Weight (g/mol):	Recommended Concentration for Use:	References:
Abietic acid	302.451		Vasconcelos, *et al.*, 2000.
Aniline	93.13		Burton, 2003.
o- & *p*-Anisidine	123.15		Niladevi and Prema, 2007.
Catechol	110.1	1 mM	Galhaup, *et al.*, 2002. Niladevi and Prema, 2007. Vasconcelos, *et al.*, 2000.
4-Chlorophenol	128.5		Vasconcelos, *et al.*, 2000. Ryan, 2003.
Coumaric acid	164.2		Vasconcelos, *et al.*, 2000.
Cresol	108.14	2.5 mM or 5%	Ryan, 2003.
$CuSO_4$	249.68	1 mM	Galhaup, *et al.*, 2002. Niladevi and Prema, 2007.
2,6-Dimethoxy benzoic acid	182.17		Vasconcelos, *et al.*, 2000.
2,3-, 2,4-, & 2,6-Dimethoxyphenol	154.16	2 mM	Burton, 2003. Vasconcelos, *et al.*, 2000. Ryan, 2003.
2,2-Dimethyl succinate	146.14	1 mM	Ryan, 2003.
Ethanol	46.07	Low concentration: 1 mM High concentration: > 2 mM	Ryan, 2003.
Ferulic acid	194.19	1 mM	Galhaup, *et al.*, 2002. Niladevi and Prema, 2007. Vasconcelos, *et al.*, 2000.
Gallic acid	170.1	1 mM	Galhaup, *et al.*, 2002. Niladevi and Prema, 2007.
Guaiacol	124.14	Low concentration: 1 mM High concentration: > 2 mM	Ryan, 2003. Galhaup, *et al.*, 2002. Niladevi and Prema, 2007. Vasconcelos, *et al.*, 2000.
8-Hydroxyquinoline	145.16		Ncanana, 2007.
Phenol	94.11		Burton, 2003. Ryan, 2003.
Pyrogallol	126.11	1 mM	Niladevi and Prema, 2007.
RBBR (Remazol Brilliant Blue R)	626.54	0.002%	Niladevi and Prema, 2007.
Syringic acid	198.17		Vasconcelos, *et al.*, 2000.
Vanillic acid	168.15	1 mM	Galhaup, *et al.*, 2002. Niladevi and Prema, 2007. Vasconcelos, *et al.*, 2000.

Veratric acid	182.18		Vasconcelos, *et al.*, 2000.
Veratryl alcohol (3,4-dimethoxybenzyl alcohol)	168.19	Low concentration: 1 mM High concentration: > 2 mM For fungi: 28 – 35 mM	Burton, 2003. Ryan, 2003. Niladevi and Prema, 2007. Vasconcelos, *et al.*, 2000.
2,5- & 2,6-Xylidine	480.42	Low concentration: 1 mM High concentration: > 2 mM	Ryan, 2003. Galhaup, *et al.*, 2002.

1.9 Laccase-catalysed polymerisation reactions

Direct laccase-catalysed oxidation of phenols generates reactive radical intermediates that undergo coupling reactions, leading to the formation of dimers, oligomers, and eventually, polymers. All of these compounds can be of synthetic importance (Riva, 2006). Cross-coupling between the reactive radicals results in the formation of C-C and C-O bonds between phenolic molecules, and C-N and N-N between aromatic amines. In phenolic cross-coupling an electron is removed from the hydroxyl group, generating an alkoxy radical. The alkoxy free radical then forms dimers in the *ortho*- and *para*-position to yield oligomeric products (Zille, 2005). There are several advantages to the use of laccases as biocatalysts for the enzymatic polymerisation of phenolic compounds (Desentis-Mendoza, *et al.*, 2006): The reaction takes place in the absence of toxic substances; laccases catalyse the polymerisation of a great variety of phenolic monomers; phenolic compounds with more than two reactive substituents can be polymerised selectively; and the structure and solubility of the polymer can be controlled by changing the conditions of the reaction (Ncanana, 2007). Examples of laccase-catalysed polymerisation reactions will be discussed further in Chapter 4.

1.10 Actinomycetes: Prokaryotes with pronounced chemical diversity and the importance of their isolation from extreme environments

Actinomycetes are filamentous, aerobic, Gram-positive bacteria, best known for their ability to produce antibiotics, and they are a potential source of many more new and useful antibiotics, as well as other metabolites, including enzymes. They are widely distributed and abundant in soil and are responsible in part for the earthy odour of soil. Actinomycetes are routinely screened for new bioactive substances as they have provided many important bioactive compounds of high commercial value, such as two-thirds of naturally occurring antibiotics, including many of medical importance (Takizawa, *et al.*, 1993; Lazzarini, *et al.*, 2001).

Streptomyces species make up approximately 95% of all actinomycetes found in soil (Lacey, 1973), and consequently this genus has been extensively studied and is thus the best characterised genus of all actinomycetes (Cross and Goodfellow, 1973). Many streptomycete strains have been shown to produce one or more antibiotics (Locci, 1989). Colonies are initially relatively smooth surfaced but later develop a weft of aerial mycelium that may appear floccose, granular, powdery, or velvety (Locci, 1989). A wide variety of pigments are produced that are responsible for the colour of the vegetative and aerial mycelia. On occasion, coloured diffusible pigments are produced. Streptomycetes do not have specific growth requirements (they are nutritionally versatile) and show tolerance of potentially inhibitory media additives (Locci, 1989).

The current need for novel bioactive compounds has prompted scientists to focus on isolating nonstreptomycete actinomycetes as sources of novel compounds, given their promising chemical diversity. Expanding the sources of actinomycetes by carrying out ecological evaluations of environments other than terrestrial soils may aid the search for novel compounds (Takizawa, *et al.*, 1993). It has been suggested that extreme environments could be a valuable source for the isolation of novel actinomycetes with the potential to yield useful new compounds (Goodfellow and Haynes, 1984). Such environments are a bountiful resource for the isolation of less exploited microorganisms with unique characteristics. The potential contribution of these microbial sources to the discovery of new bioactive molecules has been recognized and has initiated the search for new microbes from such unique niches or habitats (Goodfellow and Haynes, 1984; Moran, *et al.*, 1995; Sponga, *et al.*, 1999; Takizawa, *et al.*, 1993).

Rare genera of actinomycetes have gained increasing relevance as potential producers of new metabolites (Lazzarini, *et al.*, 2001). The development of new methodologies with which to isolate rare actinomycetes is of great importance in extending our understanding of their ecology, taxonomy and bioactivity (Otoguro, *et al.*, 2001). It has been shown that when selective isolation methods are developed and applied, some genera, such as *Amycolatopsis*, *Rhodococcus*, *Micromonospora*, and *Mycobacterium* are in fact not rare at all and can be recovered from many soil samples (Goodfellow and Haynes, 1984; Lazzarini, *et al.*, 2001).

Different species and different strains within a species are distinguished by variations in their organic molecules (Campbell, *et al.*, 1999), and hence, screening for the production of new metabolites with new isolates is one way to obtain new classes of molecules (Crueger and Crueger, 1989). Since diverse and less exploited microbes are useful sources of a variety of novel metabolites (Sponga, *et al.*, 1999), the rare genera of actinomycetes have gained increasing importance as producers of new metabolites (Lazzarini, *et al.*, 2001). In order to propose that an isolate represents a new species, its morphological and physiological characteristics should not match with the features of any known actinomycete species included in Bergey's Manual of Systematic Bacteriology (Locci, 1989) and those subsequently described in the literature.

A search of the literature and relevant databases for laccase-producing non-streptomycetes from the genera *Amycolatopsis*, *Gordonia*, *Mycobacterium*, *Micromonospora*, *Nocardia*, and *Rhodococcus* revealed no reports of laccases being produced by strains of these genera. Given the promising chemical diversity of such genera, novel laccases may be found if laccase production by these actinomycetes is investigated. Furthermore, the biological role of laccase has yet to be fully elucidated and appears to vary depending on the type of organism (Galhaup, *et al.*, 2002).

1.11 Actinomycetes as sources of novel laccases

Laccases from novel strains may possess very different properties from those of the known laccases, and thus, the same starting compounds may be converted to different products by laccases with different properties. Comparisons between known and novel laccases may also lead to insights revealing, for example, differences in catalytic mechanisms or selectivities. Further, new laccases may not catalyse entirely novel bioconversion reactions but may still possess superior properties in terms of application, and these could then be used to replace existing enzymes with the aim of improving target bioconversion reactions. Laccases from plants and fungi (both eukaryotes) have been extensively studied. However, laccases from prokaryotes, including the actinomycete bacteria, have only recently been discovered and are currently the subject of much research interest (Table 1.5). New applications can be derived from such bacteria, and comparisons with fungi can be used to identify advantages of using actinomycetes and their laccases.

Table 1.5. Properties of various fungal and bacterial laccases.

Taxonomic Group:	Example Strains:	Laccase Characteristics:				Reference:
		Extracellular or Intracellular Laccase?	Optimal pH:	Optimal Temperature:	Other:	
Fungi	Fungi in general	Predominantly extracellular.	Acidic isoelectric point of ~ pH 4.0	50 – 70°C	Molecular weight of 60 – 70 kDa. Glycosylation of 10 – 25% saccharide content.	Baldrian, 2006
	Trametes sp. AH28-2	Extracellular	Isoelectric point of pH 4.2	Thermostable at 70°C.	Molecular weight of 54 – 62 kDa. Glycosylation of 11 – 12%.	Xiao, *et al.*, 2003
	Coriolopsis rigida	Extracellular				Saparrat, *et al.*, 2002
Actinomycetes	*Streptomyces lavendulae* REN-7	Intracellular			76% amino acid sequence identity to PHS from *S. antibioticus*. Role in spore-pigmentation and melanin production.	Suzuki, *et al.*, 2003
	Streptomyces griseus' EpoA	Extracellular	6.5	40°C; Thermostable at 70°C.	Molecular weight of 100 kDa. Described as laccase-like as it did not oxidize the usual laccase substrates. Role in morphogenesis.	Endo, *et al.*, 2003; Arias, *et al.*, 2003

	Streptomyces cyaneus CECT 3335	Extracellular	Isoelectric point of pH 5.6. Optimal pH of 5.	60°C	Molecular weight of 65 kDa.	Arias, *et al.*, 2003; Berrocal, *et al.*, 1997
	Streptomyces badius	Extracellular			Role in polymerisation by radical coupling.	Borgmeyer and Crawford, 1985
Non-actinomycete bacteria	*Pseudomonas putida* F6	Intracellular	7	30°C		McMahon, *et al.*, 2007
	Bacillus subtilis' CotA	Intracellular	Isoelectric point of pH 7.7. Optimal pH of 3.		Molecular weight of 65 kDa. A spore coat protein.	Arias, *et al.*, 2003
	Azospirillum lipoferum	Intracellular	6	Thermostable at 70°C.		Diamantidis, *et al.*, 2000

Due to the many potential biotechnological applications of laccases, the optimisation of laccase production by different microbes is being carried out, and actinomycetes are a promising source of new laccases. Filamentous microbes are of increasing importance in bioprocesses, being responsible for production of many primary and secondary metabolites, such as, antibiotics, enzymes, and vitamins. Two main groups are involved; filamentous fungi and filamentous actinomycetes. The cellular growth mechanisms of these groups differ greatly (Moreira, *et al.*, 2003). However, the two groups exhibit similar morphologies, growth patterns, growth forms, and hyphal and mycelial growth kinetics, and on solid media and in liquid cultures both groups grow as dispersed mycelia and pellets (Moreira, *et al.*, 2003). Maximum specific growth rates for fungi (eukaryotes) are generally lower than for bacteria (prokaryotes). Furthermore, there are advantages to using prokaryotes instead of fungi, such as the ease of genetic manipulation of bacteria, prokaryotic enzyme expression as recombinant proteins, their rapid growth rate, and the diversity of their enzymes (Moreira, *et al.*, 2003).

The demand for laccase requires the production process to be economical. Identifying inexpensive raw materials for enzyme production, and the use of inducers, could contribute to a cost-effective approach (Cordi, *et al.*, 2007). However, the nature of the compound that induces laccase activity differs greatly with the species. In contrast to studies on fungi, comparative data on laccase induction in actinomycetes are scarce. The aromatic inducers that are generally used for laccase induction in fungi have been successfully applied to enhance laccase yield from *Streptomyces psammoticus*, an actinomycete strain (Niladevi and Prema, 2007). Comparative studies between the laccase of the bacterium *Azospirillum lipoferum* and that of the fungus *P. oryzae*, showed that their substrate ranges and sensitivities to several inhibitors were similar (Diamantidis, 2000). Laccase production can also be influenced by the nitrogen nutrient concentration in the culture medium and by the type of carbon source employed (Niladevi and Prema, 2007). Thus, with appropriate optimisation, actinomycetes may be as productive as fungi, for laccases.

The maximum activity of actinomycete laccases can occur at unusually high pH (pH of 9) suggesting the suitability of their laccases for industrial processes where alkaline conditions prevail (Machczynski, *et al.*, 2004; Niladevi and Prema, 2007; Molina-Guijarro, *et al.*, 2009). However, pH maxima near to neutrality were also observed for other bacterial laccases (McMahon, *et al.*, 2007). In contrast, the stability of fungal laccases is generally higher at acidic pH, and production of laccases from fungal sources often causes a drop in the pH of production media (Baldrian, 2006).

Most fungal laccases are extracellular enzymes but a given species may produce isozymes of both extra- and intra-cellular types. In many fungal species the presence of both constitutive and inducible laccases has been reported. Usually the enzyme originates in the cytoplasm but secretion of laccases has also been reported (Mayer and Staples, 2002; Burton, 2003). WRF oxidise aromatic compounds extracellularly whereas bacteria initiate the attack with laccases located intracellularly (Leidig, *et al*, 1999). It is notable that actinomycetes produce laccases without the addition of inducing compounds (Niladevi and Prema, 2007). In general, the bacterial laccases that have been described are intracellular or cell

bound, associated with the cell wall. Furthermore, most bacterial laccases are involved in basic cellular processes (Arias, *et al.*, 2003). The location of laccase may be connected to its physiological function and also determines the range of substrates available to the enzyme. The intracellular laccases of fungi and bacteria may participate in the transformation of low-molecular-weight phenolics *in* the cell. The cell wall and spore-associated laccases are linked to the formation of melanin and other protective cell wall compounds (Baldrian, 2006).

1.12 Development of bioprocesses for application of enzymes

As biotechnology industries develop relevant experience, industrial biocatalysis could grow rapidly (Schmid, *et al.*, 2001). The viability of new biocatalytic processes can often be determined by the availability of the biocatalyst. Regardless of how the biocatalyst is to be used, expression in a rapidly growing, robust host is required, and constitutive, rather than inducible, expression is preferable (Burton, *et al.*, 2002). Each bioprocess is constrained by a set of conditions dictated by the specific properties of the substrates, products, and the bioconversion reaction (Table 1.6). A limited number of hardware designs are found in large industrial processes, allowing the applications of biocatalysts based on only a few concepts (Schmid, *et al.*, 2001). During the course of this study, experimental decisions were made with bioprocess development in mind.

Table 1.6. Constraints influencing the operating conditions for biocatalytic reactions (modified from Burton, *et al.*, 2002).

Type of Constraint:	Specific Description of Constraints:
Reaction specific; dependent on the nature of substrates and products.	Water solubility of substrates and products; dependent on pH and temperature; Chemical nature and stability of substrates and products; Production/consumption of gases and acid/base; Reaction equilibrium; dependent on pH and temperature.
Biocatalyst specific; dependent on the properties of the biocatalyst.	The pH range over which optimal activity and stability of the biocatalyst can be maintained; Temperature range for optimal activity and stability of the biocatalyst; Concentration range of substrates and products that can be tolerated by the biocatalyst without inhibition or saturation effects.

1.12.1 Methodology of bioprocess development

In the development of a bioprocess, a bioreactor is initially used for fermentation of a microorganism, in order to produce large quantities of the enzyme of interest. Later, a bioreactor houses the biocatalyst and the environment in which the bioconversion reactions will occur (Doran, 1995). The major types of reactors, as well as their advantages and disadvantages are summarised in Table 1.7. Under optimal conditions in the bioreactor, whole-cells or enzymes perform the desired bioconversions with high efficiency. Small scale laboratory bench bioreactors can be used in initial stages, and depending on the type of reactor to be used, conditions such as gas flow rates, temperature, agitation speed, circulation rate, retention time, and volume can be determined and controlled to achieve an efficient bioprocess (Doran, 1995).

Table 1.7. The major advantages and disadvantages of continuous stirred tank reactors (CSTRs), airlift, and membrane reactors in bioprocesses (modified from Marwick, *et al.*, 1999).

Reactor Type:	Advantages:	Disadvantages:
CSTR	Nearly perfectly mixed Good oxygen transfer Extensively modelled	High shear stress High power to volume input Moving internal parts
Airlift	Lower shear stress than STR Lower energy input than STR Can have no internal parts Easier to model than a bubble column	High viscosity can limit bulk circulation
Membrane	Low shear stress Low downstream contamination High oxygenation rates	Biofilm overgrowth Membrane can rupture at high cell densities/flow rates

Preliminary small-scale culture of microorganisms is usually carried out in shake flasks. Medium composition, pH, temperature, and other environmental conditions allowing for optimal growth and productivity are determined. Calculated parameters such as cell (specific) growth rate, specific productivity, and product yield are used to describe the performance of a microbe and/or enzyme (Doran, 1995). Once suitable culture conditions for production are known, the bioprocess is scaled-up from shake flasks to a bench-top bioreactor where bioprocess variables can be monitored and/or manipulated, thus allowing for better control over the bioprocess (Doran, 1995). Important information is collected regarding bioprocess variables, such as oxygen requirements (for the cells and enzyme, in the case of laccases), and shear sensitivity (damage to the cells can result in stress and decreased production of enzyme).

The viability of a process as a commercial enterprise is of great interest, and experimental data is essential for making rational decisions regarding the bioprocess under investigation. Bioreactor types, bioprocess operation as a batch, semi-batch, fed-batch or continuous process, and other variables and parameters must be determined. However, for some targeted products, identification of high-producing strains and optimal environmental growth and enzyme production conditions are initially more valuable than improving the reactor design and operation later on (Doran, 1995; Giorno and Drioli, 2000; Mouton, 2005).

1.12.2 Native strain growth in a bioreactor for the production of laccase

As a result of the need to produce high titres of laccases, studies have focused on defining the nutritional, physiological, and environmental factors that may be related to the production of laccase (Moreira, *et al.*, 2003).

In order to achieve a suitable production of laccase the selection of an adequate bioreactor configuration is critical. Different types of reactors have been proposed for the production of laccases. The aim in most cases has been to achieve the highest possible yield of laccase titres. To do so, optimisation of the culture parameters for the overproduction of laccase involves, generally, batch operation and the use of **c**ontinuous **s**tirred **t**ank **r**eactors (CSTR) or ALRs (Domínguez, *et al.*, 2001). An **a**ir**l**ift bio**r**eactor (ALR) may be used to produce laccase due to its simplicity, reliability, low cost, low shear environment (a mechanical stirrer is not needed), low energy demand, and lower risk of contamination (Domínguez, *et al.*, 2001; Ryan, *et al.*, 2005).

Factors affecting microbial growth and pellet formation include agitation and shear, growth medium composition, aeration, and inoculation size. Although agitation generally decreases pellet size, chipping of hyphal fragments from the pellet surface or the rupturing of pellets results in a detrimental effect on enzyme production. Agitation must be gentle since mechanical deactivation of the enzyme is caused by excessive agitation (Niladevi and Prema, 2007).

Production of laccase by WRF is typically associated with their secondary metabolism, and laccase production is often followed by extracellular protease synthesis, which rapidly deactivates the produced enzyme (Moreira, *et al.*, 1998). In contrast, the increase in enzyme yield often observed in parallel with the increase in biomass suggests that laccase production by actinomycetes is growth-associated, and is a primary metabolic activity (Niladevi and Prema, 2007).

1.13 Antioxidants: Useful biological compounds

Antioxidants (AOs) provide protection against free radicals (and other reactive oxygen species) and their oxidative damage, and an increased interest in the use of natural antioxidants has developed recently (Desentis-Mendoza, *et al.*, 2006). Free radicals can be defined as molecules containing one or more unpaired electrons. The presence of unpaired electrons usually confers a considerable degree of reactivity upon a free radical. Those radicals derived from oxygen represent the most important class of free radical generated in living systems. Antioxidant (AO) defences are extremely important as they mediate the direct removal of free radicals thus providing maximal protection for biological compounds (Valko, *et al.*, 2006). Concerns over the safety of synthetic AO compounds have led to interest in natural AOs. The development of alternative AOs to the synthetic AOs, of natural origin, has received substantial attention and many researchers have focused on the discovery of new natural AOs suitable for quenching biologically harmful radicals (Balasundram, *et al.*, 2006). Some laccase-catalysed derivatives are of pharmaceutical interest because potent AOs can be obtained through laccase-oxidized polymerisation reactions.

1.13.1 Laccase-catalysed polymerisation of phenolic compounds to produce antioxidants

Of particular value to the pharmaceutical, nutraceutical, and food industries, are the reactions providing routes for the synthesis of dimeric and trimeric phenolics exhibiting biological activity, including AO activity (Burton, 2003; Chigorimbo-Murefu, *et al.*, 2009). Such polymerisation reactions may lead to higher AO activities (Hotta, *et al.*, 2002). The increasing availability of laccase biocatalysts and the improved biochemical knowledge of laccases may lead to new and promising biotechnological applications. When the number of laccase-based industrial oxidation processes expands there could be an increased interest in the synthetic exploitation of laccases, and more efficient approaches to the environmentally friendly synthesis of dimers or oligomers of phenolic derivatives may arise (Riva, 2006).

Examples of laccase-catalysed antioxidant polymers:

- The laccase-catalysed biosynthesis of resveratrol to *trans*-dehydrodimers with biological activity (Nicotra *et. al.*, 2004);
- Poly(rutin), a flavonoid polymer, has superior antioxidant properties compared to the rutin monomer (Desentis-Mendoza *et. al.*, 2006).
- The laccase-catalysed oxidation of totarol and 8-hydroxyquinoline to dimers and potent antioxidant polymers, respectively (Ncanana *et. al.*, 2007; Ncanana and Burton, 2007).

In this study, the laccase-catalysed target reaction is the dimerization of phenolic compounds (by carbon-carbon linking) to produce putative antioxidant compounds (examples are further discussed in Chapter 4). The importance of phenolic compounds as AOs, as well as the methods for assessing AO activities will be discussed in detail in Chapter 5.

1.14.1 Knowledge contribution and aims of this research

New strains are needed for the discovery of novel enzymes to be used in industrial, biotechnological processes. The Biocatalysis and Technical Biology (BTB) Research Group of Professor S. Burton have culture collections from extreme, unique environments which provide such resources (Figure 1.3). The first step in any biocatalysis application is the acquisition and development of a suitable biocatalyst, and this requires the use of various microbiological, biochemical, and engineering technologies. Novel laccases may possess new properties that can be exploited in new applications, and novel laccases possessing superior properties could replace existing enzymes used in bioconversion reactions, making the processes more efficient. The practical applications for the use of laccases are extensive, ranging from environmental to medical applications, and laccases are potentially useful in catalysing the production of new and improved antioxidants. This presents opportunities to produce pharmaceutically active compounds from natural sources, as opposed to synthetic routes.

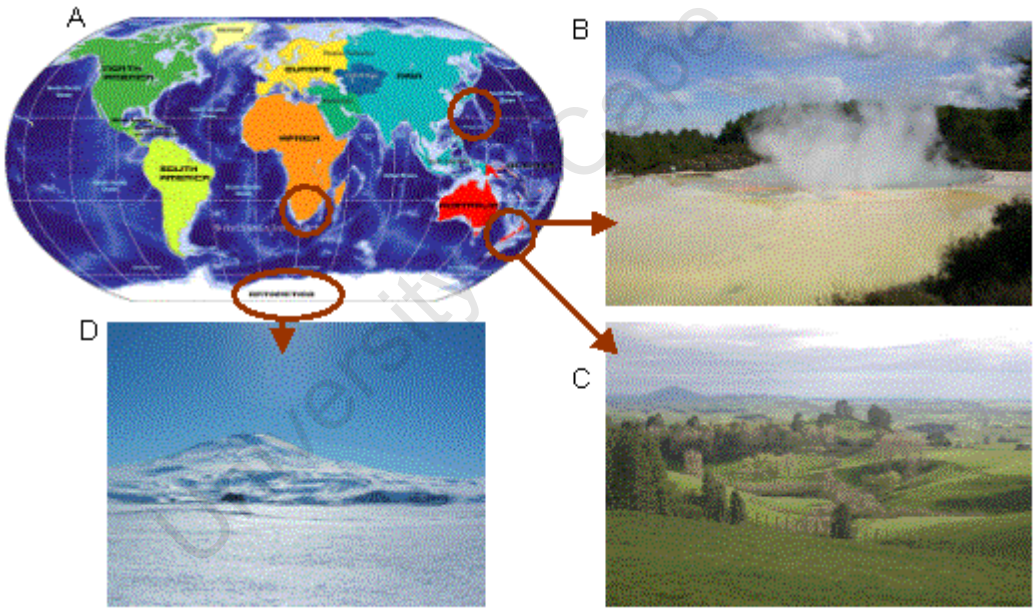


Fig. 1.3. The BTB laboratory has an extensive culture collection, with samples from environments spanning the globe, from which to screen for new strains and new enzymes. Some of the extreme environments sampled are circled and include unique locations in southern Africa, deep-sea trenches, including thermal trenches with black smokers, located in the North Pacific Ocean, locations on the continent of Antarctica, and many sites on the islands of New Zealand (A). A thermal geyser, venting, surrounded by extremely acidic environs, located in New Zealand (B). Unspoilt land in New Zealand (C). The pristine icy plains of the almost otherworldly Antarctica (D). Photos courtesy of Professor S. G. Burton and Dr M. le Roes-Hill.

This literature review has shown that while laccases are exceptionally versatile enzymes, more investigations are needed for a better understanding of their physiological importance in microbes and to further exploit their great biotechnological potential. The ongoing search for new and better biocatalysts, assisted by existing and developing technologies, promises to provide increasingly useful microbial laccases.

The methods for screening of actinomycete strains for laccase activity as well as the methods for the optimisation of laccase production used in this study are innovative approaches. Descriptions of new species of actinomycete strains, and their laccase enzymes, as well as descriptions of the products of laccase-catalysed reactions, contribute to new academic knowledge. As part of the general interest in biocatalysis, and more specifically, in enzyme-catalysed C-C bond formation, the synthetic potential of a new laccase was investigated.

1.14.2 Specific objectives of this study

The broad aim of this study was to characterize a laccase that has potential as an industrial biocatalyst for the oxidation of phenols and aromatic compounds, catalysing the oligomerisation of phenolic substrates to produce antioxidants. Many strains from the unique environments listed above were subjected to a primary screening protocol by Dr le Roes-Hill (pers. comm.). This preliminary screen demonstrated the ability of environmental actinomycete strains to produce laccase, and the most promising strains were further investigated in this study.

The specific objectives in achieving this aim were:

- Development of a secondary screening protocol to screen a selected group of actinomycete strains for the production of the enzyme of interest, laccase.
- Development of methods for enzyme production (in the native strains).
- Development of methods for improved laccase production by the native strains through optimisation of culture conditions.
- Preliminary characterisation of the laccase produced at the highest levels by a single actinomycete strain, serving as the focus for further study.
- Identification of significant substrate to product bioconversions, based on specific target reactions.
- Development of new laccase biocatalysis reactions for production of new compounds with biological activity.
- Isolation of laccase-catalysed antioxidant products and characterization of these compounds in terms of structure and antioxidant power.

1.14.3 Structure of the thesis

This thesis is comprised of six chapters, structured as follows: The first chapter has discussed the literature relevant to this study, and the background to this research. Chapter 2 discusses the screening of environmental actinomycete strains for the ability to produce the enzyme of interest, laccase, and investigates the variables influencing laccase production by the native strains. From this work, a single actinomycete strain designated as *Micromonospora* sp. strain 044 30-1, was chosen for further study. Variables influencing laccase production by this strain in flask culture and in bioreactors, as well as preliminary characterisation of strain 044 30-1's laccase are described in Chapter 3. Chapter 4 discusses the biocatalytic potential of the laccases from actinomycete *Micromonospora* sp. strain 044 30-1 and fungal strain *Trametes versicolor*. The biocatalysis reactions and the structures of the products of the laccase-catalysed oxidation of totarol, tyrosol, and monoacetylated tyrosol are presented. In Chapter 5, the biological activities of the biocatalysis products described in Chapter 4 are investigated. Preliminary antioxidant and antimicrobial activity data are presented. The general conclusions of this study and recommendations for future work are discussed in Chapter 6.

Chapter 2
Screening for Laccases from Environmental Isolates and Optimisation of Laccase Production

2.1 Introduction

This chapter documents the screening of environmental actinomycete strains for the ability to produce laccase. Variables influencing the production of laccases by these strains were investigated. The strain producing the highest levels of laccase was chosen for further study.

The majority of laccases have been obtained from lignin-degrading fungi (Claus, 2003). However, laccases are also widespread in bacteria, and a limited amount of relevant information has been published (Dubé, *et al.*, 2008). One of the most studied bacterial laccases so far is CotA, an endospore coat component of *Bacillus subtilis*, and bacterial laccases CotA, and STSL from the actinomycete *Streptomyces lavendulae* REN-7, were found to have the highest thermal stability of all laccases studied (Dubé, *et al.*, 2008).

The optimisation of physicochemical conditions is required for any bioprocess and is usually performed by altering one independent variable while fixing other variables. This conventional method can be time-consuming, and often, interaction effects are overlooked (Niladevi, *et al.*, 2007). In the present study, the experimental design of a secondary screening protocol for the detection of laccase production by native strains was particularly useful as it was used for the estimation of variables of interest. The first step involved the screening of variables and the second step involved optimisation of significant variables. Different nutrients, temperature, pH, and time allowed for growth affect enzyme production (Jaouani, *et al.*, 2006; Vasconcelos, *et al.*, 2000). In this present study, the selected variables included physical factors (such as pH, temperature, incubation period, aeration and agitation) as well as nutritional factors (different media with different carbon and nitrogen sources and salt concentration). Control of enzyme synthesis can be achieved to some degree *via* manipulation of the growth media components. A quickly metabolised substrate such as glucose may often cause maximum cell growth rates but can inhibit the production of many metabolites by catabolite repression (Marwick, *et al.*, 1999). In a similar way to carbon, the nitrogen source also regulates metabolism. Most fermentations reported in the literature have been performed in shake flasks, which, even on a small scale, may offer possibilities for bioprocess intensification (Marwick, *et al.*, 1999).

A laccase-producing strain's preferred growth conditions may be the optimal conditions for production of laccase. Laccases produced by the microorganisms can then be isolated, purified, and characterized. The liquid culture can then be scaled up in order to produce large amounts of laccase. This can be achieved using bioreactors and the best type of bioreactor to be used (such as airlift or

stirred tank) varies and is strain-specific (Chapter 3). One of the aims of this study was to achieve laccase-catalysed production of antioxidants (AO) where a monomeric AO compound could be converted to a dimeric or polymeric compound with enhanced AO capabilities (Chapters 4 and 5).

2.2 Materials and Methods

2.2.1 Growth media used in studies

The 5 types of media used in this study to investigate laccase production are described in Table 2.1. The pH of media was adjusted accordingly using HCl or NaOH. Other media used in this study were malt extract agar (Biolab), and starch-casein-nitrate (**SCN**) medium (Atlas, 1993), consisting of (in g/L): starch (10.0), casein (0.3), sodium chloride (10.0), potassium nitrate (2.0), magnesium sulphate (0.05), calcium carbonate (0.03), and iron sulphate (0.01). All media components were manufactured by Saarchem.

2.2.2 Strain maintenance

The strains used in this study are listed in Table 2.2. All fungal strains were grown at 25°C, while all actinomycete strains were grown at 30°C. Fungal strains were maintained on 2% malt extract agar (**MEA**), while actinomycete strains were maintained on yeast extract-malt extract (**YEME**). Strains were subcultured routinely and glycerol stocks made and stored at –20°C. Unless otherwise stated, liquid cultures were left shaking at 160 rpm without cotton wool bungs. The flasks were covered with foil. Streptomycete actinomycete strains were inoculated onto *ISP* Medium No. 4 in order to characterise the morphology of the aerial and substrate mycelia (Shirling and Gottlieb, 1966).

Typically, for inoculation of media, actinomycete cells or spores were scraped from agar plates using a sterile inoculating loop, and resuspended in 1 ml of water. This suspension was used to inoculate broth cultures for experimentation. For experiments requiring a small liquid culture of fungi, 1 cm $\times$ 1 cm of agar, overgrown with fungal mycelia, was cut with a sterile scalpel and transferred to broth culture. For large volumes of media, the entire fungal lawn was removed by using a sterile scalpel to lift the agar from the petri dish and transfer it to a sterile solution within an autoclaved blender (Waring Commercial Blender). The blender was used for a few seconds to break up the mycelial lawn and disperse the fungus within the solution. This solution was then added to broth for experiments.

2.2.3 Assays

2.2.3.1 Determination of laccase activity

Laccase activity was measured by monitoring the oxidation of 5 mM **ABTS** [2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid); Roche] buffered with 0.1 M sodium acetate buffer (Saarchem) adjusted to a pH of 5.0 with glacial acetic acid (Saarchem). The oxidation of ABTS was monitored by measuring the increase in absorbance of the assay mixture at 405 nm (the available excitation filter of the microtitre plate reader) and an extinction coefficient (ε) of 36 000 $M^{-1}cm^{-1}$ was used. The assay mixture contained 150 µl ABTS solution to which 50 µl of sample was added to start the reaction.

Samples were diluted, if necessary, to adjust the absorbance to within the readable range. This 200 µl assay mixture was made-up in 96-well microtitre plates (Lasec) and immediately assayed, with shaking at room temperature, using a microtitre plate reader (Anthos Zenyth 1100 with software for Anthos Zenyth Multimode Detectors). One unit of enzyme activity was defined as the amount of enzyme able to convert 1 µmole of ABTS per minute. All experiments were conducted using the necessary controls, such as the use of uninoculated liquid media samples. Laccase from *Trametes pubescens* was used as a positive control for assays (Ncanana, 2007).

2.2.3.2 Determination of tyrosinase activity

Tyrosinase activity was assayed as follows: 2.5 mM *p*-cresol (Aldrich) was buffered with 50 mM sodium phosphate buffer (Merck), pH 7.0, and 2.9 ml of this *p*-cresol solution was added to a 3 ml cuvette (Boshoff, *et al.*, 2003; Orenes-Piñero, *et al.*, 2006). 100 µl of enzyme sample was then added to the cuvette and an increase in absorbance at 400 nm (ε = 1433 $M^{-1}cm^{-1}$) was monitored spectrophotometrically for 10 minutes using a UV-Vis Heλios α Unicam Spectrophotometer. One unit of tyrosinase activity was defined as the amount of enzyme required to produce 1 µmol product per minute. Tyrosinase obtained from mushroom was used as a positive control for the assay (Ncanana, 2007). The blank contained water instead of enzyme sample. Where the microtitre plate reader was used, the protocol was as described for the ABTS assay.

2.2.3.3 Determination of protein content

Protein concentrations of samples were determined by the Bradford assay (Bradford, 1976) using Bradford reagent purchased from Sigma-Aldrich: 6.7 µl of sample was added to 200 µl Bradford reagent, the microtitre plate was allowed to stand for 5 minutes, and a colour change from brown to blue was measured at 620 nm, with shaking at room temperature, using a microtitre plate reader. Bovine serum albumin (BSA; Sigma) in phosphate-buffered saline was used as standard. Solutions of BSA between 0 and 1 mg/ml were used in every experiment and a standard curve was drawn from this data (using Microsoft Excel, Microsoft Corporation) to determine the protein concentration of experimental samples. Data analysis allowed for specific activities of enzyme samples to be calculated in U/mg protein.

2.2.4 Dye-decolourisation and the use of inducers for the detection of laccase

Dye-decolourisation experiments were conducted using the following agars: 3% MEA (pH 5.3), YEME (pH 7.3), and a low pH YEME agar (pH adjusted to 5). The following dyes were added as supplements to the media to give a final concentration of 0.002%: Poly R478 (Sigma), crystal violet (**CV**; BDH), bromophenol blue (**BB**; Merck), and Remazol Brilliant Blue R (**RBBR**; Sigma). Strains to be tested for their dye-decolourisation ability were stab-inoculated (in duplicate) into the centre of the agar plates using sterile toothpicks. Experiments were conducted for 14 days at the following temperatures: 22°C (ambient temperature), 25°C (fungal hood temperature), 30°C, and 37°C. Zones of decolourised dye indicated potential laccase production by the strains. Uninoculated control plates were used. The diameter (in mm) of each colony was measured before overlaying. The diameter (in mm) of the zone of dye-decolourisation was measured. The area of the dye-decolourisation zone in mm^2 was calculated. The arbitrary assignment of strength of dye-decolourisation was as follows: very weak

(<100 mm^2); weak (100 – 1000 mm^2); moderate (1001 – 2000 mm^2); strong (2001 – 3000 mm^2); and very strong (>3000 mm^2).

For induction experiments, 5 mM guaiacol (Sigma) or 0.05% (w/v) of *p*-cresol were added to agars to induce laccase production by the strains. Alternatively, a cresylic liquid waste (phenol: 82.8 mM, *p*-cresol: 24.99 mM, *m*-cresol: 25.8 mM, *o*-cresol: 77.03 mM), high in phenolics was used (Ryan, 2003). Two sets of 500 ml flasks were inoculated: One set containing only YEME broth and the other set containing YEME broth supplemented with cresylic waste, at a final concentration of 0.015%. Samples were taken at days 3, 5, 8, and 12, and assayed spectrophotometrically for laccase activity, using ABTS as the substrate.

2.2.5 The secondary screening protocol for detection of laccase production by native strains

Erlenmeyer flasks (50 ml), containing 10 ml of each specific medium to be tested for suitability as the best medium for laccase production, were inoculated in duplicate with the relevant microbial strain and allowed to incubate at the relevant temperature, with shaking at 160 rpm. Duplicate 1 ml samples were taken from each flask and transferred to an eppendorf on days 3, 5, and 7, except for fast growing strains, which were sampled on days 2, 4, and 6. The samples were centrifuged at 13 *g* for 5 minutes. The supernatant, containing the extracellular laccase sample, was transferred to another eppendorf. The cell mass was then resuspended in 1 ml of 0.1 M sodium acetate buffer, pH 5, and the cells were disrupted by sonication (2 $\times$ 30 s bursts with a 10 s rest period) using a Virsonic Ultrasonic Cell Disruptor 100 (by VirTis). The sonication and handling of intracellular laccase samples were performed on ice for cooling and to prevent denaturation of enzyme samples. As described in section 2.2.3, samples were added to the microtitre plates in duplicate. Thus, for each different medium or condition, results were obtained in quadruplicate. This allowed for examination of the consistency of the obtained assay data. The enzyme activity obtained using the ABTS assay was calculated in U/ml, and the protein concentration determined by the Bradford method was measured in mg/ml. Specific activities of the samples were calculated in U/mg protein and unless otherwise noted, the specific activities of samples were used for comparisons to determine favourable conditions for laccase production and to compare differences in laccase production between the strains tested.

Once analysis of data revealed the best medium and pH for production of laccase by each strain, this medium was used for subsequent experiments, including as the medium used for experiments to investigate the effects of agitation and aeration. Samples from flasks used for agitation and aeration experiments were treated as described above except that flasks were incubated with shaking at the altered speeds and some flasks were closed with cotton wool bungs, as opposed to only foil. Uninoculated culture media representing the different media and pHs examined were used as controls.

2.2.6 Representation of data

After each assay performed using the microtitre plate reader, data was transferred to Microsoft Excel Workbooks using the Anthos software. All subsequent analysis of data was performed in Excel and data were represented graphically. Please see Appendix D.

2.2.7 Aeration and agitation of cultures

Aeration and agitation experiments were performed in order to determine the optimal agitation conditions (in rpm) and aeration conditions (cotton wool bung or no bung) for laccase production. Agitation speeds used were static (*i.e.* 0 rpm), 80, 160, and 200 rpm. Samples were assayed for laccase activity and the protein concentrations of samples determined.

2.2.8 Growth curve analysis of cultures

Strains were inoculated into 50 ml of their respective best medium (section 2.2.5) in 250 ml Erlenmeyer flasks and incubated at the aeration and agitation conditions determined to be the best conditions for laccase production by the specific strain. Samples of 1 ml were taken daily from day 0 to day 11 and subjected to the protocol described above in section 2.2.5. Dried cell weight was determined as follows: 1 ml sample of liquid culture was added to a pre-weighed, dried, eppendorf tube and the cells were collected by centrifugation. The supernatant fluid was removed and the eppendorfs were allowed to dry overnight in a 60°C oven. Once the eppendorfs had cooled, they were weighed and the cell mass determined. This experiment was performed in triplicate.

2.2.9 Inhibitors and inducers of laccase in cultures

Strains were inoculated into their respective best medium (10 ml in 50 ml Erlenmeyer flasks) and incubated at the specific aeration and agitation conditions determined as the best conditions for laccase production by the specific strain. Sampling and processing of the samples was as described in section 2.2.5. The inducer or inhibitor to be tested was added to the medium of each strain at the time of inoculation to achieve a final concentration of 1 mM. Samples were taken on days 2, 6, and 8. All experiments were performed in duplicate. Ferulic acid (Fluka) and xylidine (Ponceau) were used as inducers of laccase activity, and sodium azide (BDH) as a laccase inhibitor. Solutions of inducers and inhibitors were made-up in sterile water. A control, inoculated with the strain, but containing no inducers or inhibitors, was used for each experiment.

2.2.10 Heat tolerance and storage temperature tolerance of laccase samples

Samples of intracellular and extracellular laccases obtained from the various strains (see section 2.2.5) were used in this experiment. Heat tolerance experiments were conducted in a water bath set to 55°C, and samples were taken for assaying after 1, 8, 16, and 30 min. Samples were also stored at various temperatures (–20°C, 4°C, 22°C, 25°C, 30°C, and 37°C) for 4 days and then assayed for activity. PMSF (20 mM phenylmethylsulfonyl fluoride dissolved in ethanol; Boehringer Manheim), a serine protease inhibitor, was added to a set of samples stored at 4°C to determine if a protease inhibitor prolonged the life of the laccases. All experiments were conducted in duplicate. The microtitre plate reader was used for the following assays: ABTS assay for the detection of laccase activity, the Bradford assay for determination of protein concentration, and the *p*-cresol assay for the detection of tyrosinase activity.

2.3 Results and Discussion

Screening methods were used in this study to detect the enzyme of interest, *viz*. laccase, produced by actinomycete strains. Laccase activity was assessed by various established methods, involving solid and liquid media experiments, dye decolourisation experiments, and spectrophotometric assays. Different agars, supplemented with compounds that the enzyme can act upon (in this study, dyes) are indicators of enzyme production; decolourisation of the dyes in solid media indicated laccase production by the strains.

In other studies, laccase production by fungi was correlated with dye decolourisation and sorption of dye to mycelia was reported to account for less than 3% of dye removal (Mayer and Staples, 2002). Dye degradation studies have focused mainly on the WRF although actinomycetes have also been recognized for their dye degradation abilities (Niladevi and Prema, 2007). RBBR decolourisation was linked to the time of laccase production in the actinomycete *Streptomyces psammoticus* (Niladevi and Prema, 2007). In this study, excellent decolourisation of dyes was achieved by fungal strains *Trametes pubescens* and MCTD1, as well as actinomycete strains *Streptomyces antibioticus* and *Streptomyces achromogenes* subsp. *achromogenes* strain DFNR3 (see section 2.3.3). This result was attributed to laccase production by these strains.

2.3.1 Selection of optimal media

Twelve of our laboratory's highest laccase-producing strains were chosen according to primary screening criteria suggested by another researcher (Dr M. le Roes-Hill, pers. comm.). Two fungal strains and 10 actinomycete strains were subjected to a secondary screening protocol (this study). The fungal media and the protocols used in this study were based on established media and protocols for the detection of laccase produced by fungi, as historically, fungi have been well-investigated. As the majority of strains investigated in this study were actinomycetes, documented actinomycete media and culture methodologies were used. This screening protocol examined growth and production of laccase by the strains in 5 different growth media and at 3 different pHs. Two fungal media, TDM and GYP, as well as 3 actinomycete media, YEME, Bennett's Medium containing glycerol, and Czapek-Sucrose, were used (Table 2.1). Most previous research into the production of laccase has focused on fungi, which prefer a growth medium with a pH of approximately 5. However, since this study involved the investigation of actinomycetes as well as fungi, a pH range was investigated to determine whether the microbes produce laccase under acidic, neutral, or basic conditions. Thus, the pH of each medium was adjusted to pH 5, 7, and 9. Furthermore, these media represent low, intermediate, and high glucose concentrations, and examine alternate carbon sources, in order to investigate the effects of possible glucose repression. Data were analyzed and the best medium for production of laccase by each strain was determined by calculating and comparing specific activities. Strains were ranked in order of highest laccase production (Table 2.2).

Abbreviations used for media are represented by the letter of the medium followed by the optimal pH; for example, an optimal medium of GYP at pH 9 is abbreviated as G9. Intracellular laccase is indicated by SONIC, and extracellular laccase by SNF.

2.3.2 Investigation of the factors contributing to maximal yield of laccase by the native strains

Experiments on the effects of aeration and agitation on laccase production by the strains, growth curve experiments, and the effect of laccase inducers and inhibitors on laccase production were performed using only the three highest laccase-producing actinomycete strains, as determined by the primary and secondary screening protocols (Table 2.2). Fungal strain *Trametes pubescens*, a well-known laccase producer, was used as the control strain.

Correct aeration is important as improper agitation may stress the microorganisms, resulting in limited enzyme production. In this study, static conditions (*i.e.* restricted oxygen but not an anaerobic environment), 80 rpm (slow agitation and low oxygen levels), 160 rpm (standard agitation and aeration conditions for the incubation of microbes in shake flasks), and 200 rpm (fast agitation and increased oxygenation) were examined. Flasks with cotton wool bungs (as opposed to only tinfoil covering the flasks) were used to allow for increased aeration. Aeration requirements for laccase production have been reported to vary from one fungal strain to another (Ergül, *et al.*, 2009). Interestingly, for the fungus *Neurospora crassa*, oxygen-dependent laccase production was not shown, and in static cultures, where levels of dissolved oxygen are lower, the production of laccase was found to be enhanced (Luke and Burton, 2001). Many of the actinomycete strains examined in the current study showed optimal production of laccase under static conditions, suggesting that laccase production was not necessarily oxygen-dependent. For the actinomycete *Streptomyces psammoticus*, the agitation rate influenced laccase production in such a way that the yield increased with increase in agitation up to 175 rpm and decreased at 200 rpm (Niladevi and Prema, 2007). The enhancement in laccase production at a higher agitation rate was due to better aeration in the well-agitated flasks, which was essential for the growth and enzyme production of *S. psammoticus*, an aerobic organism. The drop in enzyme yield at 200 rpm could be attributed to the possible damage (*i.e.* shear stress) that may occur in the filamentous structure of the organism thus hindering laccase production.

A study by Niladevi and Prema (2007) showed that there was a linear correlation between biomass and laccase production confirming other reports that laccase production by actinomycetes is a growth associated primary metabolic activity. In the case of *Streptomyces psammoticus*, maximum laccase yield and biomass production occurred within 48 hours, allowing for a short incubation period (Niladevi, *et al.*, 2007). A short incubation period is advantageous for an industrial process as laccase can be obtained at an early stage thereby saving time and providing for an economical process. For this reason, in the present study, growth curve analysis was only performed over an 11-day period. This time period also took into account both primary and secondary metabolism during the growth period. Dried cell mass was recorded as an indication of the growth of the strain. For the growth curve analysis experiments, the volume of the medium was scaled-up 5 times. Often, with scale-up, growth parameters of the strains cannot be easily controlled or predicted and changes in the levels of laccase production as well as the day of maximal laccase production may occur.

Table 2.1. Different types of growth media used in the secondary screening protocol.

Media:				
Different Glucose Concentrations:			Different Carbon Sources:	
Low Glucose Concentration	Intermediate Glucose Concentration	High Glucose Concentration	Glycerol as major carbon source.	Sucrose as major carbon source.
YEME (*ISP* Medium No. 2, yeast-extract-malt-extract):	**TDM (modified *Trametes* Defined Medium):**	**Modified GYP Medium:**	**Modified Bennett Medium (BM-Glycerol):**	**Czapek Solution (CZ-Sucrose):**
Ingredients (in g/L water): 4 g Glucose 4 g Yeast Extract 10 g Malt Extract pH to 5, 7, or 9 with HCl or NaOH as necessary.	Ingredients (in g/L water): 10 g Glucose 5.23 g Peptone 2 g KH_2PO_4 10 ml Trace Elements (see below) pH to 5, 7 or 9 with HCl or NaOH as necessary. Trace Elements Ingredients (in g/L water): 0.56 g Iron Sulphate 0.032 g Copper Sulphate 0.068 g Zinc Chloride 0.338 g Manganese Sulphate 0.19 g Cobalt Chloride 0.0024 g Nickel Chloride 0.818 g Ammonium molybdate	Ingredients (in g/L water): 20 g Glucose 5 g Yeast Extract 5 g Peptone 1 g $MgSO_4 \cdot 7H_2O$ pH to 5, 7 or 9 with HCl or NaOH as necessary.	Ingredients (in g/L water): 10 g Glycerol 1 g Yeast Extract 1 g Meat Extract pH to 5, 7 or 9 with HCl or NaOH as necessary.	Ingredients (in g/L water): 30 g Sucrose 2 g $NaNO_3$ 1 g K_2HPO_4 0.5 g KCl 0.5 g $MgSO_4 \cdot 7H_2O$ 0.01 g $FeSO_4 \cdot 7H_2O$ pH to 5, 7, or 9 with HCl or NaOH as necessary.
Reference: Shirling and Gottlieb, 1966	References: Addleman and Archibald, 1993 Ryan, 2003	Reference: Galhaup *et al.*, 2002	Reference: Atlas, 1993	Reference: Atlas, 1993

Abbreviations used regarding media: Y, YEME; T, TDM; G, GYP; B, BM-Glycerol; C, CZ-Sucrose; a letter followed by 5, 7, or 9 indicates the pH of that medium.

Table 2.2. Laccase-producing strains screened in this study.

Rank:	Strain Name (used in laboratory) / Extracellular or Intracellular Laccase:	Taxonomic Classification according to 16S rDNA sequence:	Maximum Specific Activity of Laccase Under Screening Conditions:				Other:
			Highest Specific Activity of Laccase (in U/µg protein):	Optimal Medium for Laccase Production:	Day of Highest Laccase Production in Optimal Medium:	Type of Growth in Optimal Medium:	
1	Tp / Extracellular	*Trametes pubescens* CBS 696.94	309	T5	7	Large, light yellow pellets; clear liquid medium.	Decolourises a wide range of dyes. Optimal aeration and agitation conditions are 160 rpm with no bung.
2	Sa / Intracellular	*Streptomyces antibioticus* NRRL B-2770	197	T7	5	Small, orange-yellow pellets; clear liquid medium.	Optimal aeration and agitation conditions for laccase are static conditions. Sticks to glass.
3	7H1 / Intracellular	*Streptomyces* spp.	80	B9	7	Fine, yellow-white mycelia; clear liquid medium.	Susceptible to glucose repression. Geosmin-producer. Optimal aeration and agitation conditions for laccase are static conditions.
4	MCTD1 / Extracellular	Unknown fungus	35	Y7	3	Large, light yellow pellets; clear liquid medium.	Strong decolourisation of bromophenol blue. Optimal aeration and agitation conditions are 160 rpm with no bung.
5	M25 / Intracellular	*Mycobacterium* spp.	30	B5	5	Large yellow 'flakes'; clear liquid medium.	Susceptible to glucose repression. Optimal aeration and agitation conditions are 160 rpm with a bung.

6	7H1 / Extracellular	As above (rank 3).	21	B9	7	Fine, yellow-white mycelia; clear liquid medium.	As above (rank 3).
7	M25 / Extracellular	As above (rank 5).	13	C9	5	Large yellow 'flakes'; clear liquid medium.	Susceptible to glucose repression. Optimal aeration and agitation conditions for laccase are static conditions.
8	Sa / Extracellular	As above (rank 2).	11	C7	7	Poor growth; clear liquid medium.	As above (rank 2).
9	DFNR8 / Intracellular	*Streptomyces* spp.	4	T9	3	Dark orange, medium-sized pellets; clear liquid medium.	Melanin producer.
10	DFNR8 / Extracellular	As above (rank 9).	2	Y9	3	Turbid medium, orange in colour.	As above (rank 9).
11	DFNR6 / Intracellular	*Streptomyces achromogenes* subsp. *achromogenes*	1.3	C9	3	White, medium-sized pellets; turbid liquid medium.	Appearance of laccase erratic. Do not recommend further studies.
12	M29 / Extracellular	*Amycolatopsis orientalis*	1.2	G7	5	Dark orange, turbid liquid medium.	Good decolourisation of crystal violet.
13	DFNR6 / Extracellular	As above (rank 11).	1.1	B7	3	White, medium-sized pellets; turbid liquid medium.	As above (rank 11).
14	DFNR3 / Extracellular	*Streptomyces achromogenes* subsp. *achromogenes*	0.68	T7	3	Medium-sized yellow pellets; clear liquid medium.	Very strong decolourisation of RBBR on malt extract agar at 30°C. Optimal aeration and agitation is 80 rpm with no bung. Sticks to glass.
15	LMP / Extracellular	*Rhodococcus ruber*	0.54	G7	4	Pink-orange appearance in	Moderate decolourisation of

						extremely turbid medium.	crystal violet.
16	BS2 / Extracellular	*Gordonia lacunae*	0.33	Y7	4	Growth shows pellets in clear liquid medium. Orange in colour.	Type strain of new species reported by le Roes *et al.*, 2007. Sticks to plastic.
17	BS2 / Intracellular	As above (rank 16).	0.24	Y5	7	Weak growth in clear liquid medium. Orange in colour.	As above (rank 16).
18	DFNR2 / Intracellular	*Streptomyces* spp.	0.14	C9	3	White, medium-sized pellet; clear liquid medium.	Melanin producer.

2.3.3 Strain descriptions

In this section, each strain investigated in this study is described. Physiological characteristics as well as factors influencing laccase production are discussed. Important findings for each strain are summarised in Table 2.2.

Fungal strains are described first [(a) and (b)], followed by actinomycete strains: Streptomycete strains [(c) to (i)], fast-growing non-streptomycete strains [(j) and (k)], and slow-growing non-streptomycete strains [(l) and (m)].

With respect to the optimal medium for highest laccase production, most strains produced laccase extracellularly, and thus it was noted whether or not intracellular laccase was detected and if it was significant (*i.e.* produced in large enough quantities to be considered useful for further study). An exception was seen in the case of *S. antibioticus*, where intracellular laccase (197 U/μg protein) was the most significant and extracellular laccase (11 U/μg protein) was not. Furthermore, in some cases, highest extracellular laccase production in a specific medium was observed to correspond to the highest intracellular value. For example, *Streptomyces* strain 7H1 produced the highest levels of extracellular and intracellular laccase in medium B9, while for other strains, extracellular and intracellular laccases were detected in different media.

(a) *Trametes pubescens* CBS 696.94

This white-rot fungus is a well-known laccase producer. During this study, the strain decolourised a wide range of dyes, preferring YEME medium for this activity. Very strong (defined in section 2.2.4) decolourisation of the dyes RBBR, BB, and Poly R, supplemented in YEME agar, was observed at 22, 25, and 30°C. The strain showed very strong decolourisation of RBBR and Poly R in low pH YEME agar at 22, 25, and 30°C. Decolourisation of BB in low pH YEME only occurred at 25°C and decolourisation of CV in low pH YEME only occurred at 30°C. Very strong decolourisation of RBBR in MEA agar was observed at 22, 25, and 30°C. No decolourisation of dyes by this strain occurred at 37°C. The strain produced more laccase when cresylic waste was used as an inducer of laccase activity (section 2.2.4). Samples of extracellular laccase in supernatant solution were still active after 4 days at 37°C and after 30 min at 55°C. Very low tyrosinase activity was detected using *p*-cresol as the substrate. Production of intracellular laccase was not significant but its presence was detected in the best medium for extracellular laccase production. Based on highest measured laccase activity, the optimal medium was found to be TDM at pH 5, which is in agreement with the literature and previous research conducted in our laboratory (Addleman and Archibald, 1993; Ryan, 2003). The growth curve for this strain is shown in Figure 2.1.

In this study, the abbreviation **Tp** is used to indicate this strain.

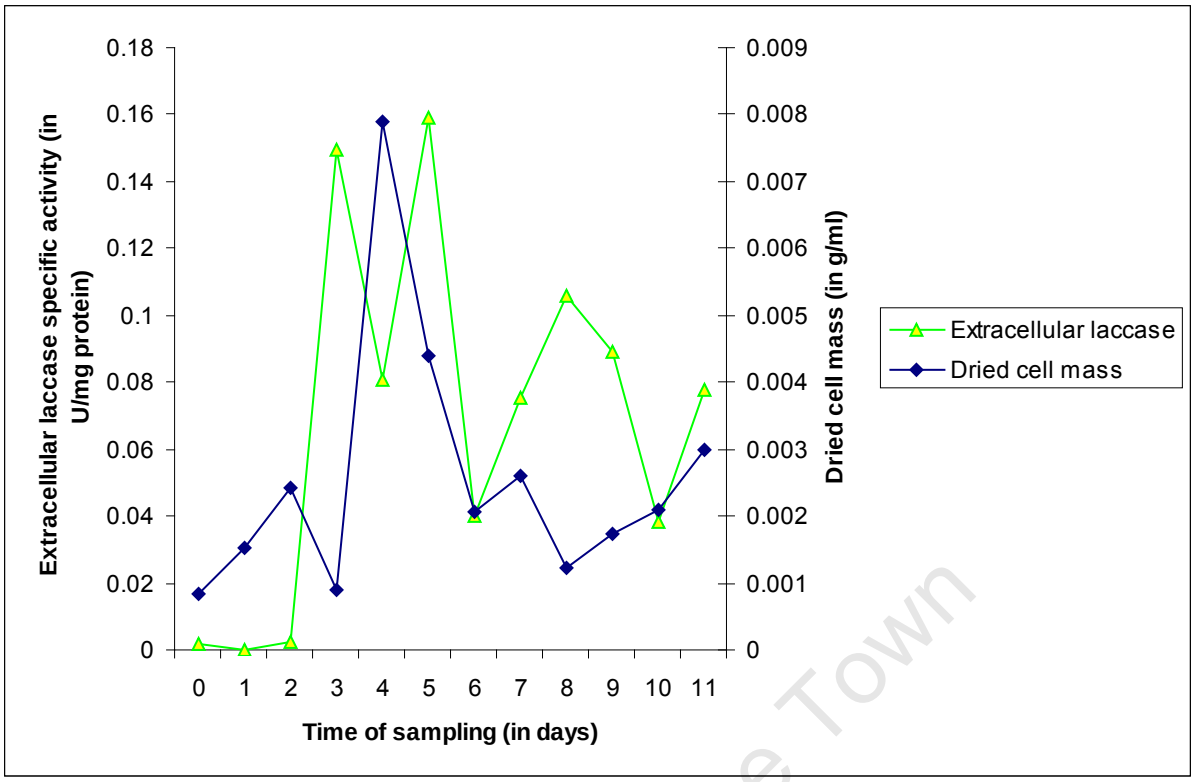


Fig. 2.1. Growth curve of *Trametes pubescens* showing the course of extracellular laccase production by the strain as well as the dried cell weights during growth. Data shown are the average of duplicate experiments.

(b) Unknown fungus: Strain MCTD1

This fungal strain was isolated from a 3-day-old air-dried soil sample obtained from the Muntiapo Chingola Tailing Dam in Zambia. The strain's appearance is *Penicillium*-like except it is white in colour. Strong decolourisation of BB on normal YEME and low pH YEME was observed at 22, 25, and 30°C. The strain produced more laccase when cresylic waste was used as an inducer of laccase. This strain was the best fungal laccase producer based on primary screens conducted in our laboratory. All other laccase producers were strains representing various genera of actinomycetes, as discussed in this chapter. Intracellular laccase production was not significant and was only detected in YEME, GYP, and TDM media. The optimal medium of YEME at pH 7 is unusual, as most fungi prefer an acidic medium for laccase production.

(c) *Streptomyces coelicolor* A3(2)

This strain is available from the NRRL and is the producer of SLAC, the small extracellular laccase missing a domain (Machczynski, *et al.*, 2004). This strain decolourised a wide range of dyes over a wide range of temperatures. The strain prefers low pH YEME medium for decolourisation of dyes. On *ISP*4 the substrate mycelium was yellow-brown and the aerial mycelium was grey-white. Diffusible pigments were produced on other media. Although this strain is a known laccase producer and dye-decolourisation studies were positive, experiments performed using YEME liquid media with and

without cresylic waste, as an inducer of laccase, revealed that no extracellular laccase could be detected using ABTS as substrate, and thus this strain was not investigated further.

(d) *Streptomyces antibioticus* IMRU 3720 ≈ NRRL B-2770

Laccase has not been previously reported to be produced by this strain but a different class of multicopper oxidase, called phenoxazinone synthase, has been reported (le Roes-Hill, *et al.*, 2009). It is intracellular, repressed by glucose and has different properties from that of laccase. During this study laccase was detected in this strain, produced both intracellularly and extracellularly. The laccase was detected in samples using ABTS as substrate and laccase production was not subject to glucose repression. To the best of the author's knowledge, this is the first report of laccase from *S. antibioticus*.

In dye-decolourisation studies, BPB was strongly decolourised in MEA at 22 and 30°C. RBBR was strongly decolourised in MEA at 22°C. BPB, supplemented in YEME, was strongly decolourised at 22, 25, and 30°C, whereas RBBR was weakly decolourised at 22, 25, and 30°C, CV was also weakly decolourised at 22 and 25°C, and BB was strongly decolourised at 25 and 30°C but weakly decolourised at 22°C. RBBR supplemented in low pH YEME was weakly decolourised at 22°C, and strongly decolourised at 25 and 30°C, whereas BB was strongly decolourised at 22 and 25°C. This strain appears to effectively decolourise BB over a wide range of conditions. Guaiacol, supplemented in 3% MEA, was an excellent inducer of laccase activity by this strain. Extracellular laccase was detected after 8 min at 55°C. Very low tyrosinase activity was detected, which is not unusual, as this strain has been documented to produce tyrosinase.

Production of intracellular laccase was most significant. Extracellular laccase production was not significant but usually corresponded to the medium in which the most intracellular laccase was produced. It is as if the strain was forced to excrete some laccase, thus the correspondence. The best medium for production of extracellular laccase was C7, and for intracellular laccase was T7, thus the best medium for obtaining maximal laccase was TDM at pH 7. Optimal aeration and agitation experiments showed that for both intracellular and extracellular laccase production static conditions were best. The growth curve for this strain is shown in Figure 2.2.

In this study, the abbreviation **Sa** is used to describe this strain.

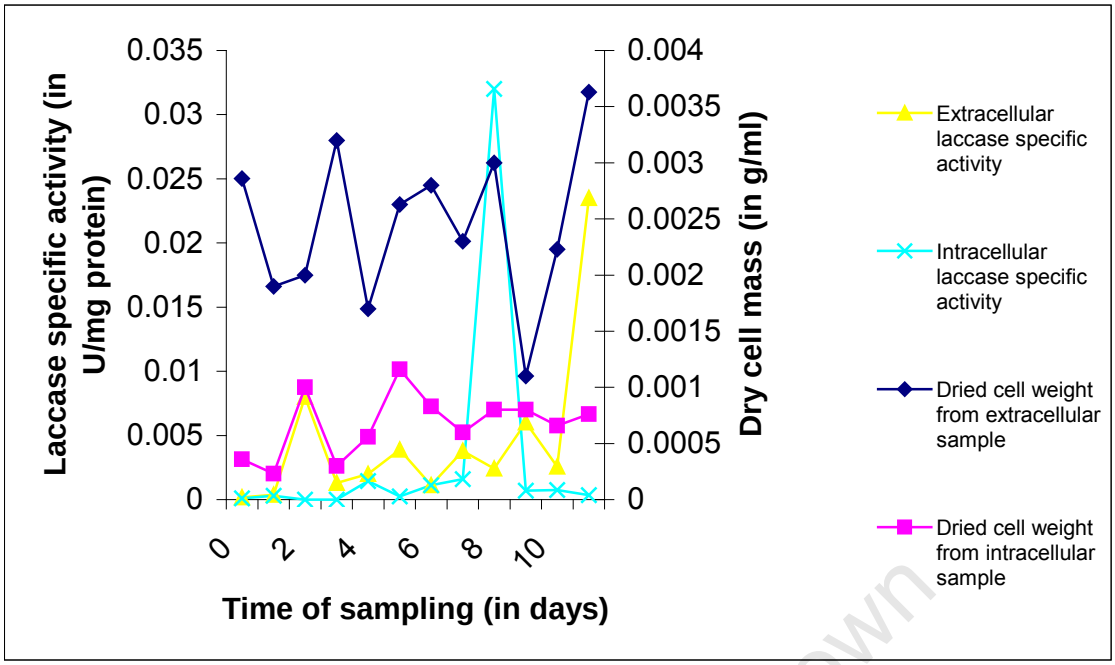


Fig. 2.2. Growth curve of *Streptomyces antibioticus*. Extracellular and intracellular laccase specific activities are shown as well as the dry cell weights. The optimal media for intracellular and extracellular laccase production are different, and therefore two dry cell weight measurements are shown.

(e) *Streptomyces achromogenes* subsp. *achromogenes* Strain DFNR3

Actinomycete strain DFNR3 is now known to be a strain of *Streptomyces achromogenes* subsp. *achromogenes* NRRL B-2120^T with 99% 16S sequence similarity (Accession No.: EF654091). (Please see Appendix B for an explanation as to the relevance of the 16S rDNA sequence.) Laccase has not been previously reported from this species. DFNR3 was isolated from farm soil on the North Island of New Zealand. On *ISP*4 the substrate mycelium is yellow-brown and the aerial mycelia grey-white. No diffusible pigments are produced. Very strong decolourisation of RBBR occurred on MEA at temperatures of 22 – 37°C, whereas weak decolourisation of CV occurred under the same conditions. Very strong decolourisation of RBBR in YEME occurred at 22, 25, and 30°C, and weak decolourisation of CV occurred at 25°C. Weak decolourisation of RBBR in low pH YEME occurred at 25 and 30°C. Cresylic waste inhibited the production of laccase by this strain. DFNR3 had a tendency to stick to glass, thereby causing difficulties in working with the strain. No intracellular laccase was detected. The DFNR strains reported in this section and in Table 2.2 are likely to be phylogenetically related. It would be of interest to determine the 16S rDNA sequences of the strains investigated in this study and determine their phylogenetic relatedness. The relatedness of the strains' laccases may be inferred from such information. It can be seen from the secondary screening protocol that the production of laccases by the *Streptomyces* DFNR strains was very different. Extracellular laccase was produced by DFNR3, intracellular laccase by DFNR2, and laccase production by DFNR6 was erratic at best, while DFNR8 produced both intracellular and extracellular laccase in large quantities relative to the other DFNR strains. The optimal media for laccase production by each strain also differed.

(f) *Streptomyces achromogenes* subsp. *achromogenes* Strain DFNR6

Actinomycete strain DFNR6 is now known to be a strain of *Streptomyces achromogenes* subsp. *achromogenes* NRRL B-2120^T with 99% 16S sequence similarity (Accession No.: EF654091). Laccase has not been previously reported to be produced by this species. DFNR6 was isolated from farm soil on the North Island of New Zealand. The strain was not further pursued due to the erratic appearance of laccase in the media tested and also due to low laccase specific activities.

(g) *Streptomyces* sp. Strain DFNR2

The 16S rDNA sequence of this strain showed 99% similarity to various streptomycetes. This strain was isolated from farm soil located on the North Island of New Zealand. Good growth occurred on YEME and melanin production was strongly evident on *ISP*6 and *ISP*7 agars. Melanin production is an indicator of tyrosinase production and of laccase (Shirling and Gottlieb, 1966). It is believed that fungal laccase is involved in the synthesis of melanin, a dark polymeric pigment protecting microorganisms against adverse environmental effects (Shutova, *et al.*, 2008). Laccase has been found in the bacteria *Streptomyces galbus*, *Azospirillum lipoferum*, and *M. mediterranea*, all of which produce a melanic pigment. This suggests a possible link between laccase activity and melanin production (Giardina, *et al.*, 2009; Molina-Guijarro, *et al.*, 2009; Hullo, *et al.*, 2001). Bacterial CotA from *Bacillus subtilis* participates in the biosynthesis of the brown spore pigment, which is also thought to be a melanin-like product (Hullo, *et al.*, 2001). CotA may be responsible for most of the protection afforded by the spore coat against UV light and hydrogen peroxide. Production of extracellular laccase was insignificant. The best medium for intracellular laccase production was C9.

(h) *Streptomyces* sp. Strain DFNR8

A 16S sequence for this strain shows 97% similarity to various streptomycetes, thus this strain may be a novel species and its laccase of interest. It was isolated from farm soil on the North Island of New Zealand. Melanin production was evident on *ISP*6 and *ISP*7 agars. Intracellular laccase production was highest in medium T9 and the best medium for extracellular laccase production was Y9.

(i) *Streptomyces* sp. Strain 7H1

This strain was isolated from a soil sample obtained at Die Hel, Gamkaskloof, in South Africa, and exposed to heat pre-treatment. The strain produced a very strong geosmin smell (*i.e.*, the chemical responsible for the smell of soil). The strain also produced green-black diffusible pigments in liquid media. Strain 7H1 may also be susceptible to glucose repression of laccase production. Glucose repression of laccase production was indicated since laccase was not produced in media containing glucose but was produced when a different carbon source was used. Highest intracellular and extracellular laccase production was in B9 and aeration and agitation experiments showed that static conditions were best for laccase production. Intracellular laccase production was very significant. Intracellular and extracellular laccase samples are best stored at –20°C or 4°C as laccase activity was lost after 4 days at higher temperatures. Addition of PMSF had no affect on samples. Intracellular

laccase activity was detected after 30 min at 55°C. The growth curve of this strain is shown in Figure 2.3.

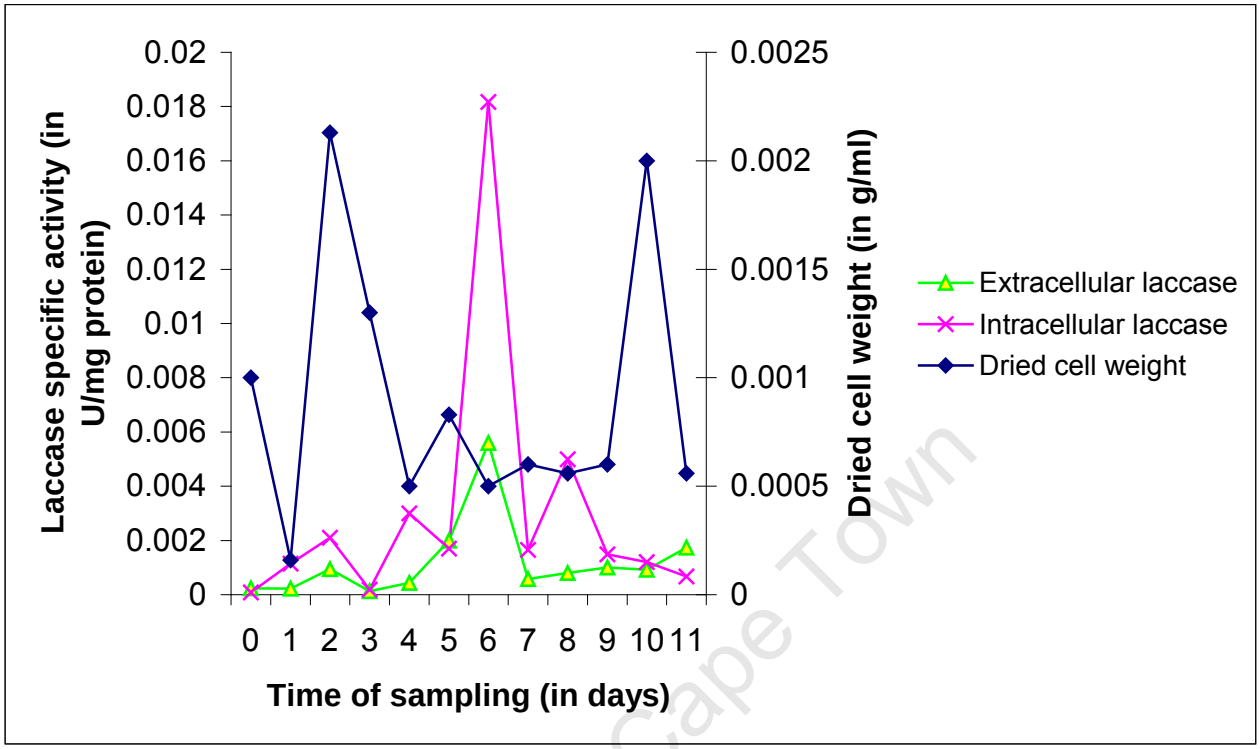


Fig. 2.3. Growth curve of *Streptomyces* sp. strain 7H1 showing the levels of intracellular and extracellular laccase produced during growth and the dried cell weight.

(j) *Gordonia lacunae* Strain BS2^T ≈ DSM 45085^T ≈ NRRL B-24551^T

It is important to note that non-streptomycete actinomycetes are rare and thus enzymes from these isolates may be equally as unique. Laccases have not been documented to have been previously produced by the genus *Gordonia*. Strain BS2 was isolated from a beach sand sample from the Beacon Isle, Plettenberg Bay, South Africa. The strain adheres to plastic thus causing difficulties when working with it. The best medium for extracellular laccase production was Y7 and for intracellular laccase production the best medium was Y5.

(k) *Rhodococcus ruber* Strain LMP

The 16S rDNA sequence of strain LMP showed 100% similarity to *Rhodococcus ruber*. Laccase has not been previously described as been produced by the genus *Rhodococcus*. Intracellular laccase production was insignificant. The optimal medium for production of extracellular laccase was G7.

(l) *Mycobacterium* sp. Strain M25

The strain was isolated from Roodepoort (South Africa) garden soil subjected to heat pre-treatment. The 16S rDNA sequence of strain M25 showed 99% similarity to various mycobacteria. Slight clearing

of RBBR and CV occurred after 21 days on dye-decolourisation media. After such a long period of time, the clearing could be due to the strain metabolising the dyes by uptake and not necessarily the enzyme breaking the dye bonds and thus causing decolourisation. Intracellular laccase was significant. This strain may be susceptible to glucose repression of laccase production. Intracellular and extracellular laccase samples remained active after 4 days at 37°C. Laccase activity was lost immediately at 55°C. Very low tyrosinase activity was detected using *p*-cresol as the substrate. The culture contains a co-culture that is possibly a *Nocardia* strain (responsible for any orange colours and white 'flakes' in the culture). A second BLAST analysis of the 16S rDNA sequence indicated that the closest relatives of strain M25 are pathogenic mycobacteria, and thus this strain was not further investigated. Laccase has not been previously documented as being produced by the genera *Mycobacterium* and *Nocardia*. The best medium for extracellular laccase production was C9 and for intracellular laccase production the best medium was B5. The growth curve of this strain is shown in Figure 2.4.

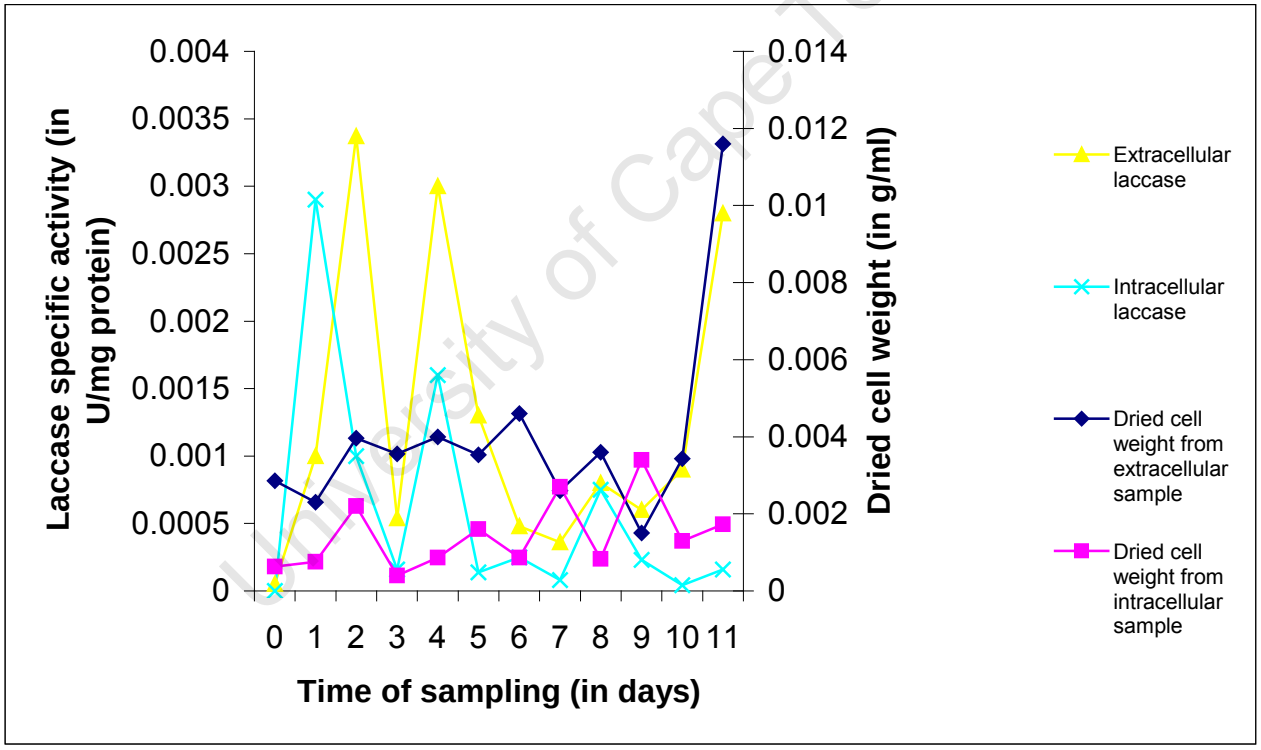


Fig. 2.4. Growth curve of *Mycobacterium* sp. strain M25 showing low levels of extracellular and intracellular laccase production and the dry cell weights from growth in the different optimal media.

The extracellular laccase produced by *Mycobacterium* sp. strain M25 in C9 medium under static conditions showed an interesting reaction with Bradford reagent. Bradford reagent is brown, turning blue when interacting with protein. However, with this laccase sample, the Bradford reagent repeatedly turned yellow-green, a colour change most likely due to interaction with the Coomassie

dye, which makes up the reagent. Neither the growth medium nor the pH was responsible for this colour change, as can be seen in Figure 2.5 below. Swamy and Ramsay (1999) simply state that "the decoloration of Coomassie blue has been used to indicate ligninolytic activity". For this strain, the laccase obtained under this specific condition showed high activity and may be capable of quickly degrading Coomassie blue, thus causing the yellow-green decolourisation of the Bradford reagent's dye. The structure of Coomassie contains no chlorine atoms, thus the colour change is not due to a bleaching reaction by peroxidase, but may be due to the action of laccase.

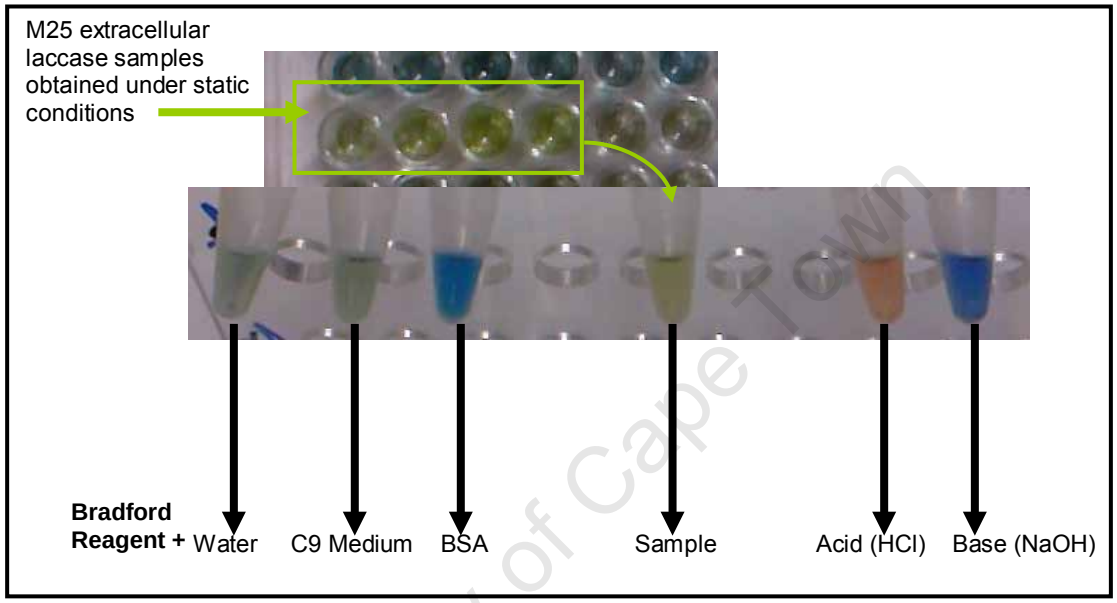


Fig. 2.5. Laccase from *Mycobacterium* sp. strain M25 may be responsible for the decolourisation of Coomassie blue.

(m) *Amycolatopsis orientalis* Strain M29

This strain was isolated from Roodepoort garden soil subjected to heat pre-treatment. The 16S rDNA sequence of strain M29 showed 100% similarity to *Amycolatopsis orientalis*. Laccase has not been previously described as being produced by the genus *Amycolatopsis*. Intracellular laccase production was insignificant. The highest levels of extracellular laccase were produced in medium G7.

2.3.4 Comparison of laccase production between strains

Based on the data shown in Table 2.2, and illustrated in Figures 2.6 and 2.7 below, the WRF *Trametes pubescens* is an excellent producer of high levels of extracellular laccase, while the fungal strain MCTD1, the genus of which has yet to be determined, is not as efficient a laccase producer.

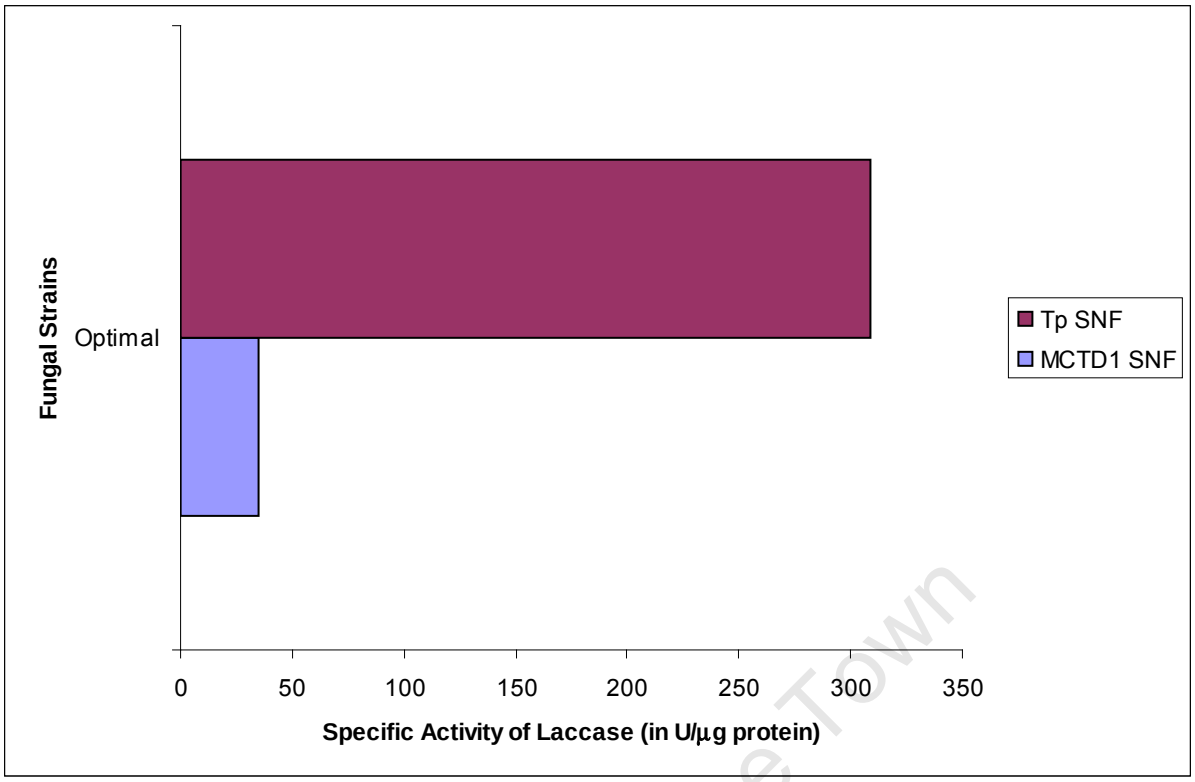


Fig. 2.6. Comparison of the laccase specific activities of the two fungal strains screened in this study. Extracellular laccase is denoted by SNF.

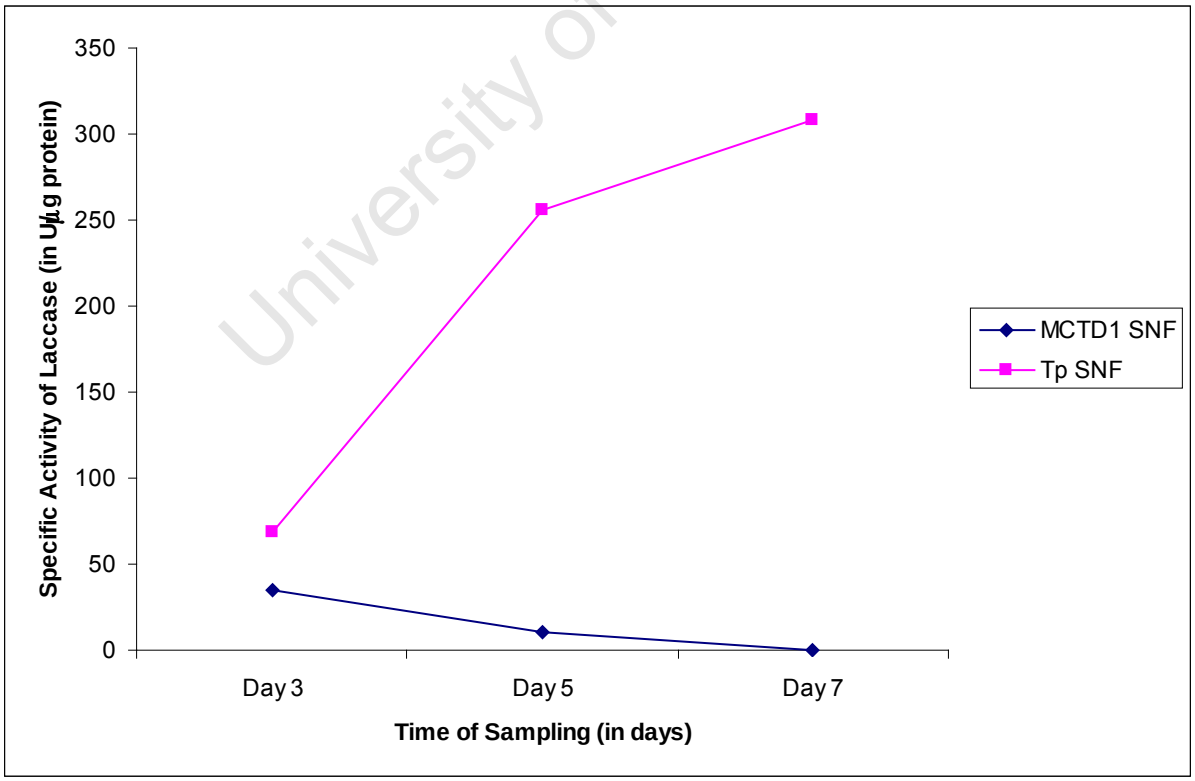


Fig. 2.7. Highest laccase production by the fungal strains over time. Extracellular laccase is denoted by SNF.

Of the non-streptomycete strains screened, *Mycobacterium* sp. strain M25 produced the highest levels of laccase, specifically intracellular laccase (Fig. 2.8). High levels of intracellular laccase as opposed to extracellular laccase are typical of bacterial strains described in the literature (Diamantidis, *et al.*, 2000; Leidig, *et al.*, 1999; McMahon, *et al.*, 2007; Suzuki, *et al.*, 2003). The production of intracellular laccase by bacteria, as opposed to extracellular laccase by fungi, might be attributable to the role of laccase in bacteria. The role, or function, of laccase in bacteria, especially actinomycetes, has yet to be conclusively determined, but it may be primarily cell-associated, while fungal laccases are excreted extracellularly to degrade material for nutrient uptake (such as lignin) or for protection by degrading toxic compounds in the surrounding environment (Arias, *et al.*, 2003; Baldrian, 2006; Solomon, *et al.*, 1996). Bacterial laccases appear to play a role in morphogenesis, sporulation processes, pigment production, and in copper homeostasis (Giardina, *et al.*, 2009; Molina-Guijarro, *et al.*, 2009). The laccase produced by *Bacillus subtilis*, a non-actinomycete bacterium, is associated with protecting the spore coat from environmental dangers such as exposure to UV (Hullo, *et al.*, 2001).

The fast-growing non-streptomycete strains examined in this study (Figures 2.9 and 2.10) were not as efficient producers of laccase as the slow-growing non-streptomycete strains (Fig. 2.11). Since laccase production has not been described by these actinomycete genera in the literature, it is unclear as to how these results should be interpreted. Fast-growing non-streptomycete strains may simply produce low levels of laccase, or high levels of laccase may have been produced within hours, as opposed to within days. In following the secondary screening protocol, samples were not taken during the first day of incubation.

Furthermore, in all the studies using the various strains, it was noted that laccase was not produced only once in the life cycle. Laccase levels would rise and fall during growth. This may be due to the formation of different laccase isoforms during the growth of the native strains (Claus, 2003).

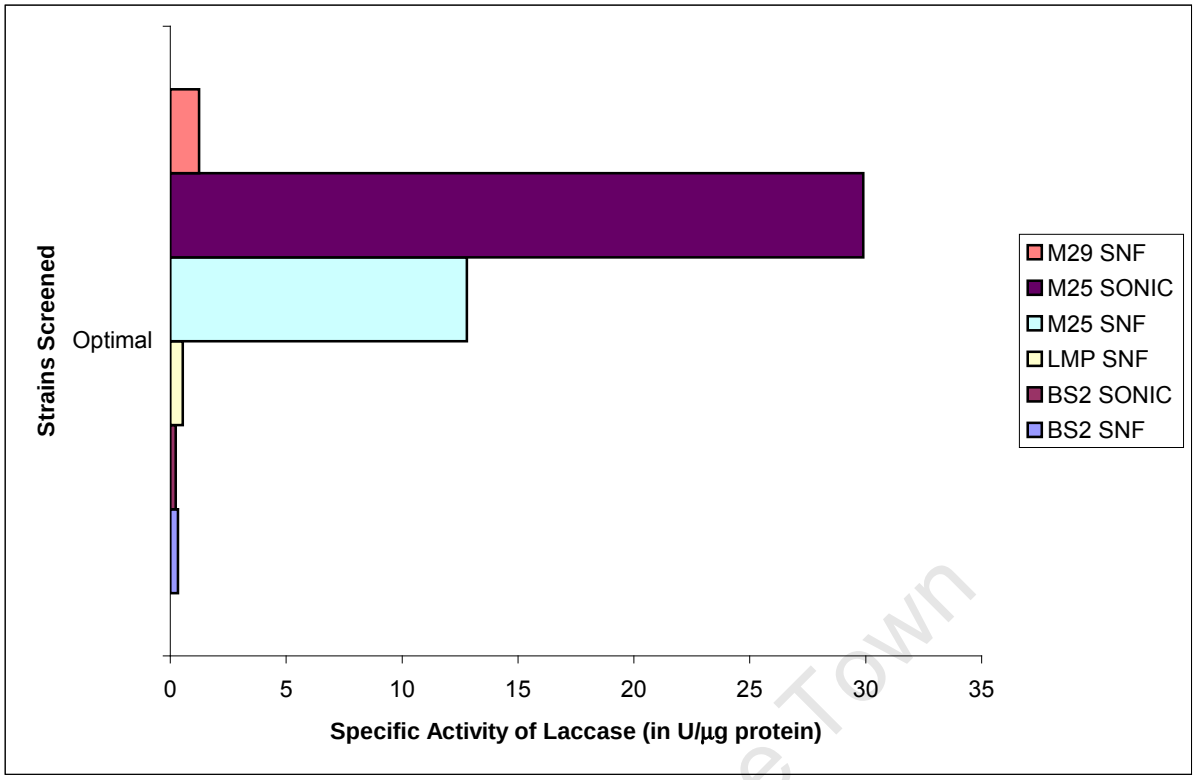


Fig. 2.8. Comparison of the laccase specific activities of the non-streptomycete strains screened in this study. Intracellular laccase is denoted by SONIC. Extracellular laccase is denoted by SNF.

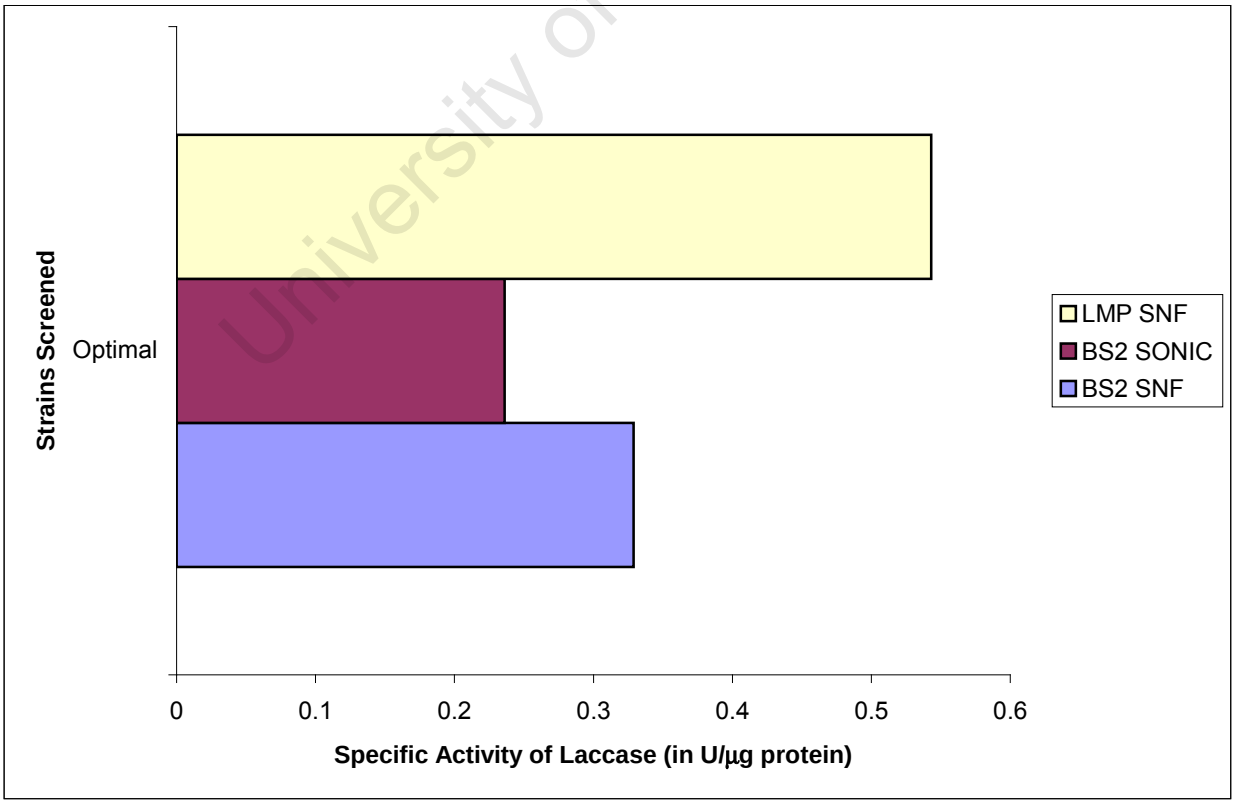


Fig. 2.9. Comparison of the laccase specific activities of the **fast**-growing non-streptomycete strains. Intracellular laccase is denoted by SONIC. Extracellular laccase is denoted by SNF.

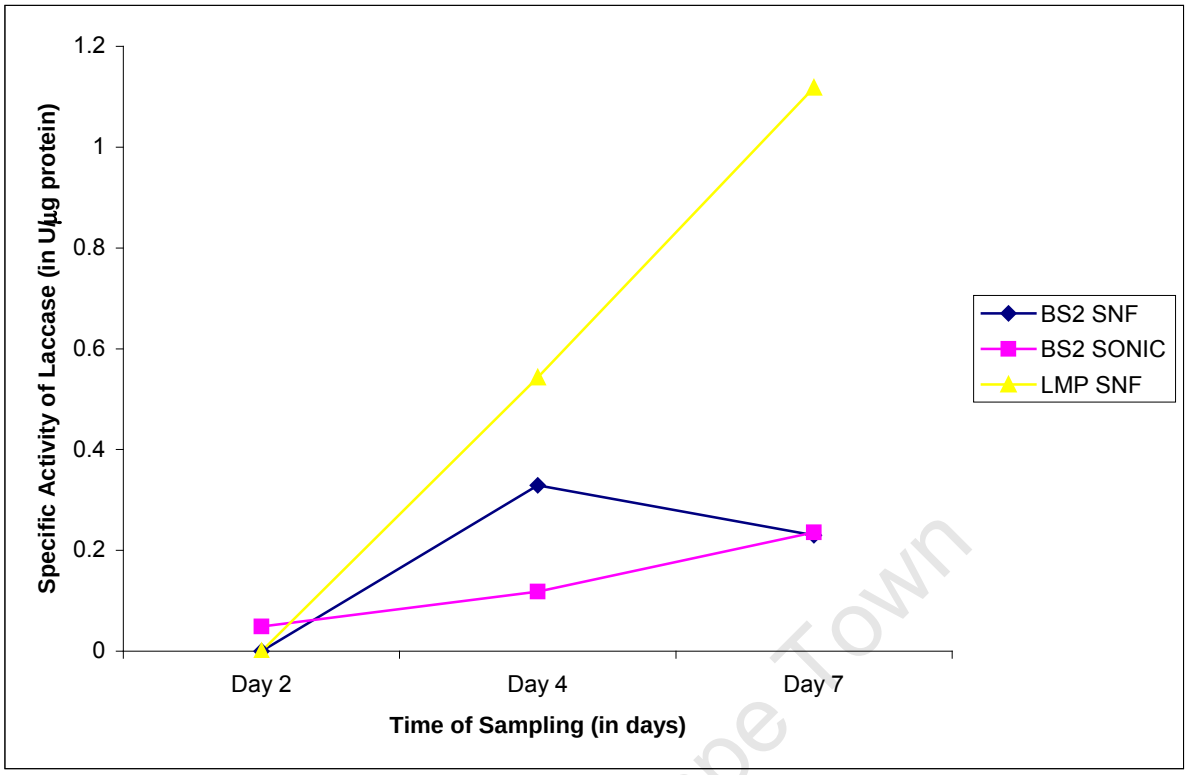


Fig. 2.10. Highest laccase production by the fast-growing non-streptomycete strains over time. Intracellular laccase is denoted by SONIC. Extracellular laccase is denoted by SNF.

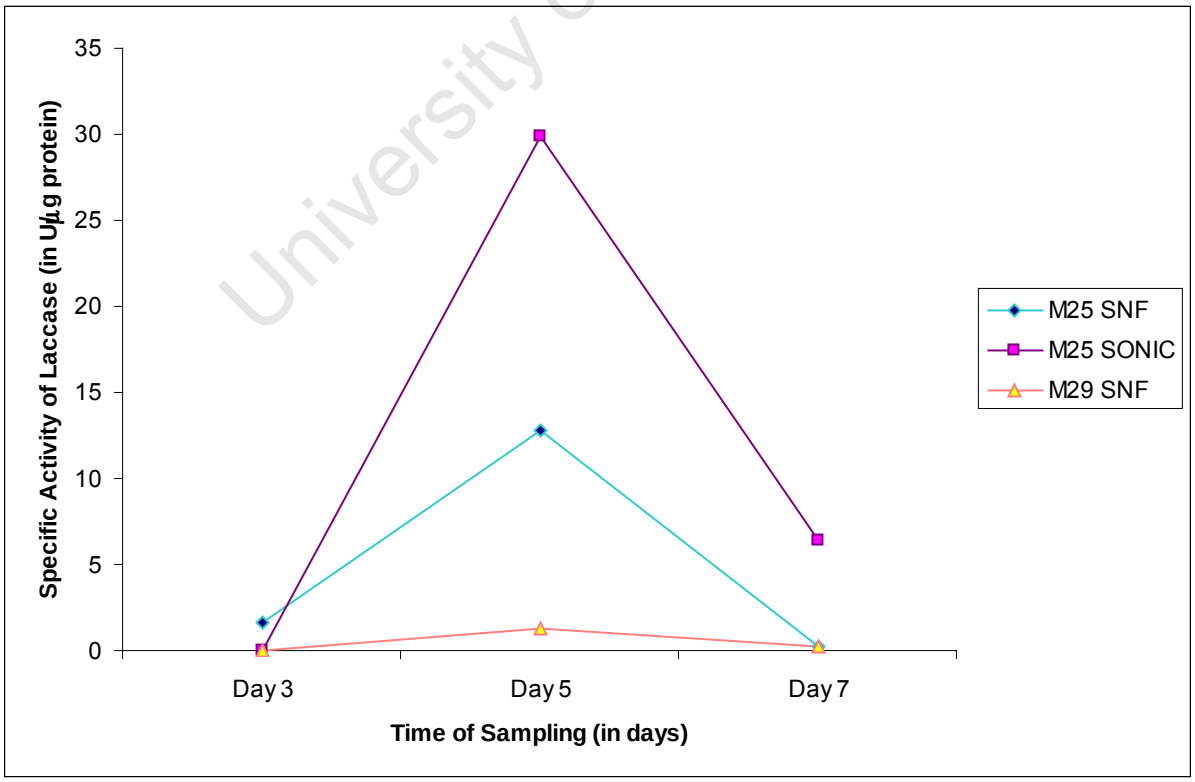


Fig. 2.11. Highest laccase production by the **slow**-growing non-streptomycete strains over time. Intracellular laccase is denoted by SONIC. Extracellular laccase is denoted by SNF.

Of the streptomycete strains screened, *Streptomyces antibioticus* produced the highest levels of laccase and again intracellular laccase was predominant (Figures 2.12 and 2.13). *S. antibioticus* is known to produce other BMCOs (le Roes-Hill, *et al.*, 2009). Of the new environmental isolates investigated in this study, strain 7H1 was the most promising streptomycete, in terms of intracellular laccase production. Isolating intracellular laccase may be a tedious process and thus extracellular laccase production would be favoured, especially for an efficient industrial process. The DFNR strains, while perhaps phylogenetically related, showed differences in their laccase production and response to optimisation variables.

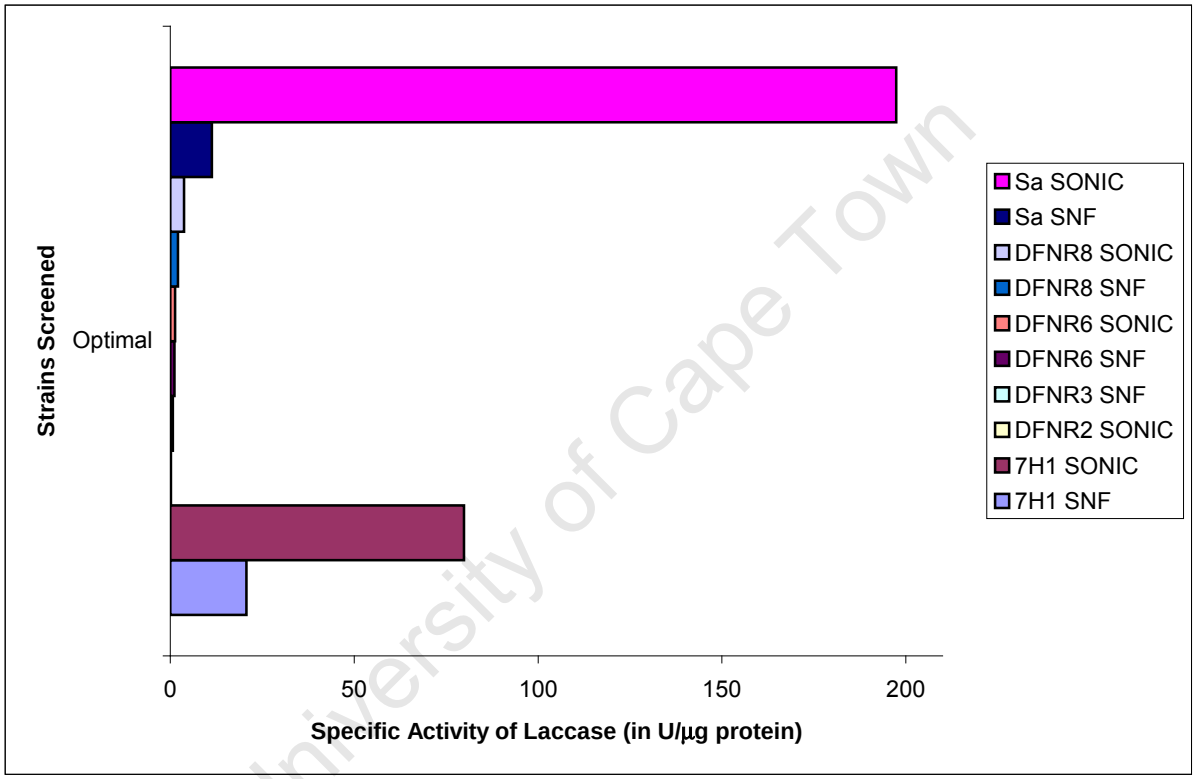


Fig. 2.12. Laccase specific activities of the streptomycete strains screened in this study. Intracellular laccase is denoted by SONIC. Extracellular laccase is denoted by SNF.

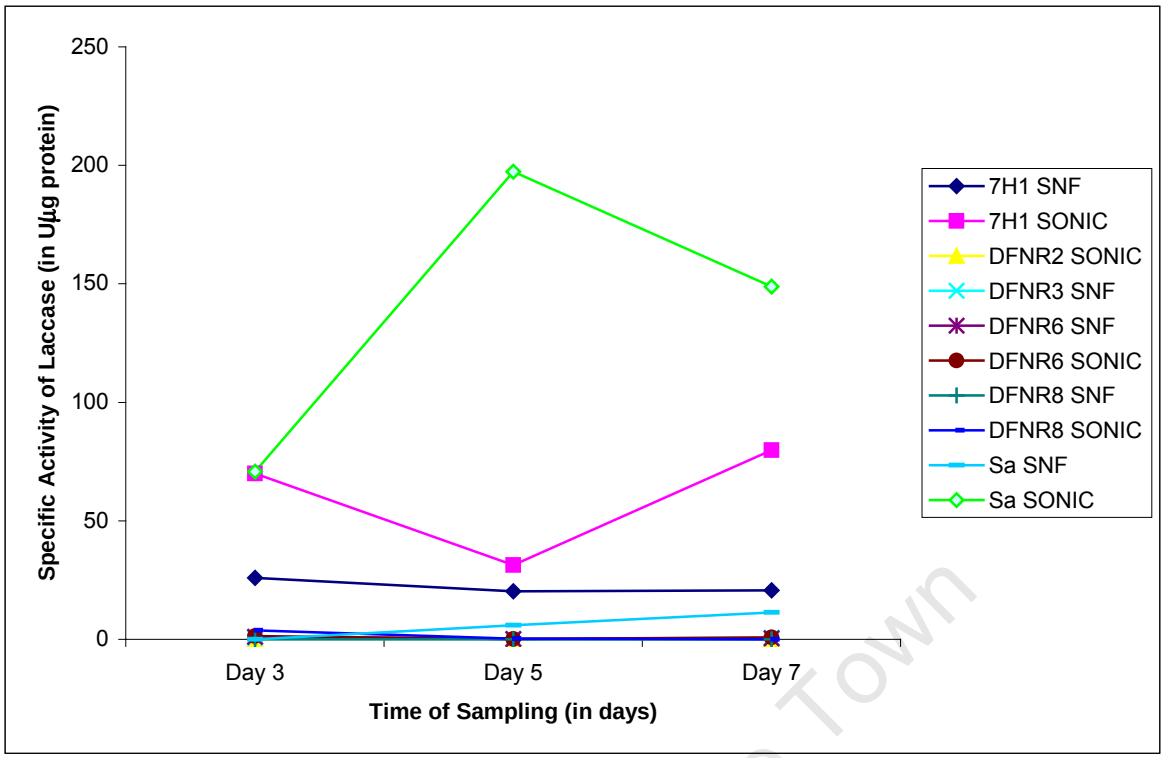


Fig. 2.13. Highest laccase production by the streptomycete strains over time. Intracellular laccase is denoted by SONIC. Extracellular laccase is denoted by SNF.

2.3.4.1 Growth curve analyses

Growth curve analysis, investigating laccase production (Fig. 2.14) and dried cell weights (Fig. 2.15) showed no definitive trend to indicate that laccase production was associated with the growth phases of the organisms. Figure 2.14 shows that the fungus *T. pubescens* produces significantly larger amounts of laccase throughout its life cycle compared to all of the actinomycete strains investigated in this study, except for strain 044 30-1 (see section 2.3.6 below).

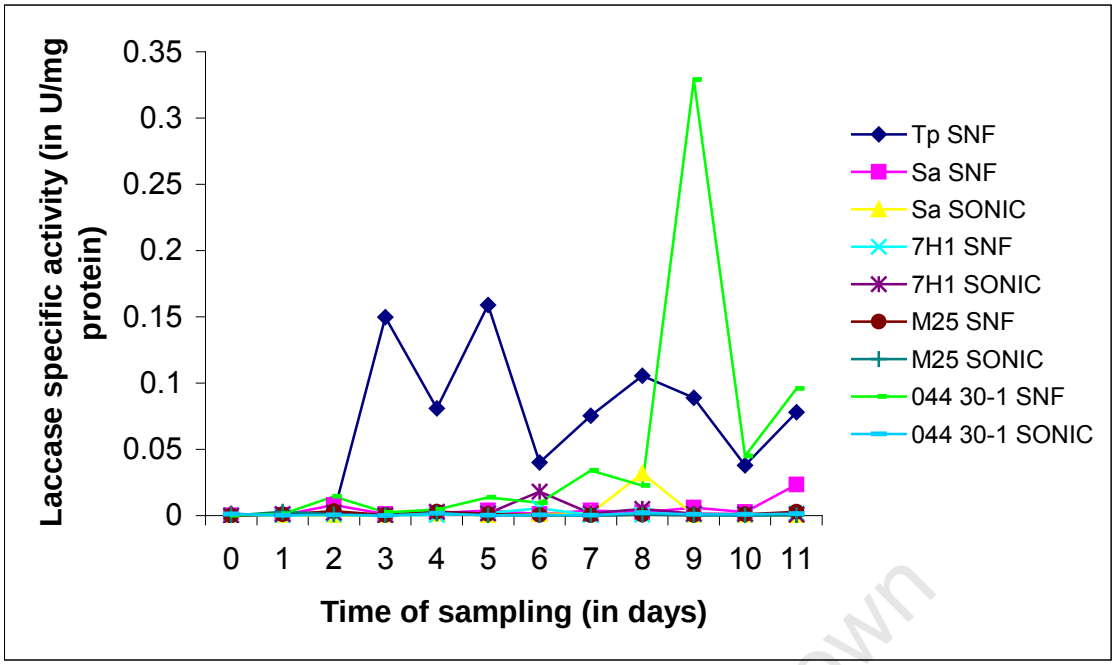


Fig. 2.14. Comparison of laccase production by the strains for which growth curve data was obtained. SNF, extracellular laccase; SONIC, intracellular laccase.

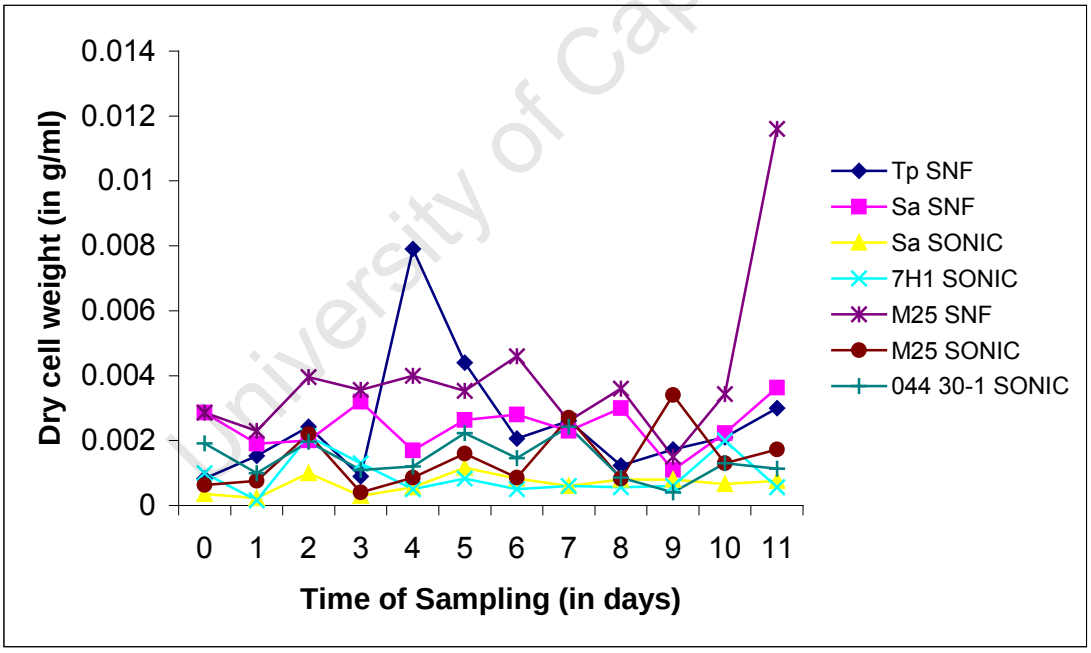


Fig. 2.15. Comparison of the dry cell weights of the strains during growth.

2.3.4.2 Inducers and inhibitors of laccase

Analysis of the growth curve of a strain and information on induction can be used to enhance laccase production. Once a suitable inducer is found (and many compounds may require testing for use as an inducer), this compound can be added at a specific time in the growth cycle of a strain in an effort to enhance production, either at a time period where laccase is already produced in large quantities

thereby increasing the yield obtained or at an earlier time to elicit laccase production at an earlier stage in the growth cycle.

The effect of laccase inducers and an inhibitor (1 mM final concentrations according to literature) on the production of laccase by the native strains is shown in Figure 2.16. The control sample was taken from a flask inoculated with the strain but the medium was not supplemented with an inhibitor or inducer. For the fungus, *T. pubescens*, sodium azide inhibited laccase production only slightly, while ferulic acid (FA) appeared to have a detrimental effect on laccase production. Xylidine was a good inducer of laccase production (enhanced by 2.2 times relative to the control) in *T. pubescens*. Extracellular laccase production by *Streptomyces* strain 7H1 was strongly induced by xylidine (enhanced 44 times). Laccase production by *Mycobacterium* strain M25 was slightly enhanced by the addition of FA and xylidine. For *S. antibioticus*, extracellular laccase production was greatly enhanced by the addition of FA (by 43 times), while intracellular laccase production was greatly enhanced by the presence of xylidine in the medium (by 33 times). Thus, in general, xylidine was an excellent inducer of laccase activity, in agreement with the literature (Giardina, *et al.*, 2009; Cordi, *et al.*, 2007; Bertrand, *et al.*, 2002; Galhaup, *et al.*, 2002; Eggert, *et al.*, 1996). FA is also a known laccase inducer (Giardina, *et al.*, 2009; Galhaup, *et al.*, 2002; Niladevi and Prema, 2007; Vasconcelos, *et al.*, 2000) and in this study, FA proved to be effective in enhancing laccase production by actinomycete strains.

Studies on laccase induction in actinomycetes are rather scarce (Niladevi and Prema, 2007). Aromatic and phenolic compounds have been widely used to elicit enhanced laccase production by different microbes and the nature of the compound that induces laccase activity differs greatly with the species. The aromatic inducers used in a study by Niladevi and Prema (2007) to enhance laccase production by *Streptomyces psammoticus* were compounds having structural similarity to lignin or polyphenols, and most of these compounds had been reported as effective laccase inducers in fungal strains. Copper sulphate also proved to be a promising inducer of laccase production by *S. psammoticus*. No other comparative data on laccase induction were found in the case of actinomycetes.

Ions that bind directly to the laccase active site, thus preventing the formation of the substrate-enzyme complex, include azide, cyanide, carbon monoxide, and halide ions. Azide inhibition in particular is attributed to its strong coordination ability with the metals in the active site, which changes the coordination number and conformation of the active site, thus inhibiting laccase activity (Shi, *et al.*, 2002). While the laccase of *Trametes versicolor* was completely inhibited by 1 mM NaN_3, the laccases of *Streptomyces coelicolor* and *Streptomyces ipomoea* were fully active under the same conditions (Dubé, *et al.*, 2008; Molina-Guijarro, *et al.*, 2009). Laccases SLAC and EpoA (showing a high amino acid sequence homology to each other) both have a higher resistance toward common, conventional laccase inhibitors. This may be due to their 3D structure, possibly the effect of a feature linked to the trinuclear Cu cluster site (Dubé, *et al.*, 2008).

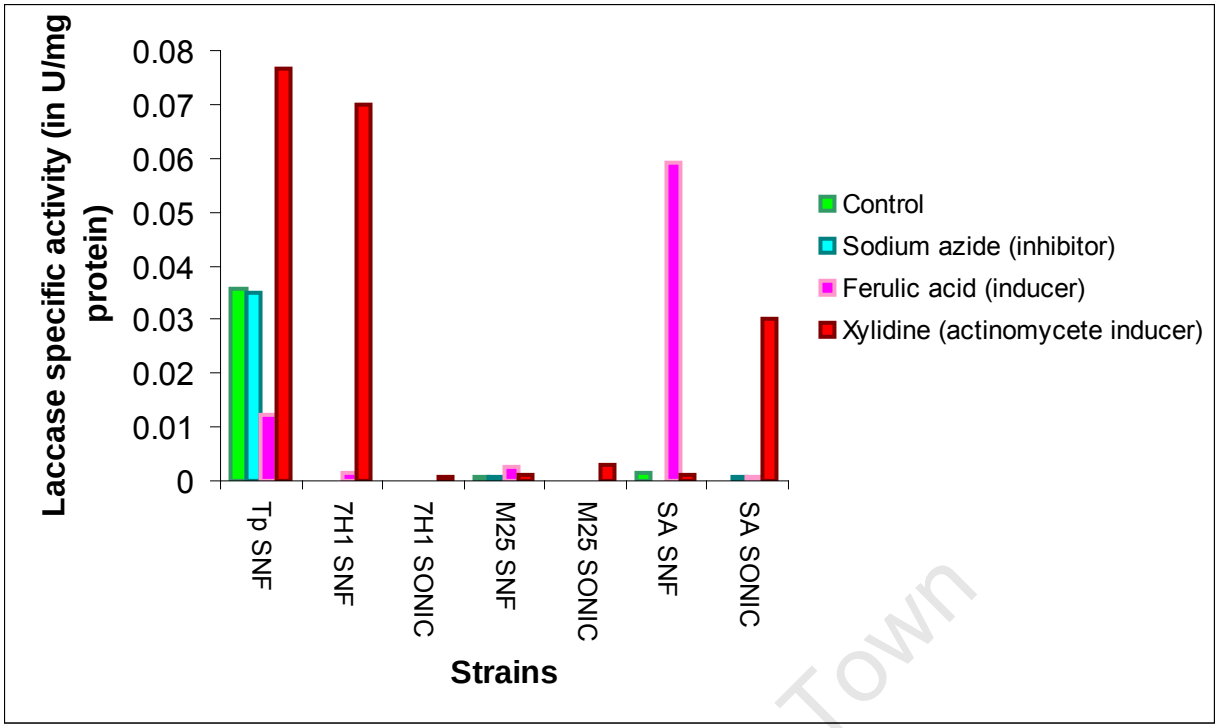


Fig. 2.16. The effect of laccase inducers and inhibitors on the production of laccase by the strains. SNF, extracellular laccase. SONIC, intracellular laccase.

2.3.5 Screening microbial strains for the detection of laccases: Effectiveness of the screening protocol

This study revealed that the optimal conditions for laccase production showed great variability amongst actinomycete strains, and between fungal and actinomycete strains (Table 2.3). It is also important to note that intracellular and extracellular laccase production, by the same strain, was often detected under very different conditions. Laccase production occurred at different times in the growth stages of the actinomycetes and researchers have yet to determine which factors trigger the onset of production of laccase by actinomycete strains in a laboratory environment. The earlier the production of laccase, the more useful the strain for industrial application, as time is an important economic concern in industry. Optimisation of culture conditions can allow for earlier production of laccase or enhanced levels of laccase production by the native strains (Chapter 3). This illustrates the usefulness of a screening protocol examining different variables affecting laccase production by the native strains.

Table 2.3. Summary of the highest activity laccase-producing strains and the most favourable conditions for production of their laccases.

Strain:	Type of Laccase:	Best Medium:	Best Aeration & Agitation:
Trametes pubescens	Extracellular	T5	160 rpm
***Streptomyces* sp. 7H1**	Extracellular & Intracellular	B9	Static
Streptomyces antibioticus	Extracellular	C7	Static
	Intracellular	T7	Static
***Mycobacterium* sp. M25**	Extracellular	C9	Static
	Intracellular	B5	160 rpm with bung

2.3.6 Strain 044 30-1

At the time that this study reached the experiments described in section 2.2.9, another researcher in our laboratory had isolated a strain which, after primary screening, produced more laccase per gram biomass than the previously screened strains (Dr M. le Roes-Hill, pers. comm.). This observation was confirmed by data illustrated in Figures 2.14 and 2.17, and it was decided that only this strain would be further pursued. Figure 2.14 shows that more laccase (in U/mg protein) was produced by this strain than by the fungus *Trametes pubescens* but that the highest level of laccase production occurred late in the growth cycle, *i.e.* 9 days after inoculation. For potential use of this strain in an industrial process, it would be preferable for high levels of laccase to be produced earlier by this strain. This might be achieved through optimisation of various parameters. Furthermore, the medium (SCN) used in this growth curve analysis was not the optimal medium but was the medium used to initially isolate this strain from an environmental sample, and the aeration and agitation conditions used were 160 rpm without a bung. The strain was designated as actinomycete strain 044 30-1, belonging to the genus *Micromonospora*. Optimisation of laccase production by strain 044 30-1 and preliminary characterisation of the laccase produced by this strain are discussed in Chapter 3.The screening process described in this chapter was applied to this strain and is also discussed in Chapter 3.

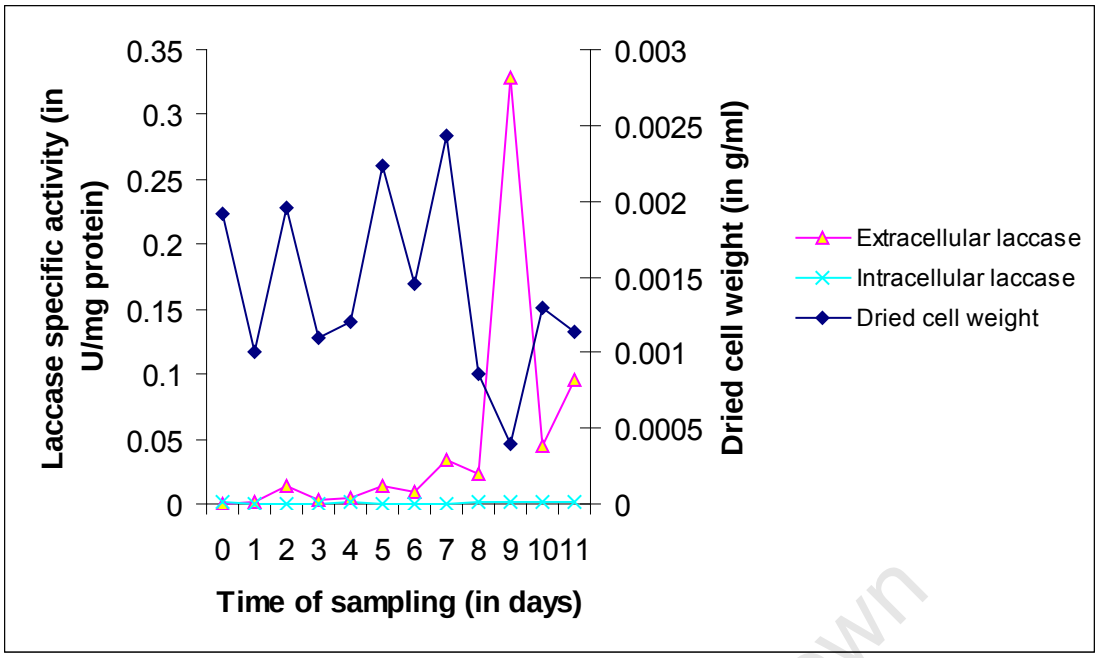


Fig. 2.17. Growth curve of *Micromonospora* sp. strain 044 30-1 in SCN medium showing high extracellular laccase production and the dry cell weight.

2.4 Conclusion

The secondary screening protocol for optimisation of maximal laccase production by the native strains identified the best strains to pursue further. This screening protocol is inexpensive, easy to perform at a laboratory scale with many strains, and provides vital information thereby saving time and effort. Specific activities of the laccases can be calculated by performing established assays. The aim of the secondary screening protocol was to determine significant variables, the manipulation of which would result in enhanced laccase production by the native strains. In this study, the variables examined included media composition, pH, temperature, incubation times, aeration and agitation, and the effect of inducers and inhibitors. The optimal medium and pH for maximum laccase production by each strain were the first variables to be determined, and laccase production was subsequently further enhanced by optimization of aeration and agitation conditions. The time of maximum production as well as the type of growth of each strain provides useful information for potential bioprocess development and for the use of the strains in bioreactors. A short fermentation time is advantageous for an industrial process as laccase can be obtained at an early stage thereby saving time and costs. Some strains cannot be used in bioreactors if they strongly adhere to parts of the reactor, thereby damaging the reactor and interfering with the required uniform distribution of cells within the reactor. Actinomycetes can produce laccases under alkaline conditions (whereas fungi prefer acidic conditions) and this may be a valuable characteristic for industrial bioprocesses (Molina-Guijarro, *et al.*, 2009). Although almost all fungi produce laccase extracellularly, it was found that actinomycetes predominantly produced larger quantities of intracellular laccase than extracellular laccase.

To the best of the author's knowledge, this work presents the first descriptions of laccases from strains representing the actinomycete genera and species listed above. Strain BS2 has recently been described as a new species (Le Roes, *et al.*, 2007) and with further investigation, its laccase may also prove to be novel. Interestingly, prior to this work, *Streptomyces antibioticus* had not been shown to produce laccase. However, it does produce other members of the multicopper oxidase family, *i.e.* tyrosinase and phenoxazinone synthase (Le Roes-Hill, *et al.*, 2009). It was also shown that dye-decolourisation experiments have value as indicators of laccase activity by environmental isolates. These experiments are currently used in the primary screening of many strains in our laboratory as laccase activity can be visually detected on agar plates supplemented with dyes.

The highest activity laccase-producing strains identified by this screening protocol will be pursued by other researchers interested in achieving maximum yield of laccase by these native strains. The laccases can be isolated, purified, and characterized and their use as biocatalysts in bioprocesses can be investigated. This work could potentially reveal the role of laccases in actinomycetes and elucidate the essential differences between prokaryotic and eukaryotic laccases and their biocatalytic capabilities.

Chapter 3

The Laccase from *Micromonospora* sp. 044 30-1: Optimisation of Laccase Production by Strain 044 30-1 and Biochemical Characterisation of the Laccase

3.1 Introduction

Micromonospora sp. strain 044 30-1 was isolated in 2004 from a sea squirt whose habitat was Algoa Bay, located off the southern coast of South Africa (Fig. 3.1). The strain is typical of the genus *Micromonospora*, having orange mycelia, and forming a black, mucoid, spore coat when subjected to stressful conditions. Strain 044 30-1 may be novel on the basis that it grows well in the presence of 5% NaCl (see Appendix B for a detailed explanation). It does not grow in the presence of 6% or 7% NaCl. Strain 044 30-1 differs from *M. siamensis* based on morphological differences, diffusible pigment production, and melanin production on *ISP*6 and 7 agars by strain 044 30-1. A dichotomous key for the identification of *Micromonospora* strains was developed (Goodwin, 2005) to facilitate the determination of novelty of a *Micromonospora* isolate in a period of 21 days from the time of inoculation of the test media. Standardized media were carefully chosen, or developed, for each physiological test to be performed for the proposed identification key. According to this key, strain 044 30-1 is a novel *Micromonospora* isolate, due to its remarkable ability to grow well in the presence of 5% NaCl, a characteristic that may be attributed to its habitat, a marine environment. Based on the absence of other reports in the literature, strain 044 30-1 is also the first *Micromonospora* isolate to have been isolated from a marine niche and another organism, the sea squirt.

The extracellular enzyme isolated from the marine actinomycete bacterium *Micromonospora* sp. 044 30-1 was studied in this chapter. The enzyme produced by this strain may be an atypical laccase. Production of laccase by this strain was investigated, with a view to its potential application in a biocatalytic process.

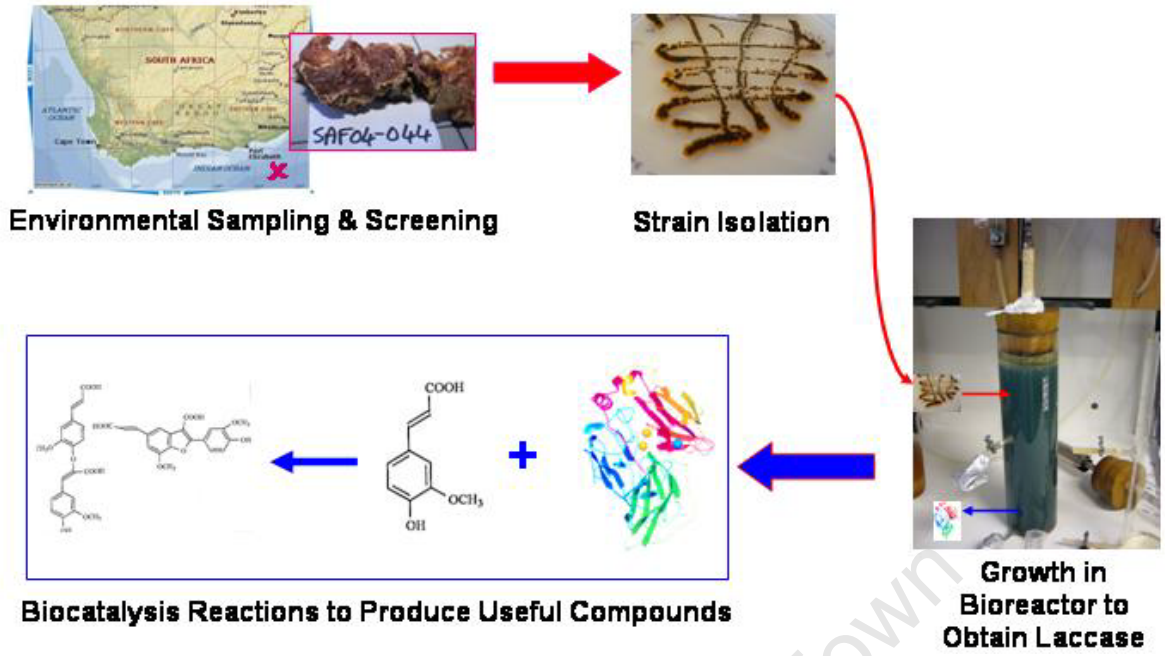


Fig. 3.1. After screening of environmental isolates (discussed in Chapter 2), strain 044 30-1 was chosen for further research. The optimisation of production of laccase by strain 044 30-1 was pursued and the biocatalysis applications of this enzyme are discussed in Chapter 4.

There is increasing interest in marine biotechnology, especially novel marine natural products, because of the potential of the untapped genetic diversity found in marine microbes to produce new compounds. Novel metabolites are gaining attention because of the growing demand for new enzymes, and many unusual enzyme types have been discovered from marine microbes (Sponga, *et al.*, 1999). One of the limitations affecting production of the desired enzymes and new compounds is the lack of understanding of the parameters involved in their biosynthesis in many microbes (Marwick, *et al.*, 1999).

There are several requirements for applications of enzymes (adapted from Gianfreda and Rao, 2004):

- An efficient enzyme production system, *i.e.* the use of a readily culturable microbial source, or of a genetically stable, non-pathogenic strain;
- The best source of such enzyme activity must be identified using selective screening by means of microbiological approaches;
- The enzyme-producing microbe must be cultured to increase the enzyme yield, and to isolate the enzyme for cell-free applications;
- An efficient biocatalyst, *i.e.* the use of resting cells or purified enzymes that are highly active in the required reaction, have flexible substrate specificity, and assure minimal side reactions;
- A stable biocatalyst, *i.e.* stable under the environmental or operational conditions it is exposed to, and adaptable to immobilisation and/or stabilization procedures, and

- Once a suitable enzyme has been identified, it must be specific for the intended chemical reaction, react with the desired substrates, and synthesise the expected products.

Bioprocess intensification is commonly used in chemical engineering to efficiently obtain higher product yields (Doran, 1995). However, bioreactors generally operate within the limits of the biocatalyst (section 1.12). Thus, bioprocess intensification focuses on optimising yields *via* media composition and feed strategies, control of physical conditions, induction, immobilisation, and bioreactor engineering (Marwick, *et al.*, 1999). Data should be collected on media and physical optima differences, along with investigations into the control mechanisms for biosynthesis, in order to recommend novel fermentation protocols (Marwick, *et al.*, 1999). Shear stress data are needed to provide bioreactor design requirements, with bioprocess scale-up in mind (Doran, 1995).

The application of bioprocess intensification methods to the production of metabolites by microbes is important for improving synthesis of natural products and in assessing their use as value added compounds (Marwick, *et al.*, 1999). In order to increase enzyme yield the fermentation must be performed on a larger scale than shake flasks. Predicting the effect of scale-up is straightforward if all parameters affecting the bacteria remain the same, or if their response to varying bioprocess parameters can be accurately controlled. However, these circumstances rarely occur, and the fact that few bacteria have been characterised in terms of their physiological response to bioprocess variables makes scale-up difficult to model effectively (Niladevi, *et al.*, 2007; Marwick, *et al.*, 1999; Doran, 1995).

3.1.1 Unusual or atypical laccases

While some fungal laccases have the typical spectroscopic properties (section 1.5), there are several reports of highly purified laccases that show different properties (Mayer and Staples, 2002; Giardina, *et al.*, 2009). There are purified laccases that are yellow or yellow-brown in colour, rather than blue (Leontievsky, *et al.*, 1997). The ratios A_{280}/A_{250} and A_{280}/A_{610} are used to determine laccase purity. The A_{280}/A_{250} ratio of proteins from fungal culture media commonly increase during purification, and has been taken as a measure of purity of laccases: 250 nm is used to detect contaminating phenolic material whereas 280 nm measures protein content (Leontievsky, *et al.*, 1997). The A_{280}/A_{610} ratio is typically 15 – 20 for blue laccases, indicating the balance between absorbance of T1 Cu at 610 nm and the combined absorbencies of tryptophan and aromatic amino acids of the protein at 280 nm. The absorbance spectra of the purified yellow-brown laccases show no significant absorbance at 610 nm, suggesting that the T1 Cu signal is absent (Giardina, *et al.*, 2009). Yellow and yellow-brown laccases, while having a copper content similar to the typical blue laccases, do not maintain their copper centres in the oxidized state in the resting enzyme under normal aerobic conditions (Leontievsky, *et al.*, 1997).

The fungus *Pleurotus ostreatus* produces a white laccase. This laccase contains only 1 copper atom per monomer instead of the usual 4, and furthermore, 2 zinc atoms and 1 iron atom are present per protein monomer (Palmieri, *et al.*, 1997). The suggestion that the enzyme belongs to the laccase family is supported by: The high degree of identity of the determined primary structures with the

corresponding sequences of known laccases; the use of oxygen as the oxidative substrate; the lack of formation of H_2O_2 as a product in the catalysed reaction; and the standard pattern of substrate specificity displayed by this enzyme if compared with that of other known laccases. This laccase from *P. ostreatus* displays unique structural characteristics and is classified as a white laccase (Palmieri, *et al.*, 1997).

Laccases, and other four-copper oxidases, contain three domains: Domains one and three contain the copper sites, while domain two forms the substrate-binding cleft (Giardina, *et al.*, 2009; Molina-Guijarro, *et al.*, 2009). In contrast to this arrangement, the genome of *Streptomyces coelicolor*, an actinomycete, was found to encode a small, four-copper oxidase that lacks the second domain (Machczynski, *et al.*, 2004). This protein is representative of a new family of enzymes, the two-domain laccases. The enzyme was named SLAC, for Small LACcase, because it lacks a domain and is thus much smaller than other laccases. It is the principal extracellular laccase produced by *S. coelicolor*. SLAC resembles the protein folds of *Coprinus cinereus* laccase, a fungal laccase, with >99% confidence (Machczynski, *et al.*, 2004). The T1 Cu site and the trinuclear cluster are intact. Despite lacking a domain, SLAC contains the normal complement of four copper ions, and is specific for the common range of laccase substrates. The enzyme is highly stable, and the maximum activity of SLAC at the unusually high pH of 9.4 suggests the suitability of the enzyme for industrial processes (Machczynski, *et al.*, 2004).

3.2 Materials and Methods

Strain maintenance was conducted as described in section 2.2.2. Sampling and preparation of samples for assaying were conducted as described in sections 2.2.5 and 2.2.8. Representation of data was as described in section 2.2.6. Aeration and agitation experiments were performed as described in section 2.2.7.

3.2.1 Media

SCN medium was used (as described in section 2.2.1) as well as modified m172F medium (**M172F**; Goodwin, 2005), consisting of (in g/L): glucose (10.0), starch (10.0), yeast extract (5.0), casein (2.5), magnesium sulfate (2.5), calcium chloride (2.0) and copper sulfate (2.0). The pH was adjusted to 5 using HCl when this medium was used as the optimal medium for laccase production. M172F was also supplemented with 2% sodium chloride when used as the optimal medium. The optimal Cu^{2+} concentration was 8 mM. All media components were supplied by Saarchem except for yeast extract, which was supplied by Biolab.

3.2.2 Laccase assays

Assays for detecting laccase activity were performed as described in section 2.2.3 for the ABTS protocol using a microtitre plate reader. Other substrates used were 2,6-dimethoxyphenol [**2,6-DMP** (450 nm; ε = 14 800 $M^{-1}cm^{-1}$)], 3-hydroxyanthranilic acid [**3-HAA** (450 nm; ε = 18 000 $M^{-1}cm^{-1}$); Katz and Weissbach, 1962], 4-aminoantipyrine phenol [**4-AAP** (492 nm excitation filter)], and syringaldazine. Solutions containing 1 mM 2,6-DMP or 1 mM 3-HAA were made in 0.2 M sodium phosphate buffer (Merck), pH 6. The Bradford assay was performed as described in section 2.2.3. For the 4-aminoantipyrine phenol assay for laccase activity a solution of 0.0025 M 4-aminoantipyrine and 0.17 M phenol was made in sterile water (ε = 7100 $M^{-1}cm^{-1}$). Oxidation of syringaldazine was followed by monitoring the increase in absorbance at 530 nm (ε = 65 000 $M^{-1}cm^{-1}$). The assay mixture contained 890 µl 0.1 M potassium phosphate buffer, pH 6.5, 100 µl of syringaldazine solution (1 mg syringaldazine dissolved in 5 ml ethanol) and 10 µl of sample. The necessary controls were used, such as the use of uninoculated solutions of relevant media. Chemicals were provided by Sigma-Aldrich, except for phenol, which was provided by Merck.

3.2.3 Airlift reactors

Airlift reactors (**ALR**s) were custom-built according to the specifications of Ryan (2003). Figure 3.2 illustrates the design and specifications of such an ALR. The reactors contained M172F medium with 0.05% antifoam. Typically, 3.5 L of the desired medium was added to the ALR and then the reactor containing the medium was autoclaved and allowed to cool overnight. The reactors were each inoculated with 300 ml of strain 044 30-1 culture grown in the desired medium in an Erlenmeyer flask. The fermentation was allowed to proceed at 30°C. Samples were aseptically taken from the top sample port. Reactors were inoculated in duplicate. Airflow rate was adjusted to 3 L/min air.

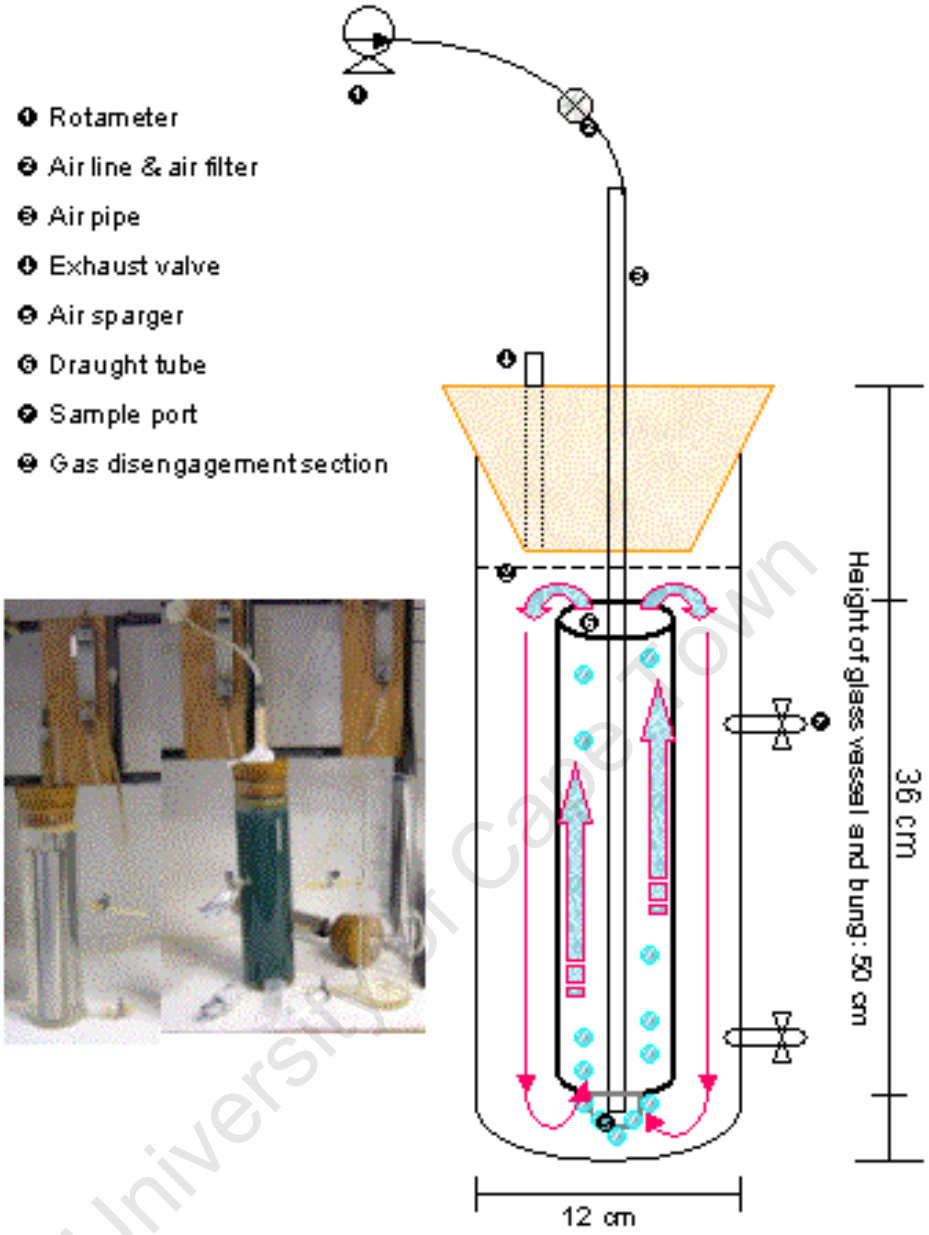


Fig. 3.2. Airlift reactor used in this study (developed by Ryan, 2003).

3.2.4 Stirred tank reactor

The continuous stirred tank reactor used was a New Brunswick Scientific Bioflo 110 Fermenter Bioreactor with external heating jacket, dissolved oxygen probe, pH probe, and sterile airflow. Agitation speed was set to 200 rpm and the temperature to 30°C. Samples were aseptically taken as required. Antifoam was added. Air pressure was adjusted to 3 L/min air.

3.2.5 Optimal medium selection

Three types of media were examined as the potential optimal medium for laccase production by strain 044 30-1: SCN, M172F, and modified M172F containing 8 mM $CuSO_4$, as described in section 3.2.1 above. The pHs of the various media were adjusted to pH 5, 7, and 9 accordingly. Samples were collected and assayed as described in sections 2.2.5 and 2.2.8. Four different laccase assay substrates were used as described in section 3.2.2 above. Experiments were performed in duplicate.

3.2.6 Optimal sodium chloride concentration

Flasks containing M172F medium at pH 5 containing 8 mM Cu were supplemented with 1.5, 2, 3, 4, and 5% NaCl. Samples were taken at days 2, 3, 4, 6, and 9 and assayed for activity using DMP and 3HAA as substrates. Experiments were performed in duplicate.

3.2.7 Optimal Cu^{2+} concentration for use in growth media for laccase production

50 ml Erlenmeyer flasks containing 10 ml of medium were inoculated in duplicate with strain 044 30-1. A series of flasks containing different concentrations of $CuSO_4$ were made: one series contained SCN medium and the other contained M172F. Both media had a pH of 5 and contained 2.0% NaCl. The concentrations of Cu examined were as follows: 0.123 mM (0.0032%), 0.5 mM (0.0125%), 1 mM (0.025%), 2 mM (0.05%), 4 mM (0.1%), and 8 mM (0.2%). 0.123 mM is the concentration of Cu used in trace salt solutions for media, specifically TDM. Samples were taken every day for 10 days and assayed with DMP and 3HAA. Protein concentration was determined using the Bradford method.

3.2.8 Laccase inducers and inhibitors

50 ml Erlenmeyer flasks containing 10 ml of the optimal medium (M172F, pH 5, + 8 mM Cu + 2.0% NaCl), SCN medium (pH 5, 2.0% NaCl) or M172F medium without Cu were inoculated in duplicate with strain 044 30-1 and allowed to incubate at 30°C with shaking at 160 rpm with bungs. Ferulic acid, 8-HQ, and xylidine were added as laccase inducers, while sodium azide and EDTA (Sigma) were investigated as inhibitors of laccase activity. The final concentration in solution of each inhibitor or inducer was 1 mM (see section 2.2.9). Samples were taken at days 2, 4, 8, 9, and 10. Combinations of inducers were also examined. The final concentration of combinations of inducers was 1 mM. Samples were taken on days 2, 3, and 6, and assayed for laccase activity.

3.2.9 Optimisation of ALR conditions to obtain laccase from strain 044 30-1

SCN medium without Cu, SCN medium without Cu but containing 1 mM 8-HQ and FA as inducers of laccase, M172F (the optimal medium), and M172F without Cu, were investigated for their effect on laccase production by strain 044 30-1 when grown in duplicate ALRs. Samples were taken at 6-hour time intervals and assayed for activity using DMP and 3-HAA as laccase substrates. The protein concentration of samples was determined using the Bradford assay. The dry cell weights and pH (CyberScan 1000 pH meter, Cape Scientific Service) of the samples was also determined.

3.2.10 Purification of extracellular laccase from strain 044 30-1 growth media

For ethanol precipitation of protein, 9 volumes of cold absolute ethanol (Merck) were added to 1 volume of protein-containing solution. For acetone precipitation, 3 volumes of cold acetone (Merck) were added to 1 volume of protein-containing solution. Ammonium sulfate (Saarchem) precipitation was performed according to the Roche Applied Science Lab FAQS manual, 2nd edition. Ammonium sulfate was either added to protein-containing solution to achieve saturation of 80%, or step-wise precipitation was performed obtaining % saturation points of 20, 40, 60, 80, and finally 100%. All protein precipitation methods were performed at 4°C and 1ml/L 20 mM PMSF was added to protein-containing solutions. For ethanol and acetone precipitation, protein had precipitated after 1 hour and the solution was then centrifuged and the pellet redissolved in 0.2 M sodium phosphate buffer, pH 6, and stored at 4°C. Ammonium sulfate precipitations were performed at 4°C with constant stirring for 8 hours. The samples were then centrifuged and stored for use as described above. Ultrafiltration of samples was performed using Nanosep Centrifugal 10K devices with a 10-kDa molecular weight cut-off (PALL Life Sciences, USA). A hydrophobic column of FP-PH12 sepabeads, supplied by Resindion (Mitsubishi), was used as specified by the manufacturer. The buffer used was 0.2 M sodium phosphate, pH 6. Cellulose membrane dialysis tubing (Sigma) and snakeskin dialysis tubing (3.5 kDa molecular weight cut-off; Pierce Separations) were used according to the manufacturer's instructions. Samples were dialysed against water containing 20% PEG (polyethylene glycol; Merck), with stirring at 4°C, for different time intervals depending on the solution to be dialysed. For example, dialysis of the crude extract required the removal of various salts, media, colour components, and copper, and dialysis would be performed for 24 – 48 hours, with fresh water containing PEG added as required. Anion-exchange chromatography was performed at the University of the Western Cape (UWC) using FPLC (fast performance liquid chromatography). The anion-exchange column used was a Q Sepharose HQ anion exchanger. A 1 M NaCl gradient was used and a single peak was detected at 36.97% NaCl. The buffer used was 0.2 M sodium phosphate, pH 6. All solutions and samples were filtered to remove impurities before being used in the system and loaded onto the column. The flow rate was 6 ml/min.

3.2.11 Spectrophotometric detection of the laccase Cu centres

Absorption peaks characteristic of laccases were detected in samples using a spectrophotometer (section 2.2.3), scanning the range 200 nm to 800 nm. Measurements were analysed using Microsoft Excel and the A_{280}/A_{610} ratio for laccase purity calculated.

3.2.12 Temperature profile

Eppendorf tubes containing extracellular laccase from strain 044 30-1 grown in M172F medium were placed in a water bath set to the specific temperature to be investigated and these tubes were sampled every 10 minutes for an 80 minute period. Samples were immediately assayed for laccase activity using DMP and 3-HAA as substrates. The protein concentration of the samples was determined using the Bradford method. Temperatures investigated were 40, 50, 60, 70, and 80°C.

3.2.13 Polyacrylamide gel electrophoresis (PAGE)

Solutions used for PAGE are listed in Appendix A. All chemicals were provided by Sigma except for Coomassie Brilliant Blue (Fluka) and acrylamide (BioRad). PAGE was performed using a 12% stacking gel and 5% separating gel. The PageRuler^{TM} prestained protein ladder from Fermentas was used as the molecular weight marker. Silver staining was performed using the PageSilver^{TM} Silver Staining Kit from Fermentas.

3.3 Results and Discussion

3.3.1 The optimal production medium and assay conditions for the detection of laccase from strain 044 30-1

The secondary screening protocol (section 2.2.5) was applied to strain 044 30-1; three types of media were investigated as the optimal medium for production of laccase by strain 044 30-1, and the pH of the various media examined was adjusted to pH 5, 7, and 9, to assess if laccase production was a function of pH. SCN medium was the medium used to initially isolate strain 044 30-1 from the marine environment. M172F is an excellent *Micromonospora* growth medium (Goodwin, 2005). M172F medium was also modified by the addition of 0.2% (8 mM) $CuSO_4$ for enhanced laccase production as $CuSO_4$ present in the growth medium of strains has been shown to be an excellent inducer of laccase activity (Arias, *et al.*, 2003). Of these three media, it was determined that the modified M172F medium, containing 8 mM Cu, and at pH 5, was the best medium for the production of laccase by strain 044 30-1 based on highest laccase specific activity (Fig. 3.3). M172F medium was further optimised by determining the optimal sodium chloride concentration of the medium (discussed in section 3.3.1.2 below). Thus, after investigation of these variables, the optimal medium for laccase production by strain 044 30-1 was determined to be M172F medium, pH 5, supplemented with 8 mM Cu and 2.0% NaCl, as the highest laccase specific activity was obtained using this medium.

Based on the laccase activity measured in flask cultures, it was found that the optimal aeration and agitation conditions for the production of extracellular laccase by strain 044 30-1 was 160 rpm with a bung. Considering that this result indicates that the strain requires high oxygenation or aeration to produce laccase, the bioreactor selected for scale-up was an airlift reactor (ALR). This is discussed in section 3.3.4 below.

Intracellular laccase production by strain 044 30-1 was barely detectable and was not further pursued (Figures 3.5, 3.6, and 3.7). Furthermore, it is more convenient and efficient to work with extracellular laccase, as it is more easily and readily isolated.

3.3.1.1 Detection of laccase activity using substrates other than ABTS

In the present study, various assay substrates were investigated for the detection of the laccase produced by actinomycete strain 044 30-1. The oxidised products of synthetic substrates for standard laccase assays, such as ABTS, have high molar absorption coefficients, and these substrates have been used in many published studies. Methoxy-substituted phenols, such as guaiacol and dimethoxyphenol (DMP) have also been commonly used to detect laccase activity (Giardina, *et al.*, 2009; Molina-Guijarro, *et al.*, 2009).

In the study by Solano and co-workers (2001), experiments using different laccase substrates demonstrated that DMP was the most suitable substrate for assaying laccase activity in microbial samples, based on a number of factors including the stability of the oxidised dimeric coloured product, its high absorption molar coefficient, weak acidic optimal pH, and oxidation efficiency for many BMCOs. ABTS requires an acidic pH for optimal enzyme-mediated oxidation, and at acidic pHs, the maintenance of the structure of some BMCOs would be compromised (Solano, *et al.*, 2001).

The 2,6-DMP assay (also used in the present study) was used by others to classify blue multicopper proteins into 3 groups, as follows (Solano, *et al.*, 2001):
(1) Laccase from the fungus *Pyricularia oryzae*, PpoA (a polyphenol oxidase) from the marine bacterium *Marinomonas mediterranea*, phenoxazinone synthase from the actinomycete *S. antibioticus*, and mammalian serum ceruloplasmin were able to oxidise DMP rapidly without Cu supplementation and were characterised by the linear accumulation of the DMP oxidised product. This behaviour should be considered as real laccase activity (Solano, *et al.*, 2001; Xu, 1997; Giardina, *et al.*, 2009). The enzyme produced by strain 044 30-1 falls within this group as evidenced by its reactivity with DMP as substrate in Cu-free media.
(2) The bacterial copper tolerance and resistance proteins CueO from *E. coli*, and CopA from *Pseudomonas syringae* and *Xanthomonas campestris,* are dependent on the addition of Cu to exhibit DMP activity. They show a burst of initial activity but are then rapidly inactivated.
(3) *Saccharomyces cerevisiae* Fet3p yeast membrane protein involved in iron transport and the CotA sporulation protein from the bacterium *B. subtilis* showed a very slow DMP oxidation rate. The assays lasted hours in the presence of Cu, and DMP oxidation was not detectable in its absence. Thus, these proteins show DMP activity dependent on Cu supplementation.

Figure 3.3 below shows that in the optimal medium (M172F, pH 5 + 8 mM Cu) maximum extracellular laccase production by strain 044 30-1 occurred at day 6. Through optimisation of the medium, the day of maximum laccase production was advantageously brought forward, from day 9 in SCN medium to day 6 in this medium (section 2.3.6). The specific activities obtained using different laccase substrates are also shown in Figure 3.3 and it can be seen that DMP was a better substrate than ABTS for the detection of laccase produced by this strain as it was more readily converted by the enzyme. 4-Aminoantipyrine phenol (4AAP) was also a good substrate and 3-HAA allowed slightly better detection than ABTS. It was thus decided that ABTS would not be used as the assay substrate of choice.

Instead, DMP and 3HAA would be used for more sensitive detection of laccase produced by strain 044 30-1. Furthermore, in the present study, controls were also used to ensure that the components of the various types of media, including Cu-containing media, did not contribute to the detected laccase activity in tested samples.

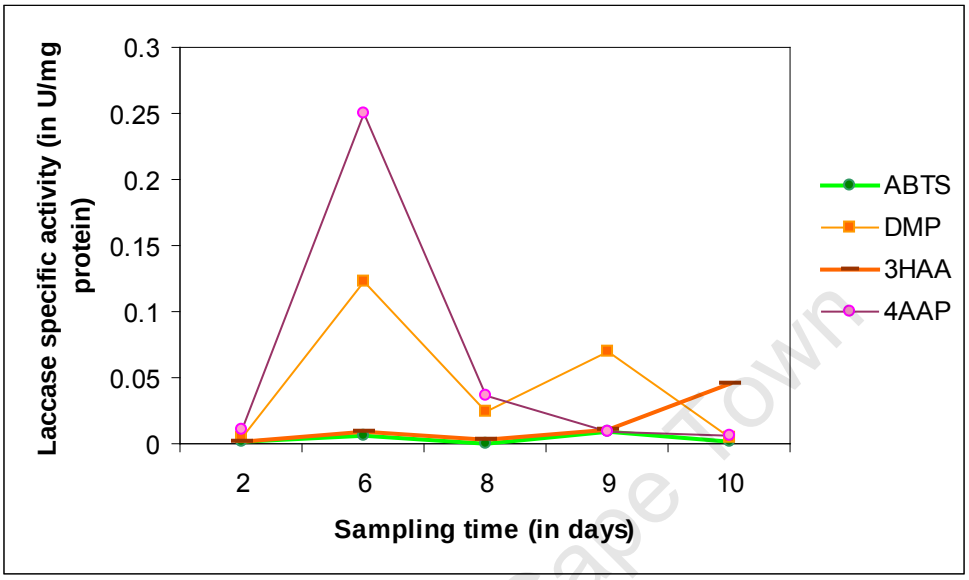


Fig. 3.3. Maximum laccase production by strain 044 30-1 was measured in M172F medium (pH 5, 8 mM Cu) on day 6 and was efficiently detected using DMP and 4AAP as assay substrates.

3.3.1.2 Optimal sodium chloride concentration

Some bacteria in the marine environment possess a requirement for salt, although the effect of salinity on metabolism has not been extensively researched. Optimal salinity levels should be established for growth and metabolite production phases (Marwick, *et al*., 1999). High salt levels may cause problems such as bioreactor corrosion and can inhibit the dissolution of sparged oxygen into aqueous media (Doran, 1995). Strain 044 30-1, a marine isolate, required a minimum of 1% NaCl for growth. Optimisation of the NaCl concentration showed that 2% NaCl was preferred for the growth of strain 044 30-1 and for the production of laccase by this strain (Fig. 3.4).

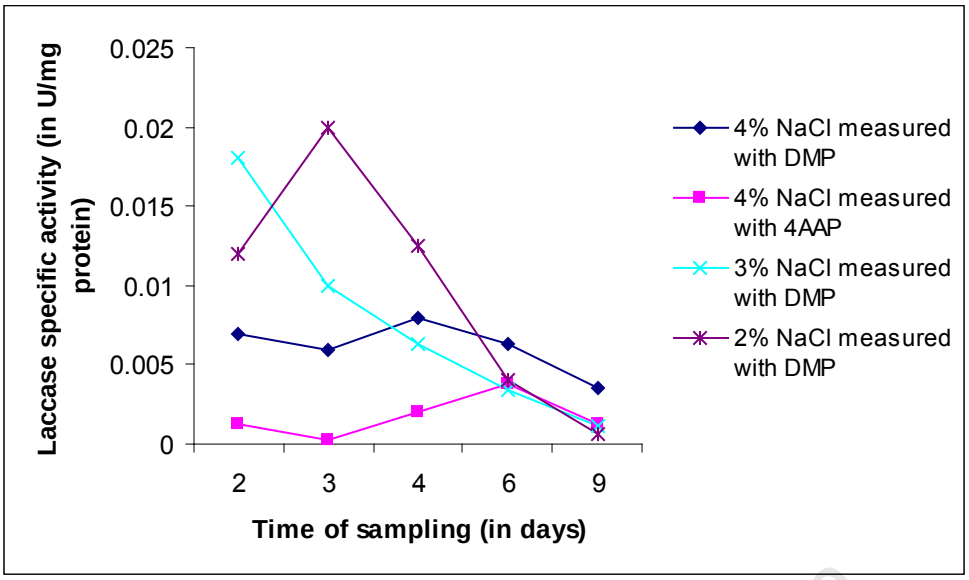


Fig. 3.4. The effect of NaCl concentration in the growth medium on the production of laccase by strain 044 30-1.

A salinity level of 2% NaCl was effective in enhancing laccase activity over 3 days, at which point laccase activity began to diminish (Fig. 3.4). However, from day 3 onwards, laccase production did not diminish in medium containing 4% NaCl. For a fed-batch process, it would be possible to ensure that the medium contains 2% NaCl for the first 3 days, and 4% NaCl thereafter, in order to enhance laccase production.

To further examine the extracellular and intracellular laccase levels produced by strain 044 30-1, and the effect of different NaCl concentrations, and to investigate different assay substrates for the detection of strain 044 30-1 laccase, the following experiments were conducted:

(1) The optimal medium (M172F + 8 mM Cu + 2.0% NaCl) was added to a flask, inoculated with strain 044 30-1, and sampled daily for 10 days (batch culture; Fig. 3.5);

(2) the optimal medium was added to the flask, the strain was inoculated and allowed to incubate for 3 days, at which point fresh medium containing M172F + 8 mM Cu + 4.0% NaCl was added to the flask ('fed-batch'; Fig. 3.6);

(3) and the use of an ALR containing the optimal medium was investigated to obtain preliminary data. The ALR was treated similarly to the fed-batch flask in (2). On day 3, half of the volume of the reactor containing the optimal medium was removed and fresh medium was added containing M172F + 8 mM Cu + 4.0% NaCl (Fig. 3.7).

The 1 L Erlenmeyer flasks containing 100 ml of media were incubated at 30°C with shaking at 160 rpm, with a bung.

In batch culture of strain 044 30-1, containing 2% NaCl supplemented in the growth medium, maximal extracellular laccase production occurred on day 4, detected with 3HAA and 4AAP as substrates (Fig. 3.5). Intracellular laccase levels were very low. In the fed-batch cultures maximum laccase production occurred at a later period, day 7, after the addition of fresh medium on day 3 (Figures 3.6 and 3.7). In

flask culture, laccase activity (detected with 4AAP) increased gradually in medium supplemented with 2% NaCl. Fresh medium supplemented with 4% NaCl was added on day 3 and a peak in activity was detected on day 7. In the fed-batch cultures, 3HAA and 4AAP were excellent substrates for the detection of extracellular laccase. Higher levels of extracellular laccase were detected using DMP as substrate (compared to data obtained from the batch culture) and a peak in extracellular laccase activity was detected on day 2 using 4AAP as substrate. The ALR experiment, with similar conditions to that of the fed-batch culture, also showed maximum extracellular laccase production on day 7, the activity of which was detected with 3HAA, 4AAP, and DMP. Another peak in laccase activity was seen on day 9. Thus, the day of maximum production of laccase by strain 044 30-1 varied according to the different conditions in which the strain grew. These experiments also illustrate that the effects of scale-up may be difficult to predict and that enzyme yield and the day of maximal enzyme production can differ in different volumes of media used. Based on the observations that 3HAA and DMP appear to be better substrates for the detection of strain 044 30-1 laccase, one may speculate that the compounds with a smaller molecular weight fit inside the catalytic pocket of this laccase better than a compound of higher molecular weight such as ABTS, and are thus readily converted showing high laccase activity levels in the respective assays.

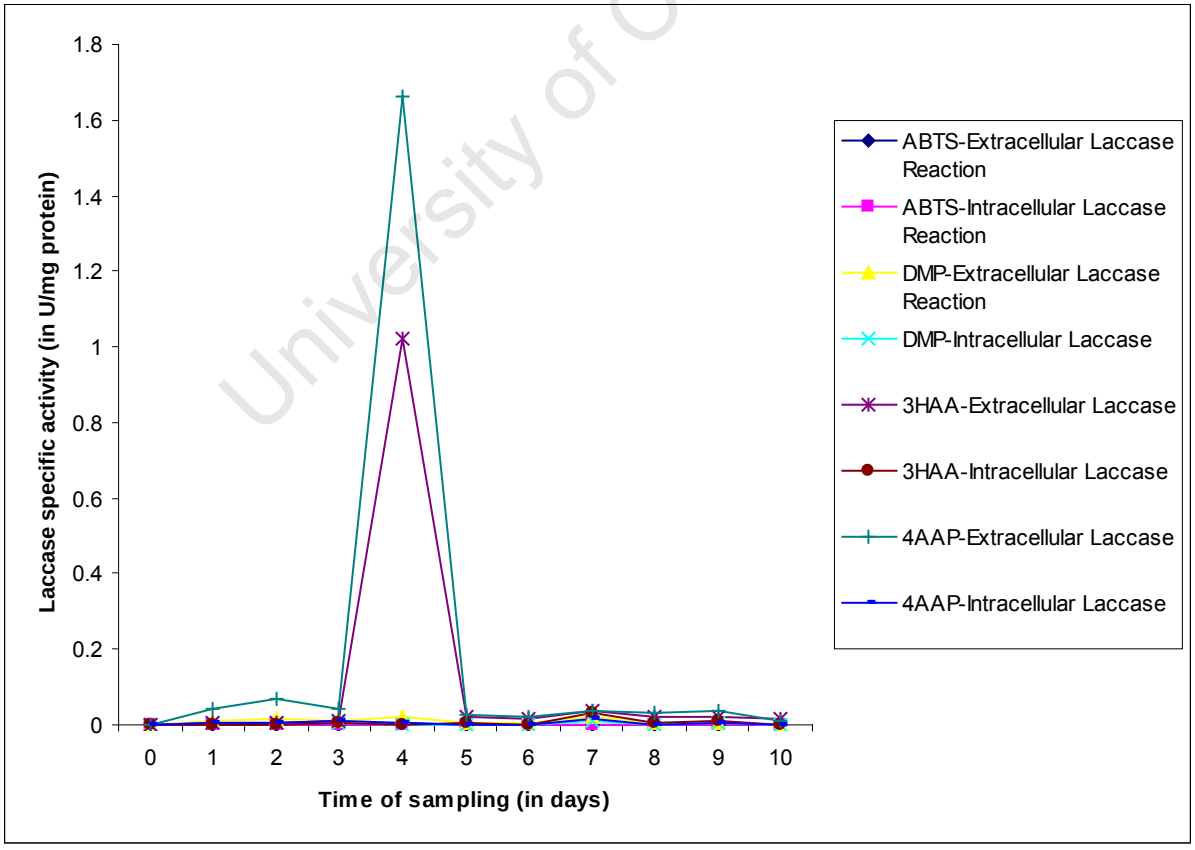


Fig. 3.5. Laccase activity levels during the growth of strain 044 30-1 under batch conditions.

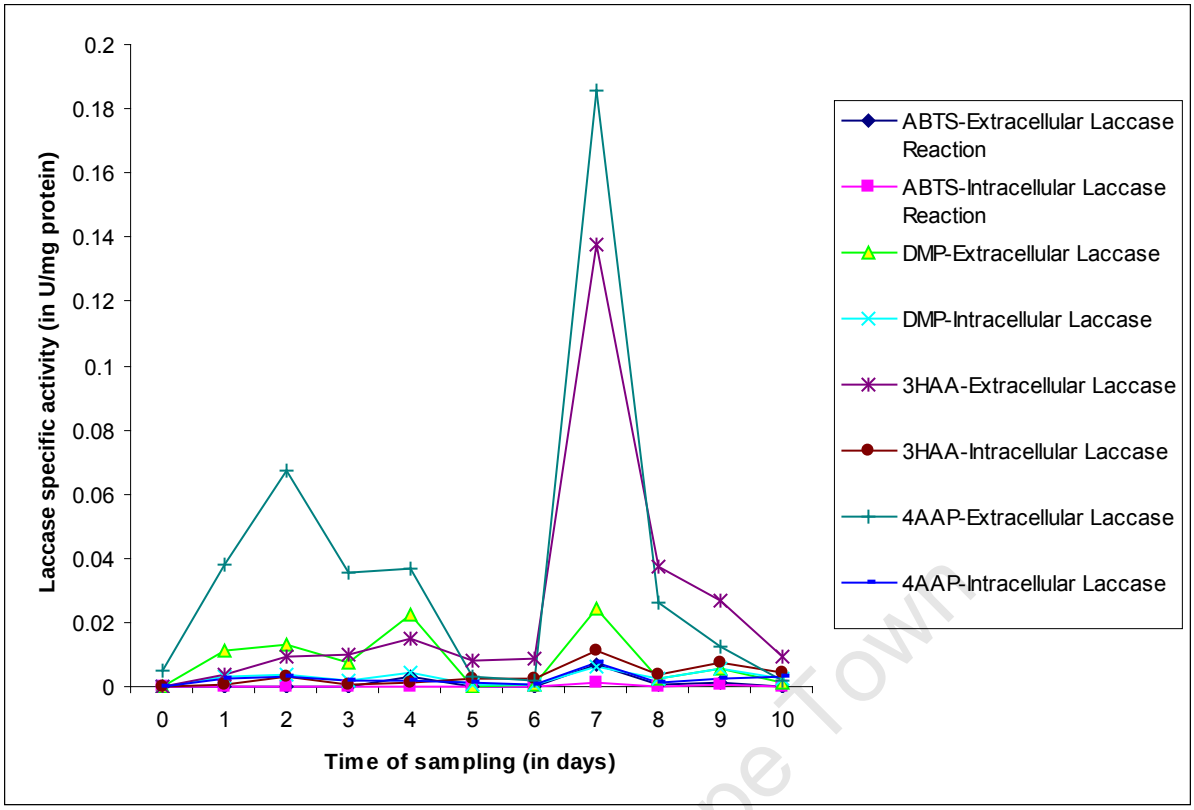


Fig. 3.6. Laccase activity levels during the growth of strain 044 30-1 under fed-batch conditions.

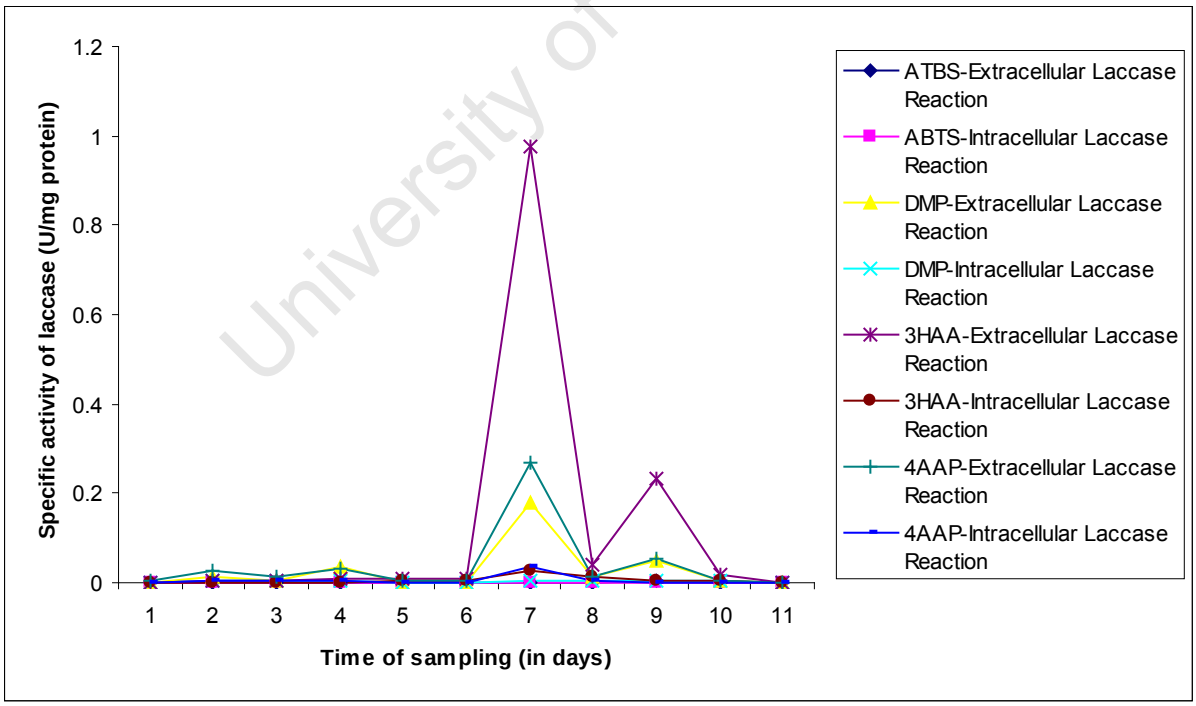


Fig. 3.7. Laccase activity levels during the growth of strain 044 30-1 in an ALR under 'fed-batch' conditions.

3.3.2 The effect of inducers and inhibitors on laccase production by strain 044 30-1

The production of laccase by strain 044 30-1 in response to various inducers and inhibitors was variable (section 3.2.8; Fig. 3.8 and Table 3.1). The inhibitors or inducers were added to M172F medium containing $CuSO_4$. In agreement with the literature and results obtained in Chapter 2 with other actinomycete strains (Fig. 2.38 and section 2.3.2.1), xylidine appeared to be a good inducer of strain 044 30-1 laccase, while sodium azide did not have an inhibitory effect on laccase production. It was also shown that the inducing compounds were not as effective in enhancing laccase activity as Cu^{2+} alone. EDTA has been documented as a laccase inhibitor due to its properties as a Cu chelator (Johannes and Majcherczyk, 2000). However, EDTA did not appear to have an inhibitory effect. The study by Molina-Guijarro and co-workers (2009) also showed that EDTA did not inhibit the activity of the laccase from the actinomycete *Streptomyces ipomoea*.

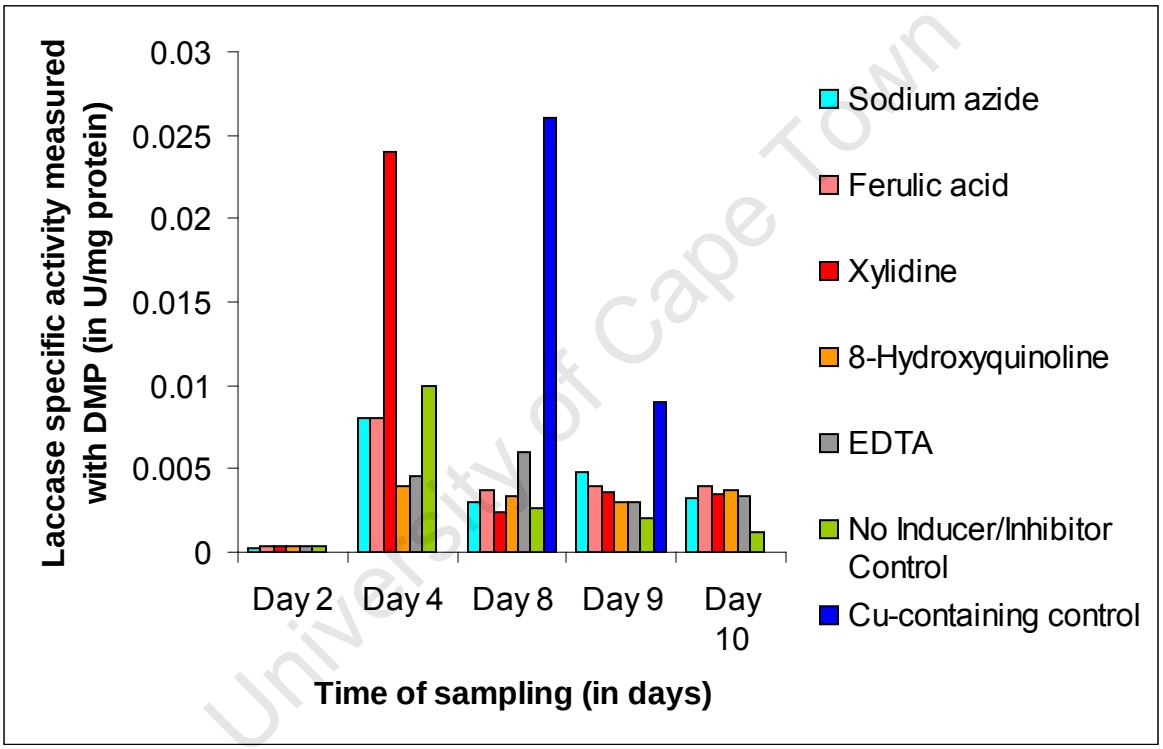


Fig. 3.8. The effect of inducers and inhibitors on the laccase produced by strain 044 30-1 in M172F Cu-containing media.

Table 3.1. The effect of known laccase inducers and inhibitors on the production of laccase by strain 044 30-1.

	Laccase inducers (1 mM)			Laccase inhibitors (1 mM)		8 mM $CuSO_4$
Day:	8-HQ	FA	Xylidine	Sodium azide	EDTA	
2	80.5 %[a]	78	85	58.5	83	100
4	40	80	240	80	46	100
8	126	137	89	111	222	963
9	150	200	180	240	150	450
10	308	333	292	275	283	100

[a]All data indicate the percentage specific activity relative to the control culture containing no inducer or inhibitor compound.

In the next experiments, the effects of inducers and inhibitors on the production of laccase by strain 044 30-1 in flask culture was investigated in SCN and M172F media, containing no Cu. In media without Cu, the levels of laccase produced were low and thus inducers were investigated with the aim of replacing the Cu if the inducers could effectively enhance laccase production by strain 044 30-1 in Cu-free medium. Inducers used alone, and in combination, were investigated.

Investigations into the effects of inducers on laccase production by strain 044 30-1 grown in media containing no Cu revealed that higher levels of laccase were detected in the SCN medium supplemented with inducers compared to the M172F medium without copper but containing inducer compounds (figures 3.9 and 3.10 below). For SCN medium, it was found that on day 3 high laccase activity of 0.0715 U/mg protein was obtained using a combination of FA and 8-HQ as inducers of laccase. Thus, production of laccase in an ALR containing SCN medium supplemented with FA and 8-HQ was later investigated and compared with that of M172F medium containing Cu (discussed in section 3.3.3 below).

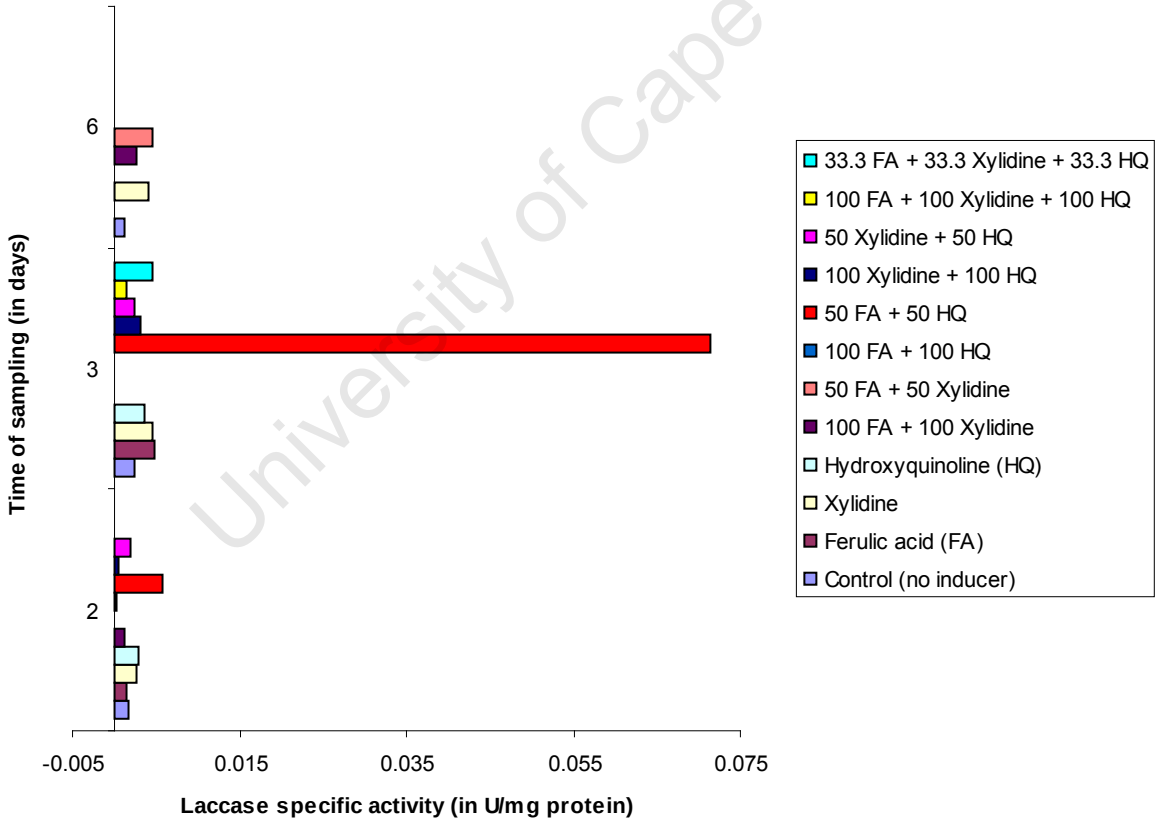


Fig. 3.9. The effect of combinations of inducers on laccase produced by strain 044 30-1 grown in **SCN medium without Cu** present. The DMP assay was used to detect laccase activities.

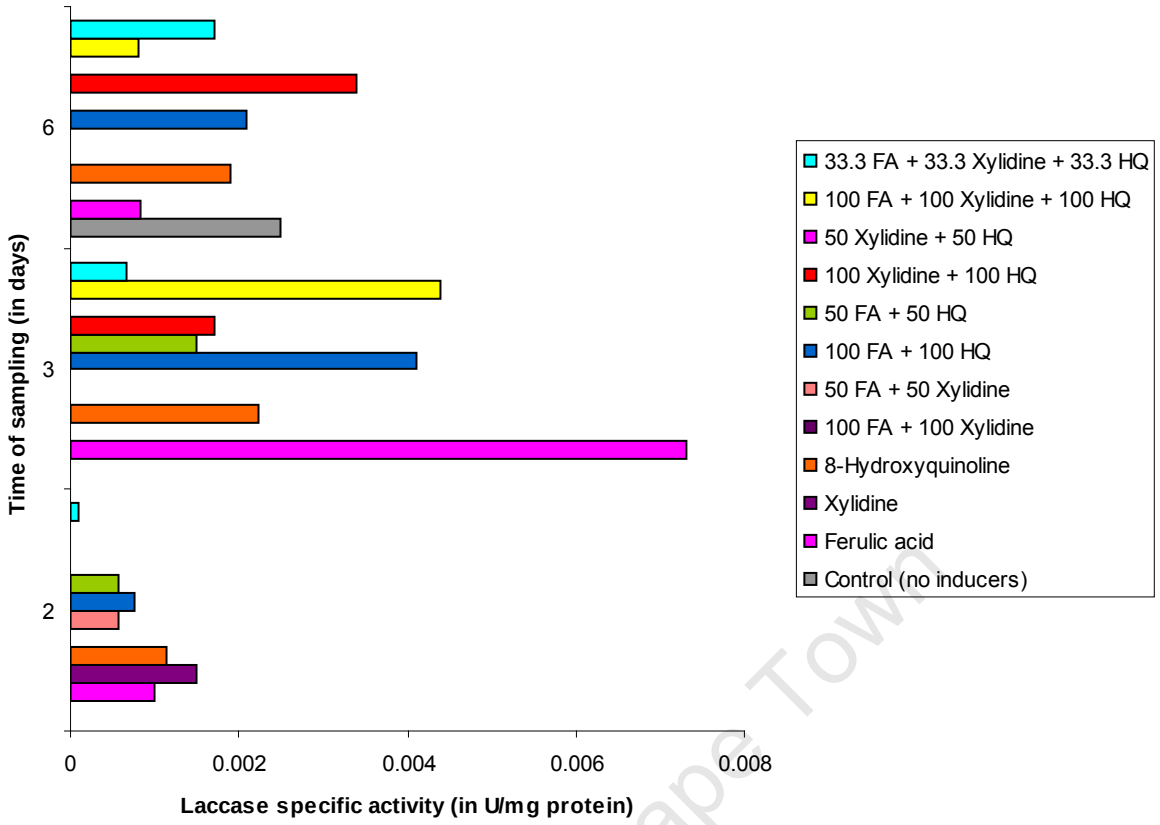


Fig. 3.10. The effect of combinations of inducers on laccase produced by strain 044 30-1 grown in **M172F medium without Cu** present. The DMP assay was used to detect laccase activities.

3.3.3 Growth of strain 044 30-1 and production of laccase in bioreactors

Growth of strain 044 30-1 in bioreactors was investigated to determine the time of maximum laccase production so that large amounts of enzyme could be obtained for use in biocatalysis reactions. The response of organisms to scale-up is difficult to predict and results are often markedly different to that seen in flask culture. The aim of these experiments was to determine the bioreactor of choice and the best medium for maximum laccase production at the earliest time. Samples were taken routinely for 10 days and laccase specific activities were calculated by using DMP and 3-HAA as laccase assay substrates and by evaluating the protein concentrations of samples. The pH of the samples as well as the dried cell weights of samples were recorded. All media used for bioreactor experiments contained 2.0% NaCl and the pH of the media was adjusted to 5. The bioreactors used in this study were airlift reactors (ALR) and a stirred tank reactor (STR).

3.3.3.1 ALRs as the bioreactors of choice

M172F medium without Cu was used in the STR, as the Cu may have damaged the stainless steel components of the reactor (section 3.2.4). Dissolved oxygen levels indicated that strain 044 30-1 entered the death phase of its growth cycle after 108 hours (4.5 days) of incubation in the STR (Fig. 3.11). In this phase, strain 044 30-1 was no longer productive and laccase levels were barely

detectable. This could be attributed to the stressful conditions experienced by the organism in a STR. Biocatalysts are often sensitive to shear fields caused by increased agitator speeds leading to a drop in productivity. CSTRs experience higher rates of shear stress compared with airlifts (Marwick, *et al.*, 1999; Niladevi and Prema, 2007). Later experiments (Fig. 3.12) showed that laccase specific activities were far higher in samples obtained from ALRs than from the STR, and thus the ALR was chosen as the bioreactor of choice for further investigations. The dried cell weights obtained from the bioreactors are shown in Figure 3.13.

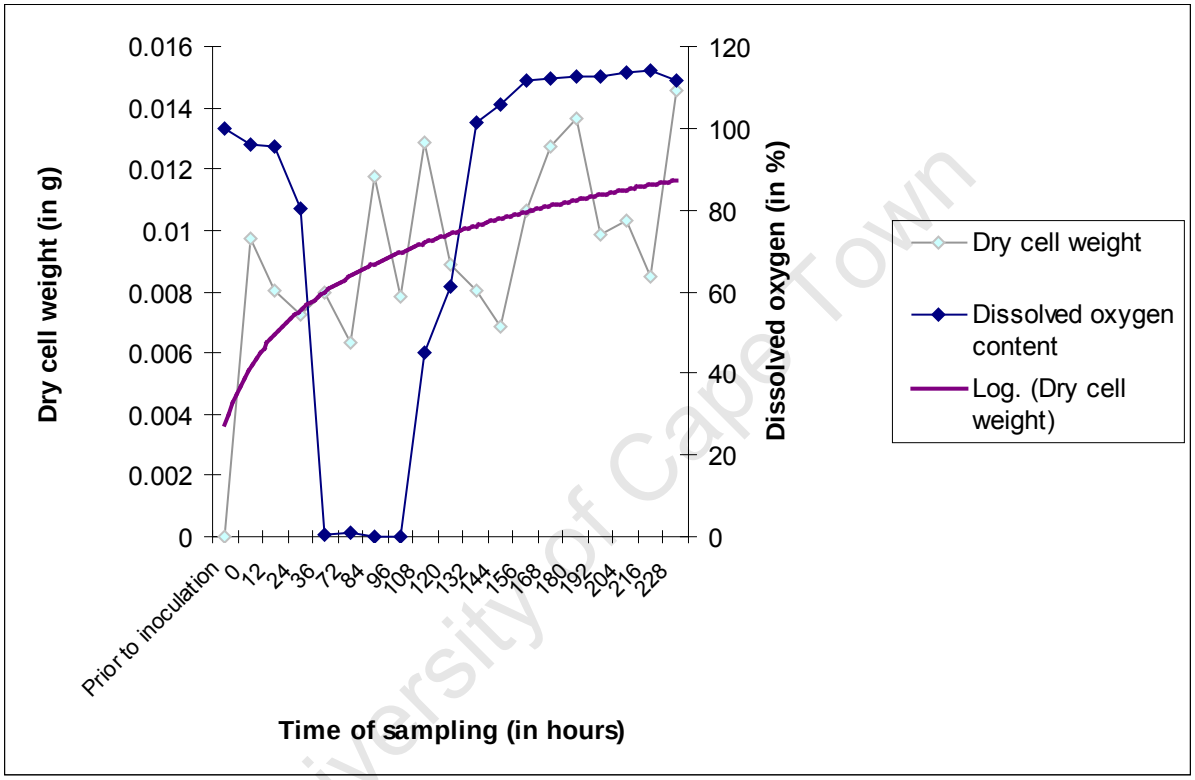


Fig. 3.11. Dissolved oxygen content and dry cell weight measurements were used to monitor the growth of strain 044 30-1 in a STR.

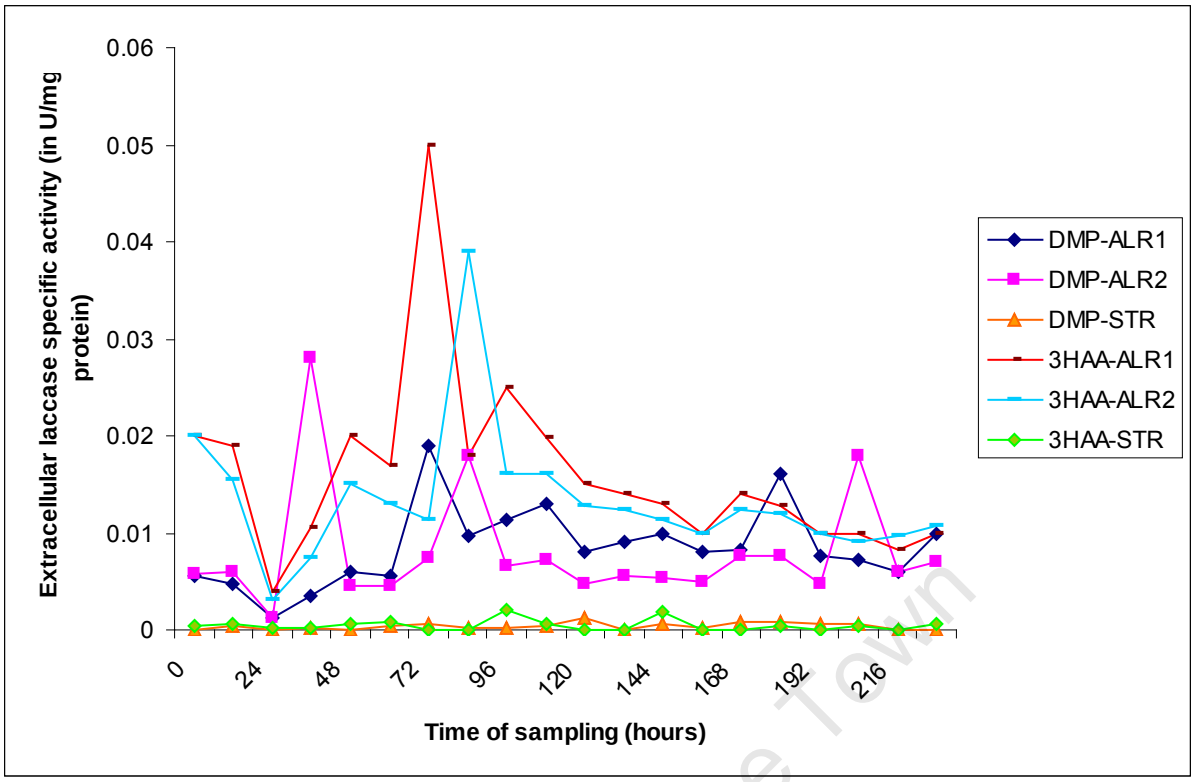


Fig. 3.12. Laccase specific activities during growth of strain 044 30-1 in ALRs and a STR. The STR contained M172F medium without Cu. The ALRs contained M172F medium with Cu.

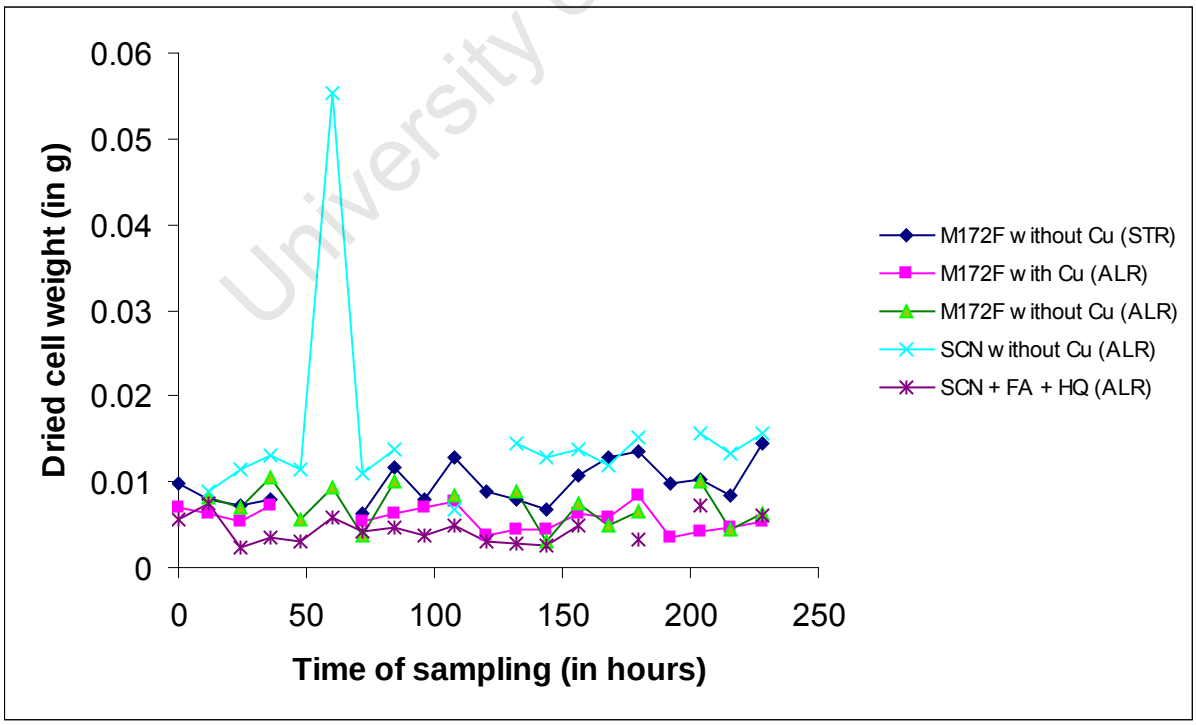


Fig. 3.13. Comparison of dry cell weights obtained using different bioreactors and media.

In this study (Fig. 3.14), the pH of the media was not influenced by laccase production nor did the pH changes influence laccase synthesis. In the study by Shutova and co-workers (2008) the addition of $CuSO_4$ caused a slight acidification of the culture medium (by 0.3 – 0.5 units of pH). Possibly Cu^{2+} ions bind OH^- ions to form the poorly soluble hydroxide $Cu(OH)_2$ thereby inducing acidification of the culture medium. In the study by Niladevi and Prema (2007), fermentation of the actinomycete *Streptomyces psammoticus* resulted in the initial pH of the media to shift towards a higher pH as laccase production started and at the time of maximum laccase production the pH shifted to 9.2. The observed result was of much significance because most of the reported laccases from fungal strains show a drop in production media pH during the course of the experiment.

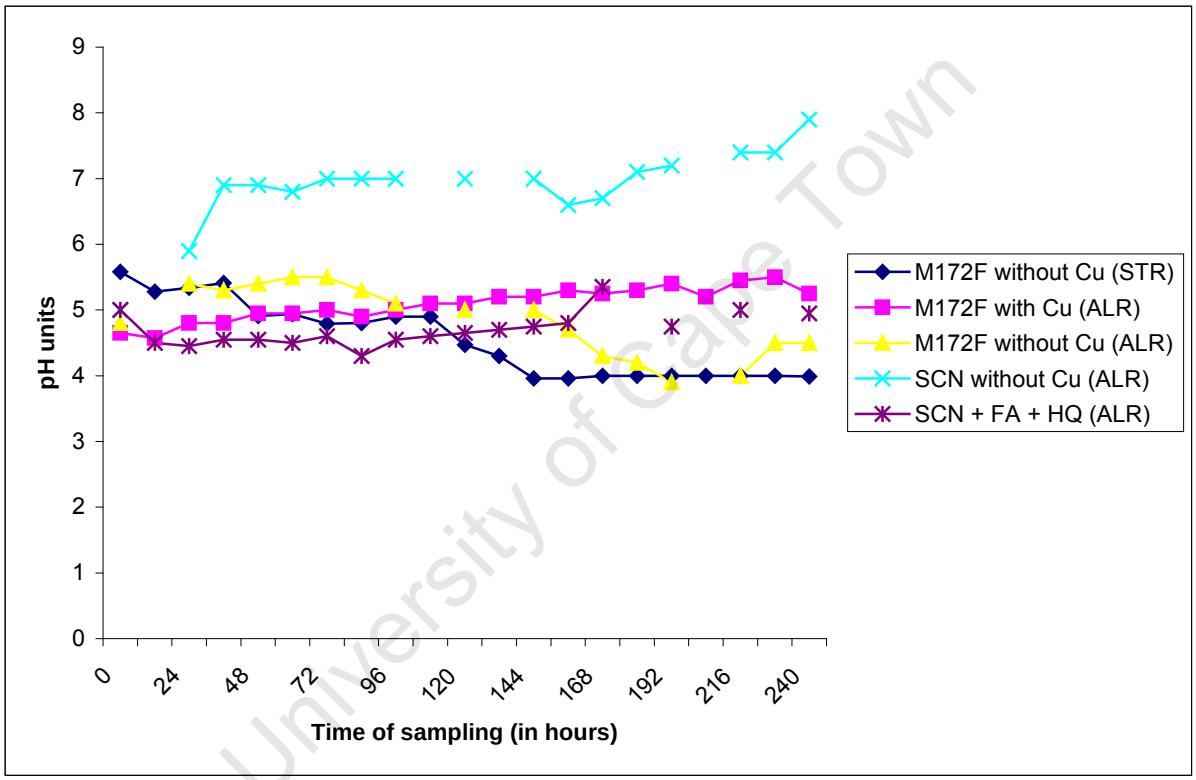


Fig. 3.14. Comparison of the pH changes of the different media during fermentation of strain 044 30-1 in different bioreactors.

3.3.3.2 The optimal medium for use in ALRs and time of maximum laccase production by strain 044 30-1 in ALRs

Laccase production by strain 044 30-1 in ALRs containing M172F medium with or without $CuSO_4$, SCN medium without $CuSO_4$, and SCN medium supplemented with the inducer compounds FA and 8-HQ were investigated. The time at which maximum laccase production occurred in each medium was determined. The criteria for choosing the best medium and bioreactor were reproducibility, early laccase production, and high laccase specific activity using both DMP and 3-HAA as substrates. It was clear that the STR resulted in inefficient laccase production due to the stress inflicted on the strain

(section 3.3.3.1). ALRs proved to be better bioreactors, where the results obtained were reproducible. In the ALRs, media without Cu gave extremely poor laccase titres, and SCN medium supplemented with FA and 8-HQ as inducers of laccase gave better laccase yields than media without copper (Fig. 3.15 and 3.16). Thus, these inducers could be used to replace Cu for stimulating laccase activity, although the inducer compounds are not as effective as Cu itself. The inducers were added at the time of inoculation, and at 90 hours in an effort to increase laccase yields, but no increase occurred after 90 hours. ALRs containing M172F with inducers were not investigated because in flask culture SCN medium with inducers had been shown to be more effective than M172F supplemented with inducers (section 3.3.2).

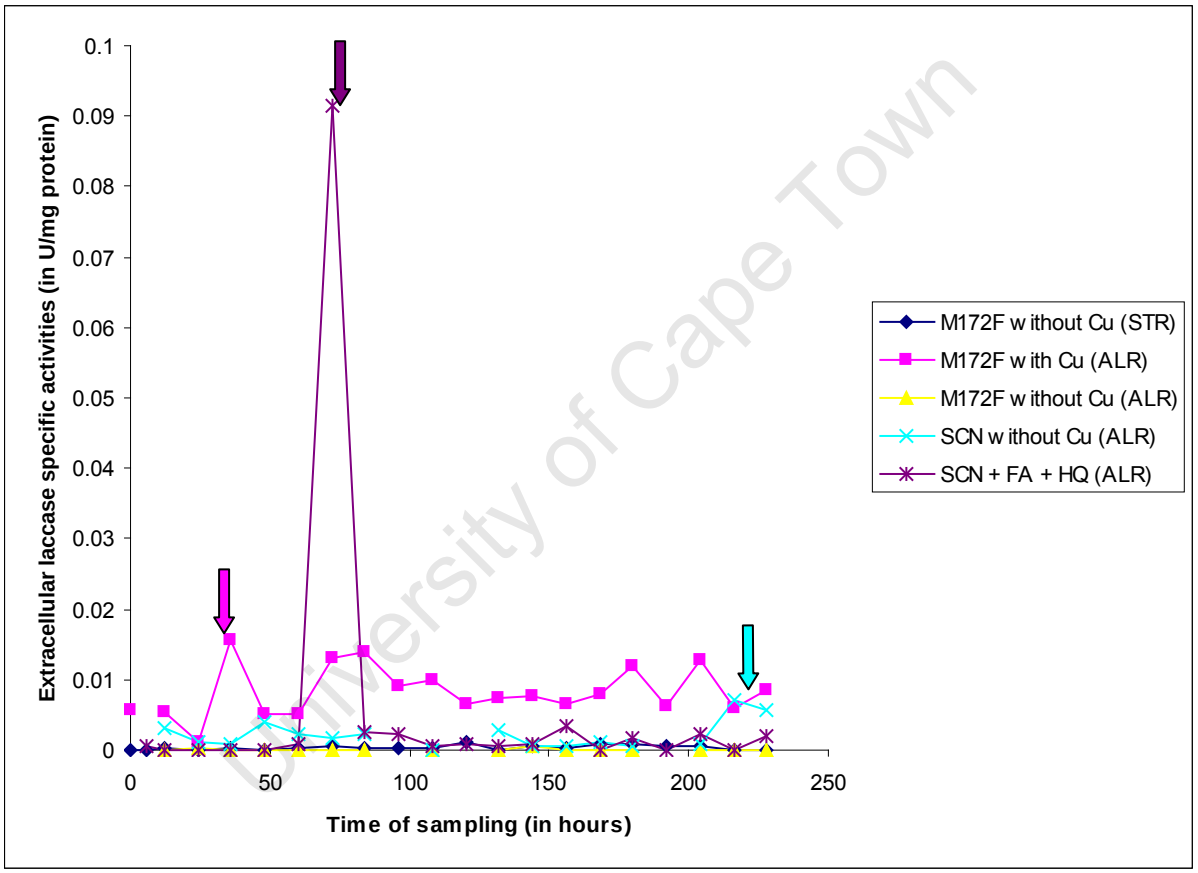


Fig. 3.15. Comparison of the DMP-laccase specific activities obtained during fermentations in different media and bioreactors. Arrows indicate the time point at which maximum laccase production occurred in a specific medium in the ALRs. Æ indicates the time of maximum laccase production in M172F medium supplemented with 8 mM $CuSO_4$.Æ indicates the time of maximum laccase production in SCN medium supplemented with FA and HQ as inducers of laccase production. Æ indicates the time of maximum laccase production in SCN medium without Cu.

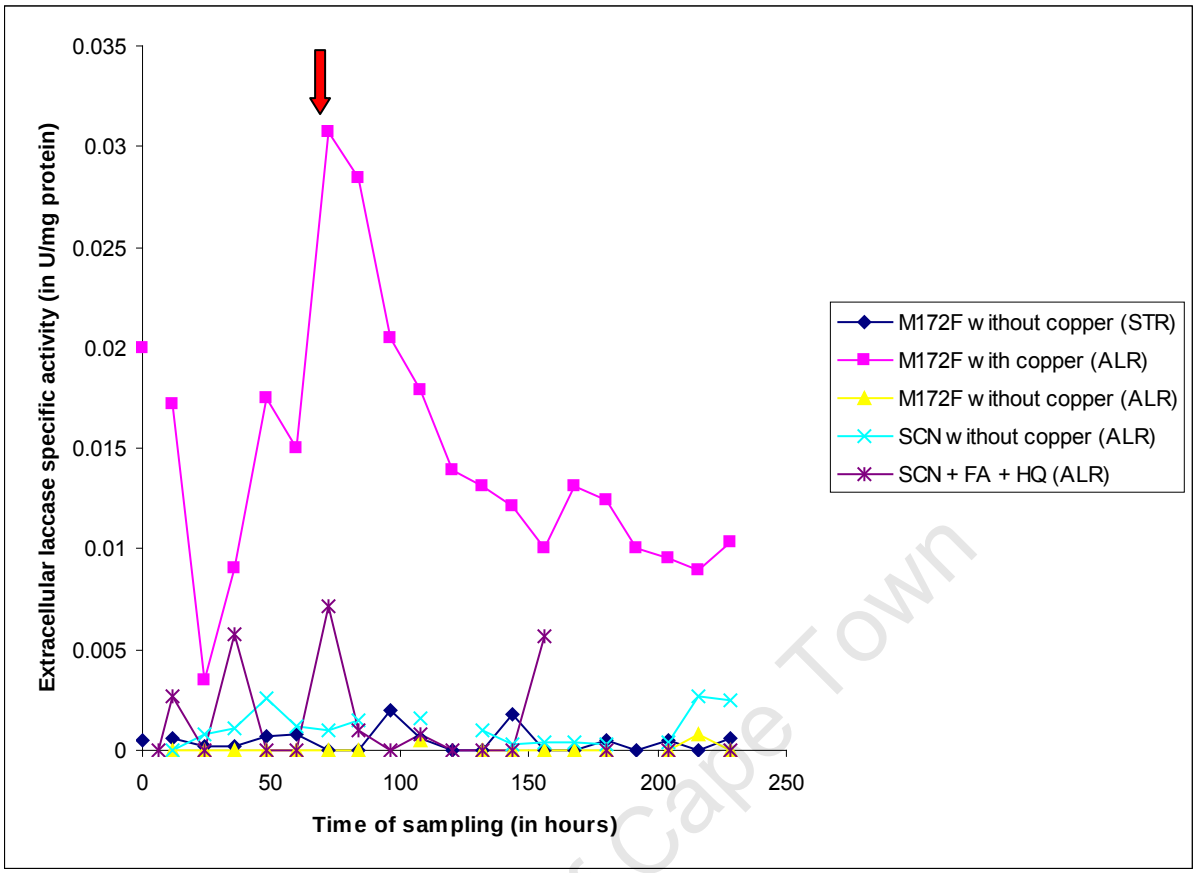


Fig. 3.16. Comparison of the 3-HAA-laccase specific activities obtained during fermentations in different media and bioreactors. The red arrow indicates the time of maximal laccase production by strain 044 30-1 in an ALR containing M172 medium supplemented with Cu.

Based on these results, the medium and conditions chosen for further study were: ALR containing M172F medium including Cu, with maximum laccase production expected at 36 hours, as evaluated with DMP as substrate (Table 3.2). Thus, for all subsequent experiments, strain 044 30-1 was inoculated in M172F medium and the fermentation allowed to proceed in the ALR for 36 hours. Activity was assayed with DMP and in cases where data was consistent with that shown in the table, the reactor was emptied. Since strain 044 30-1 laccase is produced extracellularly, the culture medium was subjected to protein isolation and purification procedures to allow isolation of the laccase (sections 3.2.10 and 3.3.7).

Table 3.2. Comparison of data related to laccase production by strain 044 30-1 in different bioreactors.

Bioreactor/Medium:	Time of Maximum Laccase Production (hours):	Assay Substrate:	Volume (ml):	Total Protein (g):	Total Activity (U):	Specific Activity (U/g):
ALR/SCN + FA + HQ	72	DMP	3800	0.23	2.09	9.1
ALR/SCN	216	DMP	3800	3.8	26.6	7
		3-HAA	3800	3.8	10.26	2.7
ALR/M172F (Cu)	36	DMP	3800	12.16	194.56	16
	72	3-HAA	3800	16.72	50.16	3

3.3.4 Optimisation of the Cu^{2+} concentration used in strain 044 30-1 growth medium for laccase production

Changes in the activity of laccase in the presence of aromatic and phenolic compounds have been extensively reported, whereas the effect of metal ions, such as Cu, on the synthesis of laccase has been less well studied. In the study by Shutova and co-workers (2008), laccase was synthesised in response to an increase in the concentration of Cu ions. It was suggested that in the presence of low concentrations of Cu^{2+}, inactive apolaccase containing no Cu ions in the active site was formed. It was shown that Cu is primarily required for the synthesis of laccase rather than for the activation of already synthesised proteins.

When laccase samples were obtained from strain 044 30-1 grown in M172F medium, containing no Cu^{2+}, laccase activity was very low. When a solution of 8 mM Cu^{2+} was added to these samples, and the samples then assayed for laccase activity, the activity was found to be remarkably higher (by as much as 300 fold). Although laccase activity was detected in the absence of Cu, the activity was clearly higher in the presence of Cu. This may suggest, in contrast to the above statement by Shutova and co-workers, that the presence of Cu enhances the catalytic function and substrate conversion rate by strain 044 30-1 laccase. Furthermore, the reaction with DMP indicates that strain 044 30-1 enzyme is an authentic laccase (section 3.3.1.1).

It was shown that for the fungi *T. versicolor*, *Ceriporiopsis subvermispora*, and *P. ostreatus* that the synthesis of laccase is regulated by Cu ions at the transcriptional level, although the precise mechanism of this regulation remains unclear (Shutova, *et al.*, 2008). In studies with *Trametes pubescens* (Galhaup, *et al.*, 2002a,b), it was shown that the addition of copper to the growth medium significantly increased laccase production. Extracellular laccase production by this fungus can be greatly stimulated by the addition of Cu in the millimolar concentration range. Several experiments suggested a stimulatory effect of Cu on phenol-oxidising enzymes in the actinomycete *Streptomyces griseus*: A novel laccase-type oxidase (37 kDa in size with 71% identity to the laccase from *S. coelicolor*) produced by this strain was repressed by glucose and induced by Cu supplied in the medium (Endo, *et al.*, 2002).

Since 8 mM Cu is a very high concentration and SCN medium supplemented with Cu had not previously been investigated, a range of different concentrations of Cu were investigated in both SCN and M172F media in flask culture, and in ALRs. Controls were used for assays representing each type of medium and concentration of Cu to ensure that activity was attributable to laccase itself. SCN medium, supplemented with Cu, was still ineffective in achieving good laccase yields. In flask culture experiments (Fig. 3.17), M172F supplemented with 0.5 mM Cu appeared to be the best medium for laccase production by strain 044 30-1 over the 10-day sampling period.

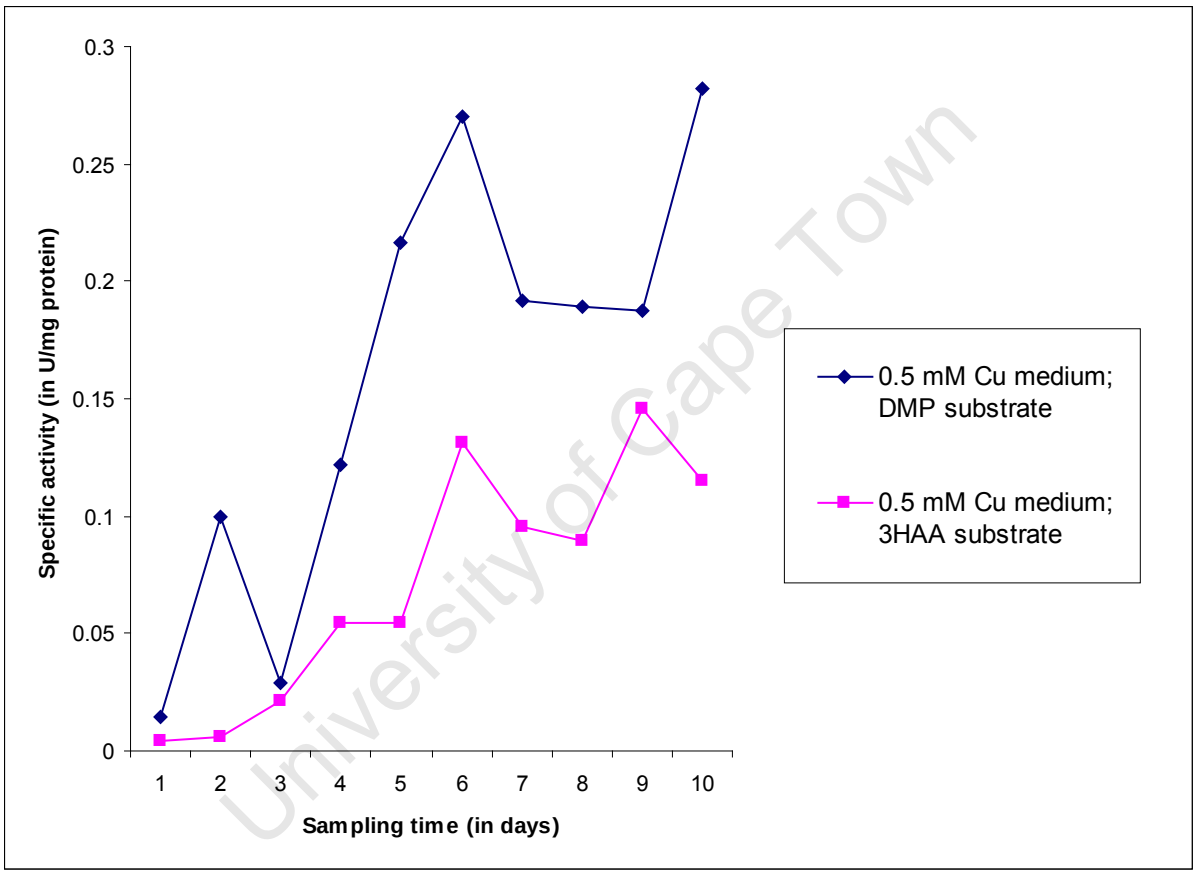


Fig. 3.17. In flask cultures containing M172F medium supplemented with $CuSO_4$, the optimal Cu concentration was 0.5 mM.

Duplicate ALRs containing M172F supplemented with 0.5 mM Cu were inoculated with strain 044 30-1 and laccase production was evaluated over 10 days. This scale-up, using this medium, was performed in order to determine if this medium gave better laccase titres than the 8 mM Cu-containing M172F medium, which had been shown to be the best medium for use in ALRs achieving the highest yield of laccase enzyme at an early time (section 3.3.3.2). The pH of the medium and dry cell weights of samples (Fig. 3.18) were also assessed.

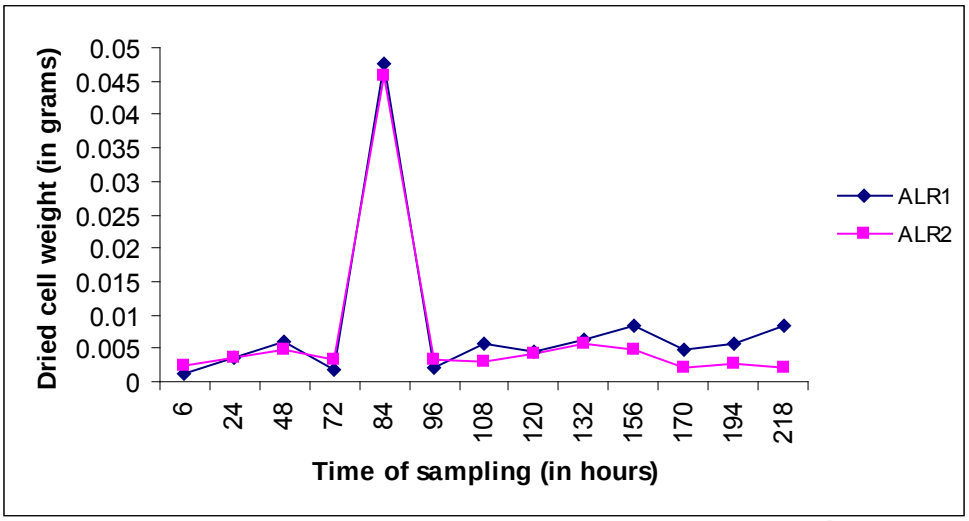


Fig 3.18. Dried cell weights of samples taken during the fermentation of strain 044 30-1 in duplicate ALRs containing M172F supplemented with 0.5 mM Cu.

Maximum laccase production occurred at 170 hours in the 0.5 mM Cu-supplemented medium and the laccase specific activity was 1.04 U/g using DMP as substrate, compared to 16 U/g achieved in the ALR containing 8 mM Cu (Fig. 3.19; Table 3.1). Thus, M172F medium supplemented with 8 mM Cu was still the best medium for synthesis of extracellular laccase by strain 044 30-1. Sporulation by strain 044 30-1 could be clearly observed, when the orange cells turned brown. Strain 044 30-1 grows well in M172F medium and sporulates very early on, perhaps due to the stress of the high Cu concentration in this medium. Furthermore, sporulation by this strain appeared to be linked to laccase production as the colour change of the cells occurred at the same time as detection of the highest laccase activity. For example, in Figure 3.17, sporulation of the strain began on day 6, when the highest laccase levels were detected. Bacterial laccases appear to have distinctive functions, playing a role in sporulation processes, including the biosynthesis of the brown spore pigment, and in resistance to copper and phenolic compounds (Giardina, *et al.*, 2009; Molina-Guijarro, *et al.*, 2009).

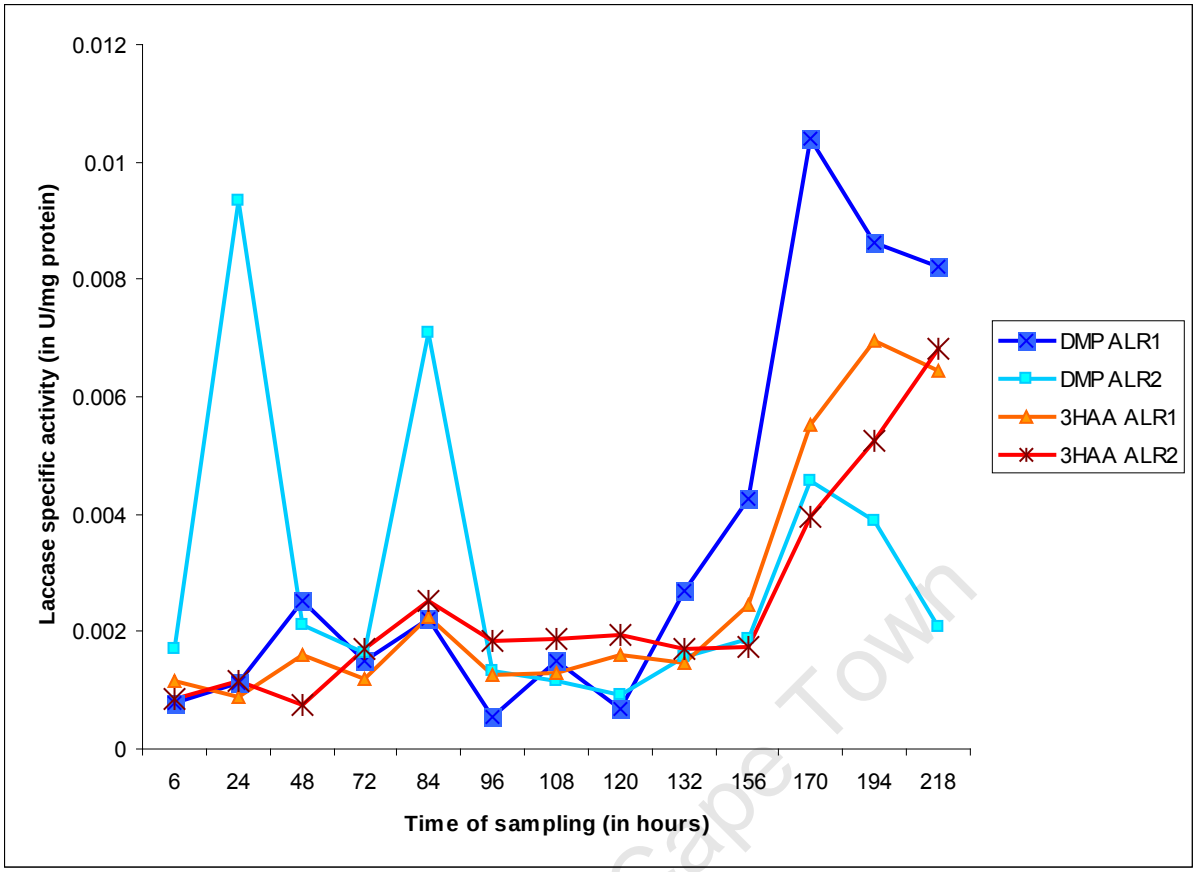


Fig. 3.19. Laccase specific activities obtained during the fermentation of strain 044 30-1 in duplicate ALRs containing M172F supplemented with 0.5 mM Cu.

3.3.5 *Micromonospora* sp. strain 044 30-1 as an extremophile

Although the synthesis of laccase by strain 044 30-1 is not dependent on the presence of Cu, laccase production was significantly increased in the presence of Cu^{2+} ions. A simple experiment whereby a gradient is created within a tube was designed by Rose (2008) in order to ascertain the distribution of a microbial population within a tube containing a sulphide/oxygen gradient. In this study, a copper/oxygen gradient was created and cells of strain 044 30-1 were shown to move towards the highest concentration of Cu in the tube (Fig. 3.20). Also, a Cu-impregnated disc was placed in the centre of an agar plate containing suspended cells of strain 044 30-1 in medium supplemented with DMP. Cells closest to the Cu disc were induced to produce laccase, as evident by the orange colour surrounding the disc. This orange colour is attributed to the oxidised dimeric DMP coloured product, formed by laccase action (Solano, *et al.*, 2001). This suggests that the presence of Cu results in the enhanced production of laccase by strain 044 30-1. Uninoculated controls were used, where no colour changes were observed.

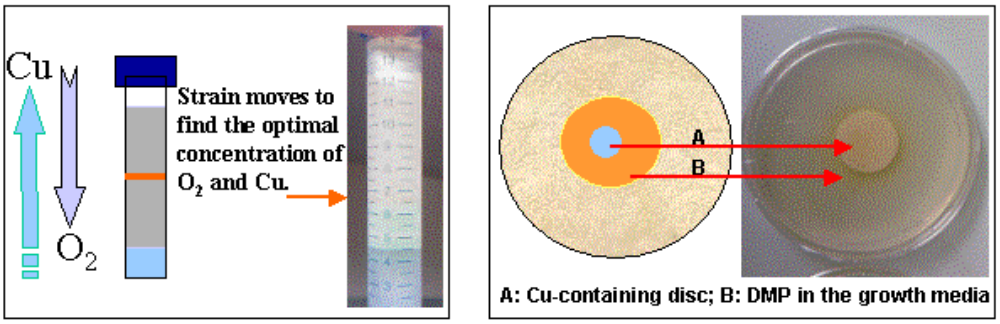


Fig. 3.20. Experiments illustrating the Cu-affinity of strain 044 30-1. Left: Orange mycelial cells of strain 044 30-1 move towards the highest concentration of Cu (8 mM). Right: Cells of strain 044 30-1 embedded in a sloppy agar supplemented with DMP move towards a 8 mM Cu-containing disc. Oxidised DMP (orange around the disc) is indicative of laccase activity by strain 044 30-1 in the presence of Cu.

Thus, strain 044 30-1 may be considered to be an extremophile, with a metal tolerance for high concentrations of Cu, such as 8 mM. It has been reported that copper tolerance is linked to acidophily (Navarro, *et al.*, 2008). Strain 044 30-1 was able to grow in the presence of 8 mM Cu^{2+} and at a pH as low as 3, making this strain a unique *Micromonospora* isolate.

3.3.6 Temperature profile of the laccase produced by *Micromonospora* sp. strain 044 30-1

The crude extracellular laccase produced by strain 044 30-1 was assessed for its stability at various temperatures using DMP and 3-HAA as assay substrates (Fig. 3.21 and 3.22). 3-HAA appeared to be the better assay substrate, being more sensitive. The enzyme appeared to be reasonably thermostable over time, which is not unusual for an actinomycete laccase (Arias, *et al.*, 2003; Molina-Guijarro, *et al.*, 2009). Thus, heat pretreatment could in future be used as a purification step, eliminating other proteins that are not as thermostable.

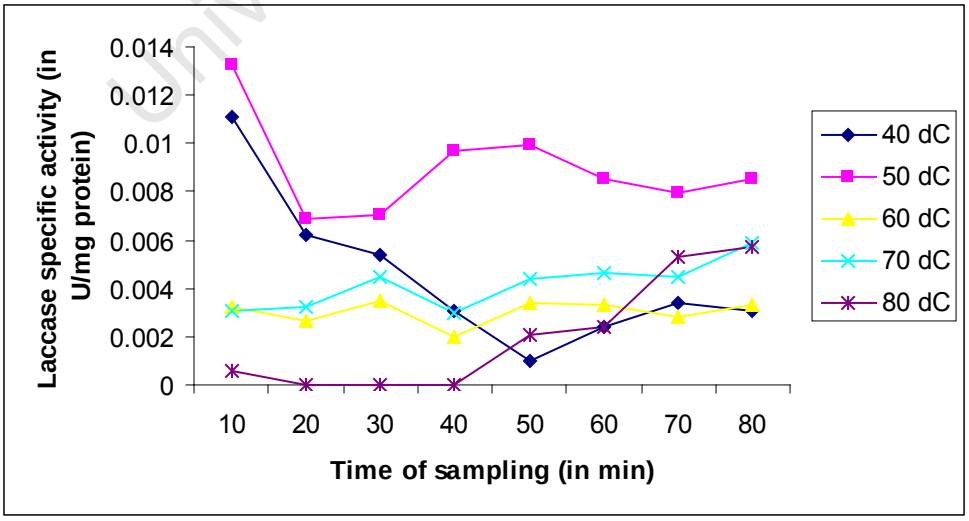


Fig. 3.21. Temperature profile of the laccase from strain 044 30-1 assessed with DMP as the assay substrate.

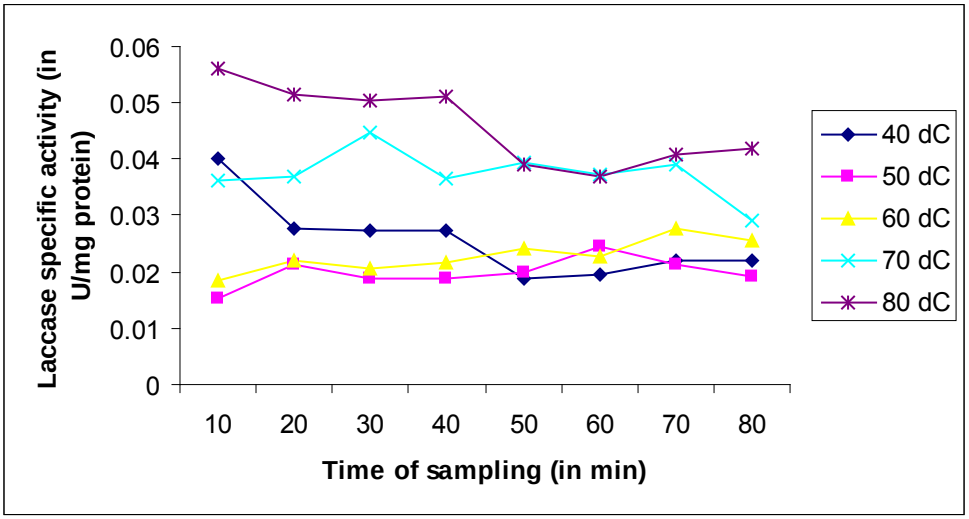


Fig. 3.22. Temperature profile of the laccase from strain 044 30-1 assessed with 3-HAA as the assay substrate.

3.3.7 Purification and characterisation of the laccase produced by *Micromonospora* sp. strain 044 30-1

Different protein isolation techniques were used to isolate the laccase from strain 044 30-1 culture medium: Acetone precipitation, ethanol precipitation, and ammonium sulfate precipitation (including step-wise fractionation using ammonium sulfate) were performed to obtain protein. Large volumes of culture fluid were freeze dried and concentrated in solution using various buffers or water. Dialysis was performed on samples precipitated with ammonium sulphate to remove the salt and the Cu from the medium, as explained in section 3.2.10. Use of a hydrophobic column and ultra filtration showed that enzyme activity was not due to copper in the solution, media components, or other contaminants. Removal of colour components, media, and other contaminants from enzyme-containing solutions by filtration and dialysis, further suggested that the enzyme activity was due to the protein in the samples. FPLC studies using an anion-exchange column and a 1 M NaCl gradient, indicated a single peak eluting at 36.97% NaCl when analysing crude samples of laccase-containing media from ALRs, and only this eluted sample exhibited laccase activity (3.2.10). The protein purification steps taken to obtain laccase are shown in Table 3.3. The activity was detected using 3-HAA as the laccase substrate in this instance.

Table 3.3. Purification table for the isolation of the extracellular laccase from strain 044 30-1 growth medium.

Laccase Sample:	Purification Step:	Volume (ml):	Total Protein (mg):	Total Activity (U):	Specific Activity (U/mg):	Purification (fold):	Yield (%):
ALR/M172F (Cu)	Crude	3500	12600	74.9	0.006	1	100
	40% Ammonium Sulphate Precipitation	3300	4950	117.7	0.024	4	157
	Dialysis	400	560	5.66	0.01	1.7	7.6
	Filtration	400	840	20.7	0.025	4.2	27.6
	Anion exchange (NaCl)	50	53.5	0.35	0.0064	1.07	0.47

Table 3.4 shows the results obtained from spectrophotometric scans of samples to detect the laccase copper centres. The $A_{280}/_{610}$ ratio is determined as a measure of laccase purity and is normally in the range of 15 – 20 for blue laccases, as can be seen for *Trametes pubescens* laccase (Leontievsky, *et al.*, 1997). However, this range will vary for the yellow or yellow-brown laccases and other atypical laccases, of which strain 044 30-1 laccase may be identified as with further study. The unusual spectral properties of atypical laccases are thought to be the result of slightly different coordination geometries of the T1 Cu and T3 Cu ions (Giardina, *et al.*, 2009) The scan to detect the Cu centres in samples of strain 044 30-1 laccase is shown in Figure 3.23.

Table 3.4. Purity of the laccase from strain 044 30-1 during purification steps, as determined by the $A_{280}/_{610}$ ratio.

Sample: $A_{280}/_{610}$ Ratio:	Crude (freeze-dried)	Crude (40% Ammonium sulphate precipitation)	Dialysis	Anion exchange (NaCl)	*Trametes pubescens* laccase
280 nm	2.0525	1.0822	0.5751	0.4387	0.6323
610 nm	0.1674	0.1208	0.1417	0.0153	0.0303
Ratio	12.26	9	4.1	28.7	20.87

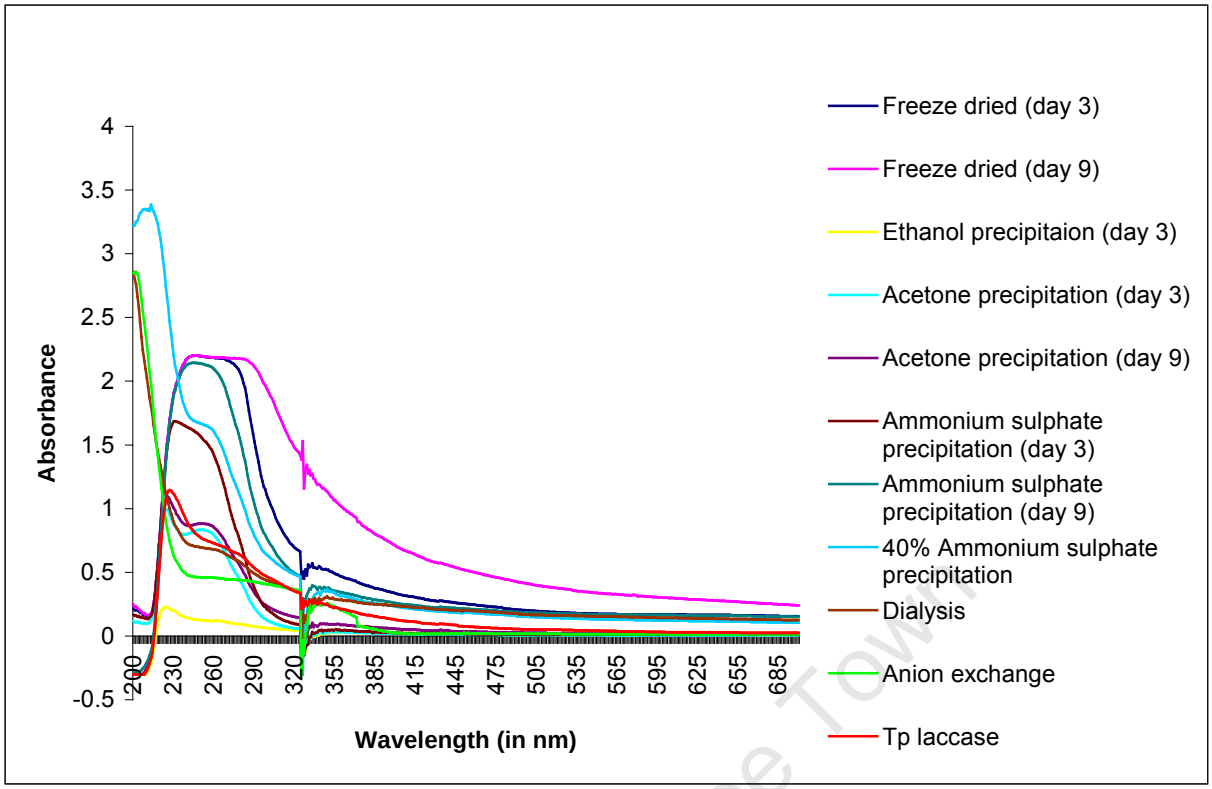


Fig. 3.23. Scan of the Cu centres of various laccase-containing samples obtained during protein purification of the laccase produced by strain 044 30-1. Tp, *Trametes pubescens*.

SDS-PAGE analysis (the denaturing gel is shown in Figure 3.24 below) showed that strain 044 30-1 laccase is approximately 49 kDa in size, an appropriate size for a laccase from a bacterium (Endo, *et al.*, 2002). Samples loaded on the gel included the concentrated crude extract and partially purified samples. Samples with the highest activity directly correlated with the increase in the presence of the 49 kDa band and a decrease in the intensity of the approximately 63 kDa band, indicating that the 49 kDa protein is the protein of interest. Silver staining and Coomassie staining were used to reveal this observation. For biocatalysis purposes, if the substrate can be converted to the desired product using the enzyme in the crude extract, further purification steps are not necessarily required. Furthermore, purification may result in significant loss of yield of the desired enzyme and in biocatalysis, a high yield of enzyme is desirable for successful biocatalytic reactions. For industrial processes, using the crude culture fluid, and not performing further protein purification or isolation techniques, saves on costs and time.

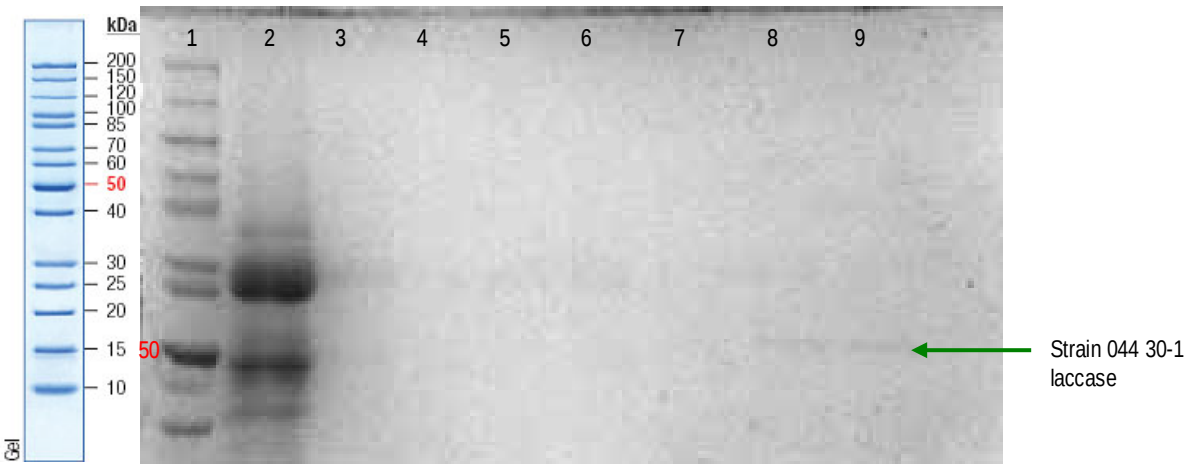


Fig. 3.24. An 8% stacking gel showing the size of strain 044 30-1 laccase (49 kDa). The PageRuler™ unstained molecular weight marker from Fermentas is shown in lane 1. Lane 2 contains concentrated crude extract. Lanes 3 – 9 show various partially purified samples obtained by ammonium sulphate precipitation (3 – 5), dialysis (6, 7), and anion exchange chromatography (8, 9) steps. The intensity of the 49 kDa band increases with each purification step. The sample in lane 9 had the highest laccase activity of all samples (specific activity of 1.57 U/mg protein using DMP as substrate), further indicating that the 49 kDa protein is the desired protein of interest.

The native gel showed that the proteins of interest (represented by the 49 and 63 kDa bands in the denaturing gel) migrated, according to charge, very differently from that of the control, *Trametes pubescens* laccase. While the laccase from *T. pubescens* migrated to about halfway down the gel, both the proteins of interest from strain 044 30-1 migrated a short distance, remaining near the top of the gel. A native (non-denaturing) gel was stained with DMP, the assay substrate of choice in this study, and laccase activity was visualised by the appearance of an orange colour. The staining reaction resulted in a diffused orange area and was not sensitive enough to distinguish between the two bands of interest, which migrated closely together. DMP has also been used as a staining agent to detect laccase in PAGE by Solano and co-workers (2001). Heat killed protein samples were used as negative controls and *Trametes pubescens* laccase was used as the positive control.

3.4 Conclusion

Micromonospora sp. strain 044 30-1 was isolated from a sea squirt whose habitat is Algoa Bay, located off the southern coast of South Africa. The strain is typical of the genus *Micromonospora*, having orange mycelia, and forming a black, mucoid, spore coat when subjected to stressful conditions. The role of laccase in this strain may be related to sporulation, and specifically, spore pigmentation, as maximal laccase production was observed to coincide with the formation of the characteristic black spores. Strain 044 30-1 may be novel on the basis that it grows well in the presence of 5% NaCl (Appendix B). Various growth requirements were investigated in order to optimise for maximal laccase production by strain 044 30-1, and the results revealed that the optimal conditions were: Growth in M172F medium supplemented with 0.2% (8 mM) $CuSO_4$, and 2.0% NaCl, at pH 5, with agitation at 160 rpm and with a cotton wool bung to enhance oxygenation. Extracellular laccase production predominated, which is unusual for a bacterial isolate. Laccase was produced in insufficient quantities in a stirred tank reactor but the strain performed well in airlift reactors. Laccase activity was efficiently detected using DMP and 3-HAA as assay substrates. To the best of the author's knowledge, this is the first report of the use of a *Micromonospora* strain in an ALR specifically for the production of laccase.

The size of strain 044 30-1 enzyme (using SDS-PAGE) was found to be approximately 49 kDa in size. There is no information on laccases from the *Micromonospora* genus of bacteria. The enzyme did not share characteristics of other blue enzymes or proteins, other oxidases, monooxygenases, dioxygenases, copper-containing enzymes, copper tolerance or copper resistance enzymes described in the literature (Adman, *et al.*, 1989; Divari, *et al.*, 2003; Djoko, *et al.*, 2008; Duffner and Müller, 1998; Howe, *et al.*, 1997; Kaizer, *et al.*, 2008; Kataoka, *et al.*, 2007; MacPherson, *et al.*, 2007; Que and Tolman, 2008; Singh, *et al.*, 2004; Solioz and Stoyanov, 2003; Sparnins and Dagley, 1975). Thus, the enzyme produced by strain 044 30-1 may be a new type of laccase, but the identity of this enzyme can only be conclusively confirmed by N-terminal amino acid sequencing of this protein, which is recommended as future work.

Strain 044 30-1 may be an extremophile, showing excellent tolerance of high concentrations of Cu^{2+} (8 mM) that are toxic to most microbes. It would be interesting to determine which mechanism within the cell is responsible for this tolerance, or resistance to, such high levels of copper. This is the first report of a *Micromonospora* strain that could be classed as an extremophile due to its copper tolerance.

Production of laccase by this strain may be linked to sporulation as evidenced by the highest detectable levels of laccase production and activity when the strain sporulates, changing colour from an orange mycelium to a black, mucoid spore layer. Laccases have been identified as having a role in sporulation; for example, much is documented in the literature about CotA, the spore coat protein of *Bacillus subtilis* that is a laccase. Laccase activity has been detected in *Bacillus sphaericus* where the presence of laccase correlates closely with spore formation and the appearance of melanin (Hullo, *et al.*, 2001).

In summary, this study has found the following evidence to suggest that the extracellular enzyme produced by strain 044 30-1 is a laccase:

- It was detected using the specific screening system developed to detect laccase in strains.
- Using spectrophotometry, the Cu centres have been detected and compared with *Trametes pubescens* and are as expected for a laccase enzyme (T1 and T3 Cus detected). Furthermore, the A_{280}/A_{610} ratio closely correlates to that typical of laccase (Leontievsky, *et al.*, 1997).
- The enzyme responds to known laccase inducers. Like other actinomycete laccases described in the literature, this enzyme is resistant to known laccase inhibitors (Molina-Guijarro, *et al.*, 2009; Dubé, *et al.*, 2008). In this study, xylidine, FA, and 8-HQ were used as inducers of laccase production by strain 044 30-1, while sodium azide and EDTA did not inhibit laccase production.
- The substrate specificity of this enzyme indicates that it is not a tyrosinase, as tyrosinase cannot convert the substrates this enzyme (a laccase) uses (Burton, 2003; Solomon, *et al.*, 1996). Furthermore, this enzyme cannot use substrates, such as cresol and L-DOPA, specifically used by tyrosinase.
- This enzyme is not a peroxidase: It does not require hydrogen peroxide to perform reactions, whereas a peroxidase requires hydrogen peroxide (Burton, 2003; Solomon, *et al.*, 1996).
- Laccase activity measured using ABTS was low. Other marine actinomycete laccases also show this (pers. comm. Dr Marilize le Roes-Hill), differing from fungal laccases, which have high ABTS activity.
- Strain 044 30-1 laccase oxidises syringaldazine, although, like ABTS, the activity is low. This enzyme cannot use L-tyrosine. The oxidation of syringaldazine and the inability to utilize L-tyrosine as a substrate is usually taken as an indication of laccase activity (Baldrian, 2006).
- It is unlikely that this enzyme is a phenoxazinone synthase as it is not subject to glucose repression (le Roes-Hill, *et al.*, 2009).
- Laccases should react with DMP, turning the solution orange and finally a red-purple precipitate forms (Solano, *et al.*, 2001). Strain 044 30-1 laccase and *Trametes versicolor* laccase catalyse this reaction although strain 044 30-1 enzyme is slower. Tyrosinases cannot use DMP.
- Strain 044 30-1 laccase catalysed the formation of polymers of 8-HQ, 3-HAA, and FA, and dimers of tyrosol and totarol, a reaction indicative of laccase action (Chapter 4).

This study has also described the production conditions for maximum synthesis of the extracellular laccase enzyme by strain 044 30-1: The use of an airlift bioreactor containing M172F medium at pH 5 supplemented with 2% NaCl and 8 mM $CuSO_4$, with maximum laccase production expected at 36 hours, as evaluated with DMP as substrate. One of the many possible uses of this enzyme is as a biocatalyst to synthesise antioxidant compounds with enhanced antioxidant power. Thus, the next aim of this study was to use the potentially novel laccase from strain 044 30-1 as a biocatalyst, producing new antioxidants (Chapters 4 and 5).

Chapter 4

Laccase Biocatalysis Applications: Focus on Tyrosol and Tyrosol-acetate Laccase-catalysed Reactions

4.1 Introduction

A wide range of reactions have been documented using laccases, from various microbial sources, for the production of value added compounds (section 1.13). Laccases are increasingly important biocatalysts in the chemical industry for organic synthesis of products such as fine chemicals, pharmaceuticals, and bulk chemicals, including polymers. Laccases are potentially important in environmental biotechnology for bioremediation or biodegradation of waste material, thus providing a cleaner environment (Riva, 2006; Ncanana, 2007).

Of particular interest in the application of laccases, is their ability to produce radicals that couple to each other by C-C or C-O bonds. Laccases are used in the synthesis of organic compounds, with target products being dimeric compounds linked by C-C or C-O bonds. Although it is generally accepted that laccases are responsible for the formation of such radicals, which then react non-enzymatically with each other to form dimers, there is little information available on the factors influencing the nature of the resulting products, and reports on the confirmed structural characterisation of products of such laccase-catalysed reactions are scarce (Ncanana and Burton, 2007).

The biocatalytic reactions catalysed by laccase are dependent on the chemical features of the phenolic substrate such as the number of –OH groups present, the nature and molecular weight of the substituents, and their position on the aromatic ring with respect to the OH groups (Gianfreda, *et al.*, 2003). The molecular structure of the substrate, the size of the substituent, and its *ortho* or *para* position may strongly influence the redox potential of the substrate, thus determining the final response to laccase oxidation (Canfora, *et al.*, 2008). Substituents that are electron-donating (*e.g.* $–CH_3$ or $–CH_2CH_3$) make the molecule more susceptible to oxidation. Substituents that are electron-withdrawing (*e.g.* $–NO_2$, –Cl, –COOH) increase the oxidation potential of the substrate and hence decrease the oxidation rate (Canfora, *et al.*, 2008). Laccase-catalysed oxidation of phenols becomes less efficient with increasing molecular weight of the substituent (Gianfreda, *et al.*, 2003).

The target substrates in a previous study conducted in our laboratory by Ncanana (2007) included tyrosol, hydroxytyrosol, 8-HQ, and totarol. All of these compounds have recognised biological activity, both as AOs and antimicrobials. The products of the laccase-catalysed reactions using these substrates were expected to have biological activities different from those of the starting materials, and in particular, the AO activity of those compounds was considered to be of interest. The 8-HQ-laccase

and tyrosol-laccase reactions resulted in the synthesis of polymeric compounds that precipitated from solution, and was recovered physically by filtration. In these reactions, 8-HQ and tyrosol were completely removed from solution (as polymers). Processes such as these were suggested to be applicable in the treatment of wastewaters contaminated with phenols (Ncanana, 2007).

Laccase-catalysed oxidations proceed *via* formation of radical cations, and subsequent deprotonation of the phenolic hydroxyl groups, to give phenoxy radicals that can undergo a broad variety of coupling reactions (Ponzoni, *et al.*, 2007). As part of the general interest in biocatalysis and in laccase-catalysed polymer formation, this present study investigated the synthetic exploitation of laccases for the oxidative dimerization of the phenolic derivatives totarol, tyrosol, hydroxytyrosol, 8-HQ, and 3-HAA. This study reports on laccase-catalysed oxidative reactions, using the laccases from *Trametes versicolor* and actinomycete strain 044 30-1, for biocatalysis of primarily tyrosol, monoacetyltyrosol (tyrosol-acetate), and to a lesser extent, reactions with totarol, 3-HAA, and 8-HQ, and reports for the first time the isolation and structure determination of some novel reaction products. The biological activities of these products are described in Chapter 5.

4.1.1 Tyrosol and the olive oil industry

Phenol-polluted waters are produced as wastes of several industrial and agricultural activities (Gianfreda, *et al.*, 2003). Biological technologies using oxidative enzymes (such as laccases) are promising technologies for the efficient cleanup of such phenol-polluted wastes (McNamara, *et al.*, 2008). Laccase-catalysed oxidative coupling reactions result in the formation of less soluble polymeric products, often less toxic than their precursors (Canfora, *et al.*, 2008; de la Rubia, *et al.*, 2008; Gianfreda, *et al.*, 2003; Gianfreda, *et al.*, 2006). As an added benefit, bioremediation may produce valuable products (McNamara, *et al.*, 2008). Olive mill waste water is considered a practical source of substrates of high added value for polymer production (Sayadi, 2009). Tyrosol and hydroxytyrosol, in particular, are found in wastewaters, and account for >80% of the olive mill waste water phenolic extract (Allouche, *et al.*, 2004). At high concentrations these compounds are toxic to organisms in water, and therefore removal of these compounds from waste water would contribute to a cleaner environment, and sustainable development (Sampedro, *et al.*, 2004; Gianfreda, *et al.*, 2006; Aranda, *et al.*, 2007).

Polyphenolic compounds produced by plants are of interest, as food ingredients and as nutraceuticals, because of their AO properties and other beneficial biological activities. Extra-virgin olive oil is the principal fat component of the Mediterranean diet, and its chemical constituents have been intensively studied (McNamara, *et al.*, 2008). The main olive oil phenols are oleuropein, tyrosol, and hydroxytyrosol. It has been reported that tyrosol has scavenging effects on ROS (Aissa, *et al.*, 2007). Studies confirm that the elevated phenolic AO content of the components of the Mediterranean diet greatly contributes to the beneficial health effects of this dietary habit (Manna, *et al.*, 1999).

4.1.2 Totarol

Totarol, a tricyclic diterpene, belongs to the terpenoid family of natural products. Terpenoids have been shown to be of pharmacological importance, having biological activities such as antimicrobial, antimalarial, anti-inflammatory, cardiovascular, and antitumoral properties (Ncanana, *et al.*, 2007). Of note, was the finding by Constantine and co-workers (2001) that totarol was active against *Mycobacterium tuberculosis*, the causative agent of the prevalent disease, tuberculosis (TB). The importance of totarol biological activity has encouraged researchers to investigate the synthesis of totarol analogues with the aim of increasing the activity or finding new applications (Evans, *et al.*, 2000). Most reports regarding the modifications of totarol have been based on chemical syntheses, and thus the use of enzymes has been proposed as an alternative method that is more selective, and would be desirable in the development of pharmaceuticals, and in the modification of natural compounds with phenolic substructures (Evans, *et al.*, 2000). The study by Ncanana (2007) reported for the first time the modification of the biologically active phenolic compound, totarol, using an enzyme, and in this study, laccase from *T. pubescens* was used for the synthesis of novel totarol dimers in organic medium. The absence of polymeric material suggested that reaction of totarol with laccase resulted in dimeric products only. The formation of two dimeric products, linked either by C-C or C-O bonds, was attributed to the coupling of the radicals formed, as illustrated in Figure 4.1 (Ncanana, 2007; Ncanana, *et al.*, 2007). Generally, the C-C linked totarol dimer was the dominant product in all the reaction conditions investigated. The results obtained in this study demonstrated that totarol dimer synthesis, catalysed by laccase, required only mild conditions, which is an important factor in the development of environmentally friendly bioprocesses.

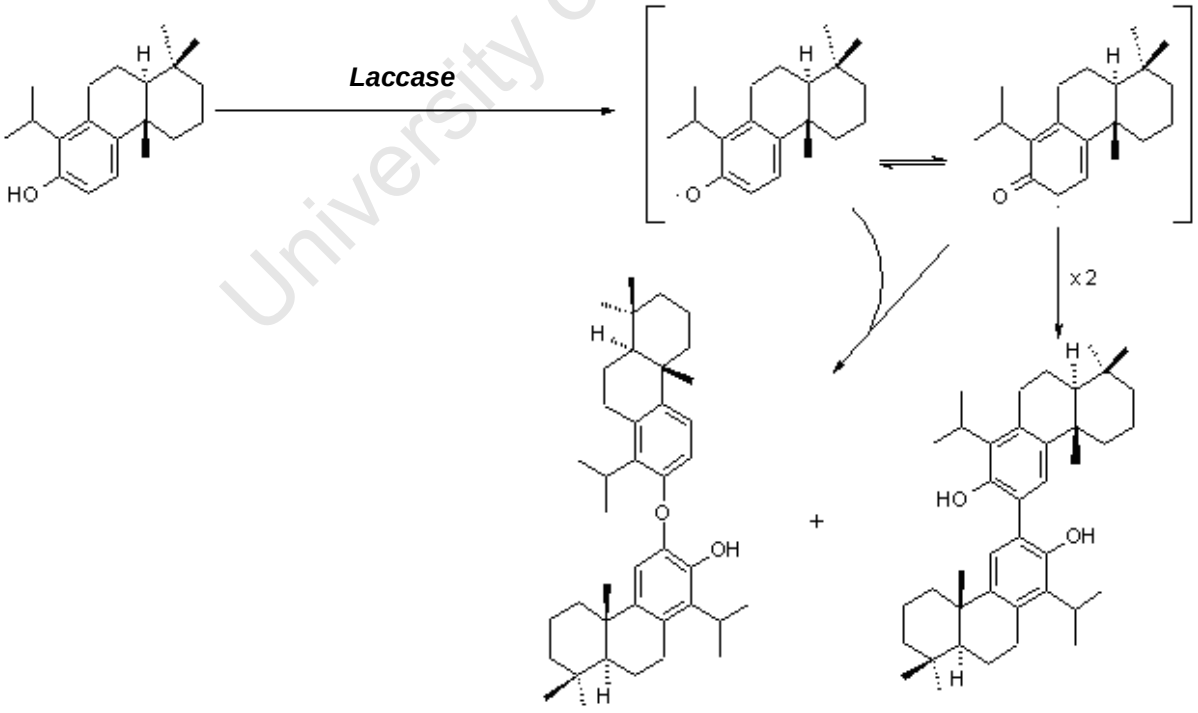


Fig. 4.1. The laccase-catalysed conversion of totarol to dimeric products. The C-C dimer is the major product of this reaction, while the C-O dimer is the minor product (Ncanana, 2007).

4.1.3 Other substrates of interest in this study: 3-hydroxyanthranillic acid, 8-hydroxyquinoline, and ferulic acid

4.1.3.1 The phenoxazinone chromophore and 3-hydroxyanthranillic acid (3-HAA)

The phenoxazinone chromophore occurs widely in nature, including in the structures of many pigments and some antibiotics. It also forms as part of a system to protect mammalian tissue from oxidative damage (Eggert, *et al.*, 1995). Upon oxidation, *o*-aminophenols are converted into their corresponding *o*-quinone imines. Because of their high reactivity, these *o*-quinone imines couple spontaneously to form derivatives such as the cyclic phenoxazinones. The phenoxazinone ring structure has been identified in a number of biological systems; for example, the *o*-aminophenol 3-HAA, a metabolite of the kynurenine pathway, is the precursor of the phenoxazinone derivative cinnabarinic acid (CA) (Eggert, *et al.*, 1995). In particular antibiotic producing bacteria, 3-HAA serves as a precursor for another phenoxazinone derivative, actinomycin. The actinomycins are chromopeptide antibiotics produced by a number of *Streptomyces* and *Micromonospora* strains (Jones, 2000). Oxidative coupling of 3-HAA occurs in reactions catalysed by the BMCOs laccase, tyrosinase, peroxidase, phenoxazinone synthase, and ceruloplasmin, and evolves along the same pathway in each case (Eggert, *et al.*, 1995, Rescigno, *et al.*, 1998). Since laccases are able to catalyse a broad range of aromatic substrates, the formation of actinocin (using aminophenol derivatives as substrates), mediated by laccases, could serve as an alternative to a chemical path for the production of novel, actinomycin-like cytotoxic drugs comprising a phenoxazinone chromophore (Osiadacz, *et al.*, 1999).

The aminophenol 3-HAA has been reported to show important AO activities in inflammatory diseases and to play a role in the tanning of proteins. Furthermore, hemoglobin is involved in 3-HAA metabolism and in the formation of phenoxazinones within the human erythrocyte (Rescigno, *et al.*, 1998). The conversion of 3-HAA to CA has received attention in clinical studies because 3-HAA is produced in large amounts by interferon-γ-primed mononuclear phagocytes and has been shown to act as a powerful scavenger of ROS (implying antioxidant power). CA is one of the major products of the peroxyl radical-mediated oxidation of 3-HAA suggested to prevent oxidative damage in mammalian tissues, and can be oxidized by the BMCO ceruloplasmin (Eggert, *et al.*, 1995; Tomoda, *et al.*, 1986).

It has been reported that in the fungus *Pycnoporus cinnabarinus*, the production of a red pigment, CA, characteristic of this fungus, is a laccase-dependent process (Fig. 4.2). Comprising a phenoxazinone chromophore, this pigment is formed *via* laccase-catalysed oxidative condensation of two molecules of 3-HAA (Osiadacz, *et al.*, 1999). A characteristic feature of the members of the genus *Pycnoporus* is the production of pigments (colouring the fungi) that contain the phenoxazinone chromophore. The members of this genus are notable laccase producers and the 3 pigments isolated from the genus (CA, cinnabarin, and tramesanguin) differ only in the oxidation state of their functional groups (Fig. 4.2). This difference in the structure of the pigments represents oxidative coupling of different *o*-aminophenol precursors, having either the alcohol, aldehyde or carboxyl group as functional groups.

Production of these precursors could be strain specific or may depend on the culture conditions (Eggert, *et al.*, 1995).

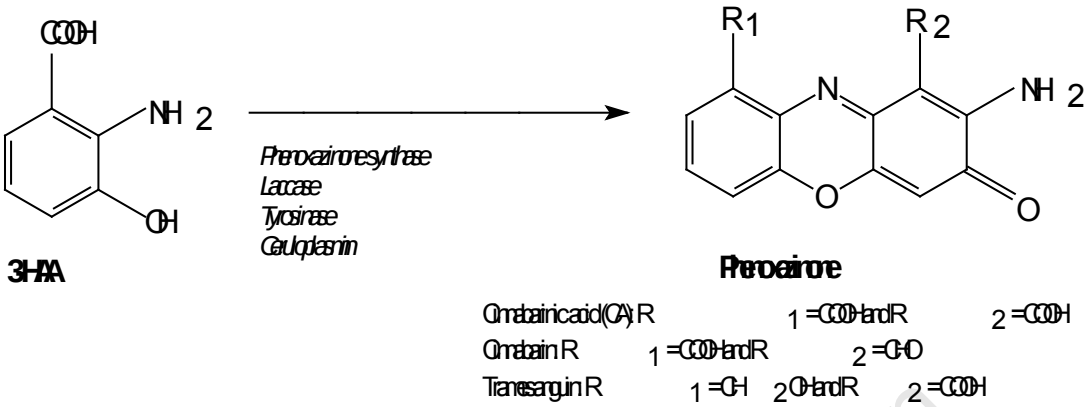


Fig. 4.2. Oxidative coupling of 3-HAA results in the formation of the phenoxazinone chromophore, which is the core ring structure of numerous pigments and certain antibiotics (le Roes-Hill, *et al.*, 2009).

4.1.3.2 Laccase-catalysed reactions of 8-hydroxyquinoline (8-HQ)

8-HQ is generally known as an antiseptic with mild fungistatic, bacteriostatic, antihelmintic, and amebicidal action and 8-HQ is also used as an AO and metal chelator (Crutchley, 1995; Barber, 2000). The accumulation of 8-HQ and other phenols in water or soil is toxic to organisms living in such environments (Jones, 1963). Thus, the removal of 8-HQ and similar pollutant compounds from water is useful and this could be achieved by polymerisation, since polymers can readily be separated from solution.

Polymerisation of 8-HQ, a low molecular weight aromatic compound bearing a reactive hydroxyl group, was achieved in reaction media containing acetone, methanol, or ethyl acetate. This result, reported by Ncanana and Burton (2007) was the first report of the enzymatic synthesis of poly(8-HQ), catalysed by laccase from the WRF *Trametes pubescens* (strain CBS696.94). The water-insoluble products obtained in this study were believed to be formed due to the laccase-catalysed oxidation of 8-HQ to form aromatic radicals, which in turn combined to form the polymeric product that precipitated spontaneously from solution due to its low solubility. This was also taken as an indication that a complex compound with higher molecular weight had been formed as a result of the high degree of polymerisation of the 8-HQ intermediate radicals. The structure of the water-insoluble product was comprised of 8-HQ monomers linked by C-C or C-O bonds to form the polymeric structure suggested in Figure 4.3 (Ncanana and Burton, 2007; Ncanana, 2007). Furthermore, it was suggested that the polymeric product mixture obtained was dominated by C-C linkages. These findings could be attributable to the structure of 8-HQ, and more specifically, the *para*-substitution of the hydroxyl group. It is generally accepted that *para*-substituted phenolic compounds are more reactive and are thus prone to polymerisation (Ncanana, 2007). Furthermore, it was suggested that the successful

polymerisation could also be due to the reaction of the 8-HQ radicals with other radicals in the medium, which was sterically possible, and hence the elongation of the chain was possible, to form a polymeric compound. The oxidation of 8-HQ by *T. pubescens* laccase presented a process that could be used in bioremediation since monomers were converted into polymers that could be recovered by filtration. Laccase from *T. pubescens* removed 100% of 8-HQ from aqueous solution (Ncanana, 2007). A further advantage of this process is that products produced in significant yields can be recovered and characterised for new properties. For example, polymers comprising 8-HQ as a building block exhibit photovoltaic properties (Xie, *et al.*, 1998).

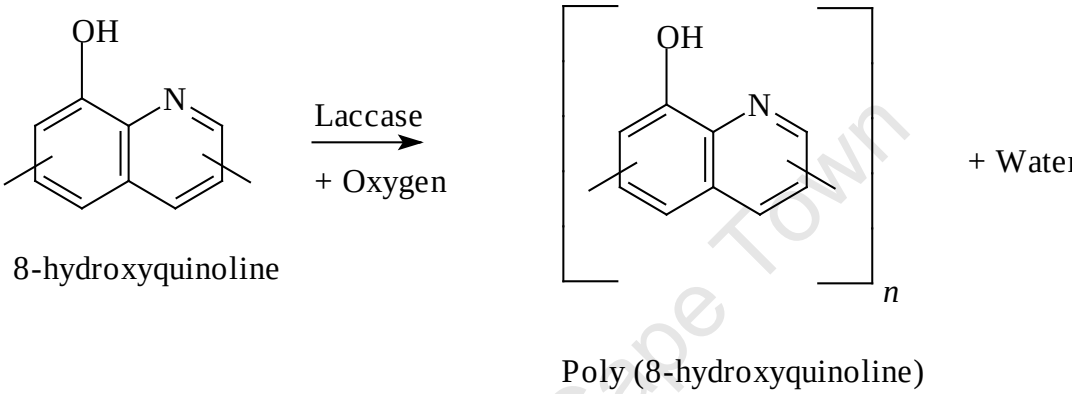


Fig. 4.3. Laccase converts 8-hydroxyquinoline to larger polymeric compounds, where *n* ranges from 6 to 23 (Ncanana, 2007).

4.1.3.3 Laccase-catalysed reactions of the antioxidant ferulic acid (FA)

Ferulic acid (FA) is the common name for 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid, and is a ubiquitous plant phenolic compound and dibasic acid with AO and potential chemopreventative properties (Chigorimbo-Murefu, 2007). The potent AO capability of FA results from the formation of a resonance-stabilised phenoxy radical, stabilised by extended side chain conjugation (Chigorimbo-Murefu, 2007).

The oxidation of phenols by laccase generates reactive species that can undergo spontaneous non-enzymatic polymerisations. Several free radicals from FA are possible depending on the position of the unpaired electron and hence different permutations and combinations can result in different polymers (Fig. 4.4). There are four possible intermediate radicals that can be formed from FA, depending on the mechanism of polymerisation (Chigorimbo-Murefu, 2007; Carunchio, *et al.*, 2001). These radicals are designated M_O, M5, M4, and M_β and there are 10 possible dimeric combinations. From each of those 10 possible combinations, 10 others are possible in the formation of the tetramer, giving a total of 55 possible tetrameric products. The types of polymers formed vary depending on the reaction medium, as polymerisation is influenced by solvent effects (Chigorimbo-Murefu, 2007). FA tetramers were formed when the reaction was catalysed by non-immobilised laccase, and dimers were formed using immobilised laccase (Carunchio, *et al.*, 2001).

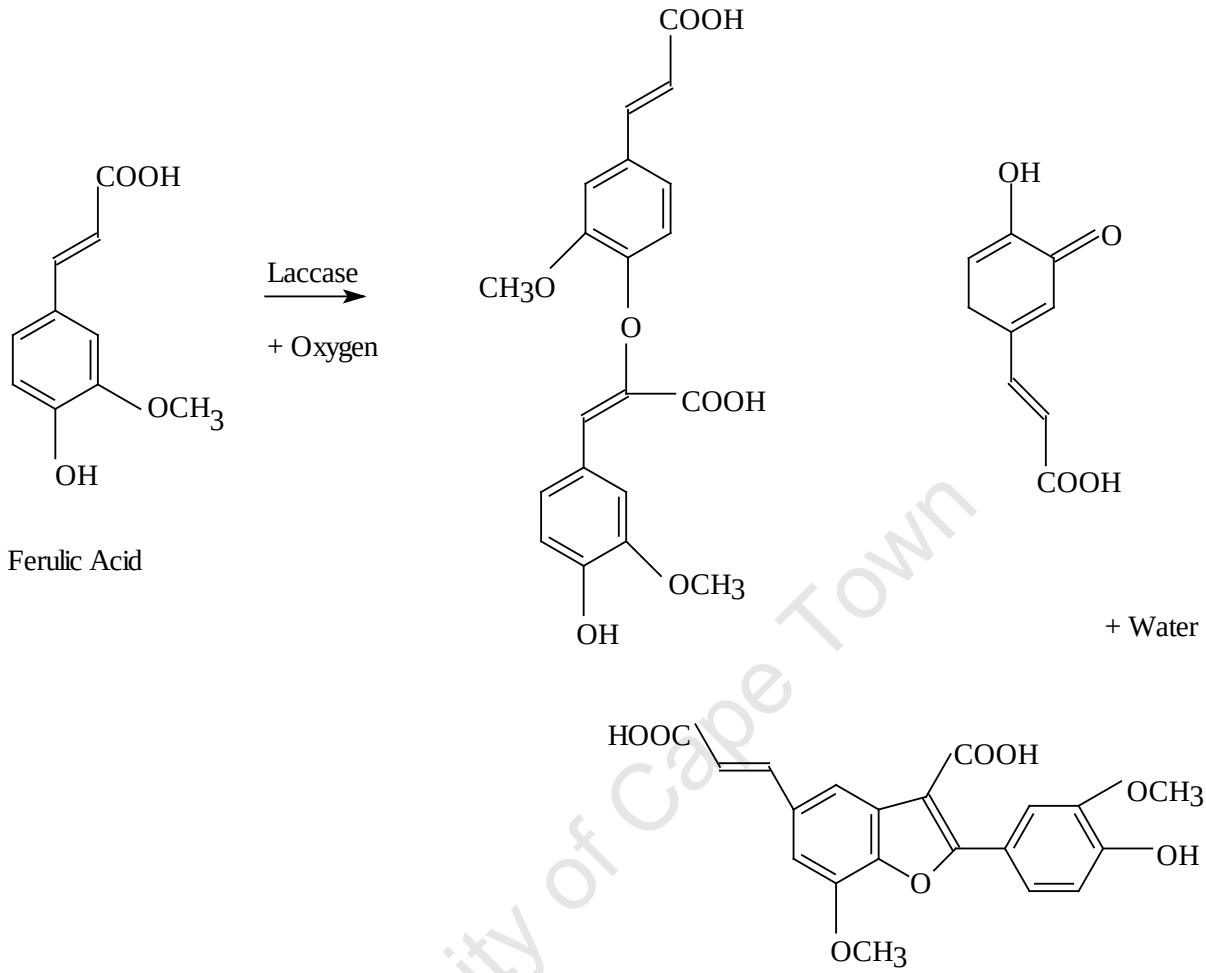


Fig. 4.4. Typical products of a laccase-catalysed biocatalytic conversion of ferulic acid (Burton, 2003).

4.2 Materials and Methods

Biocatalysis reactions using *Trametes versicolor* laccase, and tyrosinase from mushroom, were performed, in collaboration, at the ICRM, CNR, Milan, Italy.

4.2.1 Following the progress of reactions

Reactions in small volumes were used to make assessments regarding the time-course of product formation and to optimise the ratio of reaction components needed for a successful reaction. Appendix A gives details of small-scale reactions. The formation of product(s) was followed by using silica TLC plates (Merck) developed with the optimal solvent system (Table 4.1), and visualised using a UV light box at 254 nm (Desaga) followed by submersion in Pancaldi reagent (Dal Pozzo, *et al.*, 2000) and drying with a Bosch drier (PHG 600-3). Organic material stained purple-blue. Ethyl acetate was supplied by Fluka, toluene by Riedel de Haën, formic acid by AnalytiCals, and methanol, chloroform, petroleum ether, and acetone by Sigma Aldrich.

Table 4.1. Solvent systems used for the development of TLC plates for following the course of reactions.

Reaction:	Solvent Systems Used:	Reference:
Tyrosol-laccase: Product 1 Product 2	 8 Ethyl acetate : 2 Petroleum ether 10 Ethyl acetate : 0.3 Methanol	This study.
Tyrosol-tyrosinase	5 Toluene : 4 Ethyl acetate : 1 Formic acid	Ncanana, 2007.
Tyrosol-lipase	9 Petroleum ether : 1 Ethyl acetate	This study.
Tyrosol-acetate-laccase	7 Petroleum ether : 3 Ethyl acetate	This study.
Tyrosol-acetate-tyrosinase	7 Ethyl acetate : 3 Petroleum ether	This study.
Totarol-laccase	10 Petroleum ether : 0.1 Ethyl acetate	This study.

4.2.2 Large-scale reactions, isolation of product material, and purification by column chromatography

Once reactions had progressed satisfactorily (observed by assessing the colour of the reaction and precipitation of material, and by TLC) the glass vial or glass round-bottomed flask was removed from the 30°C shaker. If there had been significant precipitation of material, the precipitate was removed by filtration, usually under vacuum using a Buchner funnel and filter paper. For biphasic reactions, the two phases were then separated (thereby stopping the enzyme-catalysed reaction) using a separating funnel. All glassware was thoroughly washed with solvent (that which was used as the organic phase), usually ethyl acetate (Fluka). Ethyl acetate was added to the separating funnel approximately 3 – 6 times to ensure that all product was removed from the aqueous phase and retained in the organic phase. A few spoonfuls of sodium sulfate (Sigma Aldrich) were added to the ethyl acetate in order to remove water (anhydrification) and this was subsequently removed by filtration. Solvent was removed using a Rotavapor (Büchi). The dried product was then isolated in a preweighed round-bottomed flask

or vial. This product was then dissolved in a small volume of suitable solvent, usually methanol (Sigma Aldrich), and loaded onto a suitable column in order to be purified by flash chromatography. The size and diameter of the column was determined based on the amount of product material (in mg; see Table 4.2). A layer of sea sand (Sigma Aldrich) of approximately 2 cm was added to line the bottom of the column, followed by silica (Alltech Italia) of a height of approximately 20 cm. The chosen eluent (based on TLC solvent systems) was run through the column in order to sufficiently wet the column. The product sample was carefully added to the top of the column, followed by another layer of sea sand. Eluent was added, and under nitrogen, fractions were eluted and collected. The course of separation of products was followed by TLC (see section 4.2.1). Clean fractions, containing only the product of interest, were pooled and solvent removed by Rotavapor. The weight of each product was calculated and the purity checked by TLC (a single, clean spot must be observed) and the sample(s) were submitted for structure determination (proton NMR, carbon NMR, COSY).

Table 4.2. The type of column used for flash chromatography is determined by the mass of sample material to be loaded on the column for separation.

Column diameter (mm):	Volume of eluent for packing and elution (ml):	Sample for loading (mg):		Typical fraction size (ml):
		ΔRf≥0.2	ΔRf≥0.1	
10	100	100	40	5
20	200	400	160	10
30	400	900	260	20
40	600	1600	600	30
50	1000	2500	1000	50

4.2.3 Tyrosol-laccase biphasic reaction

The following were added to a glass round-bottomed flask and the reaction was allowed to proceed at 30°C with mild shaking: 50 mg laccase from *Trametes versicolor* (Fluka) in 50 ml 20 mM sodium acetate buffer, pH 3.5 (AnalytiCals) was added to 500 mg tyrosol [2-(p-hydroxyphenyl)-ethanol 4-hydroxyphenethyl alcohol; Sigma Aldrich] in 50 ml ethyl acetate. The reaction was considered complete after 24 hours and subjected to the protocol described in section 4.2.2. A successful reaction was characterised by the yellow colour of the ethyl acetate layer and by large amounts of orange precipitate in the aqueous phase. Only material soluble in the organic phase could be used. This procedure was suitable for obtaining PRODUCT 1, named TYR-01 (**I**). To obtain PRODUCT 2, the INTERMEDIATE of this reaction (**II**) the reaction was allowed to proceed for only 8 hours. For column chromatography, the solvent system for compound **I** was used until it was observed that this product had eluted. At this point, the eluent was changed to that of the solvent system for obtaining compound **II** (see Table 4.1). Both products were coloured orange-yellow. Structure determination of compound **I** was carried out at the ICRM, Milan, Italy, where ^{1}H-NMR, ^{13}C-NMR, and COSY spectra were

analysed. Compound **II** was believed to be an intermediate in the reaction and was observed to transform to compound **I** over time. An efficient small-scale reaction can be carried out as follows: 1 mg laccase from *Trametes versicolor* in 1 ml 20 mM sodium acetate buffer, pH 3.5, was added to 10 mg tyrosol in 1 ml ethyl acetate, was mixed, and incubated at 30°C with shaking.

4.2.3.1 Tyr01, I (43 mg, 8.6 % i.y.): ^{1}H-NMR δ (ppm, MeOD): δ = 7.21 (1H, d, J = 2.0 Hz, H-13); 7.07 (1H, dd, J_1 = 8.5 Hz, J_2 = 2.0 Hz, H-15); 6.73 (1H, d, J = 8.5 Hz, H-16); 4.80 (1H, dd, J_1 = 3.0 Hz, J_2 = 1.0 Hz, H-5); 4.15 (1H, dt, J_1 = 9.0 Hz, J_2 = 6.5 Hz, H-8_a); 4.06 (1H, dt, J_1 = 9.5 Hz, J_2 = 6.5 Hz, H-8_b); 3.95 (1H, m, H-3); 3.74 (2H, t, J = 7.0 Hz, CH_2-18); 2.86 (2H, dd, J_1 = 6.0 Hz, J_2 = 3.0 Hz, CH_2-6); 2.82 (1H, H-9_a); 2.80 (2H, t, J = 7.0 Hz, CH_2-17); 2.59 (1H, dd, J_1 = 18.0 Hz, J_2 = 3.5 Hz, H-2_a); 2.22 (1H, H-9_b); 2.21 (1H, dd, J_1 = 4.5 Hz, J_2 = 2.5 Hz, H-2_b). ^{13}C-NMR δ (ppm, MeOD): δ = 207.52 (C-1); 158.02 (C-11); 132.38 (C-14); 129.75 (C-12); 129.54 (C-15); 123.64 (C-13); 109.15 (C-16); 87.70 (C-5); 83.24 (C-3); 66.90 (C-8); 63.05 (C-18); 52.73 (C-4); 39.37 (C-6); 38.83 (C-2); 38.33 (C-17); 37.84 (C-9). m/z = 274

4.2.4 Tyrosol-tyrosinase reactions

The most successful reaction was carried out under the following conditions: 50 mg tyrosol in 8 ml chloroform was added to 5 mg tyrosinase (from mushroom; Sigma) in 8 ml 50 mM potassium phosphate buffer, pH 7 (K_2HPO_4 from Fluka; KH_2PO_4 from Merck). This reaction was allowed to proceed at 30°C with mild shaking for > 24 hours, by which time the aqueous layer was coloured dark brown and the organic layer light brown. Ascorbic acid (L+; Aldrich) was not added to this particular reaction. Normally ascorbic acid is added to such reactions in order to increase the yield of hydroxytyrosol (Appendix A). The major product of this reaction, which could be isolated by column chromatography, was named TYR-02 (**III**) and was yellow-orange in colour. Structure determination carried out at the ICRM, Italy, revealed that this product was not hydroxytyrosol.

4.2.5 Preparation of tyrosol-acetate

The hydroxyl group on the side chain of the tyrosol molecule was replaced with an acetate group by performing the following reaction: 75 mg of lipase from *Candida antarctica* (Novozyme 435; Novozymes, Denmark) was added to 200 mg of tyrosol in 5 ml of vinyl acetate (Aldrich). The reaction was allowed to proceed at 45°C with shaking at 200 rpm for 24 hours. The major product of this reaction was named TYROSOL-ACETATE (**IV**). Column chromatography was performed to separate a contaminating product. Compound **IV** was viscous and orange in colour, but resuspension in petroleum ether resulted in the formation of a dirty white solid. The structure of compound **IV** was confirmed by NMR spectra analysed at the ICRM, Italy. The molecular weight of **IV** was 180.17 g/mol by mass spectrometry.

4.2.5.1 Tyrosol-acetate, IV (173 mg, 86.4 % i.y.): ^{1}H-NMR δ (ppm, MeOD): δ = 7.05 (2H, d, J = 8.5 Hz, H-3, H-5); 6.72 (2H, d, J = 8.5 Hz, H-2, H-6); 4.21 (2H, d, J = 7.0 Hz, CH_2-8); 2.83 (2H, d, J = 7.0 Hz, CH_2-7); 2.01 (3H, s, CH_3).

4.2.6 Tyrosol-acetate-laccase reactions

The following reaction was allowed to proceed in a suitable glass vial at 30°C with mild shaking for 24 hours: 50 mg tyrosol-acetate (**IV**) in 5 ml ethyl acetate was added to 50 mg laccase from *Trametes versicolor* in 50 ml 20 mM sodium acetate buffer, pH 3.5. Two major products were isolated: TyrAc1 (the least polar; **V**) and TyrAc2 (most polar; **VI**). Structure determination of these products was performed at the ICRM, Italy. The products were light yellow in colour.

4.2.6.1 TyrAc1, V (2.7 mg, 3.4 % i.y.): ^{1}H-NMR δ (ppm, MeOD): δ = 7.19 (2H, d, J = 9.0 Hz, H-3, H-5); 6.88 (4H, m, H_{ar}); 6.77 (1H, d, J = 1.5 Hz, H-14); 4.25 and 4.17 (2H each, d each, J = 7.0 Hz, CH_2-8 and CH_2-16); 2.90 and 2.80 (2H each, d each, J = 7.0 Hz, CH_2-7 and CH_2-15); 2.01 (3H, s, CH_3); 1.96 (3H, s, CH_3). m/z = 358.

4.2.6.2 TyrAc2, VI (10 mg, 12.6 % i.y.): ^{1}H-NMR δ (ppm, MeOD): δ = 7.23 (1H, dd, J_1 = 2.0 Hz, J_2 = 0.5 Hz, H-10); 7.08 (1H, dd, J_1 = 8.0 Hz, J_2 = 2.0 Hz, H-12); 6.73 (1H, d, J = 8.0 Hz, H-13); 6.65 (1H, dd, J_1 = 10.5 Hz, J_2 = 2.0 Hz, H-3); 5.97 (1H, dd, J_1 = 10.5 Hz, J_2 = 1.0 Hz, H-2); 5.00 (1H, m, H-5); 4.32 (1H, dt, J_1 = 11.5 Hz, J_2 = 6.5 Hz, H-15$_a$); 4.25 (2H, t, J = 7.0 Hz, CH_2-17); 4.24 (1H, dt, J_1 = 14.0 Hz, J_2 = 7.0 Hz, H-15$_b$); 3.01 (1H, dd, J_1 = 17.5 Hz, J_2 = 4.0 Hz, H-6$_a$); 2.96 (1H, dd, J_1 = 3.0 Hz, J_2 = 0.5 Hz, H-6$_b$); 2.91 (2H, t, J = 7.0 Hz, CH_2-14); 2.47 (1H, dt, J_1 = 15.0 Hz, J_2 = 6.5 Hz, H-16$_a$); 2.32 (1H, dt, J_1 = 15.0 Hz, J_2 = 7.0 Hz, H-16$_b$), 2.01 (6H, s, 2 CH_3). ^{13}C-NMR δ (ppm, MeOD): δ = 150.35 (C-3); 130.76 (C-12); 127.12 (C-2); 124.91 (C-10); 110.75 (C-13); 85.73 (C-5); 66.25 (C-15); 61.75 (C-17); 38.98 (C-6); 35.42 (C-14); 35.01 (C-16); 20.70 (2 CH_3). m/z = 358

4.2.7 Tyrosol-acetate-tyrosinase reactions

In a suitable glass vial, the following reaction was allowed to proceed at 30°C with mild shaking for 24 hours: 10 mg of compound **IV** in 1.2 ml chloroform was added to 5 mg tyrosinase in 1 ml 50 mM potassium phosphate buffer, pH 7. Optimisation of reaction conditions and analysis of products is still under investigation at the ICRM, Italy.

4.2.8 Totarol-laccase reaction

The following monophasic reaction was performed: 1 mg laccase from *Trametes versicolor* in 500 µl 20 mM sodium acetate buffer, pH 3.5, was added to 3 mg totarol (4b*S*-*trans*-8,8-Trimethyl-4b,5,6,7,8,8a,9,10-octahydro-1-isopropyl-phenanthren-2-ol; Aldrich) in 500 µl acetone, and allowed to proceed at 30°C with mild shaking for 24 hours. The products of this reaction were dark orange in colour and the structures of the products have been well-documented in the literature (Ncanana, *et al.*, 2007) and by the ICRM, Italy.

4.2.9 Enzyme activity assays

The activity of laccase from *Trametes versicolor* was assayed using 5.82 mg ABTS/ml of 20 mM sodium acetate buffer, pH 3.5, as substrate. 10 µl enzyme sample was added to 890 µl buffer and 100 µl ABTS (Fluka), and monitored for an increase in absorbance at 436 nm for 200 seconds using a UV-Vis spectrophotometer. Blanks were used containing no enzyme sample. ε_{436}(ABTS) = 36000 $M^{-1}cm^{-1}$.

The activity of tyrosinase was assayed using 0.05% *p*-cresol (Aldrich) in 50 mM potassium phosphate buffer, pH 7, as substrate. 100 µl of enzyme sample was added to 2.9 ml of this cresol-buffer solution, and an increase in absorbance was monitored at 400 nm for 10 minutes. Blanks were used containing no enzyme sample. ε_{400}(*p*-cresol) = 1433 $M^{-1}cm^{-1}$.

The activity of enzymes used in biocatalysis reactions was assayed before and during reactions. If the enzyme became denatured during the reaction, and thus could no longer convert substrate to product material, the reaction was either terminated (see section 4.2.2) or more active enzyme was added to the reaction mix and the reaction allowed to continue.

4.2.10. Biocatalytic reactions using the laccase from *Micromonospora* sp. strain 044 30-1

Laccase-catalysed oxidation reactions using tyrosol, tyrosol-acetate, or totarol, were performed as described in sections 4.2.3, 4.2.6, and 4.2.8, respectively, except that the laccase from *Micromonospora* sp. strain 044 30-1 was used instead of *Trametes versicolor* laccase. NMR and mass spectrometry was provided as a service by the Stellenbosch University, South Africa. Tyrosol, compound **I**, and totarol were identified using TOF ESI-MS in the positive mode, with a HP5 (30 m × 0.25 mm) column. The totarol dimers (Ncanana, 2007), and compounds **V** and **VI** were identified using LC-MS, in negative electrospray ionization mode, with a reverse phase Waters C18 (2.1 mm × 50 mm) column, using acetonitrile as the mobile phase, with a flow rate of 0.35 ml/min. NMR of products **I**, **V**, and **VI** was conducted in *d*-MeOD, and the dimers of totarol in *d*-$CDCl_3$, at 600 MHz.

Tyrosol or totarol (2 g/L) were added to flask cultures of strain 044 30-1 grown in the optimal medium, M172F (section 3.4), and incubated at 30°C with shaking at 160 rpm. After 7 days of incubation, samples were taken directly from the culture medium and spotted on TLC plates to assess the formation of reaction products. For the tyrosol-acetate-laccase reaction, 100 µl of a 100 mg/ml solution of compound **IV** was added to 500 µl of the crude extract containing the laccase from strain 044 30-1. Crude extract was defined as the supernatant fluid containing growth medium and the extracellular enzyme, and was dialysed to remove contaminating material prior to being used in biocatalysis reactions.

4.2.11 Laccase-catalysed reactions of 3-hydroxyanthranillic acid, 8-hydroxyquinoline, and ferulic acid

Solutions of 1 mM ferulic acid (FA; Fluka) and 1 mM 3-hydroxyanthranilic acid (3-HAA; Sigma Aldrich) were made in sterile water and stored in Sterilin tubes, wrapped in foil, and kept at 4°C until needed. In a Sterilin tube, 0.25 g 8-hydroxyquinoline (8-HQ; Aldrich) was dissolved in 27.5 ml 0.1 M sodium acetate buffer (Saarchem), pH 5, 2.5 ml acetone (Merck), and 1 ml glacial acetic acid (Saarchem) according to Ncanana (2007), wrapped in foil, and kept at 4°C until needed. 50 µl of enzyme sample was added to 150 µl of each solution in wells of microtitre plates (Lasec), which were then sealed to prevent air from entering the wells and possibly causing autooxidation of substrates (especially of 3-HAA), and incubated at room temperature, in the dark, overnight. A successful reaction with 3-HAA could be assessed visually, by observing a colour change from orange to red. Reactions were left overnight to ensure completion of the reaction. For each experiment, suitable control compounds and

1 U of commercial enzymes were used. Solutions of 8-HQ were also added to flask cultures and ALRs during the growth of strain 044 30-1. Samples were taken directly from the culture media and spotted on TLC plates. Approximately 7.5 µl of each reaction was spotted on silica TLC plates with aluminium backing (Merck) and developed in the preferred solvent system: 90 benzene : 25 dioxane : 4 acetic acid for visualization of ferulic acid reactions; 4 butanol : 2 acetic acid : 1 water for 3-HAA and 8-HQ reactions. Butanol, benzene, and dioxane were supplied by Saarchem. The appearance of compounds were visualised by UV or by the use of iodine vapours, which specifically detect organic compounds containing carbon-carbon double (C=C) bonds, resulting in spots appearing brown-purple.

4.3 Results and Discussion

4.3.1. Tyrosol reactions with laccase from *Trametes versicolor*

The laccase-catalysed reaction of tyrosol led to the formation of the major product, **I**, with a yield of 8.6%. This product has been reported previously in the literature only once and was obtained using different conditions (Vinciguerra, *et al.*, 1997). Compound **II** was observed on TLC plates but could not be isolated as the compound was an unstable intermediate and was converted to compound **I** (Fig. 4.5). Compound **I** was purified and isolated by means of column chromatography and its structure confirmed by NMR (^{1}H-NMR, ^{13}C-NMR, and COSY-NMR). The purity and structure of tyrosol was also confirmed by NMR. The reaction scheme in Figure 4.6 shows the formation of compound **I** as a result of the coupling of two tyrosol radicals initiated by laccase, followed by two intramolecular Michael additions (Navarra, *et al.*, 2010; *in press*).

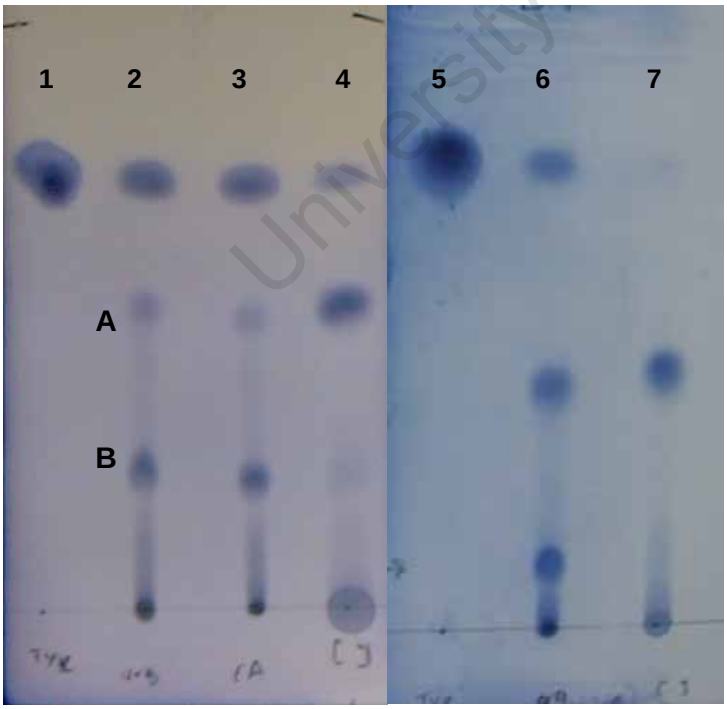


Fig. 4.5. The laccase-catalysed reaction of tyrosol. 1 and 5: Tyrosol starting material. 2, 3, and 6: Reaction products where 'A' indicates compound **I** and 'B' the unstable intermediate, **II**. 4: As the reaction progresses, compound **II** converts to product **I**. 7: Isolated product **I**.

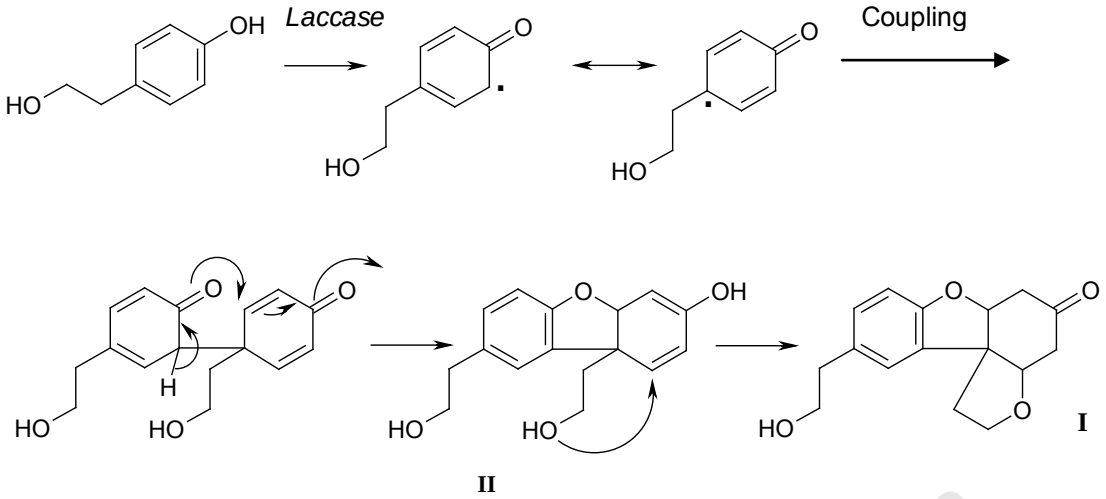


Fig. 4.6. The laccase-catalysed reaction of tyrosol brought about the formation of product **I**, a result of the coupling of laccase-catalysed radicals, forming a dimer. Compound **II** was the unstable intermediate that converts to product **I**.

The same compound (**I**) was reported by others when tyrosol was converted by laccase isolated from the fungus *Lentinus edodes*, giving the dimeric tetracyclic ketone, derived from a Pummerer's ketone-like intermediate (Vinciguerra, *et al.*, 1997). The alcohol corresponding to the above ketone was isolated after 7 days from whole cells of *Lentinus edodes* incubated with tyrosol. The formation of the alcohol may be attributed to an initial oxidation by the same laccase system that gave the ketone, followed by reduction by means of an oxidoreductase-type enzyme. Since this reduction step was not observed in the studies using isolated laccase preparation only, it appeared that the oxidoreductase enzyme was cell-associated (Vinciguerra, *et al.*, 1997). The differences between the current study and the study by Vinciguerra and co-workers can be summarised as follows: Laccase was isolated from the fungus *L. edodes*, and the reaction with tyrosol was allowed to proceed in buffer only at pH 5, and at 40°C, for 65 hours, and the yield of the dimeric ketone (identical to compound **I** by structure determination) was 6.0%. In the current study, the process to obtain this product was much faster, the temperature used was milder, and a higher yield of product was obtained: Commercial *Trametes versicolor* laccase (at a concentration of 1 mg/ml) and a biphasic reaction medium, incubated at 30°C, gave a product yield of 8.6% within 24 hours. In the study by Vinciguerra, *et al.* (1997) a different product (with a yield of 60%), the alcohol corresponding to the above ketone, was isolated from *L. edodes* culture medium after 7 days. The strain was grown in medium containing tyrosol and the product was recovered by extraction with ethyl acetate. The current study, describing the isolation of **I**, has been accepted for publication (Navarra, *et al.*, 2010; *in press*). The NMR data for product **I** were in agreement with that reported by Vinciguerra, *et al.* in 1997, and the NMR spectra are shown below in Figures 4.7 and 4.8.

Laccase-catalysed conversion of tyrosol resulted in the precipitation of a large quantity of yellow-orange product, hence the low yield of the product investigated in this study. All products examined in this study were soluble in the organic phase. The yellow-orange polymeric material was insoluble in water and organic solvents. Tyrosol had polymerised to form high molecular weight products, likely a mixture of polymeric compounds. These insoluble polymers were further researched by Ncanana (2007) but were not the focus of the present study. The polymeric material was easily removed by filtration, thus illustrating the potential use of laccase in the bioremediation of tyrosol.

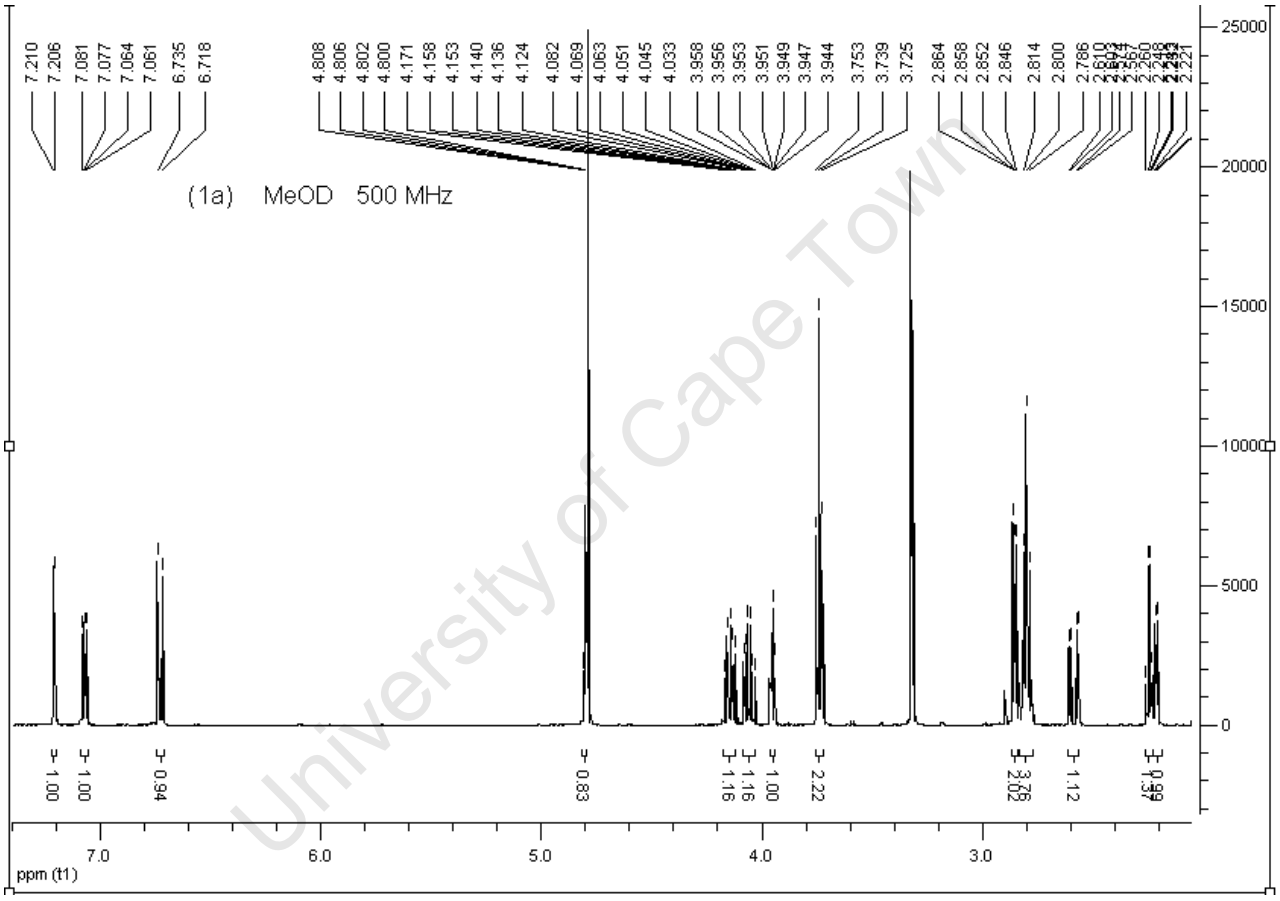


Fig. 4.7. ^{1}H-NMR spectrum of product **I**.

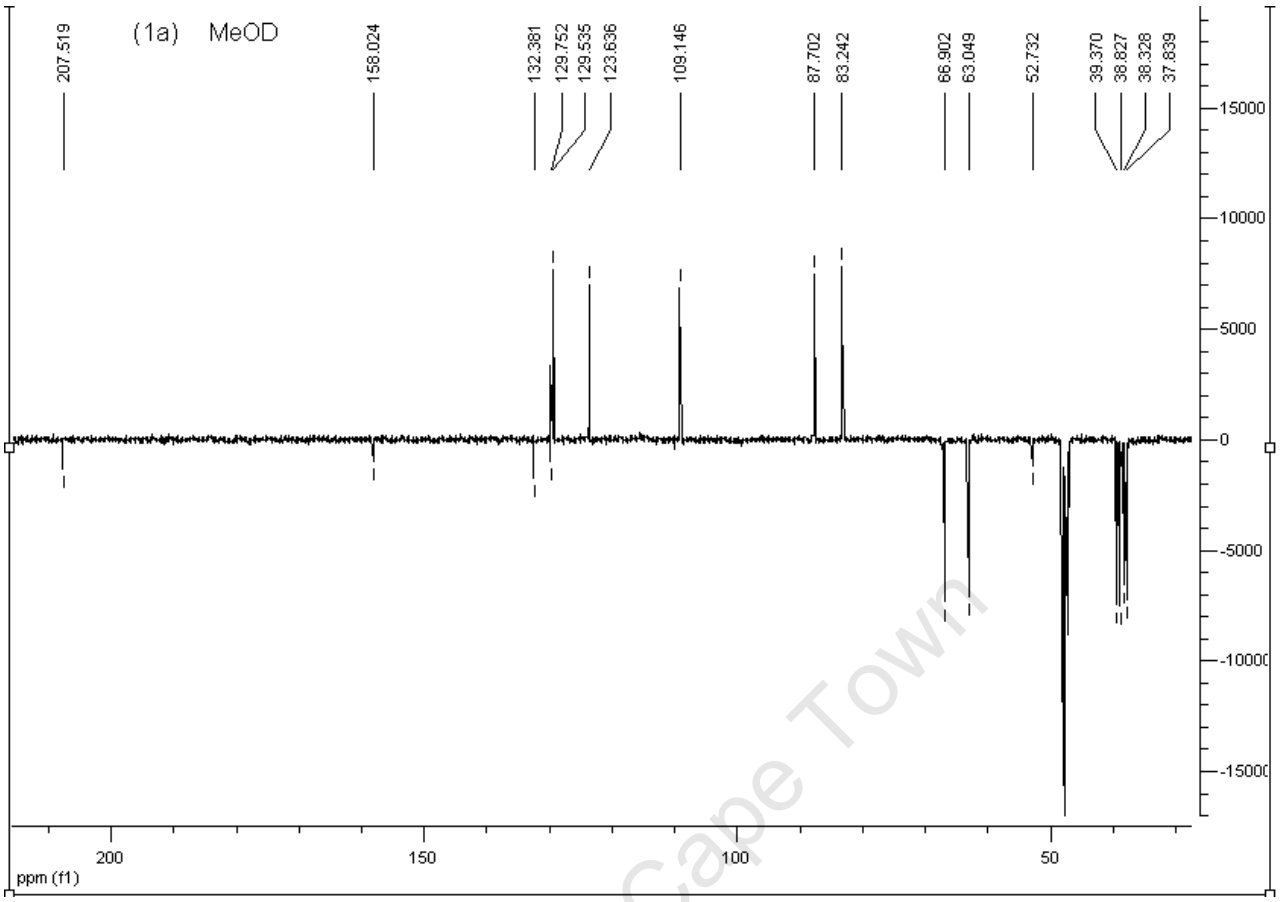


Fig. 4.8. ^{13}C-NMR spectrum of product **I**.

4.3.2 Tyrosol-tyrosinase reactions

This reaction was chosen in an effort to obtain hydroxytyrosol as documented in the literature (Kazandjian and Kilbanov, 1985; Ncanana, 2007). Hydroxytyrosol is scarcely available commercially and if it can be supplied it is extremely expensive thus researchers prefer to make hydroxytyrosol using various methods, including by means of enzymatic reactions (Espín, *et al.*, 2001; Sayadi, 2009). Methods documented in the literature (Kazandjian and Kilbanov, 1985; Ncanana, 2007) were performed in order to obtain hydroxytyrosol from tyrosol using tyrosinase as biocatalyst (Fig. 4.9). The hydroxytyrosol obtained from this reaction was to be used in reactions with laccase in order to investigate the structures of the consequent products of a hydroxytyrosol-laccase reaction and their antioxidant properties.

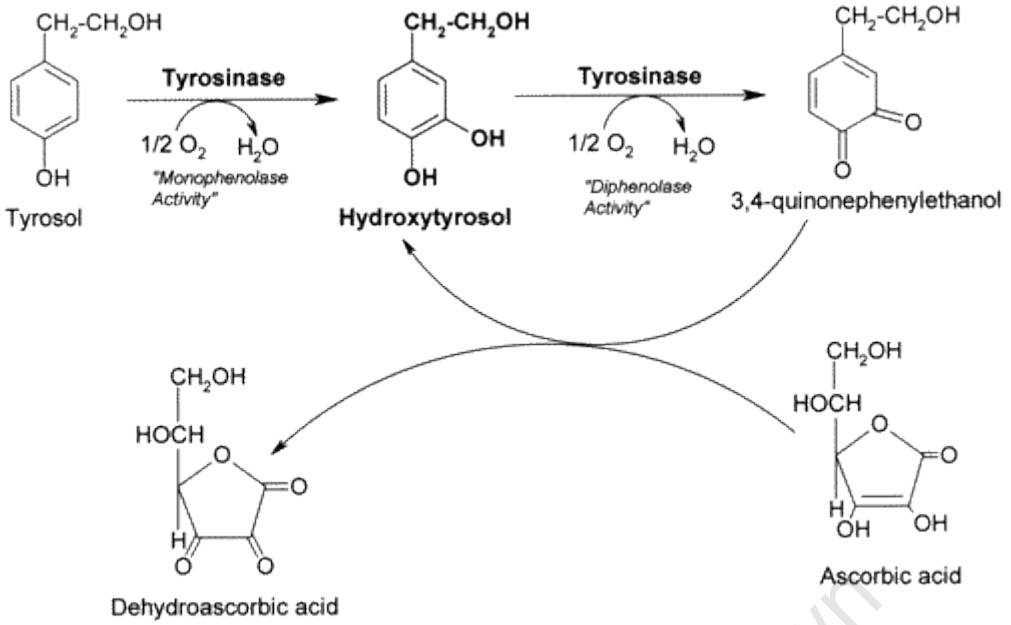


Fig. 4.9. Reaction scheme for obtaining hydroxytyrosol from tyrosol using tyrosinase as catalyst, and the role of ascorbic acid in obtaining hydroxytyrosol (from Espín, *et al.*, 2001).

Tyrosinase (monophenol, *o*-diphenol:oxygen oxido-reductase, EC 1.14.18.1) has been explored in terms of its biocatalytic potential to synthesise substituted 3,4-catechols, such as L-DOPA, the anti-Parkinson's disease drug, and other value-added products (Nolan and O'Connor, 2008). Tyrosinase is a BMCO and is ubiquitous in nature. Much of the information on the structure and function of tyrosinase has been obtained from studies of tyrosinase from mushroom species such as *Agaricus bisporus*, the actinomycete *Streptomyces antibioticus*, and the fungus *Neurospora crassa* (Rescigno, *et al.*, 1998). The biocatalytic potential of tyrosinase has received much attention as this enzyme has the advantage of being readily available, has a broad substrate range, can be used in organic media, and does not require the addition of cofactors (Nolan and O'Connor, 2008; Espín, *et al.*, 2001).

Hydroxytyrosol, a natural *ortho*-diphenolic AO with beneficial health properties, mainly occurs in virgin olive oil and olive oil mill waste waters, and can be enzymatically synthesized using mushroom tyrosinase (Espín, *et al.*, 2001). In the study by Espín and co-workers (2001), mushroom tyrosinase catalysed the *ortho*-hydroxylation of the monophenol tyrosol (monphenolase activity) to form the *o*-diphenol hydroxytyrosol, which was oxidised in a reaction also catalysed by tyrosinase (diphenolase activity) to form the corresponding *o*-quinone (3,4-quinonephenylethanol) (Fig. 4.9). The oxidation of the catechol product to *o*-quinones by the diphenolase activity of tyrosinase is adverse. The *o*-quinone is highly unstable and reacts nonenzymatically to yield brown pigments. This oxidation step can be prevented by the addition of reducing agents, such as ascorbic acid, resulting in the accumulation of the desired catechol, although, such measures do increase the overall cost of the process (Nolan and O'Connor, 2008). Hydroxytyrosol accumulates as long as ascorbic acid is present in the medium (Brooks, *et al.*, 2006).

Although, in the current study, mushroom tyrosinase from Sigma was also used, hydroxytyrosol was not obtained, in contrast to the results obtained by Espín and co-workers (2001). The reaction medium used by Espín and co-workers contained only 0.1 M sodium phosphate buffer, whereas the most efficient reaction medium used in the current study to obtain products was a biphasic system consisting of potassium phosphate buffer and chloroform (section 4.2.4). Instead, a new product, **III**, was obtained with a yield of 14% (Fig. 4.10). This product was isolated by means of column chromatography and its structure was determined by NMR. Compound **III** was observed to be produced in reactions with or without ascorbic acid in the reaction mixture.

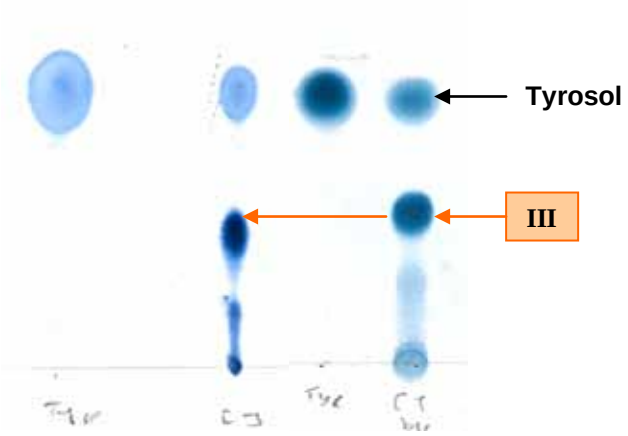


Fig. 4.10. TLC plate of the tyrosol-tyrosinase reaction showing the major product of the reaction, **III**.

The reason for obtaining compound **III**, as opposed to hydroxytyrosol, is unknown but a reaction scheme depicting what was thought to have occurred is proposed in Figure 4.11. It was suggested that the major contributing factor to the formation of compound **III** was that the side chain of the tyrosol molecule formed a second ring structure. To the best of the author's knowledge, having consulted the literature and chemical databases, this is the first account of compound **III**, a novel compound. The biological activity of compound **III**, a molecule possessing two hydroxyl groups, was investigated in Chapter 5 where its antioxidant activity was compared with that of tyrosol.

In an effort to further understand the mechanism of this reaction and to provide an answer for why hydroxytyrosol was not obtained, it was theorised that replacing the hydroxyl group on the side chain of the tyrosol molecule with an acetate molecule, may prevent the formation of a ring structure. This work is discussed in the next section.

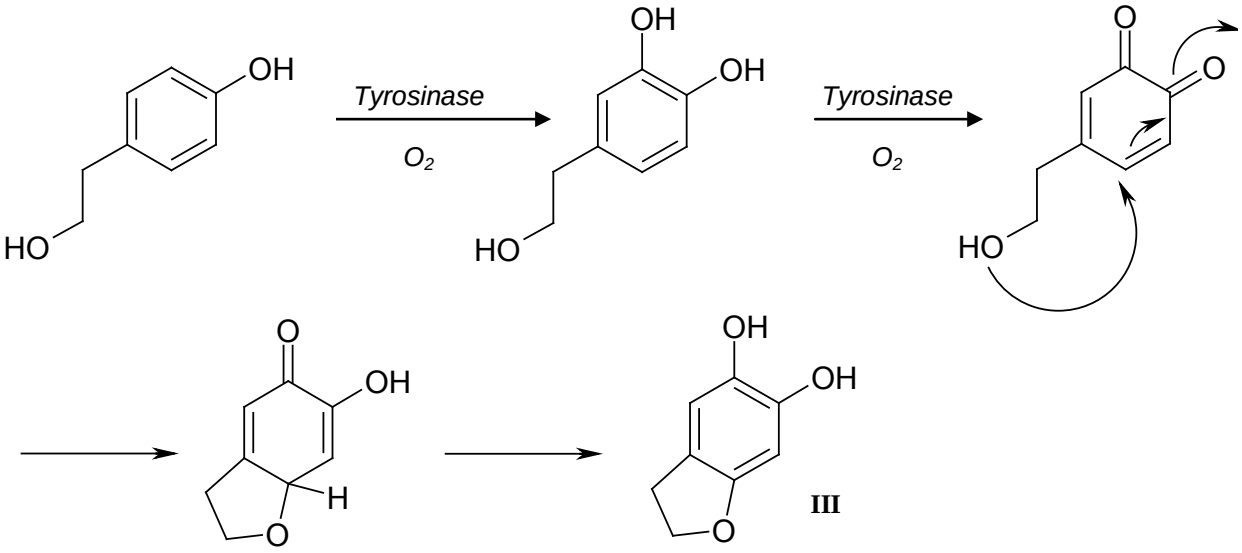


Fig. 4.11. Reaction scheme depicting the formation of product **III** as a result of the tyrosol-tyrosinase reaction.

4.3.3 Tyrosol-lipase reaction to produce tyrosol-acetate (IV)

Lipase, immobilised on beads, submerged in vinyl acetate, was reacted with tyrosol to produce compound **IV** (Fig. 4.12) with a yield of 86.4%. A contaminant was also produced with a yield of 10.7% (Fig. 4.13). Column chromatography was performed to remove the contaminant. The structure of compound **IV** was confirmed by NMR. Lipase selectively removed the hydroxyl group on the chain of the tyrosol molecule and replaced it with an acetate group. This change of functional group was expected to prevent the chain of the molecule from forming ring structures, as seen with the tyrosol molecule, when reacted with laccase and tyrosinase. The proton NMR spectrum of compound **IV** is shown in Figure 4.14.

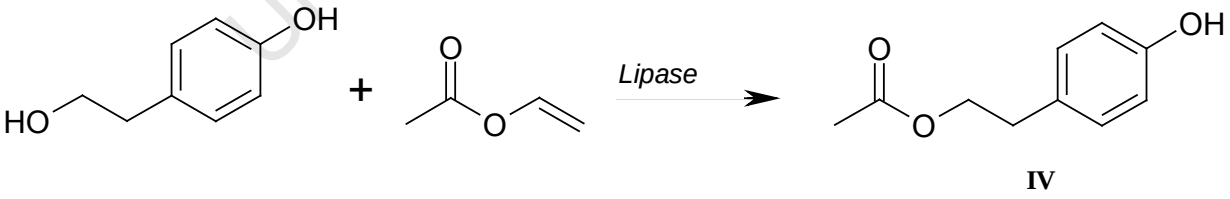


Fig. 4.12. Conversion of tyrosol to compound **IV** using lipase and vinyl acetate.

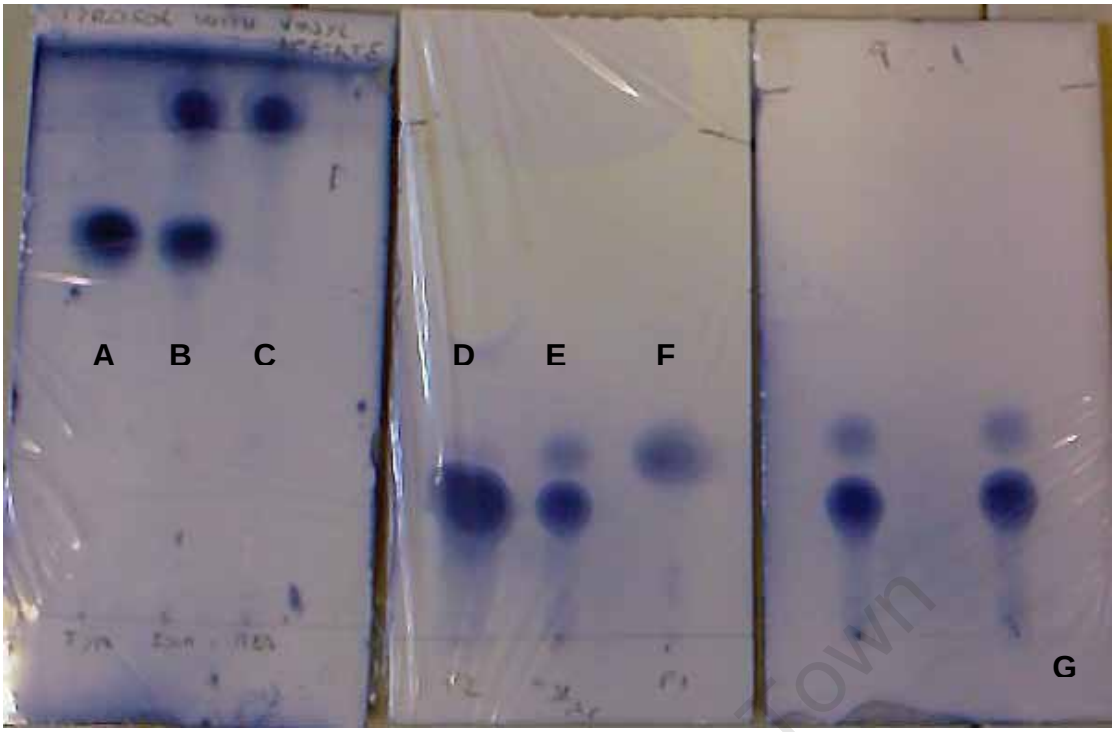


Fig. 4.13. TLC plates showing the results of the conversion of tyrosol to compound **IV** by a lipase-catalysed reaction. A small amount of contaminating material had to be separated by column chromatography in order to obtain pure compound **IV**. A. Tyrosol (starting material). B. Tyrosol and product **IV**. C. **IV**, the major product of the reaction. D. Pure product **IV** after column chromatography. E. Compound **IV** and a contaminant (before column chromatography). F. The isolated contaminating material (after column chromatography). G. **IV**, the major product (bottom, dark spot) and the contaminant (top, lighter spot).

A non-commercial immobilised lipase from the bacterium *Staphylococcus xylosus* was used to catalyse the transesterification of tyrosol in the presence of ethyl acetate to form mono- and di-acetylated tyrosol derivatives (Aissa, *et al.*, 2007). In the current study, commercial lipase from the fungus *Candida antarctica*, immobilised on beads in vinyl acetate as the reaction medium, was used to synthesise monoacetylated tyrosol (**IV**) as the major product. Tyrosol and its monoacetylated derivative showed similar antiradical activity with DPPH (2,2-diphenyl-1-picrylhydrazyle), thus the radical scavenging activity of tyrosol is not notably influenced by the presence of an acyl group (Aissa, *et al.*, 2007). Lipophilic derivatives of phenols with AO activity obtained *via* esterification of the hydroxyl groups with aliphatic molecules can be used to increase lipophilicity and therefore improve their intestinal absorption and cell permeability *in vivo*. Furthermore, this lipophilic analogue of tyrosol could be used as a food AO in oil-based formulae or cosmetic fields (Aissa, *et al.*, 2007). The biological activities of compound **IV** are investigated in Chapter 5.

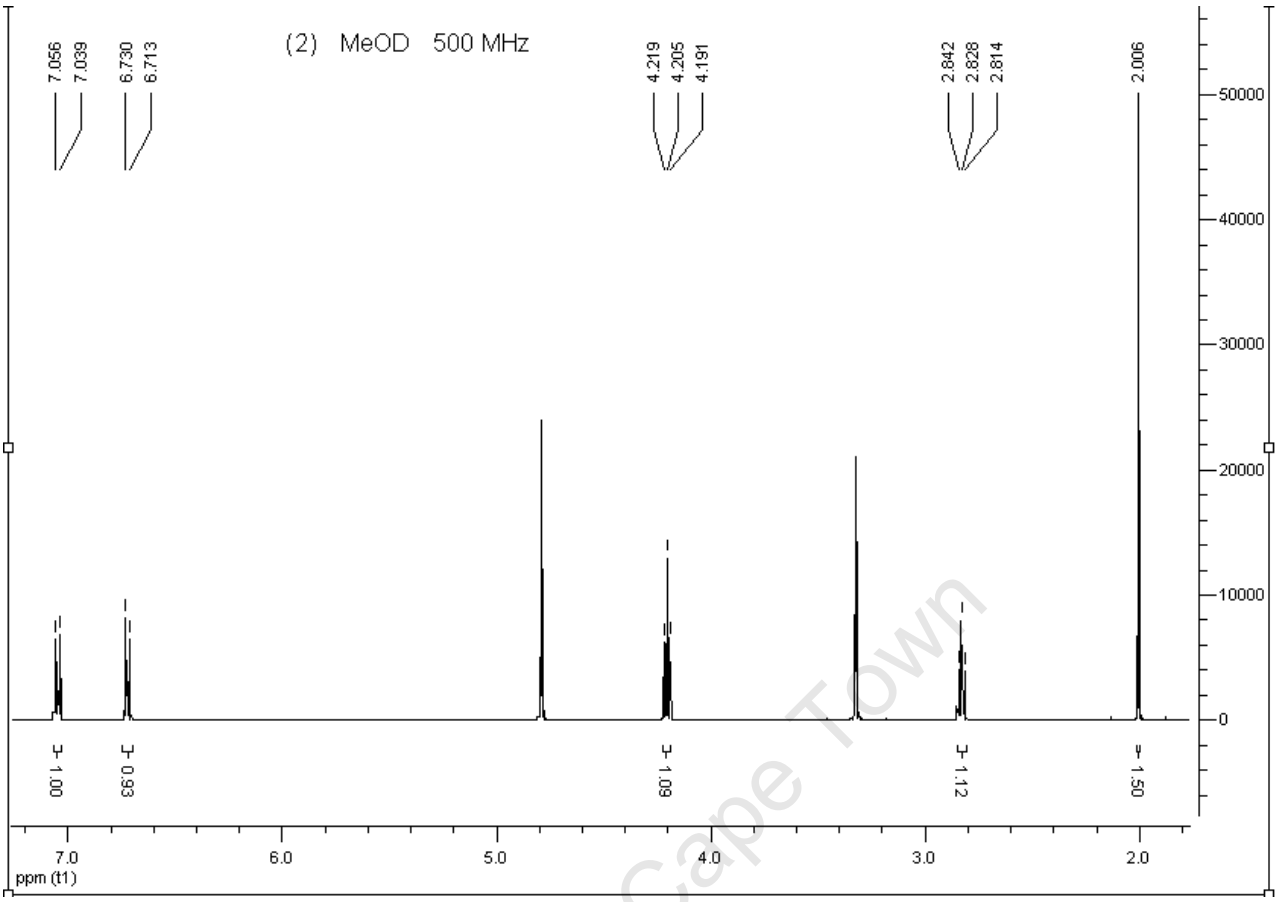


Fig. 4.14. ^{1}H-NMR spectrum of product **IV**.

4.3.4 Tyrosol-acetate reactions with laccase from *Trametes versicolor*

Laccase successfully reacted with tyrosol-acetate to produce two products, **V** and **VI** (Fig. 4.15). These products were separated and isolated by column chromatography. Product **V** (the least polar) was isolated with a yield of 3.4%, while product **VI** was isolated with a yield of 12.6% (Fig. 4.16). The structures were determined at the ICRM, Italy, by analysis of NMR spectra. Mechanisms of reaction describing the formation of products **V** and **VI** due to laccase action are shown in Figures 4.17 and 4.18 respectively, as well as the proton NMR spectra for each in Figures 4.19 and 4.20, respectively. **V**, the minor product, was easily identified as the C-O dimer by mass spectrometry and its ^{1}H-NMR spectrum. **VI**, the major product, displayed the following properties: The presence of an olefinic double bond was clearly shown by the two doublets at 6.65 and 5.97 ppm (J = 10.5 Hz), while the other signals between 7.2 and 6.7 ppm were diagnostic for a three-substituted aromatic ring. COSY and HMQC analysis allowed for the complete assignments of all the other signals of the ^{1}H-NMR and ^{13}C-NMR spectra to the corresponding proton and carbon atoms, thus confirming the proposed structure of product **VI**. This is the first account of a laccase-catalysed reaction using compound **IV** as substrate, and the structures of products **V** and **VI** have not previously been described. Furthermore, this work has been accepted for publication (Navarra, *et al.*, 2010; *in press*). The biological activities of these compounds are investigated in Chapter 5. The addition of the acetate group, replacing the hydroxyl group, on the side chain of the tyrosol molecule did prove to prevent the additional ring structure from forming.

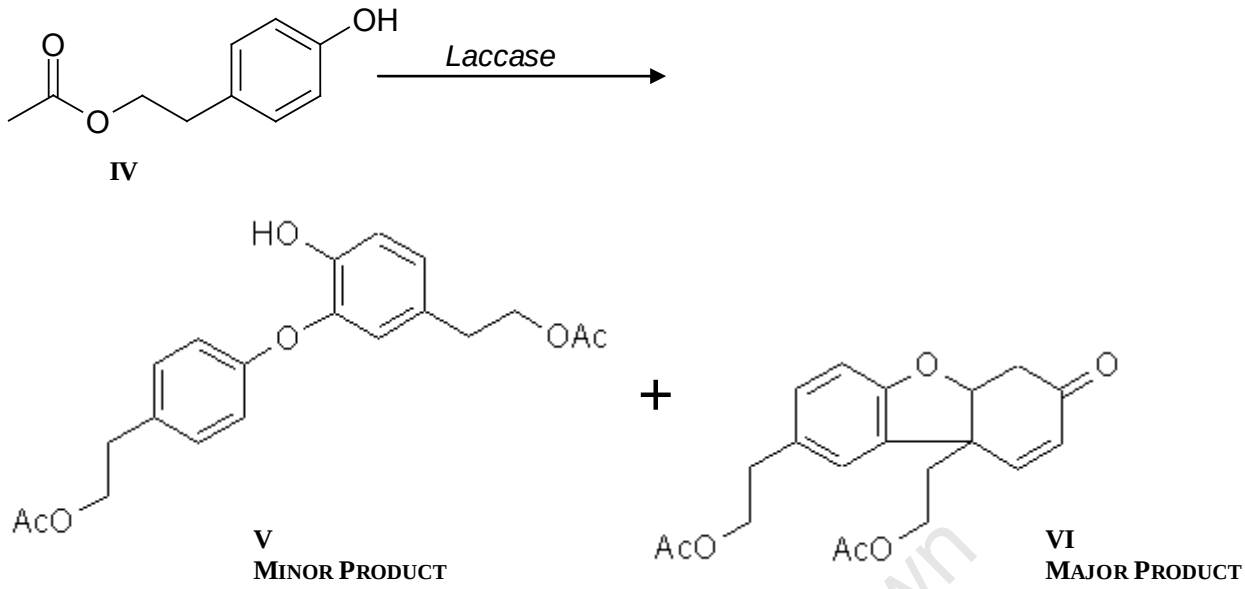


Fig. 4.15. Laccase was reacted with compound **IV** to produce two products, **V** and **VI**.

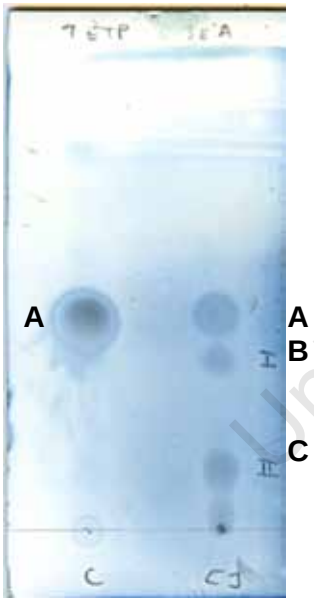


Fig. 4.16. Laccase-catalysed reactions of compound **IV** resulted in the formation of two products: **V** (B) and **VI** (C). Compound **IV** starting material is indicated as 'A'.

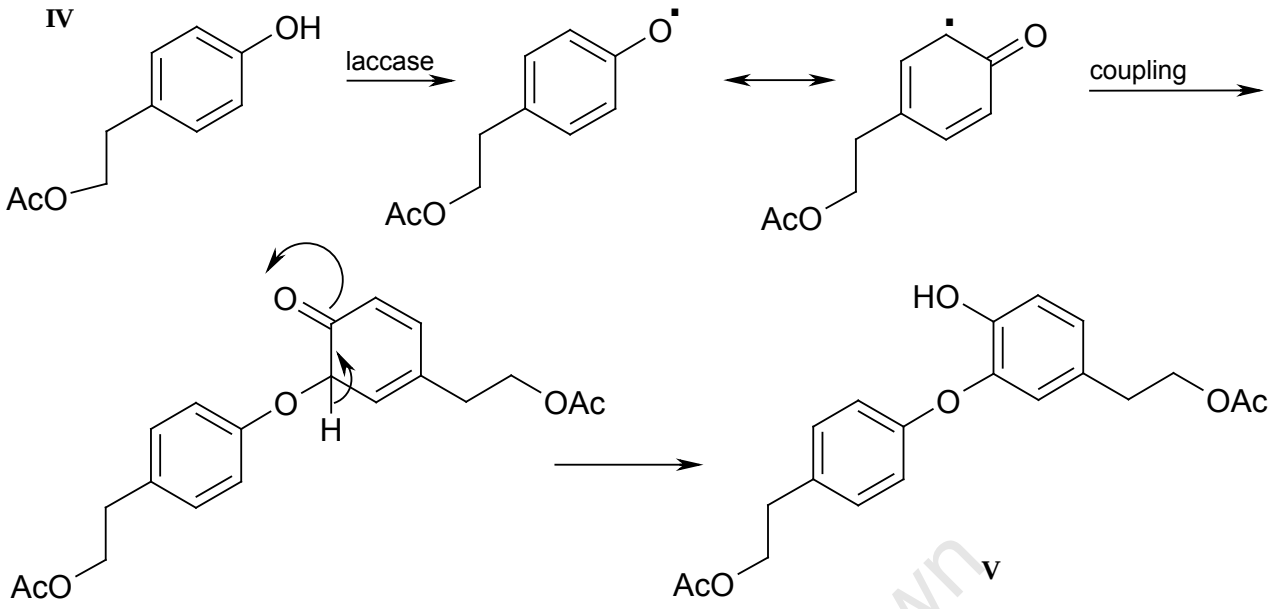


Fig. 4.17. Reaction scheme depicting the formation of product **V**, as a result of the coupling of laccase-catalysed radicals.

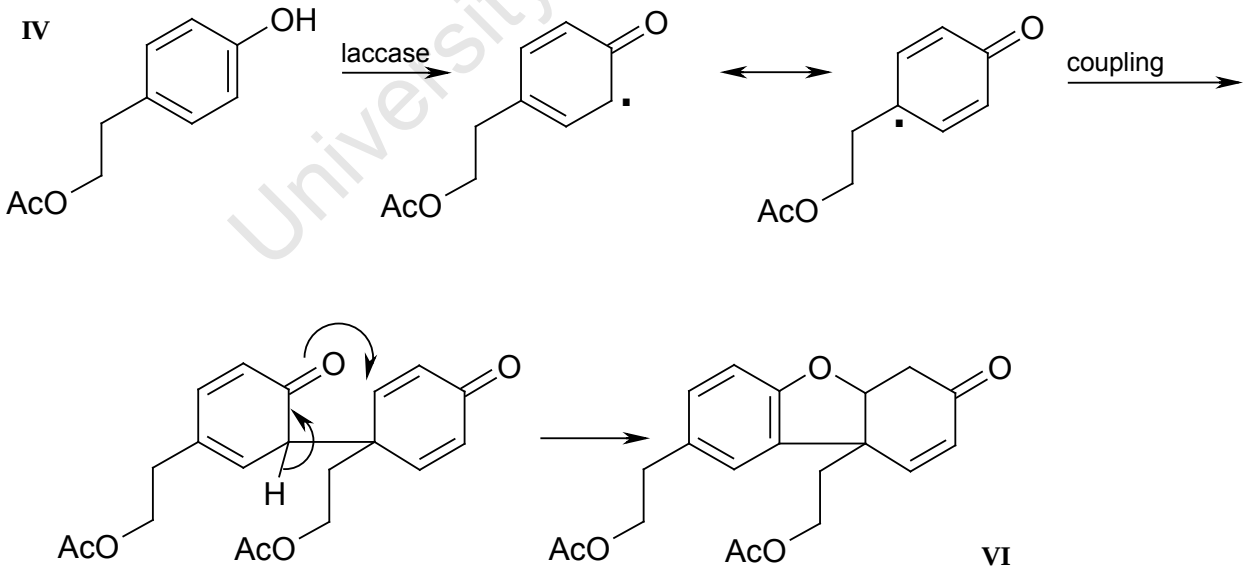


Fig. 4.18. The laccase-catalysed reaction of compound **IV** brought about the formation of product **VI**, a result of the coupling of laccase-catalysed radicals.

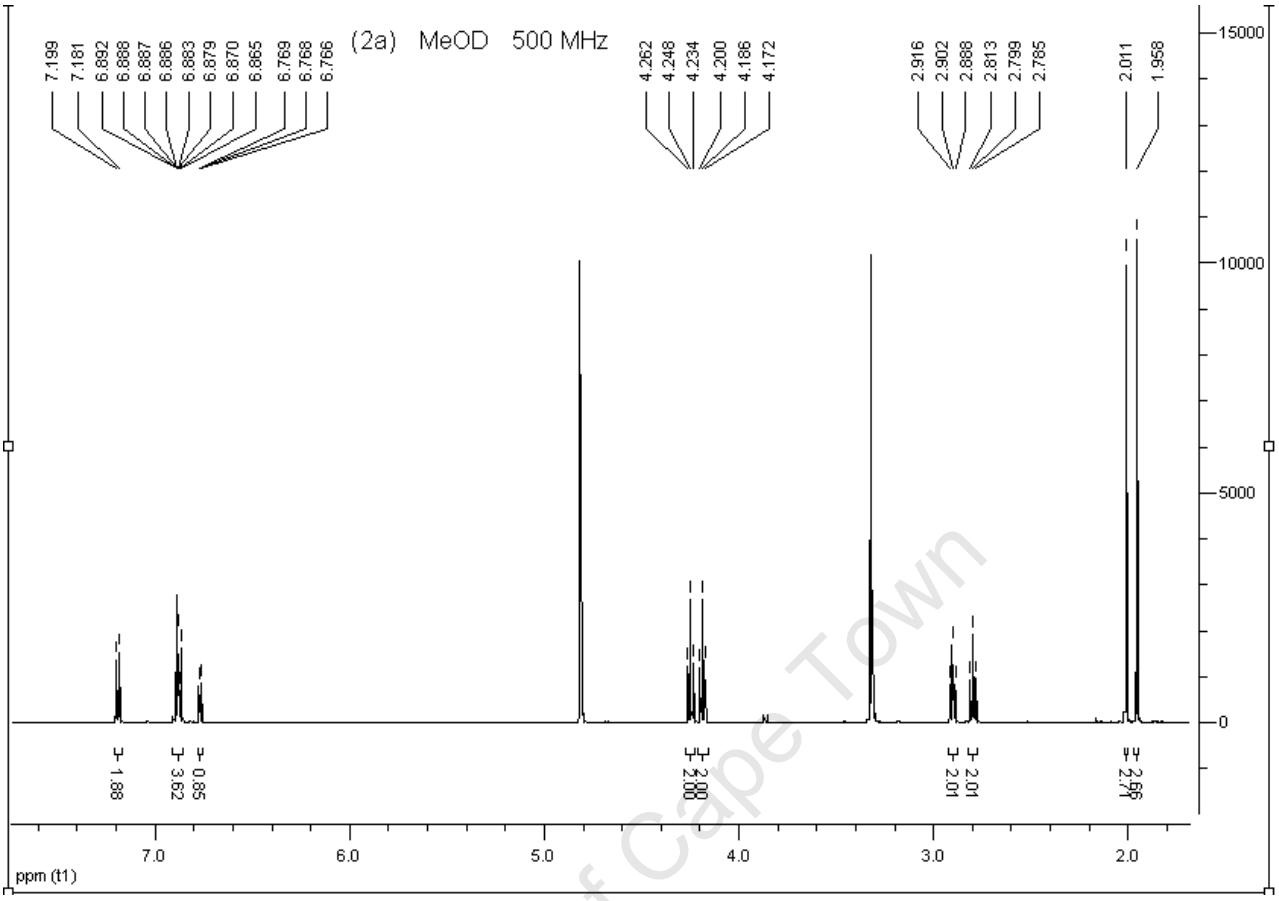


Fig. 4.19. ^{1}H-NMR spectrum of product **V**.

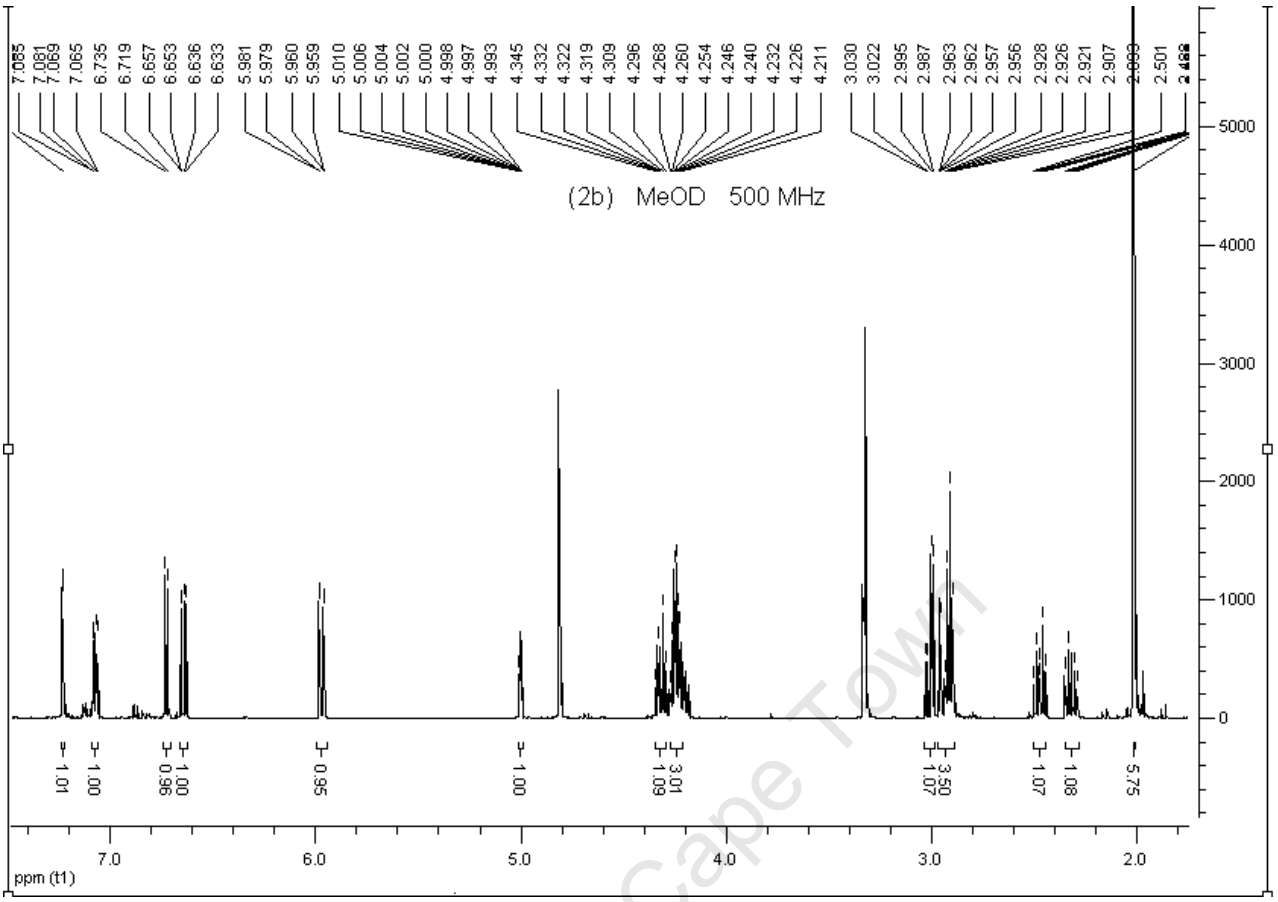


Fig. 4.20. ^{1}H-NMR spectrum of product **VI**.

4.3.5 Tyrosol-acetate-tyrosinase reactions

Compound **IV** was reacted with tyrosinase (Fig. 4.21) in reaction vials, with and without ascorbic acid. The aim was to obtain a product with hydroxyl groups on positions 3 and 4 of the ring, thus resembling hydroxytyrosol. The acetate molecule would later be removed and replaced with a hydroxyl group, thereby obtaining hydroxytyrosol.

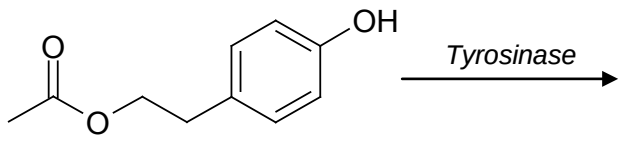


Fig. 4.21. **IV** was reacted with tyrosinase.

The presence of ascorbic acid in reactions appeared to adversely affect the yield of any products formed and was thus was not added to subsequent reactions. Various reaction products were observed by TLC in both the organic and aqueous phases (Fig. 4.22). Isolation of products and analysis of structures is ongoing at the ICRM, Italy.

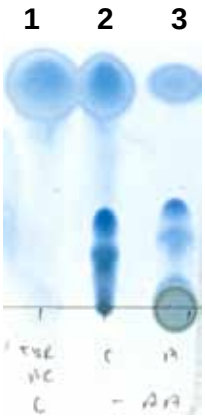


Fig. 4.22. Products formed as a result of the tyrosol-acetate-tyrosinase reaction (without ascorbic acid). 1. Compound **IV** (unreacted). 2. Products of the organic phase (chloroform). 3. Products of the aqueous phase.

4.3.6 Totarol reactions with laccase from *Trametes versicolor*

The laccase-catalysed reaction of totarol results in the formation of two dimeric products that can be observed by TLC (Fig. 4.23). These are the well-characterised C-C and C-O dimers (Ncanana, *et al.*, 2007). The accumulation of these products is due to the fact that, in contrast to other dimers (such as those obtained with the laccase-tyrosol reaction), they are "dead-end" products, which cannot be further oxidised by the enzyme (Ncanana, 2007; Navarra, *et al.*, 2010).

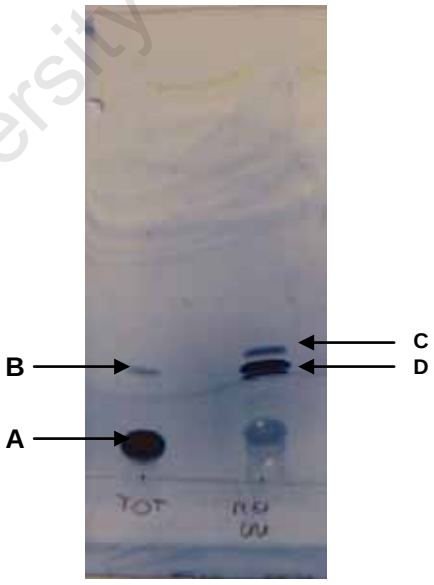


Fig. 4.23 The laccase-catalysed reaction of totarol results in the formation of two dimeric products, the major product a C-C dimer (D), and the minor product a C-O dimer (C). Unreacted totarol is indicated as 'A' and an unidentified contaminant as 'B'.

4.3.7 Biocatalytic capability of the laccase from *Micromonospora* sp. strain 044 30-1

The extracellular laccase excreted by actinomycete strain 044 30-1 into its growth medium was used as a biocatalyst in reactions using tyrosol, tyrosol-acetate (**IV**), or totarol as substrates. The laccase-catalysed dimerization of radicals is predicted to occur due to the same mechanism by all laccases, and hence it was expected that the products of these reactions, using strain 044 30-1 laccase, would be identical to those obtained using *Trametes versicolor* (fungal) laccase, as reported earlier in this chapter.

Substrates to be transformed by strain 044 30-1 laccase were added to the culture medium as this method had proved successful in the studies described by Allouche and co-workers (2004) and by Vinciguerra and co-workers (1997). In the current study, samples of the medium were taken and spotted on TLC plates to detect the formation of products. This was successfully applied for the formation of the totarol dimers, product **I** from tyrosol (section 4.2.10) and for the observation of polymers of 8-HQ and 3-HAA catalysed by strain 044 30-1 laccase (section 4.2.11).

Since totarol and tyrosol were both transformed to the respective products when supplemented to the growth medium while strain 044 30-1 was growing in shake flasks, one could not be certain whether the cells, the extracellular enzyme, or both, were responsible for this transformation. Thus, the extracellular enzyme was examined using the crude extract, and the cells were also examined after separating them from the culture fluid, washing them with water, and resuspending them in buffer.

In a preliminary experiment, samples were assessed for laccase activity before their use in biocatalysis reactions. Cells (100 mg/ml) and crude extract, obtained from the same culture, had laccase-DMP activities of 0.57 U/ml and 2.28 U/ml, respectively. The cells were most likely still secreting laccase, even after separation from the culture fluid and after the washing steps. It was interesting to note that the coloured oxidation products of both DMP and 3-HAA adsorbed to the cells showing laccase activity. Cells that had been separated from the culture fluid, washed, and left resting at room temperature for 7 days (called 'old' cells) were still active and the coloured oxidation products of the assay substrates adsorbed to the cells. The laccase activity of the 'old' cells (100 mg/ml) was 0.34 and 0.98 U/ml with DMP and 3-HAA, respectively. 'Old' cells showed conversion of tyrosol and totarol to the respective products (observed by TLC).

Strain 044 30-1 laccase could be used for these biocatalysis reactions under mild conditions. While *Trametes* laccase was used at pH 3.5 to catalyse these reactions, strain 044 30-1 laccase was used at pH 6. However, the biocatalysis reactions with strain 044 30-1 laccase progressed slowly (7 days) compared to *Trametes* laccase (1 day). Optimisation of the reaction conditions should be pursued in the future so that biocatalysis reactions and conversion efficiencies using the laccase from strain 044 30-1 would be improved.

Table 4.3. The productivity of the biocatalysis reactions using the laccases from the fungus *Trametes versicolor* and the actinomycete bacterium *Micromonospora* sp. strain 044 30-1.

Source of Laccase:	*Trametes versicolor*	044 30-1 Crude Extract	044 30-1 Cells	*Trametes versicolor*	044 30-1 Crude Extract	044 30-1 Cells	*Trametes versicolor*	044 30-1 Crude Extract	044 30-1 Cells
Laccase-DMP Activity (in U/ml):	0.47	0.68	0.23	0.47	0.68	0.23	0.47	0.68	0.23
Wet Weight of Cells (in g/ml):	NA	NA	0.11	NA	NA	0.11	NA	NA	0.11
Reaction Mixture and Conditions:	50 ml aqueous solution containing laccase added to 500 mg **tyrosol** dissolved in 50 ml ethyl acetate. Reaction allowed to proceed at 30°C with mild shaking.			25 ml aqueous solution containing laccase added to 150 mg **totarol** dissolved in 25 ml acetone. Reaction allowed to proceed at 30°C with mild shaking.			1 ml aqueous solution containing laccase added to 10 mg **tyrosol-acetate** dissolved in 1 ml ethyl acetate. Reaction allowed to proceed at 30°C with mild shaking.		
Product Yield (in mg and %):	153.8 mg; 30.8%	111.8 mg; 22.4%	136.0 mg; 25.2%	62.2 mg; 41.5%	9.2 mg; 6.1%	7.33 mg; 4.9%	6.57 mg; 65.7%	4.9 mg; 49.0%	1.0 mg; 10.0%
Time for product formation (in days):	1	7	7	1	7	7	1	7	7
Units of Enzyme:	23.5	33.4	11.4	11.8	16.7	5.7	0.47	0.67	0.23
Conversion Efficiency (mg product/U enzyme):	6.54	3.35	11.93	5.29	0.55	1.29	13.98	7.34	4.39

NA, not applicable.

Under the same large-scale reaction conditions, *Trametes* laccase showed better product conversion (in mg product/U enzyme) than strain 044 30-1 laccase (Table 4.3). Notably, the cells of strain 044 30-1 converted tyrosol to product **I** with higher efficiency than the extracellular enzymes.

4.3.7.1 Conversion of tyrosol by strain 044 30-1 laccase

The small-scale tyrosol reaction was performed as described in Appendix section A2, except that 1 ml of cells resuspended in 0.1 M sodium acetate buffer, or 1 ml of crude extract containing strain 044 30-1 laccase was used. Crude extract was the supernatant fluid consisting of growth medium and the extracellular enzyme, dialysed to remove contaminating material prior to being used in biocatalysis reactions. The crude extract converted tyrosol to product **I** (detected in the organic phase), as did the cells.

Samples from the experiment using the laccase produced in strain 044 30-1 growth medium supplemented with tyrosol (section 4.2.10) were spotted on TLC, developed with the desired solvent system, and visualised using iodine vapours. Results showed that strain 044 30-1 laccase catalysed the conversion of tyrosol to product **I**, the same product as seen for reactions using *Trametes versicolor* laccase. Unconverted tyrosol and the unstable intermediate (**II**) were also detected by TLC. The presence of product **I** in ethyl acetate extracts was further confirmed by mass spectrometry and NMR (Fig. 4.24 A, B; section 4.2.3.1).

4.3.7.2 Conversion of tyrosol-acetate (IV) by strain 044 30-1 laccase

Compound **IV** was converted to the novel products **V** and **VI** by the crude extract containing strain 044 30-1 laccase (section 4.2.10). The appearance of the products was confirmed by TLC observations and by the use of iodine vapours. The presence of products **V** and **VI** in ethyl acetate extracts was confirmed by TLC and by mass spectrometry and NMR (Fig. 4.24 C; section 4.2.6). No other experiments were performed due to the limited availability of **IV**.

4.3.7.3 Conversion of totarol by strain 044 30-1 laccase

The small-scale totarol reaction was performed as described in section 4.2.8, except that 0.5 ml of cells resuspended in buffer, or 0.5 ml of crude extract containing strain 044 30-1 laccase was used. The crude extract converted totarol to the dimeric products, as observed by TLC, while the cells did not. The dimers were detected in the growth medium of strain 044 30-1, supplemented with totarol (section 4.2.10), after 7 days of incubation.

Samples spotted on TLC, developed with the desired solvent system, and visualised using Pancaldi or iodine vapours, showed that strain 044 30-1 laccase catalysed the conversion of totarol to form the C-C dimer (major dimer) and the C-O dimer, as seen for reactions using *Trametes versicolor* laccase. The C-C dimer was produced in a larger quantity than the C-O dimer and no contaminant was visible (as shown in Fig. 4.23 above). The presence of both dimers was confirmed by mass spectrometry (Fig. 4.25 A, B). NMR data was in agreement with that obtained by Ncanana (2007).

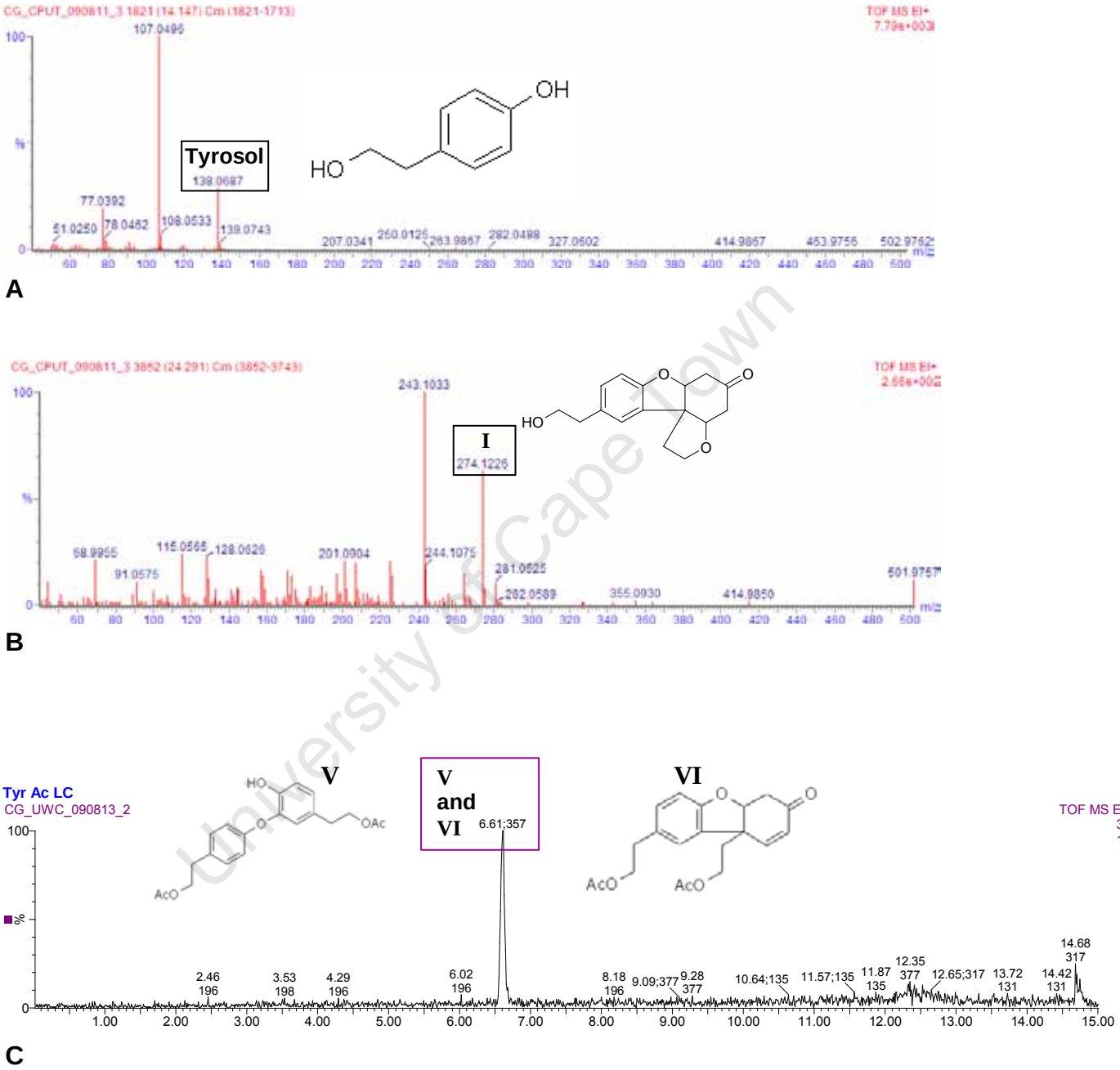
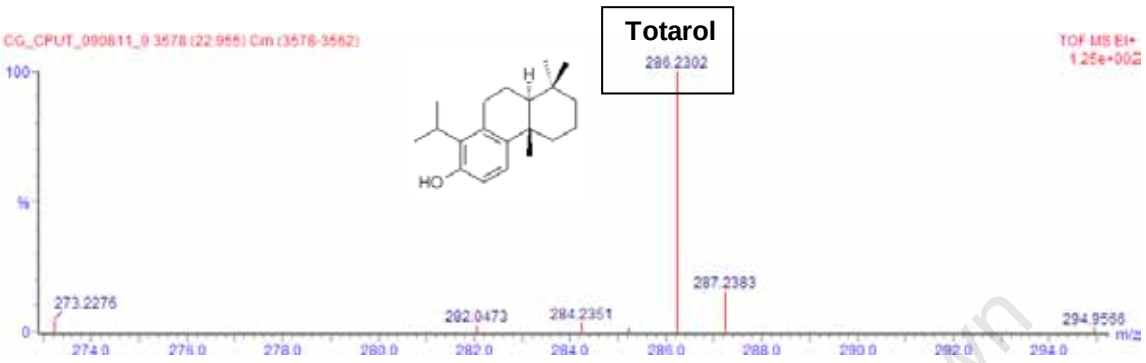
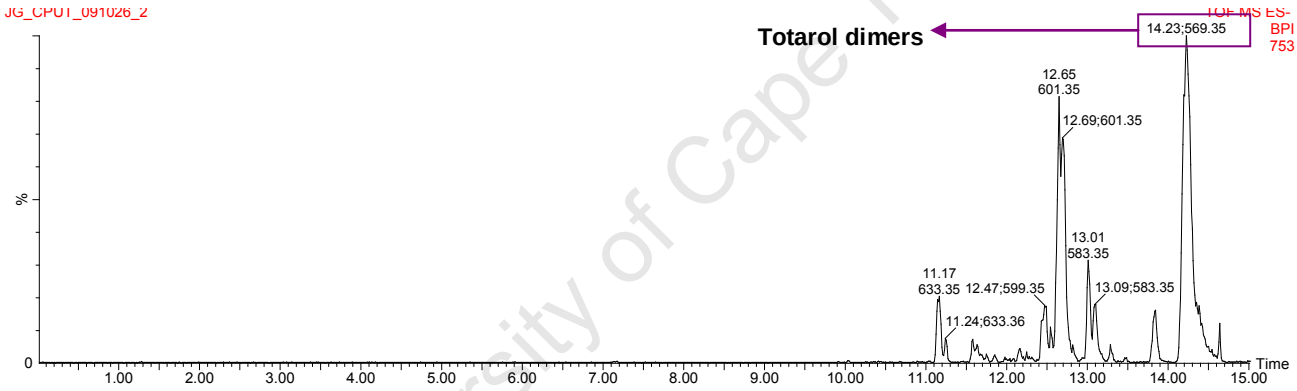


Fig. 4.24. Mass spectra indicating the products formed through biocatalysis of tyrosol and monoacetylated tyrosol using strain 044 30-1 laccase. A. Detection of tyrosol. B. Detection of product **I**. C. Detection of the dimeric products, **V** and **VI**.



A



B

Fig. 4.25. Mass spectra indicating the products formed through biocatalysis of totarol using strain 044 30-1 laccase. A. Detection of totarol. B. Detection of the dimeric products of the totarol-laccase reaction.

4.3.7.4 Conversion of 3-HAA by strain 044 30-1 laccase

Figure 4.26 shows that strain 044 30-1 laccase converted 3-HAA to CA but also produced products that were not formed by the fungal laccases examined. These compounds, marked by X in the figure, are possibly aromatic intermediates of the laccase-mediated 3-HAA oxidation. Such aromatic compounds have been resolved by HPLC but remain unidentified (Eggert, *et al.*, 1995). The orange-red reaction solution can contain a complex mixture of 450 nm-absorbing compounds, one of which is CA (Rescigno, *et al.*, 1998). Since the starting substrate, 3-HAA, has excellent antioxidant capabilities, it may be possible that product X is a polymeric compound with greater antioxidant efficiency, as polymeric derivatives of laccase-catalysed reactions with phenolic compounds often possess enhanced AO capabilities (section 1.13). It would be of interest to determine the structure of product X. Furthermore, it is of great interest that the fungal laccases do not produce this product. It is not known at this time whether or not strain 044 30-1 laccase is the only laccase to produce this product. It can also be seen that enzyme samples obtained from growth of strain 044 30-1 under optimised conditions in an ALR (C in the figure) completely converted 3-HAA whereas enzyme samples obtained from the stressful environment of a STR (D in the figure) do not complete the conversion of 3-HAA.

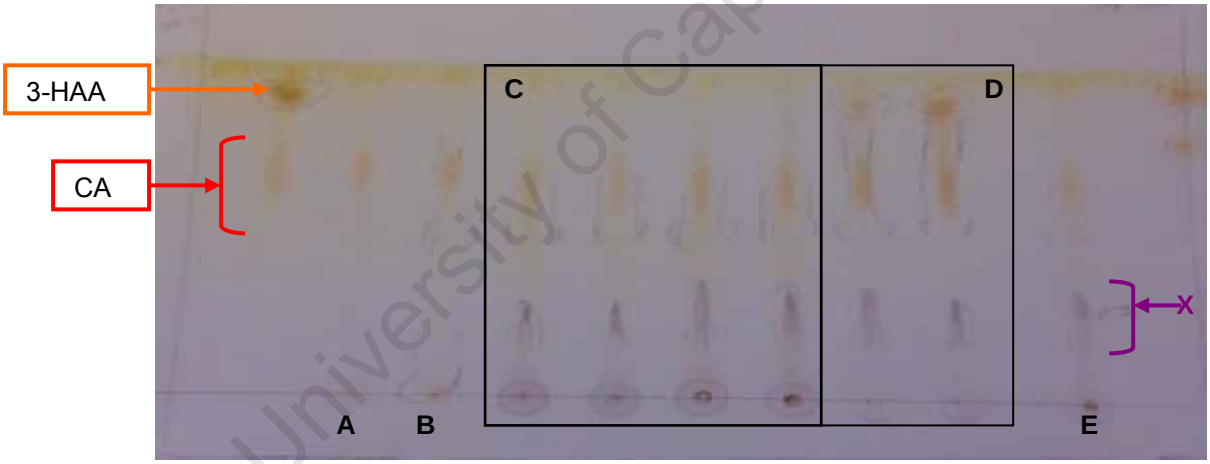


Fig. 4.26. TLC plate showing the outcome of laccase-catalysed reactions of 3-HAA. The migration of 3-HAA and CA are indicated. A. Commercial laccase from *Coriolus* (*Trametes*) *versicolor*. B. *Trametes pubescens* laccase. C. Strain 044 30-1 laccase obtained from ALRs. D. Strain 044 30-1 laccase obtained from STRs. E. Partially purified laccase from strain 044 30-1. X indicates unknown product(s) produced by strain 044 30-1.

4.3.7.5 Conversion of 8-HQ by strain 044 30-1 laccase

During growth of strain 044 30-1 in flask cultures and in ALRs containing 8-HQ as an inducer of laccase production (see 4.2.11 and Fig. 4.27), a colour change was observed in the medium after 48 hours. The yellow 8-HQ solution took on a blackish colour and a product precipitated out of the medium by 48 hours. It is presumed that strain 044 30-1 laccase converted 8-HQ to polymers, which precipitated out of solution (Ncanana, 2007). Samples were taken from flasks and ALRs and the organic compounds were examined by TLC (Fig. 4.28).

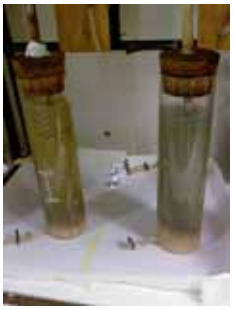


Fig. 4.27. In fermentations of strain 044 30-1 in ALRs with 8-HQ added to the medium, a colour change from yellow to black-grey was observed.

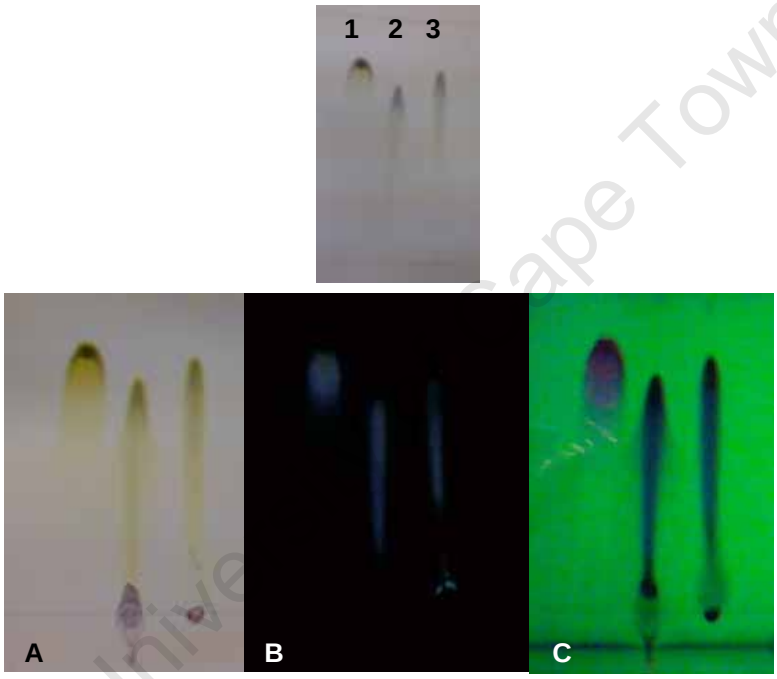


Fig. 4.28. TLC observations of 8-HQ reactions with laccase from strain 044 30-1. 1. 8-HQ (unreacted). 2. Sample of 8-HQ converted by the laccase of strain 044 30-1 grown in M172F medium. 3. Sample of 8-HQ converted by the laccase of strain 044 30-1 grown in SCN medium. A. Iodine vapours. B. Long wave UV (365 nm). C. Short wave UV (254 nm).

TLC results showed that the starting monomer material, 8-HQ, had been converted to large polymeric material by strain 044 30-1 laccase (Dr S. Riva, pers. comm.). It had been previously shown that laccase-catalysed polymers of 8-HQ had higher antioxidant activity than the monomer parent compound (Ncanana, 2007). This was assessed in Chapter 5.

4.3.8 Summary of this study's biocatalysis research findings

The products obtained from laccase-catalysed oxidation reactions, and reactions catalysed with tyrosinase, using tyrosol and tyrosol-acetate (**IV**) as substrates are illustrated in Figures 4.29 and 4.30, respectively. A summary of all of the substrates investigated in this study, and by others, and the resultant products of lipase-, laccase-, and tyrosinase-catalysed reactions are listed in Table 4.4.

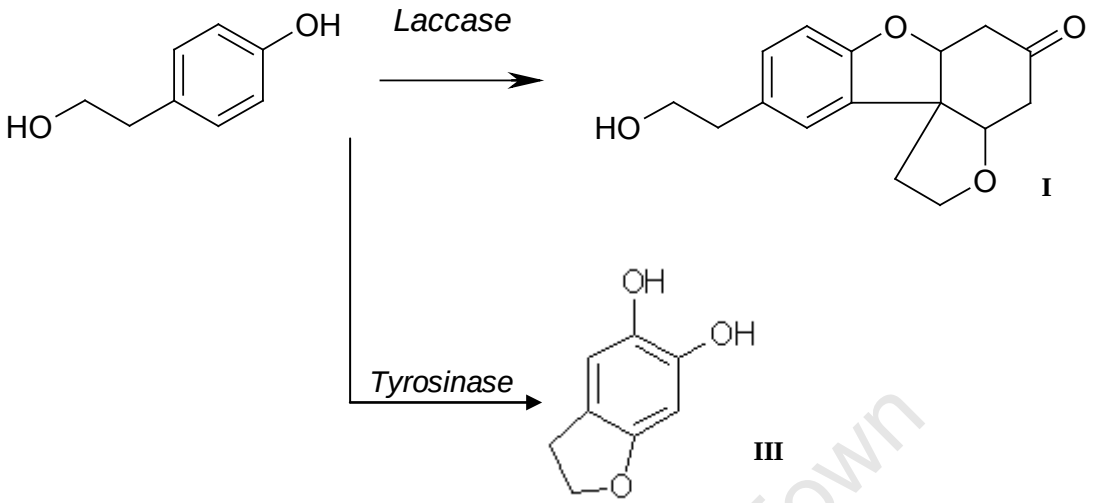


Fig. 4.29. Summary of tyrosol reactions with oxidase enzymes laccase and tyrosinase, and the respective products, **I** and **III**, of these reactions. Laccase from *Trametes versicolor* and strain 044 30-1 laccase convert tyrosol to product **I**.

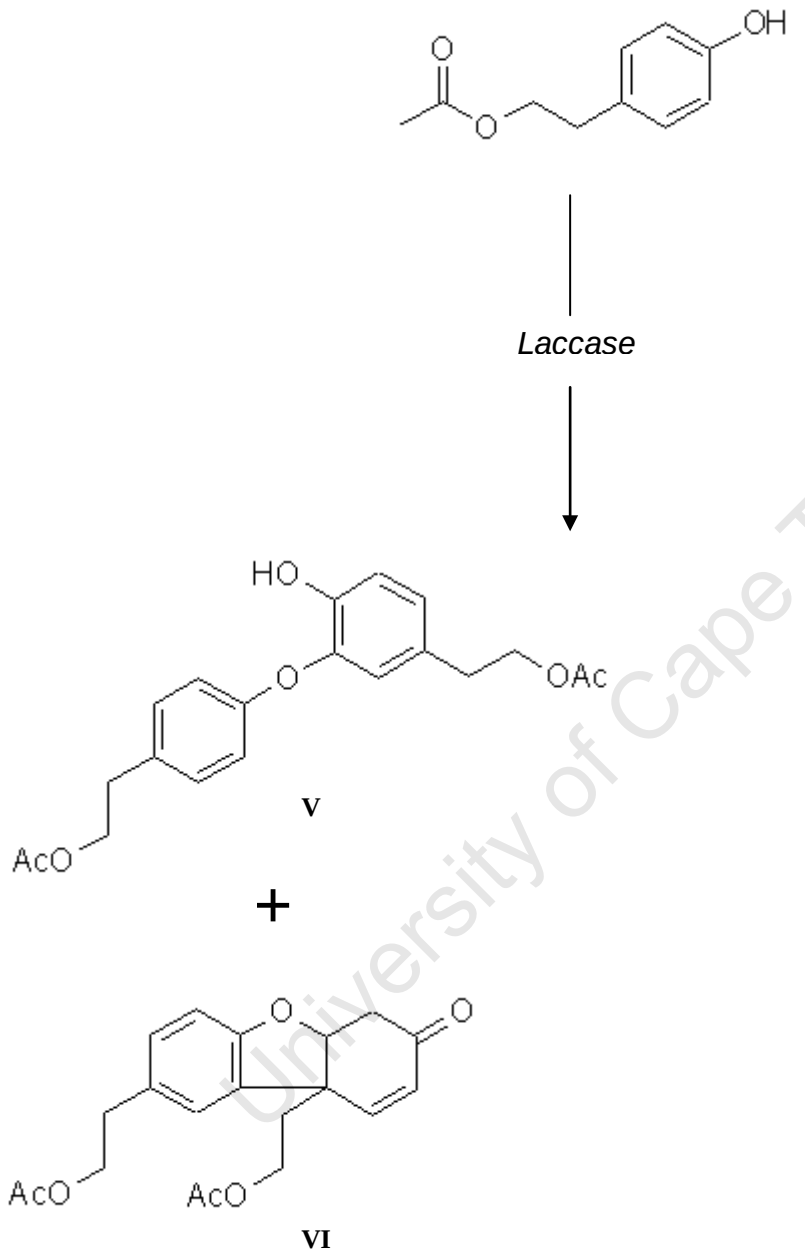
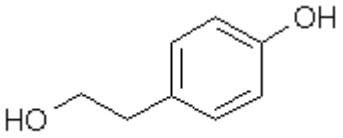
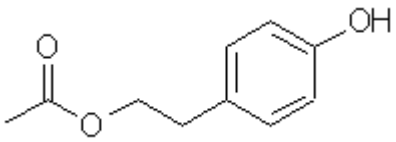
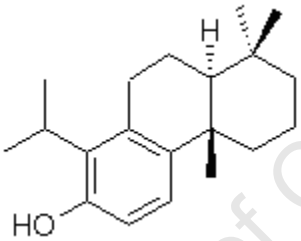
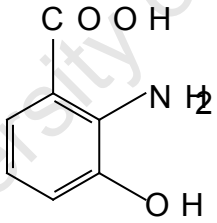
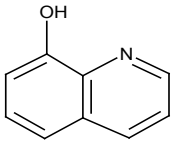
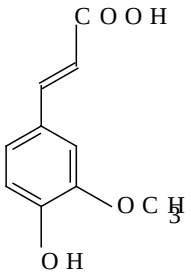


Fig. 4.30. Summary of the tyrosol-acetate reaction with laccase (*Trametes versicolor* and *Micromonospora* sp. 044 30-1 laccases) and the products of this reaction.

Table 4.4. Summary of the biocatalysis reactions investigated in this study and by others.

Enzyme:	Substrate:	Structure:	Molecular Weight (g/mol):	Product:	Reference:
Laccase	Tyrosol		138.17	Product 1 (Tyr-01; major product; **I**) Product 2 (Intermediate; **II**)	Vinciguerra, *et al.*, 1997. This study.
Tyrosinase	Tyrosol			Tyr-02 (**III**)	This study.
Lipase	Tyrosol			Tyrosol-acetate (**IV**)	This study. Aissa, *et al.*, 2007.
Laccase	Tyrosol-acetate		180.17	Two products: TyrAc1 (**V**) and TyrAc2 (**VI**; major product and most polar)	This study.
Tyrosinase	Tyrosol-acetate			Many products.	This study.
Laccase	Totarol		286.45	Major product: C-C dimer Minor product: C-O dimer	Ncanana, *et al.*, 2007.
Laccase	3-HAA		153.14	Cinnabarinic acid Unidentified compounds	Eggert *et al.*, 1995. This study.
Laccase	8-HQ		145.16	Polymeric compounds with repeat units (6 – 23)	Ncanana, 2007.
Laccase	Ferulic acid		194.19	Dehydrodimers	Ward, *et al.*, 2001.

4.4 Conclusion

The aim of the current study was to investigate laccase-catalysed oxidation reactions in search of novel reaction products with potential biological activity, specifically enhanced antioxidant capability. The dimeric reaction products of the laccase-catalysed oxidation of compound **IV** are novel and their biological activities are investigated in Chapter 5. The work describing the laccase-tyrosol and laccase-**IV** biocatalysis reactions, and the novel products **V** and **VI**, has been accepted for publication (Navarra, *et al.*, 2010; *in press*).

The structure of the tyrosol molecule proved to be problematic as the side chain formed ring structures under the reaction conditions investigated during this study. To prevent the formation of a ring by the side chain, lipase was used to replace the hydroxyl group on the side chain of the tyrosol molecule with an acetate group, thereby synthesising monoacetylated tyrosol (**IV**). This study reports for the first time the laccase-catalysed dimerization of monoacetylated tyrosol to the novel products **V** and **VI** under environmentally benign reaction conditions, using the laccases from the fungus *Trametes versicolor* and the actinomycete *Micromonospora* sp. 044 30-1.

Published methods (Kazandjian and Kilbanov, 1985; Ncanana, 2007) for obtaining hydroxytyrosol were used but hydroxytyrosol was not isolated during this study. The tyrosol-tyrosinase reaction produced compound **III** instead of the expected hydroxytyrosol structure. Researchers in our laboratory had previously successfully synthesised hydroxytyrosol from tyrosol using tyrosinase isolated and purified from shop-bought mushrooms (Ncanana, 2007). The tyrosinase used in this study was commercial mushroom tyrosinase from Sigma. Thus, the possibility exists that tyrosinases from different sources, under the same reaction conditions, could catalyse the formation of different reaction products.

A further aim of the current study was to assess the biocatalytic capability of the potentially novel laccase from the actinomycete strain *Micromonospora* sp. 044 30-1. As predicted for biocatalysis reactions where tyrosol, compound **IV**, or totarol were used as substrates, the dimeric products obtained using the laccase from strain 044 30-1 appeared to be identical to those obtained using laccases from fungal strains such as *Trametes* species and *Lentinus edodes* (Ncanana, 2007; Ncanana and Burton, 2007; Vinciguerra, *et al.*, 1997). Although optimisation of the reaction conditions for use of the laccase from strain 044 30-1 is recommended for future research, it was noted that both the crude extract from the growth medium of strain 044 30-1 and the cells of the strain could be used for biocatalysis reactions.

Investigations into the polymers of laccase-catalysed reactions of 8-HQ have been limited due to the complex nature of the polymeric products (Ncanana, 2007). It is believed that strain 044 30-1 laccase also mediates the formation of such complex polymers. The antioxidant 3-HAA is an interesting molecule that is converted by BMCOs to other useful compounds. Strain 044 30-1 laccase converts 3-

HAA to CA and TLC observations showed that other products are also formed during this reaction. These products were not shown to be produced by fungal laccases and are thus of interest as they may be structurally interesting.

Further, from this work we may infer which solvents the enzymes can tolerate in biphasic reaction media: *Trametes* laccase and strain 044 30-1 laccase can tolerate a range, including acetone and ethyl acetate. Reactions performed in biphasic media offer easy separation of products and to an extent, the organic solvent allows for some control of polymerisation, thereby avoiding undesirable reactions (Díaz-García and Valencia-Gonzáez, 1995).

A survey of the literature on applications of phenolics, relevant to this work, showed that researchers not only test for antioxidant efficiency but also antimicrobial activity. The reason for this interest is that antioxidants are not only excellent additions to foods for their health benefits but they also act as preservatives, preventing the growth of bacteria in food stuffs. In Chapter 5, the products of the reactions described in this chapter were evaluated for antioxidant efficiency and also as antimicrobials.

Chapter 5
Biological Activity of Laccase-catalysed Reaction Products: Antioxidant and Antimicrobial Properties

5.1 Introduction

Laccases are attractive, useful tools in the synthesis of fine chemicals with biological activity. However, there are few reports related to the biological activity of products obtained from laccase biocatalysis processes. Thus, in many cases, the relationship between the structure of biocatalysis products and their biological activity still needs to be determined.

5.1.1 Phenolic antioxidants

Antioxidants (AOs) inhibit or retard the oxidation of other compounds, which is often caused by the effects of reactive oxygen species (ROS). Oxidation of biological molecules causes deleterious effects, such as cancer in humans, as well as normal physiological phenomena such as aging. Oxidation also affects food, and almost half of worldwide fruit and vegetable spoilage is due to post-harvest degenerative oxidation reactions (Chigorimbo-Murefu, 2007). Similarly, the oxidation of fats and oils in food results in rancid odours and flavours leading to a deterioration of the nutritional quality, colour, texture and flavour of the foodstuff and potentially toxic compounds are formed. AOs are also used to prevent deterioration of other oxidisable goods, such as cosmetics, plastics, and pharmaceuticals. Furthermore, the protection that fruits and vegetables provide against certain diseases has been shown to be due to the presence of various AOs (Moure, *et al.*, 2001). AOs are also useful if they are stable and can be used as industrial preservatives, as nutraceuticals, and for use in medicine, and the fine chemicals industry (Moure, *et al.*, 2001).

A major group of naturally occurring AOs are the phenolics, such as gallic acid, ferulic acid, isoeugenol, and resveratrol. These phenolic compounds can be converted to dimers or short oligomers by laccase and the products have been suggested to be more effective antioxidants than the parent compounds (Chigorimbo-Murefu, 2007; Ncanana, 2007; Ponzoni, *et al.*, 2007). Structurally, phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl substituents, and range from simple phenolic molecules to highly polymerised compounds. The diverse group of phenolic compounds derived from biological sources are often referred to as polyphenols (Balasundram, *et al.*, 2006). The AO activity of phenolic compounds depends on the structure and in particular the number and position of the hydroxyl groups and the nature of the substitutions on the aromatic rings. Phenolic compounds exhibit a wide range of physiological properties such as anti-allergenic, anti-artherogenic, anti-inflammatory, antimicrobial, antioxidant, anti-thrombotic, cardioprotective, and vasodilatory effects (Moure, *et al.*, 2001). The beneficial effects derived from phenolic compounds have been attributed to their AO activity, and phenolic-rich extracts have shown AO activities comparable to that of synthetic AOs (Balasundram, *et al.*, 2006). Phenolic antioxidants (PhOH) interfere with the oxidation of lipids

and other molecules by the rapid donation of hydrogen atoms to radicals, shown in the following equation:

$$ROO^{\bullet} + PhOH \rightarrow ROOH + PhO^{\bullet}$$

The phenoxy radical intermediates are relatively stable so they do not initiate (propagate) further radical reactions. It has been reported that the AO activity of phenolic compounds may result from the neutralization of free radicals that would otherwise initiate oxidation processes, or from the termination of radical chain reactions, due to their hydrogen donating ability (Balasundram *et al.*, 2006).

The AO capacity of any compound, as demonstrated by its ability to scavenge free radicals, is dependent on a combination of factors including its electronic structure and hence its ability to abstract or donate hydrogen atoms, the ionisation potential of the reactive hydroxyl groups present in the compound, and the potential for stabilisation of the resulting radical by charge delocalisation (Ncanana and Burton, 2007). Higher stability is conferred by electron delocalisation, and in aromatic polymeric structures, free radical scavenging activity is correlated to the molecular weight of the polymers and the extent of π-polyconjugated systems in the structure (Ncanana and Burton, 2007).

An example of the phenolic structure-AO activity relationship is that of hydroxytyrosol [2-(3,4-dihydroxyphenyl) ethanol; two hydroxyl groups on the phenolic ring], the natural AO obtained from olive oil (Table 5.1). It is known for its biological properties that may contribute to the reduced risk of cardiovascular diseases in the populations of Mediterranean countries (Galli and Visioli, 1999). The analogue of hydroxytyrosol, tyrosol (having only one hydroxyl group on the phenolic ring), has lower AO activity. AO activity of a phenolic compound may be influenced by both hydroxyl and methoxyl groups in the molecule. The AO activity of phenolic acids increase with increasing hydroxylation but the substitution of hydroxyl groups with methoxyl groups reduces activity. Ferulic acid, bearing both hydroxyl and methoxyl groups in the aromatic ring, has greater AO activity than that of *p*-coumaric acid, bearing only a hydroxyl group (Sánchez-Moreno, *et al*., 1999). Generally, hydroxyl groups present in the *ortho* position, to give a catechol ring, increase AO capacity due to resonance stabilization and increases the rate of H-atom transfer to ROS (Chen and Ho, 1997; Torres de Pinedo, *et al.*, 2007).

A number of studies indicate that olive oil phenolic constituents have AO and antimicrobial properties contributing to the reduction of ROS-RNS (Grasso, *et al.*, 2007, and references therein). Studies on olive phenols have shown the importance of the lipophilic character of the AO with regards to the dispersion medium (bulk oil, emulsions), to cell uptake, membrane crossing, and to the substrate to be protected (LDL or membrane constituents).

Table 5.1. Antioxidant power (in decreasing order) of natural phenolic compounds and commercial antioxidants (adapted from Garrote, *et al.*, 2004).

Assay:	**Antioxidant Power:**
Data concerning the inhibition of oil or fatty acid oxidation in lipophilic phase, emulsions, liposomes, or microsomes:	
Refined olive oil	**HT** > CaA > BHT > PCA > SA > pHBA > **T** > pCA > oCA ≥ VA
Data concerning the inhibition of free radical formation and scavenging:	
$ABTS^{\bullet+}$ scavenging	**HT** > CaA > VA > **T** > V
$DPPH^{\bullet}$ scavenging	SpA > αToc > **HT** > CaA > PCA > SA > VA = **T**
Abbreviations: BHT, butylhydroxytoluene; CaA, caffeic acid; HT, hydroxytyrosol; oCa, *o*-coumaric acid; pCA, *p*-coumaric acid; PCA, protocatechuic acid; pHBA, p-hydroxybenzoic acid; SA, syringic acid; SpA, sinapic acid; T, tyrosol; αToc, α-tocopherol; VA, vanillic acid; V, vanillin.	

Mixtures of polyphenols have been shown to exhibit higher AO activity as compared with their respective individual components (Ncanana, 2007), which suggests synergy between the AO components in AO reactions. It has been shown that various compounds demonstrate synergism in their AO capacity, although the mechanism of this synergy is not completely understood (Castro, *et al.*, 2005). Synergism shown by mixtures of products could be useful, especially since it is often difficult to isolate the individual components, for example in mixtures of natural products. Furthermore, this could be considered economically useful in industry, as the separation step in downstream processing of product mixtures could be avoided.

5.1.2 Benefits of phenolics other than as antioxidants

In addition to their beneficial AO properties, phenolic compounds contribute to the sensory properties of foods, particularly colour (Mustafa, *et al.*, 2005). The beneficial effects of dietary fibre from whole grain products are attributed to the effects of phenolic acids (Garrote, *et al.*, 2004). Colonic microflora transform flavonoids into low-molecular-weight phenolics, which have protective biological activities in the colon (Garrote, *et al.*, 2004). Caffeic acid and FA are protective against the nitrite ion, and *in vivo* they can inhibit or block the formation of carcinomas (Garrote, *et al.*, 2004). FA esters are active agents in cosmetics due to their AO and UV radiation absorption properties (Garrote, *et al.*, 2004; Mustafa, *et al.*, 2005). Phenolic acids such as ferulic, coumaric, sinapic or caffeic acid add a pleasant flavour to beverages and show antimicrobial activity comparable or superior to those of mixtures of benzoic acid-sorbic acid (Balasundram, *et al.*, 2006; Garrote, *et al.*, 2004).

5.1.3 Antimicrobial activity of phenolic compounds

Although less potent than clinical antibiotics, phenolics are interesting antimicrobial agents used alone or in combination with synthetic additives (Kubo, *et al.*, 1992). Their mode of action is concentration-dependent and comparatively high concentrations (up to 200 ppm) are allowed (Garrote, *et al.*, 2004). Phenols affect microbial membrane permeability, decrease electron transport and nutrients uptake, interfere with the synthesis of macromolecules and nucleic acids, and can penetrate the spore coat thereby inhibiting the growth of spores, and serving as a preservative (Bernabeu, *et al.*, 2002; Garrote, *et al.*, 2004). The dissociation of the acid moiety and the presence of one or more reactive double bonds contribute to the antimicrobial activity of phenolic compounds. In addition, a linear relationship exists between the number of carbon atoms in an alkyl chain and the antimicrobial activity, and the number of hydroxyl groups and the oxidation level also increase toxicity (Garrote, *et al.*, 2004).

5.1.4 The role of actinomycete metabolites in combating disease

Actinomycetes are routinely screened for new bioactive substances as they have provided many bioactive compounds of great value, including many of medical importance. Presently, there is a growing problem of bacterial resistance to antibiotics used to treat medically important diseases. In a world that expects effective antibiotics available for every disease, the rapid spread of antibiotic resistant bacteria is of critical concern (Bush, 2004; Thomson, *et al.*, 2004). Furthermore, disregard of the necessity for new antibiotics to treat bacterial infections, and the departure of many large pharmaceutical companies from the field of antibiotic development, have added to the problem (Payne and Tomasz, 2004).

The emergence of multidrug-resistant (MDR) bacteria is of concern to the clinician and the pharmaceutical industry, as it is the major cause of failure in the treatment of infectious diseases (Davies, 1994; Levy, 1998). Drug resistance poses a serious threat to tuberculosis control programmes in the Western Cape province of South Africa, contributing to the increasing tuberculosis (TB) epidemic in the region (Weyer, *et al.*, 1995; Caelers, 2004). An increase in the number of drug-resistant TB cases (especially MDR-TB cases) has been observed both locally and internationally due to ineffective treatment, patient non-compliance, and to the spread of HIV (Mukadi, *et al.*, 2001). The advent of the HIV epidemic in sub-Saharan Africa has seen not only a dramatic increase in the incidence of TB cases in the region, but also a dramatic increase in the number of people dying from what is a curable disease (Caelers, 2004). HIV-compromised immunity increases the susceptibility of individuals to develop TB either from a reactivation of latent infection or a rapid progression of recent infection by the opportunistic *M. tuberculosis* (Caelers, 2004; Mukadi, *et al.*, 2001). Insidiously, the host's immune response to *M. tuberculosis* enhances HIV replication and might accelerate the natural progression of HIV infection (Mukadi, *et al.*, 2001). Although education and prevention do help to combat TB, novel antitubercular antibiotics are required to combat the threat posed by MDR *M. tuberculosis* strains.

In the current study, *Micromonospora* sp. strain 044 30-1, a marine actinomycete strain, was investigated for the production of antibiotics against clinically relevant bacterial strains, including *Mycobacterium aurum*, a strain closely related to *Mycobacterium tuberculosis*. Further, the biocatalysis products of the reactions described in Chapter 4 were also investigated as potential antibiotics with antitubercular activity. The antioxidant activities of the biocatalysis products were also assessed using the 2,2-diphenyl-1-picrylhydrazyle (DPPH) and low density lipoprotein (LDL) assays, to be discussed below. This chapter describes an investigation into the biological activities of the compounds obtained through laccase-catalysed oxidation reactions, as described in Chapter 4.

5.1.5 Types of antioxidant assays

Major AO capacity assays can be divided into two categories: Hydrogen atom transfer (HAT) reaction based assays, and single electron transfer (ET) reaction based assays. The ET-based assays involve one redox reaction with the oxidant (which is also the probe for monitoring the reactions) as an indicator of the reaction endpoint. Most HAT-based assays monitor competitive reaction kinetics, and the quantitation is derived from the kinetic curves. HAT-based methods generally involve a synthetic free radical generator, an oxidizable molecular probe, and an AO. HAT- and ET-based assays are intended to measure the radical, or oxidant, scavenging capacity, instead of the preventative AO capacity of the sample (Huang and Prior, 2005).

5.1.5.1 ET-based assays

These methods involve two components in the reaction mixture: Antioxidant, and oxidant (also the probe). They are based on the following electron-transfer reaction:

Probe (oxidant) + electron (from antioxidant) $\rightarrow$ reduced probe + oxidized antioxidant.

The probe itself is an oxidant that extracts an electron from the AOs, causing colour changes of the probe. The degree of the colour change is proportional to the active AO concentrations. The reaction endpoint is reached when colour change stops. The change of absorbance is plotted against the AO concentration to give a linear curve. The slope of the curve indicates the AO's reducing capacity (Huang and Prior, 2005). It is assumed that AO capacity is equal to reducing capacity. Although the reducing capacity of a sample is not directly related to its radical scavenging capacity, it is an important parameter of AOs (Huang and Prior, 2005). The DPPH assay, discussed below, is an ET assay.

5.1.5.2 DPPH radical scavenging capacity assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) is one of a few stable and commercially available organic nitrogen radicals and has a UV-visible absorption maximum at 515 nm (Fig. 5.1A). Upon reduction, the solution colour fades, and the reaction progress is conveniently monitored spectrophotometrically. The DPPH assay is the most common laboratory method for measuring AO activity (Chigorimbo, *et al.*, 2009). The DPPH radical has been widely used to evaluate the radical scavenging activity of a variety of natural polyphenols including tyrosol and hydroxytyrosol (Grasso, *et al.*, 2007). The basic method is to measure the decay of visible absorption of the DPPH radical ($DPPH^{\bullet}$) due to its conversion into a

colourless hydrazine (DPPH-H) when an H-donor (for example, a phenol) causes the H-atom transfer reaction, under standardised conditions (Es-Safi, *et al.*, 2007). The more rapidly the DPPH absorbance decreases, the more potent is the AO activity of the sample (Es-Safi, *et al.*, 2007).

The percentage of the DPPH remaining is calculated as (Huang and Prior, 2005):
$\%DPPH_{rem} = 100 \times [DPPH]_{rem}/[DPPH]_{T=0}$

$\%DPPH_{rem}$ is proportional to the AO concentration, and the concentration that causes a decrease in initial DPPH concentration by 50% is defined as EC_{50}. The time needed to reach the steady state with EC_{50} concentration is calculated from the kinetic curve and defined as T_{EC50}. The kinetic behaviour of an AO compound can be classified as follows (Huang and Prior, 2005): <5 min (rapid), 5 – 30 min (intermediate), and >30 min (slow). A parameter, called antiradical efficiency, AE, is used to express the AO capacity of a certain AO compound, and can be calculated as follows: $AE = (1/EC_{50})T_{EC50}$.

5.1.5.3 Trolox Equivalent Antioxidant Capacity (TEAC) or ABTS assay

The TEAC assay (Re, *et al.*, 1999) is an AO assay utilizing both HAT and SET mechanisms of quenching free radicals. Decolourisation of the $ABTS^{\bullet+}$ chromophore due to quenching of the radical by AOs, forms the basis of the assay. Unlike the DPPH assay, the radical chromophore is not commercially available and is generated by reacting ABTS with potassium persulphate. AOs reduce the radical to ABTS, in direct proportion to their AO activity, concentration, and duration of the reaction (Fig. 5.1C). The extent of decolourisation, measured at 734 nm and calculated as percentage inhibition of $ABTS^{\bullet+}$ is determined as a function of concentration and time (Chigorimbo-Murefu, 2007). The reactivity of the test AOs is calculated relative to the reactivity of the synthetic AO Trolox, a water-soluble vitamin E analogue, used as a reference (Fig. 5.1B).

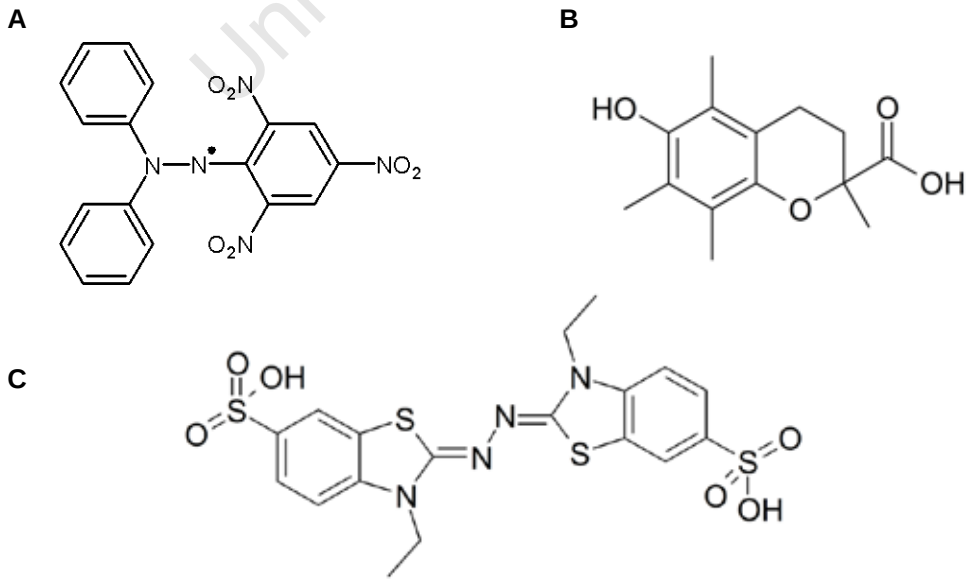


Fig. 5.1. Test compounds used in AO assays. A. The DPPH radical. B. Trolox. C. ABTS.

5.1.5.4 The Low Density Lipoprotein (LDL) assay

Oxidative damage to lipids, initiated by ROS, occurs as follows (Meral, *et al.*, 2000): Hydrogen abstraction of a CH_2 group within the lipid occurs due to ROS attack, resulting in the formation of a fatty acid radical. This radical is then stabilised by the rearrangement of its molecular structure to form a conjugated diene. When oxygen is present in sufficient concentrations, the fatty acid radical reacts with O_2 to form a peroxy radical ($ROO^{\bullet}$) capable of abstracting another hydrogen from a neighbouring fatty acid molecule, resulting in a chain reaction that can lead to the peroxidation of all lipids within the entire membrane. An AO that can bring this reaction to a halt, usually by intervening during the initiation step by reacting with the radicals, is termed a chain-breaking, or primary, AO (Chigorimbo-Murefu, 2007; Teissedre, *et al.*, 1996; Wright, *et al.*, 2001).

Low density lipoproteins (LDLs) are lipid-protein complexes and are the major carriers of cholesterol in the blood. The inhibition of LDL oxidation by AO compounds is useful, since the formation of artheromas (the major cause of coronary heart disease) is believed to result from the oxidation of LDLs (Teissedre, *et al.*, 1996). Furthermore, it has been shown that dietary phenolic compounds are associated with reduced coronary heart disease mortality, and that phenolic compounds known to occur in wine and grapes are powerful AOs in inhibiting LDL oxidation (Teissedre, *et al.*, 1996). The LDL assay may provide a more appropriate indication of the potential use of an AO *in vivo*, for example, in protecting physiological lipids or cell membranes, while the DPPH and TEAC assays are considered more useful in assessing the AO capacity of compounds to be used as, for example, food preservatives (Chigorimbo-Murefu, *et al.*, 2009).

The ability of AO compounds to inhibit LDL oxidation is assessed by adding the compounds to a reaction mixture containing LDL and copper (II) ions (used to initiate oxidation), and then spectrophotometrically monitoring the compounds' effect on lipid peroxidation-associated diene conjugation after 4 hours (Chigorimbo-Murefu, *et al.*, 2009). An increase in absorbance at 234 nm indicates diene conjugation, while a decrease in absorbance is attributed to AO activity, resulting in a decrease in conjugated diene formation of LDL.

5.2 Materials and Methods

5.2.1 Solid media overlays: The sloppy-agar method

The antimicrobial activity exhibited by strain 044 30-1 was assessed by the use of the sloppy-agar overlay technique. This experiment was performed in duplicate. By using sterile toothpicks, the strain was stab-inoculated into the centre of agar plates and incubated at 30°C for 11 days. The antimicrobial activity of the strain was tested against each of four test bacteria: *Escherichia coli* ATCC 25922 (a standard antibiotic-susceptibility testing strain; Gram-negative), a vancomycin resistant clinical isolate of *Enterococcus faecium* (Van A; Gram-positive), a *Micrococcus* sp. strain (Gram-positive), and *Mycobacterium aurum* A+ (acid fast). On day 10, each test bacterium was inoculated

into 10 ml 2YT (Atlas, 1993) broth and incubated overnight at 37°C with shaking. The test bacteria were Gram stained to verify the purity of the cultures. The optical density at 600 nm (OD_{600}) of each culture was measured using a spectrophotometer. Cultures were diluted, before taking the reading, as follows: *E. coli*: diluted 1-in-10 in sterile 2YT broth; *E. faecium, Micrococcus*: diluted 1-in-4 in sterile 2YT broth; and *M. aurum*: diluted 1-in-6 in sterile 2YT broth. Empirically determined calculations were used to ensure that there would be the same concentration of test bacterium cells in the sloppy-agar from one overlay experiment to the next. The number of microlitres of bacterial culture used per sloppy-agar test tube was calculated as follows (solve for ***x***): $OD_{600} \times$ test bacterium's dilution factor $\times$ (***x*** µl) = test bacterium's constant. The values vary for the different test bacteria: *E. coli*: $OD_{600} \cdot 10\boldsymbol{x} = 4$; *E. faecium, Micrococcus*: $OD_{600} \cdot 4\boldsymbol{x} = 160$; *M. aurum*: $OD_{600} \cdot 6\boldsymbol{x} = 160$. The calculated volume (in µl) of test culture was pipetted into test tubes containing 6 ml 2YT sloppy-agar, vortexed gently, and poured onto the 11 day old stab inoculated plates, ensuring that the sloppy-agar was distributed evenly over the entire surface of the plate. These plates were incubated overnight (14 – 18 h) at 37°C for *E. coli, E. faecium*, and *Micrococcus*, and for 40 – 45 h at 37°C for *M. aurum*. The diameter (in mm) of each colony was measured before overlaying. The diameter (in mm) of the zone of growth inhibition of the test bacteria was measured if a strain showed antimicrobial activity. The area of the inhibition zone in mm^2 was calculated. The arbitrary assignment of strength of antimicrobial activity was as follows: very weak (<100 mm^2); weak (100 – 1000 mm^2); moderate (1001 – 2000 mm^2); strong (2001 – 3000 mm^2); and very strong (>3000 mm^2).

The degree of antimicrobial activity against the four test bacteria was determined on the optimised M172F medium, *i.e.* containing 8 mM Cu and 2% NaCl, and on Bennett's (Atlas, 1993) and 2YT agars.

5.2.2 Thin layer chromatography

Thin Layer Chromatography (TLC) plates used in this research were TLC aluminium-backed silica gel 60 F_{254} (Merck; Catalogue Number: 1.05554.0001). The appearance of compounds were visualised by UV or by the use of iodine vapours, which specifically detect organic compounds containing carbon-carbon double (C=C) bonds, resulting in spots appearing brown-purple. TLC plates were developed in glass beakers covered with tinfoil.

5.2.3 Starting substrates and products used in this study

The compounds used in this study were: tyrosol, compounds **I**, **II**, **III**, **IV**, **V**, and **VI**, totarol, the dimers of totarol, FA, 8-HQ, 3-HAA, and Trolox. The concentrations used were 1, 2, 5, 10, 20, 30, 40, 50, and 100 mg/ml. This information is illustrated in Appendix C. All compounds were dissolved in methanol. Trolox was supplied by Sigma.

5.2.4 DPPH assay

The DPPH assay was performed using a microtitre plate reader (section 2.2.3) and the decrease in absorbance at 492 nm was monitored for 150 minutes. Readings were recorded every 30 seconds. 150 µl of 0.06 mM methanolic DPPH was added to 50 µl of sample to be tested, and assayed immediately.

5.2.5 Bioautography for the visualisation of antimicrobially active spots

Cultures were grown as described in section 5.2.1. The cultures were diluted with sterile 2YT broth to give an OD_{600} of 0.5. Samples (section 5.2.3) were spotted on TLC plates and/or spotted, dried, and developed in a solvent system (Table 4.1 and section 4.2.11). Once the solvent had evaporated, the test bacteria were applied to the TLC plate by dabbing with sterile cotton wool until the entire plate was covered with test bacterium. The TLC plates were transferred to a plastic container, the bottom of which was lined with wet paper towel to create a moist environment, and placed face up on the paper towel. The container was sealed and incubated at 37°C for at least 6 hours. TLC plates were removed from the container and dabbed with 0.25% (w/v) MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (Sigma) dissolved in phosphate buffered saline (pH 7.3), using sterile cotton wool. The paper towel was removed and the TLC plates were returned to the container, which was sealed and left at 37°C for at least 45 min, so that a colour change was observed. The activity spots on TLC plates were visualized by using MTT, which acts as an oxidising agent, serving as the final electron acceptor in the bacterial electron transport chain. If MTT is reduced, a colour change from yellow to purple-blue is observed. The presence of the purple-blue colour indicates where the test bacteria are actively respiring, whereas areas lacking the purple-blue colour indicate the existence of an antibiotically active spot that the test bacteria are sensitive to. Methanol was used as a control.

5.2.6 Visualisation of antioxidant active spots using DPPH for detection

Samples (section 5.2.3) were spotted on TLC plates and/or spotted, dried, and developed in a solvent system (Table 4.1 and section 4.2.11). Once the solvent had evaporated, TLC plates were submerged briefly in DPPH. The antioxidant activity spots on TLC plates are visualized by using 0.06 mM methanolic DPPH and observing a colour change from purple to yellow. This colour change usually occurred immediately or within a few minutes but was monitored visually over 3 hours. Methanol was used as a control. This experiment was performed in duplicate.

5.2.7 LDL oxidation

Oxidation of LDL (from human plasma; Merck) was performed according to the method of Nardini, *et al.* (1995). LDL was dialysed in a 200-fold volume of PBS, pH 7.4, at 4°C in the dark for 18 hrs. 100 μg/ml LDL (determined by the Bradford method) was then oxidised using 5 μM $CuCl_2$ for 4 hrs at 37°C in the presence and absence of test antioxidant. 100 μl $CuCl_2$ and 10 μl of sample to be tested was added to 890 μl of a 100 μg/ml solution of LDL in PBS. A control, containing no sample was used. The experiment was performed in duplicate. Conjugated diene formation was measured spectrophotometrically at 234 nm.

5.3 Results and Discussion

In this study, the parent compounds used in biocatalysis reactions, and the subsequent products obtained through laccase-catalysed oxidation reactions (described in Chapter 4; listed in Appendix C) were investigated for their biological activities: Antioxidant activities using the DPPH radical and the LDL assay, as well as antimicrobial activity were determined.

Staining TLC plates with DPPH allows one to quickly and easily assess whether a sample compound has AO activity (Fig. 5.2). Other observations made can be the determination of the intensity of the radical quenching capability (by visual observation of the extent of colour change from purple to yellow), and the minimal concentrations of sample required to detect AO activity can be ascertained. Samples can be spotted on the TLC plate or can be developed using an appropriate solvent system, thereby allowing for the separation of products in reaction mixtures and detecting individual AO activity of the products. Staining of TLC plates with the DPPH radical in order to detect the formation of hydroxytyrosol was successfully achieved by Espín and coworkers (2001). The DPPH assay, using a spectrophotometer, must be performed if kinetic data are to be obtained. The benefits of using a microtitre plate system for the DPPH assay are as follows: Many samples can be processed simultaneously (other methods are time consuming and laborious), small volumes of the sample compounds can be used, as the use of a microtitre plate system allows for high throughput screening (Moolla, 2007).

The bacterial strains used for antibiotic and/or AO susceptibility testing were: *E. coli* ATCC 25922, a standard antibiotic susceptibility testing strain (Gram negative), *E. faecium* VanA, a vancomycin resistant clinical isolate (Gram positive), a *Micrococcus* isolate (morphologically identified as *M. luteus*; Gram positive), and *M. aurum* A+ (Gram positive and acid fast). *Mycobacterium aurum* is closely related to *Mycobacterium tuberculosis*, but is non-pathogenic and grows faster (Goodwin, 2005). Its antibiotic susceptibility profile is similar to that of *M. tuberculosis* and thus activity (be it antimicrobial or AO) against *M. aurum* is of the most value as the antibiotics/AOs could be further pursued as potential antitubercular agents. Micrococci can be opportunistic pathogens, particularly in hosts with compromised immune systems, such as HIV patients. *M. luteus* causes clinically significant infections in humans (Smith, *et. al.*, 1999). It is difficult to compare the antimicrobial activity results obtained in this study with reports in the literature as data relating to many of the compounds tested, and the strains used in this study, have not been previously reported.

5.3.1 Antimicrobial activity of *Micromonospora* sp. Strain 044 30-1

Antibiotic production by strain 044 30-1 was evaluated on 3 different types of media: 2YT, Bennett's medium (BM-Glucose), a *Micromonospora* medium, and M172F, the optimal medium for laccase production by strain 044 30-1. The results of the sloppy-agar overlay method for the detection of antibiotic activity revealed that the best medium among those tested for production of an antibiotic by strain 044 30-1 was 2YT medium. No activity was detected against the Gram-negative bacterium *E. coli*. For the other bacteria tested, all of which are Gram-positive microbes, weak antimicrobial activity exhibited by strain 044 30-1 was detected after 11 days. For the vancomycin-resistant *E. faecium* clinical isolate, zones of inhibition were detected on BM-Glucose medium (226 mm^2) and on 2YT (364 mm^2). For the clinically relevant *Micrococcus* strain, zones of inhibition were detected on 2YT agar (262 mm^2). Of most significance, strain 044 30-1 exhibited activity (286 mm^2) against *M. aurum* on 2YT.

5.3.2 Antimicrobial activity of the test compounds

In this section, the antimicrobial activity of the test compounds, evaluated using bioautography as described in section 5.2.5, are discussed and are summarised in Table 5.2. The antimicrobial activity of the control compound, trolox, a Vitamin E analogue, is also discussed in this section.

5.3.2.1 Antimicrobial activity of tyrosol and its derivatives

Both trolox and tyrosol showed strong antimicrobial activity against *E. coli*, *E. faecium*, *Micrococcus*, and *M. aurum* over the entire concentration range tested (100 – 1 mg/ml). It is documented in the literature that tyrosol, found in wastewaters, is an antimicrobial agent (Gianfreda, *et al.*, 2006; Grasso, *et al.*, 2007). Monoacetylated tyrosol (**IV**) was effective against *E. coli* and *E. faecium* over the concentration range 100 mg/ml – 1 mg/ml. The minimal inhibitory concentration (MIC) against *Micrococcus* was 5 mg/ml of compound **IV** and the MIC against *M. aurum* was 20 mg/ml. For product **I**, the product of the tyrosol-laccase reaction, samples of 100 mg/ml and 1 mg/ml were tested. These concentrations were clearly effective in killing the four test bacteria. For product **III**, the product of the tyrosol-tyrosinase reaction, samples of 100 mg/ml and 1 mg/ml were also tested. Product **III** killed all the test bacteria at these concentrations, except for *Micrococcus*, which was not affected by the 1 mg/ml sample. Samples of tyrosol, compounds **I**, **III**, **IV**, the **V**-**VI** mixture, **VI**, and trolox were further diluted to achieve concentration ranges of 0.75, 0.5, 0.25, and 0.1 mg/ml. These compounds were still effective against *E. coli* and *E. faecium* at these concentrations and thus the MIC for all of these compounds against these test bacteria was < 0.1 mg/ml. For *Micrococcus* and *M. aurum*, the **V**-**VI** mixture, and compound **VI** itself, spotted and developed on TLC, was effective up until 1 mg/ml and not at lower concentrations. This shows that the dimers of compound **IV** are better antimicrobials against these Gram-positive bacteria than compound **IV** alone. For trolox, the MIC against *Micrococcus* was 1 mg/ml. Trolox, tyrosol, and the derivatives of tyrosol achieved by biocatalysis were effective against both Gram-positive and Gram-negative organisms and should be investigated as potential antitubercular agents.

5.3.2.2 Antimicrobial activity of totarol and its derivatives

(+)-Totarol is the major chemical constituent in the hexane extract of the heartwood of the New Zealand tree *Podocarpus totara* G. Benn, and can also be isolated from a variety of other sources (Evans, *et al.*, 1999). The extract is presently used in a variety of cosmetic and personal care formulations (*e.g.* toothpaste and mouthwash) in New Zealand and Australia (Ncanana, *et al.*, 2007). Totarol is effective against Gram positive bacteria, with *Propionibacterium acnes* being the most sensitive bacterium. This bacterium causes skin and teeth problems and thus totarol is considered potent enough for practical use in hygiene products (Kubo, *et al.*, 1992). The antimicrobial activity of totarol can also be enhanced when used in combination with several other natural products (Kubo, *et al.*, 1992).

(+)-Totarol contains a phenolic moiety with an isopropyl group *ortho* to the hydroxyl group. Totarol is active against β-lactam-resistant bacteria and this diterpenoid, used either alone or in combination

with other molecules, may prove useful in antimicrobial chemotherapy, since its cytotoxicity towards human cell cultures is relatively mild (Bernabeu, *et al.*, 2002). Structure-bioactivity relationships of totarol and derivatives have shown that the phenolic moiety is essential for its potent antibacterial effects (Evans, *et al.*, 1999; Evans, *et al.*, 2000; Kubo, *et al.*, 1992). The underlying mechanism(s) for totarol's antibacterial and AO activities are incompletely understood, although results obtained suggest that its site of action could be the cell membrane. Totarol incorporates very efficiently into phospholipid systems due to its hydrophobic nature and in so doing, totarol perturbs interactions between membrane phospholipids (Bernabeu, *et al.*, 2002).

Resistance to multiple antibiotics by strains of Gram positive bacteria such as *Enterococcus faecalis*, methicilin resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pneumoniae* are significant problems amongst hospitalised patients. Totarol has been shown to have antibacterial activity against Gram-positive bacteria and in particular, MRSA (Evans, *et al.*, 1999). Totarol inhibits oxygen consumption and respiratory-driven proton translocation in whole cells of Gram negative *Pseudomonas aeruginosa* and also NADH oxidation in membrane preparations from this bacterium (Evans, *et al.*, 2000).

The totarol dimers mixture (samples of each purified dimeric product were not available), spotted on TLC, was effective against all the test bacteria over the concentration range 100 mg/ml – 1 mg/ml. The totarol dimers, developed on TLC and thus separated, were also each effective in killing all four test bacteria over the concentration range tested. Samples of totarol, and the totarol dimers were further diluted to achieve concentration ranges of 0.75, 0.5, 0.25, and 0.1 mg/ml. These compounds were still effective against all the test bacteria (except *Micrococcus*) at these concentrations, thus the MIC for all of these compounds against the test bacteria was < 0.1 mg/ml. For *Micrococcus*, the MIC is 1 mg/ml. Totarol and the dimers showed extremely potent antibacterial activity against *E. coli*, *E. faecium*, *Micrococcus*, and *M. aurum*. In accordance with studies reported in the literature, totarol was effective against both Gram-negative and Gram-positive bacteria. Furthermore, the activity against *M. aurum* supports the result obtained by Constantine and co-workers (2001) who reported the activity of totarol against *M. tuberculosis*.

5.3.2.3 Antimicrobial activity of 8-HQ, FA, and 3-HAA

Samples of FA, 3-HAA, and 8-HQ were active against all the test bacteria over the concentration range of 100 mg/ml – 1 mg/ml. Samples of FA, 3-HAA, and 8-HQ were further diluted to achieve concentration ranges of 0.75, 0.5, 0.25, and 0.1 mg/ml. These compounds were still effective against all the test bacteria (except *Micrococcus*) at these concentrations, thus the MIC for all of these compounds against these test bacteria was < 0.1 mg/ml. For *Micrococcus*, the MIC was 1 mg/ml. 3-HAA, 8-HQ, poly(8-HQ), and CA showed effective antimicrobial activity against *E. coli*, *E. faecium*, *Micrococcus*, and *M. aurum*. These compounds thus showed excellent antimicrobial activity against Gram-positive and Gram-negative microbes and should be pursued as potential antitubercular agents, providing that their toxicity towards human cells is within a reasonable limit.

In summary, all of the test compounds investigated showed antimicrobial activity against the test bacteria used in this study (Table 5.2). The product of the laccase-catalysed oxidation of tyrosol-acetate, dimeric product **VI**, showed a more effective MIC against *Micrococcus* and *M. aurum* than the parent compound, tyrosol-acetate. Totarol and its derivatives, and FA, 3-HAA, 8-HQ, and trolox were all more active against *M. aurum* than tyrosol and its derivatives. Further quantitative and structure-activity relationship analyses are suggested as future work.

Table 5.2. Summary of the antimicrobial activities of the test compounds investigated in this study.

Compounds tested:	Minimal Inhibitory Concentration (in mg/ml) against:			
	E. coli	*E. faecium*	*Micrococcus*	*M. aurum*
Tyrosol	< 0.1	< 0.1	1	1
Product **I** (of the tyrosol-laccase reaction)	< 0.1	< 0.1	1	1
Tyrosol-tyrosinase reaction mix containing product **III**	< 0.1	< 0.1	1	1
Tyrosol-acetate (compound **IV**)	< 0.1	< 0.1	5	20
Product **VI** (of the tyrosol-acetate-laccase reaction)	< 0.1	< 0.1	1	1
IV-**V**-**VI** mixture	< 0.1	< 0.1	1	1
Totarol	< 0.1	< 0.1	1	< 0.1
Totarol dimers	< 0.1	< 0.1	1	< 0.1
Ferulic acid	< 0.1	< 0.1	1	< 0.1
8-Hydroxyquinoline	< 0.1	< 0.1	1	< 0.1
3-HAA	< 0.1	< 0.1	1	< 0.1
Trolox	< 0.1	< 0.1	1	< 0.1

5.3.3 Antioxidant activity of the test compounds

Results of the use of the TLC method (section 5.2.6; Figures 5.2 to 5.5) for the visualisation of antioxidant active spots using DPPH for detection are described in this section. This analysis is qualitative in nature and was performed to obtain preliminary data regarding the antioxidant effectiveness of the test compounds and to ascertain the concentration to be used for the DPPH and LDL assays, which provide quantitative data (described in section 5.3.5 below). Trolox, a Vitamin E analogue, was used as the control in antioxidant studies.

When developing mixtures on TLC, and thus allowing for the separation of the products, this was performed in duplicate for all experiments, so that one TLC could be stained with DPPH, and the other exposed to iodine vapours so that the position of the compounds of interest could be visualised clearly.

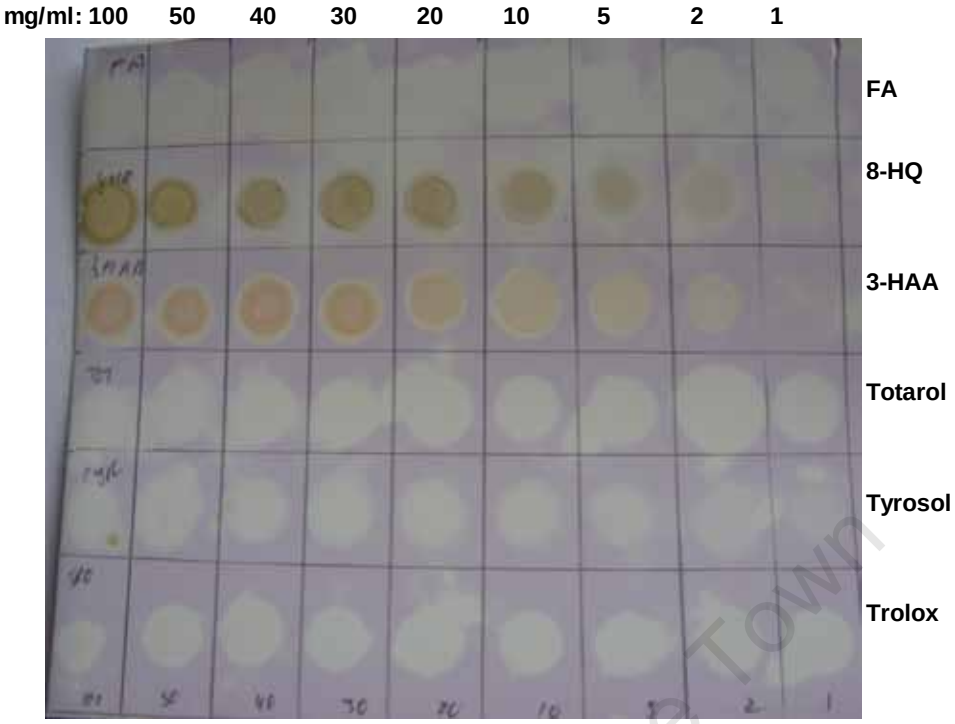


Fig. 5.2. Compounds tested for antioxidant activity were spotted on TLC plates and stained with the DPPH radical. Yellow-white spots indicate antioxidant activity.

Fig. 5.3. A mixture containing the totarol dimers was spotted on TLC plates and stained with the DPPH radical. Yellow spots indicate antioxidant activity. Totarol (far right) was used as a control.

Fig. 5.4. The mixture (**1**) containing **IV**, **V**, and **VI** gave a more potent DPPH reaction than **IV** (**3**) or **VI** (**2**). The antioxidant effectiveness can be stated as follows, in decreasing order: **1 > 3 > 2**. These samples were all of a concentration of 100 mg/ml.

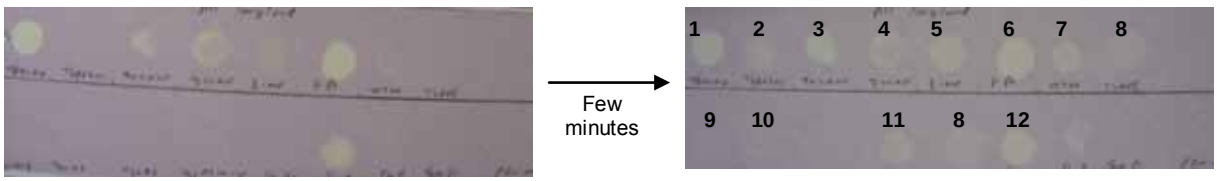


Fig. 5.5. One mg/ml concentrations of all the compounds used in this study were spotted and the reaction with DPPH assessed. Illustrated is the change in colour and thus antioxidant capability of the samples over time. Trolox (**1**) = FA (**6**) = **III** (**12**) > 3-HAA (**4**) = 8HQ (**5**) = totarol (**3**) > totarol dimers (**7**) = **IV-V-VI** mixture (**11**) > tyrosol (**2**) = **IV** (**8**) > **I** (**10**) = **VI** (**9**).

5.3.3.1 Antioxidant activity of tyrosol and its derivatives

The study by Ncanana (2007) showed that the tyrosol radicals generated by the action of laccase were very reactive and polymerised rapidly under the conditions used. The high reactivity of tyrosol radicals was attributed to the *para* position of the hydroxyl group (Ncanana, 2007). Laccase-catalysed oxidation of tyrosol to a dimeric or polymeric product resulted in compounds that were more active AOs than tyrosol. AO studies by Ncanana (2007), suggested that DPPH radical scavenging by tyrosol was based on HAT. It was determined that the hydroxyl group in tyrosol donated a hydrogen atom to the DPPH radical. For compound **I** (also isolated by Ncanana, 2007), it was proposed that DPPH radical scavenging was based on the electron-coupled proton transfer (ET) method rather than HAT, attributed to the fact that product **I** bears no phenolic hydroxyl group in its structure that could donate hydrogen by HAT. The conclusions drawn from the study by Ncanana (2007) were that laccase reactions yield new structures showing different AO activities when compared to their parent compounds, and that the increase or decrease in activity was influenced by the structural configuration of the products.

In this study, tyrosol, spotted on TLC plates (section 5.2.6), reacted slowly with DPPH, requiring several minutes for the reaction to occur. The concentrations of 5 and 2 mg/ml were particularly slow and the 1 mg/ml sample was weakly positive after a few hours. The most effective concentration range was 100 mg/ml to 10 mg/ml for tyrosol. The product of the laccase-catalysed oxidation of tyrosol, **I**, was also investigated. Compound **I**, alone or in a mixture, spotted or developed on TLC, showed strong antioxidant activity when the TLC plates were stained with DPPH. It was clear that compound **I** was a better antioxidant than tyrosol at the same concentration (100 mg/ml), as the reaction of compound **I** with DPPH was immediate and a strong colour change was observed compared to tyrosol. This is in agreement with the literature (Ncanana, 2007).

Tyrosol-acetate (**IV**), spotted on TLC plates, reacted very slowly with DPPH, requiring several minutes for the reaction to occur. The concentration of 5 mg/ml was particularly slow and the 2 and 1 mg/ml samples were weakly positive after a few hours. The most effective concentration range was 100 mg/ml to 10 mg/ml for compound **IV**, similar to that of tyrosol, supporting previous reports in the literature. In the study by Aissa and co-workers (2007), tyrosol and its monoacetylated derivative (**IV**) showed similar antiradical activity of DPPH, thus the radical scavenging activity of tyrosol was not markedly influenced by the presence of an acyl group. The novel dimeric products (**V** and **VI**) of the laccase-catalysed oxidation of compound **IV** were also spotted on TLC plates and allowed to react with DPPH in solution. Compounds **IV**, **VI**, and a mixture containing compounds **IV**, **VI**, and **V** were tested. The mixture exhibited more potent DPPH activity than compounds **IV** or **VI** alone (Fig. 5.4). Compound **IV** was also more effective than compound **VI**. Unfortunately, a pure sample of **V** was unavailable. However, the development of the mixture on TLC revealed that **V** did show weak DPPH activity similar to that of **VI**. The strong activity of the mixture may be due to synergism.

The tyrosol-tyrosinase reaction mixtures (containing the major product **III** and other minor reaction products) showed strong DPPH activity, spotted or developed on TLC, where it was clear that compound **III** had antioxidant activity. Antioxidant activity is influenced by the degree of hydroxylation and this compound contains two hydroxyl groups on the phenolic ring.

Trolox was used as a positive control for antioxidant experiments. Trolox showed strong DPPH activity with an immediate reaction when samples over a concentration range were spotted on TLC and the TLC plate stained with DPPH [100 mg/ml (0.4 M) to 1 mg/ml (0.004 M)].

5.3.3.2 Antioxidant activity of totarol and its derivatives

The increased number of hydroxyl groups in the C-C totarol dimer did not result in an increase in AO activity (Ncanana, 2007). It was suggested that the decreased AO activity might be attributable to the bulky structure and conformation of the C-C dimer, which would confer poorer availability of hydroxyl groups able to donate the hydrogens required to quench the radicals. Furthermore, the bulky dimeric reaction products did not react further with other radicals in the medium, due to steric hindrance. Although the biological activity of the C-O totarol dimer was not evaluated in the study by Ncanana (2007), it was suggested that on the basis of the C-C dimer properties, that its AO activity would be lower than that of the C-C totarol dimer. The reason for this assumption is that the C-C totarol dimer possesses two hydroxyl groups whereas the C-O totarol dimer has only one hydroxyl group.

As illustrated in the study by Ncanana (2007), increasing the number of hydroxyl groups by dimerisation or polymerization of phenolic structures does not necessarily lead to higher AO activity. Nevertheless, AO activity is linked to structural configuration. The structure of a compound with higher AO activity should possess low steric hindrance, thereby ensuring the availability of hydroxyl groups that can donate hydrogens. Laccase reactions favouring the formation of C-C linkages rather than C-O linkages of monomers may be preferred as they may allow more electron delocalisation, a characteristic that may lead to higher AO activity (Ncanana, 2007).

Staining of TLC plates using DPPH showed that totarol was a very potent antioxidant as the reaction with DPPH occurred immediately and the colour change was intense. The concentrations tested [100 mg/ml (0.17 M) to 1 mg/ml (0.0017 M)] were strongly positive with DPPH. All samples reacted immediately except for the sample of 1 mg/ml, which reacted slower, giving a positive reaction within several minutes (Fig. 5.3). Samples containing both the C-C and C-O dimers of totarol were spotted on TLC and developed using the desired solvent system. When stained with DPPH, it was clear that both dimers exhibited AO activity over the concentration range tested (100 – 1 mg/ml) with the colour change caused by the C-C dimer occurring before that of the C-O dimer, supporting the assumption by Ncanana (2007) that the C-O dimer possesses lower AO activity than the C-C dimer. The C-C dimer, being the major product of the reaction, is found in a greater quantity than the C-O dimer. The colour change initiated by the dimers required several minutes. Spotted samples of the dimers mixture showed that 1 mg/ml was the slowest to cause a colour change of the DPPH.

5.3.3.3 Antioxidant activity of 8-HQ, FA, and 3-HAA

Soluble phenolics, with AO and antimicrobial activity, are being utilised as food additives (Garrote, *et al.*, 2004). Laccases can also be used in the food or pharmaceutical industries for the production of phenolic colorants. Mustafa and co-workers (2005) synthesised ferulic acid (FA) colorants by using an industrial laccase named Suberase® isolated from the fungus *Myceliophthora thermophila* (Novo Nordisk A/S, Bagsvaerdt, Denmark) in aqueous and biphasic reaction media. The properties of these compounds suggest their use as food colorants for the replacement of synthetic colorants like tartrazine. Furthermore, the stability of these compounds also allows for their use in cosmetics (Mustafa, *et al.*, 2005). Laccase-catalysed oxidation of ferulic acid also results in the production of dehydrodimers, which can be oxidised further. Certain dehydrodimers formed from oxidative coupling of FA have been reported to be more effective AOs than FA itself (Ward, *et al.*, 2001). 3-HAA is also a powerful radical scavenger (Christen, *et al.*, 1992) and both 3-HAA and CA have been shown to be potent antibacterial agents (Hiramatsu, *et al.*, 2008).

In the study by Ncanana and Burton (2007) quenching of DPPH indicated that the kinetic behaviour of poly(8-HQ) was rapid in terms of AO activity relative to other known AOs. Monomeric AOs are currently used in the manufacturing of materials such as plastics but their preservative properties are known to be lost over time due to physical loss by leaching and evaporation. It is likely that a less mobile AO such as poly(8-HQ) would be less readily lost and may enhance the stability of such materials (Ncanana and Burton, 2007).

Staining of TLC plates with DPPH showed that 8-HQ, 3-HAA, and FA have antioxidant activity, in accordance with literature reports. Spots of FA [100 mg/ml (0.51 M) to 1 mg/ml (0.0051 M)] were strongly positive, showing an immediate reaction with DPPH. 8-HQ yellow spots were visible within a few minutes, with the concentrations ≤ 10 mg/ml becoming visible later. Spots of 3-HAA reacted immediately except for the concentration of 1 mg/ml, which was weakly positive after a few hours.

Samples of poly(8-HQ) were developed on TLC plates using the desired solvent system, after which the TLC plates were submerged in DPPH. The polymeric material was weakly positive after a few hours. Samples of poly(8-HQ) were also spotted on TLC, undeveloped, and gave a weakly positive result with DPPH.

Samples of laccase-catalysed reactions of 3-HAA were also developed on TLC plates. Unconverted starting material, *i.e.* 3-HAA, was immediately reactive with DPPH, turning yellow. As previously illustrated in Figure 4.24, the oxidative coupling of 3-HAA to form CA, a 3-HAA dimer, was catalysed by laccase, and a polymeric compound of unknown structure was formed as a product when strain 044 30-1 laccase was used. The polymers may be a combination of 3-HAA trimers and tetramers (Iwahashi, 1999). Neither CA (an AO), nor the polymers, gave a positive reaction with DPPH using this method. When these samples were only spotted on TLC, and not developed using a solvent system,

the reaction with DPPH was positive. This may be due to only the presence of the unconverted 3-HAA since, when the material was separated, as discussed above, only 3-HAA gave a positive result.

Mixtures of polymers of 3-HAA and 8-HQ were not used for the antioxidant assays (LDL and DPPH assay) or for some antimicrobial tests as samples of the products were in aqueous solution and their masses could not be determined. All other samples could be used as they could be dried and dissolved in organic solvent. FA, 3-HAA, 8-HQ, and trolox are all good antioxidants, as shown by their ability to inhibit LDL oxidation and quench the DPPH radical (to be discussed in 5.3.5 below).

The concentration allowing for the best visual discrimination between antioxidant samples when spotted on TLC and stained with DPPH was 1 mg/ml (Fig. 5.5). Based on the time taken for the colour change to occur, it was concluded that the antioxidant scavenging capability of the test compounds was in the order: Trolox = FA = **III** > 3-HAA = 8-HQ = totarol > totarol dimers = **IV-V-VI** mixture > tyrosol = **IV** > **I** = **VI**.

5.3.4 Comparison of antioxidant activities of test compounds: The DPPH and LDL assays

5.3.4.1 The DPPH assay

For the DPPH assay, all of the test compounds showed slow AO kinetic behaviour (Figures 5.6 and 5.7), *i.e.* the kinetics of the AO reaction with the DPPH radical shows that steady state is only reached after 1 – 6 hours (Brand-Williams, *et al.*, 1995). Researchers have identified FA and other phenolic compounds as belonging to the group of AO compounds displaying slow kinetic behaviour. The kinetics of such reactions involving phenolic compounds was found to be complex. It has been suggested that compounds with slower reaction kinetics, which take longer to reach steady state, are more beneficial as more free radicals are quenched. This is shown in Table 5.3 below for the compounds investigated in this study. Furthermore, it has been proposed that slower reaction kinetics are more beneficial *in vivo*, as AOs that possess activity over a longer period of time would provide sustained AO protection against a variety of cellular ROS (Chigorimbo-Murefu, 2007).

Based on the time taken to reach steady state in the DPPH assay, the following can be concluded regarding the AO effectiveness of the test compounds (in increasing AO capability): **III** B < FA < **IV** < Trolox = totarol dimers < **I** < 8-HQ = totarol = tyrosol = **VI** = **IV-V-VI** mix < 3-HAA < **I** mix.

In general, the products of the laccase-catalysed biocatalysis reactions continued reacting with the DPPH radical for longer than the parent compounds. The reaction involving the totarol dimers reached steady state before totarol, the parent compound, suggesting that the totarol dimers were less effective AOs than totarol itself. Compound **IV** reached steady state before compound **VI** and the **V-VI** mix, suggesting that the dimeric products of the laccase-catalysed oxidation reaction of compound

IV are more effective AOs. The **I** mixture, containing tyrosol and product **I**, reached steady state later than tyrosol or purified compound **I** alone. This result may be due to synergism. Compound **III** mixes reached steady state far sooner than the other compounds tested, suggesting that compound **III** cannot quench as much DPPH radical as the other compounds tested and may be a weaker AO as evaluated by this assay.

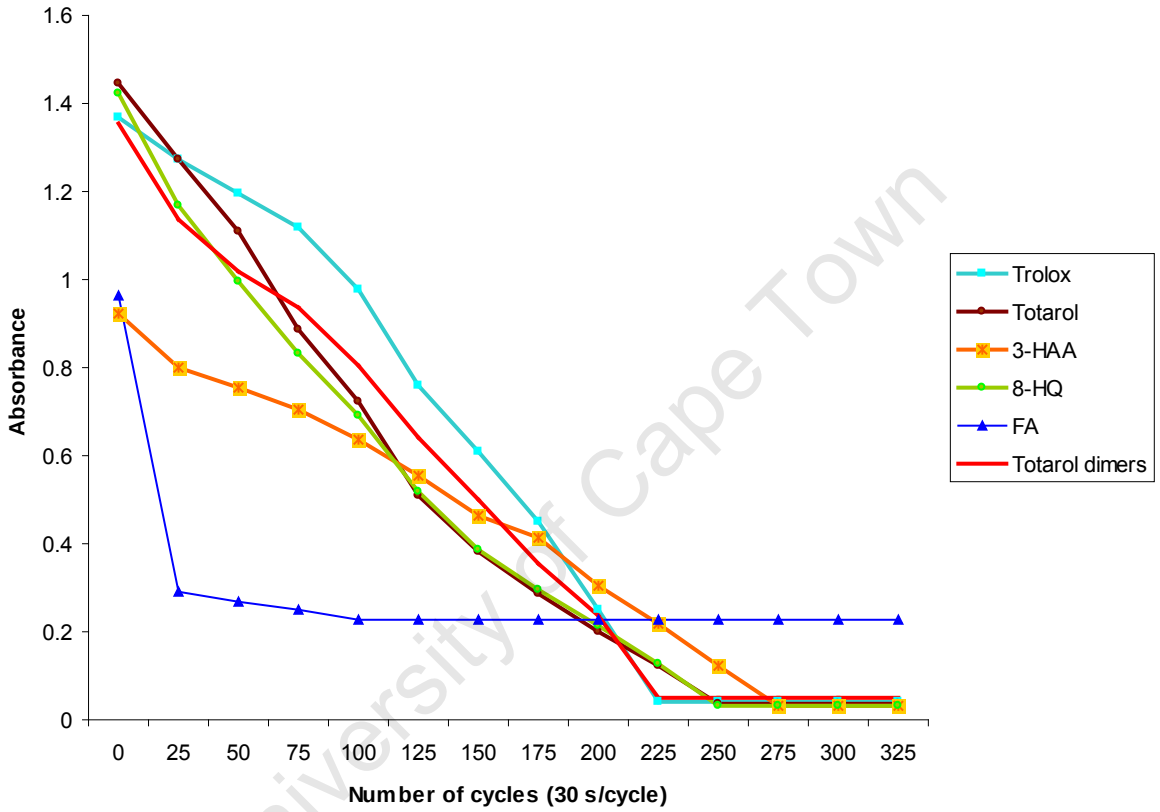


Fig. 5.6. Quenching of the DPPH radical by test compounds at a concentration of 1 mg/ml. A microtitre plate reader was used for this assay and readings were taken every 30 seconds.

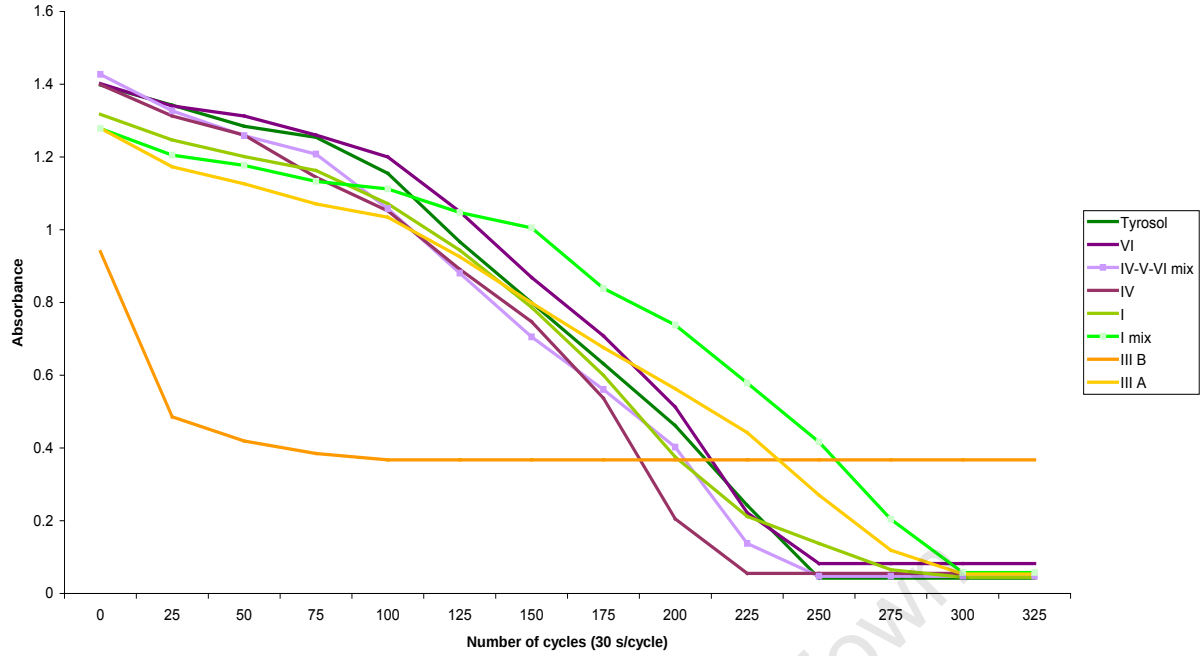


Fig. 5.7. Quenching of the DPPH radical by test compounds (at a concentration of 1 mg/ml) as measured using a microtitre plate system.

Table 5.3. Summary of the DPPH radical quenching activities of the test compounds.

Test compound:	Time taken to reach steady state (in min):	% DPPH remaining:
Trolox	112.5	3
3-HAA	137.5	3.6
8-HQ	125	2.2
FA	50	23.4
Totarol	125	2.5
Totarol dimers	112.5	3.5
Tyrosol	125	3
I	120	3.4
I mix	150	4.5
IV	106	4
VI	125	5.9
IV-V-VI mix	125	3.3
III A	150	4.2
III B	20	39

5.3.4.2 The LDL oxidation assay

For the LDL assay, all of the compounds tested, except for totarol, had an absorbance decreasing effect and thus were able to limit lipid peroxidation and conjugated diene formation at varying degrees (Fig. 5.8). The LDL assay may provide a more appropriate indication of the potential use of an AO *in vivo*, for example, in protecting physiological lipids or cell membranes, while the DPPH assay is considered useful in assessing the AO capacity of compounds to be used as, for example, food preservatives (Chigorimbo-Murefu, *et al.*, 2009). Totarol displays AO activity when quenching the DPPH radical but was ineffective in preventing the oxidation of LDL. Thus totarol, as an AO, would not be used for *in vivo* applications but rather as a food preservative. Furthermore, totarol has excellent antimicrobial activity, which makes its use as a food preservative that much more effective. Interestingly, the totarol dimers show very good inhibition of LDL oxidation, but have been shown to be poor antimicrobials due to their bulky structures. Totarol incorporates very efficiently into phospholipid systems, disturbing interactions between membrane phospholipids, and it is because of this mechanism of action that totarol is ineffective in preventing LDL oxidation (Bernabeu, *et al.*, 2002). However, the dimeric products are inefficient at perturbing phospholipids, due to their bulky structures.

As seen with the dimers of totarol, the dimer of **IV** (**VI**) also showed better inhibition of LDL oxidation than the parent compound. It was also apparent that the mixtures of compounds containing **I** and the mixtures containing both **V** and **VI** showed antagonist behaviour, where the mixtures were less efficient at preventing LDL oxidation than the parent compounds or pure product compounds alone.

Naturally occurring AO phenolics strengthen the resistance of LDL to oxidation both *in vitro* and *in vivo*, and the presence of a second phenolic hydroxyl group is responsible for the enhanced inhibitory effect on LDL oxidation by these compounds (Nardini, *et al.*, 1995). This was observed to be true for the mixtures that contained compound **III**, a compound containing two phenolic hydroxyl groups (mix A contained more of compound **III** than mix B). Although **III** was not more effective than the parent compound, tyrosol, in inhibiting LDL oxidation, the **III** mixes were more effective than the other tyrosol biocatalysis derivatives. Phenolic compounds possessing multiple hydroxyl groups, particularly 3′,4′ *o*-dihydroxy groups (such as compound **III**), are generally the most effective AOs in preventing LDL oxidation (Meyer, *et al.*, 1998). However, little is known about how the variations in the remaining molecular structure of such phenols affect AO activity towards LDL. Furthermore, it has been noted that the different AO capacity of phenolics decreases with their esterification (Nardini, *et al.*, 1995). This was observed for compound **IV**, the monoacetylated derivative of tyrosol, which was a less effective AO than tyrosol.

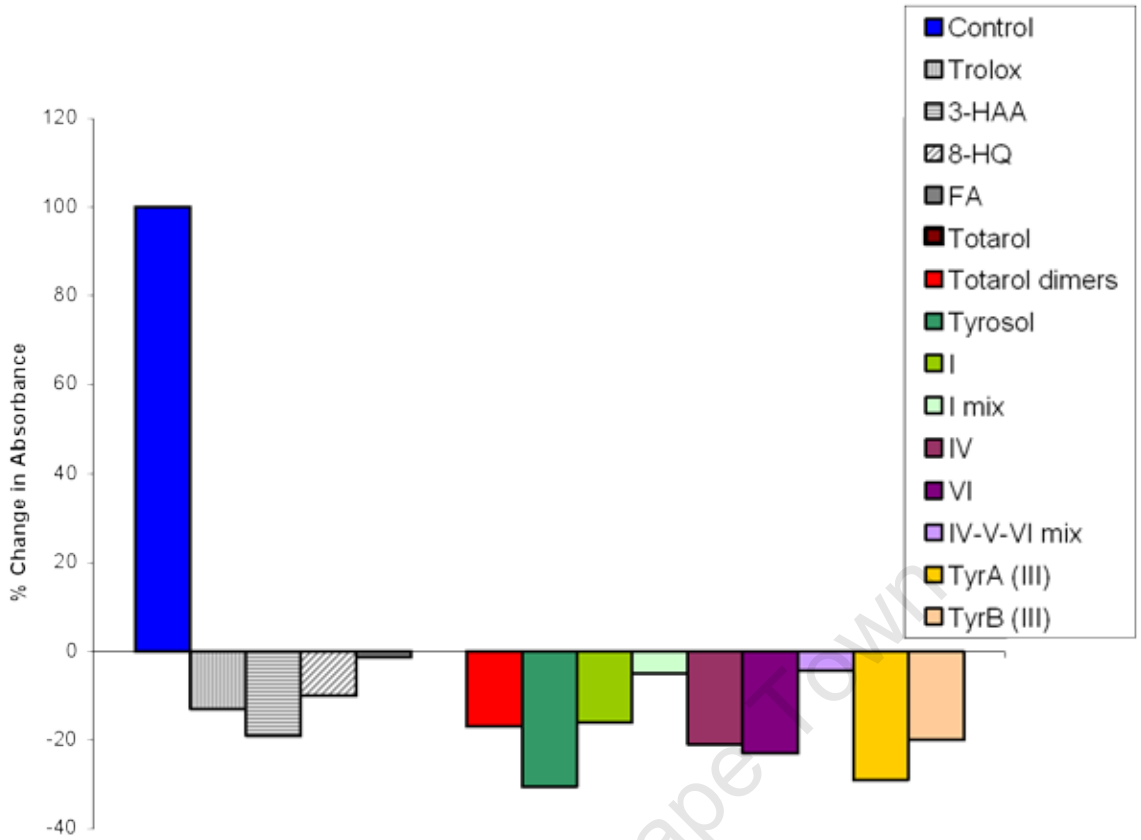
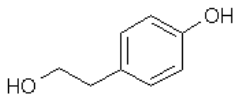
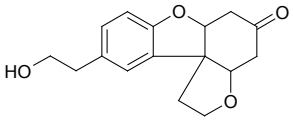
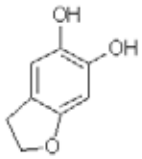
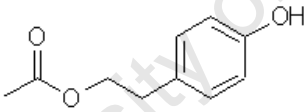
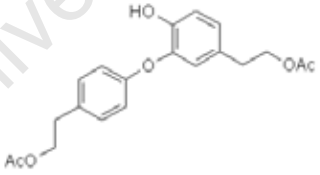
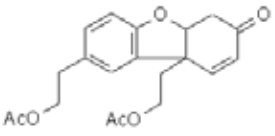


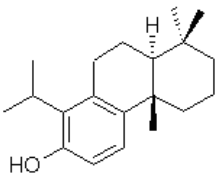
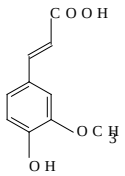
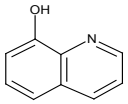
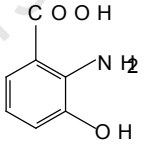
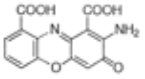
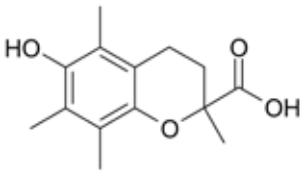
Fig. 5.8. All of the test compounds (except totarol) at a concentration of 1 mg/ml were able to prevent the oxidation of LDL (*i.e.* prevent conjugated diene formation). The control contained methanol in place of antioxidant test sample. This experiment was performed in duplicate.

5.3.5 Summary of the biological activities of the compounds investigated during this study

The aim of this study was to perform a preliminary assessment of the biological activities of the various compounds investigated throughout this study. The antioxidant and antimicrobial properties of the products of laccase-catalysed oxidation reactions, as well as the parent compounds used as the substrates for these reactions, are described in Table 5.4 below.

Table 5.4. Summary of the biological activity of the compounds investigated in this study.

Compounds tested:	Structure:	Biological Activities:
Tyrosol MW: 138.17 g/mol		Antimicrobial activity. MIC against *E. coli* and *E. faecium* is < 0.1 mg/ml. MIC against *Micrococcus* and *M. aurum* is 1 mg/ml. AO activity: Effective at inhibiting LDL oxidation. Slow but effective AO kinetics in quenching the DPPH radical.
I MW: 274 g/mol		Antimicrobial activity. MIC against *E. coli* and *E. faecium* is < 0.1 mg/ml. MIC against *Micrococcus* and *M. aurum* is 1 mg/ml. Inhibition of LDL oxidation poorer than tyrosol. Better quenching of the DPPH radical than tyrosol.
Tyrosol-tyrosinase reaction mix containing **III**		Antimicrobial activity. MIC against *E. coli* and *E. faecium* is < 0.1 mg/ml. MIC against *Micrococcus* and *M. aurum* is 1 mg/ml. Better inhibition of LDL than tyrosol attributed to two phenolic hydroxyls. Less effective at quenching the DPPH radical.
IV MW: 180.17 g/mol		Antimicrobial activity. MIC against *E. coli* and *E. faecium* is < 0.1 mg/ml. MIC against *M. aurum* is 20 mg/ml and 5 mg/ml against *Micrococcus*. AO activity: Effective at inhibiting LDL oxidation. Slow but effective AO kinetics in quenching the DPPH radical.
V MW: 358 g/mol		No sample of pure TyrAc1. Antimicrobial and AO activity observed when mixture developed on TLC.
VI MW: 358 g/mol		Better antimicrobial activity than tyrosol-acetate. MIC against *E. coli* and *E. faecium* is < 0.1 mg/ml. MIC against *Micrococcus* and *M. aurum* is 1 mg/ml. Better DPPH radical quenching and inhibition of LDL oxidation than tyrosol-acetate.
IV-**V**-**VI** mixture		Better antimicrobial activity than tyrosol-acetate. MIC against *E. coli* and *E. faecium* is < 0.1 mg/ml. MIC against *Micrococcus* and *M. aurum* is 1 mg/ml. Better DPPH radical quenching and inhibition of LDL oxidation than tyrosol-acetate.

Totarol MW: 286.45 g/mol		Antimicrobial activity. MIC against *E. coli*, *E. faecium*, and *M. aurum* is < 0.1 mg/ml. MIC against *Micrococcus* is 1 mg/ml. AO activity: Cannot inhibit LDL oxidation. Slow but effective AO kinetics in quenching the DPPH radical.
Totarol C-C and C-O dimers mixture MW of each dimer: 593.43 g/mol		Antimicrobial activity. MIC against *E. coli*, *E. faecium*, and *M. aurum* is < 0.1 mg/ml. MIC against *Micrococcus* is 1 mg/ml. AO activity: More effective at inhibiting LDL oxidation than totarol. Slow but effective AO kinetics in quenching the DPPH radical.
Ferulic acid MW: 194.19 g/mol		Antimicrobial activity. MIC against *E. coli*, *E. faecium*, and *M. aurum* is < 0.1 mg/ml. MIC against *Micrococcus* is 1 mg/ml. AO activity: Effective at inhibiting LDL oxidation. Slow but effective AO kinetics in quenching the DPPH radical.
8-Hydroxyquinoline MW: 145.16 g/mol		Antimicrobial activity. MIC against *E. coli*, *E. faecium*, and *M. aurum* is < 0.1 mg/ml. MIC against *Micrococcus* is 1 mg/ml. AO activity: Effective at inhibiting LDL oxidation. Slow but effective AO kinetics in quenching the DPPH radical.
8-HQ polymers		Antimicrobial activity. Effective against all test bacteria. AO activity: Quenches the DPPH radical.
3-HAA MW: 153.14 g/mol		Antimicrobial activity. MIC against *E. coli*, *E. faecium*, and *M. aurum* is < 0.1 mg/ml. MIC against *Micrococcus* is 1 mg/ml. AO activity: Effective at inhibiting LDL oxidation. Slow but effective AO kinetics in quenching the DPPH radical.
3-HAA products mixture		Antimicrobial activity. CA effective against all test bacteria. AO activity: Quenches the DPPH radical.
Control: Trolox (Vitamin E analogue) MW: 250.3 g/mol		Antimicrobial activity. MIC against *E. coli*, *E. faecium*, and *M. aurum* is < 0.1 mg/ml. MIC against *Micrococcus* is 1 mg/ml. AO activity: Effective at inhibiting LDL oxidation. Slow but effective AO kinetics in quenching the DPPH radical.

5.4 Conclusion

The aim of this study was to investigate the biological activities, specifically the antioxidant and antimicrobial activities, of compounds of interest. These compounds were those used as substrates in laccase-catalysed oxidation reactions, as well as the products of the biocatalysis reactions described in Chapter 4. It is possible that the laccase-catalysed dimerization of phenolic compounds with documented antioxidant activity leads to the formation of reaction products with enhanced antioxidant activities, as seen with the novel products, **V** and **VI**, the resultant dimers of the laccase-**IV** reaction.

The biocatalysis reaction products of the laccase-catalysed oxidation of tyrosol and monoacetylated tyrosol (**IV**) showed better antioxidant activity than the parent compounds, as determined by the DPPH and LDL assays, showing that the dimeric derivatives of laccase-catalysed reactions with phenolic compounds may have enhanced AO capabilities. In agreement with the literature, the DPPH radical activity of tyrosol and compound **IV** were similar (Aissa, *et al.*, 2007), however, compound **IV** did exhibit poorer inhibition of LDL oxidation. The novel dimers, **V** and **VI**, showed better antimicrobial activity than the parent compound, **IV**, revealing that these dimeric products could be used as antibiotics as well as antioxidants.

According to the Tuberculosis Antimicrobial Acquisition and Coordinating Facility of the Southern Research Institute (www.taacf.org), Trolox is listed in the library of compounds with antibacterial activity against *Mycobacterium tuberculosis* H37Rv, but none of the other compounds in this study have been previously investigated. Thus, based on the results of this study, it would be of interest to investigate these compounds further given their potential as antitubercular agents. Interestingly, the clinical isolate belonging to the genus *Micrococcus* showed the most resistance to the compounds tested as antimicrobials in this study, illustrating that *Micrococcus* is also of pertinent concern as a clinical pathogen.

Chapter 6
General Discussion, Conclusions, and Recommendations for Future Work

The focus of this study was to investigate an environmental actinomycete strain capable of producing a laccase enzyme. The fermentation conditions were optimised for maximal laccase production by the chosen native strain. The end goal of this study was to obtain novel antioxidant compounds achieved through laccase biocatalysis.

The specific objectives of this study were:

(a) To develop a screening protocol for the detection of the production of laccase by actinomycete strains isolated from unique environments.
(b) To investigate the variables influencing laccase production by the native strains.
(c) To investigate laccase production by a single actinomycete isolate (*Micromonospora* sp. 044 30-1); to determine the variables influencing laccase production by this strain, and to characterise the laccase.
(d) To investigate biocatalysis reactions using a fungal laccase and the laccase from actinomycete strain 044 30-1, with the aim of comparing them, and acquiring novel dimeric reaction products with potential antioxidant capabilities.
(e) To determine the biological activities of the biocatalysis products of laccase-catalysed oxidation reactions.

The objectives were accomplished, and the major findings of this study are summarised as follows:

(a) A screening protocol for the detection of laccase production by environmental isolates was developed. This initial screen investigated laccase production by the strains in 5 different types of media, each medium also being made to investigate the influence of a range of pH (pHs 5, 7, and 9). Fungal strains, as well as actinomycete strains from unique habitats, were investigated for their ability to produce intracellular or extracellular laccase. The strains differed greatly in their preferences for type of medium and pH. Based on searches of the literature and of databases, including BRENDA (www.brenda.uni-koeln.de), the comprehensive enzyme information system, laccase production by members of the rare actinomycete genera (*Amycolatopsis*, *Gordonia*, *Rhodococcus*, *Mycobacterium*, and *Micromonospora*) investigated in this study had not been previously reported.

(b) The variables influencing laccase production by the native strains included oxygenation and induction. Oxygenation was investigated by altering the agitation speeds of the shakers holding the shake flask cultures, as well as by the use of cotton wool bungs for increased aeration of cultures, and

static conditions. The strains' requirements for oxygenation differed. Many of the actinomycete strains examined in the current study showed optimal production of laccase under static conditions, suggesting that oxygen-dependent production of laccase did not occur (Luke and Burton, 2001). Induction was investigated by determining the effects of low molecular weight inducing compounds, supplemented to the cultures, on laccase production by the strains. In agreement with the literature (Giardina, *et al.*, 2009; Cordi, *et al.*, 2007; Bertrand, *et al.*, 2002; Galhaup, *et al.*, 2002; Eggert, *et al.*, 1996), xylidine was found to be an excellent inducer of laccase production by the actinomycete strains investigated in this study, while sodium azide did not inhibit laccase production by the strains (Dubé, *et al.*, 2008; Molina-Guijarro, *et al.*, 2009). Laccase production by *Streptomyces* strain 7H1 and *Streptomyces antibioticus* was enhanced by 44- and 43-fold, respectively, when xylidine was added to cultures as an inducer.

(c) A single actinomycete strain was chosen for further study. This strain, designated *Micromonospora* sp. 044 30-1, was isolated from a unique marine environment. The best medium for the production of extracellular laccase by this strain was M172F medium, at pH 5, supplemented with 2.0% NaCl, and 8 mM $CuSO_4$. The strain was grown under these conditions in airlift reactors to attain large quantities of the extracellular laccase enzyme. This is the first report of the application of a *Micromonospora* strain in an ALR specifically for the production of laccase. The role of laccase in this strain may be linked to sporulation. Bacterial laccases have been documented to play a role in sporulation processes, including the biosynthesis of the brown spore pigment, and in resistance to copper and phenolic compounds (Giardina, *et al.*, 2009; Molina-Guijarro, *et al.*, 2009). The role of Cu^{2+} in the enhanced activity of *Micromonospora* sp. 044 30-1 laccase is unclear and it would be of interest to pursue this further.

(d) Biocatalysis reactions were investigated, using the laccases from the fungus *Trametes versicolor* and the actinomycete *Micromonospora* sp. 044 30-1 as biocatalysts. The target compounds for conversion were tyrosol, monoacetylated tyrosol, and totarol, and the major reaction products obtained for each target reaction using the different laccases were identical. The laccase-catalysed oxidation of monoacetylated tyrosol was a novel reaction and two dimeric reaction products of novel structure were obtained.

(e) The reaction products of the laccase-catalysed biocatalysis reactions, as well as the parent compounds used as laccase substrates, were investigated for biological activity, specifically antioxidant and antimicrobial activities. All the compounds investigated showed promise as potential antitubercular agents as they exhibited antimicrobial activity against *Mycobacterium aurum*. All the compounds examined had antioxidant activities, as revealed by the DPPH and LDL assays, indicating the potential use of these compounds as antioxidants for medicinal purposes as well as preservatives in foods. The dimeric products **V** and **VI** of the novel reaction involving the laccase-catalysed dimerization of monoacetylated tyrosol showed improved antioxidant capability, and antimicrobial activity, compared to the parent compound, illustrating that laccase-catalysed reactions targeting known antioxidant compounds can produce new compounds with enhanced biological activity.

6.1 General discussion

The secondary screening protocol makes use of relevant variables to be investigated in order to assess whether a strain producing laccase should be further pursued. The protocol is useful at laboratory scale where many strains are screened.

The majority of past research on laccases has involved the study of fungal laccases. Only recently have laccases from actinomycetes received attention, and little is known concerning the factors influencing laccase production by these microbes. While the fungal strains secrete laccase reproducibly and consistently in large quantities, with high activity, and are stable in their extracellular form, the actinomycete bacterial laccases investigated in this study were produced in small quantities with low activities and were fairly unstable, making purification difficult. Reproducible production of laccases by the actinomycetes investigated in this study was difficult to achieve in shake flask cultures. For actinomycete strain 044 30-1, production of laccase in ALRs was found to be reproducible.

During this project, initial difficulties obtaining laccase from the native actinomycete bacterium *Micromonospora* sp. 044 30-1 resulted in biocatalysis pursuits using *Trametes versicolor* (fungal) laccase. Novel dimeric products were obtained during biocatalysis studies and new information describing the biological activities of these and other biocatalysis products are presented.

At a later time in the course of this project, biocatalysis reactions using the laccase from the desired strain, *Micromonospora* sp. 044 30-1, were performed and the same novel dimeric products were obtained. Furthermore, the laccase from this actinomycete strain was used as a biocatalyst in the same reactions investigated using *Trametes versicolor* laccase, and the same reaction products were obtained for these various reactions. This may suggest that the active site and mechanism of action of the laccase from bacterial strain 044 30-1 may be similar to that of the laccase from fungal strain *Trametes versicolor*. It must be noted that the use of *Micromonospora* sp. 044 30-1 laccase in such biocatalysis reactions has yet to be optimised. This would involve the investigation of many factors including kinetics, the time course of conversion to product, the yield of product obtained (assessed using HPLC), solvent engineering, and the proportions of components (aqueous and organic) to be used in biphasic reactions, including the amount of starting material to be used. Furthermore, immobilisation techniques could be exploited. The use of the extracellular enzyme only, cells only, or both components would need to be further clarified for use in such applications. This further research would provide information determining the type of reactor configuration to be used (*e.g.* continuous) and immobilisation technique.

The structure of Tyr01 (**I**) had been previously reported in the literature but the structures of TyrAc1 (**V**) and TyrAc2 (**VI**) had not. Furthermore, in the current study, the process to obtain product **I** was more favourable than that previously reported by Vinciguerra and co-workers (1997). The temperature used was milder, and a higher yield of product was obtained at an earlier time. Searches of the

literature and chemical databases confirmed that compounds **III**, **V**, and **VI** had not previously been described. New biological data are reported revealing that these compounds possess good antioxidant and antimicrobial capabilities. Antimicrobial activities reveal the potential use of these compounds as antitubercular agents. Further studies may reveal the potential applications of these compounds in the fields of medicine and food science (as preservatives).

6.2 Recommendations for a pilot bioprocess

This study has revealed that actinomycete strain *Micromonospora* sp. 044 30-1 could be used in a bioprocess for the production of laccase enzyme to be used as a biocatalyst for the synthesis of dimeric phenolic compounds with enhanced antioxidant properties. Many bioprocess variables would need to be further investigated and optimised for a full-scale industrial bioprocess. For use in a pilot bioprocess, strain 044 30-1 could be inoculated into an airlift reactor containing modified M172F medium at pH 5 supplemented with 2.0% NaCl and 8 mM $CuSO_4$. As demonstrated in this study, the fermentation in the 4 L ALR should proceed for 36 hours, at which point laccase activity should be checked using DMP as the assay substrate. Then the contents of the ALR should be emptied into a suitable container and centrifuged to remove the cells from the growth medium (supernatant). The extracellular laccase, found in the supernatant, is the crude extract and can be used in biocatalysis reactions. This extracellular laccase sample can be dialysed against water to remove the Cu, salts and other media components. In this study, the novel reaction of the laccase-catalysed oxidation of monoacetylated tyrosol to two dimeric reaction products was described. The crude extract described above would be added to monoacetylated tyrosol dissolved in ethyl acetate, and the reaction allowed to proceed at 30°C with mild shaking. The dimeric products of this reaction are obtained by extraction with ethyl acetate, and are separated (and purified) using flash chromatography (7 petroleum ether: 3 ethyl acetate). This study has shown that these dimeric products have enhanced antioxidant properties compared to the parent compound and could find application as antioxidants and as antimicrobials.

Since much of this study has focused on achieving the optimal medium for laccase production by the native strain, some comments can be made about the media components and their use on an industrial scale. The medium is currently a rich medium, which could be very expensive. Creating a continuous system, as opposed to the batch culture used now, would allow for reuse of the starch and salt. A less rich medium could be used but finding this medium might take months of testing and should prove better than the current medium if it is to be used. The copper in the medium is of a high quantity but it is well-documented in the literature that the Cu^{2+} can be removed from the aqueous medium and reused. These techniques are cost effective, easy to use, fast, and commercially available, and thus deal with the issue of environmental hazards and green processes (He, *et al.*, 2008, and references therein; Piacquadio, *et al.*, 1997).

Despite the numerous advantages that prokaryotic (bacterial) enzyme production may offer over eukaryotic (fungal) production, industrial use of bacterial laccase is limited by extremely low production yield (Dubé, *et al.*, 2008). Improvement of production yield would have a notable impact on bacterial laccase use in a variety of applications. To further enhance the production of *Micromonospora* sp. 044 30-1 laccase, the gene for the enzyme should be cloned. Some primers for laccases, and BMCOs, have been suggested in the literature (Giardina, *et al.*, 2009; Molina-Guijarro, *et al.*, 2009; Dubé, *et al.*, 2008; Solioz and Stoyanov, 2003) as well as *Micromonospora-Streptomyces*, *Micromonospora-E. coli*, and *Streptomyces-E. coli* cloning vectors (Li, *et al.*, 2003, and references therein). Cloning vectors have been developed from several actinomycete plasmids, and these vectors have been extensively used as they can integrate into numerous actinomycete genera and species (Alexander, *et al.*, 2003; Hosted, *et al.*, 2005; Oshida, *et al.*, 1986). The rare actinomycetes (other than *Streptomyces*) are continually screened for useful bioactive compounds, such as extracellular enzymes, and *Micromonospora* species are a thriving resource for industrial screening programs as they are abundant producers of many interesting natural products (Li, *et al.*, 2003). Gene cloning systems for these microbes could allow for the overproduction of useful or novel compounds, as well as improved productivity of useful metabolites (Li, *et al.*, 2003). Cloning and expression of genomic related information in heterologous, fast-growing mesophiles such as *E. coli* has resulted in an increased, considerably cheaper commercial production of enzymes (Gianfreda and Rao, 2004). Cloning generally allows for greater expression or over expression of the enzyme, thereby allowing biocatalysis research to be performed on a large scale, as more enzyme will be available. If the enzyme cannot be cloned from the strain, it is an option to immobilize the whole cells.

Immobilisation of the whole cells would suit a continuous process. Furthermore, if cloning of the laccase gene into a bacterium such as *E. coli* is successful, a simple, inexpensive medium can be used and the enzyme will be produced very quickly, due to the nature of *E. coli*. This will allow for a highly efficient process, obtaining large quantities of the desired enzyme (Dubé, *et al.*, 2008).

The overall aim of this study was accomplished, showing that actinomycete bacteria from unique environments are a useful source of laccases for biocatalysis applications. Actinomycete laccases could be used in the laccase-catalysed conversion of phenolic substrates to value-added compounds possessing enhanced biological activity, such as antioxidant capability.

Appendix A: Solutions

A1 SDS-PAGE Solutions

Running Buffer (4 X Stock Solution):

Adjust pH with HCl. Trizma base: Tris(hydroxymethyl)aminomethane.

Ingredients:	Stock Concentration:	Quantity:	Final Concentration:
Trizma base	1.5 M, pH 8.8	181.5 g/l	0.375 M, pH 8.8
Sodium azide	0.1% w/v	1 g/l	0.025 % w/v

Stacking Buffer (4 X Stock Solution):

Adjust pH with HCl.

Ingredients:	Stock Concentration:	Quantity:	Final Concentration:
Trizma base	0.5 M, pH 6.8	60.5 g/l	0.125 M, pH 6.8
Sodium azide	0.1% w/v	1 g/l	0.025 % w/v

10 ml 1 X Denaturing Sample Buffer (for samples which need dilution ≥ 1:4):

β-mercaptoethanol: 50 µl/ml sample buffer.

Ingredients:	Stock Concentration:	Quantity/Volume:	Final Concentration:
4 X Stacking Buffer	0.5 M, pH 6.8	1.25 ml	0.0625 M, pH 6.8
Glycerol	87% v/v	1.15 ml	10% v/v
SDS		200 mg	2% w/v
Bromophenol blue	0.05% w/v	0.2 ml	0.001% w/v
Water		7.4 ml	

10 ml 2 X Denaturing Sample Buffer (1:1):

β-mercaptoethanol: 100 µl/ml sample buffer.

Ingredients:	Stock Concentration:	Quantity/Volume:	Final Concentration:
4 X Stacking Buffer	0.5 M, pH 6.8	2.5 ml	0.0625 M, pH 6.8
Glycerol	87% v/v	2.3 ml	10% v/v
SDS		400 mg	2% w/v
Bromophenol blue	0.05% w/v	0.4 ml	0.001% w/v
Water		4.8 ml	

10 X Electrode Buffer Stock:

Use 40 ml stock + 360 ml water + 400 mg SDS (i.e. 0.1% w/v) = 400 ml total. Shake vigorously immediately.

Ingredients:	Stock Concentration:	Quantity:	Final Buffer Concentration:
Trizma base	0.25 M	30.28 g/l	25 mM, pH 8.5
Glycine	1.92 M	144 g/l	0.192 M
Sodium Azide	0.1% w/v	1 g/l	0.01% w/v

Running Gel (4 ml per gel):

Solution can be degassed with stirring using a vacuum to remove all air.

Pour a layer of isopropanol over the running gel to prevent air entering while the gel sets. Remove the isopropanol before adding the stacking gel.

Add the ingredients listed as 'A' first. Once the ingredients listed as 'B' are added, the gel will begin to set.

Order:	Ingredients:	Stock Concentration:	Quantity:	Gel Concentration:
A	Acrylamide stock	30% T, 4% C	1.33 ml	10% T, 4% C
A	4 X Running Buffer	1.5 M, pH 8.8	1 ml	0.375 M, pH 8.8
B	SDS	4% w/v	100 µl	0.1% w/v
B	APS	40% w/v	16 µl	0.04% w/v
B	TEMED		4 µl	0.1% v/v
A	Double distilled Water		1.57 ml	

APS: Ammonium persulphate

TEMED: N,N,N',N'-tetramethyl ethylenediamine

Stacking Gel (2 ml per gel):

Order:	Ingredients:	Stock Concentration:	Quantity:	Gel Concentration:
A	Acrylamide stock	30% T, 4% C	260 µl	4% T, 4% C
A	4 X Stacking Buffer	0.5 M, pH 6.8	0.5 ml	0.125 M, pH 6.8
B	SDS	4% w/v	50 µl	0.1% w/v
B	APS	40% w/v	8 µl	0.04% w/v
B	TEMED		2 µl	0.1% v/v
A	Double distilled Water		1.19 ml	

Staining Solution:

Coomassie Blue (0.1% w/v):	0.5 g
Ethanol:	200 ml
Double distilled water:	250 ml
Acetic acid:	50 ml

Destaining Solution:

Acetic acid:	0.5 L
Ethanol:	1.5 L
Water:	3 L

A2 Small-scale Organic Chemistry Reactions

Unless otherwise stated all reactions were carried out in the appropriately sized glass vial, with lid secured with Parafilm, and shaken gently at 30°C. These various reactions were carried out in order to assess the formation of reaction products using TLC and to optimise reaction conditions.

Tyrosol-laccase Biphasic Reaction:

Tyrosol (starting substrate material)	10 mg
Laccase (from *Trametes versicolor*)	1 mg
Buffer (Sodium acetate, 20 mM, pH 3.5)	1 ml
Ethyl acetate	1 ml

Tyrosol-tyrosinase Reactions:

Experimental Reaction 1: Aqueous (Ncanana, 2007)

Tyrosol (starting substrate material)	5 mg
Tyrosinase (from mushroom)	1 mg
Buffer (Potassium phosphate, 50 mM, pH 7)	4 ml
Ascorbic acid	34 mg

Experimental Reaction 2: Monophasic

Tyrosol	5 mg
Tyrosinase	1 mg
Buffer	1.6 ml
Acetone	400 µl
Ascorbic acid	34 mg

Experimental Reaction 3: Biphasic (Kazandjian and Kilbanov, 1985)

Tyrosol	5 mg
Tyrosinase	1 mg
Buffer	800 µl
Chloroform	800 µl
Ascorbic acid	34 mg

Experimental Reaction 4: Biphasic Without Ascorbic Acid

Tyrosol	5 mg
Tyrosinase	1 mg
Buffer	800 µl
Chloroform	800 µl

Experimental Reaction 5: Scaled-up Monophasic

Tyrosol	100 mg
Tyrosinase	5 mg
Buffer	32 ml
Acetone	8 ml
Ascorbic acid	680 mg

Experimental Reaction 6: Scaled-up Biphasic

Tyrosol	100 mg
Tyrosinase	5 mg
Buffer	16 ml
Chloroform	16 ml
Ascorbic acid	680 mg

Tyrosol-tyrosinase and Tyrosol-acetate-tyrosinase Reactions

Reaction 1:

Tyrosol (starting substrate material)	50 mg
Tyrosinase	5 mg
Buffer	8 ml
Chloroform	8 ml

Reaction 2:

Tyrosol	60 mg
Tyrosinase	7 mg
Buffer	10 ml
Chloroform	10 ml

Reaction 3:

Tyrosol-acetate (starting substrate material)	5 mg
Tyrosinase	500 µg
Buffer	800 µl
Chloroform	800 µl

Reaction 4:

Tyrosol-acetate	5 mg
Tyrosinase	500 µg
Buffer	800 µl
Chloroform	800 µl
Ascorbic acid	34 mg

Reaction 5: With and without ascorbic acid

Tyrosol-acetate	5 mg
Tyrosinase	1 mg
Buffer	800 µl
Chloroform	800 µl
Ascorbic acid	34 mg

Reaction 6: With and without ascorbic acid:

Tyrosol-acetate	10 mg
Tyrosinase	5 mg
Buffer	1 ml
Chloroform	1.2 ml
Ascorbic acid	34 mg

Tyrosol-acetate-laccase Reactions:

Reaction 1:

Tyrosol-acetate (starting substrate material)	5 mg
Laccase	500 µg
Buffer	500 µl
Ethyl acetate	500 µl

Reaction 2: Scaled-up	
Tyrosol-acetate	100 mg
Laccase	12 mg
Buffer	12 ml
Ethyl acetate	10 ml

Appendix B: The *Micromonospora* Identification Key

Modern bacterial systematics makes use of a polyphasic approach towards taxonomic determinations. This means that an isolate's physiological characters, further descriptive information (such as chemotaxonomy and morphological features by scanning electron microscopy, in the case of actinomycetes), and 16S rDNA sequence analysis all contribute to determining the novelty of an isolate.

When screening environmental samples for new actinomycete species, the novelty of an isolate can be suggested by the sequencing of the isolate's 16S rDNA (a sequence of more than 1450 nucleotides is sufficient, as it represents more that 90% of the length of 16S rRNA genes). However, even a short, partial 16S rDNA sequence is enough to inform a researcher of the genus to which an isolate belongs. For actinomycete researchers in developing countries, such as South Africa, there are difficulties, such as limited financial and technological resources. Due to the number of potentially novel actinomycete isolates obtained from environmental samples, it would be extremely costly to obtain such sequences for all the isolates.

A less expensive option is to examine an isolate's physiological characters. The genus *Streptomyces* has 139 recommended characters that could be examined (Locci, 1989). The genus *Micromonospora* could also be examined to such an extent. However, it is unnecessary to use so many characters to determine the novelty of environmental *Micromonospora* isolates. A few discerning characters can be used.

Using physiological testing to discern quickly between species may be the answer to the problem. Having a defined set of physiological characters to examine would be inexpensive, easy to perform, and could allow one to differentiate between *Micromonospora* isolates. It would be important that for these physiological characters to be tested, the media used be standardized and that every strain be tested on the specific standardized medium for a specific physiological character. Furthermore, the selected tests should allow one to determine whether a newly isolated strain is likely to be a strain of a known species, or a probable new species.

A dichotomous key for the identification of *Micromonospora* strains was developed to allow one to determine easily and inexpensively the novelty of a *Micromonospora* isolate in a period of 21 days from the time of inoculation of the test media. Due to the lack of attention attributed to the genus *Micromonospora*, standardized media were carefully chosen, or developed, for each physiological test to be performed for the proposed identification key.

B1 Materials and Methods

B1.1 Strains

Micromonospora aurantiaca NRRL B-16091^T, *Micromonospora carbonacea* subsp. *carbonacea* NRRL 2972^T, *Micromonospora chalcea* subsp. *chalcea* NRRL B-2344^T, *Micromonospora citrea* NRRL B-16101^T, *Micromonospora echinaurantiaca* NRRL B-16102^T, *Micromonospora echinofusca* NRRL B-

16695^T, *Micromonospora echinospora* subsp. *echinospora* NRRL 2985^T, *Micromonospora fulviviridis* NRRL B-16104^T, *Micromonospora halophytica* subsp. *halophytica* NRRL 2998^T, *Micromonospora inositola* NRRL B-16095^T, *Micromonospora nigra* NRRL 3097^T, *Micromonospora olivasterospora* NRRL 8178^T, *Micromonospora echinospora* subsp. *pallida* NRRL 2996^T, *Micromonospora purpureochromogenes* NRRL B-16094^T, and *Micromonospora rosaria* NRRL 3718^T were kindly provided by Dr David P. Labeda, curator of the Actinobacterial Culture Collection at the U.S. Department of Agriculture Agricultural Research Service, Peoria, Illinois, United States of America. *Micromonospora chersina* DSM 44151^T, *Micromonospora coerulea* DSM 43143^T, and *Micromonospora matsumotoense* DSM 44100^T were purchased from the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. *Micromonospora endolithica* strain AA-459^T was kindly provided by Prof. emer. Dr. Peter Hirsch, Institut für Allgemeine Mikrobiologie, Christian-Albrechts-Universität Kiel, Germany. The following strains could not be obtained and thus information from the publications describing these species was used (see Table B1): *Micromonospora auratinigra, M. chaiyaphumensis, M. chokoriensis, M. coriariae, M. coxensis, M. eburnea, M. krabiensis, M. lupini, M. mirobrigensis, M. narathiwatensis, M. pattaloongensis, M. rifamycinica, M. saelicesensis,* and *M. siamensis*. No sodium chloride tolerance data is available for the following strains (Kroppenstedt, *et al.*, 2005) and these strains were also unavailable to us, thus they were not included in the Key: *M. citrea, M. echinaurantiaca, M. echinofusca, M. fulviviridis, M. inyoensis, M. peucetia, M. sagamiensis,* and *M. viridifaciens*.

Table B1. Validly published *Micromonospora* species used in this study.

	***Micromonospora* Strains:**	**Reference:**
1	*M. aurantiaca*	Goodwin, 2005.
2	*M. auratinigra*	Thawai, *et al.*, 2004.
3	*M. carbonacea* subsp. *carbonacea*	Goodwin, 2005.
4	*M. chaiyaphumensis*	Jongrungruangchok, *et al.*, 2008.
5	*M. chalcea*	Goodwin, 2005.
6	*M. chersina*	Goodwin, 2005.
7	*M. chokoriensis*	Ara and Kudo, 2007.
8	*M. citrea*	Kroppenstedt, *et al.*, 2005.
9	*M. coerulea*	Goodwin, 2005.
10	*M. coriariae*	Trujillo, *et al.*, 2006.
11	*M. coxensis*	Ara and Kudo, 2007.
12	*M. eburnea*	Thawai, *et al.*, 2005.
13	*M. echinaurantiaca*	Kroppenstedt, *et al.*, 2005.
14	*M. echinofusca*	Kroppenstedt, *et al.*, 2005.
15	*M. echinospora* subsp. *echinospora*	Goodwin, 2005.
16	*M. endolithica*	Goodwin, 2005.
17	*M. fulviviridis*	Kroppenstedt, *et al.*, 2005.

18	*M. halophytica* subsp. *halophytica*	Goodwin, 2005.
19	*M. inositola*	Goodwin, 2005.
20	*M. inyonensis*	Kroppenstedt, *et al.*, 2005.
21	*M. krabiensis*	Jongrungruangchok, *et al.*, 2008.
22	*M. lupini*	Trujillo, *et al.*, 2007.
23	*M. matsumotoense*	Goodwin, 2005.
24	*M. mirobrigensis*	Trujillo, *et al.*, 2005.
25	*M. narathiwatensis*	Thawai, *et al.*, 2008.
26	*M. nigra*	Goodwin, 2005.
27	*M. olivasterospora*	Goodwin, 2005.
28	*M. pallida*	Goodwin, 2005.
29	*M. pattaloongensis*	Thawai, *et al.*, 2007.
30	*M. peucetia*	Kroppenstedt, *et al.*, 2005.
31	*M. purpureochromogenes*	Goodwin, 2005.
32	*M. rifamycinica*	Huang, *et al.*, 2008.
33	*M. rosaria*	Goodwin, 2005.
34	*M. saelicesensis*	Trujillo, *et al.*, 2007.
35	*M. sagameinsis*	Kroppenstedt, *et al.*, 2005.
36	*M. siamensis*	Thawai, *et al.*, 2006.
37	*M. viridifaciens*	Kroppenstedt, *et al.*, 2005.

B1.2 Physiological Testing and Media Used Specifically for the *Micromonospora* Identification Key (Table B2)

All cultures were incubated at 28°C – 30°C (unless otherwise stated) for 3 weeks, and were examined at 7, 14, and 21 days. Maximum sodium chloride tolerance (% w/v) was performed as recommended by Locci, 1989, on Bennett's agar (BM) (Atlas, 1993), containing (g/L): glucose (10.0), casitone (Difco; 2.0), yeast extract (1.0), beef extract (1.0), agar (20.0), pH 7.0, and the required amount of sodium chloride. Growth at 37°C was determined on Bennett's agar as recommended by Locci, 1989. The production of diffusible pigments were tested on *ISP* Medium No. 2 (Shirling and Gottlieb, 1966), Bennett's-glycerol agar (10 g/L glucose was replaced with 10 g/L glycerol), and on modified 172F agar, containing (g/L): glucose (10.0), yeast extract (5.0), starch (10.0), casitone (Difco; 5.0), $MgSO_4 \cdot 7H_2O$ (2.5), $CaSO_4 \cdot 2H_2O$ (2.0), agar (15.0), pH 7.2. The utilization of compounds as sole carbon sources (1.0% w/v) was performed as recommended by Locci, 1989, and Shirling and Gottlieb (1966). Growth on *ISP* Medium No. 4 was determined according to the methods of Shirling and Gottlieb (1966).

Table B2. A list of selected physiological tests to be performed and the corresponding standardized media to be used for identification of *Micromonospora* strains.

Physiological Characters to be Tested:	Standardized Media to be Used (Abbreviations in parentheses):
Maximum Sodium Chloride Tolerance (% w/v): 1.5%, 2.0%, 3.0%, 4.0%, 5.0%, 6.0%, and 7.0%. Growth at 37°C.	Bennett's Agar (BM-Glucose)
Production of Diffusible Pigments and, Diagnostic Mycelial Pigment Colour.	Bennett's Agar, with glucose replaced by glycerol (BM-Glycerol); *ISP* Medium No. 2 Agar (YEME); Modified 172F Agar (m172F).
Growth and Colour of Mycelia.	*ISP* Medium No. 4 Agar (*ISP*4)
Utilization of Glycerol (1.0% w/v) and *myo*-Inositol (1.0% w/v) as Sole Carbon Sources [a negative control containing no carbon source, and a positive control, glucose (1.0% w/v) must be included].	*ISP* Medium No. 9 Agar (*ISP*9)

B2 Results and Discussion

B2.1 The Identification Key

The Identification Key is presented in Figure B1 and Tables B3 – B6 below. The Key is based on the 37 validly published members of the genus *Micromonospora* as at January 05, 2010 (Euzéby, 2010).

Before using the *Micromonospora* Identification Key, strains must be identified as members of the genus. Environmental isolates can be identified as strains of the genus *Micromonospora* on the basis of the distinctive colony morphology of this genus.

Maximum sodium chloride tolerance is a very important physiological characteristic of the genus *Micromonospora* as this is the character that most readily differentiates strains into groups. The diagnostic mycelial pigment colour, as well as diffusible pigment colour, are also very significant characters. Since the mycelial pigment colour, prior to sporulation, of the majority of *Micromonospora* strains, is orange, a different mycelial pigment colour is very useful in distinguishing species. The diagnostic mycelial pigment colours of *M. coerulea*, *M. echinospora*, *M. olivasterospora*, and *M. pallida*, and the diffusible pigments of *M. aurantiaca*, *M. carbonacea*, *M. chalcea*, *M. halophytica*, *M. olivasterospora*, *M. purpureochromogenes*, and *M. rosaria*, are well-known by taxonomists as easily identifiable characters of these species. To date, *M. inositola* is the only *Micromonospora* species able to utilize inositol as a sole carbon source, and *M. purpureochromogenes* is the only species able to

utilize glycerol as a sole carbon source. Thus, it was important that these significant characters be included in the identification key. *M. matsumotoense* is the only species that does not sporulate. This is also a significant determinant and was included in the identification key.

1. Maximum Sodium Chloride Tolerance (% w/v) is:				
1.5%: If YES, go to Table B3.	**2.0%:** If YES, go to Table B4.	**3.0%:** If YES, go to Table B5.	**4.0%:** If YES, go to Table B6.	**Less than 1.0% or greater than 5.0%** If YES: Probable New Species
VPM of the 1.0% Group: *M. coriariae* **VPM of the '1.5% Group':** *M. echinospora* *M. purpureochromogenes*	**VPM of the '2.0% Group':** *M. auratinigra* *M. chersina* *M. coerulea* *M. endolithica* *M. inositola* *M. matsumotoense* *M. lupini* *M. saelicesensis*	**VPM of the '3.0% Group':** *M. aurantiaca* *M. carbonacea* *M. nigra* *M. olivasterospora* *M. pallida* *M. rosaria* *M. chaiyaphumensis* *M. chokoriensis* *M. coxensis* *M. krabiensis* *M. mirobrigensis* *M. pattaloongensis* *M. rifamycinica*	**VPM of the '4.0% Group':** *M. chalcea* *M. halophytica* *M. eburnea* *M. narathiwatensis* **VPM of the 5.0% Group:** *M. siamensis*	

Fig. B1. The crucial first step of the identification key is to determine an isolate's maximum sodium chloride tolerance (on BM-Glucose). Illustrated in this scheme is to which 'maximum sodium chloride tolerance group', the validly published members (VPM) of *Micromonospora* belong.

Table B3. Dichotomous key for the identification of *Micromonospora* strains of the '**1.5% NaCl Group**'.

2.1 Is the diagnostic mycelial pigment a **purple** colour on BM-Glucose?	
(a) Yes	Probably *M. echinospora*.
(b) No	Go to 2.2.
2.2 Produces a ***dark* brown** diffusible pigment on BM-Glucose?	
(a) Yes	Go to 2.3.
(b) No	Probable New Species.
2.3 Is glycerol (1.0% w/v) utilized as a sole carbon source?	
(a) Yes	Probably *M. purpureochromogenes*.
(b) No	Probable New Species.

Table B4. Dichotomous key for the identification of *Micromonospora* strains of the '**2.0% NaCl Group**'.

3.1 Growth on *ISP*4?	
(a) Yes	Probably *M. auratinigra*.
(b) No	Go to 3.2.
3.2 Is there an absence of sporulation?	
(a) Yes	Probably *M. matsumotoense*.
(b) No	Go to 3.3.
3.3 Is the diagnostic mycelial pigment a **blue-black** colour?	
(a) Yes	Probably *M. coerulea*.
(b) No	Go to 3.4.
3.4 Is *myo*-inositol (1.0% w/v) utilized as a sole carbon source?	
(a) Yes	Go to 3.6.
(b) No	Go to 3.5.
3.5 Growth at 37°C?	
(a) Yes	Go to 3.7.
(b) No	Probably *M. chersina*.
3.6 Produces **brown** to **orange-brown** diffusible pigments?	
(a) Yes	Probably *M. saelicesensis*.
(b) No	Probably *M. inositola*.
3.7 Is there no growth on Bennett's Medium **and** growth on 2% NaCl is variable?	
(a) Yes	Probably *M. lupini*.
(b) No	Probably *M. endolithica*.

Table B5. Dichotomous key for the identification of *Micromonospora* strains of the '**3.0% NaCl Group**'.

4.1 Produces a **bright yellow** diffusible pigment on m172F?

(a) Yes	Probably *M. aurantiaca*.
(b) No	Go to 4.2.

4.2 Produces a **brown** (but *not* reddish-brown) diffusible pigment on m172F?

(a) Yes	Probably *M. carbonacea*.
(b) No	Go to 4.3.

4.3.Produces a **wine-red** diffusible pigment on m172F **and** YEME agars?

(a) Yes	Probably *M. rosaria*.
(b) No	Go to 4.4.

4.4 Is the diagnostic mycelial pigment an **olive-green** colour on BM-Glucose?

(a) Yes	Go to 4.5.
(b) No	Go to 4.6.

4.5 Produces an **olive-green** diffusible pigment on BM-Glucose **and** YEME agars?

(a) Yes	Probably *M. olivasterospora*.
(b) No	Probable New Species.

4.6 Is the diagnostic mycelial pigment a **pale white/ivory** colour on BM-Glucose?

(a) Yes	Probably *M. pallida*.
(b) No	Go to 4.7.

4.7 Is the diagnostic mycelial pigment a **yellow-white to pale orange** colour on YEME agar?

(a) Yes	Probably *M. pattaloongensis*.
(b) No	Go to 4.8.

4.8 Grows on *ISP*4 producing **orange** mycelia but **does not** produce melanin on *ISP*6 and 7?

(a) Yes	Probably *M. nigra*.
(b) No	Probably *M. krabiensis*.

4.9 Produces scanty, white aerial mycelia?

(a) Yes	Probably *M. rifamycinica*.
(b) No	Go to 4.10.

4.10 Grows at 45°C?

(a) Yes	Probably *M. chaiyaphumensis*.
(b) No	Go to 4.11.

4.11 Is negative for nitrate reduction ***and*** tyrosine degradation?

(a) Yes	Probably *M. mirobrigensis*.
(b) No	Go to 4.12.

4.12 Produces light brown diffusible pigments?

(a) Yes	Go to 4.13.
(b) No	Go to 4.2.

4.13 Is glycerol used as a sole carbon source?

(a) Yes	Probably *M. coxensis*.
(b) No	Probably *M. chokoriensis*.

Table B6. Dichotomous key for the identification of *Micromonospora* strains of the '**4.0% NaCl Group**'.

5.1 Produces a **yellow** diffusible pigment on m172F?

(a) Yes	Probably *M. chalcea*.
(b) No	Go to 5.2.

5.2 Produces a **reddish-brown** diffusible pigment on m172F?

(a) Yes	Probably *M. halophytica*.
(b) No	Probable New Species.

5.3 Is the diagnostic mycelial pigment ivory or yellow-white?

(a) Yes	Go to 5.4.
(b) No	Got to 5.1.

5.4 Does growth occur at 45°C?

(a) Yes	Probably *M. narathiwatensis*.
(b) No	Probably *M. eburnea*.

B3 Conclusion

This study has shown that a *Micromonospora* Identification Key based on a small number of phenetic characteristics is an easy and inexpensive method to determine the potential novelty of *Micromonospora* isolates obtained from environmental samples. As a result of this study, standardized media have been developed for testing maximum sodium chloride tolerance and for the production of diffusible pigments by *Micromonospora* strains. This Identification Key is proposed to be used to decide whether an isolate is worth investigating further, and is thus not intended as a stand-alone taxonomic tool. It is recommended that when *Micromonospora* strains are isolated, the isolates' sodium chloride tolerance be determined first. If the maximum sodium chloride tolerance is less than 1.0% or greater than 5.0%, the isolate is presumptively identified as a novel species and is worth investigating further. If the maximum sodium chloride tolerance is 1.5%, 2.0%, 3.0%, or 4.0%, the other physiological tests should be carried out.

It must also be emphasized that a strain having a diagnostic mycelial pigment colour other than orange is significant. If a strain is isolated with an olive-green mycelial pigment colour and its maximum sodium chloride tolerance is *not* 3.0%, it cannot be *M. olivasterospora* according to the Identification Key, and thus is a potentially novel species. *M. olivasterospora* is currently the only species having an olive-green mycelial pigment.

Appendix C: Summary of the biological activities of the compounds investigated in this study

Table C1. Antioxidant and antimicrobial activity of compounds spotted on TLC plates.

Compounds tested:	**100** mg/ml	**50** mg/ml	**40** mg/ml	**30** mg/ml	**20** mg/ml	**10** mg/ml	**5** mg/ml	**2** mg/ml	**1** mg/ml
Tyrosol MW: 138.17 g/mol	0.72 M D++ *Ec, Ef, Ma, Mc*	0.36 M D++ *Ec, Ef, Ma, Mc*	0.29 M D++ *Ec, Ef, Ma, Mc*	0.22 M D++ *Ec, Ef, Ma, Mc*	0.14 M D++ *Ec, Ef, Ma, Mc*	0.072 M D++ *Ec, Ef, Ma, Mc*	0.036 M D+ *Ec, Ef, Ma, Mc*	0.014 M D+ *Ec, Ef, Ma, Mc*	0.0072 M D+, L+ *Ec, Ef, Ma, Mc*
TYR01, **I** MW: 274 g/mol	0.36 M D++ *Ec, Ef, Ma, Mc*	0.18 M ND	0.15 M ND	0.11 M ND	0.073 M ND	0.036 M ND	0.018 M ND	0.0073 M ND	0.0036 M D+, L+ *Ec, Ef, Ma, Mc*
Tyrosol-**I**-**II** mixture_ Developed on TLC Spotted on TLC	100 mg/ml of mixture: D++, *Ec, Ef, Ma, Mc* D++, *Ec, Ef, Ma, Mc*			1 mg/ml: D+, L+, *Ec, Ef, Ma, Mc*					
Tyrosol-tyrosinase reaction mix A é Developed on TLC Spotted on TLC	100 mg/ml of mixture: D++, *Ec, Ef, Ma, Mc* D++, *Ec, Ef, Ma, Mc*			1 mg/ml: D+, L+, *Ec, Ef, Ma*					
Tyrosol-tyrosinase reaction mix B é Developed on TLC Spotted on TLC	100 mg/ml of mixture: D++, *Ec, Ef, Ma, Mc* D++, *Ec, Ef, Ma, Mc*			1 mg/ml: D++, L+, *Ec, Ef, Ma*					
TYROSOL-ACETATE, **IV** MW: 180.17 g/mol	0.56 M D++ *Ec, Ef, Ma, Mc*	0.28 M D++ *Ec, Ef, Ma, Mc*	0.22 M D++ *Ec, Ef, Ma, Mc*	0.17 M D++ *Ec, Ef, Ma, Mc*	0.11 M D++ *Ec, Ef, Ma, Mc*	0.056 M D++ *Ec, Ef, Mc*	0.028 M D++ *Ec, Ef, Mc*	0.011 M D+ *Ec, Ef*	0.0056 M D+, L+ *Ec, Ef*
TYRAC1, **V** MW: 358 g/mol	ND								

TyrAc2, **VI** MW: 358 g/mol Developed on TLC Spotted on TLC	0.28 M D++, *Ec, Ef, Ma, Mc* D++, *Ec, Ef, Ma, Mc*	0.14 M ND	0.11 M ND	0.084 M ND	0.056 M ND	0.028 M ND	0.014 M ND	0.0056 M ND	0.0028 M D+, L+, *Ec, Ef, Ma, Mc*
IV-**V**-**VI** mixture_ Developed on TLC Spotted on TLC	100 mg/ml of mixture: D++, *Ec, Ef, Ma, Mc* D++, *Ec, Ef, Ma, Mc*			1 mg/ml: D+, L+, *Ec, Ef, Ma, Mc*					
Totarol MW: 286.45 g/mol	0.35 M D++ *Ec, Ef, Ma, Mc*	0.17 M D++ *Ec, Ef, Ma, Mc*	0.14 M D++ *Ec, Ef, Ma, Mc*	0.1 M D++ *Ec, Ef, Ma, Mc*	0.07 M D++ *Ec, Ef, Ma, Mc*	0.035 M D++ *Ec, Ef, Ma, Mc*	0.017 M D++ *Ec, Ef, Ma, Mc*	0.007 M D++ *Ec, Ef, Ma, Mc*	0.0035 M D++, L– *Ec, Ef, Ma, Mc*
Totarol C-C and C-O dimers mixture MW of each dimer: 593.43 g/mol Developed on TLC Spotted on TLC	0.17 M D++, *Ec, Ef, Ma, Mc* D++, *Ec, Ef, Ma, Mc*	0.084 M D++, *Ec, Ef, Ma, Mc* D++, *Ec, Ef, Ma, Mc*	0.067 M D++, *Ec, Ef, Ma, Mc* D++, *Ec, Ef, Ma, Mc*	0.05 M D++, *Ec, Ef, Ma, Mc* D++, *Ec, Ef, Ma, Mc*	0.034 M D++, *Ec, Ef, Ma, Mc* D++, *Ec, Ef, Ma, Mc*	0.017 M D++, *Ec, Ef, Ma, Mc* D++, *Ec, Ef, Ma, Mc*	0.0084 M D++, *Ec, Ef, Ma, Mc* D++, *Ec, Ef, Ma, Mc*	0.0034 M D++, *Ec, Ef, Ma, Mc* D++, *Ec, Ef, Ma, Mc*	0.0017 M L+ D++, *Ec, Ef, Ma, Mc* D++, *Ec, Ef, Ma, Mc*
Ferulic acid MW: 194.19 g/mol	0.51 M D++ *Ec, Ef, Ma, Mc*	0.26 M D++ *Ec, Ef, Ma, Mc*	0.21 M D++ *Ec, Ef, Ma, Mc*	0.15 M D++ *Ec, Ef, Ma, Mc*	0.1 M D++ *Ec, Ef, Ma, Mc*	0.051 M D++ *Ec, Ef, Ma, Mc*	0.026 M D++ *Ec, Ef, Ma, Mc*	0.01 M D++ *Ec, Ef, Ma, Mc*	0.0051 M D++, L+ *Ec, Ef, Ma, Mc*
8-Hydroxyquinoline MW: 145.16 g/mol	0.69 M D++ *Ec, Ef, Ma, Mc*	0.34 M D++ *Ec, Ef, Ma, Mc*	0.28 M D++ *Ec, Ef, Ma, Mc*	0.21 M D++ *Ec, Ef, Ma, Mc*	0.14 M D++ *Ec, Ef, Ma, Mc*	0.069 M D++ *Ec, Ef, Ma, Mc*	0.034 M D++ *Ec, Ef, Ma, Mc*	0.014 M D++ *Ec, Ef, Ma, Mc*	0.0069 M D++, L+ *Ec, Ef, Ma, Mc*
8-HQ polymeric mixture_	Developed on TLC: D+; *Ec, Ef, Ma, Mc* Spotted on TLC: D+; *Ec, Ef, Ma, Mc*								

3-HAA MW: 153.14 g/mol	0.65 M D++ *Ec, Ef, Ma, Mc*	0.33 M D++ *Ec, Ef, Ma, Mc*	0.26 M D++ *Ec, Ef, Ma, Mc*	0.2 M D++ *Ec, Ef, Ma, Mc*	0.13 M D++ *Ec, Ef, Ma, Mc*	0.065 M D++ *Ec, Ef, Ma, Mc*	0.033 M D++ *Ec, Ef, Ma, Mc*	0.013 M D++ *Ec, Ef, Ma, Mc*	0.0065 M D+, L+ *Ec, Ef, Ma, Mc*
3-HAA products mixture_	**Developed on TLC:** Unconverted 3-HAA: D+; *Ec, Ef, Ma, Mc* CA: *Ec, Ef, Ma, Mc* Product X: No activities. **Spotted on TLC**, *i.e.* undeveloped: D+; *Ec, Ef, Ma, Mc*								
Control: Trolox (Vitamin E analogue) MW: 250.3 g/mol	0.4 M D++ *Ec, Ef, Ma, Mc*	0.2 M D++ *Ec, Ef, Ma, Mc*	0.16 M D++ *Ec, Ef, Ma, Mc*	0.12 M D++ *Ec, Ef, Ma, Mc*	0.08 M D++ *Ec, Ef, Ma, Mc*	0.04 M D++ *Ec, Ef, Ma, Mc*	0.02 M D++ *Ec, Ef, Ma, Mc*	0.008 M D++ *Ec, Ef, Ma, Mc*	0.004 M D++, L+ *Ec, Ef, Ma, Mc*
<Compounds tested:	**100** mg/ml	**50** mg/ml	**40** mg/ml	**30** mg/ml	**20** mg/ml	**10** mg/ml	**5** mg/ml	**2** mg/ml	**1** mg/ml

M, molarity in mol/L. ND, not determined. D, positive reaction with DPPH, where D++ is a strongly positive reaction and D+ is a weakly positive reaction. L+, prevention of LDL oxidation, and L-, ineffective at preventing LDL oxidation. *Ec*, *Ef*, *Mc*, or *Ma* indicates the specific bacterium against which antimicrobial activity was detected.
_Molecular weights and molarity not determined for products in mixtures.
é Contains TYR02 (**III**) within the mixture.

Appendix D: Representation and Reproducibility of Data

After each assay performed using the microtitre plate reader, data was transferred to Microsoft Excel Workbooks using the Anthos software. All subsequent analysis of data was performed in Excel and data were represented graphically.

An example of the interpretation of data is discussed here. Figure D1 below is shown on page 83 of this thesis and shows the experimental data of identical experiments performed at the same time in duplicate bioreactors, so that the variation in assay results could be illustrated. Other graphs depicted in sections of this thesis are the average of such duplicate experiments.

Samples were assayed for laccase activity as discussed in section 2.2.5. Although, at some data points there is much variation in the specific activities at particular time points (*e.g.* at 25 and 84 hours), the overall trend in laccase production is reproducible. It was stated that maximum laccase production occurred at 170 hours and that the laccase specific activity was 1.04 U/g using DMP as substrate.

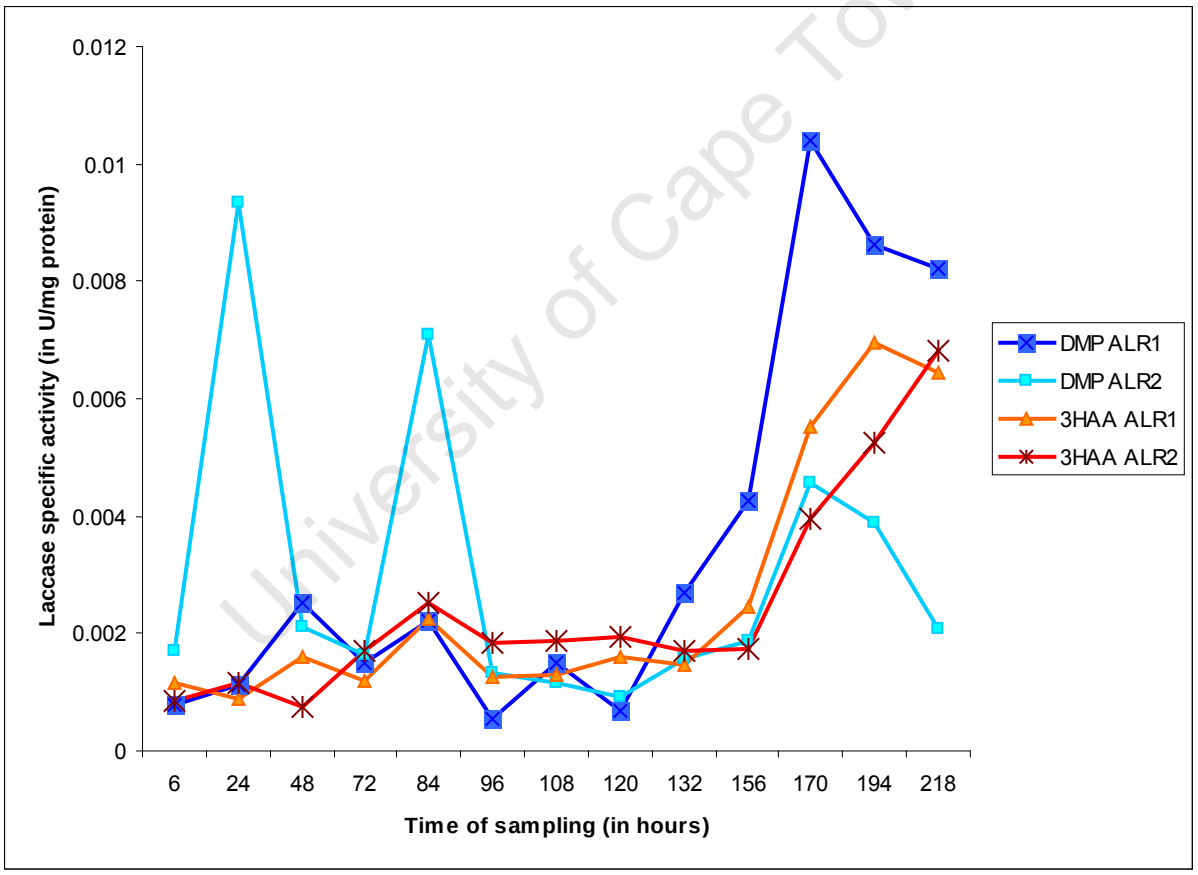


Fig. D1. Laccase specific activities obtained during the fermentation of strain 044 30-1 in duplicate ALRs containing M172F supplemented with 0.5 mM Cu.

The data shown in Figure D1 was determined as described in section 2.2.5. Samples of culture fluid were added to the microtitre plate in duplicate and the assay performed using DMP or 3-HAA as substrate. The Anthos software generated data indicating the laccase activity in terms of units of substrate converted by the samples. In Figure D2 below, the average laccase activity (in units) of the duplicate assay samples is shown, as well as the standard deviation. An aberrant experimental error is likely responsible for the deviation seen in the laccase assay performed with the samples obtained at 24 hours.

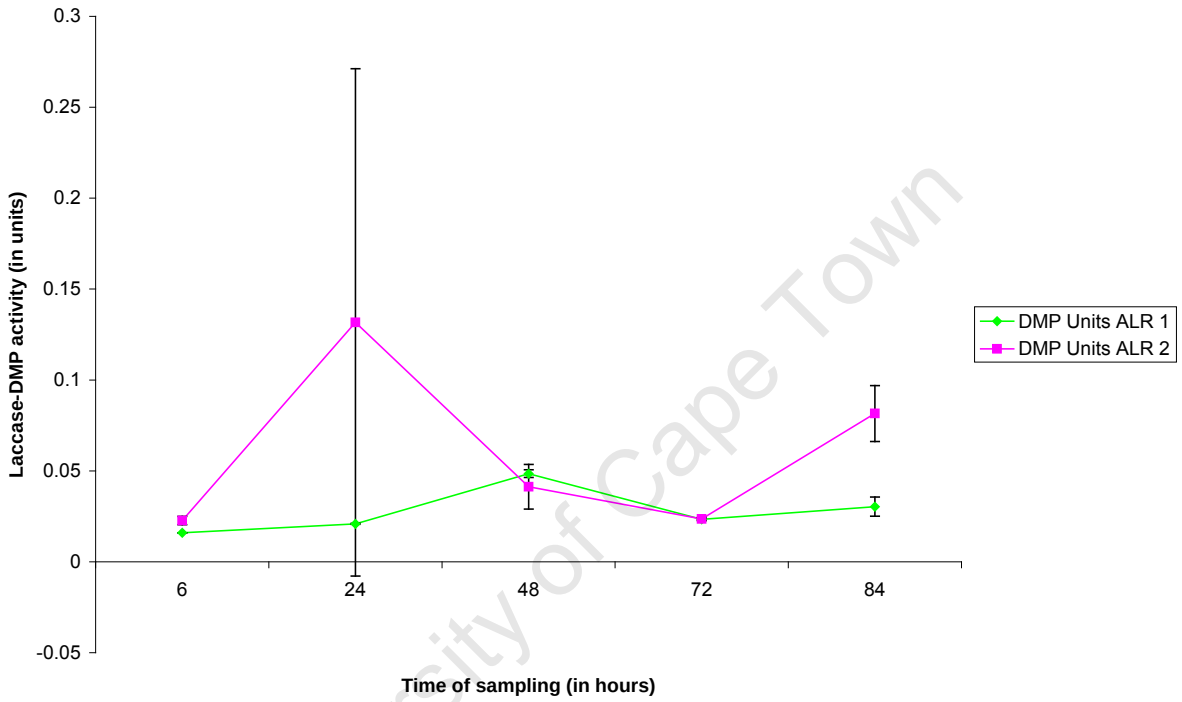


Fig. D2. Laccase activity is measured in units by the microtitre plate reader. The data shown are the average of duplicate samples assayed using DMP as laccase substrate.

The specific activities (in U/mg protein) shown in Figure D1 and laccase activity shown in Figure D2 are combined in Figure D3 below. To obtain specific activities, protein content was determined. The trend in laccase activity is reproducible, for example, laccase activity increases in both reactors from 120 – 218 hours using both DMP and 3-HAA as assay substrates (Fig. D1).

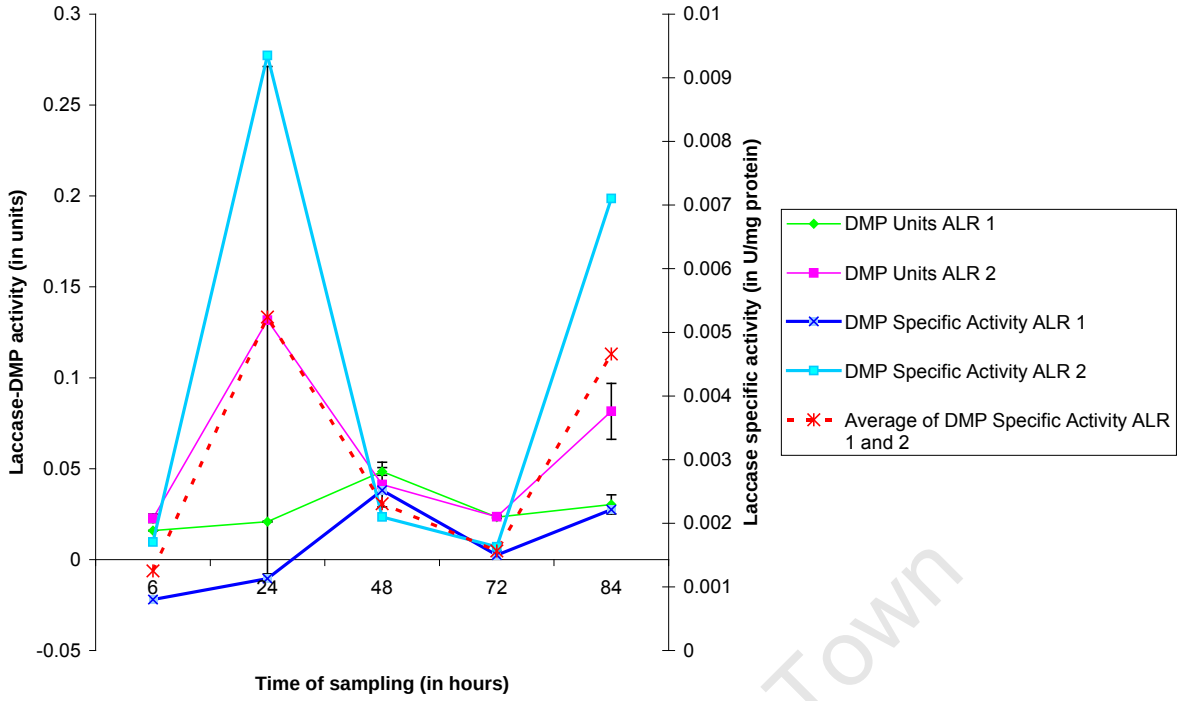


Fig. D3. Units of laccase activity, determined using the microtitre plate reader, and specific laccase activity (calculated in U/mg protein) for duplicate experiments carried out in ALRs. Standard deviation is shown for the samples assayed in duplicate using the microtitre plate reader. The average of the specific activities is also shown.

Project Outputs

Publications:

Le Roes, M., **Goodwin, C. M.**, and P. R. Meyers. 2008. *Gordonia lacunae* sp. nov., isolated from an estuary. *Systematic and Applied Microbiology.* 31:17-23.

Le Roes-Hill, M., **Goodwin, C. M.**, and S. G. Burton. 2009. Phenoxazinone synthase: What's in a name? *Trends in Biotechnology*. **27:**248-258.

Navarra, C., **Goodwin, C.**, Burton, S., Danieli, B., and S. Riva. 2010. Laccase-mediated oxidation of phenolic derivatives. *Journal of Molecular Catalysis B: Enzymatic*. doi: 10.1016/j.molcatb.2009.12.016.

Conference Presentations:

South African Women in Engineering National Conference, Cape Town, South Africa, 2007.

BIO-08, Grahamstown, South Africa, 2008. SPEAKER & POSTER PRESENTATION.

Goodwin, C.M., le Roes-Hill, M., and S. G. Burton. 2008. Novel laccases from unique strains for use as biocatalysts to produce useful products. Page 102. BIO-08 Conference, South Africa.

Extremophiles 2008, Somerset West, South Africa, 2008. POSTER PRESENTATION.

Goodwin, C.M., le Roes-Hill, M., and S. G. Burton. 2008. Optimisation of laccase production by *Micromonospora* sp. strain MB for biocatalysis applications. Page 204. Extremophiles 2008, South Africa.

Cape Biotechnology Forum, Somerset West, South Africa, 2008. SPEAKER & POSTER PRESENTATION.

Goodwin, C.M., le Roes-Hill, M., and S. G. Burton. 2008. Optimisation of laccase production by *Micromonospora* sp. strain 044 30-1 for biocatalysis applications. Page SP27. Cape Biotechnology Forum, South Africa.

The 15th International Symposium on the Biology of Actinomycetes, Shanghai, China, 2009. POSTER.

Goodwin, C. M., le Roes-Hill, M., Harrison, S. T. L., and S. G. Burton. 2009. Optimisation of laccase production by *Micromonospora* sp. strain MB for biocatalysis applications. 15th International Symposium on the Biology of Actinomycetes. Book of Abstracts, page 79. Shanghai, China.

Please note that strain MB = strain 044 30-1.

References

Addleman, K., and F. Archibald. 1993. Kraft pulp bleaching and delignification by dikaryons and monokaryons of *Trametes versicolor*. *Applied and Environmental Microbiology*. **59(1):**266-273.

Adman, E. T., Turley, S., Bramson, R., Petratos, K., Banner, D., Tsernoglou, D., Beppu, T., and H. Watanabe. 1989. A 2.0-Å structure of the blue copper protein (cupredoxin) from *Alcaligenes faecalis* S-6. *The Journal of Biological Chemistry*. **264(1):**87-99.

Aissa, I., Bouaziz, M., Ghamgui, H., Kamoun, A., Miled, N., Sayadi, S., and Y. Gargouri. 2007. Optimization of lipase-catalyzed synthesis of acetylated tyrosol by response surface methodology. *Journal of Agricultural and Food Chemistry*. **55:**10298-10305.

Alexander, D. C., Devlin, D. J., Hewitt, D. D., Horan, A. C., and T. J. Hosted. 2003. Development of the *Micromonospora carbonacea* var. *africana* ATCC 39149 bacteriophage pMLP1 integrase for site-specific integration in *Micromonospora* spp. *Microbiology*. **149:**2443-2453.

Allouche, N., Damak, M., Ellouz, R., and S. Sayadi. 2004. Use of whole cells of *Pseudomonas aeruginosa* for synthesis of the antioxidant hydroxytyrosol via conversion of tyrosol. *Applied and Environmental Microbiology*. **70(4):**2105-2109.

Ara, I., and T. Kudo. 2007. Two new species of the genus *Micromonospora*: *Micromonospora chokoriensis* sp. nov., and *Micromonospora coxensis* sp. nov., isolated from sandy soil. *Journal of General and Applied Microbiology*. **53:**29-37.

Aranda, E., García-Romera, I., Ocampo, J. A., Carbone, V., Malorni, A., Sannino, F., De Martino, A., and R. Capasso. 2007. Reusing ethyl acetate and aqueous exhausted fractions of dry olive mill residue by saprobe fungi. *Chemosphere*. **66:**67-74.

Arias, M. E., Arenas, M., Rodríguez, J., Soliveri, J., Ball, A. S., and M. Hernández. 2003. Kraft pulp bioleaching and mediated oxidation of a nonphenolic substrate by laccase from *Streptomyces cyaneus* CECT 3335. *Applied and Environmental Microbiology*. **69(4):**1953-1958.

Atlas, R. M. 1993. *In* L. C. Parks (Ed.), Handbook of Microbiological Media. CRC Press, Inc., Boca Raton.

Balasundram, N., Sundram, K., and S. Samman. 2006. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry*. **99:**191-203.

Baldrian, P. 2006. Fungal laccases – occurrence and properties. *FEMS Microbiology Reviews*. **30:**215-242.

Barber, M. S., McConnell, V. S., and B. S. DeCaux. 2000. Antimicrobial intermediates of the general phenylpropanoid and lignin specific pathways. *Phytochemistry*. **54:**53-56.

Bernabeu, A., Shapiro, S., and J. Villalaín. 2002. A MAS-NMR study of the location of (+)-totarol, a diterpenoid bioactive molecule, in phospholipid model membranes. *Chemistry and Physics of Lipids*. **119:**33-39.

Berrocal, M. M., Rodríguez, J., Ball, A. S., Pérez-Leblic, M. I., and M. E. Arias. 1997. Solubilisation and mineralisation of [^{14}C]lignocellulose from wheat straw by *Streptomyces cyaneus* CET 3335 during growth in solid-state fermentation. *Applied Microbiology and Biotechnology*. **48:**379-384.

Bertrand, T., Jolivalt, C., Briozzo, P., Caminade, E., Joly, N., Madzak, C., and C. Mougin. 2002. Crystal structure of a four-copper laccase complexed with an arylamine: Insights into substrate recognition and correlation with kinetics. *Biochemistry*. **41:**7325-7333.

Borgmeyer, J. R., and D. L. Crawford. 1985. Production and characterization of polymeric lignin degradation intermediates from two different *Streptomyces* spp. *Applied and Environmental Microbiology*. **49(2):**273-278.

Boshoff, A., Burton, M. H., and S. G. Burton. 2003. Optimization of catechol production by membrane-immobilized polyphenol oxidase: a modelling approach. *Biotechnology and Bioengineering*. **83(1):**1-7.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. **72:**248-254.

Brand-Williams, W., Cuvelier, M. E., and C. Berset. 1995. Use of a free radical method to evaluate antioxidant activity. *Lebensm. Wiss. Technol.* **28:**25-30.

Brooks, S. J., Doyle, E. M., and K. E. O'Connor. 2006. Tyrosol to hydroxytyrosol biotransformation by immobilised cell extracts of *Pseudomonas putida* F6. *Enzyme and Microbial Technology*. **39:**191-196.

Burton, S. G. 2001. Development of bioreactors for application of biocatalysts in biotransformations and bioremediation. *Pure and Applied Chemistry*. **73:**77-83.

Burton, S. G., Cowan, D. A., and J. M. Woodley. 2002. The search for the ideal biocatalyst. *Nature Biotechnology*. **20:**37-45.

Burton, S. G. 2003. Laccases and Phenol Oxidases in Organic Synthesis – a Review. *Current Organic Chemistry*. **7:**1317-1331.

Bush, K. 2004. Why it is important to continue antibacterial drug discovery. *ASM News*. **70:**282–287.

Caelers, D. November 17, 2004. 'Cape of Bad Coughs' a fertile breeding ground for TB. Cape Argus, p. 16.

Campbell, N. A., J. B. Reece, and C. G. Mitchell. 1999. Chapter 4: Carbon and the molecular diversity of life, p. 48–57. *In* Biology (5th edition). Benjamin/Cummings, CA, USA.

Canfora, L., Iamarino, G., Rao, M. A., and L. Gianfreda. 2008. Oxidative transformation of natural and synthetic phenolic mixtures by *Trametes versicolor* laccase. *Journal of Agricultural and Food Chemistry*. **56:**1398-1407.

Carunchio, F., Rescans, C., Pirelli, A. M., Messina, A., and A. M. Tarola. 2001. Oxidation of ferulic acid by laccase: identification of the products and inhibitory effects of some dipeptides. *Talanta*. **55:**189-200.

Castro, I. A., Moraes Barros, S. B., Lanfer Marquez, U. M., Motizuki, M., and T. C. Higashi Sawad. 2005. Optimization of the antioxidant capacity of a mixture of carotenoids and α-tocopherol in the development of a nutritional supplement. *Food Research International*. **38:**861-866.

Charcosset, C. 1998. Review: Purification of proteins by membrane chromatography. *J. Chem. Technol. Biotechnol.* **71:**95-110.

Chen, J. H., and C. T. Ho. 1997. Antioxidant activities of caffeic acid and its related hydrocinnamic acid compounds. *Journal of Agricultural and Food Chemistry.* **45:**2374-2376.

Chigorimbo-Murefu, N. T. L. 2007. Production of bio-active compounds from ferulic acid using biocatalysis. PhD Thesis. University of Cape Town. South Africa.

Chigorimbo-Murefu, N. T. L., Riva, S., and S. G. Burton. 2009. Lipase-catalysed synthesis of esters of ferulic acid with natural compounds and evaluation of their antioxidant properties. *Journal of Molecular Catalysis B: Enzymatic.* **56:**277-282.

Christen, S., Southwell-Keely, P. T., and R. Stocker. 1992. Oxidation of 3-hydroxyanthranilic acid to the phenoxazinone cinnabarinic acid by peroxyl radicals and by compound I of peroxidase or catalase. *Biochemistry*. **31:**8090-8097.

Claus, H. 2003. Laccases and their occurrence in prokaryotes. *Archives Microbiology*. **179:**145-150.

Claus, H. 2004. Laccases: structure, reactions, distribution. *Micron*. **35:**93-96.

Constantine, G. H., Karchesy, J. J., Franzblau, S. G., and L. E. LaFleur. 2001. (+)-Totarol from *Chamaecyparis nootkatensis* and activity against *Mycobacterium tuberculosis*. *Fitoterapia*. **72:**572-574.

Cordi, L., Minussi, R. C., Freire, R. S., and N. Durán. 2007. Fungal laccase: copper induction, semi-purification, immobilization, phenolic effluent treatment and electrochemical measurement. *African Journal of Biotechnology*. **6(10):**1255-1259.

Cross, T., and M. Goodfellow. 1973. Taxonomy and classification of the actinomycetes, p. 11–112. *In* G. Sykes and F. A. Skinner (ed.), *Actinomycetales*: characteristics and practical importance. Academic Press, Inc., London.

Crueger, W., and A. Crueger. 1989. Biotechnology: a textbook of industrial microbiology (2nd edition). Sinauer Associates, Inc., Sunderland, USA.

Crutchley, D. J., and B. G. Que. 1995. Copper-induced tissue factor expression in human monocytic THP-1 cells and its inhibition by antioxidants. *Circulation*. **92:**238-243.

D'Annibale, A., Stazi, S. R., Vinciguerra, V., Mattia, E. D., and G. G. Sermanni. 1999. Characterization of immobilized laccase from *Lentinula edodes* and its use in olive-mill wastewater treatment. *Process Biochemistry*. **34:**697-706.

D'Annibale, A., Celletti, D., Felici, M., DiMattia, E., and G. Giovannozzi-Sermanni. 1996. Substrate specificity of laccase from *Lentinus edodes*. *Acta Biotechnology*. **16:**257-270.

Dal Pozzo, A., Fagnoni, M., Bergonzi, R., Vanini, L., de Castiglione, R., Aglio, C., and S. Colli. 2000. Synthesis and anti-aggregatory activity of linear retro-inverso RGD peptides. *Journal of Peptide Research*. **55:**447-454.

Davies, J. 1994. Inactivation of antibiotics and the dissemination of resistance genes. *Science*. **264:** 375–382.

de la Rubia, T., Lucas, M., and J. Martínez. 2008. Controversial role of fungal laccases in decreasing the antibacterial effect of olive mill waste-waters. *Bioresources Technology*. **99:**1018-1025.

Desentis-Mendoza, R. M., Hernández-Sánchez, H., Moreno, A., del C., E. R., Chel-Guerrero, L., Tamariz, J., and M. E. Jaramillo-Flores. 2006. Enzymatic Polymerization of Phenolic Compounds Using Laccase and Tyrosinase from *Ustilago maydis*. *Biomacromolecules*. **7:**1845-1854.

Diamantidis, G., Effosse, A., Potier, P., and R. Bally. 2000. Purification and characterization of the first bacterial laccase in the rhizospheric bacterium *Azospirillum lipoferum*. *Soil Biology & Biochemistry*. **32:**919-927.

Díaz-García, M. E., and M. J. Valencia-Gonzáez. 1995. Enzyme catalysis in organic solvents: a promising field for optical biosensing. *Talanta*. **42:**1763-1773.

Divari, S., Valetti, F., Caposio, P., Pessione, E., Cavaletto, M., Griva, E., Gribaudo, G., Gilardi., G., and C. Giunta. 2003. The oxygenase component of phenol hydroxylase from *Acinetobacter radioresistens* S13. *European Journal of Biochemistry*. **270:**2244-2253.

Djoko, K. Y., Xiao, Z., and A. G. Wedd. 2008. Copper resistance in *E. coli*: Multicopper oxidase PcoA catalyzes oxidation of copper(I) in Cu^{I}-Cu^{II}-PcoC. *ChemBioChem*. **9:**1579-1582.

Domínguez, A., Couto, S. R., and A. Sanromán. 2001. Amelioration of ligninolytic enzyme production by *Phanerochaete chrysosporium* in airlift bioreactors. *Biotechnology Letters*. **23:**451-455.

Doran, P. M. 1995. Bioprocess engineering principles. Academic Press Limited, London.

Dubé, E., Shareck, F., Hurtubise, Y., Daneault, C., and M. Beauregard. 2008. Homologous cloning, expression, and characterization of a laccase from *Streptomyces coelicolor* and enzymatic decolourisation of an indigo dye. *Applied Microbiology and Biotechnology*. **79:**597-603.

Duffner, F. M., and R. Müller. 1998. A novel phenol hydroxylase and catechol 2,3-dioxygenase from the thermophilic *Bacillus thermoleovorans* strain A2: nucleotide sequence and analysis of the genes. *FEMS Microbiology Letters*. **161:**37-45.

Durán, N., Rosa, M. A., D'Annibale, A., and L. Gianfreda. 2002. Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: a review. *Enzyme and Microbial Technology*. **31:**907-931.

Eggert, C., Temp, U., and K-E L. Eriksson. 1996. The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: Purification and characterization of the laccase. *Applied and Environmental Microbiology*. **62(4):**1151-1158.

Eggert, C., Temp, U., Dean, J. F. D., and K-E L. Eriksson. 1995. Laccase-mediated formation of the phenoxazinone derivative, cinnabarinic acid. *FEBS Letters*. **376:**202-206.

Endo, K., Hayashi, Y., Hibi, T., Hosono, K., Beppu, T., and K. Ueda. 2003. Enzymological characterization of EpoA, a laccase-like phenol oxidase produced by *Streptomyces griseus*. *Journal of Biochemistry (Tokyo)*. **133(5):**671-677.

Endo, K., Honsono, K., Beppu, T., and K. Ueda. 2002. A novel extracytoplasmic phenol oxidase of *Streptomyces*: its possible involvement in the onset of morphogenesis. *Microbiology*. **148:**1767-1776.

Enguita, F. J., Martins, L. O., Henriquest, A. O., and M. A. Carrondo. 2003. Crystal structure of a bacterial endospore coat component: A laccase with enhanced thermostability properties. *The Journal of Biological Chemistry*. **278(21):**19416-19425.

Ergül, F. E., Sargin, S., Öngen, G., and F. V. Sukan. 2009. Depholisation of olive mill wastewater using adapted *Trametes versicolor*. *International Biodeterioration & Biodegradation*. **63:**1-6.

Es-Safi, N-E., Kollmann, A., Khlifi, S., and P-H. Ducrot. 2007. Antioxidative effect of compounds isolated from *Globularia alypum* L. structure-activity relationship. *LWT*. **40:**1246-1252.

Espín, J. C., Soler-Rivas, C., Cantos, E., Tomás-Barberán, F. A., and H. J. Wichers. 2001. Synthesis of the antioxidant hydroxytyrosol using tyrosinase as biocatalyst. *Journal of Agricultural and Food Chemistry*. **49:**1187-1193.

Euzéby, J. P. List of Bacterial Names with Standing in Nomenclature. http://www.bacterio.cict.fr/

Evans, G. B., and R. H. Furneaux. 2000. The synthesis and antibacterial activity of totarol derivatives. Part 2: Modifications at C-12 and O-13. *Bioorganic & Medicinal Chemistry*. **8:**1653-1662.

Evans, G. B., Furneaux, R. H., Gravestock, M. B., Lynch, G. P., and G. K. Scott. 1999. The synthesis and antibacterial activity of totarol derivatives. Part 1: Modifications of ring-C and pro-drugs. *Bioorganic & Medicinal Chemistry*. **7:**1953-1964.

Galhaup, C., Wagner, H., Hinterstoisser, B., and D. Haltrich. 2002a. Increased production of laccase by the wood-degrading basidiomycete *Trametes pubescens*. *Enzyme and Microbial Technology*. **30:**529-536.

Galhaup, C., Goller, S., Peterbauer, C. K., Strauss, J., and D. Haltrich. 2002b. Characterization of the major laccase isoenzyme from *Trametes pubescens* and regulation of its synthesis by metal ions. *Microbiology*. **148:**2159-2169.

Galli, C., and F. Visioli. 1999. Antioxidant and other activities of phenolics in olives/olive oil, typical components of the Mediterranean diet. *Lipids*. **34:**S23-S26.

Garrote, G., Cruz, J. M., Moure, A., Domínguez, H., and J. C. Parajó. 2004. Antioxidant activity of byproducts from the hydrolytic processing of selected lignocellulosic materials. *Trends in Food Science & Technology*. **15:**191-200.

Gianfreda, L., Iamarino, G., Scelza, R., and M. Rao. 2006. Oxidative catalysts for the transformation of phenolic pollutants: a brief review. *Biocatalysis and Biotransformation*. **24(3):**177-187.

Gianfreda, L., and M. A. Rao. 2004. Potential of extra cellular enzymes in remediation of polluted soils: a review. *Enzyme and Microbial Technology*. **35:**339-354.

Gianfreda, L., Sannino, F., Rao, M. A., and J-M. Bollag. 2003. Oxidative transformation of phenols in aqueous mixtures. *Water Research*. **37:**3205-3215.

Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S., and G. Sannia. 2009. Laccases: a never-ending story. *Cellular and Molecular Life Sciences*. doi 10.1007/s00018-009-0169-1

Giorno, L., and E. Drioli. 2000. Biocatalytic membrane reactors: applications and perspectives. *TIBTECH*. **18:**339-349.

Goodfellow, M., and J. A. Haynes. 1984. Actinomycetes in marine sediments, p. 453–472. *In* L. Ortiz-Ortiz, L. F. Bojalil, and V. Yakoleff (ed.), Biological, biochemical, and biomedical aspects of actinomycetes. Academic Press, Inc., Orlando, Fla.

Goodwin, C. M. 2005. Investigations into actinomycetes isolated from coastal environments with a special emphasis on the genus *Micromonospora*. Master of Science thesis. University of Cape Town. South Africa.

Grasso, S., Siracusa, L., Spatafora, C., Renis, M., and C. Tringali. 2007. Hydroxytyrosol lipophilic analogues: Enzymatic synthesis, radical scavenging activity and DNA oxidative damage protection. *Bioorganic Chemistry*. **35:**137-152.

He, Z-Y., Nie, H-L., Branford-White, C., Zhu, L-M., Zhou, Y-T., and Y. Zheng. 2008. Removal of Cu^{2+} from aqueous solution by absorption onto a novel activated nylon-based membrane. *Bioresource Technology*. **99:**7954-7958.

Hiramatsu, R., Hara, T., Akimoto, H., Takikawa, O., Kawabe, T., Isobe, K., and F. Nagase. 2008. Cinnabarinic acid generated from 3-hydroxyanthranilic acid strongly induces apoptosis in thymocytes through the generation of reactive oxygen species and the induction of caspase. *Journal of Cellular Biochemistry*. **103:**42-53.

Hosted Jr, T. J., Wang, T., and A. C. Horan. 2005. Characterization of the *Micromonospora rosaria* pMR2 plasmid and development of a high G + C codon optimised integrase for site-specific integration. *Plasmid*. **54:**249-258.

Hotta, H., Nagano, S., Ueda, M., Tsujino, Y., Koyama, J., and T. Osakai. 2002. Higher radical scavenging activities of polyphenolic antioxidants can be ascribed to chemical reactions following their oxidation. *Biochimica et Biophysica Acta*. 1572:123-132.

Howe, R., Evans, R. L., and S. W. Ketteridge. 1997. Copper-binding proteins in ectomycorrhizal fungi. *New Phytologist*. **135:**123-131.

Huang, H., Jiasen, L., Hu, Y., Fang, Z., Zhang, K., and S. Bao. 2008. *Micromonospora rifamycinica* sp. nov., a novel actinomycete from mangrove sediment. *International Journal of Systematic and Evolutionary Microbiology*. **58:**17-20.

Huang, D., Ou, B., and R. L. Prior. 2005. The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*. **53:**1841-1856.

Hullo, M-F., Moszer, I., Danchin, A., and I. Martin-Verstraete. 2001. CotA of *Bacillus subtilis* is a copper-dependent laccase. *Journal of Bacteriology*. **183(18):**5426-5430.

Iwahashi, H. 1999. 3-Hydroxyanthranilic acid-derived compounds formed through electrochemical oxidation. *Journal of Chromatography B*. **736:**237-245.

Jaouani, A., Tabka, M. G., and M. J. Penninckx. 2006. Lignin modifying enzymes of *Coriolopsis polyzona* and their role in olive oil mill wastewaters decolourisation. *Chemosphere*. **62:**1421-1430.

Johannes, C., and A. Majcherczyk. 2000. Laccase activity tests and laccase inhibitors. *Journal of Biotechnology*. **78:**193-199.

Jolivalt, C., Brenon, S., Caminade, E., Mougin, C., and M. Pontié. 2000. Immobilization of laccase from *Trametes versicolor* on a modified PVDF microfiltration membrane: characterization of the grafted support and application in removing a phenylurea pesticide in wastewater. *Journal of Membrane Science*. **180:**103-113.

Jones, G. H. 2000. Actinomycin production persists in a strain of *Streptomyces antibioticus* lacking phenoxazinone synthase. *Antimicrobial Agents and Chemotherapy*. **44(5):**1322-1327.

Jones, O. T. G. 1963. The inhibition of bacteriochlorophyll biosynthesis in *Rhodopseudomonas* spheroids by 8-hydroxyquinoline. *Journal of Biochemistry*. **88:**335-340.

Jongrungruangchok, S., Tanasupawat, S., and T. Kudo. 2008. *Micromonospora chaiyaphumensis* sp. nov., isolated from Thai soils. *International Journal of Systematic and Evolutionary Microbiology*. **58:**924-928.

Jongrungruangchok, S., Tanasupawat, S., and T. Kudo. 2008. *Micromonospora krabiensis* sp. nov., isolated from marine soil in Thailand. *Journal of General and Applied Microbiology*. **54:**127-133.

Kaizer, J., Baráth, G., Csonka, R., Speier, G., Korecz, L., Rockenbauer, A., and L. Párkányi. 2008. Catechol oxidase and phenoxazinone synthase activity of a manganese(II) isoindoline complex. *Journal of Inorganic Biochemistry*. **102:**773-780.

Kataoka, K., Komori, H., Ueki, Y., Konno, Y., Kamitaka, Y., Kurose, S., Tsujimura, S., Higuchi, Y., Kano, K., Seo, D., and T. Sakurai. 2007. Structure and function of the engineered multicopper oxidase CueO from *Escherichia coli* – deletion of the methionine-rich helical region covering the substrate-binding site. *Journal of Molecular Biology*. **373:**141-152.

Katz, E., and H. Weissbach. 1962. Biosynthesis of the actinomycin chromophore; Enzymatic conversion of 4-methyl-3-hydroxyanthranilic acid to actinocin. *The Journal of Biological Chemistry*. **237(3):**882-886.

Kazandjian, R. Z., and A. M. Klibanov. 1985. Regioselective oxidation of phenols catalyzed by polyphenol oxidase in chloroform. *Journal of the American Chemical Society*. **107:**5448-5450.

Kroppenstedt, R. M., Mayilraj, S., Wink, J. M., Kallow, W., Schumann, P., Secondini, C., and E. Stackebrandt. 2005. Eight new species of the genus *Micromonospora, Micromonospora citrea* sp. nov., *Micromonospora echinaurantiaca* sp. nov., *Micromonospora echinofusca* sp. nov., *Micromonospora fulviviridis* sp. nov., *Micromonospora inyoensis* sp. nov., *Micromonospora peucetia* sp. nov., *Micromonospora sagamiensis* sp. nov., and *Micromonospora viridifaciens* sp. nov. *Systematic and Applied Microbiology*. **28:**328-339.

Kubo, I., Muroi, H., and M. Himejima. 1992. Antibacterial activity of totarol and its potentiation. *Journal of Natural Products*. **55(10):**1436-1440.

Lacey, J. 1973. Actinomycetes in soils, composts and fodders, p. 231–251. *In* G. Sykes and F. A. Skinner (ed.), *Actinomycetales*: characteristics and practical importance. Academic Press, Inc., London.

Lazzarini, A., L. Cavaletti, G. Toppo, and F. Marinelli. 2001. Rare genera of actinomycetes as potential producers of new antibiotics. *Antonie Van Leeuwenhoek*. **79:**399–405.

Le Roes, M., Goodwin, C. M., and P. R. Meyers. 2007. *Gordonia lacunae* sp. nov., isolated from an estuary. *Journal of Systematic and Applied Microbiology*. doi:10.1016/j.syapm.2007.10.001

Le Roes-Hill, M., Goodwin, C. M., and S. G. Burton. 2009. Phenoxazinone synthase: What's in a name? *Trends in Biotechnology*. **27:**248-258.

Leidig, E., Prüsse, U., Vorlop, K-D., and J. Winter. 1999. Biotransformation of poly R-478 by continuous cultures of PVAL-encapsulated *Trametes versicolor* under non-sterile conditions. *Bioprocess Engineering*. **21:**5-12.

Leontievsky, A. A., Vares, T., Lankinen, P., Shergill, J. K., Pozdnyakova, N. N., Myasoedova, N. M., Kalkkinen, N., Golovleva, L. A., Cammack, R., Thurston, C. F., and A. Hatakka. 1997. Blue and yellow laccases of ligninolytic fungi. *FEMS Microbiology Letters*. **156:**9-14.

Levy, S. B. 1998. The challenge of antibiotic resistance. *Scientific American*. March: 32–39.

Li, X., Zhou, X., and Z. Deng. 2003. Vector systems allowing efficient autonomous or integrative gene cloning in *Micromonospora* sp. strain 40027. *Applied and Environmental Microbiology*. **69(6):**3144-3151.

Locci, R. 1989. Streptomycetes and related genera, p. 2451–2508. *In* S. T. Williams, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 4. The Williams & Wilkins Co., Baltimore.

Luke, A. K., and S. G. Burton. 2001. A novel application for *Neurospora crassa*: Progress from batch culture to a membrane bioreactor for the bioremediation of phenols. *Enzyme and Microbial Technology*. **29:**348-356.

Machczynski, M. C., Vijgenboom, E., Samyn, B., and G. W. Santers. 2004. Characterisation of SLAC: a small laccase from *Streptomyces coelicolor* with unprecedented activity. *Protein Science*. **13(9):**2388-2397.

MacPherson, I. S., and M. E. P. Murphy. 2007. Review: Type-2 copper-containing enzymes. *Cellular and Molecular Life Sciences*. **64:**2887-2899.

Majcherczyk, A., Johannes, C., and A. Hüttermann. 1999. Oxidation of aromatic alcohols by laccase from *Trametes versicolor* mediated by the 2,2`-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) cation radical and dication. *Applied Microbiology and Biotechnology*. **51:**267-276.

Manna, C., Galletti, P., Cucciolla, V., Montedoro, G., and V. Zappia. 1999. Olive oil hydroxytyrosol protects human erythrocytes against oxidative damages. *Journal of Nutritional Biochemistry*. **19:**159-165.

Marwick, J. D., Wright, P. C., and J. G. Burgess. 1999. Bioprocess intensification for production of novel marine bacterial antibiotics through bioreactor operation and design. *Marine Biotechnology*. **1:**495-507.

Mayer, A. M., and R. C. Staples. 2002. Laccase: new functions for an old enzyme. *Phytochemistry*. **60:**551-565.

McMahon, A. M., Doyle, E. M., Brooks, S., and K. E. O'Connor. 2007. Biochemical characterization of the coexisting tyrosinase and laccase in the soil bacterium *Pseudomonas putida* F6. *Enzyme and Microbial Technology*. **40:**1435-1441.

McNamara, C. J., Anastasiou, C. C., O'Flaherty, V., and R. Mitchell. 2008. Review: Bioremediation of olive mill wastewater. *International Biodeterioration & Biodegradation*. **61:**127-134.

Meral, A., Tunkel, P., Surmen-Gur, E., Ozbek, R., and U. Guncey. 2000. Lipid peroxidation and antioxidant status in beta-thalassemia. *Pediatric Thermatology Oncology*. 17:687-693.

Meyer, A. S., Heinonen, M., and E. N. Frankel. 1998. Antioxidant interactions of catechin, cyaniding, caffeic acid, quercetin, and ellagic acid on human LDL oxidation. *Food Chemistry*. **61:**71-75.

Molina-Guijarro, J. M., Pérez, J., Muñoz-Dorado, J., Guillén, F., Moya, R., Hernández, M., and M. E. Arias. 2009. Detoxification of azo dyes by a novel pH-versatile, salt-resistant laccase from *Streptomyces ipomoea*. *International Microbiology*. **12:**13-21.

Moolla, A., Van Vuuren, S. F., Van Zyl, R. L., and A. M. Viljoen. 2007. Biological activity and toxicity profile of 17 *Agathosma* (Rutaceae) species. *South African Journal of Botany*. **73:**588-592.

Moran, M. A., L. T. Rutherford, and R. E. Hodson. 1995. Evidence for indigenous *Streptomyces* populations in a marine environment determined with a 16S rRNA probe. *Applied and Environmental Microbiology*. **61:**3695–3700.

Moreira, M. Y., Palma, C., Feijoo, G., and J. M. Lema. 1998. Strategies for the continuous production of ligninolytic enzymes in fixed and fluidised bed bioreactors. *Journal of Biotechnology*. **66:**27-39.

Moreira, M. T., Feijoo, G., and J. M. Lema. 2003. Fungal bioreactors: Applications to white-rot fungi. *Reviews in Environmental Science and Bio/Technology*. **2:**247-259.

Moure, A., Cruz, J. M., Franco, D., Domínguez, J. M., Sineiro, J., Domínguez, H., Núñez, J., and J. C. Parajó. 2001. Natural antioxidants from residual sources. *Food Chemistry*. **72:**145-171.

Mouton, J. 2005. How to succeed in your Master's and Doctoral studies: A South African guide and resource book. Van Schaik Publishers, South Africa.

Mukadi, Y. D., D. Maher, and A. Harries. 2001. Tuberculosis case fatality rates in high HIV prevalence populations in sub-Saharan Africa. *AIDS*. **15:**143–152.

Mustafa, R., Muniglia, L., Rovel, B., and M. Girardin. 2005. Phenolic colorants obtained by enzymatic synthesis using a fungal laccase in a hydro-organic biphasic system. *Food Research International*. **38:**995-1000.

Nardini, M., D'Aquino, M., Tomassi, G., Gentili, V., Di Felice, M., and C. Scaccini. 1995. Inhibition of human low-density lipoprotein oxidation by caffeic acid and other hydroxycinnamic acid derivatives. *Free Radical Bio. Med.* **19:**541-552.

Navarra, C., Goodwin, C., Burton, S., Danieli, B., and S. Riva. 2010. Laccase-mediated oxidation of phenolic derivatives. *Journal of Molecular Catalysis B: Enzymatic*. doi: 10.1016/j.molcatb.2009.12.016.

Navarro, C., Orell, A., and C. A. Jerez. 2008. Copper resistance mechanisms of extremophilic bacteria and archae living under exceptionally high concentrations of metals. Page 67. Extremophiles 2008, South Africa.

Ncanana, S. W. 2007. Biocatalytic studies of phenol oxidases producing antioxidants. PhD Thesis. University of Cape Town. South Africa.

Ncanana, S., Baratto, L., Roncaglia, L., Riva, S., and S. G. Burton. 2007. Laccase-mediated oxidation of totarol. *Advanced Synthesis & Catalysis*.

Ncanana, S., and S. Burton. 2007. Oxidation of 8-hydroxyquinoline catalyzed by laccase from *Trametes pubescens* yields an antioxidant aromatic polymer. *Journal of Molecular Catalysis B: Enzymatic*. **44:**66-71.

Nicotra, S., Cramarossa, M. R., Mucci, A., Pagnoni, U. M., Riva, S., and L. Forti. 2004. Biotransformation of resveratrol: synthesis of *trans*-dehydrodimers catalyzed by laccases from *Myceliophtora thermophyla* and from *Trametes pubescens*. *Tetrahedron*. **60:**595-600.

Niladevi, K. N., Sukumaran, R. K., Jacob, N., Anisha, G. S., and P. Prema. 2007. Optimization of laccase production from a novel strain – *Streptomyces psammoticus* using response surface methodology. *Microbial Research*. doi:10.1016/j.micres.2006.10.006

Niladevi, K. N., and P. Prema. 2007. Effect of inducers and process parameters on laccase production by *Streptomyces psammoticus* and its application in dye decolourisation. *Bioresource Technology*. doi:10.1016/j.biortech.2007.06.056

Nolan, L. C., and K. E. O'Connor. 2008. Dioxygenase- and monooxygenase-catalysed synthesis of *cis*-dihydrodiols, catechols, epoxides and other oxygenated products. *Biotechnology Letters*. **30:**1879-1891.

Orenes-Piñero, E., García-Carmona, F., and A. Sánchez-Ferrer. 2006. Kinetic characterisation of cresolase activity of *Streptomyces antibioticus* tyrosinase. *Enzyme and Microbial Technology*. doi: 10.1016/j.enzmictec.2006.03.001

Oshida, T., Takeda, K., Yamaguchi, T., Ohshima, S., and Y. Ito. 1986. Isolation and characterisation of plasmids from *Micromonospora zionensis* and *Micromonospora rosaria*. *Plasmid*. **16(1):**74-76.

Osiadacz, J., Al-Adhami, A. J. H., Bajraszewska, D., Fischer, P., and W. Peczyñska-Czoch. 1999. On the use of *Trametes versicolor* laccase for the conversion of 4-methyl-3-hydroxyanthranilic acid to actinocin chromophore. *Journal of Biotechnology*. **72:**141-149.

Otoguro, M., M. Hayakawa, T. Yamazaki, and Y. Iimura. 2001. An integrated method for the enrichment and selective isolation of *Actinokineospora* spp. in soil and plant litter. *Journal of Applied Microbiology*. **91:**118–130.

Palmieri, G., Giardina, P., Bianco, C., Scaloni, A., Capasso, A., and G. Sannia. 1997. A novel white laccase from *Pleurotus ostreatus*. *Journal of Lipid Research*. **272(50):**31301-31307.

Payne, D., and A. Tomasz. 2004. The challenge of antibiotic resistant pathogens: the medical need, the market and prospects for new antimicrobial agents. *Current Opinion in Microbiology*. **7:**435–438.

Piacquadio, P., De Stefano, G., Sammartino, M., and V. Sciancalepore. 1997. Phenols removal from apple juice by laccase immobilized on Cu^{2+}-chelate regenerable carrier. *Biotechnology Techniques*. **11(7):**515-517.

Ponzoni, C., Beneventi, E., Cramarossa, M. R., Raimondi, S., Trevisi, G., Pagnoni, U. M., Riva, S., and L. Forti. 2007. Laccase-catalysed dimerization of hydroxystilbenes. *Advanced Synthesis and Catalysis*. **349:**1497-1506.

Que Jr, L., and W. B. Tolman. 2008. Biologically inspired oxidation catalysis. *Nature*. **455:**333-340.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., and C. Rice-Evans. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **26:**1231-1237.

Rescigno, A., Sanjust, E., Soddu, G., Rinaldi, A. C., Sollai, F., Curreli, N., and A. Rinaldi. 1998. Effect of 3-hydroxyanthranilic acid on mushroom tyrosinase activity. *Biochimica et Biophysica Acta*. **1384:**268-276.

Riva, S. 2006. Laccases: blue enzymes for green chemistry. *TRENDS in Biotechnology*. **24(5):**219-226.

Rodakiewicz-Nowak, J., Kasture, S. M., Dudek, B., and J. Haber. 2000. Effect of various water-miscible solvents on enzymatic activity of fungal laccases. *Journal of Molecular Catalysis B: Enzymatic*. **11:**1-11.

Rose, P. 2008. Life at the air/water interface. Page 23. Extremophiles 2008, South Africa.

Ryan, D. 2003. Reactor development and process optimisation for the bioremediation of phenolic wastewaters by *Trametes* species. PhD Thesis. University of Cape Town. South Africa.

Ryan, D. R., Leukes, W. D., and S. G. Burton. 2005. Fungal bioremediation of phenolic wastewaters in an airlift reactor. *Biotechnol. Prog.* **21:**1068-1074.

Sánchez-Moreno, C., Larrauri, J. A., and F. Saura-Calixto. 1999. Free radical scavenging capacity and inhibition of lipid oxidation of wines, grape juices and related polyphenolic constituents. *Food Research International.* **32:**407-412.

Saparrat, M. C. N., Guillén, F., Arambarri, A. M., Martínez, A. T., and M. J. Martínez. 2002. Induction, Isolation, and Characterization of Two Laccases from the White Rot Basidiomycete *Coriolopsis rigida*. *Applied and Environmental Microbiology*. **68(4):**1534-1540.

Sampedro, I., Romero, C., Ocampo, J. A., Brenes, M., and I. García. 2004. Removal of monomeric phenols in dry mill olive residue by saprobic fungi. *Journal of Agricultural and Food Chemistry*. **52:**4487-4492.

Sayadi, S. 2009. The use of olive oil processing wastes for the production of chemicals and polymers: a review. sami.sayadi@cbs.rnrt.tn

Schmid, A., Dordick, J. S., Hauer, B., Kiener, A., Wubbolts, M., and B. Witholt. 2001. Industrial biocatalysis today and tomorrow. *Nature*. **409:**258-268.

Shi, C., Liu, O., Dai, Y., Xie, Y., and X. Xu. 2002. The mechanism of azide activation of polyphenol oxidase II from tobacco. *Acta Biochim. Polonica.* **49:**1029-1035.

Shirling, E. B., and D. Gottlieb. 1966. Methods for characterization of *Streptomyces* species. *International Journal of Systematic Bacteriology*. **16:**313–340.

Shutova, V. V., Revin, V. V., and Y. A. Myakushina. 2008. The effect of copper ions on the production of laccase by the fungus *Lentinus (Panus) tigrinus*. *Applied Biochemistry and Microbiology*. **44(6):**619-623.

Singh, S. K., Grass, G., Rensing, C., and W. R. Montfort. 2004. Cuprous oxidase activity of CueO from *Escherichia coli*. *Journal of Bacteriology*. **186(22):**7815-7817.

Smith, A. W., Camara-Artigas, A., Olea Jr, C., Francisco, W. A., and J. P. Allen. 2004. Crystallization and initial X-ray analysis of phenoxazinone synthase from *Streptomyces antibioticus*. *Acta Crystallographica*. **D60:**1453-1455.

Smith, A. W., Camara-Artigas, A., Wang, M., Allen, J. P., and W. A. Francisco. 2006. Structure of phenoxazinone synthase from *Streptomyces antibioticus* reveals a new Type 2 copper center. *Biochemistry*. **45:**4378-4387.

Smith, K. J., Neafie, R., Yeager, J., and H. G. Skelton. 1999. *Micrococcus* folliculitis in HIV-1 disease. *British Journal of Dermatology*. **141:**558-561.

Solano, F., Lucas-Elío, P., López-Serrano, D., Fernández, E., and A. Sanchez-Amat. 2001. Dimethoxyphenol oxidase activity of different microbial blue multicopper proteins. *FEMS Microbiology Letters*. **204:**175-181.

Solioz, M., and J. V. Stoyanov. 2003. Copper homeostasis in *Enterococcus hirae*. *FEMS Microbiology Reviews*. **27:**183-195.

Solomon, E. I., Sundaram, U. M., and T. E. Machonkin. 1996. Muliticopper oxidases and oxygenases. *Chemical Reviews*. **96:**2563-2605.

Sparnins, V. L., and S. Dagley. 1975. Alternative routes of aromatic catabolism in *Pseudomonas acidovorans* and *Pseudomonas putida*: Gallic acid as a substrate and inhibitor of dioxygenases. *Journal of Bacteriology*. **124(3):**1374-1381.

Sponga, F., L. Cavaletti, A. Lazzarini, A. Borghi, I. Ciciliato, D. Losi, and F. Marinelli. 1999. Biodiversity and potentials of marine-derived microorganisms. *Journal of Biotechnology*. **70:**65–69.

Suzuki, T., Endo, K., Ito, M., Tsujibo, H., Miyamoto, K., and Y. Inamori. 2003. A thermostable laccase from *Streptomyces lavendulae* REN-7: purification, characterization, nucleotide sequence, and expression. *Bioscience Biotechnology Biochemistry*. **67(10):**2167-2175.

Swamy, J., and J. A. Ramsay. 1999. The evaluation of white rot fungi in the decoloration of textile dyes. *Enzyme and Microbial Technology*. **24:**130-137.

Takizawa, M., R. R. Colwell, and R. T. Hill. 1993. Isolation and diversity of actinomycetes in the Chesapeake Bay. *Applied and Environmental Microbiology*. **59:**997–1002.

Teissedre, P. L., Frankel, E. N., Waterhouse, A. L., Peleg, H., and J. B. German. 1996. Inhibition of *in vitro* human LDL oxidation by phenolic antioxidants from grapes and wines. *Journal of Science Food Agriculture.* **70:**55-61.

Terrón, M. C., González, T., Carbajo, J. M., Yagüe, S., Arana-Cuenca, A., Téllez, A., Dobson, A. D. W., and A. E. González. 2004. Structural close-related aromatic compounds have different effects on laccase activity and on *lcc* gene expression in the ligninolytic fungus *Trametes* sp. I-62. *Fungal Genetics and Biology*. **41:**954-962.

Thawai, C., Tanasupawat, S., Itoh, T., Suwanborirux, K., and T. Kudo. 2004. *Micromonospora aurantionigra* sp. nov., isolated from a peat swamp forest in Thailand. *Actinomycetologica*. **18:**8–14.

Thawai, C., Tanasupawat, S., Itoh, T., Suwanborirux, K., and T. Kudo. 2005. *Micromonospora siamensis* sp. nov., isolated from Thai peat swamp forest. *Journal of General and Applied Microbiology*. **51:**229-234.

Thawai, C., Tanasupawat, S., Itoh, T., Suwanborirux, K., Suzuki, K., and T. Kudo. 2005. *Micromonospora eburnea* sp. nov., isolated from a Thai peat swamp forest. *International Journal of Systematic and Evolutionary Microbiology*. **55:**417-422.

Thawai, C., Tanasupawat, S., Suwanborirux, K., Itoh, T., and T. Kudo. 2007. *Micromonospora narathiwatensis* sp. nov., from Thai peat swamp forest soils. *Journal of General and Applied Microbiology*. **53:**287-293.

Thawai, C., Tanasupawat, S., and T. Kudo. 2008. *Micromonospora pattaloongensis* sp. nov., isolated from a Thai mangrove forest. *International Journal of Systematic and Evolutionary Microbiology*. **58:**1516-1521.

Thomson, C. J., E. Power, H. Ruebsamen-Waigmann, and D. Labischinski. 2004. Antibacterial research and development in the 21st Century – an industry perspective of the challenges. *Current Opinion in Microbiology*. **7:**445–450.

Tomoda, A., Yamaguchi, J., Kojima, H., Amemiya, H., and Y. Yoneyama. 1986. Mechanism of *o*-aminophenol metabolism in human erythrocytes. *FEBS Letters*. **196(1):**44-48.

Torres de Pinedo, A., Peñalver, P., and J. C. Morales. 2007. Synthesis and evaluation of new phenolic-based antioxidants: Structure-activity relationship. *Food Chemistry*. **103:**55-61.

Tranchimand, S., Tron, T., Gaudin, C., and G. Iacazio. 2006. Synthesis of bis-lactone lignans through laccase catalysis. *Journal of Molecular Catalysis B: Enzymatic*. **42:**27-31.

Trujillo, M. E., Fernández-Molinero, C., Velázquez, E., Kroppenstedt, R. M., Schumann, P., Mateos, P. F., and E. Martínez-Molino. 2005. *Micromonospora mirobrigensis* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*. **55:**877-880.

Trujillo, M. E., Kroppenstedt, R. M., Schumann, P., Carro, L., and E. Martínez-Molina. 2006. *Micromonospora coriariae* sp. nov., isolated from root nodules of *Coriaria myrtifolia*. *International Journal of Systematic and Evolutionary Microbiology*. **56:**2381-2385.

Trujillo, M. E., Kroppenstedt, R. M., Fernández-Molinero, C., Schumann, P., and E. Martínez-Molina. 2007. *Micromonospora lupini* sp. nov. and *Micromonospora saelicensis* sp. nov., isolated from root nodules of *Lupinus angustifolius. International Journal of Systematic and Evolutionary Microbiology*. **57:**2799-2804.

Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M., and M. Mazur. 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions*. **160:**1-40.

van Beilen, J. B., and E. G. Funhoff. 2005. Expanding the alkane oxygenase toolbox: new enzymes and applications. *Current Opinion in Biotechnology*. **16:**308-314.

Vasconcelos, A. F. D., Barbosa, A. M., Dekker, R. F. H., Scarminio, I. S., and M. I. Rezende. 2000. Optimization of laccase production by *Botryosphaeria* sp. in the presence of veratryl alcohol by the response-surface method. *Process Biochemistry*. **35(10):**1131-1138.

Vinciguerra, V., D'Annibale, A., Gàcs-Baitz, E., and G. D. Monache. 1997. Biotransformation of tyrosol by whole-cell and cell-free preparation of *Lentinus edodes*. *Journal of Molecular Catalysis B: Enzymatic*. **3:**213-220.

Ward, G., Hadar, Y., Bilkis, I., Konstantinovsky, L., and C. G. Dosoretz. 2001. Initial steps in ferulic acid polymerization by lignin peroxidase. *The Journal of Biological Chemistry*. **276(22):**18734-18741.

Weyer, K., P. Groenewald, M. Zwarenstein, and C. J. Lombard. 1995. Tuberculosis drug resistance in the Western Cape. *South African Medical Journal*. **85:**499–504.

Wright, J. S., Johnson, E. R., and G. A. DiLabio. 2001. Predicting the activity of phenolic antioxidants: theoretical method, analysis of substituent effects, and application to major families of antioxidants. *Journal of the American Chemical Society*. **123:**1173-1183.

Xiao, Y. Z., Tu, X. M., Wang, J., Zhang, M., Cheng, Q., Zeng, W. Y., and Y. Y. Shi. 2003. Purification, molecular characterization and reactivity with aromatic compounds of a laccase from basidiomycete *Trametes* sp. strain AH28-2. *Applied Microbiology and Biotechnology*. **60(6):**700-707.

Xie, T. F., Wang, D. J., Chen, S. M., and T. J. Li. 1998. Photovoltaic properties of novel conjugated oligomer/n-Si(111) heterostructrues. *Thin Solid Films*. **327-329:**415-418.

Xu, F., Kulys, J., J., Duke, K., Li, K., Krikstopaitis, K., Deussen, H-J W., Abbate, E., Galinyte, V., and P. Schneider. 2000. Redox chemistry in laccase-catalyzed oxidation of *N*-hydroxy compounds. *Applied and Environmental Microbiology*. **66(5):**2052-2056.

Xu, F. 1997. Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases. *The Journal of Biological Chemistry*. **272:**924-928.

Zille, A. 2005. Laccase reactions for textile applications. Universidade do Minho. Portugal.