# BIOCATALYTIC STUDIES OF PHENOL OXIDASES PRODUCING ANTIOXIDANTS

By

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# DECLARATION

I, **Sandile Welcome Ncanana**, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgments indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any matter whatsoever.

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**Abstract**

In recent years there has been increasing interest in the production of oligomers and polymers of economic importance using biocatalysts; the application of enzymes in dimerisation or polymerisation processes can lead to the synthesis of unique compounds, with novel properties, that could not be easily achieved by conventional methods. Further, the application of biocatalysts in polymerization processes can be exploited in development of bioremediation systems, and there is a demand for new technologies that can be utilized in the removal of organic pollutants such as phenolics from contaminated environment. This study reports on the potential application of laccase, obtained from the white rot fungi *Trametes pubescens,* in the synthesis of organic compounds which are dimers or polymers, and in the development of bioprocesses of potential economic importance. The focus of this study is, particularly, on the effect of organic solvents and the structure of the substrates on the nature of products formed. The thesis also gives some insight into the relationship between the structure of laccase products and their biological (antioxidant and antimalarial) activity.

The compounds tyrosol, hydroxytyrosol, 8-hydroxyquinoline, and totarol were selected as the model compounds for laccase reactions. Tyrosol was oxidised by laccase, yielding dimeric and polymeric compounds which were identified by LC-MS and $^{1}$H-NMR. As a comparative study, hydroxytyrosol was also oxidised by laccase yielding dimeric, trimeric and polymeric compounds which were identified by LC-MS. Manipulation of this biocatalytic system resulted in development of an efficient process that allows for selectivity with respect to the products. A system was then developed whereby oxidation of hydroxytyrosol by laccase would selectively yield either dimers or oligomeric products. Thus, use of 50 % acetone in the reaction medium favoured the synthesis of dimeric products, and 20 % methanol resulted in the formation of a polymeric product. These results showed that hydroxytyrosol-laccase reactions were more readily controlled than tyrosol-laccase reactions, and this difference was attributed to structural configuration of these substrates.

The compound 8-hydroxyquinoline was polymerised in a reaction medium that contained 8 % acetone in sodium acetate buffer. The average molecular weight of the polymeric product obtained, as determined by matrix-assisted laser desorption/ionization coupled with time-of-flight mass spectrometry (MALDI-TOF) analysis, was 789 *m/z*. The degree of polymerisation of 8-hydroxyquinoline was found to be controllable by using acetone at different concentrations. Furthermore, the particle size of this polymer was influenced by the organic solvents used, in that use of acetone resulted in particles that were larger than those obtained using methanol or aqueous medium.

The compound totarol was also oxidised by laccase in a monophasic or biphasic medium. The totarol-laccase reaction yielded C-C linked and C-O linked totarol dimers, and again, the nature of the products was influenced by the type of organic solvent used. The dominant product was a C-C linked dimer. However, C-O linked dimer formation was increased in a reaction where the medium contained *t*-amyl alcohol.

The products of the laccase reactions were evaluated for antioxidant activity as compared with that of the starting materials. The DPPH radical quenching method for measuring antioxidant activity was used to demonstrate that the laccase reaction products reacted differently, compared with their parent compounds, and that the polymers of 8-hydroxyquinoline and tyrosol had higher antioxidant activity than the corresponding monomers. The biological activity of laccase reaction products was explained in terms of differences in their chemical structures.

The potential application of the biocatalytic systems developed in this study was investigated. Oxidation of 8-hydroxyquinoline and tyrosol by laccase yielded the polymeric products that precipitated out of the solution, and these products were physically removed from aqueous solution by filtration. The remaining solution was analysed by HPLC and it was found that residual concentration of 8-hydroxyquinoline (9.6 mg/ml) and tyrosol (4 mg/ml) in solution were significantly decreased with up to 100 % of these substrates being removed from the aqueous solution. Therefore, these biocatalytic reactions could be used as bioremediation systems for removal of 8-hydroxyquinoline or tyrosol from polluted water.

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## List of Figures

## List of Tables

## List of Publications

The following is a list of publications and conference proceedings resulting from the work presented in this thesis.

### Publications

**Sandile Ncanana** and Stephanie Burton. Towards the development of multi-enzyme biotransformation systems for production of target chemical compounds of economic importance. Chemical Technology, *Dec edition 2004.*

**Sandile Ncanana** and Stephanie Burton. Oxidation of 8-hydroxyquinoline catalyzed by laccase from *Trametes pubescens* yields an antioxidant aromatic polymer. Journal of Molecular Catalysis B: Enzymatic 44 (2006) 66-71,

**Sandile Ncanana** and Stephanie Burton. Enzymatic synthesis of poly (8-hydroxyquinoline) by *Trametes pubescens* laccase as a method to remove 8-hydroxyquinoline from aqueous solution, a bioremediation model. Proceedings. South African Chemical Engineering Congress 2006.

**Sandile Ncanana,** Lara Baratto, Lucia Roncaglia, Sergio Riva, and Stephanie Burton. Laccase-mediated oxidation of totarol. Advanced Synthesis and Catalyisis (2007) submitted.

## Conferences

Cape Biotech 2003 Conference, University of Cape Town, South Africa: Development of Multi-Enzyme Biotransformation Processes (MEBP) for the production of target chemical compounds of economic importance. Sandile Ncanana, Steven Makhongela, and Stephanie Burton.

Multi-step Enzyme Catalysed Processes (MECP'06) Graz/Austria 18-21 April 2006: Sequential Biocatalytic oxidation steps in a process producing novel antioxidants compounds. Burton, SG, Chigorimbo-Murefu, N, and Ncanana, S.

South African Chemical Engineering Congress 2006. "Engineering Africa in the 21st Century". 20 to 22 September 2006. International Convention Centre Durban, South Africa. Enzymatic synthesis of poly (8-hydroxyquinoline) by *Trametes pubescens* laccase as a method to remove 8-hydroxyquinoline from aqueous solution, a bioremediation model. Sandile Ncanana and Stephanie Burton.

## Table of Contents

**Chapter 2**

**Investigations on the synthesis and antioxidant activity of the products obtained from tyrosol-laccase and hydroxytyrosol-laccase reactions.**

**Chapter 3**

**Oxidation of 8-hydroxyquinoline by laccase from Trametes pubescens**

**Chapter 4**

**Laccase-catalyzed dimerisation of the natural compound totarol**

# Chapter 1

# Literature review and background to the research

## 1.1 General introduction

This thesis reports an investigation into the application of the enzyme laccase, from the white rot fungus *Trametes pubescens,* in the synthesis of organic compounds derived from selected phenolic substrates. At the heart of this thesis is the relationship between the structure of the substrates, the reaction medium used, and the nature of the products formed. The thesis also gives some insight into the relationship between the structure of laccase products and their biological activity with respect to antioxidant and antimalarial activity properties.

## 1.2 Biocatalysis

Enzymes as biocatalysts can play an important role in the synthesis of chemical compounds with significant biological activity which cannot readily be synthesized by conventional chemical methods, especially compounds that resemble natural products. The difficulty in conventional chemical synthesis of these products arises because their structures are complex, and their synthesis would require precise chiral selectivity, regioselective or site-specific substitution steps, addition of functionality, and/or formation carbon-carbon bonds in specific positions (Roberts, 1998). Biocatalytic reactions are well-recognized for their potential in providing such highly selective synthesis routes (Bull *et al.*, 1999). Enzymes are able to catalyze stereo-and regio-selective reactions, usually without the need for chemical protecting groups, and can be used to produce pure isomers as opposed to racemic mixtures (Bull *et al.*, 1999).

A vital factor in biocatalytic processes is the availability of a suitable biological catalyst. Most enzymes of industrial importance have been derived from species that are Generally Regarded as Safe (GRAS). These include bacterial species and fungi (Dalboge and Lange, 1998). Hydrolases are the most common biocatalysts but redox enzymes are increasingly being used for chiral synthesis in the pharmaceutical industry, in medicinal chemistry, agriculture, cosmetic and food industries. The development of a biocatalytic process is generally based on the requirement for

the specific target product. On the basis of the reactions required for production of these target compounds, the required biocatalysts can be defined, screened for, characterized and modified (Burton, 2001).

Constituting the next essential step in of the biocatalyst development is the characterization of the selected enzyme, in terms of enzyme activity, inhibition and substrate specificity studies and stability (e.g. temperature and pH dependence) (Saito *et al.*, 2003). The biocatalyst characteristics can also, potentially, be modified by means of biochemical or protein engineering (Burton *et al.*, 2002). It is believed that protein or biochemical engineering allows introduction of specific changes in the biocatalyst that cannot be achieved by molecular evolution, such as surface structure modification for immobilization (Reetz and Jaeger, 1999; Wilks and Holbrook, 1991).

The biocatalyst can be further developed by immobilization. According to Walsh (2002) enzyme immobilization is a technique specifically designed to restrict the freedom of movement of an enzyme, allowing it to be stably retained and recovered at the end of the biocatalytic process. This facilitates the reutilization of enzymes which is of obvious economic benefit. The more expensive the enzyme preparation is, the greater the motivation to utilize it in an immobilized form. In some cases, the stability of the enzyme can be enhanced by immobilization. For example, a significant increase in operational and thermal stability was observed for FEAGE (Fuller's earth-adsorbed gelatin-entrapped) tyrosinase (Sharma *et al.*, 2003).

Subsequent to biocatalyst development is its application in the biotransformation process, and the isolation and characterization of the products.



### 1.2.1 Applications of enzymes in biocatalysis

The potential benefits of utilizing enzymes or whole - cell biocatalysts in industrial processes arise because of the following:

- Enzymes are very efficient catalysts

The rates of enzymatic reaction are increased by $10^8$-$10^{10}$ when compared with non-enzymatic reactions

- Utilization of enzymes leads to development of environmental benign processes
- Enzymes act under mild conditions of temperature, pH and pressure
- Enzymes catalyze a broad spectrum of reactions
- Enzymes show three major types of selectivity: chemoselectivity, regioselectivity and enantioselectivity (Faber, 1994).

Hence, enzymes are increasingly important catalysts in the chemical industry, for synthesis of products ranging from fine chemicals and pharmaceuticals to bulk chemicals such as polymers. They are also equally important in the environmental biotechnology sector for bioremediation or biodegradation of waste material.



#### 1.2.1.1 Application of biocatalysts in bioremediation

The pollution of water or soil by various industries is a major concern not only to industrialists who pay major fines for discharging toxic substances to the environment, but also to environmentalists and nature conservation programmes. It is generally understood that organic pollutants such as phenols are challenging to remove from contaminated environments. Thus there is a demand for new technologies that can be utilized in the removal of these pollutants.

Removal of phenols from wastewater can be achieved through conventional remediation methods such as solvent extraction, chemical oxidation and adsorption on activated supports) (Klibanov *et al.*, 1983). These methods may be effective, but some of these methods present a number of disadvantages such as high costs, time-consuming procedures and formation of toxic residues. Biological technologies involving the use of phenol oxidases, for instance, laccases, peroxidases and tyrosinases, may represent an efficient, alternative way to address the clean-up of phenol-polluted wastewater (Arseguil and Baboulene, 1994; Atlow *et al.*, 1984).

Laccases, in particular, are known for their ability to degrade certain polymers such as lignin, and hence their application in textile finishing and dye removal. Lignin removal, the key step for

natural biodegradation of lignocellulose, is required both for converting wood into paper pulp and for bleaching pulp fibers (Sigoillot *et al.*, 2005). Bioremediation processes often involve redox reactions, using both native and engineered enzymes of microorganisms. For instance, the oxidation of aromatic hydrocarbons including polyaromatic hydrocarbons (PAHs), haloaromatics including polychlorinated biphenyls (PCBs), and insecticides such as lindane, and textile dyes, mediated by peroxidases and laccases, have been reported by several authours (Burton, 2003). Polycyclic aromatic hydrocarbons (PAHs) together with other xenobiotics are a major source of contamination in soil; therefore, their degradation is of great importance for the environment. Laccases were used to mediate the coupling of reduced 2, 4, 6-trinitrotoluene (TNT) metabolites to an organic soil matrix, which resulted in detoxification of the residue (Durán and Esposito, 2000). Luke and Burton (2001) reported the degradation of phenol and *p*-cresol by phenol oxidase from *Neurospora crassa*. Recently, Ryan *et al.* (2005) showed that a laccase from *Trametes pubescens* was effective in the removal of phenol and *p*-, *m*- or *o*-cresol. Most of the studies have focused on the degradation of the substrates, and with little or no explanation on the fate of bioremediation products.



### 1.2.1.2 Application of biocatalysts in organic synthesis

Besides playing a vital role in degradation or polymerisation of toxic substances in water or soil, thus rendering a cleaner environment, oxidative enzymes such as laccase and tyrosinase are also used in organic synthesis. From the literature, various organic compounds have been synthesized using phenol oxidases. For instance, laccases and tyrosinase are involved reactions where novel biologically active products such as the antibiotic phenoxazinone derivative actinocin (Osiadacz, *et al.*, 1999), poly-hydroxybenzoic acids and polyphenolic polymers (Uyama and Kobayashi, 2002) among others. Of great interest in the utilization of laccases, in particular, is their ability to produce radicals that couple to each other by C-C or C-O bonds (Kobayashi and Higashimura, 2003). Through these characteristics, various organic molecules have been synthesized such as biaryls (Ciecholewski, 2005); and 17 *beta*-estradiol dimer (Nicotra *et al.*, 2004) among others.

Chemoselectivity of laccase towards certain substrates is increasingly being noted by phenol oxidase researchers. Some examples of regioselective oxidations include the oxidation of the

primary OH's of natural glycosides (thiocolchicoside, colchicoside, amygdalin, asiaticoside, ginsenoside (Baratto *et al.*, 2006). These reactions have been performed on a preparative scale by exploiting the laccase-2, 2, 6, 6-tetramethyl-1-piperidinyloxy (TEMPO) methodology (Baratto *et al.*, 2006). TEMPO acts as a mediator in the oxidation of the natural glycosides mentioned above. It is initially oxidised by laccase and then itself catalyses the oxidation of the glycosides (Barrato *et al.*, 2006). Laccases can also be used in food or pharmaceutical industries for production of phenolic colourants. Recently, Mustafa *et al.* (2005) synthesised phenolic colourants by using an industrial laccase named Suberase® (Novo Nordisk A/S, Bagsvaerdt, Denmark).

The importance of biocatalysts in organic synthesis has also been noted with respect to hydroxylation of various aromatic compounds. Efficient and specific insertion of one oxygen atom into an organic substrate is a reaction that is difficult to perform using conventional methods of organic synthesis. Although some catalysts have been developed that are able to catalyze specific oxygenations, the specificity of enzymes performing monooxygenations, such as phenol oxidase or tyrosinase, is still remarkable (Berkel *et al.*, 2006).

Most of the chemical reactions performed using conventional methods are conducted in organic solvents, with the primary aim being to increase the solubility of the substrates. Thus, successful practical application of enzymes as catalysts for chemical reactions depends on their ability to tolerate the operational conditions, which includes their ability to retain activity in both aqueous and organic media. Immobilization of enzymes has been reported to be one of the means of developing a biocatalyst to allow it to tolerate adverse conditions.



### 1.2.1.3 Immobilization of a biocatalyst

Immobilization is a technique specifically designed to restrict the freedom of movement of an enzyme, allowing it to be stably retained and recovered at the end of the biocatalytic process. Enzyme immobilization involves the attachment or incorporation of enzyme molecules on to or into large structures, via simple adsorption, covalent attachment, or encapsulation (Tischer and Kasche, 1999; Livage *et al.*, 2001). Benefits that can be obtained from enzyme immobilization

include the improvement in enzyme stability that can allow successful practical applications. Immobilization can also reduce the amount of enzyme required, prolong the lifetime of enzyme reactors, and increase the potential for enzyme reuse (Kim *et a*l., 2006). Dodor and coworkers reported that laccase from *Trametes versicolor*, immobilized in kaolinite, showed increased resistance to acid and base denaturation (Dodor *et a*l., 2004). Similar results were reported by Yinghui and coworkers who reported the improved stability of laccase immobilized on carboxylated polyvinyl alcohol under extreme conditions of pH (Yinghui *et al.*, 2002). Immobilization of *Lentinula edodes* laccase on Eupergit C increased its pH, thermal and proteolytic stability, with only slight modifications in laccase oxidation efficiency (D'Annibale *et al.*, 2000). The other benefits that can be derived from use of an immobilized enzyme include uniformity of conversions and less complex downstream processing or recovery of products (Komaraiah *et al.*, 2003). However, there are some disadvantages of immobilization that include loss of enzyme activity that can occur during and after immobilization, and complications related to diffusion of substrates and products have also been reported (Dayal and Godjevargova, 2006).



### 1.2.1.4 Biocatalysis in organic solvents

There is a growing interest in using enzymes as catalysts in reaction medium containing organic solvents. The main reason for choosing a 'non-conventional' medium is to shift the thermodynamic equilibrium to favour synthesis over hydrolysis. For instance hydrolases such as lipase and proteases can catalyze synthetic reactions in organic media (Kaur *et al.*, 1997). Several authors have reported the potential application of lipases in esterification and transesterification (Cao *et a*l., 1992; Mustranta *et al.,* 1993), reactions that are only possible in organic media.

The use of organic solvents is not restricted to hydrolases but can also be used with other enzymes. With regard to phenol oxidases, organic solvents may play an important role in determining the nature of the end products. Studies of oxidation of ferulic acid by laccase from *Myceliophthora thermophila* in biphasic hydro-organic medium showed significant advantages such as increased effectiveness in the prevention of browning and an increased solubility of the resulting colored products (Mustafa *et al.*, 2005). The oxidation of ferulic acid by laccase in a biphasic aqueous-organic system consisting of ethyl acetate and sodium phosphate buffer resulted in intermediate stable yellow products. These authors asserted that organic solvent

decreased the activity of the laccase and the rate of non-enzymatic reactions led to the stability of these intermediate products which otherwise would be unstable in aqueous medium (Mustafa *et al.*, 2005). Furthermore, the biphasic medium facilitated the separation of these products which were soluble only in the organic phase of this system (Mustafa *et al.*, 2005). In similar work, Tranchimand *et al.* (2006) asserted that a biphasic system can be used to improve the yield of and sinapinic acid products in laccase catalysed-reactions. These authors reported that in the absence of co-solvent, only 24 % yield of bis-lactone was obtained as the product of sinapinic-laccase reactions wheareas ethyl-acetate containing medium resulted in 85 % yield of bis-lactone (Tranchimand *et al.*, 2006).

It has been reported that the use of organic media has an effect on the activity of biocatalyst. According to the studies of Tominaga *et al.* (2004) a thermostable laccase from *Trametes sp.* showed the highest catalytic activity for the oxidation of 4-hydroxybiphenyl (4-HB) when dimethylsulfoxide (DMSO) was employed as a co-solvent. Kazandjian and Klibanov (1985) reported that tyrosinase is more active in organic solvent media as indicated by the greater number of phenolic substrates quantitatively converted to stable *o*-quinones (Kazandjian and Klibanov, 1985). Chloroform is a particularly suitable medium for tyrosinase, since oxygen has higher solubility in it and is therefore not a limiting factor in the reaction (Burton *et al.*, 1993; Kermasha and Tse, 2000).

Depending on the aim of the experiment, various organic media that can be used for enzymatic biocatalysis in three types of systems:

- biphasic system: this medium consists of two phases, namely the aqueous phase containing enzyme, and a second phase of a non-polar organic solvent where most of the product and substrate are located
- monophasic organic system: this system is characterised by bulk water replaced by water-immiscible organic solvents
- monophasic aqueous-organic system: the substrate and or product are dissolved in a monophasic solution consisting of water and a water-miscible organic co- solvent (Faber, 1994).

Although the evidence of the effects of organic solvents in biocatalysis is increasingly becoming available to researchers, there are relatively few studies that have been reported in literature regarding oxidases. Thus in the current study, the effects of organic solvents on the selectivity of product formed in laccase reactions was evaluated.

### 1.2.1.5 Application of biocatalysts in bioreactors

Bioreactors are used to culture and/or contain microorganisms, such as yeasts and bacteria, for production of useful chemical compounds or enzymes. The main objective of designing a reactor is to produce a specified product at a given rate from known reactants under appropriate conditions. Bioreactors can be classified according to various criteria:

(a) Type and form of biocatalyst: free cells in submerged cultures; carried bound or immobilized cells/enzymes; retention or recirculation of the biocatalyst

(b) Configuration: tank (height/diameter <3), column (height/diameter > 3)

(c) Energy input and aeration: liquid phase; gas phase; combined

(d) Hydrodynamics: perfect mixing; partial mixing; no mixing

(e) Mode of operation: batch; continuous; fed-batch (Coulson *et al.*, 1982)

The choice of a reactor is dependent on its cost and ease of operation, among other considerations. The most widely used reactors in biotransformation processes include packed bed reactors, fluidized bed reactors and membrane reactors (Coulson *et al.*, 1982).

#### 1.2.1.5.1 Membrane reactors

Membrane bioreactors contain semi-permeable membranes which allow the free passage of the product molecules but contain the soluble or adsorbed enzyme molecule. The typical example of such a membrane is dialysis membrane, used for removing low molecular weight species from

protein preparations. In a membrane bioreactor, the enzyme is either immobilized by absorption or entrapment within the membrane itself to form a catalytic membrane or is immobilized on another carrier, which is then packed inside the membrane (Curcio *et al.*, 2006; Burton *et al.*, 1998). The disadvantage of these reactors is that membranes are expensive and need to be replaced at regular intervals (http://www.lsbu.ac.uk/biology/enztech/membrane.html).

#### 1.2.1.5.2 Fluidized bed bioreactors (FBB)

Fluidized bed bioreactors (FBB) have received increased attention in the recent years due to their advantages over other types of reactors. Most of the FBBs developed have been for biological systems involving cells as biocatalysts. The FBBs are generally operated in co-current upflow with liquid as the continuous phase. Usually, fluidization is achieved either by external liquid re-circulation or by gas fed to the reactor. In the case of immobilized enzymes the usual situation is a two-phase system involving solid and liquid but the use of cells as biocatalysts may necessitates introduction of gas (air) as the third phase. Couto and coworkers reported the decolourization of different synthetic dyes (Indigo Carmine, Bromophenol Blue, Methyl Orange and Poly R-478) by the white-rot fungus *Trametes hirsuta* at bioreactor scale under solid-state conditions, operating with ground orange peelings as a support-substrate (Couto *et al.*, 2006).

#### 1.2.1.5.3 Packed-bed or fixed-bed bioreactors

In these systems, the immobilized biocatalyst is packed in the column and fed with reactants either from top or bottom depending on the substrate and/or product solution flow properties. A continuous packed bed reactor has the advantage over a batch reactor because it is easy to control and operate, there is a reduction of labour costs, stabilization of operating conditions, and easy quality control of products. In literature, few studies have been reported on the use of packed-bed reactors in the bioremediation process or in the production of organic compounds using biocatalysts. For instance, crude laccase covalently immobilized to Eupergit® C was used in the continuous elimination of 2,6-dimethoxyphenol carried out in a packed bed reactor followed by filtration of the precipitate formed (Hublik and Schinner, 2000). More recently, a packed backed

reactor showed some advantages over a batch reactor in the production of L-DOPA by tyrosinase immobilised on Cu- alginate gels, where the productivity obtained in the packed -bed reactor was higher than that of the batch reactor (Ates *et al.*, 2006). These authors asserted that undesirable losses of L-DOPA can be prevented by the continuous presence of ascorbic acid and substrate solution, and this was pumped to a packed bed reactor continuously in their study. Continuous supply of ascorbate in the reactions that involve tyrosinase is imperative when the desired product is catechol rather than quinones or polymeric compound. Ascorbate reduces quinones into their corresponding catechols in such reactions (Chapter 2). Thus in a continuous packed-bed reactor, reagents are easily supplied on the continuous basis as required by the process.

To this point, this review has covered the general aspect of biocatalysis which included the advantages of utilizing biocatalysts in various chemical reactions (i.e bioremediation and organic synthesis), and application of biocatalyst in organic solvents and/ or in bioreactors. It is clear that biocatalysts are increasingly becoming a recognised tool in chemical processes. The next section of this review gives deeper insight into the specific biocatalysts (laccase and tyrosinase) which were used in this study.



## 1.3 Laccases

### 1.3.1 Structure and reaction mechanism of laccase

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is a multi-copper-bearing lignolytic enzyme which catalyzes the one-electron oxidation of aromatic phenolic compounds with concomitant reduction of oxygen to water. Laccases often occur as isoenzymes with a molecular mass of monomer ranging from about 50 to 100 kDa, that oligomerize to form multimeric complexes (Claus, 2004). Laccase catalytic activity is believed to involve a minimum of four copper atoms per active site arranged as three types:

Type 1: paramagnetic copper which confers blue colour to multicopper proteins
Type 2: paramagnetic non-blue copper
Type 3: diamagnetic spin-coupled copper- copper pair (Figure 1.1).

Type 1 copper has trigonal coordination, with two histidines and a cysteine as conserved ligands. The type 1 copper provides the site for the oxidation of substrate and this has been attributed to its high redox potential. Type 2 and type 3 copper ions in laccase form a trinuclear cluster, where reduction of molecular oxygen and release of water take place. It has been suggested that the oxygen molecule binds to type 2 and one of the type 3 copper ions, for activation. The various copper centers of laccase transfer electrons from a reducing substrate to molecular oxygen, a phenomenon achieved by four electronic oxidations of the substrate catalysed by type 1 copper. Electrons are then transferred to type 2 and type 3 clusters (Figure 1.1).The oxidation of the reducing substrate results in the formation of reactive radicals that can undergo non-enzymatic reactions (Burton, 2003). The structure and mechanism of laccase allow for a very broad substrate range (discussed in Section 1.3.2).

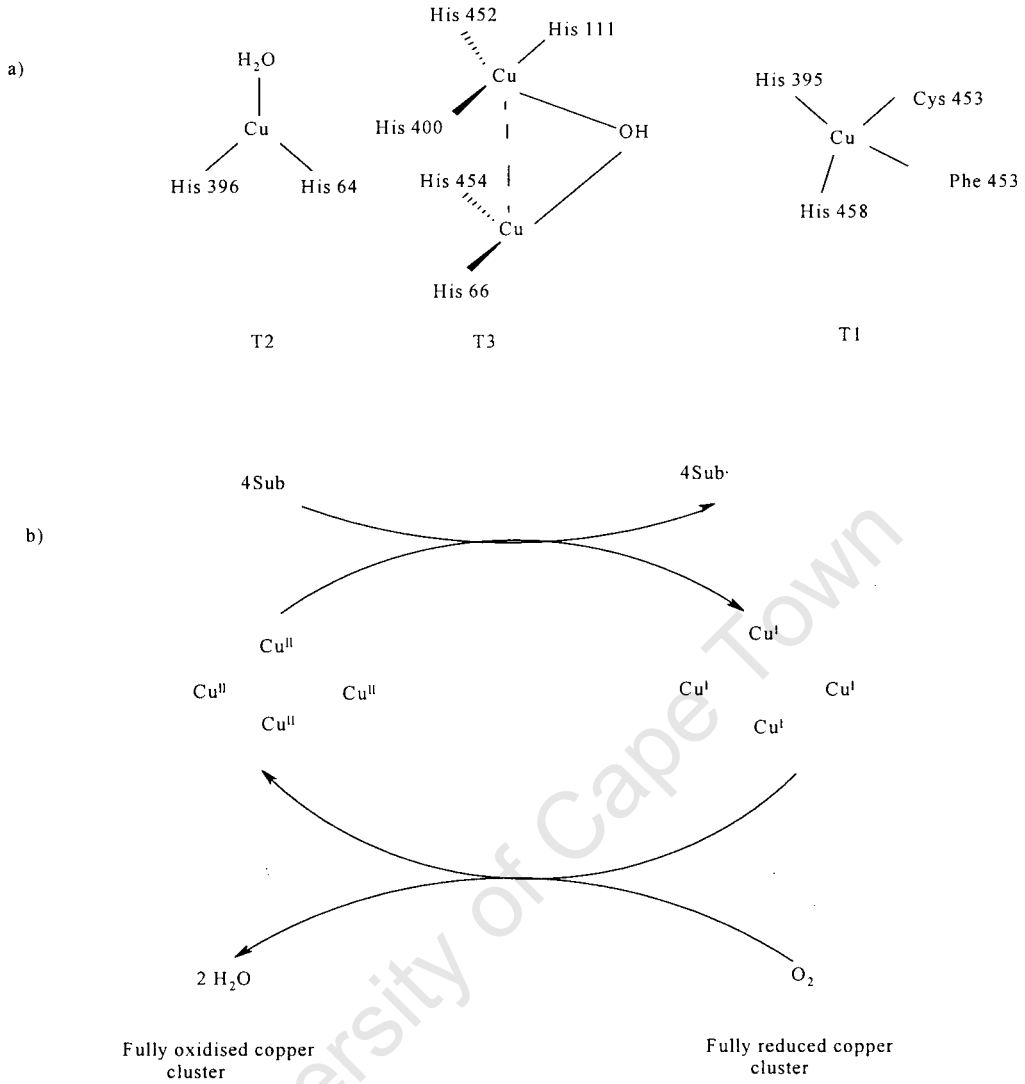





**Figure 1.1 Laccases: active-site structure and catalytic cycle:**

(a) Catalytic cluster of the laccase from *Trametes versicolor* with 4 copper atoms. Type 1 (T1) copper confers the typical blue colour to the protein and is the site where substrate oxidation takes place. Type 2 (T2) and Type 3 (T3) coppers form a trinuclear cluster, where reduction of molecular oxygen and release of water take place.

(b) Schematic representation of the laccase catalytic cycle producing 2 molecules of water from reduction of one oxygen molecule, and the concomitant oxidation of 4 substrate molecules (Sub) to the corresponding radicals (Riva, 2006).

### 1.3.2 Substrates of laccases

Laccases can be applied in the synthesis of chemicals or in the degradation of complex natural polymers. In syntheses, enzymatic oxidation of aromatic compounds by laccases generate radicals that react with each other to form dimers, oligomers or polymers, covalently coupled by C-C, C-O and C-N bonds (Claus, 2004). Laccases are reported to be quite non-specific as compared with other enzymes (Thurston, 1994). Laccases catalyze the one-electron oxidation of a wide variety of organic substrates, including mono-, di- and polyphenols, aminophenols, methoxyphenols, aromatic amines, polyamines and lignin, and aryl diamines with the concomitant four electron reduction of oxygen to water (Thurston, 1994; Höfer and Schlosser, 1999; Burton, 2003).

In the presence of mediators such as 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) or 1-hydroxybenzotriazole (HBT), laccase was found to degrade lignin model compounds and delignify kraft pulp (Kawai *et al.*, 1999; Bourbonnais *et al.*, 1997). Daina *et al.* (2002) reported the degradation of phenolic lignin dimer by laccase obtained from *Ceriporiopsis subvermispora* (Figure 1.2).

Among other studies, it was reported that laccase from the white rot fungus *Pycnoporus cinnabarinus* SBUG-M catalyzed oxidative dimerization of salicylic esters, in which a carbon-carbon bond was formed, resulting in the formation of biaryl products (Figure 1.3) (Ciecholewski, 2005).

Laccases are also known for their ability to oxidize compounds bearing benzoic acid functional groups. A fungal laccase from *Trametes villosa* (EC 1.10.3.2 *p*-phenoloxidase) was used to mediate the oxidation and cross-coupling of two *para*-dihydroxylated benzamide derivatives with 4-aminobenzoic acid (Figure 1.4) (Manda *et al.*, 2005).

The diverse nature of laccase substrates was further demonstrated in the work reported by Nicotra *et al.* (2004) where it was shown that laccase could mediate the oxidation of the steroid hormone 17 *beta*-estradiol, **10** to form C-C, **11** and C-O, **12** dimers (Figure 1.5).

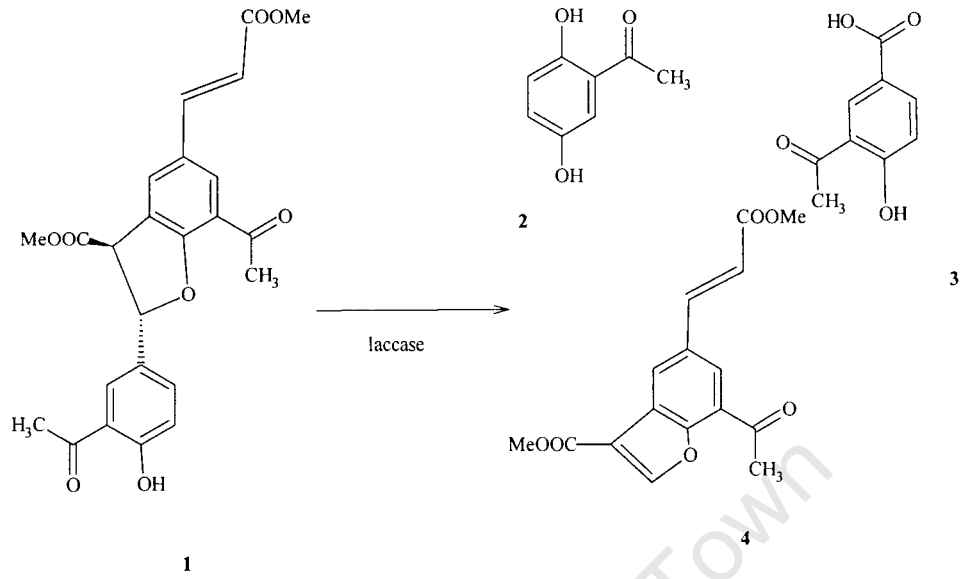



**Figure 1.2 Products obtained from fungal laccase degradation of phenolic beta -5 lignin, a lignin model compound; the major product recovered was compound 2, while the other products were not quantified (Daina *et al.*, 2002).**

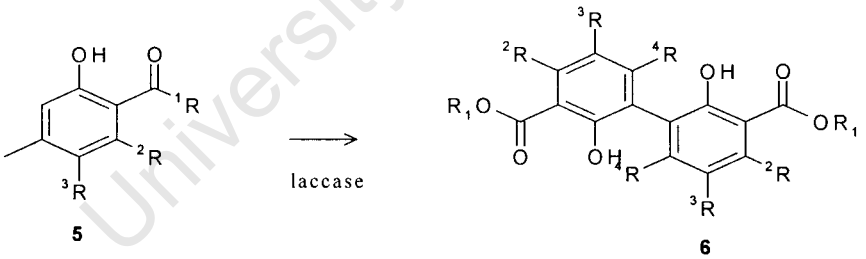



**Figure 1.3 Dimerization of salicylates by laccase (Ciecholewski, 2005).**

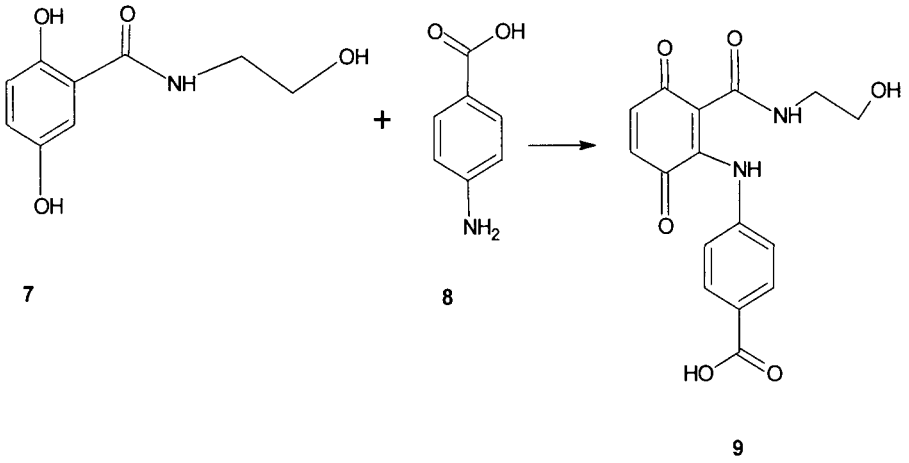



**Figure 1.4 Reaction pathway scheme for laccase-mediated cross-coupling of 2, 5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide (7) and 4-aminobenzoic acid (8), yielding the product 2-(4-carboxy-anilino)-*N*-(2-hydroxyethyl)-3, 6-dioxo-1,4-cyclohexadien-1-carboxamide (9) (Manda *et al.*, 2005).**

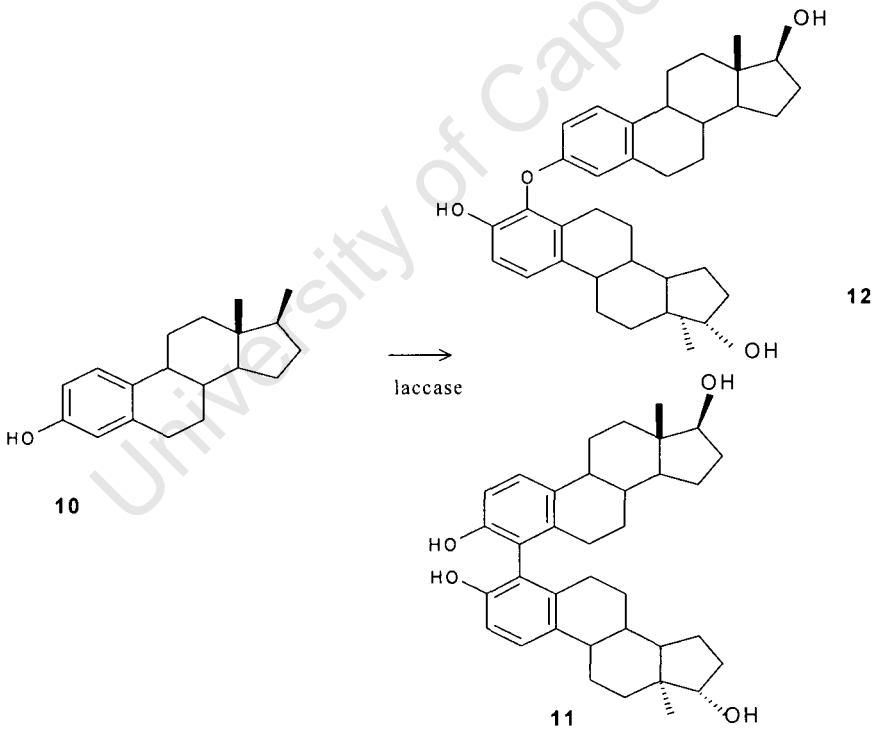



**Figure 1.5 Laccase-mediated oxidation of *beta*-estradiol 1, 10 to form dimers (Nicotra *et al.*, 2004).**

Compounds with redox potentials comparable to that of laccase are less favourable as laccase substrates, but these compounds can be oxidised by laccase in the presence of other, small, redox-active substrates (Kulys and Vidziunaite, 2005). In laccase reactions containing mediator, the mediator can be oxidized by the enzyme and subsequently oxidize another compound that is either a substrate or non-substrate resulting in the formation of oxidized product(s), and regeneration of the mediator. The effectiveness of mediators is attributed to their lower redox potentials as compared to fungal laccases. The redox potentials of mediators are lower than of T1 copper (laccase) therefore the change in redox potential becomes higher (equation below), and hence the higher driving force. The higher driving force translates into a higher rate of electron transfer or oxidation of the unnatural substrate present in the reaction medium (Kurniawati and Nicell, 2007).

$$\textit{Change in } E^0 = E^0 \textit{(T1 copper)} - E^0 \textit{(substrate)}$$

In essence, these small molecules are mediators and can be used to expand the substrate range for laccases. Several authors have reported on the role of mediators in enzymatic oxidation with laccases. Examples of substrates that can be oxidized by laccase in the presence of mediators include anthracene and benzo[a]pyrene, in the presence of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Dodor, 2004), and ethers of various structures in the presence of 1-hydroxybenzotriazole (HBT), *N*-hydroxyphthalamide (NHPI) and 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO) (D'Acunzo *et al.*, 2003; Galli and Gentili, 2004). Examples of mediators that can be used in laccase reactions are shown in Figure 1.6.

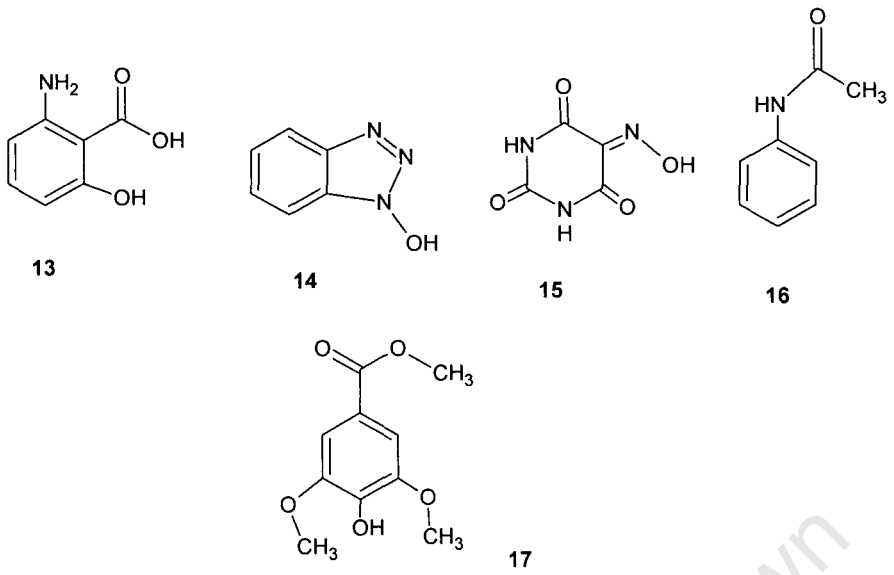



**Figure 1.6 Examples of laccase mediators. 3-hydroxyanthranilic acid (HAA) (13), *N*-hydroxybenzotriazole (HBT) (14), violuric acid (VLA) (15), *N*-hydroxyacetanilide (NHA) (16), methyl ester of 4-hydroxy-3, 5-dimethoxy-benzoic acid (syringic) (17).**



### 1.3.3 Sources and production of laccase

The recognition of laccase as an important biocatalyst for the chemical industry has prompted the need to find new laccases from various sources, and to improve laccase production methods. Laccases can be divided into two major groups which show clear differences, namely those from higher plants and those from fungi (Harvey and Walker, 1999). The presence of laccase-like enzymes has been reported in bacteria (Claus and Filip, 1997; Givaudan *et al.*, 1993) and to some extent, insects (Kramer *et al.*, 2001). In this study, the laccase used was obtained from a white-rot fungus. Thus the focus of this review will be limited to the fungal laccases.

Usually laccase originates in the cytoplasm, but many instances of secretion of laccases as extracellular enzymes have also been reported. Most white-rot fungi apparently contain several laccases, which may be referred to as laccase 1 or laccase 2, and often differ in molecular weight

(Kim *et al.*, 1995). Four different laccases have been detected in *Rhizoctonia solani* (Wahleithner *et al.*, 1996) and *Fusarium proliferatum* (Kwon and Anderson, 2001), and there appear to be at least three laccases in *Botrytis cinerea* (Marbach *et al.*, 1984; Pezet, 1998). White-rot fungi, in particular, are believed to have evolved complex enzymatic machinery capable of degrading lignin, and they are only organisms able to degrade whole wood (Kirk and Fernn, 1982). White-rot fungi produce laccases that form part of this machinery, having an ability to oxidize phenolic lignin-typel compounds by C-α - C-β cleavage of the side chain (Kawai *et al.*, 1999). Specific fungi that produce such laccases include *Lentinula edodes* (Leatham, 1986), *Aspergillus nidulans* (Aramayo and Timberlake, 1990), and the Basidiomycetes *Trametes versicolor* or *T. pubescens* (Collins and Dobson, 1997; Ryan *et al.*, 2005) among others.

The white-rot fungal Basidiomycetes *T. versicolor or T. pubescens,* in particular, have been reported to be the excellent producers of laccase (Jang *et al.*, 2002; Galhaup and Haltrich, 2001). To increase the production of laccase from these species some researchers have used different inducers in the culture medium. For instance, laccase production can be enhanced by the addition of compounds such as 2, 5-xylidine, veratryl alcohol (3,4-dimethoxibencyl alcohol) and ethanol. Laccase production by *T. versicolor* and *T. hirsuta* grown on barley bran was improved by supplementing the cultures with 2,5-xylidine and copper sulphate (Couto *et al.*, 2004). The effect of metal ions such as copper was also studied by Lorenzo *et al.* (2002). These authors discovered that the activity of laccase from *T. versicolor* can be increased up to 12-fold in the presence of copper. Studies by Galhaup *et al.* (2002) revealed that extracellular laccase production can be highly enhanced by addition of copper II in the millimolar range, in glucose-based culture. The work by Ryan *et al.* (2005) concurred with other authors, that *Trametes* species were excellent producers of laccase. Further, Ryan *et al.* (2005) reported that *T. trametes* and *T. pubescens* are resilient to the effect of aromatic compounds which are usually toxic to most other microorganisms. This was proved by growing these organisms in media containing phenolic effluent, which led to increases in biomass and laccase activity (Ryan *et al.*, 2005).

## 1.4 Tyrosinase

### 1.4.1 Overview

Tyrosinase (EC 1.14.18.1), like laccase, is a phenol oxidase enzyme, and it is also known as polyphenol oxidase (PPO). Tyrosinases are copper-containing monooxygenases known to catalyze two reactions, viz., the *ortho*-hydroxylation of phenols to catechols (1,2-dihydroxybenzene) by means of cresolase activity and subsequent oxidation of catechols to *ortho*-quinones by means of catecholase activity using molecular oxygen (Figure 1.7) (Sánchez-Ferrer *et al.*, 1995; Boshoff *et al.*, 2003). The reactive quinones polymerize non-enzymatically to form macromolecular melanins (Burton, 1994).

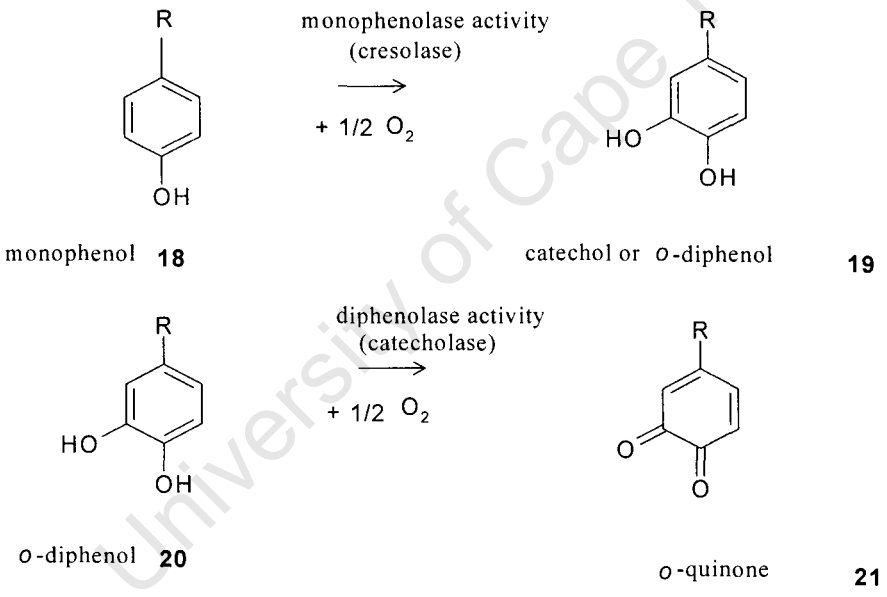



**Figure 1.7 Cresolase and catecholase activity of tyrosinase.**

The known reaction products of tyrosinase include the natural pigments of mammalian hair, eye, and skin (Yasunobu and Gordon, 1959; Raper, 1928), and browning of fruits and vegetables during post-harvest handling (Martinez and Whitaker, 1995). Tyrosinase was first discovered by Schoenbein (1856) from *Agaricus bisporus* and subsequently by Bourquelot and Bertand (1895) from the mushroom *Russula nigricans*. Mushroom tyrosinase, from *Agaricus bisporus*, in

particular, is a tetramer, comprising two H subunits (43 kDa) and two L subunits (13 kDa), with a molecular mass of 120 kDa containing two active sites (Strothkamp *et al.*, 1976; Burton 1994). Tyrosinase has two copper ions per active site, and each copper is coordinated by histidine residues in the active sites. These two coppers are known to be essential for catalytic activity of the enzyme (Solomon *et al.*, 1996).

### 1.4.2 Reaction mechanism of tyrosinase

Tyrosinase catalyzes two reactions, utilizing its cresolase and catecholase activities respectively. The reaction mechanisms for *ortho*-hydroxylation of monophenols, or oxidation of the diphenols, are based on the associative ligand substitution at the tyrosinase site. The phenolic substrate initially coordinates to an axial position of one of the coppers of oxytyrosinase. Rearrangement through a trigonal bipyramidal intermediate leads to *ortho*-hydroxylation by the bound peroxide, followed by loss of water, and coordination of the newly formed diphenol. Subsequent oxidation of the diphenol to a quinone results in a reduced, binuclear cuprous site which releases the quinone and then binds dioxygen to regenerate oxytyrosinase (Wilcox *et al.*, 1985; Claus and Decker, 2006).



### 1.4.3 The lag phase of tyrosinase activity

A significant feature of tyrosinase is its ability to exist in an inactive, or latent, state. Tyrosinase exists in either a latent or an active form, as well as in both forms simultaneously, in many sources (Whitaker, 1995). According to Yamaguchi *et al.* (1970) and Van Leeuwen and Wichers (1999), the latent mushroom (*Agaricus bisporus*) tyrosinase represents around 98-99% of total activity. The lag phase in tyrosinase activity is attributed to competition between the phenolic and catecholic substrates leading to an inactive enzyme known as *met*-enzyme. The addition of L-DOPA, in particular, activates *met*-enzyme into oxytyrosinase, the state required for the *ortho*-hydroxylation of monophenols (Naish-Byfield *et al.*, 1994; Riley, 2000). It has been suggested that the lag phase can be shortened by the addition of small amounts of reducing agent such as L-DOPA or metal ions such as ferrous ions (Ros *et al.*, 1993). The activation of latent tyrosinase

has also been achieved by addition of sodium dodecyl sulfate (SDS) (Pérez-Gilabert *et al.*, 2004; Moore and Flurkey, 1990 among others).

### 1.4.4 Inactivation of tyrosinase in a reaction

Tyrosinase is one of a small number of enzymes that undergo suicide inactivation. The earliest report of suicide inactivation occurring during tyrosinase-catechol oxidation was by Asimov and Dawson (1950). Subsequently, many researchers have investigated different aspects, attempting to understand the mechanism or reasons for the inactivation. For instance, Haghbeen *et al.* (2004) acylated the residues of mushroom tyrosinase and proposed that the interruption in the conformational changes in the tertiary and quaternary structures of tyrosinase, triggered by the substrate then mediated by the solvent molecules, might be the cause for the suicide inactivation of the enzyme.

### 1.4.5 Substrates of tyrosinase

Tyrosinase oxidizes a very broad range of substrates. The most researched natural compounds that can be oxidised by tyrosinase are tyrosine and DOPA (Burton, 1994). Tyrosine is transformed by tyrosinase into L-DOPA, **22** and dopachrome, **23** (Figure 1.8). This reaction has been used by many researchers in assaying the activity of polyphenol oxidase (Burton, 1994; Boshoff *et al.*, 1998). L-DOPA is also used for treating Parkinson's diseases (Held *et al.*, 1998).

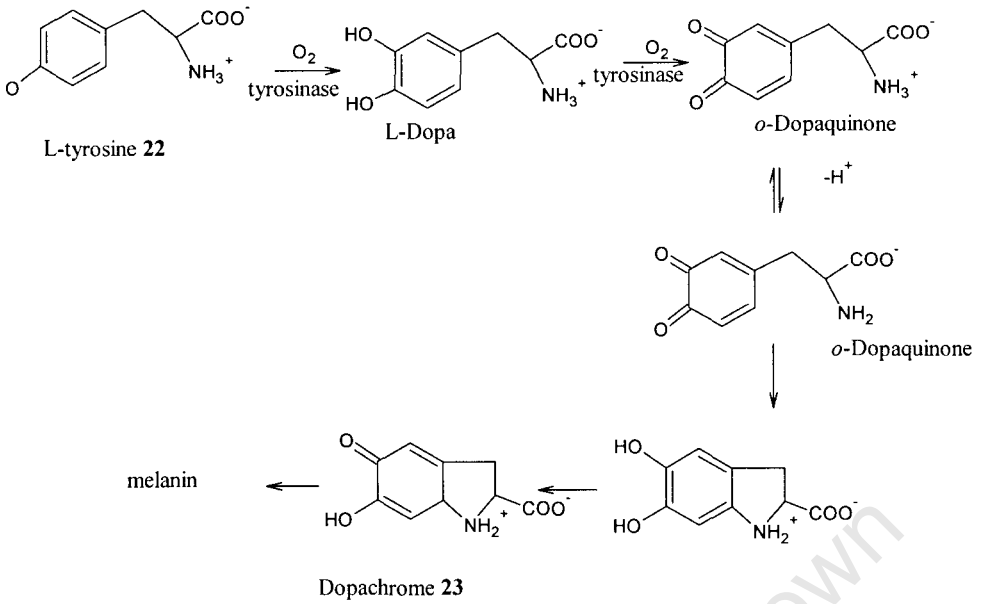



**Figure 1.8 Melanogenesis pathway from L-tyrosine, 22 to dopachrome, 23 (Burton, 1994; Boshoff *et al.*, 2003).**

Tyrosinase is also known for its ability to oxidize monophenols such as *p*-cresol, *m*-cresol, phenol, 4-chlorophenols and 4-methoxyphenol for the production of catechols (Boshoff *et al.*, 2003). In the present study, tyrosinase from mushroom was used to hydroxylate tyrosol, **24,** leading to the production of hydroxytyrosol, **25,** a reaction that was first reported by Espín *et al.* (2001) (Figure 1.9).

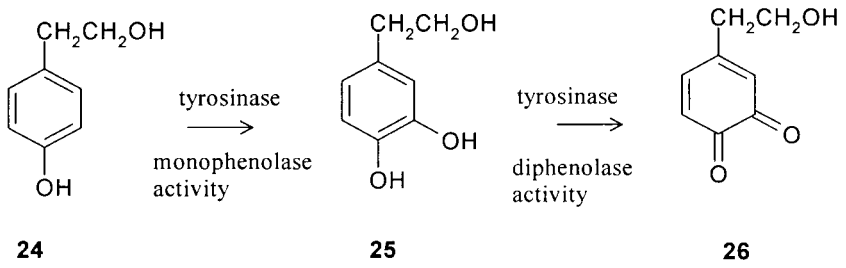



**Figure 1.9 Hydroxylation of tyrosol (24) to hydroxytyrosol (25) by tyrosinase (Espín *et al.*, 2001).**

Catechols can also act as substrates for tyrosinase. For instance quercetin, **27**, a catechol, is oxidized by mushroom tyrosinase to the corresponding *o*-quinone and subsequent isomerization to *p*-quinone methide type intermediates (Figure 1.10) (Kubo *et al.*, 2004).

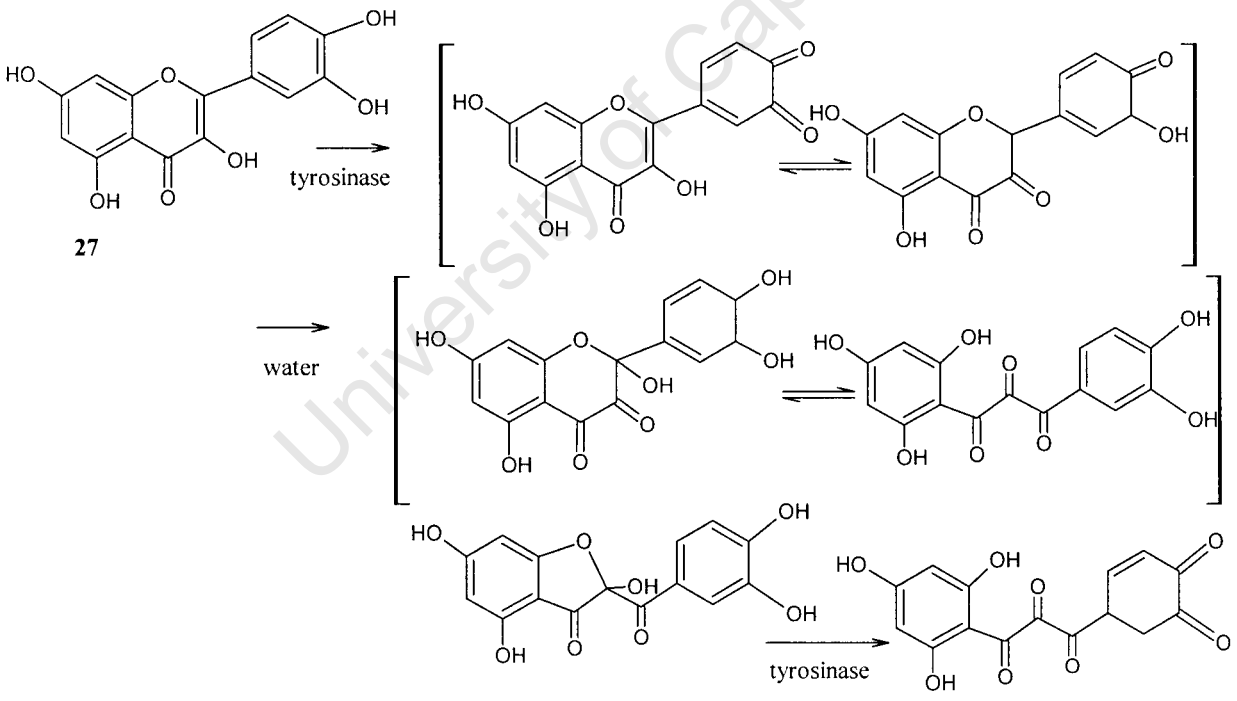



**Figure 1.10 Proposed mechanism for the degradation of quercetin (27) catalyzed by mushroom tyrosinase (Kubo *et al.*, 2004).**

In addition to the examples mentioned above, polyphenol oxidase from mushroom was recently used to oxidize the flavonoid eriodictyol, **28**, (Figure 1.11) directly leading to the formation of eriodictyol-*o*-quinone (Jiménez-Atiénzar *et al.*, 2005).

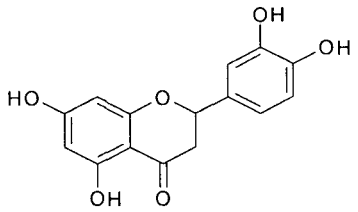



**Figure 1.11 Structure of a flavonoid eriodictyol (28).**



### 1.4.6 Inhibitors of tyrosinase

Inhibitors of polyphenol oxidase fall into three main groups: i) small molecules or ions that bind to the copper in the active site, ii) aromatic inhibitors which compete for phenolic substrates in the binding to the active site and iii) compounds which reduce or oxidize the copper ions (Burton, 1994). Ions that bind directly to an enzyme active site, thus preventing the formation of substrate-enzyme complex, include azide, cyanide, carbon monoxide, halide ions and tropolone. Azide inhibition in particular is attributed to its strong coordination ability with the metal in the active site, which changes the coordination number and conformation of the active site (Shi *et al.*, 2002).

A second group of inhibitors, which consists of aromatic carboxylic acids of the benzoic and cinnamic series, has been reported (Janovitz-Klapp *et al.* 1990). The compounds of this group behave as competitive inhibitors of polyphenol oxidase due to their structural similarity with phenolic substrates. Furthermore, benzaldehydes (Huang *et al.*, 2003) and alkoxybenzoic acids (Chen *et al.*, 2005) have been shown to inhibit both diphenolase activity and monophenolase activity of mushroom tyrosinase. The effects of cinnamic acid and its derivatives (2-hydroxycinnamic acid, 4-hydroxycinnamic acid and 4-methoxycinnamic acid) on the activity of

mushroom tyrosinase were also reported in the studies of Chen *et al.* (2005). It was shown that cinnamic acid, 4-hydroxycinnamic acid and 4-methoxycinnamic acid strongly inhibited the diphenolase activity of mushroom tyrosinase and the inhibition was reversible.

The inhibition of tyrosinase can also be achieved by reduction of copper in the active site (Burton, 1994). Reducing agents play a role in the prevention of enzymatic browning either by reducing *o*-quinones to colourless diphenols, or by reacting irreversibly with *o*-quinones to form stable colourless products. Bisulphate inhibition for instance is due to the reaction of sulphites with intermediate quinones, resulting in the formation of sulphoquinones, which irreversibly inhibit polyphenol oxidase, causing complete inactivation (Ferrer *et al.*, 1989b). The studies of the effect of ascorbic acid on tyrosinase have yielded different results. According to Yağar and Sağiroğlu (2002) polyphenol oxidase extracted from quince (*Cydonia oblonga*) is inhibited by ascorbic acid due to dehydroascorbic acid, the oxidation product of ascorbic acid which reacts with amino groups in close proximity to the active site(s) of the enzyme through Strecker degradation. However, Ros *et al.* (1996) reported that ascorbic acid has been used as a reducing agent for *o*-quinones, with no obvious effect on the active site.



### 1.4.7 Sources of tyrosinase

Tyrosinase can be found in plants, animals and microorganims including fungi and bacteria. Tyrosinases from various sources may show different characteristics or properties. For instance tyrosinase extracted from quince (*Cydonia oblonga)* is inhibited by ascorbic acid (Yağar and Sağiroğlu, 2002). In contrast, tyrosinase from mushroom is not inhibited (Rose *et al.*, 1996). Although it is generally accepted that all tyrosinases have both cresolase and catecholase activity, some authors hold a different view that distinguishes those containing cresolase activity from those that do not (Marusek *et al.*, 2006; Ramírez *et al.*, 2003). The presence or absence of cresolase activity may be the explanation for the fact the polyphenol oxidases from most organisms are able to oxidize L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) but many show variable specificity for the different monophenols and *o*-diphenols, as reported by Duckworth *et al.* (1970) and Pomerantz (1963).

### 1.4.7.1 Plant tyrosinase

Tyrosinase in plants can be found in plastids and chloroplasts and also exits in the cytoplasm of senescing or ripening plants (Vaughn and Duke, 1984). The role of tyrosinase in plants is associated with its ability to catalyse the production of quinones from phenols. Enzymatic browning is the main outcome of tyrosinase activity in fruit and vegetables, which is often undesirable and responsible for unpleasant sensory qualities and losses in nutrient quality (Espin *et al.*, 1996). Tyrosinase has been purified from plants that include tobacco (*Nicotiana tobacum*) (Shi *et al.*, 2002) and truffle ascocarps of *Tuber melanosporum* (Zarivi *et al.*, 2003), among others.

### 1.4.7.2 Bacterial tyrosinase

The first bacterial tyrosinases were purified from cell extracts of *Streptomyces nigrifaciens* Nambudiri and Bhat (1972) and *Streptomyces glaucescens* (Lerch and Ettlinger, 1972). A list of various sources of bacterial tyrosinase is given Table 1.1. For most bacterial tyrosinases, it is not known whether they are produced constitutively or their production is due to induction (Claus and Decker, 2006).

**Table 1.1: Bacterial tyrosinases (Claus and Decker, 2006)**

| Source | Molecular weight (Da) |
|---|---|
| *Rhizobium etli* | 7.28 |
| *Marinomas mediterranea* | 74.469 |
| *Bacillus cereus* | 28.509 |
| *Streptomyces antibioticus* | 30.608 |
| *Streptomyces griseus* | 8.90 |

Besides bacteria, tyrosinase has also been isolated from fungal species such as *Agaricus bisporus*. The tyrosinase from *Agaricus bisporus* has been found in active and latent forms in the ratio that may range from 3 to approximately 20 (Van Gelder *et al.*, 1997). Activation *in vitro* can be effected, for instance, by detergents such as SDS or by protease treatment (Burton and Duncan, 1995b; Espín *et al.*, 1999). Mushroom or fungal polyphenol oxidases have also been isolated from *Neurospora crassa* and *Asperigillus oryzae* (Van Gelder *et al.*, 1997).



### 1.4.7.3 Animal tyrosinase

Tyrosinase exists in insects and crustaceans as a zymogen or propolyphenol oxidase form, and is thought to confer disease resistance in animals, insects and crustaceans. The polyphenol oxidase from an insect *Sarcophaga buflata,* in particular, can oxidize a number of o-diphenols such as catechol, 3-methylcatechol, 4-methylcatechol, 3-dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, 3,4- dihydroxyphenylglycol, 3,4-dihydroxyphenethyl alcohol, caffeic acid, 3,4-dihydroxyphenylpropionic acid, chlorogenic acid, DOPA, dopamine, epinephrine and norepinephrine (Sugumaran and Lipke, 1983).

## 1.5 The biological activity of selected phenolic compounds

The ultimate aim of studying laccase reactions is to produce new compounds (dimeric or oligomeric in nature) with improved biological activities as compared to their monomers. In this section of the study, biological activities (antioxidant and antimalarial activities) of various phenolic compounds are reviewed.

### 1.5.1 Antioxidant activity

#### 1.5.1.1 Overview

Antioxidants are substances which can prevent or slow the oxidative damage caused by oxidizing agents such as free radicals. There are at least four general sources of antioxidants: (1) enzymes, for example, superoxide dismutase, glutathione peroxidase, and catalase; (2) large molecules (albumin, ceruloplasmin, ferritin, other proteins); (3) small molecules [ascorbic acid, glutathione, uric acid, tocopherol, carotenoids, (poly) phenols]; and (4) some hormones such as estrogen, angiotensin, and melatonin (Prior *et al.*, 2005). When mammalian cells use oxygen, they naturally produce free radicals which can cause damage when in excess. Antioxidants act as "free radical scavengers" and hence prevent and repair damage done by these free radicals (Prior *et al.*, 2005).



#### 1.5.1.2 Free radicals

Free radicals are defined as atoms or molecules which contain one or more unpaired electrons. Many of these free radical molecular species are oxygen-centered, and hence they are referred to as reactive oxygen species (ROS) (Halliwell and Gutteridge, 1999; Miller *et al.*, 1990). There are various types of free radicals that can be produced by living organisms. The most common ROS include the superoxide anion ($O_2$-), the hydroxyl radical (OH ·), singlet oxygen ($O_2$), hydrogen peroxide ($H_2O_2$), and nitric oxide (NO) (Valko *et al.*, 2006). ROS are generated in mitochondria

during normal cellular metabolism as oxygen is reduced along the electron transport chain (Valko *et al.*, 2006). Alternatively, ROS formation is elicited by external factors.

Examples of situations in which oxygen radicals are generated in cells include:

- **White blood cells** such as neutrophils, monocytes, macrophages and eosinophils produce oxygen radicals for defense purpose against infectious organisms
- **Cells exposed to abnormal environments** such as hypoxia or hyperoxia generate reactive oxygen species. A number of drugs have oxidizing effects on cells and lead to production of oxygen radicals
- **Ionizing radiation** is well known to generate oxygen radicals within biological systems (Valko *et al.*, 2006).

Under normal conditions free radicals or ROS produced in the body are quenched by the antioxidants which are also available in the cells. However, the failure of antioxidants to act on the free radicals results into an excess of free radicals that can cause oxidative stress to normal cells (Kovacic, and Jacintho, 2001).



### 1.5.1.3 Effects of free radicals within the body system

One of the best known oxidative effects of oxygen radicals is damage to cellular membranes (plasma, mitochondrial and endomembrane systems), which is initiated by a process known as lipid peroxidation (Valko *et al.*, 2006). A common target for peroxidation is unsaturated fatty acids present in membrane phospholipids as depicted in the Figure 1.12. It is notable that hydrogen is abstracted from the fatty acid by a hydroxyl radical, leaving a carbon-centered radical as part of the fatty acid. This radical then reacts with oxygen to yield the peroxy radical, which can then react with other fatty acids or proteins Peroxidation of membrane lipids can have numerous effects, including increased membrane rigidity, decreased activity of membrane-bound enzymes, and altered activity of membrane receptors.

(http://www.vivo.colostate.edu/hbooks/pathphys/misc_topics/radicals.html).

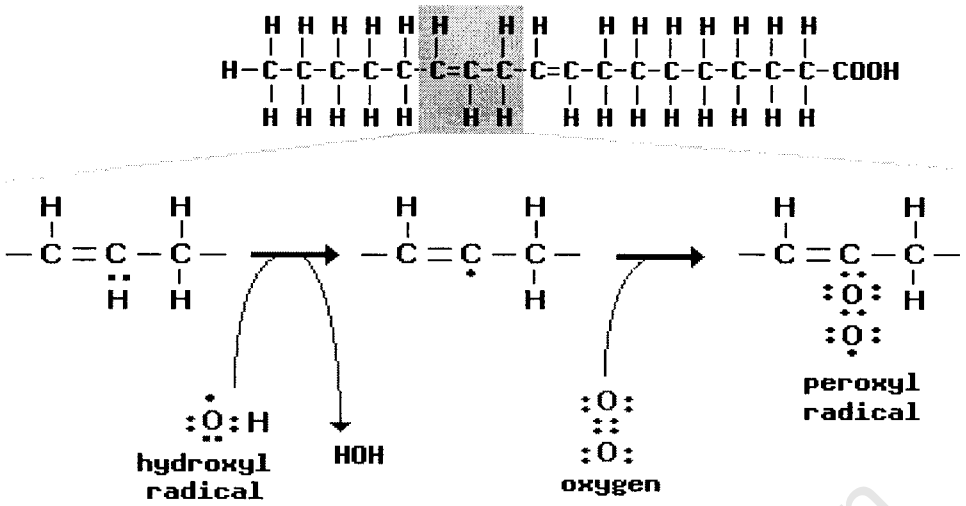
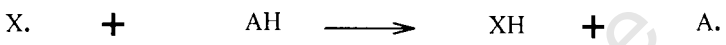



**Figure 1.12 Reactions involving radicals occur in chain reactions** (http://www.vivo.colostate.edu/hbooks/pathphys/misc_topics/radicals.html).

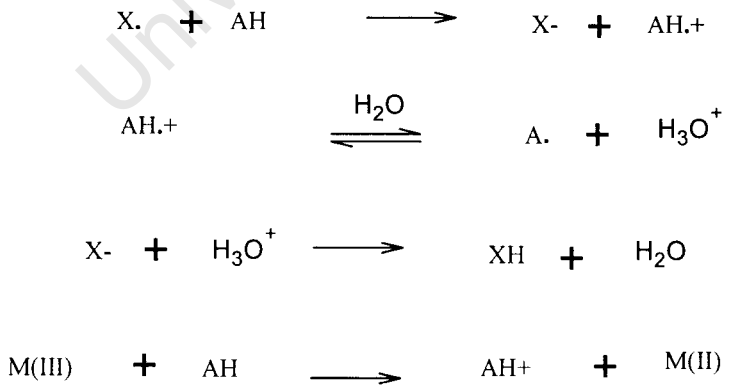
Another oxidative effect of free radicals is damage to the DNA in cells. It is generally accepted that damage to DNA by ROS causes cancer; ROS modify DNA and cause mutations that include a range of specifically oxidized purines and pyrimidines, alkali labile sites, single strand breaks and instability formed directly or by repair processes (Wang *et al.*, 1998; Dizdaroglu *et al.*, 2002). Some of these modified bases have been found to possess mutagenic properties such that when they are not repaired they lead to carcinogenesis. High levels of modified bases in cancerous tissue may be due to the production of large amount of hydrogen peroxide, which has found to be characteristic of human tumor cells (Szatrowski and Nathan, 1991; Olinski, 1998). Generally, the excess production of free radicals leads to various diseases or complications associated with oxidative stress including aging, inflammatory diseases, and Parkinson's diseases (Valko *et al.*, 2006).

### 1.5.1.4 Scavenging of free radicals by antioxidants

In the cellular defense system, free radicals are quenched by antioxidants to avert the oxidative stress and conditions associated with it. Antioxidants can deactivate radicals by two major mechanisms, hydrogen atom transfer (HAT) and single electron transfer (SET) (Prior *et al.*, 2005). Proton-coupled electron transfer and HAT reactions may occur in parallel, and the mechanism dominating in a given system is determined by antioxidant structure and properties. In *vitro* system, solubility, partition coefficient, and system solvent may influence these mechanisms. The HAT reaction mechanism is based on the classical ability of an antioxidant to quench free radicals by hydrogen donation:




The SET reaction mechanism is based on the ability of a potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls, and radicals (Prior *et al.*, 2005):



The radical A formed in these reactions could possibly dimerise or polymerise resulting in a new antiradical species which could further scavenge radical X.

### 1.5.1.5 Phenolic compounds as antioxidants

Polyphenolic antioxidants are increasingly attracting the attention of, particularly, consumers and food manufacturers (Morillas-Ruiz *et al.*, 2006). Phenolic compounds play an important role as antioxidants due to the presence of hydroxyl substituents and their aromatic structure, which enables them to scavenge free radicals. It has been suggested that the antioxidant activity is determined by factors that include electron delocalisation across the phenolic compound, and the contribution to stability of the aroxyl radicals (Chen and Ho, 1997). Thus it has been generally accepted that the activity of antioxidant is related to its structure. For instance, molecules bearing *ortho*-dihydroxyl or 4-hydroxy-3-methoxyl groups possess significantly higher antioxidants activity than those bearing no such functionalities (Cheng *et al.*, 2006). Cheng and coworkers demonstrated this phenomenon using resveratrol (3,5,4'-trihydroxy-trans-stilbene), **29**, (Figure 1.13), a naturally occurring phytoalexin present in grapes, nuts and other plants with health benefits associated with low incidence of heart disease (Arnous *et al.*, 2001; Da Porto *et al.*, 2000). Cheng and coworkers discovered that some synthetic resveratrol analogues bearing *ortho*-dihydroxyl or methoxy functionality (Figure 1.13) exhibit significantly higher antioxidant and cytotoxic activity against HL-60 cancer cells than resveratrol and other analogues bearing no such functionality (Fang *et al.*, 2002).

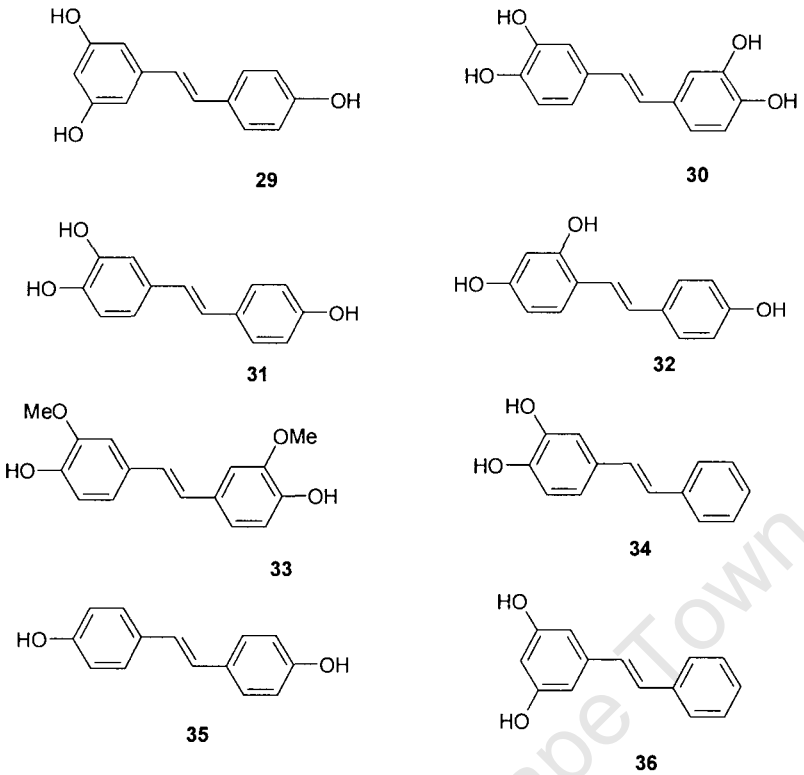
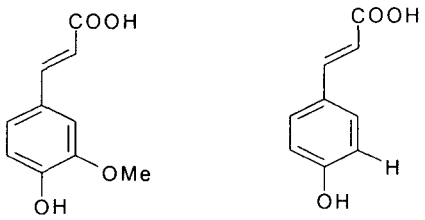



**Figure 1.13 Structures of resveratrol (29) and its analogues 3,4,3',4'-tetrahydroxy-trans-stilbene (3,4,3',4'-THS) (30), 3,4,4-trihydroxy-trans-stilbene (3,4,4'- THS) (31), 2,4,4'-trihydroxy-trans-stilbene (2,4,4'-THS) (32), 3,3'-dimethoxy-4,4-dihydroxy-trans-stilbene (3,3'-DM-4,4'-DHS) (33), 3,4-dihydroxy-trans-stilbene (3,4-DHS) (34), 4,4-dihydroxy-trans-stilbene (4,4'- DHS) (35) and 3,5-dihydroxy-trans-stilbene (3,5-DHS) (36) (Cheng *et al.*, 2006).**



Another example of antioxidant activity-phenolic structure relationship is that of hydroxytyrosol, 2-(3,4-dihydroxyphenyl) ethanol, **25**, the natural antioxidant obtained from olive oil. It is known for its biological properties that may contribute to the reduced risk of cardiovascular diseases and malignant neoplasms in the populations of Mediterranean countries (Owen *et al.*, 2004; Tuck *et al.*, 2002). The analogue of hydroxytyrosol, tyrosol, **24,** shown in Figure 1.9 has lower antioxidant activity. Thus the antioxidant activity of a phenolic compound may be influenced by both hydroxyl and methoxyl groups in the molecule. Ferulic acid, **37**, bearing both hydroxyl and methoxyl group in the aromatic ring (Figure 1.14), has more antioxidant activity than that of *p*-

coumaric acid, **38**, (Sánchez-Moreno *et al.*, 1999). Generally, hydroxyl groups present in the *ortho* position of aromatic ring increase antioxidant capacity due to resonance stabilization and formation of *o*-quinone and *p*-quinone structures (Chen and Ho, 1997).




37 38

**Figure 1.14 Phenolic compounds (ferulic acid, 37 and *p*-coumaric acid, 38) which can be obtained from grapes and wine.**



### 1.5.1.6 Measurement of antioxidant activity

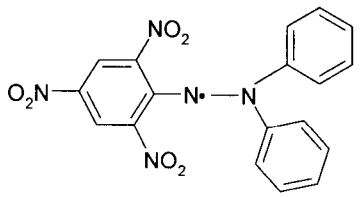
The analytical methods used to assess the biological activity of a particular extract or compound are as important as the properties of the compounds in question. There are many assay methods for evaluating antioxidant activity of foodstuff (Antolovich, 2002). According to Fernández-Pachón and the coworkers (2004) the choice of using a particular method is determined by the experimental conditions and methodological approach involved. From these reports, similar results were obtained with 2,2-Diphenyl-1-picrylhydrazyl (DPPH), **39**, (Figure 1.15), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), **40**, (Figure 1.16) and oxygen-radical absorbing capacity (ORAC) methods. These methods were therefore recommended as methods for evaluation of antioxidant activities of wines. In this study, we review several of the more commonly used methods for measuring antioxidants.

### 1.5.1.6.1 DPPH radical method

The DPPH radical (Figure 1.15) is a stable organic nitrogen-containing radical which bears a deep purple color. This assay is based on the measurement of the reducing activity of antioxidants toward DPPH. The activity can be evaluated by electron spin resonance (EPR) or by measuring the decrease of its absorbance. The decoloration assay was first reported by Brand-Williams and co-workers (Brand-Williams *et al.*, 1994). Antioxidant assays are based on measurement of the loss of DPPH colour at 515 nm after reaction with test compounds; the solution changes from a deep purple blue/red solution to colourless, and the reaction is monitored by a spectrometer (Bondet *et al.*, 1997). The DPPH assay is considered to be based mainly on an electron transfer reaction, and hydrogen-atom abstraction is a minor reaction pathway. From the reaction shown below, stable radical reacts with (AH), the antioxidant molecule, to form the DPPH-H molecule.

$$DPPH^{\bullet} + AH \quad \rightarrow \quad DPPH\text{-}H + A^{\bullet}$$

Total H atom-donating capacities are evaluated by using the $EC_{50}$ index, defined as the concentration needed to reduce 50 % of DPPH•. The time needed to reach the steady state at the concentration $EC_{50}$ is named $TEC_{50}$, which indicates the rate of reaction towards the free radical. The test is simple and rapid and needs only a UV-vis spectrophotometer. However, interpretation is complicated when the test compounds have spectra that overlap with that of DPPH at 515 nm; for example, carotenoids, in particular, interfere with the reaction (Noruma *et al.*, 1997). This method was used in this study to evaluate the antioxidant activity of various compounds.

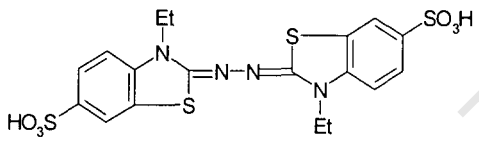



**39**

**Figure 1.15 Structure of 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$).**

### 1.5.1.6.2 TEAC or ABTS assays

The TEAC assay was first reported by Miller and Rice-Evans (Miller *et al.*, 1993). It is based on the scavenging ability of antioxidants to the long-life radical anion ABTS (Figure 1.16). The pre-formed radical monocation of ABTS•+ is generated by oxidation of ABTS with potassium persulfate or other oxidants, and is reduced in the presence of hydrogen-donating antioxidants (Re *et al.*, 1999). ABTS assays can be used in multiple media to determine both hydrophilic and lipophilic antioxidant capacities of extracts and body fluids (Awika *et al.*, 2003).




40

**Figure 1.16 Structure of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$).**

### 1.5.1.6.3 ORAC method for determination of antioxidant activity

The ORAC assay measures antioxidant inhibition of peroxyl radical-induced oxidations and thus reflects classical radical chain breaking antioxidant activity by H atom transfer (Ou *et al.*, 2001). For wide application, the ORAC assay has been adapted to measure lipophilic as well as hydrophilic antioxidants (Wu *et al.*, 2004).

## 1.5.2 Antimalarial activity of compounds

### 1.5.2.1 Malaria

Malaria is a mosquito-borne disease caused by the protozoan parasite *Plasmodium*, and causes an estimated one million deaths each year globally, mainly because of severe complications of

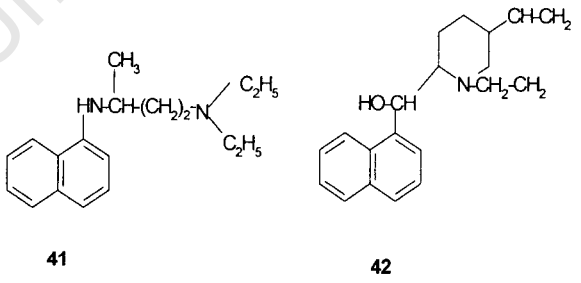
*Plasmodium falciparum* infection (Breman, 2001). The majority of malaria infections occur in sub-Saharan Africa, South and Southeast Asia (Medana and Turner, 2006). The adverse effects of malarial infection have prompted many researchers in the field of medicinal chemistry to devote much of their efforts to the synthesis of new antimalarial compounds.

### 1.5.2.2 Antimalarials

There are several classes of antimalarial drugs, with different structural features, which have been synthesized. These drugs target different stages of the malaria parasite's life cycle. Among these drugs, chloroquine or quinoline-related drugs are the most researched and successful antimalarial drugs. They are believed to be in involved in the inhibition of *beta*-hematin formation (Egan *et al*; 1994). Hematin is therefore the molecular drug target for chloroquine, **41**, (Figure 1.17) and other 4-aminoquinoline and quinoline methanol antimalarials (Cohen *et al.*, 1964; Chou *et al.*, 1980).

As a result of emerging multidrug resistance in malaria, combination chemotherapy has become rational approach to avert the problem (White, 1987). For instance, chlorpheniramine showed synergistic effect with chloroquine, mefloquine, quinine, **42**, (Figure 1.17) or pyronaridine against both multidrug-resistant *P. falciparum* and chloroquine resitant *P. falciparum* (Nakornchai and Konthiang, 2006).

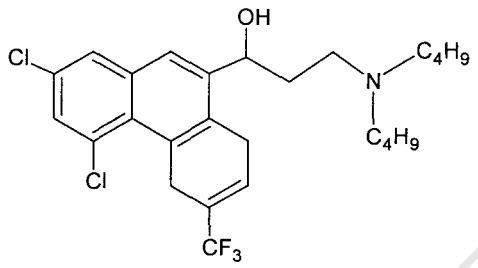





41 42

**Figure 1.17 Structures of chloroquine (41) and quinine (42), both antimalarial drugs.**

The problem of multidrug resistance reported in *P. falciparum* strains can also be solved by utilization of halofantrine, **43**, (Figure 1.19), an effective drug for the treatment of malaria which was first synthesized by Colwell *et al.* (1972). This compound is often used together with chloroquine and/or pyrimethamine resistant *P. falciparum* (Watkins *et al.*, 1988; Cosgriff *et al.*, 1982).




**43**

**Figure 1.18 Structure of halofantrine**

Xanthones, **44** to **46**, (Figure 1.19), the natural compounds sourced from *Calophyllum* and *Garcinia* species of the *Clusiaceae* family, are also known for their antimalarial properties (Hay *et al.*, 2004). In the elucidation of antimalarial activity of xanthoses related compounds, it has been suggested that structural features play a vital role on the activity against malaria. The 1,4,5-trihydroxyxanthone, **45**, and 1,6-dihydroxyxanthone, **44**, showed better activities compared to the 1,3,5-trihydroxylated structure , **46**, (Hay *et al.*, 2004) (Figure 1.19). These findings suggest that the activity of a compound is indeed related to the structure of the compound. Further, the increased activity is not only dependent on the number functional groups present but also on the structure configuration or positions of those functional groups (OH).

Another natural compound with antimalarial activity is totarol, **62,** (see Chapter 4). Totarol derivatives synthesis was reported by Clarkson and co-workers who reported the synthesis of totarol amino alcohol derivatives, and they also demonstrated that all of the derivatives had an improved antiplasmodial activity as compared to totarol (Clarkson *et al.*, 2003). In this current

study we have considered the effect of dimerisation (by laccase) of totarol on its antimalarial activity.

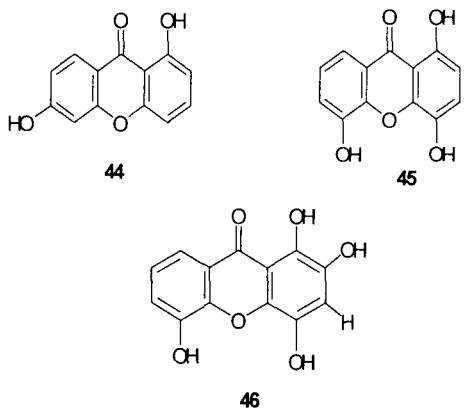



**Figure 1.19 Xanthone compounds with antimalarial properties**



## 1.6 Conclusion

Chemical methods may be used for modification of phenolic substructures to the corresponding derivatives, but these methods are often complex, costly, and difficult to predict or control (Manda *et al.*, 2005). Thus, alternative methods which are more selective and require more gentle conditions are desirable (Manda *et al.*, 2005). Biotransformation processes utilize biological agents in the form of whole cells or isolated enzymes, to catalyze chemical reactions. Such processes may be utilized for environmentally benign biocatalysis of synthetic reactions or for bioremediation of polluted environments (Burton, 2001).

Isolated enzymes, in particular, are increasingly becoming an important tool in the oxidation of various phenolic substrates to produce novel compounds for application as pharmaceuticals and to modify natural compounds with phenolic structures. Among the group of enzymes that can be used in oxidation reactions, laccases catalyze the one-electron oxidation of a wide variety of organic substrates with the concomitant four-electron reduction of oxygen to water (Burton, 2003; Ryan *et al.*, 2005). At present, laccases are used largely in biocatalytic oxidations of dyes, polymerization of lignin and lignosulphonates, bioremediation and bleaching (Lorenzo *et al.*,

2002; Tzanov *et al.*, 2003; Riva, 2006). Bioremediation processes involving laccases are focused on the degradation of phenolics in wastewaters. The main goal of this process is to degrade toxic waste into small molecular weight non-toxic products. However, there is little or no explanation as to what happens to the final products. Furthermore, degradation processes do not necessarily translate into complete removal of toxic waste and small product residues could still be toxic. Therefore new approaches are required that would compliment the existing approaches.

There is increasing interest in the use of laccase 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 radicals that react non-enzymatically with each other to form dimers, there is little information available on what factors influencing the nature of the products. Thus, biocatalytic systems need to be developed for laccase biotransformations reaction using model phenolic as substrates, with focus on the influence of organic solvents, the structure of the substrate and the nature of the products.

It is also clear that enzymes, laccases in particular, are becoming useful tools in the synthesis of fine chemicals with biological activity relevant for pharmaceutical industries. However, there are few reports related to the biological activity of products obtained from biotransformation processes. Thus, the relationship between the structure of biocatalyst products and their biological activity would need to be determined.

Furthermore, the current application of phenol oxidases in organic synthesis is largely aimed at producing a single product in one chemical transformation step. However, the synthesis of organic product may require more than one chemical transformation step, and hence more than one enzyme is required to produce the target product. In order to generate these target products, a multi-enzyme process is required, in which the steps are integrated in terms of kinetics and productivity. However, biocatalysts vary widely with respect to both their kinetics and optimal reaction conditions. Thus, it is often impractical to use them in single reactor processes. Multi-enzyme biotransformation systems may offer a mechanism to address the limitations of single-enzyme biocatalysis and those of whole-cell biotransformations. Thus, this study investigates

also the potential of employing more than one enzyme in the synthesis of a compound with biological activity.

## 1.7 Objectives of this study

The broader objective of this study was to investigate the potential of using laccase obtained from the white rot fungus *Trametes pubescens* in the synthesis of organic compounds with useful biological activity.

**Specific objectives**

1) Production of laccase in airlift bioreactors. Laccase was to be used for all biocatalytic reactions in this study. The method for producing laccase had been previously developed by Ryan *et al.* (2005). This method as reported by these authors leads to the production of stable laccase with higher activity as compared to commercial laccase, and it is cheaper to produce laccase using this method.

2) Produce new products with improved biological activity using laccases. The substrates for laccase were phenolic structures with known biological activities. The susbtrates included *p*-tyrosol, hydroxytyrosol, 8-hydroxyquinoline, and a natural compound, totarol. The fundamental aim was to investigate the new substrates for laccase. The effect of the structure of these substrates on the final product was elucidated.

3) Since it is widely accepted that laccase does not necessarily influence the nature of products formed, this study investigated the effect of organic solvents in the selectivity of laccase reaction products. The focus was on controlling the degree of polymerization and coupling (whether it could be C-C or C-O bond formation during the coupling of monomers, and the particle size of the polymers). The reaction conditions such as buffer salts, pH, and temperature aimed at enhancing the yields of the products were also investigated.

4) The production of biological active compounds using laccases has not been reported extensively in literature. Thus, this study was also aimed at developing new understanding of the structure-function relationships of the products from these reactions. This was done by testing the antioxidant and antimalarial of the products.

5) The application of biocatalysts can be exploited in the development of various processes, and hence this thesis also described:
   - the development of a potential bioremediation system for treatment of wastewater contaminated with tyrosol or 8-hydroxyquinoline and
   - the development of a multi-enzyme biotransformation system.

# Chapter 2

## Investigations on the synthesis and antioxidant activity of the products obtained from tyrosol-laccase and hydroxytyrosol-laccase reactions

### 2.1 Introduction

The aim of the work described in this Chapter was to investigate the synthesis and antioxidant activity of tyrosol-laccase and hydroxytyrosol-laccase reactions products. Tyrosol (*p*-hydroxyphenylethanol) **24** and hydroxytyrosol (3,4-(dihydroxyphenylethanol)) **25** are found in virgin olive oil and table olives (Brenes *et al.*, 1999; Servili *et al.*, 1999). Hydroxytyrosol, in particular, is known for its high antioxidant activity (Grasso *et al.*, 2006; Roche *et al.*, 2005), and hence there is growing interest in studying this compound and its oxidation products.

Synthesis of hydroxytyrosol was necessary as part of this study because it is not commercially available. From literature, several methods of production of this compound have been proposed. These methods include chemical synthesis (Baraldi *et al.*, 1983) usually utilizing 3,4-dihydroxyphenylacetic acid as precursor (Bai *et al.*, 1998; Capasso *et al.*, 1999) or hydrolysis of oleuropein (García *et al.*, 1996). Other authors have isolated the compound after several chromatographic steps from vegetative waste waters (Capasso *et al.*, 1999; Capasso *et al.*, 1992). In enzymatic reactions, Espin and coworkers used free mushroom tyrosinase to hydroxylate tyrosol (Espín *et al.*, 2001) while more recently Brooks and coworkers synthesised hydroxytyrosol from tyrosol using *Pseudomonas putida* F6 immobilised cells (Brooks *et al.*, 2006). In this study, hydroxytyrosol was produced from tyrosol following the method of Espin *et al.* (2001). This method was preferred because hydroxytyrosol is prepared for immediate use from the more stable and readily available tyrosol (Grasso *et al.*, 2006).

The development of an enzyme (tyrosinase)-based method was also motivated by the obvious potential of developing a multi-enzyme biotransformation for synthesis of products with biological activity. It was also envisaged that, as a long term objective, the multi-enzyme concept

could include a kinetic modeling approach to integrate consecutive enzyme-catalyzed reactions into a system which can readily be scaled-up, potentially leading to the demonstration of feasibility of the process. The advantage of this system is that each reaction step could be individually optimised to increase the selectivity of the products and/or to improve the yields of the desired products. This method can be an alternative to a system where multi-enzymatic reactions are performed in one reactor, a system generally known as one-pot synthesis.

Despite the availability of methods there is a need to further develop the existing methods for large scale production of hydroxytyrosol. Ultimately, large scale production of hydroxytyrosol would result in high yields, thereby ensuring the availability of hydroxytyrosol which could be used for further manipulation (e.g. oxidation by laccase). Thus the work described in this chapter was also conducted with the aim of improving hydroxytyrosol production.

Although there is an increasing interest of studying the oxidative products of these compounds (tyrosol and hydroxytyrosol) there is little work reported in literature involving enzymatic oxidation of these compounds. Few reported work on these compounds include that of Vinciguerra and coworkers who reported that *p*-tyrosol is converted by laccase from *Lentinus edodes* into a dimeric tetracylic ketone (Vinciguerra *et al.*, 1997). Gianfreda and coworkers reported the oxidation of *m*-tyrosol by *Rhus vernificera laccase* and hydroxytyrosol, but the structures of the oxidative products were not elucidated (Gianfreda *et al.*, 2003). However, there has been no work reported in literature elucidating the effects of organic solvents on the selectivity of the products from tyrosol or hydroxytyrosol-laccase reactions. Thus the broader objective of work described in this chapter was to compare *p*-tyrosol-laccase and hydroxytyrosol-laccase reactions and the emphasis was on the effect of organic solvents on the selectivity of the reactions products.

The specific objectives of this chapter were:

- Development of a hydroxytyrosol production method and hence its increased availability as a substrate for laccase
- evaluation of the ability of immobilised tyrosinase to transform tyrosol to hydroxytyrosol in a fixed-bed reactor

- oxidisation of tyrosol and hydroxytyrosol by laccase and characterisation of the products, and
- evaluation of the antioxidant activity of hydroxytyrosol/tyrosol-laccase products to obtain some insight into the relationship between phenolic structure and antioxidant activity.

## 2.2 Materials and Methods

First, in this section of the study, was the production of laccase from white rot fungi, and extraction of tyrosinase from white mushrooms. Tyrosinase was then used in production of hydroxytyrosol from tyrosol, and laccase in the oxidation of both tyrosol and hydroxytyrosol.

### 2.2.1 Production of laccase

#### 2.2.1.1 Strain preservation

A culture of *T. pubescens* was (CBS 696.94) was obtained from the Boku Institute in Austria. This culture was maintained on 2 % malt extract agar slants and subcultured every 60 d.

#### 2.2.1.2 Growth of *Trametes* in agar plates

*T. pubescens* was grown on agar plates containing 50g/l malt extract agar, supplemented with a laccase inducer [1% phenol mixture (phenol: 82.8 mM, *p*-cresol: 24.99 mM, *m*-cresol: 25.8 mM, *o*-cresol: 77.03 mM)] (Ryan *et al.*, 2005). *T. pubescens* mycelial blocks were aseptically inoculated on the plates and incubated at 28 °C for 6 d.

#### 2.2.1.3 Growth of *T. pubescens* in liquid medium

##### 2.2.1.3.1 Growth of *T. pubescens* for laccase production in flasks

*T. pubescens* was cultured in *Trametes* Defined Medium (TDM), a liquid medium containing 10 g glucose, 10 g bacteriological peptone, and 2 g $KH_2PO_4$ per litre (Addleman and Archibald, 1993). Two portions of 200 ml of sterilised TDM medium were inoculated aseptically with homogenised (Sorvall benchtop homogenizer) mycelial plug taken from a solid Petri dish preculture (see above). Cultures were incubated in 1000 ml Erlenmeyer flasks at 28 °C with agitation at 180 rpm, for 9 d.

#### 2.2.1.3.2 Large scale production of laccase in airlift bioreactors

This was performed according to the method of Ryan *et al.* (2005). *T. pubescens* was grown in a 4 l airlift bioreactor. A 400 ml production flask culture (9 d old) was used to inoculate two 3.6 l TDM in the airlift bioreactors each containing TDM supplemented with 0.03 % antifoam. The laccase activity was then monitored daily; 2 ml samples were taken from the bioreactor and centrifuged at 5 000 rpm for 5 min. The supernatant was assayed for laccase activity. After 5 d of incubation the medium in the bioreactors was supplemented with 30 ml phenol inducing mixture (see above in 2.2.2) and 1g glucose (previously filter-sterilised). Subsequently, further portions of 30 ml phenol mixture and 1g glucose were added to the medium daily. After 13 d, the medium containing laccase displaying activity of 2-3 U/ml, was harvested by centrifugation at 10 000 rpm for 10 min.

#### 2.2.1.4 Isolation of laccase

The laccase in the culture medium (see above in Section 2.2.1.3.2) was precipitated using acetone or ammonium sulphate. For acetone precipitation, 300 ml of cold acetone was added to 300 ml sample (culture medium containing laccase). The mixture was then kept at -20 °C for 30 min. The precipitated protein was recovered by centrifugation at 10 000 rpm for 15 min at 4 °C. The pellet was resuspended in 0.1 M sodium acetate buffer, pH 5 and kept at -20 °C or 4 °C. For ammonium sulphate precipitation, 400 ml of 100 % saturated ammonium sulphate was added to 100 ml sample to give 80 % ammonium sulphate. The mixture was kept at 4 °C overnight. The precipitated proteins were recovered by centrifugation as above. The pellet was resuspended in 0.1 M sodium acetate buffer, pH 5 and kept at -20 °C or 4 °C. The protein solution was then dialyzed against the 0.1 M sodium acetate buffer (pH 5) for 2 d. The dialyzed protein solution was aliquoted into Eppendorfs tubes or freeze dried and then stored at -20 °C or 4 °C.

### 2.2.1.5 Protein determination

The protein concentration in the extracts was determined using the method of Bradford (Bradford, 1976). The analysis gives a linear response from 0.2 mg/ml to 1.0 mg/ml protein using bovine serum albumin (BSA) as a standard protein. 3 ml volumes of Bradford reagent were added to 100 µl of protein solution. The absorbances were read at 595 nm using a Unicam UV–vis spectrophotometer, after 5 min of incubation at room temperature. The standard curve is shown in Figure 2.1. The laccase protein concentration was determined graphically.

### 2.2.1.6 Enzyme activity assay

Laccase activity was determined with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) (Roche) as the substrate (Wolfenden and Willson, 1982). The assay mixture contained 0.33 ml of 5 mM ABTS, 2.5 ml of 0.1 M sodium acetate buffer (pH 5.0), and 0.17 ml aliquots of culture supernatant or enzyme solution. Oxidation of ABTS was monitored by following the increase in absorbance at 420 nm ($\varepsilon$ = 36 000 $M^{-1}cm^{-1}$) using a Unicam UV–vis spectrophotometer. One unit (U) of laccase activity was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per min at 25 °C.



### 2.2.1.7 SDS-PAGE gel analysis of the protein

The partially purified laccase (50 µl) was loaded on to a non-denaturing polyacrylamide gel (Laemmli, 1970). PAGE was performed using a 20 % separating gel and 6.5 % stacking gel (Appendix A). Gels were run at 120 V constant voltage according to manufacturer's instructions, stained with Coomassie brilliant blue, and completely destained using 7 % (v/v) acetic acid containing 25 % (v/v) ethanol. The activity of the major protein was demonstrated by immersing the gel into a mixture of ABTS (5 mM) in 0.1 M sodium acetate buffer (pH 5). The gel was incubated in this mixture for 3 d at room temperature. The laccase enzyme was stored at 4 °C till needed for biotransformation reactions.

### 2.2.2 Extraction of tyrosinase from mushrooms

The extraction was performed according to the method of Burton *et al.* (1994). Fresh mushrooms (*Agaricus bisporus*) (1 kg) (obtained from local supermarket) were frozen and then homogenized in cold acetone (2.5 l) using a blender. The resulting slurry was filtered rapidly on a Buchner funnel, and the residual pulp was air dried briefly before being frozen with liquid nitrogen. The pulp was mixed with polyvinylpolypyrrolidone (PVPP) and stirred in cold water (500 ml), and the mixture allowed to stand overnight at 4 °C. The paste was filtered through cheesecloth, and the filtrate was stood in ice while nitrogen gas bubbled gently through the solution for 3 h to remove residual acetone. The protein content of the filtrate was determined using Bradford method. The activity of tyrosinase was determined (Section 2.2.2.1). The filtrate was partially purified using ammonium sulphate. 54.2 g of ammonium sulphate was added to 200 ml the filtrate to achieve 40 % saturation. The solution was allowed to stand overnight at 4 °C. The precipitate was separared by centrifugation (10000 g, 10 min). The supernatant was mixed with ammonium sulphate (46.3 g in 100 ml) to bring it to 52 % saturation. The solution was again allowed to stand overnight and centrifuged as above. L-DOPA activity assays were performed as described in Section 2.2.2.1.



#### 2.2.2.1 Measurement of tyrosinase activity

The activity of tyrosinase was determined by monitoring the production of dopachrome at 475 nm in 3 ml 10 mM L-DOPA in a potassium phosphate buffer (50 mM, pH 7) (Burton *et al.*, 1993). One unit of enzyme was defined as the amount of biocatalyst that catalyses the formation of dopachrome from L-DOPA at a rate of 1 $\mu mol.min^{-1}$ where the extinction coefficient is 3600 $M^{-1}min^{-1}$. A Unicam Merck UV/VIS spectrophotometer was used for all spectrophotometric assays.The protein concentration in the extracts was determined using method of Bradford (Bradford, 1976) (in Section 2.2.1.5).

### 2.2.3 Bioconversion of tyrosol by free tyrosinase in aqueous medium to yield hydroxytyrosol

A solution containing 20 ml of potassium phosphate buffer (50 mM, pH 7), 25 mg tyrosol, 170 mg ascorbic acid and 5 ml tyrosinase (8 U/ml) was incubated in a 200 ml Schott bottle at 30 °C and agitated at 180 rpm for 6 h. The reaction was monitored by HPLC. The mobile phase was methanol-acetic acid-water (20:2.5:80) with a flow rate 1 ml/min, and using a C18 Waters (250 mm x 4.6 nm) reverse phase column and UV detection at 280 nm. Peaks were analysed using HPLC Manager, Merk Hitachi model D 700 data software. The percentage conversion was obtained by comparing the peak area of reaction sample with that of a control. The progress of the reaction mixture was also monitored using TLC analysis (eluent used was toluene-ethyl acetate-formic acid solution (5:4:1).

### 2.2.4 Optimization of hydroxytyrosol production

#### 2.2.4.1 The effect of pH on the bioconversion of tyrosol by tyrosinase

The pH of the reaction medium was varied from 6 to 7.5 using 50 mM potassium phosphate buffer. The reaction mixture contained 6 mg tyrosol and 40 mg ascorbic acid dissolved in 5 ml of 50 mM potassium phosphate buffer. The reaction was initiated by adding 1.2 ml (0.1 U/ml) of tyrosinase. The reactions which were performed in duplicate and were incubated at 30 °C for 5 h. The reaction was monitored by HPLC (see Section 2.2.3).

#### 2.2.4.2 The effect of SDS on the bioconversion of tyrosol by tyrosinase

The reaction mixture contained 6 mg tyrosol, 40 mg ascorbic acid dissolved in 5 ml of 50 mM potassium phosphate buffer pH 7 and SDS at various concentrations (0.5, 1.0, 2.0, 3.0 mM). The reaction was initiated by adding 1.2 ml (0.1 U/ml) of tyrosinase. The reactions which were performed in duplicate were incubated at 30 °C for 4 h with agitation at 160 rpm. The reaction was monitored by HPLC (see Section 2.2.3).

### 2.2.4.3 The effect of temperature on the bioconversion of tyrosol by tyrosinase

In order to determine the impact of temperature on tyrosol conversion by tyrosinase, reactions mixtures were incubated at various temperatures. The reaction mixture contained 6mg tyrosol and 40 mg ascorbic acid dissolved in 5 ml of 50 mM potassium phosphate buffer pH 6. The reaction was initiated by adding 1.2 ml (0.1 U/ml) of tyrosinase. The reactions which were performed in duplicate were incubated at 20, 30, 40, and 50 °C for 6 h with agitation at 160 rpm. The reaction was monitored by HPLC (see Section 2.2.3).

### 2.2.5 LC-MS analysis of hydroxytyrosol and hydroxytyrosol-laccase reaction products

Hydroxytyrosol was identified using a Full Scan Chromatograpahic-Electron Spray Mass Spectrophotometry (LC-ESMS) system, in negative Electrospray Ionization (ESI) mode, with a reverse-phase C18 Waters (250 mm x 4.6 nm) column and a mobile phase was methanol-acetic acid-water (20:2.5:80) with a flow rate 1 ml/min, and using a UV detection at 280 nm.

### 2.2.6 Extraction of hydroxytyrosol from aqueous reaction media

The organic product was recovered from the reaction mixture using ethyl acetate (3 x 100 ml). 100 ml ethyl acetate was mixed with the equal volume of the reaction mixture. The mixture was shaken vigorously, and the phases were then allowed to separate. The organic phase was recovered, dried using the rotary evaporator, and then resuspended in 1 ml methanol. Samples were then applied on the TLC for analysis and separation. The silica aluminum plates were used to run TLC using as mobile phase a mixture of toluene-ethyl acetate-formic acid, (50:40:10). Hydroxytyrosol was isolated by scratching out the band corresponding to hydroxytyrosol standard observed under UV light, and extracted from the silica using ethyl acetate. The sample was dried and redissolved in *d*-chloroform for $^{1}$H-NMR analysis.

### 2.2.7 Determination of hydroxytyrosol concentration

Hydroxytyrosol was prepared as stated in Section 2.2.3. It was then extracted from aqueous reaction medium and purified as stated above (Section 2.2.6). The purity was checked by HPLC analysis. The purified sample was then dried and weighed. Hydroxytyrosol was then dissolved in methanol at various concentrations and the solution used to construct a hydroxytyrosol standard curve (Appendix B). In a calibration curve, the HPLC peak area was plotted against the known concentration of hydroxytyrosol. The concentrations of hydroxytyrosol fractions were calculated from the equation of this curve.

### 2.2.8 Determination of initial rates for hydroxytyrosol production

Experiments were conducted in batch cultures of 3.1 ml to determine the initial reaction rates of tyrosinase and tyrosol in mushroom tyrosinase extract. 3 mg Tyrosol and 2 mg ascorbic acid were dissolved in 2.5 ml potassium phosphate buffer (50 mM, pH 7) containing 2 mM SDS. The reaction was initiated by addition of 0.6 ml tyrosinase (0.1 U/ml). The reaction mixtures were incubated at 30 °C with agitation at 200 rpm. Reactions were performed in sets of triplicate, and were set to end at 1 h intervals from 0 to to 8 h. The rates of formation of the product hydroxytyrosol were determined from the product yield at hourly intervals up to 8 h. The product concentrations were determined from the hydroxytyrosol HPLC standard curve.



### 2.2.9 Hydroxytyrosol production using tyrosinase immobilized on sodium aluminosilicate (zeolite) molecular sieve

#### 2.2.9.1 Immobilization of tyrosinase on the zeolite

Immobilization was performed according to the method of Seetharam and Saville (2002). A glutaraldehyde/buffer solution was prepared by mixing glutaraldehyde in 0.05 M phosphate buffer (pH 7) to achieve a 1 % glutaraldehyde solution. 3 g of the zeolite (molecular sieves, 0.4 nm) support (Merck) was mixed with glutaraldehyde/buffer solution and incubated for 4 h at

room temperature. The zeolite, modified with glutaraldehyde, was recovered by filtration and washed with distilled water. An enzyme solution was prepared by resuspending the enzyme in 0.05 M citrate buffer solution (pH 6). This solution (5 ml) was mixed with 3 g of zeolite and incubated at room temperature for 48 h under gentle stirring using a magnetic stirrer. The resulting immobilized enzyme biocatalyst was recovered by vacuum filtration, and dried overnight at room temperature. The fraction of the available tyrosinase transferred onto the zeolite support was determined by comparing the initial tyrosinase activity in the immobilization solution with the activity after the immobilization process was complete.

The fraction of residual enzyme (R) is defined as
R = cresolase activity at 48 h/ cresolase activity at 0 h
Therefore, the maximum immobilization yield or fraction of enzyme activity transferred to the support would be (1-R) (Seetharam and Saville, 2002).



**2.2.9.2 Hydroxylation of tyrosol using immobilized tyrosinase in a batch reaction**

5 ml of 50 mM potassium phosphate buffer pH 7 containing 4 mg tyrosol, 21 mg ascorbic acid and 1.5 g enzyme biocatalyst (16 U/g zeolite) was incubated in at 30 °C and agitated at 235 rpm for 77 h. After the reaction the biocatalyst was washed with buffer, air dried overnight and reused under the same reaction conditions. The reaction was monitored by HPLC as described in Section 2.2.3.

**2.2.9.3 Hydroxylation of tyrosol using immobilized tyrosinase in a continuous fixed-bed reactor**

The packed-bed reactor used was 145 mm long, with an 80 mm capacity, a 20 mm internal diameter, and a 34 mm external diameter (Figure 2.1) (Makhongela, 2005). The inlet point was fitted with a 200 μm sinter disk for fixing of the biocatalyst and the outlet was fitted with a Teflon plunger which could be used to adjust the working volume. The reactor was constructed using borosilicate 3.3 glass. To pump water and the substrate to the reactor, two Masterflex

console drive pumps were used. A water bath was used for the temperature control. Substrate was contained in 1 l Schott bottle, and was kept at the water bath with controlled temperature. Another 500 ml Schott bottle was used as a product collection vessel. The reactor was packed with 15 g of biocatalyst (tyrosinase immobilized on sodium aluminosilicate in Section 2.2.9.1). The reaction mixture (320 mg tyrosol, 1600 mg ascorbic acid dissolved in 0.05 M potassium phosphate buffer pH 7 with temperature controlled at 30 °C) was pumped over the biocatalyst housed in a column reactor at a rate of 2.5 ml/min. The eluent (3 ml) collected was analyzed using HPLC at 45 min interval for 270 min.

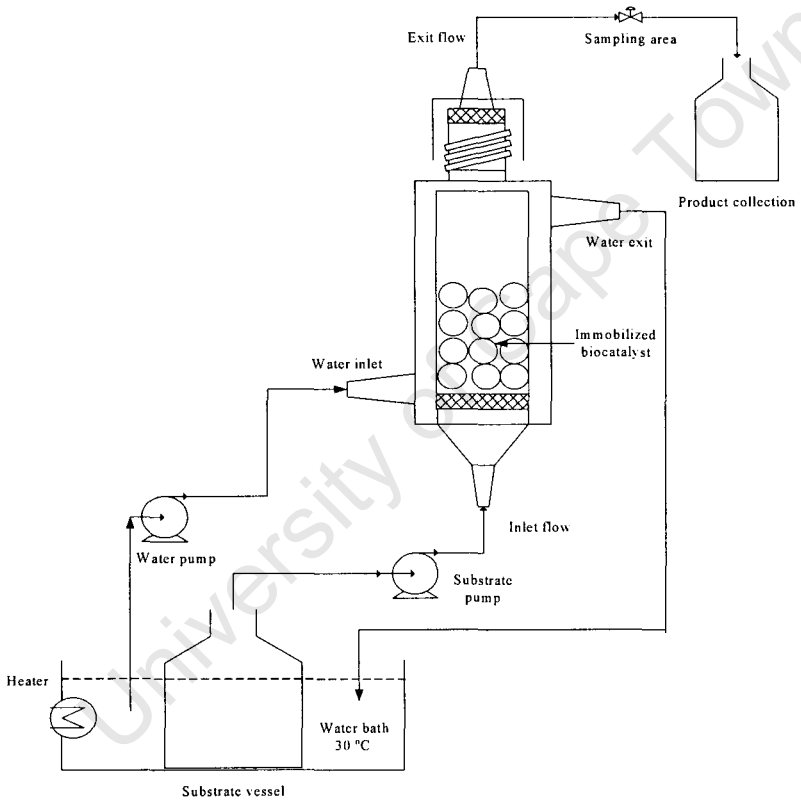





**Figure 2.1 Fixed-bed reactor for the production of hydroxytyrosol using immobilised tyrosinase**

### 2.2.10 Oxidation of tyrosol and hydroxytyrosol catalysed by laccase

Laccase from *Trametes pubescens* was produced using airlift reactors and partially purified using ammonium sulphate (in Section 2.2.1). Hydroxytyrosol was obtained from the reaction of tyrosol with tyrosinase and was purified as described previously in Section 2.2.6.

#### 2.2.10.1 Oxidation of hydroxytyrosol catalysed by laccase in 20 % methanol

A typical reaction mixture contained 1 mg hydroxytyrosol dissolved in 1 ml methanol and 1 U laccase in 4 ml sodium acetate buffer (0.1 M pH 5.0). The control reaction mixture contained no enzyme. Reaction vials were covered with foil and incubated for 24 h at 30 °C in the dark with shaking at 180 rpm. The reaction mixture was monitored by HPLC and the molecular weight of the products determined using LC-MS (Section 2.2.5).

#### 2.2.10.2 Oxidation of hydroxytyrosol catalysed by laccase in 50 % methanol or acetone or ethyl acetate

Hydroxytyrosol (5 mg) dissolved in 1 ml methanol or acetone or ethyl acetate (2.5 mg/ml, final concentration) was mixed with 1 ml sodium acetate buffer (O.1 M pH 5) containing 1 U of laccase. Reaction vials were covered with foil and incubated at 30 °C in the dark with shaking at 180 rpm. The reaction was sampled at 30 min intervals and analysed using HPLC as above in Section 2.2.3.

### 2.2.10.3 Characterisation of hydroxytyrosol-laccase reaction products by TLC and NMR

A preparative reaction was conducted using hydroxytyrosol (10 mg) which was dissolved in 4 ml methanol (2.5 mg/ml, final concentration) and then mixed with 4 ml sodium acetate buffer (0.1 M, pH 5) containing 36 U of laccase. Oxidation of hydroxytyrosol by laccase was also performed in acetone reaction medium (40 mg of hydroxytyrosol was dissolved 1 ml acetone and then mixed with 1 ml sodium acetate buffer (0.1 M, pH 5) containing 12 U of laccase). The reactions were incubated at 30 °C in the dark, with shaking at 180 rpm, and monitored by HPLC (Section 2.2.3). When hydroxytyrosol had been completely converted (after 24 h), the reaction products were recovered by ethyl acetate extraction as described in Section 2.2.6. The sample obtained was applied to the preparative TLC Silica gel 60 F254 TLC aluminium plates 20 x 20 cm (Merck) plates and products separated using the solvent mixture toluene-ethyl acetate-formic acid (5:4:1). The individual bands representing pure products were scratched from TLC plate and then the organic compounds recovered by extraction with ethyl acetate.



### 2.2.11 Oxidation of tyrosol by laccase

Tyrosol (Merck) was oxidised by laccase obtained from *T. pubescens*, under the following conditions:

**A) Biphasic system**

The reaction mixture contained 100 mg tyrosol, 20 mg laccase (24 U) prepared as described in section 2.2.1.4, 5 ml ethyl acetate and 5 ml sodium acetate buffer 20 mM, pH 4.5. The second reaction contained 200 mg tyrosol, 40 mg laccase (48 U) dissolved in 10 ml of 0.1 M sodium acetate buffer, pH 5 and 40 ml ethyl acetate (80 % ethyl acetate). The reactions were performed in duplicate. In each case, the mixture was incubated at 25 °C with agitation at 250 rpm for 16 h. The progress of the reaction was monitored by thin layer chromatography (TLC) (eluent, toluene-ethyl acetate-formic acid, 5:4:1).

**B) Aqueous media**

The reaction mixture contained 5 mg tyrosol, 2 mg (2.4 U) laccase and 1 ml sodium acetate buffer 20 mM, pH 4.5. The mixture was incubated at 25 °C with agitation at 250 rpm. The progress of the reaction was monitored by TLC (eluent, toluene-ethyl acetate-formic acid, 5:4:1). The reaction products were recovered by ethyl acetate extraction. The ethyl acetate was evaporated using the rotary evaporator and the residue was weighed, and redissolved in methanol and then analysed by HPLC (The mobile phase was methanol-acetic acid-water (20:2.5:80) with a flow rate 1 ml/min, and using a C18 Waters (250 mm x 4.6 nm) reverse phase column and UV detection at 280 nm).

**C) Miscible organic aqueous medium**

Tyrosol was oxidised by laccase under three different of reaction conditions. The first reaction mixture contained 200 mg tyrosol dissolved in 10 ml acetone and 40 mg laccase (48 U) dissolved in 40 ml of 0.1 M sodium acetate buffer, pH 5. A second reaction mixture contained 50 % acetone with other reaction conditions remaining the same. In a third reaction, 200 mg tyrosol was dissolved in a 50 ml of acetone and 40 mg laccase (48 U) dissolved in 10 ml sodium acetate buffer (0.1M, pH 5). The reaction mixture was incubated at 25 °C with agitation at 250 rpm for 16 h. The progress of the reaction was monitored by TLC (eluent, toluene-ethyl acetate-formic acid, 5:4:1). The reaction products were recovered by ethyl acetate extraction. The ethyl acetate was evaporated using the rotary evaporator and the residue was weighed, and redissolved in methanol and then analysed by HPLC (mobile phase methanol-acetic acid-water (20:2.5:80) with a flow rate 1 ml/min, and using a C18 Waters (250 mm x 4.6 nm) reverse phase column and UV detection at 280 nm).



### 2.2.11.1 Purification of tyrosol derivatives from biphasic medium using flash chromatography

The ethyl acetate phase (organic phase) from the 10 ml reaction mixture (in Section 2.2.11 B) was decanted into a round bottom end flask. The organic compounds in the aqueous phase were

extracted using the ethyl acetate (3 x equal volume) and the extracts were combined. The ethyl acetate was evaporated using the rotary evaporator and the residue was weighed, and redissolved in 3 ml ethyl acetate. The concentrated sample was loaded on a flash chromatography column and tyrosol and products were eluted using petroleum ether-ethyl acetate, 5:8 containing 0.1 % methanol. The purified samples were dried and weighed, before analysis as described in Section 2.2.11.2 below. This procedure was also repeated for other reactions in Section 2.2.11.

#### 2.2.11.2 Nuclear magnetic resonance (NMR) analysis

For tyrosol reaction products, $^{1}$H-NMR was conducted with *d*-chloroform at 300 MHz.

#### 2.2.11.3 Liquid chromatography-mass spectrometry (LC-MS) analysis of tyrosol products

Full Scan Liquid Chromatography-Electron Spray Mass Spectrophotometry (LC-ESMS) was performed in the negative or positive mode between 0-1000 mV. The mobile phase was a mixture of formic acid (0.1 % v/v) (solvent A) and acetonitrile (solvent B) with a flow rate 0.7 ml/min, and using a Luna 5 µm C18 Waters (250 mm x 4.6 nm) reverse phase column and UV detection at 280 nm. The gradient elution programm was: 95 % A and 5 % B (0-2 min), 20 % A and 80 % B (2-25 min), 100 % B (25-30 min), and 95 % A (30-40 min) and 5 % B (30-40 min).

### 2.2.12 DPPH radical assay of laccase-hydroxytyrosol/tyrosol reaction products

#### 2.2.12.1 DPPH radical assay for a mixture of products from hydroxtyrosol-laccase reactions

The method used to determine the antioxidant activity was a modified version of the methods of Siddhuraju and Becker (2003) and Sánchez-Moreno *et al.* (1998). Hydroxytyrosol (5 mg) dissolved in 0.5 ml methanol (2 mg/ml, final concentration) was mixed with 2 ml sodium acetate buffer (O.1 M pH 5) containing 12 U of laccase. Reaction vials were covered with foil and incubated at 30 °C in the dark with shaking at 180 rpm for 2 h or until the 100 % conversion of hydroxytyrosol had been achieved as confirmed by HPLC. The control reaction was prepared as above but contained no laccase. 100 µl volumes of these samples were reacted with 3.9 ml of DPPH solution (6 x $10^{-5}$ M in methanol). The decrease in the absorbance at 515 nm was

monitored using a Unicam UV-visible spectrophotometer, until the reaction reached the steady state.The percentage of remaining DPPH was calculated as follows:

% DPPH remaining = [DPPH]t / [DPPH]t = 0 x 100
where [DPPH]t was the concentration of DPPH. at the time of steady state and
[DPPH]t = 0 was the concentration of DPPH at time zero.

The concentration of the antioxidant (sample) required to decrease the initial DPPH concentration by 50 % (the $EC_{50}$) was determined graphically (Sánchez-Moreno *et al.*, 1998). The experiment was performed in duplicate. The percentage DPPH remaining for a product sample was compared with that of control solution. This procedure was reapeted for the solutions of purified hydroxytyrosol dimer **57** and a trimer **59** with the original concentration of 2 mg/ml.

Furthermore, tyrosol was oxidised by laccase in medium containing 80 % ethyl acetate and the products (compound **49** (poly (tyrosol)), and compound **50** (a tyrosol dimeric product) obtained were also evaluated for antioxidant activity. A stock solutions 2.5 mg/ml of tyrosol **24**, compound **49**, and compound **50** were prepared in the mixture of 1,4-dioxane and methanol (1:1, v/v). The decrease in the absorbance was measured as stated above.

## 2.3 Results and Discussion

### 2.3.1 Production, purification and characterization of laccase

Laccase for use in the biocatalytic conversion of tyrosol and hydroxytyrosol was isolated and partially purified. Extracellular laccase was produced by culture of *Trametes pubescens* in an airlift reactor according to the method established by Ryan *et al.* (2005). Thus, *T. pubescens* was cultured in *Trametes* Defined Medium (TDM) inoculated with homogenized mycelial plug taken from a solid Petri dish preculture. These cultures were used to inoculate 4 l airlift bioreactors each containing TDM. The laccase from *T. pubescens* is excreted to the medium and has previously been shown to be the major component of the extracellular protein in the medium (Ryan *et al.*, 2005). A single activity band that correlated with laccase (Sigma) was obtained for the enzyme purified using acetone or ammonium sulphate, on a non-denaturing PAGE gel stained with ABTS solution. The protein in the medium was partially purified using acetone or ammonium sulphate, and the protein concentration was measured using Bradford's method (Figure 2.2) (Appendix A). The 100 % pure enzyme is not necessarily required for biocatalytic processes if product can be isolated pure from the reaction medium. Thus, no further purification of laccase was performed. The purified laccase showed a specific activity of 1.8 U/mg. This laccase was used in bioconversion of hydroxytyrosol and tyrosol. The production of laccase from *T. pubescens* was repeated several times during this study, and the laccase was subsequently used in the oxidation of tyrosol and hydroxytyrosol.

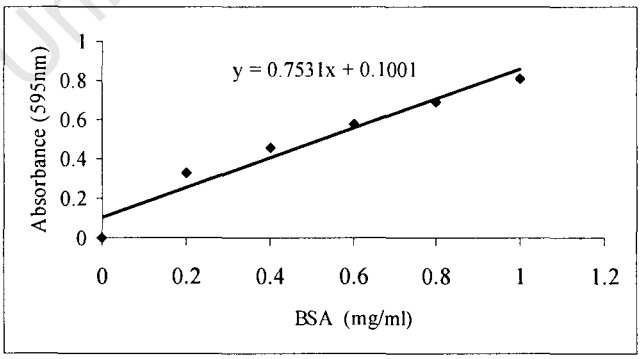



**Figure 2.2 Standard curve for protein determination using Bradford's assay.**

### 2.3.2 Oxidation of tyrosol by laccase

Tyrosol was oxidised by laccase from *T. pubescens* (Section 2.2.11) in reaction mixtures containing sodium acetate and 80 % ethyl acetate, 83 % acetone, aqueous medium, 50 % acetone and 50 % ethyl acetate. Precipitation was observed within 3 h from all media used. In a reaction mixture that contained 200 mg tyrosol dissolved in 40 parts ethyl acetate to 10 parts sodium acetate buffer, 100 mg of a yellow water-insoluble product, **49**, was obtained (50 % yield and 40 % conversion of tyrosol as determined by HPLC). Since in polymerisation reactions the molecular weight of the product is not necessarily well defined, mass ratio was used instead of molar ratios, and the conversion was calculated on the basis of percentage substrate converted. This polymeric product did not dissolve in acetone, methanol, ethyl acetate, *n*-hexane, dichloromethane or chloroform. However, it was poorly soluble in a mixture of 1,4 dioxane and methanol (1:1) or diethyl ether-chloroform-tetrahydrofuron (1:1:1). The insolubility of this polymer in water and the organic solvents listed above, was an indication that tyrosol had polymerised to form a group of products with higher molecular weight as depicted in Figure 2.3a and Figure 2.3b.



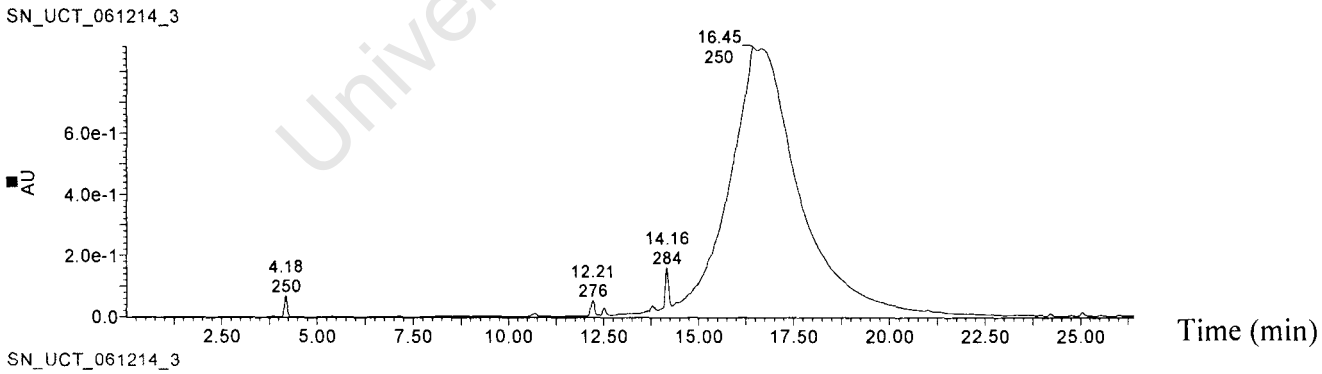



**Figure 2.3a LC-MS total ionisation chromatogram of yellow water-insoluble product, 49 obtained from biotransformation tyrosol with laccase from *T. pubescens*.**

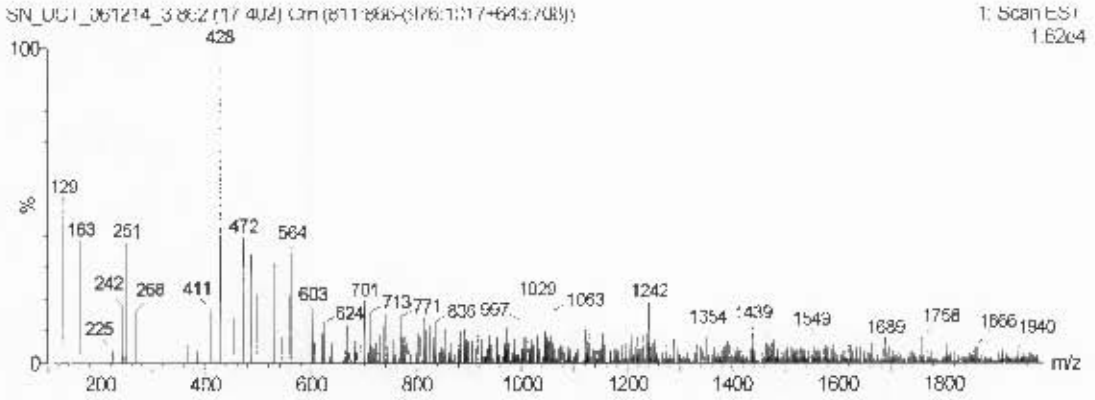



**Figure 2.3b LC-MS of tyrosol product mixture 49, corresponding to peak with retention time 16.45 min in Figure 2.3a, indicating the presence of higher molecular weight products.**

Further characterisation of the tyrosol-laccase reaction products obtained from same medium (ethyl acetate-buffer mixture, (40:10)) was performed using TLC analysis. The TLC plate (Figure 2.4) showed the presence of three new products, **50, 51** and **52,** with $R_f$ values of 0.41, 0.33 and 0.26 respectively. The product on the base line of the TLC plate when analysed by HPLC co-eluted with product **49,** and thus it is likely that it is the same polymeric product.

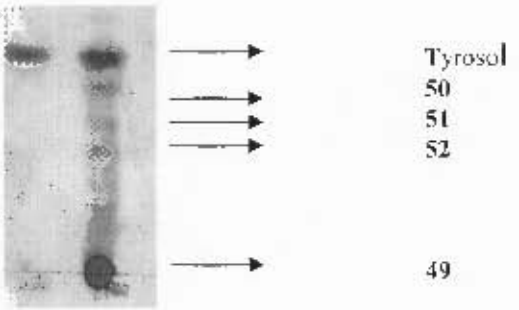



**Figure 2.4 TLC plate showing the spots representing the products 49-52 of the laccase tyrosol reactions. The solvent system used was a mixture of toluene-ethyl acetate-formic acid, (5:4:1). The TLC plate was stained with Pancaldi solution (Gerber-Lemaire *et al.*, 2006) (Appendix E) and then dried in the oven for visualization of spots.**

Product **50** was purified from the reaction mixture in a flash chromatography column;further purified on a preparative TLC plate, and then analysed using $^{1}$H-NMR analysis to complete the structural characterisation. The $^{1}$H-NMR spectral data (Appendix F) were characterised by peaks similar to those reported by Vinciguerra *et al.* (1997). Vinciguerra and coworkers reported that tyrosol is converted by laccase from *Lentinus edodes* into a dimeric tetracylic ketone. As proposed by Vinciguerra *et al.* (1997), the formation of this dimer is attributed to the laccase being capable of generating, from tyrosol, radicals such as **b**, **c**, **d** (Figure 2.5). The coupling of **c** and **d** resulted in a product **e**, an intermediate. The closure of the neighbouring alcohol function on the double bond of the cyclo hexonone ring resulted in a final product, **50,** a dimer (Vinciguerra *et al.*, 1997). It is notable that there could be other possibilities of coupling of radicals, for instance, the coupling could have been by C-C bond formation. Cermola and coworkers (2004) reported the photooxidation of tyrosol which resulted in radicals formed by C-C coupling, **53**, as depicted in Figure 2.6 (Cermola *et al.*, 2004). Such variation of coupling can lead to various products, as depicted by TLC plate in Figure 2.4. This may explain the low yield of 50 (7 %) and the other two products obtained that were not characterised in this study because of low yields. Product **50** was also analysed using LC-MS and the lower-intensity peak with molecular weight 952.45 m/z (Figure 2.7) was obtained suggesting this product also contained polymeric structure. The presence of a high-intensity peak with molecular ion mass of 276 m/z in the same Figure was an indication of the dimer of tyrosol that had been anticipated. However, it is acknowledged that to obtain complete structure of product **50** further chemical characterization is necessary.

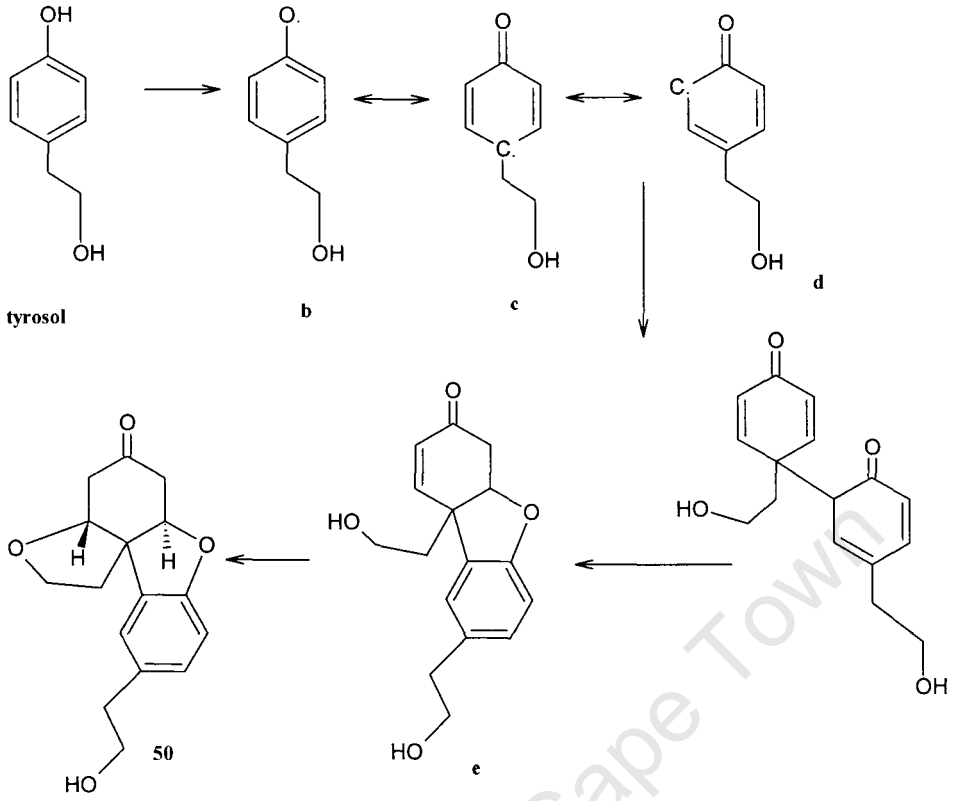



**Figure 2.5 Oxidation of tyrosol by laccase resulted in a formation of radicals that couple with each other to form a dimeric compound (Vinciguerra *et al.*, 1997).**

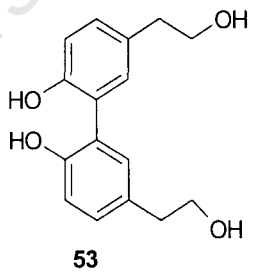



**Figure 2.6 Photooxidation of tyrosol resulted into radicals that coupled to C-C dimeric compound (Cermola *et al.*, 2004).**

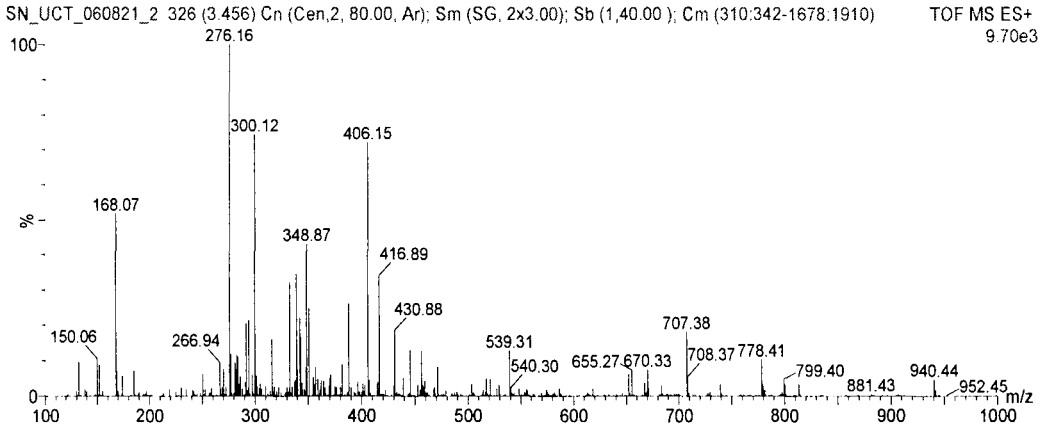



**Figure 2.7 LC-MS of tyrosol product 50 indicating the presence of a dimerised tyrosol product with molecular weight 276 m/z. This reaction was performed in ethyl acetate containing medium.**

From this study, it was clear that tyrosol radicals as generated by laccase were very reactive and polymerised rapidly to form a precipitate in the reaction media containing various organic solvents. It can be concluded that organic solvents used had no influence in formation of the precipitate or polymeric products. The high reactivity of tyrosol radicals was attributed to the position of the hydroxyl group which is *para*, since attachment of hydroxyl group in the *para* position confers high reactivity to the compound (Gianfreda *et al.*, 2003). The hydroxyl group confers polarity in tyrosol, therefore causing the radicals generated to be more reactive. This explanation is consistent with literature where it has been reported that functional groups could increase the polarity, and hence leading to high reactivity (Braunecker and Matyjaszewski, 2006). However, these results are not consistent with the concept that organic solvents in the reaction medium decrease the hydrophobic interactions of molecules (see Chapter 3), and hence prevent the self-aggregation of radicals leading to products with lower molecular weight (Oguchi *et al.*, 2002). The implication of this is that products with higher molecular weight could be formed even in the presence of organic solvents in the reaction medium.The products obtained were evaluated for antioxidant activity using DPPH radical assay.

### 2.3.3 Antioxidant activity of tyrosol-laccase products

The antioxidant activity of tyrosol, **24**, was compared with that of the dimeric product of tyrosol, **50**, and the polymeric product, **49** (Section 2.2.12). These products were dissolved in a mixture of methanol and 1,4 dioxane (1:1) and reacted with DPPH in methanol. The remaining DPPH was measured in a spectrophotometrically. In equivalent reactions, tyrosol quenched 3 % of the total DPPH radical while products **50** and **49** had quenched 45 % and 14 % respectively of the total DPPH radical present (Figure 2.8). Although concentrations of these products were expressed in terms of mass/volume rather than molar concentration, it is apparent that the new products obtained from the laccase reactions had higher antioxidant activity as compared to the parent compound tyrosol. Thus, oxidising tyrosol with laccase to a dimeric product or polymeric product resulted in compounds that were more active than tyrosol, and are stable antioxidants (no increase in absorbance reading was observed).

The elucidation of the radical-scavenging pathways was based on two existing different routes for antioxidant to scavenge peroxide radical. One is a direct H-atom-abstraction process and the other is stepwise electron transfer/proton transfer process (see Chapter 1, Section 1.5.1.6.1). For tyrosol, it is proposed that DPPH radical scavenging was based on the H-abstration method. It is suggested that the hydroxyl group in this compound donated a hydrogen atom to the DPPH radical. It is generally known that both the number and configuration of H-donating hydroxyl groups are major structural features influencing the antioxidant capacity of phenolics (Cao *et al.*, 1997; Pannala *et al.*, 2001). Thus for compound 53, H-abstraction method could be expected since this compound is characterised by increased number of hydroxyl groups that could readily donate hydrogens. For compound **50**, DPPH radical scavenging method could be based on the electron-coupled proton transfer method rather than hydroxyl groups donating hydrogens. The reason for this is based on the fact that the compound 50 bears no hydroxyl group that could donate hydrogen in the H-abstration method. A similar conclusion was reached by Wang *et al.* (2006) and Zoete *et al.* (1999) who suggested that the possible mechanism for the reaction of indomethacin with trichloromethyl peroxyl or peroxyl radicals was one electron reduction, since the compound indomethecin contained no hydroxyl group in its structure. In addition to the structure of a compound **50**, the reaction medium, in conjuction with ionization potential,

possibly played an important role in the antioxidant activity of product **50**. It has been reported that single electron-transfer reactions are more likely to occur in predominantly aqueous solution, since the polar solvents will stabilize the charged molecules present as products of ionization (Nakanishi *et al.*, 2002; Wright *et al.*, 2001). In literature, it has been reported that for the single electron transfer system to occur, the ionization potential (IP) of an antioxidant must be lower than that of the corresponding radical (Wright *et al.*, 2001). Wright *et al.* (2001) calculated the IPs of several compounds and showed that as the IP drops to below that of a phenol, compounds tend to follow single-electron transfer reactions. Above this point, the reactions undergo hydrogen atom transfer reactions. In this study, the DPPH radical and product **50** were dissolved in methanol, a polar solvent, which could have favoured electron transfer system.

The stability of the phenoxy radicals involved in the reaction of the products **49** and **50** with the DPPH radical could be explained in terms of the availability of conjugated double bonds that allow electron delocalisation across the molecule thus stabilizing the phenoxyl radical (Ariga *et al.*, 1988; Dizhbite *et al.*, 2004).

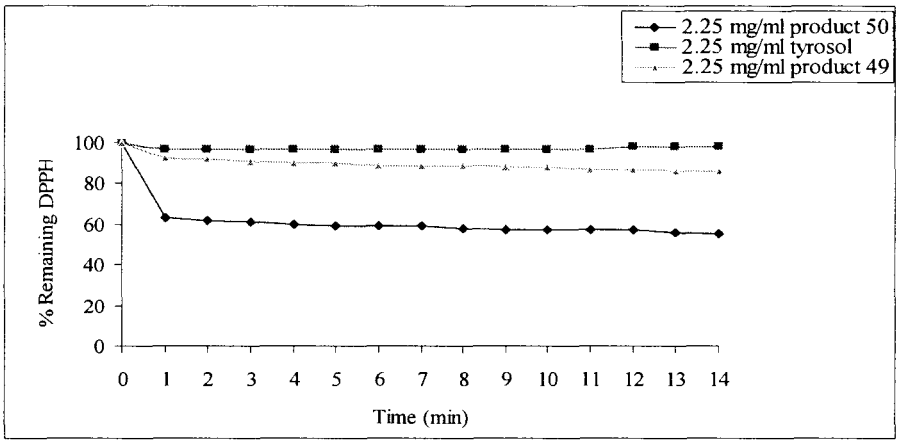



**Figure 2.8 Antioxidant activity of tyrosol, product 50, and polymeric product 49 obtained from the same reaction.**



### 2.3.4 Alternative application of tyrosol-laccase biocatalytic reaction: Bioremediation of tyrosol

Phenols are widely known as one of the main compounds discharged to the environment from industrial processes. Many of these compounds are considered toxic and some are carcinogenic. It is also believed that they are main compounds responsible for destruction of plants (Wang *et al.*, 2002a). Thus the removal of phenols from industrial waste is of great importance (Klibanov *et al.*, 1980). The physical and chemical methods used to remove phenols are generally technically and economically not feasible (Ramos-Cormenzana *et al.*, 1995). In the light of this background, there is growing interest in using biological agents, and in particular fungal systems, in bioremediation process (Ryan *et al.*, 2005). Laccase has been investigated widely in bioremediation studies (Ryan *et al.*, 2005) and references therein, but tyrosol has not been described in this context. In this study, tyrosol was considered as a model compound for developing a bioremediation process using laccase from *T. pubescens*

Thus, further investigation was performed to evaluate the potential of laccase to remove *p*-tyrosol from aqueous solutions. Tyrosol was added to a Schott bottle containing 50 ml of acetone, 10 ml

acetate buffer (0.1M pH 5) (Section 2.2.11), and laccase. The reaction mixture was incubated at 25 °C with shaking at 200 rpm. The removal of *p*-tyrosol in the reaction medium was monitored by HPLC analyses as shown in Figure 2.9. The presence of laccase in aqueous solution resulted in a disappearance of a tyrosol peak, **24**, with retention time 7.5 min. Within 48 h of incubation of the solution, HPLC analysis further showed the appearance a new peak, (retention 4.2 min), which was identified by LC-MS as polymeric compound with molecular weight 962.8 m/z (Figure 2.10). This polymeric compound of tyrosol (**54**) was constituted from 7 monomers of tyrosol. Further analysis after 48 h, showed the presence of another new peak, **50**, with retention time 1.9 min. This peak was a dimeric product reported previously in Section 2.3.2.

The detection of the polymeric product before the dimeric product does not necessarily mean laccase first produces a polymer and then a dimer. However, the results confirm that tyrosol is highly reactive and polymerises rapidly to form products with higher molecular weight. The absence of a peak representing the dimer within 24 h could simply be due to lower levels of this dimeric product that were not be detected, or were superceded by large peak area of the polymer.

The disappearance of tyrosol was accompanied by appearance of the precipitate (80 mg) which was also a polymeric product, **49,** reported in Section 2.3.2. This was an indication that polymeric structures had aggregated in the solution forming particles that were easily removed by filtration. There was 95 % tyrosol removal within 24 h and complete removal was achieved after 48 h (Figure 2.9). From these results it is apparent that laccase from *T. pubescens* is suitable for the removal of many phenolic compounds, including tyrosol, from solution.

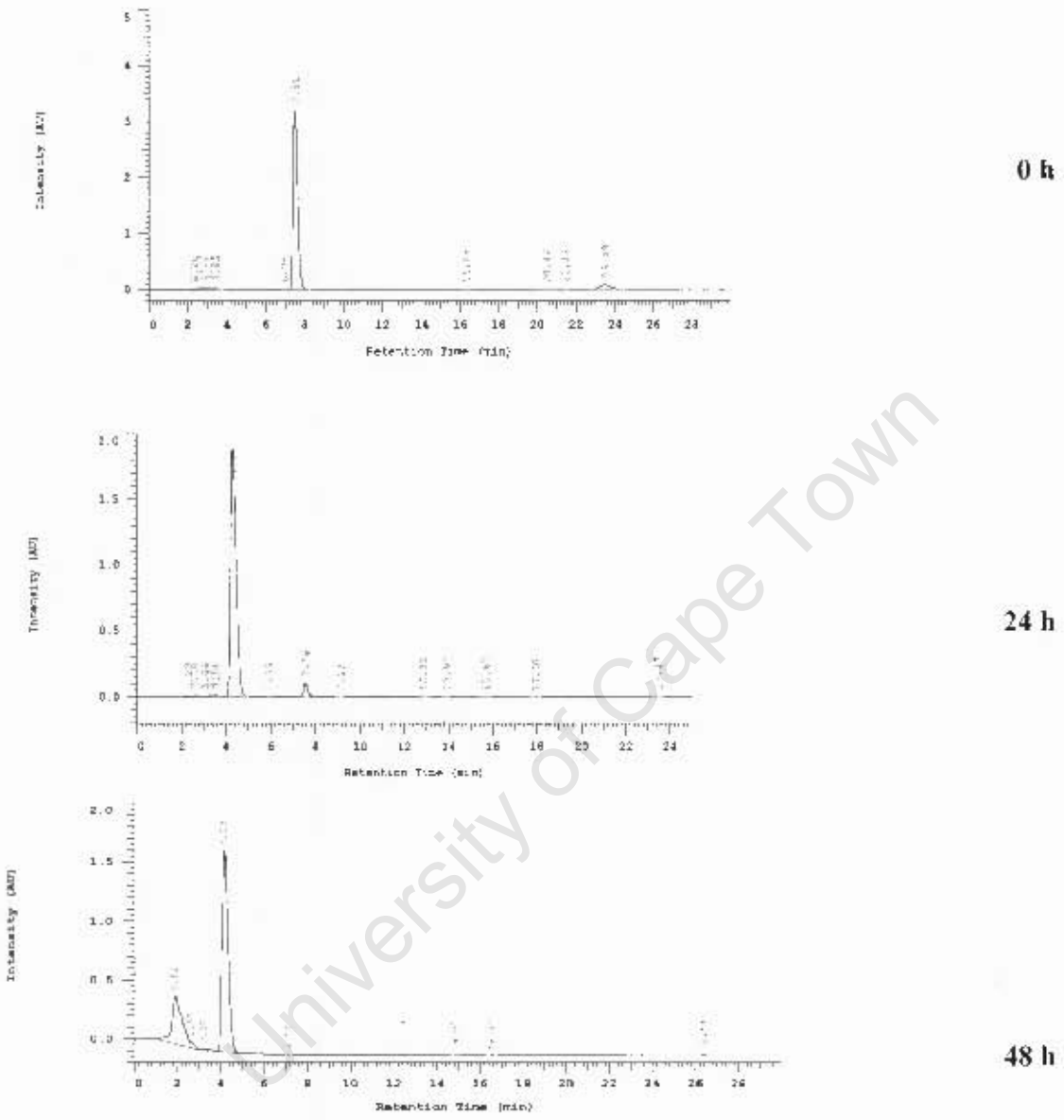





**Figure 2.9 Removal of tyrosol from an aqueous medium by laccase. Disappearance of a tyrosol (peak with retention time 7.5 min) is accompanied by appearance of a new peak after 24 h with retention time 4.2 min, which is the polymeric product 54, and a peak with retention 1.92 min which represented a product 50, a dimer of tyrosol, after 48 h.**

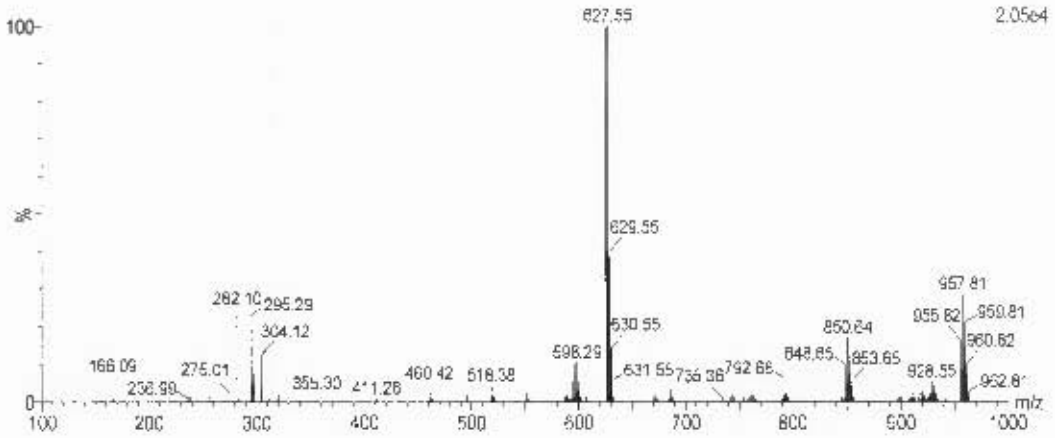



**Figure 2.10 LC-MS profile: The removal of tyrosol from an acetone containing medium by laccase resulted in an appearance of a new peak with retention time 4.2 min (product 54). The LC-MS profile of this peak demonstrates the production of a polymer comprised of 7 monomers of tyrosol (962.8 m/z).**

To our knowledge, there is also no work that has been reported on the removal of *p*-tyrosol by laccase from *T. pubescens*. However, Gianfreda *et al.* (2003) reported on the transformation of a mixture of four phenols (catechol, methylcatechol, *m*-tyrosol and hydroxytyrosol) by laccase from *Rhus vernificera*. These authors reported that *m*-tyrosol was less reactive as compared to other phenols in the mixture (i.e 65 % *m*-tyrosol was still in solution after 24 h (Gianfreda *et al.*, 2003)). Besides the possibility of the competitive effects that may arise when more than one phenol is present in the reaction solution, the lower reactivity of *m*-tyrosol was based on its structure (argued by Gianfreda *et al.* (2003)), particularly, the *meta* position of hydroxyl group in a tyrosol aromatic ring. Thus it is difficult to compare our results in terms of monomer removal efficiency with those of Gianfreda *et al.* (2003), since the tyrosol used in the current study had the hydroxyl group in the *para* position. However, it is clear from these results, and also based on the comparisons with the results of (Gianfreda *et al.*, 2003), that the reactivity of laccase with phenolic substrates is strongly be influenced by structural configuration. The *para* position of hydroxyl group contributed to higher reactivity of laccase with tyrosol. These results also affirm the assertions made in Section 2.3.3 that the reactivity of *p*-tyrosol was attributable to its structure. The results are also consistent with the work of Shuttleworth and Bollag (1986) who

showed that the ability of laccase to remove cresol from solution was dependent on the position of the methyl group with *meta*-substituted cresol being least oxidised and *para*-cresol the most.

The results obtained in this study are of great importance since the predictions on the performances of a bioremediation model involving laccase can be made, particularly, in terms of the ability of laccase to oxidise compounds within an effluent. Further work that could be done to give more insight into the use of laccase in bioremediation of tyrosol would include: testing the stability of laccase under adverse conditions that include low pH, high temperature, and the presence of many other compounds that might be present in polluted water.

In the work described in the next sections of this chapter, hydroxytyrosol was also oxidized by laccase. However, first, the extraction of tyrosinase, needed for production of hydroxytyrosol from tyrosol, was performed.



### 2.3.5 Extraction of tyrosinase from white mushrooms

Extraction of tyrosinase from mushrooms (*Agaricus bisporus*) was a performed using methodology previously established by Burton (1994) and references therein. Fresh mushrooms were homogenized in acetone to remove lipids and the homogenate filtered though a Buchner funnel. The liquid nitrogen-dried residue was mixed with water to form a slurry, from which tyrosinase was extracted with water. The extraction mixture was then centrifuged, and the extract, which contained the tyrosinase activity, was collected. The extract was purified using ammonium sulphate fractionation. The activity of tyrosinase and concentration of the protein in the purified and crude fractions were determined (Section 2.2.2). The highest specific activity of tyrosinase was found in the 52 % ammonium sulphate saturated pellet (Table 2.1). The extraction procedure was repeated several times during the study and the tyrosinase obtained was used in hydroxylation of tyrosol to yield hydroxytyrosol.

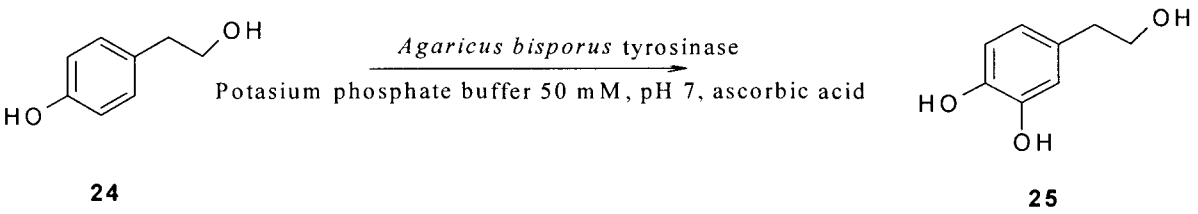
**Table 2.1 The purification table for tyrosinase extracted from mushrooms.**

| Fraction | Volume (ml) | Protein (mg/ml) | Activity (U/ml) | Specific Activity (U/mg) | Total activity (U) | Total protein (mg) |
|---|---|---|---|---|---|---|
| **Crude** | 500 | 4 | 6.4 | 1.6 | 3200 | 2000 |
| **40 % pellet** | 500 | 1.4 | 2.3 | 1.6 | 1150 | 700 |
| **52 % pellet** | 20 | 2.9 | 6 | 2.06 | 300 | 145 |



### 2.3.6 Production of hydroxytyrosol using tyrosinase in aqueous media

Hydroxytyrosol, **25**, was produced from tyrosol, **24**, based on a previously reported enzymatic procedure employing a tyrosinase-catalysed hydroxylation of the aromatic ring in the presence of ascorbic acid (Espín *et al.*, 2001). Tyrosinase (prepared as described in Section 2.3.5) was used to hydroxylate tyrosol in a reaction medium that contained ascorbic acid and tyrosol dissolved in phosphate buffer as described in Section 2.2.3 and as depicted in Figure 2.11. Sample aliquots were withdrawn from the reaction mixture periodically, and analyzed by HPLC. The retention times of ascorbic acid, hydroxytyrosol and tyrosol were 3.12, 4.18 and 5.42 min respectively (Figure 2.12), identified on the basis of comparisons with standards compounds. The reaction products were also analyzed using TLC. The band with $R_f$ value of 0.58 represented hydroxytyrosol, (identified by comparing with standard hydroxytyrosol). Further characterization of the product was achieved using LC-MS, where a molecular weight of 154 m/z was found (Figure 2.13). The structure was confirmed using $^1$H-NMR (Appendix C). The results were in agreement with those previously reported by Capasso *et al.* (1999) and Espín *et al.* (2001), and therefore the compound obtained was confirmed to be hydroxytyrosol. An attempt was then made to optimize the production of hydroxytyrosol (see below in Section 2.3.7).




24 25

**Figure 2.11 Hydroxylation of tyrosol by tyrosinase to form hydroxytyrosol.**

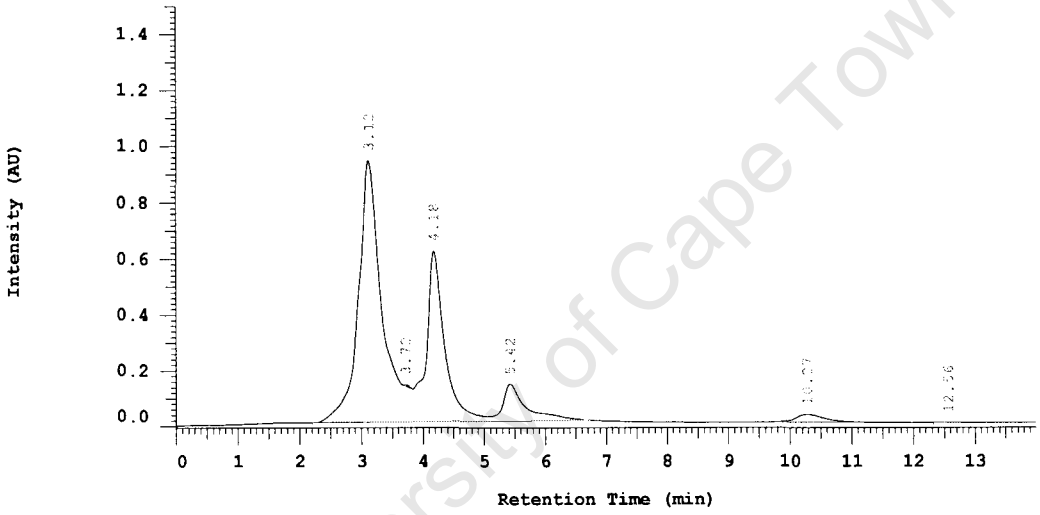





**Figure 2.12 HPLC profile of tyrosol-tyrosinase reaction after 5 h. Tyrosol was oxidized by tyrosinase in a reaction mixture that contained ascorbic acid dissolved in 50 mM potassium phosphate buffer. The first peak is ascorbic acid (retention time 3.12 min), the second peak is the new product, hydroxytyrosol (25) (retention 4.18 min) and tyrosol, 24, is represented by peak retention 5.42 min.**

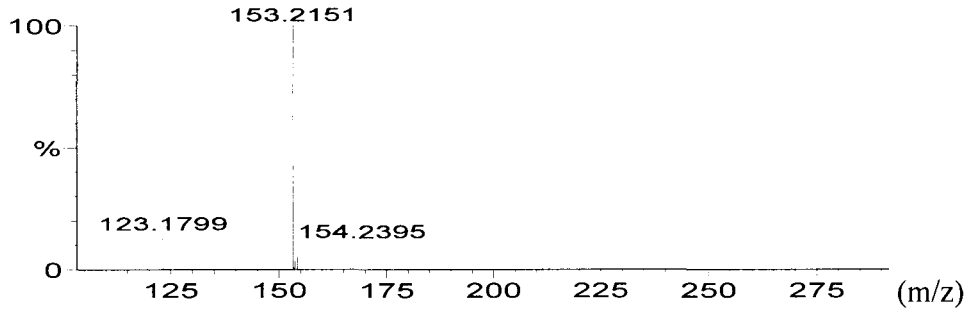



**Figure 2.13 LC-MS profile of hydroxytyrosol (25) synthesized using tyrosinase in buffer medium containing ascorbic (see Section 2.2.3).**



### 2.3.7 Optimization of hydroxytyrosol production

#### 2.3.7.1 The effect of pH on the conversion of tyrosol by tyrosinase

The effect of pH on the production of hydroxytyrosol was evaluated. This was achieved by setting up a reaction mixture that contained ascorbic acid and tyrosol dissolved in potassium phosphate buffer of pH varying between 6 and 7.5, and the reaction was initiated by addition of tyrosinase (see Section 2.2.4.1) The reaction was monitored by HPLC to measure the conversion after 5 h. The highest conversion of tyrosol was achieved with pH 7 where 74 % conversion was obtained. The results of the investigation of the effect of pH on tyrosol conversion are shown in Figure 2.14A. From these results, it can be concluded that the optimal pH for conversion of the tyrosol was 7; increasing the pH to 7.5 resulted in a decrease in conversion. These results are in agreement with those of Ikehata and Nicell (2000) who reported that the maximum activity of mushroom tyrosinase is observed at neutral pH and that tyrosinase is not significantly active at basic pH. All subsequent experiments were conducted at pH 7.

#### 2.3.7.2 The effect of temperature on the bioconversion of tyrosol by tyrosinase

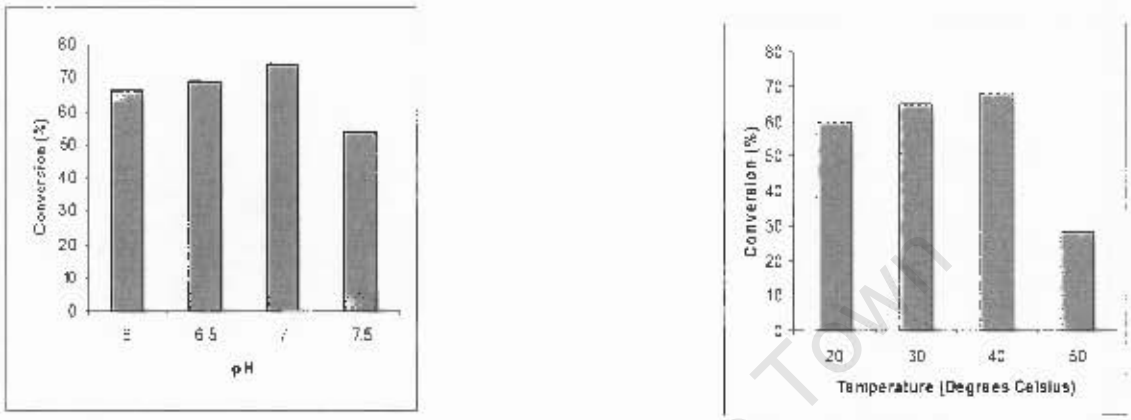
The effect of temperature on the production of hydroxytyrosol was evaluated. This was achieved by setting up a reaction mixture that contained ascorbic acid and tyrosol dissolved in potassium

phosphate buffer at pH 7 and the reaction was initiated by addition of tyrosinase (see Section 2.2.4.2). The reaction mixtures were incubated at various temperatures and monitored using HPLC. The conversion of tyrosol after a treatment time of 5 h, at various temperatures, is shown in Figure 2.14B. The data show that the conversion of tyrosol achieved in the 5 h reaction time increased with temperature up to an optimum achieved at approximately 40 °C, above which conversion decreased. It is clear that above 40 °C, tyrosinase started to lose its activity, presumably due to denaturation. Thus the optimum temperature for conversion of tyrosol by tyrosinase is between 30 and 40 °C as depicted in Figure 2.14B, and a temperature of 30 °C was chosen for subsequent experiments.

### 2.3.7.3 The effect of detergent SDS on the bioconversion of tyrosol by tyrosinase

The activation of tyrosinase by SDS has been reported by many researchers (Burton, 1994 and Espín *et al.*, 1999, among others). In this study, the effect of SDS on the bioconversion of tyrosol by tyrosinase was evaluated. The potential benefit using SDS was tested by treating tyrosol with various concentration of SDS and comparing the conversion achieved in each case to the conversion achieved when no SDS was present. The conversion of tyrosol by tyrosinase in the medium containing SDS was higher than the conversion in the medium with no SDS (Figure 2.15A). Under these conditions the conversion rate increased with an increase in SDS concentration till the optimum concentration was reached (2 mM), and thereafter conversion rate decreased (Figure 2.15). The results obtained in this study suggest that the increase of tyrosol conversion is linked to the presence of SDS in the reaction medium. The inference made is that SDS activated tyrosinase, more particularly the catecholase activity of this enzyme which represented 98-99 % of the mushroom tyrosinase activity (Van Leeuwen and Wichers, 1999). The results obtained in this study are in agreement with those from other studies of Burton (1994) and Witteberg and Triplett (1985), among others. The activation of tyrosinase is related to conformational changes due to SDS binding a phenomenon that was described by Espín *et al.* (1999).

It is apparent from this study that highest conversion of tyrosol to hydroxytyrosol by tyrosinase is achieved when the pH of potassium phosphate buffer is 7, the reaction the reaction medium is supplemented with 2 mM SDS and when the reaction mixture is incubated between 30 to 40 °C.




A B

**Figure 2.14 The effects of pH (A) and temperature (B) on the conversion of tyrosol by tyrosinase in a medium containing ascorbic acid.**

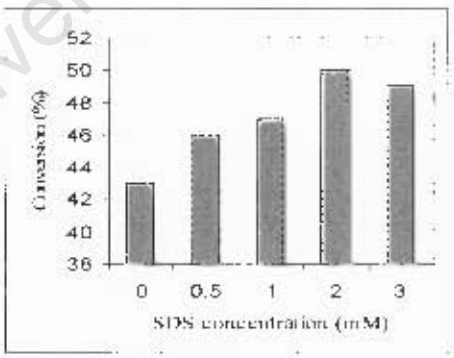



**Figure 2.15 The effect of SDS on the conversion of tyrosol by tyrosinase in buffer containing ascorbic acid.**

### 2.3.8 Determination of initial rates of reaction for soluble tyrosinase enzyme

Since the ultimate aim of this study was to produce hydroxytyrosol in relatively large quantity in a bioreactor system, the rates for soluble tyrosinase, under typical bioreactor conditions, were determined in order to evaluate its potential performance in a reactor system. To determine the reaction rate for hydroxytyrosol production, tyrosol (3 mg) and ascorbic acid (20 mg) were dissolved in 50 mM potassium phosphate buffer containing 2 mM SDS and the reaction was initiated by addition of tyrosinase (0.1 U). The conversion of tyrosol by tyrosinase to hydroxytyrorosol was monitored by HPLC, and a 70 % conversion was achieved after 4 h. The rate of hydroxytyrosol production was then determined graphically (Figure 2.16). It was found that, over these time periods, hydroxytyrosol was produced at a rate of 0.14 mM/min (obtained by expressing the slope of the tangent line to the saturation curve in Figure 2.16 in terms of concentration of hydroxytyrosol produced per min). A total concentration of 14.0 mM of hydroxytyrosol per 0.1 U biocatalyst was produced over 100 min (91 % yield calculated based on the number of moles). The yields obtained in this study are comparable with those of Espín *et al.* (2001) who reported that yields of hydroxytyrosol produced using mushroom tyrosinase were between 80 to 90 %.



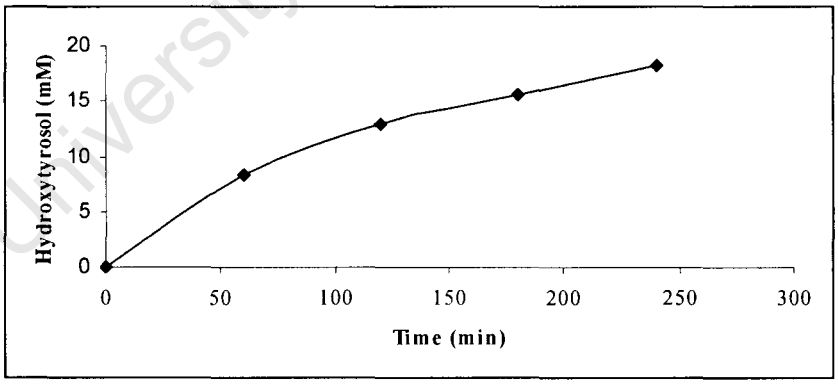



**Figure 2.16 Initial rate of hydroxytyrosol production by tyrosinase in the presence of 2 mM SDS.**

### 2.3.9 Production of hydroxytyrosol in a batch reactor system using immobilized tyrosinase

This study reports for the first time, to our knowledge, the use of tyrosinase immobilized on zeolite, in the production of hydroxytyrosol. Tyrosinase was immobilized on zeolite molecular sieve granules, which are composed of zeolite sodium aluminosilicate, according to the method of Seetharam and Saville (2002). Enzyme uptake on the zeolite support previously modified with 1 % glutaraldehyde was measured by comparing the soluble tyrosinase activity in solution before and after immobilization. The uptake was found to be 89 %, giving an activity of 16U/g support. Based on these results showing high uptake of tyrosinase by the support, no further optimization of the immobilization was warranted, and the immobilized biocatalyst was used in the production of hydroxytyrosol from tyrosol.

The biocatalyst was used in repeated reactions, using fresh substrate in each cycle. In a first reaction cycle, hydroxytyrosol was produced in a potassium phosphate reaction medium that contained 5.8 mM tyrosol, 21 mg ascorbic acid and 1.5 g of immobilized biocatalyst (16 U/g support). As monitored by HPLC, 69 % conversion of tyrosol was achieved after 77 h, and the hydroxytyrosol concentration accumulated was 1.1 mM (19 % yield, calculated based on moles) as presented in Table 2.3. These results indicate that the hydroxytyrosol yield using immobilised tyrosinase was less than that obtained with the non-immobilised tyrosinase. The lower yield could be attributed to the accessibility of substrate to the immobilised biocatalyst, in that limitations of mass transfer or substrate diffusion could have resulted in less tyrosol being converted into hydroxytyrosol. Furthermore, it was also noted that the yield of hydroxytyrosol was lower relative to tyrosol conversion, and this could be attributed to following:

- it is likely that some hydroxytyrosol were further oxidised to quinones or
- the hydroxytyrosol produced interacted with the zeolite (and was fixed on the zeolite material) and therefore could not be quantified.

However, these assertions would need to be investigated further in the future studies.

To test the reusability and stability of the biocatalyst, the same immobilized enzyme used in the first reaction, was rinsed with 0.01 M potassium phosphate buffer and then used in the second reaction (second cycle) under the same conditions. It was noted that hydroxytyrosol produced (18

% yield) was comparable to that of the first cycle (Table 2.2), indicating that the immobilised biocatalyst was still active after 154 h of operation, albeit at a lower level of activity.

In a further experiment, tyrosinase immobilized on zeolite was mixed with phosphate buffer and incubated for 24 h. The immobilised biocatalyst was then removed by filtration and the activity of tyrosinase in the buffer was then evaluated. There was no significant activity of tyrosinase detected in potassium phosphate buffer. It was concluded that the leaching of tyrosinase from the support was negligible and therefore did not affect the total hydroxytyrosol production, and hence it was concluded that zeolite is a suitable material for immobilization of tyrosinase for production of hydroxytyrosol. The retained cresolase activity of the tyrosinase in the biocatalyst was 89 %, a result that is comparable to that of Seetharam and Saville (2002).



**Table 2.2 Yields of hydroxytyrosol produced in a batch reactor system containing immobilized tyrosinase.**

| Cycles | Duration (h) | Reaction volume (ml) | Hydroxytyrosol concentration (mM) | Hydroxytyrosol yield (%) |
|---|---|---|---|---|
| **1st Cycle: 5.8 mM tyrosol, 21 mg ascorbic acid and 1.5g zeolite tyrosinase (16 U/g zeolite)** | 77 | 5 | 1.1 | 19 |
| **2nd Cycle: 5.8 mM tyrosol, 21 mg ascorbic acid and the same 1.5g zeolite tyrosinase (16 U/g zeolite)** | 77 | 5 | 1.0 | 18 |

### 2.3.10 Hydroxytyrosol production in a packed-bed reactor system using immobilized tyrosinase

The packed-bed reactor system described in Section 2.2.9.3 was used for the continuous production of hydroxytyrosol using tyrosinase as a biocatalyst. The biocatalyst was immobilized on zeolite (as described in Section 2.2.10.1) and then packed in a packed-bed reactor. The reaction medium, containing ascorbic acid and tyrosol, dissolved in a potassium phosphate buffer (see Section 2.2.9.3), was pumped from the separate reservoir to the packed-bed reactor. The hydroxytyrosol concentration in samples collected at interval of 45 min was determined using HPLC. The production yield of hydroxytyrosol over time in the packed-bed bioreactor was determined using HPLC (Figure 2.17). The hydroxytyrosol concentration in the 3 ml samples collected after 45 min was 0.4 mM and at 180 min was 0.5 mM. The concentration started to drop after this time, indicating that the biocatalyst was losing the activity. The total amount of hydroxytyrosol produced in the packed-bed reactor was calculated using the area under the curve in Figure 2.9 (MathCAD 2001, Appendix D), and was found to be 227 moles per 15 g biocatalyst (16 U/g zeolite) after 38 % conversion of tyrosol. This conversion is lower than that obtained using non-immobilized tyrosinase or the biocatalyst in batch reactions. The lower conversions of hydroxytyrosol in a continuous packed-bed reactor system are suggested to be attributable to the mass transfer and diffusion limitations.

In order to improve the conversions or performance of batch and/or packed-bed reactors, various parameters would need to be optimized. These parameters include increasing the specific activity of enzyme attached on the support. In our study, it is apparent that although the uptake (89 %) of the enzyme was very high, the activity of the biocatalyst was low. In the case of the continuous bioreactor, flow rates control the residence time of the substrate in bioreactor, and this would need to be evaluated; a higher conversion rate of tyrosol to hydroxytyrosol might be achieved by decreasing the flow rate and thus increasing the residence time of the substrate (tyrosol) in the reactor. However, this may then be complicated by the product inhibition known to be

characteristic of tyrosinase (Wood *et al.*, 2004). Thus, optimisation of the production of hydroxytyrosol in the bioreactor was acknowledged to be beyond the scope of this project.

The hydroxytyrosol obtained in this study was used as the substrate for laccase, with the aim of comparing laccase/tyrosol reactions with laccase/hydroxytrosol reactions. Further, the next section describing work aimed at incorporating the laccase as the second enzyme in a multi-enzyme biotransformation system.

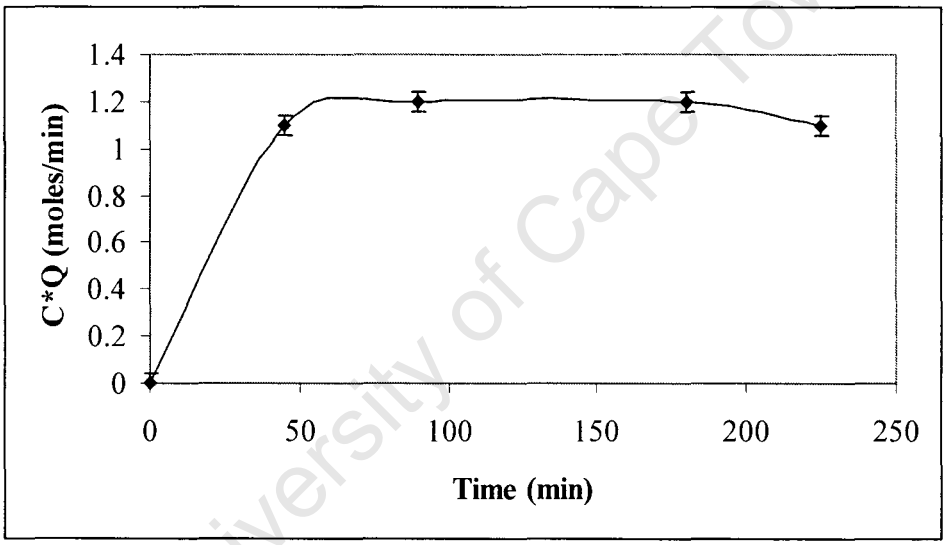





**Figure 2.17 Yields of hydroxytyrosol produced in a packed-bed reactor (C: concentration, Q: flow rate, C*Q = Yield). The area under the curve was used to estimate the total hydroxytyrosol produced over 240 min).**

### 2.3.11 Oxidation of hydroxytyrosol by laccase

In work described in a previous section of this study, tyrosol was oxidatively coupled by laccase in a sodium acetate buffer medium containing methanol, acetone or ethyl acetate at 20 to 80 % concentration. For the purpose of comparison, the same reaction conditions were used for hydroxytyrosol-laccase reactions. Hydroxytyrosol, synthesized using tyrosinase as a biocatalyst, and then purified (by preparative TLC plate, initially) was oxidized by laccase obtained from *T. pubescens,* in sodium acetate buffer medium containing 20 % methanol (Section 2.2.10.1). The reaction was monitored by HPLC as described in Section 2.2.5. After 6 h, 80 % conversion of hydroxytyrosol (represented by the peak with retention time 4.8 min) was achieved, and 6 product peaks were observed (Figure 2.18). The identification of the new product peaks was achieved using LC-MS. These peaks represented various products of the hydoxytyrosol-laccase reaction. A product with retention time 2.45 min was a polymeric product **55** (Figure 2.19). Product **55** (constituting 27 % of the total peak area) comprised 8 monomers (1202.5 m/z) of hydroxytyrosol, presumably linked either by C-C bonds or C-O bonds. Product **57** (39 %) with retention 3.8 min contained 2 monomers (306.08 m/z) of hydroxytyrosol, thus forming a dimeric product (Figure 2.20). Product **59** (1 %) with retention 7.6 min (Figure 2.21) and product **60** (20 (Figure 2.22) were also presumed to be oligomeric products of hydroxytyrosol. However, it is acknowledged that these products would need to be fully characterized for their structures to be proposed.

These results show that tyrosol-laccase and hydroxytyrosol-laccase reaction have similar characteristics, since both reactions resulted in a mixture of products with higher molecular weight, in a reaction mixture containing 20 % methanol. These results are consistent with many studies reported from the literature stating that phenolics are 'natural' substrates for phenol oxidases such as laccase, and that they are readily polymerized when exposed to these oxidase enzymes (Uyama and Kobayashi, 2002 and references therein). The effect of organic solvents on the nature of the hydroxytyrosol product was then evaluated as described in Section 2.3.11.1

below, and the structures of these products was considered further as described in Section 2.3.11.2.

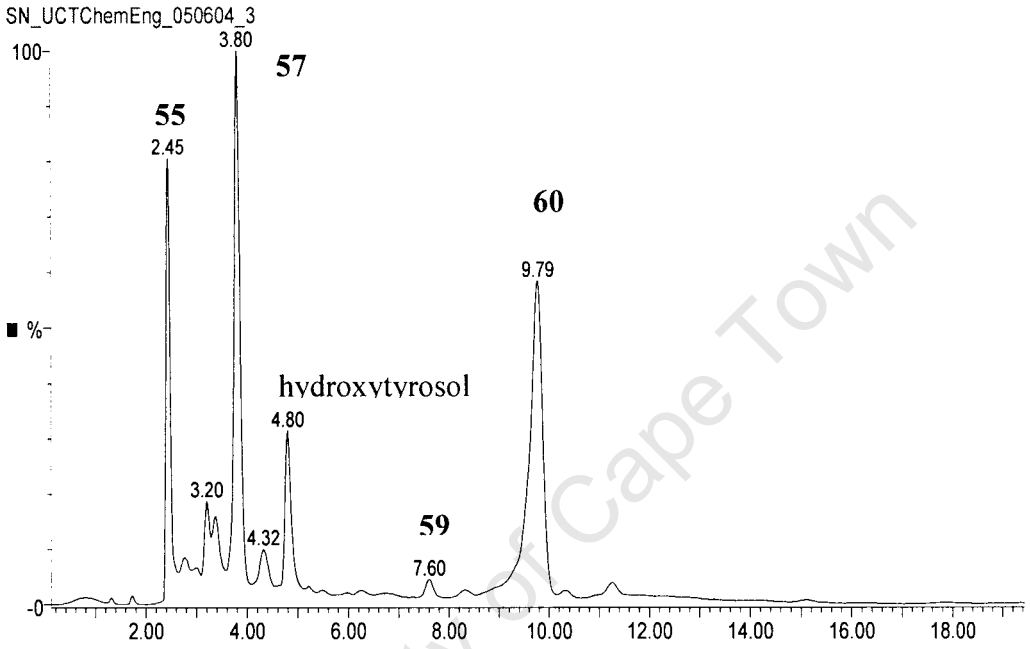





**Figure 2.18 HPLC profile showing bioconversion of hydroxytrosol by laccase obtained *T. pubescens* in 20 % methanol. The products identified by LC-MS are product 55 (polymeric product) with retention time 2.45 min; product 57 (dimeric product) with retention time 3.80 min and products 59 (7.60 min) and 60 (9.79 min) (unknown products). Hydroxytyrosol, 25, is represented by the peak with retention time 4.80 min.**

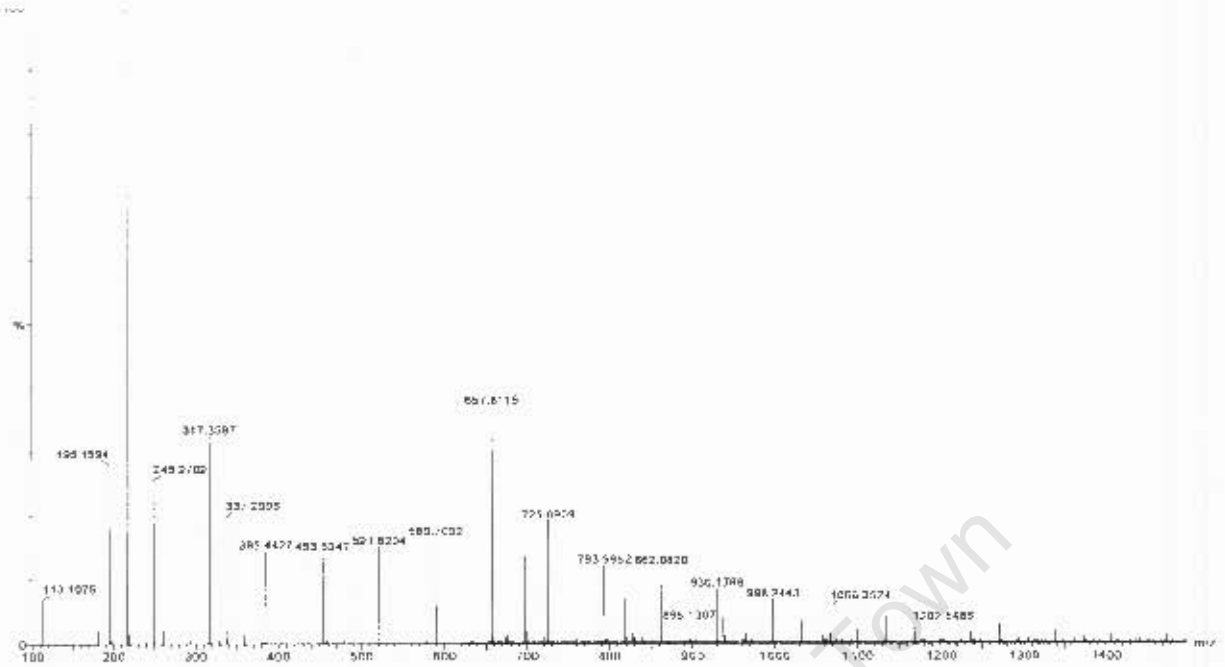


**Figure 2.19 LC-MS profile of product 55, indicating the presence of a polymer obtained from bioconversion of hydroxytyrosol by laccase in reaction medium containing 20 % methanol.**

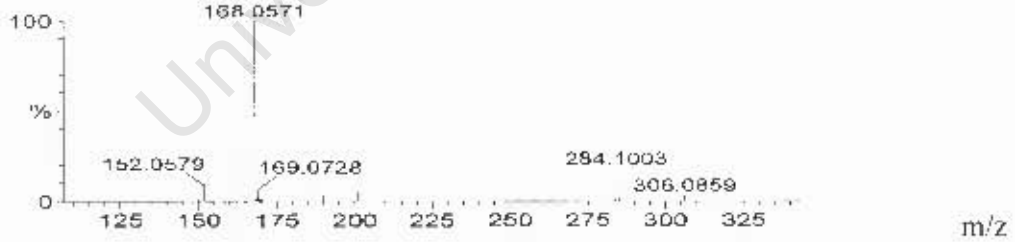



**Figure 2.20 LC-MS profile of product 57, indicating the presence of a dimeric product obtained from bioconversion of hydroxytyrosol by laccase in reaction medium containing 20 % methanol.**

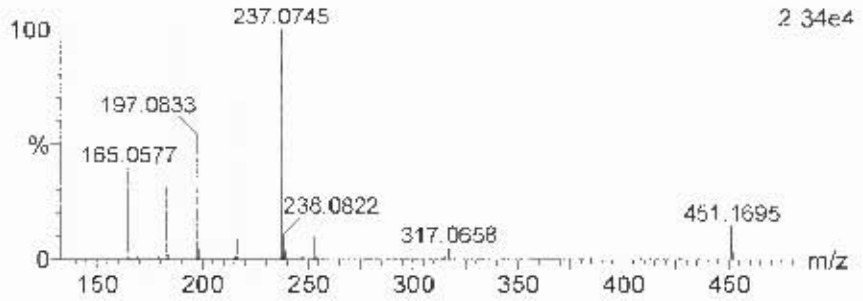



**Figure 2.21 LC-MS profile of product 59 with retention time 7.60 min, indicating the presence of the oligomeric compounds obtained from bioconversion of hydroxytyrosol by laccase in reaction medium containing 20 % methanol.**

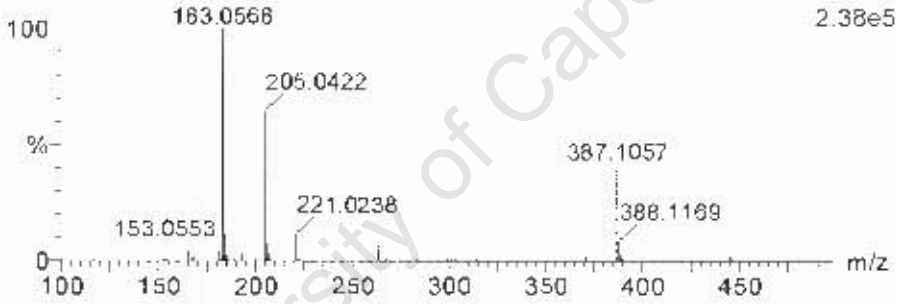



**Figure 2.22 LC-MS profile of product 60, with retention time 9.79 min, indicating the presence of oligomeric compounds obtained from bioconversion of hydroxytyrosol by laccase in reaction medium containing 20 % methanol.**



### 2.3.11.1 The effects of organic solvents on the oxidation of hydroxytyrosol by laccase

The influence of organic solvents on the selectivity of the hydroxytyrosol-laccase reactions was investigated, to compare with the results previously obtained using tyrosol (Section 2.3.2). Thus, hydroxytyrosol was oxidized by laccase in sodium acetate reaction medium containing 50 % acetone, 50 % methanol or 50 % ethyl acetate (biphasic system). For the purpose of comparison,

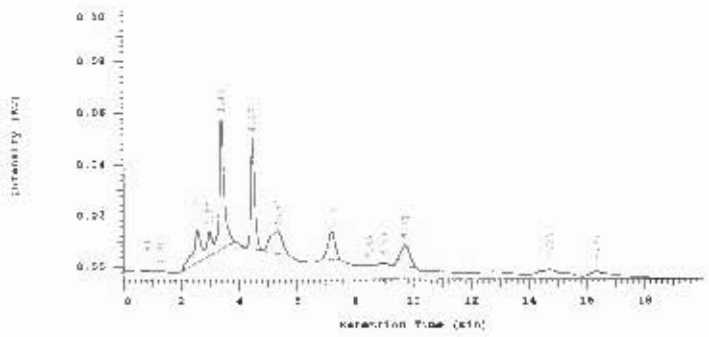
these reactions conditions were similar to those of tyrosol-laccase reactions (Section 2.2.11). In the analysis of the hydroxytyrosol-laccase reaction mixtures by HPLC, it was noted that the nature of products differed, and this was dependent on the type of type of organic solvents used (Figure 2.23). For instance, (shown in Table 2.3), a dimer of hydroxytyrosol, product **57**, was the main product (52 % yield) in a reaction mixture containing 50 % acetone whereas from the same reaction mixture yields of the other products (**55**, **59** and **60**) were very low (3 % yield). For reaction medium containing 50 % methanol, the main product obtained was product **59**, and for the reaction mixture containing ethyl acetate, product **60** was the main product.

**Table 2.3 Conversions of hydroxytyrosol by laccase detected by HPLC in the presence of each cosolvent after 6 h.**

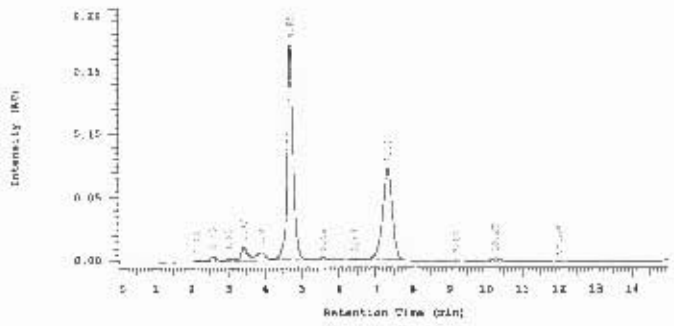
| Organic solvent* | Hydroxytyrosol conversion based on the HPLC peak area comparison (%) | Hydroxytyrosol not reacted (%) | Product 55 (%)* | Product 57 (%) | Product 59 (%) | Product 60 (%) | Product 61 (%) |
|---|---|---|---|---|---|---|---|
| Acetone 50 % | 60 | 40 | 3 | 52 | 3 | 3 | - |
| Methanol 50 % | 96 | 4 | 3 | 18 | 69 | 2 | - |
| Ethyl acetate 50 % | 95 | 5 | 3 | 0.5 | 1 | 50 | 40 |

* used as cosolvent with sodium acetate buffer (0.1 M, pH 5)

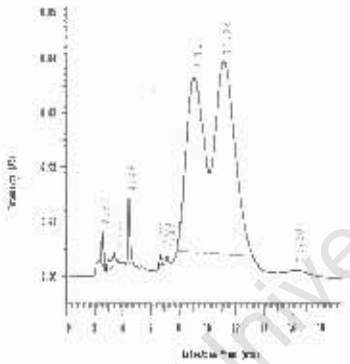
** determined on the basis of HPLC peak areas.



A



B



C



**Figure 2.23 HPLC profile showing bioconversion of hydroxytrosol by laccase, A: reaction medium containing 50 % acetone, the main product is a dimer, retention time 3.40 min; B: reaction medium containing 50 % methanol, the main product is product 59, retention time 7.33 min and C: the reaction medium containing 50 % ethyl acetate, the main product is product 60 represented by a peak with retention time 9.12 min. A peak with retention time 11.24 min is also an unknown product.**

It was notable that the intensity of the peak representing the polymeric product **55,** in reaction mixtures containing acetone, ethyl acetate or 50 % methanol, was decreased significantly as compared to the same peak in the reaction containing 20 % methanol. Polymeric product formation decreased with an increase in the amount of organic solvent in the reaction medium. This was attributed to the fact that organic solvents decrease the hydrophobic interaction of molecules in solution (see Chapter 3). Thus, the results obtained clearly show that organic solvents have an influence on the nature of hydroxytyrosol-laccase reactions products. Further, from these results, it is clear that hydroxytyrosol-laccase reactions differ with those of tyrosol-laccase reactions. The summary of tyrosol-laccase and hydroxtyrosol-laccase reaction products is provided in Table 2.4.

**Table 2.4 Summary of the products of tyrosol-laccase and hydroxytyrosol-laccase reactions.**

| Laccase substrate | Organic solvent* | Product |
|---|---|---|
| tyrosol | 50 % acetone | Major product was a polymer with molecular weight of 962 m/z |
| hydroxytyrosol | 50 % acetone | Major product was a dimer with molecular weight of 306 m/z |
| tyrosol | 50 % or 80 % ethyl acetate | Major product was a polymer with molecular weight of 1940 m/z |
| hydroxytyrosol | 50 % ethyl acetate | Major product was an unknown product **60** |
| tyrosol | 50 % methanol | Major product was a polymer with molecular weight of 665 m/z |
| hydroxytyrosol | 50 % methanol | Major product was an unknown product **59** |

* used as cosolvent with sodium acetate buffer (0.1 M, pH 5)

As already described above, hydroxytyrosol reactions were more readily controlled using organic solvents, contrary to the tyrosol-laccase reactions which yielded largely polymeric compounds

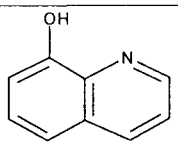
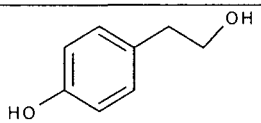
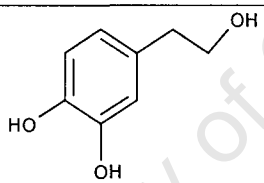
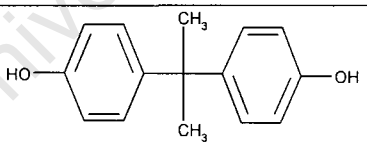
(Section 2.3.2). The difference in terms of ability to control polymerisation in the hydroxytyrosol-laccase and tyrosol-laccase reactions could be attributed to the structures of these compounds. The difference between tyrosol and hydroxytyrosol is increased polarity/hydrophilic nature as well as increased steric size in hydroxytyrosol. Although increased polarity of a compound could result in more reactivity, this effect could be nullified by increased steric hindrance and decreased hydrophobic interactions (effected by organic solvents). For instance, hydroxylation of tyrosol near the *para*-hydroxyl group might have resulted in some steric hindrance, in that two hydroxytyrosol radicals couple to form a dimeric structure (Figure 2.23d) but due to spatial limitation in this dimer it could be sterically impossible to attach more radicals. Thus, the steric effects in conjuction with the effects of organic solvents (i.e decreasing the hydrophobic interactions of molecules) in the reaction medium might have resulted in the production of low molecular weight compounds. Besides steric and hydrophobic interaction effects, the ability to control polymerization of hydroxytyrosol could also be attributed to that fact that:

- the hydroxyl group could alter the delocalization of the unpaired electrons in the radical intermediate and hence, possibly, stabilize the radical and lead to lower molecular weight products or
- the radical intermediates of tyrosol and hydroxytyrosol could also interact differently with the organic solvents therefore resulting into different products.

It is clear that the presence of the additional hydroxyl group in hydroxytyrosol has a profound effect (i.e. reactions of hydroxytyrosol with laccase were more controllable as compared to tyrosol-laccase reactions). However, the mechanism whereby it has this effect is not entirely clear, and it would require further study of the reaction mechanism to elucidate this. In contrast, the high reactivity of tyrosol as attributed to its structural configuration is consistent with many laccase polymerization reactions reported in literature, for instance, polymerization of bisphenol A by laccase reported by Uchida *et al.* (2001). Bisphenol A compound (Table 2.5) has no substitution adjacent to its reactive hydroxyl group, and therefore it is very reactive and polymerizes rapidly (Uchida *et al.*, 2001). Similarly, 8-hydroxyquinoline, also containing unhindered hydroxyl group at the *para* position (see Chapter 3) polymerises rapidly in both aqueous reaction medium and organic solvent-containing reaction medium (Ncanana and Burton, 2006). Thus, the inference that could be made from this study is that structural configuration of

laccase substrates has an influence on the reactivity of the radicals generated. Furthermore, the interactions of these radicals in the reaction medium can be influenced by nature of organic solvents used. This section of this study has therefore demonstrated that the reaction medium can potentially be 'engineered' or manipulated, for the purpose of selecting the desired product. The significance of this result is that the desired product can be selectively produced and its purification process could then readily be optimized.

**Table 2.5 Showing laccase substrates structure-product relationship**

| Laccase substrate | Structure | Molecular weight (m/z) | Product | Reference |
|---|---|---|---|---|
| 8-hydroxyquionoline |  | 145 | A polymer comprised of 15 monomers was obtained as major product. | Ncanana and Burton (2006) and the current study (Chapter 3) |
| tyrosol |  | 138 | A polymer comprised of 14 monomers was obtained as major product | Current study |
| hydroxytyrosol |  | 154 | Polymer (comprised of 8 monomers) formation controllable to favour dimer formation | Current study |
| Bisphenol A |  | 251 | Oligomers comprised of 3 and 4 monomers as major products | Uchida *et al.* (2001) |

### 2.3.12 Isolation and characterisation by NMR of the hydroxytyrosol-laccase reactions products

Hydroxytyrosol was treated with laccase in a reaction medium containing 50 % methanol (Section 2.2.10.2). The reaction was monitored by HPLC and TLC until 100 % conversion of hydroxytyrosol had been achieved. Upon TLC analysis, two products, with $R_f$ values 0.30 and 0.20 were obtained. The product with $R_f$ 0.3, which gave a prominent band on the TLC plate, was purified and re-analyzed using HPLC. This product (2.5 mg obtained from a starting material of 10 mg) corresponded to the HPLC peak with retention time 7.2 min, previously identified as product (**59**) (Section 2.3.11.1) using HPLC. An attempt was made to elucidate by NMR the structure of this product, but the results were inconclusive. This was attributed to the fact that there was no sufficient material available for analysis.

In a separate reaction, hydroxytyrosol was treated with laccase in a reaction mixture that contained 50 % acetone and the reaction monitored by HPLC and TLC as above. Three products with $R_f$ values 0.2 (the main product) and 0.3 (minor product **59**) were identified by TLC. The third product on the base of the TLC plate was presumably a polymeric product **55**. The main product ($R_f$ value of 0.2, 10 mg obtained from a starting material of 40 mg) of this reaction was analysed by $^1$H-NMR which showed a spectrum with two single peaks at 6.8 ppm and 6.9 ppm of the aromatic region, different from that of hydroxytyrosol which showed 2 doublets and a singlet at the aromatic region (Appendix C). However, the peaks representing protons of the chain $CH_2CH_2OH$ were present in the spectra of both the product and hydroxytyrosol, and thus the two spectra were interimposable. It was suggested that the product was a symmetric dimeric structure (**57**) linked by C-C bond (as shown in Figure 2.23d), and this was confirmed by LC-MS which showed molecular ion peak of 306 m/z. The formation of dimeric product is attributed to coupling of the radical intermediates produced by the laccase-mediated oxidation of phenolic substrates (Burton, 2003; Riva, 2006). The products obtained were evaluated for antioxidant activity.

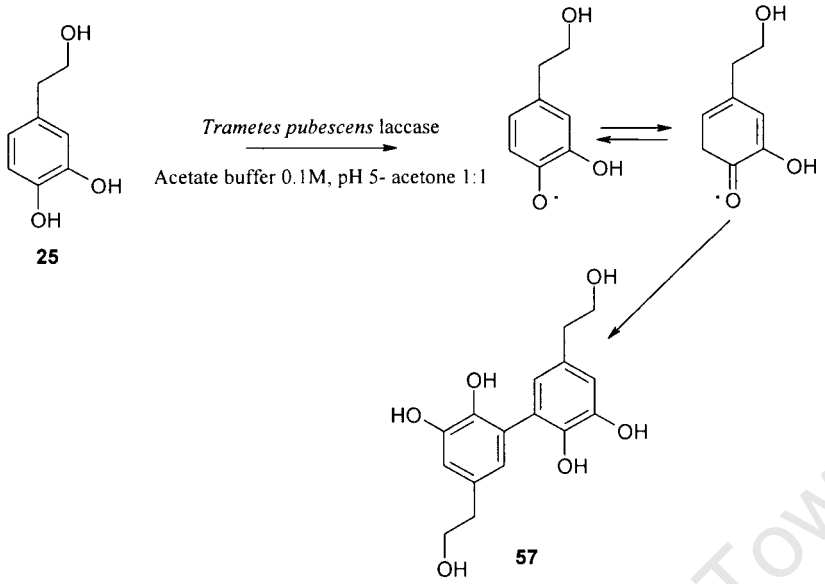



**Figure 2.23d Structure of dimeric product 57 obtained from hydroxytyrosol-laccase reactions.**



### 2.3.13 DPPH radical quenching assay to measure the antioxidant activity of hydroxytyrosol laccase products

Hydroxytyrosol (1mg/ml) was treated with laccase in a reaction medium containing 20 % methanol until the 100 % conversion had been achieved as dectected by HPLC. The control reaction was conducted with no laccase present (see Section 2.2.12.1). The mixture of products included polymer (**55**), dimer (**57**), (**59**) and (**60**), in a ratio of (53:35:1:7). Since separation of these products had been found to be problematic, this mixture of products was used in a DPPH assay and compared with hydroxytyrosol which had not been treated with laccase. The untreated hydroxytyrosol solution quenched 94 % of the DPPH radical within 1 min while the reaction product mixture had quenched 47 % within 100 min (Figure 2.24). The reaction of DPPH with hydroxytyrosol-laccase products was continued until 100 % DPPH radical had been quenched, after 4 h. From these results, it is clear that the reaction of hydroxytyrosol (untreated with laccase) with DPPH radical was faster as compared with hydroxytyrosol-laccase product mixture.

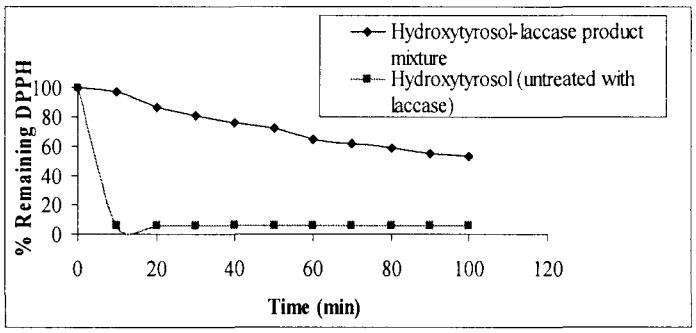



**Figure 2.24 DPPH radical quenching assay of hydroxytyosol-laccase reaction products.**



The stoichiometric ratio (i.e the amount of the antioxidant needed to scavenge a particular dose of DPPH radical) was not determined in this case, since the reaction products were not separated. The stochiometric ratio would have provided the information related to efficiency of new products as compared to hydroxytyrosol. However, from these results it is apparent that a reaction involving DPPH and hydroxytyrosol-laccase products is slower than that of pure hydroxytyrosol. The slower reaction mixture does not necessary means the antioxidant is inefficient. For instance, Bondet *et al.* (1997) reported that 1 mol of BHT reduces 3 mol of radical in a reaction that takes 5 h to reach steady state. In comparison, 1 mole of isougenol reduces one of DPPH radical with the steady state being reached within 0.5 min (Bondet *et al.*, 1997). Thus, BHT was a better antioxidant because a smaller amount of this compound quenched relatively higher quantities of DPPH radicals as compared to isoeugenol. Bondet *et al.* (1997) asserted that such an inverse relation between reaction stochiometry and rate indicates a slower reaction rate which could be due to a more complex reaction mechanism which may involve more than one secondary reaction. The first step to occur in such mechanisms is suggested to be hydrogen donation by the antioxidant to the DPPH radical, and this step is subsequently followed by donation of the second hydrogen atom. In the last step, the antioxidant reacts with the DPPH radical forming a bi-quinoid structure. These reactions may result in coupling of two bi-quinoid structures which form a complex structure with the DPPH, thus resulting in scavenging of many molecules of DPPH, but in a slow process.

In the context of the current study, it is likely that complex reaction mechanisms could have occurred in the presence of the mixture of products (polymeric, and dimeric products). It is suggested that hydroxytyrosol dimers and oligomers (products **57** and **59** respectively) donate hydrogens to DPPH radicals, and this reaction is subsequently followed by dimerisation of these products forming larger molecules, thus leading to a complex slow reaction with DPPH.

To investigate this further, the dimeric product **57** and the product **59,** produced in separate reaction and purified on a TLC plate, were evaluated for antioxidant activity (Figure 2.25.). It is apparent the DPPH reactions of dimeric product **57** and product **59** were similar and faster, as compared to the product mixture (containing polymeric **55,** dimeric **57** and **59**) reported above, with the steady state being reached within 3-4 minutes. However, the radical scavenging ability of these dimeric and product **59** was lower (only 23-30 % DPPH radicals scavenged at the steady state) as compared to both product mixture and hydroxytyrosol. This was an indication that the mixture of polyphenols has higher antioxidant activity as compared to the individual component. The higher activity could be attributed to product **55**, **57** or **59** individually. However, the occurance of synergism is also possible. These results could be in consistent with literature reports which suggest synergy in antioxidant reactions. For instance, a mixture of carotenoids and alpha-tocopherol yielded a product with a higher antioxidant activity when compared with individual components (Castro *et al.*, 2005). It has been shown that various compounds demonstrate synergism in their antioxidant capacity, although the mechanism is not completely understood (Castro *et al.*, 2005 and references therein). Thus, this synergism shown by the mixture of products could be more useful, especially when it is difficult to isolate the individual components, and it could also be considered to be economically useful in pharmaceutical industry, as the separation step in downstream processing could be eliminated.

The lower reactivity of dimeric product **57** as compared to hydroxytyrosol, could be due to the possible disruption or steric hindrance in the catechol moiety of the hydroxytyrosol by oligomerisation. For instance, the attachment of another hydroxytyrosol molecule at the carbon atom of the aromatic ring near catechol moiety to form a C-C or C-O dimer **57** (Figure 2.23d), and subsequently a trimer, would result in a sterically bulky product **59a or 59b** (Figure 2.26), therefore making these products less reactive. Thus, in spite of the lack of success in determining

the complete structure of the polymeric products, this study provides further evidence for the already recognised importance of the catechol moiety in antioxidant compounds (Zhang and Wang, 2004). Furthermore, this study proves that the number of hydroxyl groups in a compound may not necessarily lead to an improved antioxidant activity of that particular compound. For antioxidant activity to be increased, correct spatial arrangement or position of the hydroxyl groups could also be a significant requirement.

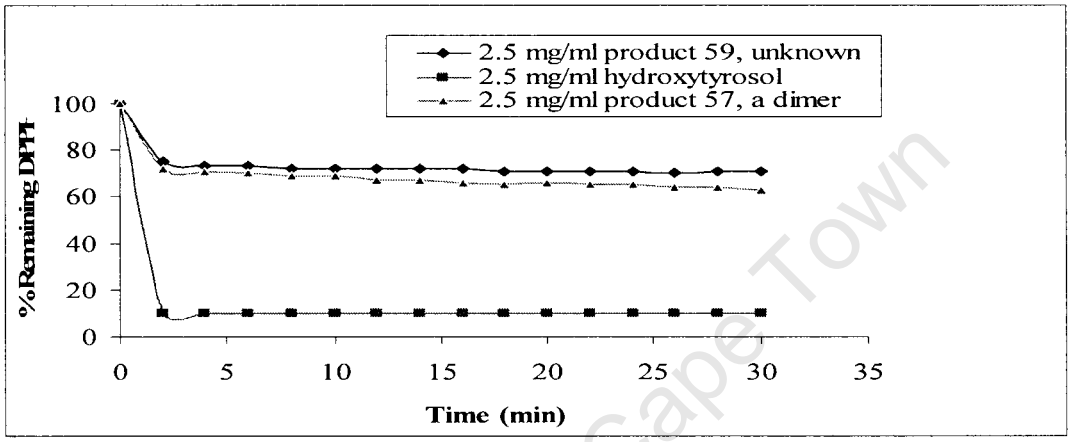
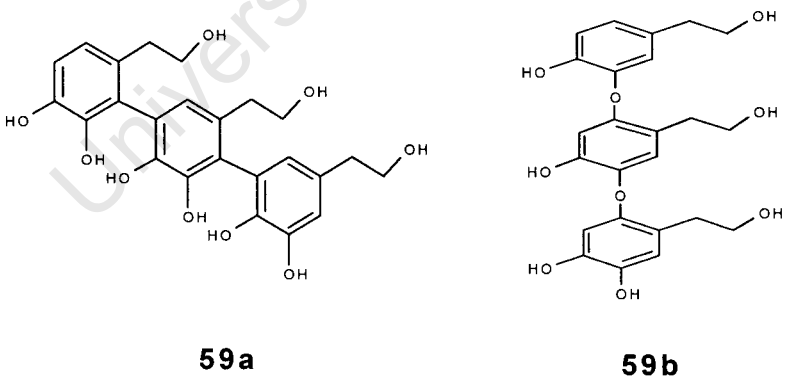



**Figure 2.25 DPPH radical quenching assay of hydroxytyosol-laccase reaction products**




**59a** **59b**

**Figure 2.26 The possible structures of hydroxytyosol-laccase products**

### 2.3.14 Other implications of this study: multi-enzyme synthesis

In this study, tyrosinase was used to hydroxylate tyrosol for the production of hydroxytyrosol. The hydroxytyrosol produced was in turn purified and then treated with laccase for production of hydroxytyrosol dimers **57** or product **59**. Thus, new antioxidant products were synthesized using two different enzymes in separate reactions, representing a multi-enzyme biotransformation system. To our knowledge, there are no studies reported in literature dealing with multi-enzyme synthesis involving phenol oxidases (i.e tyrosinase and laccase) in the production of a biologically active compound. Thus, this study is the first to report the sequential biocatalytic oxidations steps leading to production of new compounds with antioxidant activities different from the parent compound. The significance of this study is that the synthetic route was developed by identification of the suitable enzymes for each step, optimization of each biocatalytic step and incorporation of the steps as unit operations, to build a multi-enzyme biotransformation process for synthesis of the target organic product.



## 2.4 Conclusion

Biotransformation of tyrosol and hydroxytyrosol was achieved using tyrosinase from *A. bisporus* and laccase from *T. pubescens* respectively. The objective was to compare the laccase reactions involving tyrosol with those involving hydroxytyrosol. Hydroxytyrosol, in particular, is known for its high antioxidants activity and hence there is great interest in studying this compound and the products formed by its reactions with laccase.

Firstly, tyrosol was successfully modified using tyrosinase from *A. bisporus*. The structure of the product, hydroxytyrosol, was confirmed using LC-MS and $^{1}$H-NMR and the results were similar to those of Espín *et al.* (2001). The reaction of tyrosol and tyrosinase was optimised in terms of temperature, pH and sodium dodecyl sulphate (SDS), the highest conversion was achieved when the reaction was performed under the conditions: pH 7, 30 - 40 °C and the reaction medium supplemented with 2 mM SDS.

Tyrosinase was successfully immobilized on glutaraldehyde-modified zeolite. The retained cresolase activity of the tyrosinase in the biocatalyst was 89 %, showing that covalent cross-linking of tyrosinase on the support was an efficient method for immobilization. The immobilized biocatalyst was found to be stable and could be re-used without a significant loss of its activity. The tyrosinase-zeolite biocatalyst was successfully used to produce hydroxytyrosol, and to our knowledge no work has been reported related to this. Although the production yield of hydroxytyrosol using the tyrosinase-zeolite biocatalyst was significantly less that from free tyrosinase, the results of this preliminary study provide a route for the synthesis of hydroxytyrosol in a reactor system using an immobilized biocatalyst.

Production of hydroxytyrosol was further developed by using immobilised tyrosinase in batch and continuous packed-bed reactors. The best results were obtained using a continuous packed-bed reactor with reaction mixture containing tyrosol and ascorbic acid.

In a separate reaction, hydroxytyrosol was oxidised by laccase in reaction mixtures containing acetone or methanol or ethyl acetate. The nature (whether the product is dimeric, trimeric or polymeric) of laccase products was strongly influenced by the nature of the reaction medium. The use of various organic solvents resulted in formation of various products, with 50 % acetone favouring the synthesis of dimeric product **57**, and 50 % methanol favouring the production of a product **59**. Lower concentration of organic solvents (20 % methanol) in the reaction medium favoured the increased production of polymeric products **55**. Thus oxidation of hydroxytyrosol by laccase could be controlled using various organic solvents to yield the desired product. As a comparative study, tyrosol was also oxidised by laccase in a similar reaction conditions. The products of tyrosol-laccase reactions were largely polymeric and the selectivity of the nature products was not achieved using organic solvents. Thus these results showed that hydroxytyrosol-laccase reactions were readily controllable than tyrosol-laccase reactions. The difference between tyrosol-laccase reactions and hydroxytyrosol-laccase reactions was attributable to the structural configuration of these compounds. The hydroxyl group adjacent to the *para* positioned hydroxyl group in hydroxytyrosol might have affected the binding of the

radical intermediates (steric effects), and hence hydroxytyrosol radicals were less reactive as compared with tyrosol radicals.

The products obtained from hydroxytyrosol-laccase reactions and tyrosol-laccase reactions were evaluated for antioxidant activity using DPPH assay. The hydroxytyrosol-laccase product mixture reacted slowly with DPPH radical as compared to the reaction of pure hydroxytyrosol. However in both samples, 100 % reduction of DPPH radicals was achieved. It was deduced that reaction mechanism of the product mixture obtained from hydroxytyrosol laccase reaction with DPPH radicals was complex, and hence slow. Dimeric hydroxytyrosol product **57** and hydroxytyrosol product **59** were isolated, purified and assayed for antioxidant activity. In comparison with the mixture of products, pure compounds **57** and **59** had a weaker antioxidant activity, and the higher antioxidant activity in product mixture as compared to individual components was attributed to synergistic effects.

The products of tyrosol-laccase reactions (dimer **50**) and a polymer **49** had higher antioxidant activity as compared to tyrosol, and this was attributable to the structural configuration of these products. The conclusion that can be made from this study is that laccase reactions yield new structures showing different antioxidant activity when compared to their parent compounds, and the increase or decrease in activity is influenced by the nature of the product structures. For instance, it was suggested that the products structures with increased delocalisation of electrons are characterised by stable antioxidant radical (formed after hydrogen abstraction) therefore favouring the forward reaction (i.e scavenging of more DPPH radicals).

The influence of organic solvents in laccase reactions was further explored in the work described in Chapter 3, with the emphasis on the effect of organic solvents in the degree of polymerisation of 8-hydroxyquinoline, and the size of polymer particles formed

# Chapter 3

## Oxidation of 8-hydroxyquinoline by laccase from *Trametes pubescens*

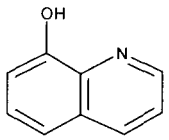
### 3.1. Introduction

In work described in this Chapter, the effect of organic solvents on the nature of laccase reaction products was further evaluated. The specific objective of this work was to evaluate the effect of organic solvents on the degree of polymerisation of 8-hydroxyquinoline, and polymer particle size. Polymerisation of 8-hydroxyquinoline was achieved in a reaction mixture that contained acetone, ethyl acetate or methanol. The polymeric product obtained was analysed using matrix-assisted laser desorption/ionization coupled with time-of-flight mass (MALDI-TOF) spectrometry and Fourier transform infrared (FTIR) spectroscopy.

In recent years there has been increasing interest in the production of polymers of economic importance using biocatalysts; the application of enzymes in polymerisation processes can lead to the synthesis of unique polymeric compounds, with novel properties, that could not be easily achieved by conventional methods (Aktas and Tanyolac, 2003; Akkara, 1996).

Enzymes which have been used in biocatalytic polymerisation reactions include, among others, laccase, tyrosinase and horseradish peroxidase (Kobayashi *et al.*, 1995). Horseradish peroxidase, in particular, was used to synthesize poly (8-hydroxyquinoline-hydroquinone), a conjugated polymer that has been shown to have photovoltaic properties (Xie *et al.*, 1998). However, the disadvantage of using horseradish peroxidase is that the reaction requires hydrogen peroxide which can inactivate the biocatalyst (Aktas *et al.*, 2000) and this also adds cost constraints in the application of the process. Laccases provide an alternative method that does not require the hydrogen peroxide but, rather, uses oxygen. The laccases produced by *Trametes* strains are generally stable and some are readily available commercially, but these enzymes can also be produced simply by fermentation on a laboratory scale (Burton, 2003; Ryan *et al.*, 2005).

This study describes the polymerisation of 8-hydroxyquinoline, **47**, a low molecular weight aromatic compound bearing a reactive hydroxyl group. This compound was dissolved in various reaction media, and the reaction was initiated by addition of laccase from *T. pubescens*. The effect of organic solvents was also evaluated.




8-hydroxyquinoline **47**

8-Hydroxyquinoline is generally known as an antiseptic with mild fungistatic, bacteriostatic, antihelmintic, and amebicidal action. It is also used as an antioxidant reagent and metal chelator (Crutchley, 1995; Barber, 2000). The effect of 8-hydroxyquinoline on the metabolism of microorganisms was reported by Jones (1963) who showed that 8-hydroxyquinoline inhibits the formation of bacteriochlorophyll by *Rhodopseudomonas spheroids* and causes the formation of several unusual pigments (Jones, 1963). It has also been reported that 8-hydroxyquinoline has strong toxic properties and that it may cause substantial DNA-strand breakage and lipid peroxidation in mammalian cells (Leanderson and Tagesson, 1996). The accumulation of this organic compound and other phenols in water or soil would be toxic to organisms living in such environmental systems. Thus, the removal of 8-hydroxyquinoline and similar pollutant compounds from water is important and this could be achieved by polymerization.

This study reports for the first time the enzymatic synthesis of poly (8-hydroxyquinoline) catalysed by laccase from the white rot fungus *Trametes pubescens* (strain CBS 696.94). The synthesis of conjugated oligomers such as poly (8-hydroxyquinoline-hydroquinone), poly (8-hydroxyquinoline-1,5-hydroxynaphthalene) and poly (8-hydroxyquinoline-4-phenylphenol) using horseradish peroxidase has been reported by Liu and co-workers (Liu *et al.*, 1995), and poly (8-hydroxyquinoline-5,7-dimethylene) has been synthesized non-enzymatically (Ebraheem *et al.*, 1998). The application of the polymers comprising 8-hydroxyquinoline as a building block was investigated by Xie and the co-workers (Xie *et al.*, 1998) who reported that polymers such as

poly (8-hydroxyquinoline-4-phenylphenol), among others, exhibit photovoltaic properties. Poly (8-hydroxyquinoline-5,7-dimethylene) was also shown to possess chelation properties which suggests the potential application of these polymers as cytotoxic agents (Shen *et al.*, 1999). To further explore the properties of the 8-hydroxyquinoline-derived polymer produced in the present study, an investigation was conducted into the activity of product as an antioxidant, comparing it with the monomeric starting material. This work has recently been published (Ncanana and Burton, 2006).

## 3.2 Methods and Materials

### 3.2.1 Bioconversion of 8-hydroxyquinoline using laccase

A typical reaction mixture contained 0.25 g 8-hydroxyquinoline (Sigma) dissolved in 1 ml glacial acetic acid, 3 U laccase (partially purified using acetone as described above) in 5 ml sodium acetate buffer (0.1 M, pH 5.0), 2.5 ml acetone (final concentration 8 %) and 22.5 ml sodium acetate buffer (0.1 M pH 5.0). Reaction vials were covered with foil and incubated at 30 °C in the dark with shaking at 180 rpm. Control vials contained no enzyme. Polymerised product was recovered by filtration using No. 1 Whatman filter paper on a Buchner funnel. The polymerised product was washed with 3 x 10 ml portions of acetone and then air-dried. In other reactions, acetone strength was increased to 50 % which other reaction conditions remaining the same. The product was analysed as described in section 3.2.6.

### 3.2.2 Effect of organic solvents on the production of the polymeric product of 8-hydroxyquinoline

To evaluate the effect of solvents on the production of a polymeric product, 0.25 g 8-hydroxyquinoline dissolved in 1 ml glacial acetic acid, was added to a reaction mixture containing 3 U laccase in 5 ml sodium acetate buffer (0.1 M pH 5.0), 2.5 ml methanol (8 %) or no organic solvent and 22.5 ml sodium acetate buffer (0.1 M pH 5.0). Alternatively, the reaction mixture contained ethyl acetate in equal volume with sodium acetate buffer (biphasic aqueous-organic medium) which other reaction conditions remaining the same.

### 3.2.3 Effect of incubation time on the molecular weight of the polymeric product of 8-hydroxyquinoline

To study the effect of reaction time on the molecular weight of the polymerised product, the reaction, using acetone at 8 %, was repeated as described above and 3 ml samples were withdrawn from the reaction mixture after 24, 48 and 72 h. In each case, the polymerised product was recovered as described above and its molecular weight was determined by matrix-assisted laser desorption/ionization coupled with time-of-flight mass (MALDI-TOF) spectrometry. The activity of laccase in the filtrate of each sample was also determined as described in Chapter 2 Section 2.2.1.6.

### 3.2.4 Effect of temperature on the bioconversion of 8-hydroxyquinoline

In order to determine the effect of temperature on 8-hydroxyquinoline conversion by the laccase, 0.25 g 8-hydroxyquinoline, dissolved in 1 ml glacial acetic acid, was added to a reaction mixture containing 3 U laccase in 5 ml sodium acetate buffer (0.1 M pH 5.0), 2.5 ml acetone (8 %) and 22.5 ml sodium acetate buffer (0.1 M pH 5.0). Reactions were conducted at temperatures of 20, 30, 40, 50 and 65 °C, while other conditions remained the same. Samples were analysed using high performance liquid chromatography (HPLC) as described in section 3.2.6.1 after 24 h reaction time.



### 3.2.5 Effect of pH on the stability of laccase

To evaluate the effect of acidic pH on the stability of laccase, 10 U of laccase was dissolved in 30 ml of sodium acetate buffer (0.1 M) and the pH was adjusted to pH 3.5 or pH 5 with glacial acetic acid. The samples were incubated at 30 °C with agitation at 180 rpm. Activity of the laccase in solution was determined at times 0, 24, 48 and 72 h.

### 3.2.6 Analysis of the polymeric products of laccase reactions with 8-hydroxyquinoline

#### 3.2.6.1 HPLC analysis

The conversion of 8-hydroxyquinoline by laccase was monitored using HPLC (Merck La Chrom). The mobile phase was methanol-acetic acid-water (60:2:40) with a flow rate 1 ml/min, and using a C18 Waters (250 mm x 4.6 nm) reverse phase column and UV detection at 240 nm. Peaks were analysed using HPLC Manager, Merk Hitachi model D 700 data software. The percentage conversion was obtained by comparing the peak area of the substrate in the reaction sample with that of a control.

#### 3.2.6.2 Mass spectrophotometry (MS) analysis

##### 3.2.6.2.1 MALDI-TOF analysis

MALDI- TOF mass spectrometry was used to determine the molecular weight of compounds obtained from laccase 8-hydroxyquinoline reactions. MALDI–TOF mass spectra were obtained using a Perseptive Biosystems DE-PRO MALDI mass spectrometer equipped with a TOF analyser operated in positive ion mode. Sample aliquots (1 ml) in 50% chloroform were mixed with 1 ml of 2,5 dihydroxybenzoic acid matrix and applied to the gold sample plate. Analyses were performed as a service in the Department of Molecular and Cellular Biology, University of Cape Town.



##### 3.2.6.2.2 Liquid chromatography-mass spectrometry (LC-MS) analysis

Full Scan Liquid Chromatography-Electron Spray Mass Spectrophotometry (LC-ESMS) was performed in the negative mode between 0-1000 mv. The mobile phase was methanol-acetic acid-water (60:2:40) with a flow rate 1 ml/min, and using a C18 Waters (250 mm x 4.6 nm) reverse phase column and UV detection at 240 nm).

### 3.2.6.3 Infrared spectroscopic analysis

For infrared (IR) spectroscopic analysis, a 1 mg sample (oven dried for approximately 6 h) of a polymeric product obtained from laccase 8-hydroxyquinoline reaction was dissolved in dichloromethane and analysed for functional groups using a Perkin Elmer Spectrum One FT-IR spectrometer.

### 3.2.6.4 Analysis of polymeric product by Scanning Electron Microscopy (SEM)

The polymeric product of 8-hydroxyquinoline was produced in acetone (8 %) or methanol (8 %) or in aqueous medium as described previously. The air dried polymeric product was fixed with colloidal graphite in the sputter coater holder. The samples were then subjected to critical point drying process in a closed CPD020 chamber. The specimens were then removed from the holder and examined using Leica Stereo S440 scanning electron microscope which was operated according manufacturer's instructions.



### 3.2.7 Measurement of antioxidant activity of the polymeric products of 8-hydroxyquinoline using the DPPH free radical method

The method used to determine the antioxidant activity was a modified version of the methods of Siddhuraju and Becker (2003) and Sánchez-Moreno *et al.* (1998). A stock solution 400 mg/l of poly (8-hydroxyquinoline) was prepared in the mixture of 1,4-dioxane and methanol (3:7, v/v). An aliquot of the poly (8-hydroxyquinoline) solution (100 µl) of different concentrations (267, 200 and 100 mg/l) was added to 3.9 ml DPPH solution (6 x $10^{-5}$ M in methanol). The decrease in the absorbance at 515 nm was monitored using a Unicam UV-visible spectrophotometer, until the reaction reached the steady state. A control experiment was conducted using the same reaction mixture in the absence of poly (8-hydroxyquinoline) to confirm that the dioxane did not affect the DPPH assay. The same experiment was conducted using 8-hydroxyquinoline to determine the antioxidant activity of the monomeric starting material. The percentage of remaining DPPH was calculated as follows:

% DPPH remaining = [DPPH]t / [DPPH]t = 0 x 100
where [DPPH]t was the concentration of DPPH. at the time of steady state and
[DPPH]t = 0 was the concentration of DPPH at time zero.

The concentration of the antioxidant [poly (8-hydroxyquinoline)] required to decrease the initial DPPH concentration by 50 % (the $EC_{50}$) was determined graphically (Sánchez-Moreno *et al.*, 1998). The experiment was performed in duplicate.

## 3.3 Results and discussion

### 3.3.2 Bioconversion of 8-hydroxyquinoline by laccase

8-Hydroxyquinoline was treated with laccase in a medium of sodium acetate buffer containing 8 % acetone. From this reaction mixture containing 250 mg starting material, 119 mg of water-insoluble product **48** was obtained by filtration. In a separate experiment, 8-hydroxyquinoline was treated with laccase in buffer containing 50 % ethyl acetate or 8 % methanol. The summary of the product yields from these reactions is shown in Table 3.1 below. The highest mass yield was obtained with the reaction medium being sodium acetate buffer containing no organic solvents (153 mg per 30 ml volume of reaction was obtained, 80 % conversion of 8-hydroxyquinoline). This result is consistent with the suggestion by Faber (1994) that polymerisation is favoured in aqueous medium.

**Table 3.1 Treatment of 8-hydroxyquinoline (250 mg) with laccase (3 U) in a sodium acetate buffer medium containing various solvents resulted in production of the water-insoluble product 48.**

| **Medium** | **8-hydroxyquionoline converted (%) after 24 h** | **Mass of product (mg)** |
|---|---|---|
| 8 % acetone | 60 | 119 |
| 8 % methanol | 65 | 140 |
| 50 % ethyl acetate | 63 | 135 |
| aqueous medium | 80 | 153 |

The water-insoluble products were believed to be formed due to laccase oxidising 8-hydroxyquinoline to form aromatic radicals, which in turn combined to form the polymeric product. The polymeric product precipitated spontaneously from solution due to its low

solubility. It was not soluble in water, the sodium acetate buffer, acetone or ethyl acetate, and only poorly soluble in methanol, but completely soluble in 1, 4-dioxane. This was also an indication that a complex compound with higher molecular weight had been formed as a result of high degree of polymerisation of 8-hydroxyquinoline intermediate radicals.

### 3.3.2.1 Characterization of the polymeric product

The oxidation of 8-hydroxyquinoline by laccase resulted in the formation of water-insoluble products as described in Section 3.3.2 above. It is well-recognised that the mechanism of action of laccase involves radical-forming steps which can result in the formation of carbon-carbon or carbon-oxygen linked polymeric products (Chapter 1). Thus, further investigation was necessary to determine the nature of these products, and this was undertaken using MALDI-TOF and IR spectrometry. MALDI-TOF mass spectrometry is generally used to obtain information such as the average molar mass, average molecular weight, and molar mass distribution of a polymer. MALDI-TOF spectrum is characterized by peaks which orginate from ions of a polymer, and the individual ion peaks represent a particular compound or structure in a polymer sample. Furthermore, the peak-to-peak mass increment gives information on polymer composition (Pasch *et al.*, 1995; Montaudo *et al.*, 2006).

In this study, the oxidation of 8-hydroxyquinoline in acetone by laccase resulted into polymeric products as depicted by the MALDI-TOF spectrum (Figure 3.1). The compounds detected, varied from dimers (289 m/z) and trimers (431 m/z) to a polymer (2149 m/z) of 8-hydroxyquinoline, among others. The mass difference between the peaks representing respective oligomeric structures was 143 m/z which is the mass of a monomer (145) - 2H. This indicates that the loss of 2 hydrogens resulted in linking with another monomer. The peak distribution in a MALDI-TOF spectrum reflects the quantity of the various oligomers present in the sample. In the reaction product, tetramer (574 m/z) and hexamer (861 m/z) products (oligomers containing 4 and 6 monomers of 8-hydroxyquinoline respectively) gave the greatest signal heights, representing the most abundant oligomers in the sample. The peak average molecular weight or number average molecular weight $M_n$ was found to be 789 m/z as calculated using the formula:

$M_n$ = Total weight ($N_iM_i$) / Number of Polymers ($N_i$)

where $N_i$ is the number of polymer molecules (i.e number of ion peaks in the MALDI-TOF spectrum with mass $M_i$ (Montaudo *et al.*, 2006).

The type of linkages between the monomers could not be conclusively determined from the MALDI-TOF spectrum obtained, since, for example, the four monomers constituting a tetramer (574 m/z) could be joined by either C-O or C-C linkage, with the same concomittant loss of a total of 6 hydrogen atoms. Furthermore, the spectrum gave no information on the regioselectivity of these linkages. In this study, further characterization of the product by nuclear magnetic resonance (NMR) spectroscopy of the product was largely unhelpful due to the polymerisation resulting in a mixture of oligomeric and polymeric structures which were not found to be separable. As reported by Reed *et al.* (2005) and Foo *et al.* (2000b) such complications associated with the interpretation of the NMR spectrum can be attributed to the fact that positions of monomer attachment may differ among oligomers of the same degree of polymerization.

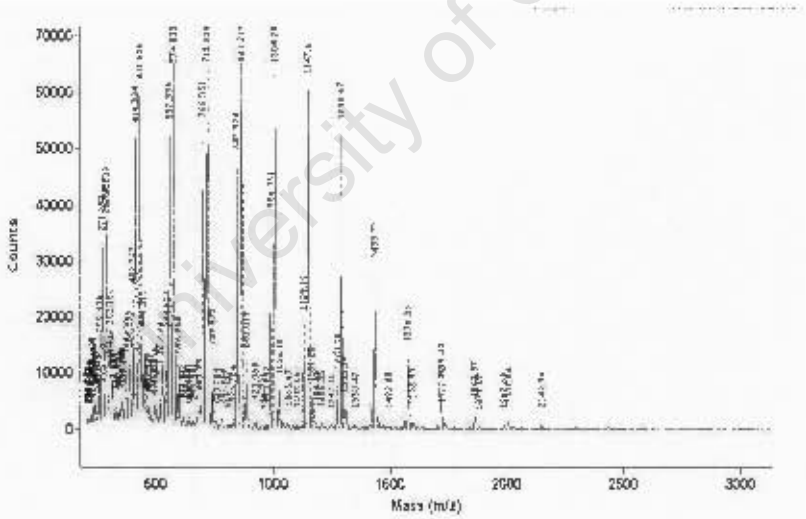



**Figure 3.1 MALDI–TOF mass spectrum of a poly (8-hydroxyquinoline) obtained from a reaction medium containing 8 % acetone and laccase.**

Further characterisation of the poly (8-hydroxyquinone) product included the use of infrared spectroscopy to identify the functional groups present. The infrared spectrum, measured in the region of 4000 400 $cm^{-1}$, showed the presence of a peak at the region of 3686.37 $cm^{-1}$ due to the hydroxyl group (O-H) vibrations in the compound (Figure 3.2). It was unlikely that this peak was due to some traces of water since the sample analysed was dried adequately. The absence of this peak would imply that the monomers are linked by C-O bonds. Other bonds observed were C-N at 1274.91, C-H at 3004.90, C-C at 1605.68 and C-O at 1252.99 $cm^{-1}$ region. These IR results were also similar to those obtained by Krishnakumar and Ramasamy (2005) who reported the vibrational spectra of isoquinoline and 8-hydroxyquinoline; this, together with our findings that the increment between the peaks in the MALDI-TOF spectrum was 143 mass units (equivalent to 8-hydroxyquinoline molecular weight), supports our conclusion that the product obtained comprised polymerised 8-hydroxyquinoline (Neanana and Burton, 2006).

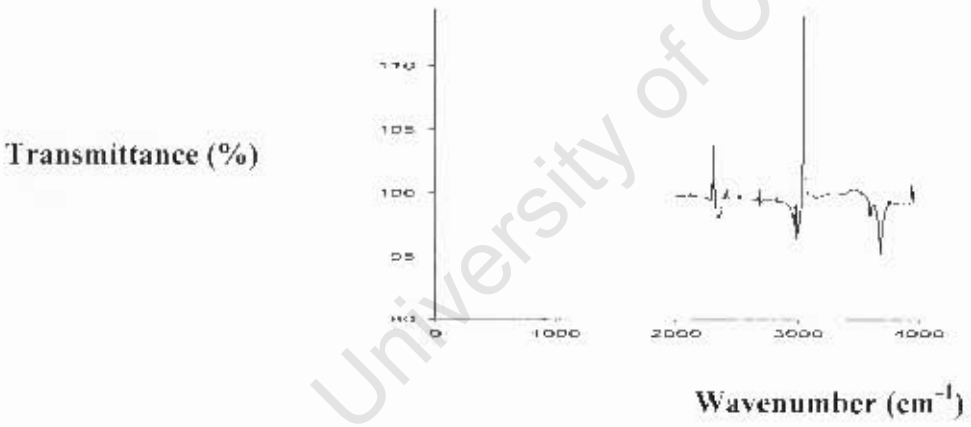



**Figure 3.2 FT-IR spectrum of poly (8-hydroxyquinoline) obtained by laccase oxidation in reaction medium containing 8 % acetone.**

The structure of the water-insoluble product **48** was thus confirmed to comprise 8-hydroxyquinoline monomers linked by C-C or C-O bonds to form the polymeric structure suggested in Figure 3.3. It can be postulated that the polymeric product mixture obtained was dominated by C-C linkages. This type of linkage is generally associated with acidic reaction

medium; a similar result was obtained by Ikeda and co-workers who reported that C-C coupling occurred during the laccase-catalyzed polymerization of 2,6-dimethyl-1,4-phenylene oxide under acidic conditions (Ikeda *et al.*, 1996).

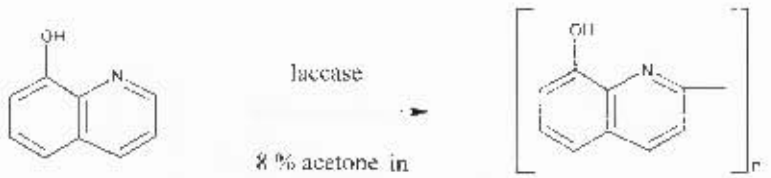



**Figure 3.3 Suggested structure of the poly (8-hydroxyquinoline) product obtained from a reaction medium containing 8 % acetone and laccase.**

### 3.3.2.2 The effect of accumulation of polymeric product poly (8-hydroxyquinoline) in the reaction medium containing laccase and 8 % acetone.

In an investigation of the time course of the reaction, the polymeric product was isolated from the reaction mixture after periods of 24, 48 and 72 h, and the laccase activity in the reaction mixture was monitored during this time. The laccase activity was found to decrease steadily and the amount of polymeric product being produced in each time period decreased correspondingly as the activity of laccase in the reaction mixture decreased (Figure 3.4). The highest amount of product (85 mg per 30 ml reaction volume, initially containing 3 U laccase) was obtained within 24 h, correlating with laccase activity being relatively high over this period; a further 20 mg of the polymeric product was obtained from the same reaction mixture after the reaction had run for 48 h. The reaction was continued until the activity of the laccase was zero and no further product was obtained. The total yield of the polymeric product after 72 h was 50 % (mass of the product/mass of the starting material x 100). Upon addition of 2 U of fresh laccase to the same reaction mixture, a further 9 mg product was obtained within a further 24 h, confirming that the formation of polymeric product was dependent on the presence of active laccase in the reaction

medium, although this additional yield of product, obtained after the second dose of laccase, represents only 10 % of the 100 mg theoretically possible.

Approximately half of the original laccase activity was lost within the first 24 h of reaction, and this loss of the enzyme activity was at least in part attributable to the acidic conditions. This was proved by incubating the enzyme at the acid conditions during which time activity was monitored. The low pH was due to the fact that the orginal reaction was conducted in acidic conditions brought about by the acetic acid used in dissolving 8-hydroxyquinoline. As depicted in Figure 3.5, 22 % activity was lost after 24 h due to acidic pH of the reaction mixture. The loss of enzyme activity may also have been due to accumulating polymeric product. However, this effect was not investigated in this study.

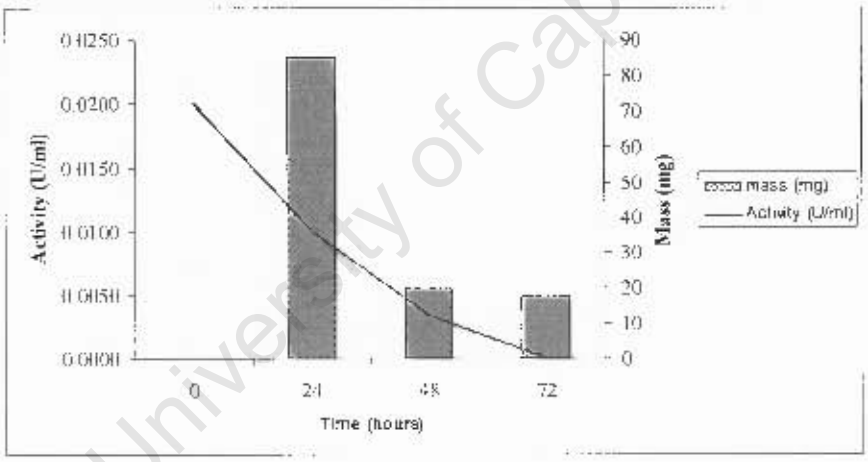



**Figure 3.4 Activity of laccase in the reaction mixture containing 8 % acetone, acetic acid, 8-hydroxyquinoline and sodium acetate buffer at pH 3.5, showing the relationship between laccase activity and the amount of polymeric product produced.**


A B

**Figure 3.5 Effect of acidic pH on the activity of laccase. The activity of laccase in buffer solution adjusted to various pH levels was monitored after 24, 48, and 72 h. A) Shows that laccase activity was unstable at pH 3.5. B) Shows that activity of laccase was stable in pH 5.**

### 3.3.2.3 The effect of temperature on the bioconversion of 8-hydroxyquinoline by laccase

The effect of temperature on the bioconversion of 8-hydroxyquinoline by laccase was investigated by incubating reaction mixtures at various temperatures. The optimal temperature was found to be 30 °C, and a conversion of 76 % was achieved after 24 h at this temperature (Figure 3.6). This gives a clear indication that operation at higher temperature, with the objective of enhancing reaction rate, or at lower temperature to enhance protein stability, would not necessarily be advantageous in terms of product yield. We acknowledge that there is a need for optimisation of the reaction process and this work will be reported in due course. Nevertheless, the highest conversion of 8-hydroxyquinoline was achieved at 30 °C.

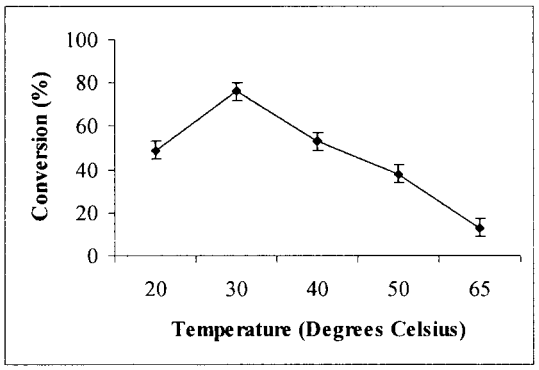



**Figure 3.6 Effect of temperature on the bioconversion of 8-hydroxyquinoline by laccase in medium containing 8 % acetone monitored using HPLC.**



### 3.3.2.4 The effect of organic solvents on the laccase-catalysed production of polymeric product 48 from 8-hydroxyquinoline

To evaluate the effect of organic solvents on the degree of polymerisation or distribution molecular weight, bioconversion of 8-hydroxyquionoline by laccase was performed in the medium that contained 8 % methanol, 8 % acetone or 50 % ethyl acetate. The obtained products were analysed using MALDI-TOF mass spectrometry as described previously. From all conditions tested, the MALDI-TOF spectra were characterised by similar pattern in which individual products (peaks) existed in a sample. For instance, in a medium containing 8 % acetone as described previously, the dominant peaks ranged from tetramers to decamers (Figure 3.7). The dimers (271 m/z) and product peaks with molecular weight greater than 1575 m/z formed the minor part of the sample, as depicted by low peak intensity. Similar results were also obtained for a polymeric product synthesized in a biphasic medium containing ethyl acetate (Figure 3.7) or monophasic medium containing methanol (not shown). Thus it can be postulated that peak molecular weight distribution of this polymeric product was not strongly influenced by the nature of organic solvents used.

However, the type of organic solvent used had an effect on the particle size of a polymeric product. The polymeric product obtained from the reactions in aqueous or 8 % methanol medium could not be recovered by filtration using No. 1 Whatman paper as was done with the polymeric product obtained from acetone-sodium acetate buffer or ethyl acetate media. This was an indication that the particle sizes of the polymer synthesized in aqueous or aqueous methanol medium differed from that of the polymer obtained from the 8 % acetone medium. This hypothesis was validated by characterisation of the polymeric products using scanning electron microscopy. From Figure 3.8 it is apparent that polymeric product obtained from 8 % acetone differed from those obtained from 8 % methanol or aqueous medium. The polymer particles synthesized in the presence of methanol, for instance, were smaller (particle size ranging from 0.5 to 1 μm) than those synthesized in the 8 % acetone medium (particle size ranging from 1.1 to 1.4 μm); and these results confirm the observations made during the filtration process. Thus organic solvents could be used to control the particle size of the polymer. Similar experiences have been reported by Pringle *et al.* (2006) who reported that particle size of polymers such as poly (pyrrole), poly (thiophene) and poly (terthiophene) was influenced by reaction conditions. Smaller polymer particles are highly sought after for a range of electrochemical device applications because they give a larger surface area and thus improved charge accessibility (Pringle *et al.*, 2006).

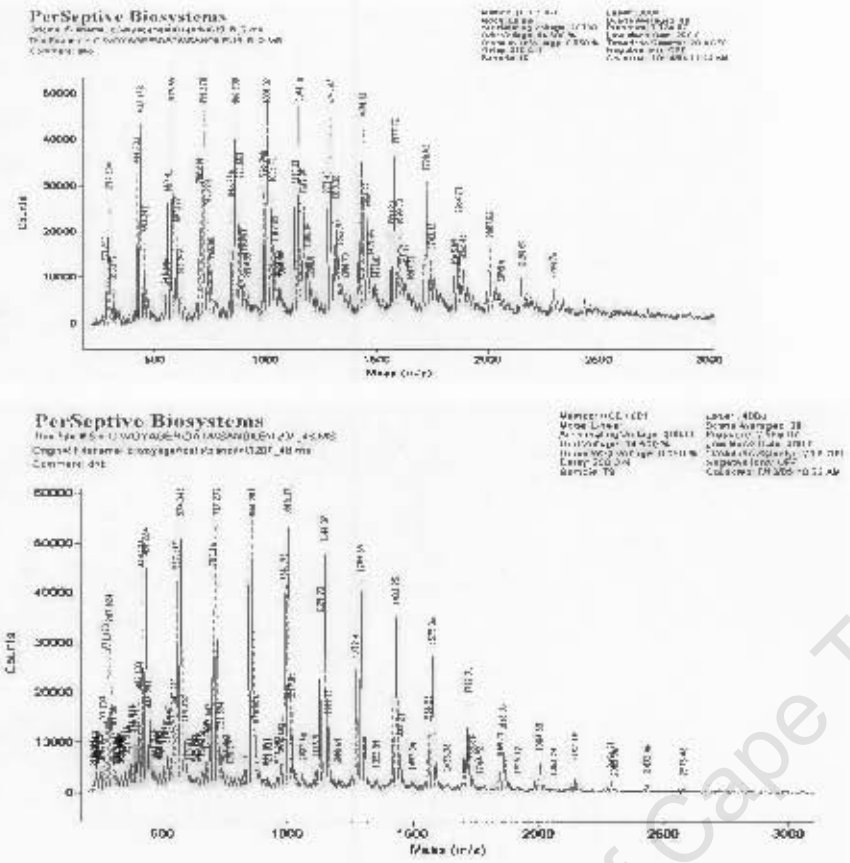
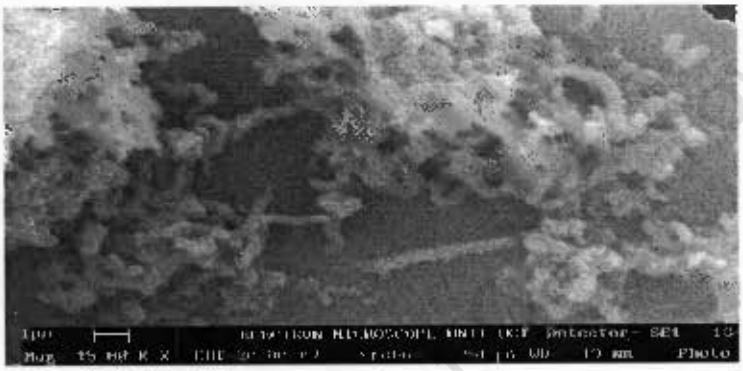



**Figure 3.7 MALDI-TOF spectra showing similar peak-molecular weight distribution of poly (8-hydroxyquinoline) synthesized using laccase in A) ethyl acetate-sodium acetate buffer medium and B) acetone-sodium acetate buffer medium.**

A



B

C

**Figure 3.8 SEM images of poly (8-hydroxyquinoline) agglomerated particles: (A) synthesized in 8 % acetone; (B) synthesized in 8 % methanol and (C) synthesized in aqueous medium (magnification 15 000 X).**

The effect of organic solvent on the nature of the polymeric product of 8-hydroxyquinoline was further considered with respect to degree of polymerisation. The highest degree of polymerisation was attained in aqueous medium (with no organic solvent added); a polymeric product obtained from this medium contained up to 23 monomers. However, addition of acetone (final concentration 8 %) reduced the degree of polymerisation, and the polymeric product contained up to 15 monomers. Further reduction was observed when oxidation of 8-hydroxyquinoline by laccase was performed in the reaction medium containing 50 % acetone. From this medium, there was no formation of a water-insoluble product. In this reaction, products were separated by HPLC (Figure 3.9) and their masses determined using liquid chromatography-mass spectrometry (LC-MS) (Figure 3.10). Three peaks at retention time 3.2, 7.7, 5.4 min showing the presence of higher molecular weight peak ions of 1145 m/z (8 monomers), 1077 m/z (7 monomers), and 877 m/z (6 monomers) respectively. Thus, the observed trend was that increasing the concentration of organic solvents in a reaction medium resulted in a decrease in the polymerisation of 8-hydroxyquinoline. The decrease in degree of polymerisation can be attributed to the retarding of molecular self-assembling prosesses which could be caused by increased organic solvent in the reaction medium. It has reported from literature that self-assembling of molecules to form polymers is enhanced by the hydrophobic, hydrogen-bonding, dipole-dipole interactions among other interactions (Oguchi *et al.*, 2002). The hydrophobic interaction, in particular, was demonstrated in the work of Wakisaka *et al.* (1995) where it was proved that bonding of phenol and pyridine was dependent on the organic solvent used. These authors provided evidence for hydrophobic effects on the self-binding of molecules. It has been suggested that at low concetration of water in the reaction medium, hydrophobic interactions to do not occur as the hydrogen bonding of water is compromised by presence of organic solvents (Oguchi *et al.*, 2002). Thus, in this study we postulate that the hydrophobic interaction is reduced since hydrogen bonding of water could have been affected in the presence of high concentration of acetone in the reaction medium, and hence coupling of 8-hydroxyquinoline radicals was impeded. This work is consistent with that of Oguchi *et al.*, (2002) who reported that oxidative polymerisation of phenols using horseradish peroxidase was dependent on the organic solvents. These authors asserted that the degree of polymerisation of *m*-cresol decreased with an increase in organic solvent (1.4 dioxane) content in the reaction medium.

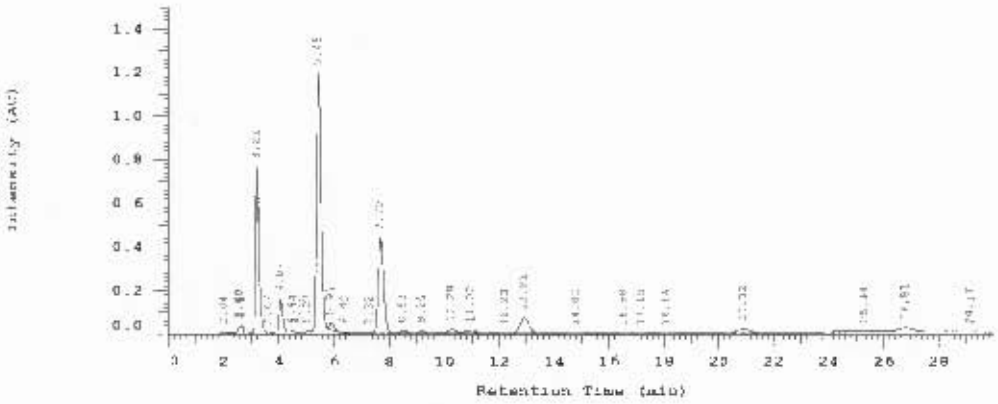



**Fig 3.9 HPLC profile of the 8-hydroxyquinoline reaction products produced using laccase in a reaction medium containing 50 % acetone. Peaks with retention time 3.21, 5.45 and 7.70 min represented new products 48(I), 48(II), and 48(III) respectively, and the peak with retention time 12.92 min represented 8-hydroxyquinoline.**

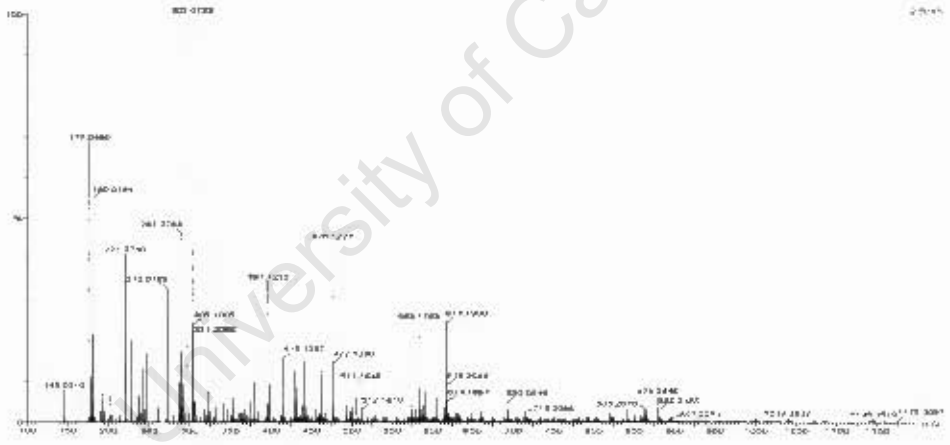


**Figure 3.10a LC-MS of the 8-hydroxyquinoline product 48(I), peak with retention time 3.2 min, produced using laccase in reaction medium containing 50 % acetone, indicating the presence of a product with a higher molecular weight of 1145 m/z.**

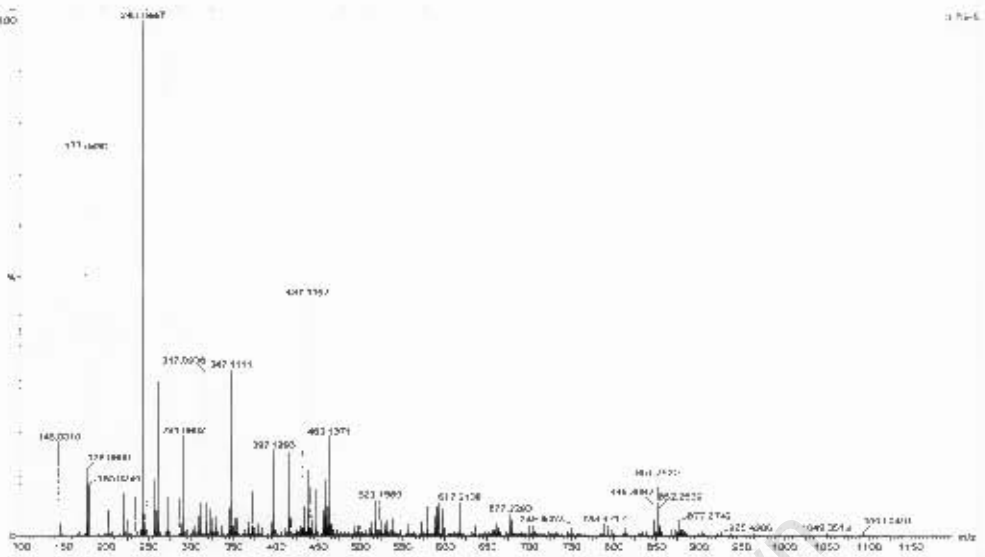


**Figure 3.10b LC-MS 8-hydroxyquinoline product 48(II), peak with retention time 5.4 min, produced using crude laccase in reaction medium containing 50 % acetone, indicating the presence of a product with a molecular weight of 877 m/z.**



It is also important to note that the products 48(I) and 48 (II) shown in Figure 3.10a and Figure 3.10b are largely composed of relatively lower molecular weight compounds. This is indicated by the presence of high abundance peak ions with masses ranging from 177 m/z to 476 m/z.

These results demonstrate that the reaction of laccase with 8-hydroxyquinoline results in the production of a mixture of compounds with a range of molecular weights, as characterised using MALDI-TOF and HPLC analysis. The summary of the results is shown in Table 3.2. From these results, it was apparent that the degree of polymerisation could be controllable by selection of appropriate organic solvents. The use of organic solvents in laccase reactions is increasingly being investigated. Previously, Carunchio *et al.* (2001) had reported that oxidation of ferulic acid in acetate buffer containing 9 % ethanol resulted in the production of mixture of dimeric and polymeric products. More, recently, Mustafa *et al.* (2005) reported that polymerisation of the same compound by laccase was prevented in the presence of organic solvent and particularly, a

sodium acetate-ethyl acetate biphasic system (80 % ethyl acetate). However, polymerisation of 8-hydroxyquinoline, both in the absence or presence of organic solvent, did occur under the conditions tested in the present study. These findings could be attributable to the structure of 8-hydroxyquinoline, and more specifically the *para*-substitution with the hydroxyl group. It is generally accepted that the *para*-substituted phenolic compounds are more reactive and could be prone to polymerisation (Gianfreda *et al.*, 2003; Shuttleworth and Bollag, 1986). Further, the successful polymerisation could also be due to the fact that the reaction of the 8-hydroxyquinoline radicals with other radicals in the medium is apparently sterically possible, and hence the elongation of chain is possible, to form a polymeric compound.

**Table 3.2 Laccase-catalysed polymerisation of 8-hydroxyquinoline in different reaction conditions**

| Organic solvent | Product size (number of monomers) | Product Particle size (µm) | Product solubility |
|---|---|---|---|
| Aqueous (no organic solvent) | Product **$48_{23}$** | 0.2 - 0.9 | Soluble in 1,4 dioxane-methanol (1:1) |
| 8 % acetone | Product **$48_{15}$**, same as the above product but the number of monomers was lower. | 1.1 – 1.4 | Soluble in 1,4 dioxane-methanol (1:1) |
| 50 % acetone | Product **$48(I)_8$** **$48\ (II)_7$** **$48(III)_6$** | Not determined | Soluble in 1,4 dioxane, methanol, acetone |
| 8 % methanol | Product **$48_{20}$** | 0.5 - 1 | Soluble in 1,4 dioxane-methanol (1:1) |



### 3.3.3 Potential application of the laccase reaction products

#### 3.3.3.1 Antioxidant activity of the poly (8-hydroxyquinoline)

In view of the potential of the poly (8-hydroxyquinoline) (see Section 3.1) as an antioxidant, the antioxidant capacity of the product was evaluated by monitoring the quenching of the stable free

radical DPPH (Sánchez-Moreno *et al.*, 1998) (described in Section 3.2.7). Addition of the poly (8-hydroxyquinoline) solution to the purple coloured DPPH solution caused a colour change from purple to yellow, a positive indication of quenching of the free radical DPPH (Espín *et al.*, 2000). The antioxidant activity is indicated by the amount of DPPH radical reduced in a solution and is expressed in terms of percentage. As poly (8-hydroxyquinoline) was dissolved in 1,4 dioxane, and DPPH in methanol (Section 3.2.7), the effect of these solvents on the antioxidant activity was also determined. It was observed that in the presence of the solvents but absence of poly (8-hydroxyquinoline) the amount of DPPH radical did not decrease (there was no change in colour or absorbance reading). Therefore, the decrease in DPPH radical was attributable to the poly (8-hydroxyquinoline). The percentage of DPPH remaining in the presence of poly (8-hydroxyquinoline) at different concentrations (400, 267, 200 and 100 mg/l) are shown in Figure 3.11. When the same experiment was conducted with equivalent masses of unpolymerised 8-hydroxyquinoline monomer in place of the polymer, the antioxidant activity, defined as the ability of a compound to scavenge DPPH radical, was found to be lower by 10 %. The DPPH remaining at a steady state is directly related to antioxidant activity where lower DPPH remaining is indicative of higher antioxidant activity of the compound. The percentages of the DPPH remaining at the steady state decreased with an increase in the concentration of the polymer, and the time required to reach the steady state varied between 1 and 3 min. This kinetic behaviour would be classified as rapid in terms of antioxidant activity relative to other known antioxidants (previously classified by Sánchez-Moreno *et al.* (1998). According the report of Sánchez-Moreno *et al.* (1998), gallic acid took 14.69 min to scavenge 50 % of the DPPH in the same reaction, and this was classified as intermediate reaction kinetic behaviour.

The concentration of the antioxidant poly (8-hydroxyquinoline) required to decrease the initial DPPH concentration by 50 % ($EC_{50}$) was obtained by plotting the percentages of DPPH remaining against the concentration of the polymer per gram DPPH (Figure 3.12). The $EC_{50}$ of poly (8-hydroxyquinoline) was found to be 250 mg antioxidant/g DPPH, $EC_{50}$ was determined graphically by finding the correlation between remaining DPPH and concentration of antioxidant (Figure 3.12) (Sánchez-Moreno *et al.*, 1998). The results obtained in this study showed that 250 mg of poly (8-hydroxyquinoline) was needed to scavenge 1 g of DPPH. This value is

comparable to the $EC_{50}$ of DL-α-tocopherol which is 200 mg antioxidant /g DPPH. as reported by Sánchez-Moreno *et al.* (1998).

The antioxidant 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 ionization potential of the reactive hydroxyl groups present in the molecule, and the potential for stabilisation of the resulting radical by charge delocalisation (Prior *et al.*, 2005; Dizhbite *et al.*, 2004). In aromatic polymeric structures such as lignin, free radical scavenging activity has been correlated with the molecular weight of the polymer and the extent of π-polyconjugated systems in the structure. It is suggested that antioxidant power is enhanced by the presence of extended π-polyconjugation systems as well as the presence of numerous specific groups such as phenolic hydroxyls and benzylic hydrogen moieties (Dizhbite *et al.*, 2004). Correspondingly, in the present study, the scavenging activity of the poly (8-hydroxyquinoline) can be attributed to the increased additional conjugation of the radical product due to the extended aromatic structure which will contribute to π-polyconjugation and hence favour the antioxidant quenching reaction with free radical agents.

Uses of antioxidant products extend across a very broad range of applications, from medical products and nutraceuticals to industrial preservatives. The results obtained in this study suggest that poly (8-hydroxyquinoline) has potential for application as an antioxidant in a number of areas such as these. For example, the anti-bacterial properties of compounds with structures similar to poly (8-hydroxyquinoline), including lignins (Barber *et al.*, 2000; Dizhbite *et al.*, 2004) and derivatives of 8-hydroxyquinoline itself (Kayirere *et al.*, 1998) are attributed to their ability to inhibit free radical reaction processes. Similarly, aromatic antioxidants such as butylated hydroxytoluene (BHT) are well-known for their application as stabilisers in resins and plastics (Boersma, 2006). Our results indicate that the poly (8-hydroxyquinoline) has an improved antioxidant properties as compared to those of the monomer. It is also notable that the decrease of DPPH radical due to poly (8-hydroxyquinoline) was a stable reaction as there was no subsequent increase of this radical. The stability of the antioxidant could prove to be important particularly in prolonging the life of polymeric materials, since it is known that, in polymeric materials, the efficiency of antioxidants is lost over time, largely as a result of physical loss e.g. by leaching and

evaporation (Boersma, 2006). Thus, it is likely that a less mobile antioxidant would be less readily lost from the material and may thus prolong the life of the polymeric material.

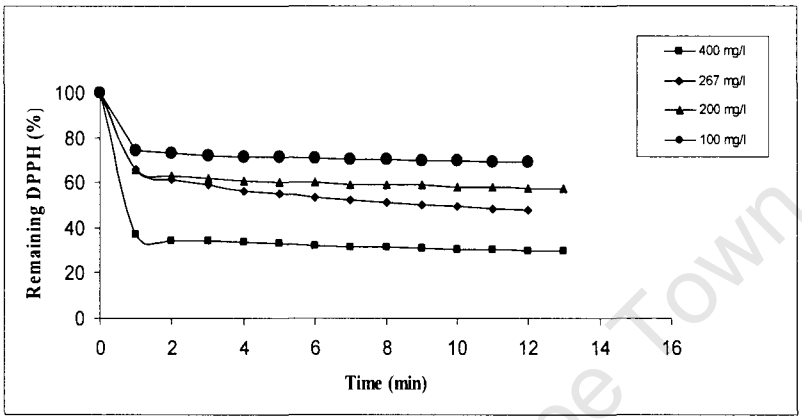



**Figure 3.11 Time courses of DPPH quenching due to poly (8-hydroxyquinoline) antioxidant activity using varying amounts of poly (8-hydroxyquinoline).**

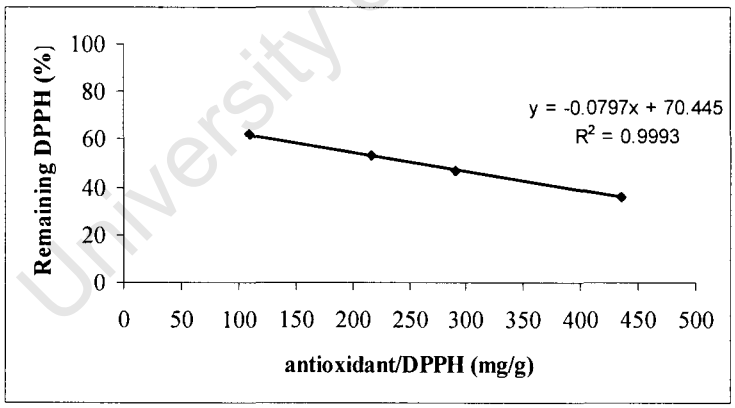



**Figure 3.12 Graph of DPPH remaining in solution as a function of amount of poly (8-hydroxyquinoline) added per gram of DPPH, used to determine $EC_{50}$ (Sánchez-Moreno *et al.*, 1998)**

### 3.3.3.2 Other applications of products formed by oxidation of 8-hydroxyquinoline

Although there is a great deal of literature on the use laccase as a bioremediation agent, 8-hydroxyquinoline has not been described in this context. Thus, in this study, 8-hydroxyquinoline was also considered as model compound for developing a bioremediation process using laccase from *T. pubescens*. To quantify the removal of 8-hydroxyquinoline from aqueous medium, the compound 8-hydroxyquinoline was added to a Schott bottle containing 8 % acetone, acetic acid, sodium acetate buffer and laccase. The samples were taken over time for analysis using HPLC. The decrease in the concentration of 8-hydroyxquinoline from the solution was determined by comparing the peak areas of 8-hydroxyquinoline incubated with laccase and that of 8-hydroxyquinoline incubated in the absence of laccase (control). It was observed that the disappearance of 8-hydroxyquinoline was accompanied by appearance of the precipitate in the Schott bottle, and 80 % of the 8-hydroxyquinoline was removed from solution, as previously observed. Since the activity of laccase was lost over time due to acidic conditions (see previously shown Figure 3.4 in Section 3.3.2.2), fresh laccase was added to the medium after 2 d. Upon further analysis of the aqueous solution, it was observed that 8-hydroxyquinoline peak had completely disappeared, and therefore 100 % removal 8-hydroxyquinoline had been achieved. To our knowledge this study is the first to report on the removal of 8-hydroxyqquinoline from aqueous solution using laccase from *T. pubescens*. As a result, there was no data from literature that could be compared. However, it is clear that the results obtained from this study are consistent with other studies that generally assert that the laccase from *Trametes* species, or *Trametes* as whole cells is suitable for bioremediation of phenolic compounds as reported by Ryan *et al.* (2005) and Kim and Nicell (2006), among others. The method developed for removal of 8-hydroxyquinoline from solution can be further improved, for example, by immobilization of laccase in a membrane system, a process that would allow retention of enzyme activity. This process could be further developed by designing a continuous membrane reactor in which effluent containing 8-hydroxyquinoline could fed be continuously in to reactor, the polymeric product formed as a precipitate removed, and where enzyme can be reused.

### 3.4 Conclusion

In this study, 8-hydroxyquinoline was selected as a model compound for the investigation of the potential of laccase to yield products with biological activity. The oxidation of 8-hydroxyquinoline yielded poly (8-hydroxyquinoline) of average molecular weight 780 m/z using laccase from a *T. pubescens* strain (Ncanana and Burton, 2006). The polymerisation was catalyzed by laccase in acetone-sodium acetate buffer at ambient temperature and pressure. This reaction, yielding an antioxidant polymer, using a sustainable catalyst in an aqueous based, low temperature system, represents a feasible, environmentally benign alternative to conventional polymerisation methods.

A loss of laccase activity during the production of this polymer was observed; however, this is not regarded as problematic because fungal laccases can be readily and cheaply produced. Thus, we envisage that, in an optimised process, fresh laccase could be fed into a reactor as necessary, until the desired level of conversion has been achieved.

The degree of polymerisation of hydroxyquinoline by laccase was controllable using organic solvents. The highest degree of polymerisation was attained in aqueous medium (with no organic solvent added), and the highest molecular weight product obtained was composed of 23 monomers of hydroxyquinoline. Addition of acetone (final concentration 8 %) reduced the degree of polymerisation to 15 monomers. Further reduction to 8 monomers was observed when oxidation of 8-hydroxyquinoline by laccase was performed in the reaction medium containing 50 % acetone.

The effects of organic solvents were further observed with varying particle size of polymers of 8-hydroxyquinoline, where larger particles were obtained in acetone medium and smaller particles methanol medium.

The products obtained were assayed for antioxidant activity. It was established that poly (8-hydroxyquinoline) can act as a potent antioxidant with an antiradical efficiency comparable with,

or better than that of other compounds commonly used as antioxidants, but its polymeric nature may confer additional advantages in terms of stability.

Oxidation of 8-hydroxyquinoline by laccase from *T. pubescens* yielded a process that could be used as a bioremediation model since monomers were converted into polymers that can recovered by filtration. Laccase from *T. pubescens* removed 100 % of 8-hydroxyquinoline from aqueous solution. An advantage of this process is that products produced in significant yields can be recovered and characterised for new properties. Therefore, value added products could be obtained from wastewaters. Future work that should be done would involve the modelling of the bioremediation process, to generate the information required for scaling up of the process for application in bioreactors.

Generally, it was apparent that oxidation of phenolic compounds by laccase could be controlled by using organic solvents, and that the reactivity of the substrate was attributable to its structural configuration. The next Chapter describes work in which the aim was to obtain some insight into the influence of organic solvents in the selectivity of totarol-laccase reactions products with respect to coupling of monomers.

# Chapter 4

## Laccase-catalyzed dimerisation of the natural compound totarol

### 4.2 Introduction

The broader objective of work reported in this chapter was to compare the laccase reactions involving a relatively complex natural product compound, totarol, with those reported in the previous chapters, which had involved lower molecular weight substrates. Thus, in this chapter, totarol was used as the model compound to study the reaction of laccase with substrates of higher molecular weights. Further, the effect of organic solvents on the nature of the products was evaluated.

Totarol, **62**, a tricyclic diterpene, belongs to the terpenoid family of natural products. The primary source of totarol is the *Podocarp Totara* tree (*Podocarpus totara*), native to New Zealand. Totarol can be extracted from the heartwood of decayed *totara* trees. It has also been extracted from other plant species such *Thuja plicata (Cupressaceae*) (Sharp *et al.*, 2001*)* among other sources. Terpenoids have been shown to be of ethno-pharmacological importance. The biological activities such as antimicrobial, inflammatory, cardiovascular and antitumoral properties of these natural products have already been reported by several authors including Urzua *et al.* (1998); Fatope *et al.*(1996) and Benrezzouk *et al.* (1999) among others. Totarol was found to be active against *Mycobacterium tuberculosis* (Constantinea *et al.*, 2001). The significance of totarol biological activity in the field of organic chemistry and ethno-pharmacology prompted several researchers to start investigating the synthesis of its analogues with the hope of increasing the activity or finding new applications. For instance, Evans and co-workers reported the methylation and acetylation of a phenolic group in totarol to give *o*-methyltotarol and totaryl acetate respectively, and glycosylation of totarol which led to the improvement in its solubility in water and would potentially better the bioavailability (Evans *et al.*, 1999). Further work on synthesis of totarol derivatives was reported by Clarkson and co-workers who reported the synthesis of totarol amino alcohol derivatives, and they also demonstrated that all of the derivatives have an

improved antiplasmodial activity as compared to totarol (Clarkson *et al.*, 2003). All the reports regarding the modifications of totarol have been based on chemical syntheses.

According to Manda and co-workers, chemical methods may be used for modification of phenolic substructures to the corresponding derivatives, but their use often leads to an irreversible destruction of the desired substructures (Manda *et al.*, 2005). Therefore, alternative methods which are more selective and gentle are desirable to develop pharmaceuticals and to modify natural compounds with phenolic substructures (Manda *et al.*, 2005). To our knowledge, no work has been reported on the modification of totarol using enzymes. Thus this study reports for the first time the synthesis of novel totarol dimers using laccase from *T. pubescens* in organic medium. The reactions were performed in organic media and the reaction products were characterized using HPLC, NMR, MALDI-TOF analysis and ESI-MS. The product of totarol-laccase reaction was evaluated for antioxidant and antimalarial activity to obtain the deeper insight into the relationship between phenolic structure and biological activity.

## 4.2 Materials and Methods

### 4.2.1 Oxidation of totarol by laccase in a monophasic system

The first batch reaction contained totarol (200 mg) (Sigma) dissolved in 32 ml methanol, which was added to 32 ml sodium acetate buffer (20 mM pH 4.5) in which the laccase from *T. pubescens* (100 mg, 60 U) had been previously dissolved. The milky solution was incubated at 25 °C under mild shaking, following the conversion by TLC (eluent: petroleum ether-ethyl acetate, 19: 1) and HPLC (eluent: acetonitrile-$H_2O$ 80: 20 for 10 min, then 100 % acetonitrile; flow rate 1 ml/min). After 48 h the solution was extracted with ethyl acetate and the solvent was evaporated to give a residue that showed a composition similar to the precipitate. The crude product material (200 mg) was purified by flash chromatography (eluent: petroleum ether) to give the products **63** (37 mg) and **64** (2.5 mg), together with unreacted totarol (109 mg).

The second batch reaction contained 100 mg totarol, 15 mg (300 U) *T. pubescens* laccase, 16 ml acetone or acetonitrile and 16 ml acetate buffer 20 mM pH 4.5.

The third batch reaction contained contained 10 mg totarol, 100 mg (6 U) *T. pubescens* laccase, 10 ml ethanol and 10 ml sodium acetate buffer with concentration of 20 mM at pH 4.5. These reactions were incubated and monitored as described above.

### 4.2.2 Oxidation of totarol by laccase in a biphasic system

The reaction mixture contained 10 mg totarol, 3.4 mg (80 U) *T. pubescens* laccase, 0.5 ml ethyl acetate or chloroform or methyl *t*-butyl ether or *t*-amyl alcohol or toluene and 0.5 ml sodium acetate buffer (20 mM pH 4.5). The reactions were incubated at 30 °C with shaking at 200 rpm. The progress of the reaction was monitored by TLC (eluent: petroleum ether-ethyl acetate, 19:1) and the conversion was measured by HPLC.

The reaction with *t*-amyl alcohol was scaled-up. Totarol (100 mg) was dissolved in 8 ml *t*-Amyl alcohol and added to 16 ml acetate buffer 20 mM pH 4.5 with 20 mg of the laccase from *T.*

*pubescens* (400 U). The reaction was incubated at 30 °C by shaking at 220 rpm. After 4 d the reaction was stopped by extracting the solution with chloroform, and the solvent was evaporated. The crude material (100 mg) was purified by flash chromatography (eluent: petroleum ether - ethyl acetate 25:1) to give a mixture of **63** and **64** (34 mg).

### 4.2.3 Effect of buffer salts concentration on the bioconversion of totarol

The effect of buffer concentration on the conversion of totarol was investigated. The buffer concentrations were varied from 20 to 100 mM. The reaction mixture contained 1 mg totarol, 10 mg (6 U) *T. pubescens* laccase, 1 ml methanol and 1 ml sodium acetate buffer with concentration of 20, 50 or 100 mM, at pH 5. The reactions were incubated at 30 °C with shaking at 200 rpm. The progress of the reaction was monitored by HPLC.

### 4.2.4 Effect pH on the bioconversion of totarol

The pH of the reaction medium was varied from 4 to 7 using 20 mM sodium acetate buffer adding acetic acid to adjust the pH. The reaction mixture contained 1 mg totarol, 10 mg (6 U) *T. pubescens* laccase, 1ml ethanol and sodium acetate buffer.

### 4.2.5 Effect of reaction temperature on the bioconversion of totarol

In order to determine the effect of temperature on the totarol conversion, reactions were conducted at various temperatures. The reaction mixture contained 1 mg totarol, 10 mg (6 U) *T. pubescens* laccase, 1ml methanol and 1 ml 20 mM sodium acetate pH 4.5. The reactions were incubated at 20, 30, 40, and 50 °C with all other conditions remaining the same.

### 4.2.6 HPLC analysis of totarol-laccase products

Unless otherwise stated, all totarol products were analyzed using HPLC as follows:
The conversion of totarol by laccase was monitored using HPLC (Merck La Chrom).The mobile phase was acetonitrile 100 % with a flow rate 0.5 ml/min, and using a C18 Waters (250 mm x 4.6 nm) reverse phase column and UV detection at 280 nm. Peaks were analyzed using HPLC Manager, Merck Hitachi model D 700 data software. The percentage conversion was obtained by comparing the peak area of reaction sample with that of a control containing no enzyme.

### 4.2.7 MALDI-TOF analysis and ESI-MS of totarol-laccase products

MALDI- TOF mass spectrometry was used to determine the molecular weight of the compound. MALDI–TOF mass spectra were obtained using a Perspective Biosystems DE-PRO MALDI mass spectrometer equipped with a TOF analyser. Sample aliquots (1 ml) in 50 % chloroform were mixed with 1 ml of 2,5-dihydroxybenzoic acid matrix and applied to the gold sample plate. The spectrometer was operated in positive- and negative-ion modes (service rendered by Department of Molecular and Cellular Biology, University of Cape Town).



### 4.2.8 Biological activity of product 63, the C-C totarol dimer

#### 4.2.8.1 Antioxidant activity

Totarol, **62,** and totarol dimer, **63,** were dissolved in methanol (initial concentration 0.7 or 1.7 mM). 100 µl of totarol or totarol dimer solution was added to 3.9 ml of DDPH solution 6 x $10^{-5}$ M. The decrease in absorbance at 515 nm was measured and the % DPPH remaining calculated and plotted against time.

#### 4.2.8.2 Antimalarial activity of totarol and product 63

The work described in this section was done by Dr Clarkson of University of Cape Town, Pharmacology department. All samples were tested, in duplicate, on one occasion, against a chloroquine sensitive (CQS) strain of *Plasmodium falciparum* (D10). Continuous *in vitro* cultures of asexual erythrocyte stages of *P.falciparum* were maintained using a modified method of Trager and Jensen (1976). Quantitative assessment of antiplasmodial activity *in vitro was* determined via the parasite lactate dehydrogenase assay using a modified method described by Makler (1993). The samples were prepared to a 2 mg/ml stock solution in 10 % methanol and were tested as a suspension if not properly dissolved. Chloroquine (CQ) was used as the reference drug. Test compounds were stored at -20 °C until use. A full dose-response was performed with a starting concentration of 100 µg/ml, which was serially diluted 2-fold in complete medium to give 10 concentrations; with the lowest concentration being 0.195 µg/ml. CQ was tested at a starting concentration of 100 ng/ml using same dilution technique. The highest concentration of solvent to which the parasites were exposed to had no measurable effect on the parasite viability (data not shown). The 50 % inhibitory concentration ($IC_{50}$) values were obtained using a non-linear dose-response curve fitting analyses via GraphPad Prism v.4.0 software.

## 4.3. Results and Discussion

### 4.3.1 Biotransformation of totarol in homogenous aqueous-organic system

Extracellular laccase was produced by culture of *T. pubescens* in an airlift reactor (Chapter 2). The laccase was then used in a biotransformation system in which the enzyme catalyzed the oxidation of totarol, **62**. The reaction was monitored using TLC analysis (eluent used was the mixture of petroleum ether and ethyl acetate, 19:1). The TLC analysis showed two products (**63**, TLC $R_f$: 0.15 and **64**, TLC $R_f$: 0.19) which were also observed within 1 h from other reactions that contained acetonitrile or acetone or ethanol. The conversion of totarol by laccase was further monitored using HPLC (Merck La Chrom). The HPLC profile (Figure 4.1) confirmed that the reaction of totarol with laccase resulted into two products as observed on the TLC plate. The polar compound **63**, was the main product of the reaction and was obtained at a ratio of 8:1 with the minor product **64.** The two products obtained were purified by flash chromatography and analysed using NMR and mass spectrometry.

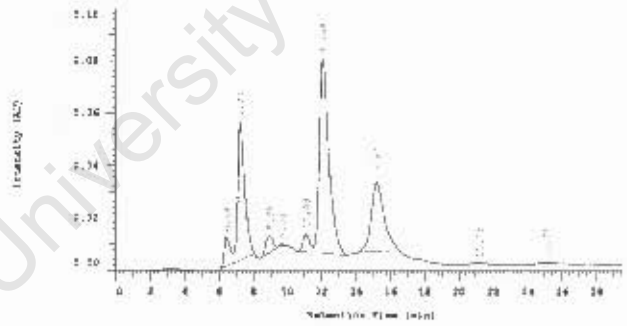
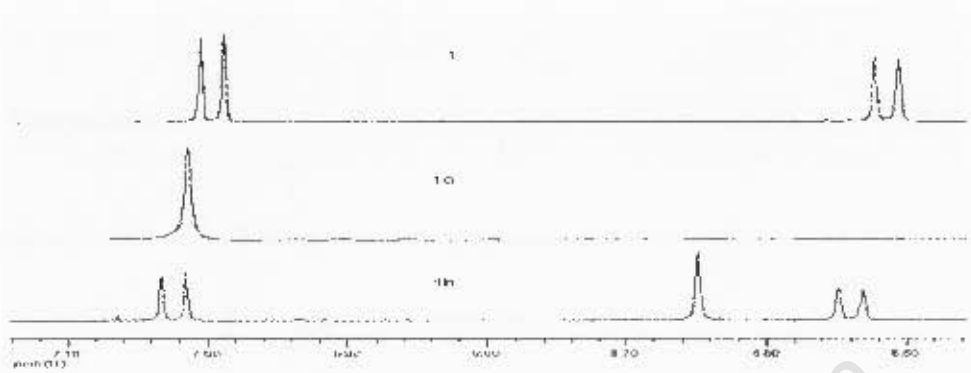


**Figure 4.1 HPLC profile of product mixtures (63 with retention time 12.09 min and 64 with retention time 15.21 min) obtained from reactions of totarol (62 with retention time 7.24 min) catalyzed by *T. pubescens* laccase.**

To determine the structure of the compounds **63** and **64**, the products of the reaction mixture (Section 4.2.1) were purified by flash chromatography. The purified compounds were dried and analyzed using $^{1}$H-NMR and $^{13}$C-NMR (Appendix G). The $^{1}$H-NMR spectra of the products are shown in Figure 4.2. In this figure the signals due to the aromatic protons (Figure 4.2A) and some of the aliphatic protons (Figure 4.2B) of totarol and of the two dimers, are compared. The $^{1}$H-NMR spectrum representing compound **62** was characterized by the presence of the presence of two doublets at 7.03 and 6.54 ppm) which represented 2 hydrogens in the aromatic ring of totarol, in contrary the spectrum of the totarol-laccase product **63,** shows the presence of singlet at 7.03 ppm. This was an indication that product **63** had lost two hydrogens as a result of coupling by C-C bonds of two radicals of totarols. The aliphatic protons of totarol, **62**, and product **63** as represented by the signals between 1.90 and 3.60 ppm, were similar, and hence confirming the presence of the symmetric C-C dimeric structure showed in Figure 4.4. In contrast, the aromatic portion of the $^{1}$H-NMR spectrum of **64** showed both the presence of two doublets and of one singlet (overall three protons), whereas all the signals between 1.90 and 3.60 ppm were duplicated, thus suggesting the formation of a C-O linkage between two totarol units. The $^{13}$C-NMR spectra of **63** and **64**, further supported the proposed structures.

A)



62

63

64

B)

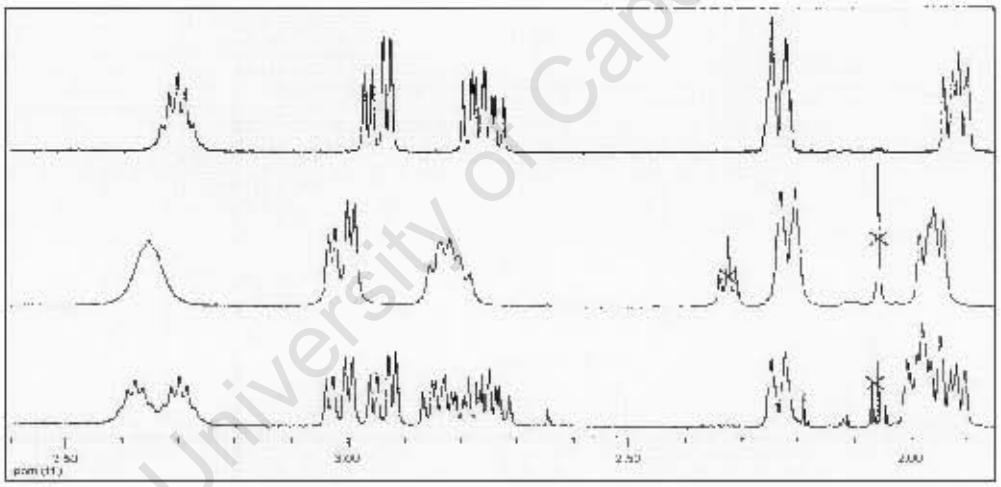




**Figure 4.2 $^{1}$H-NMR spectra of compounds 62, 63, and 64, showing (A) the aromatic protons and (B) a portion of the aliphatic protons.**

The molecular mass of compound **63** was determined using ESI-MS (Table 4.1). The molecular ion peak with mass 569 m/z, determined under negative mode, was a further confirmation that totarol (286 m/z) was dimerised by laccase. To test whether the polymerization of totarol had taken place, the crude reaction products of laccase and totarol were analyzed using MALDI-TOF analysis (Figure 4.3) which showed a molecular peak with mass of 571 m/z corresponding to the mass of two monomers of totarol. No high molecular weight ion was observed. The absence of polymeric product suggests that reaction of totarol with laccase resulted in dimeric products only. The formation of dimeric products is attributed to the coupling of radicals, the oxidation intermediate products of totarol laccase reaction. It is suggested that laccase oxidized totarol to form aromatic radicals, which in turn combined to form dimeric structures linked through C-C bond or C-O bond formation (Figure 4.4).

**Table 4.1 ESI -MS results for compound 63**

| ion | m/z calculated | m/z experimental | Error (ppm) |
|---|---|---|---|
| $[M + Na]^+$ | 593.43 | 593.43 | 1.70 |
| $[M - H]^-$ | 569.43 | 569.43 | 1.50 |

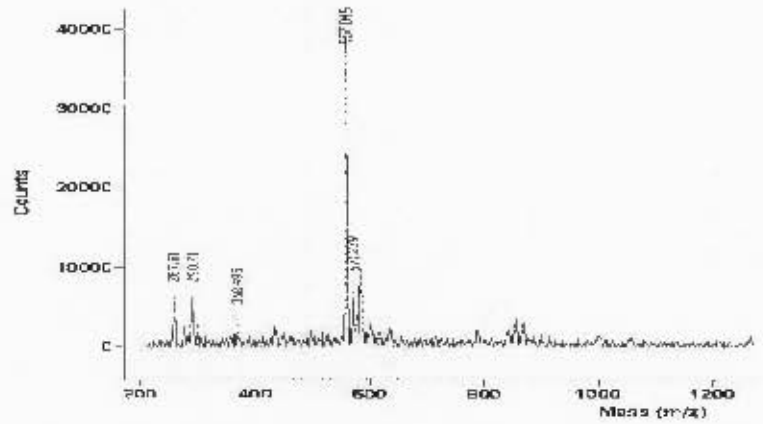



**Figure 4.3 MALDI-TOF analysis of compound 63 obtained from the reaction of totarol catalyzed by *T. pubescens* laccase.**

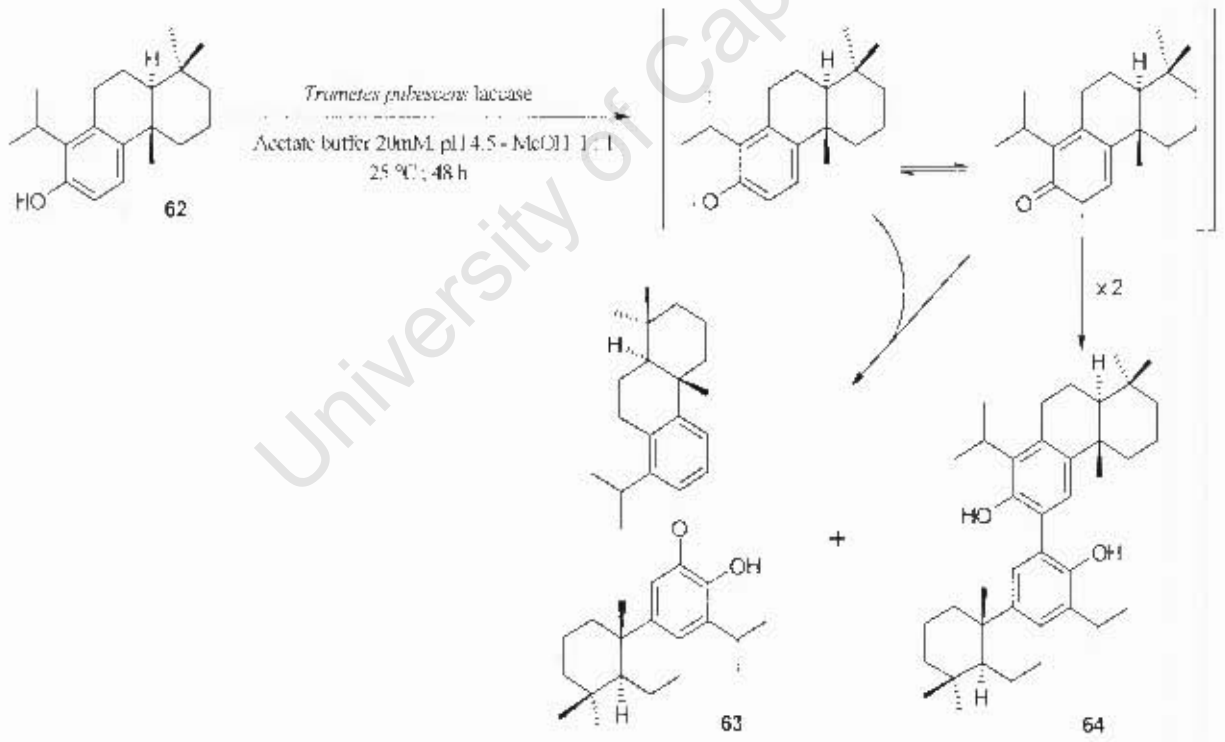



**Figure 4.4 Structures of totarol 62 and dimeric products 63 and 64 obtained from totarol-laccase reactions.**

The totarol-laccase reactions were compared with tyrosol, hydroxytyrosol and 8-hydroxyquinoline-laccase reactions as shown in Table 4.2, all the reactions having been conducted under similar conditions. The reactions of laccase with substrates of relatively lower molecular weight (tyrosol and 8-hydroxyquinoline) yielded largely polymeric products, whereas laccase reactions with hydroxytyrosol or totarol, the substrates with relatively higher molecular weight, yielded dimeric products. The bulky nature of the dimeric products from hydroxytyrosol and totarol reactions is suggested to cause steric hindrance, and therefore further reaction with other monomers could be prevented as described previously in Chapter 2. In this study it is therefore clear that the structure of the substrate has a profound effect on the nature of the products formed from laccase reactions. Molecular modelling to obtain more precise information on this effect would be a useful future study.

**Table 4.2 The laccase reaction products obtained from different substrates in similar reactions conditions.**

| **Substrate** | **Organic solvent used** | **Organic solvent Concentration (%)** | **Major products of the reaction (molecular weight) m/z** |
|---|---|---|---|
| tyrosol (MW:138 m/z) | acetone | 50 | 962 (polymer) |
| 8-hydroxyquinoline (MW: 145 m/z) | acetone | 50 | 1145 (polymer) |
| hydroxytyrosol (MW: 154 m/z) | acetone | 50 | 306 (dimer) |
| totarol MW: 286 m/z) | acetone | 50 | 571 (dimer) as the sole product |

### 4.3.2 Biotransformation of totarol by laccase in a biphasic reaction system

Totarol was treated with laccase in reaction mixtures that contained ethyl acetate, toluene, chloroform or methyl-*t*-butyl ether. The formation of the laccase reaction products in reaction media that contained either ethyl acetate, chloroform or toluene was slow (with about 7 % conversion achieved after 4 d). The slow rate of oxidation in the biphasic systems could be attributed to insufficient mass-transfer of the reactants to and products from the enzyme and between two phases, as suggested by Faber (1994). No new products were observed in the reaction using methyl-*t*-butyl ether. The highest conversion (56.1 %) was achieved in the reaction using *t*-amyl alcohol. Qualitatively, the products obtained in the biphasic media were the same those of the monophasic system as confirmed by HPLC.

### 4.3.3 Effect of organic solvent on the nature of products obtained

The products of the reactions using laccase and totarol in various organic solvent systems were analyzed by HPLC and the ratio of **63** (C-C dimer) to **64** (C-O dimer) formation was compared. It was observed that C-C dimer was the favoured reaction product in monophasic systems. For instance, in acetone, 85 % of the reaction product was the C-C dimer whereas the C-O dimer was only present to 3 % in this medium (Table 4.3). However, in biphasic systems, particularly using *t*-amyl alcohol, there was an increase in the quantity of C-O dimer. The reaction products were 38.6 % C-C dimer and 9.9 % C-O dimer as reported in Table 4.4. Generally, C-C linked totarol dimer was a dominant product in all reactions conditions used. This could be attributed either to electronic or steric effects; the formation of C-C totarol dimer could be more sterically favoured than formation of C-O-C linked totarol dimer, while the altered ratio of C-C totarol dimer/C-C totarol dimer obtained by changing the solvent could be due polarity differences. Recently, Nicotra *et al.* (2004) observed a similar effect in the oxidation of tetrahydro-2-napthol or 17β-estradiol catalyzed by the laccases from *T. pubescens* or *Myceliopthora thermophyla*: the relative ratios of the dimeric products were strongly affected by the nature of the organic solvent present in the reaction solution.

**Table 4.3 Conversions of totarol by laccase in the presence of different cosolvents as detected by HPLC after 24 h.**

| Cosolvent (50% v/v) | Conversion % | % | | | C-O dimer/C-C dimer ratio |
|---|---|---|---|---|---|
| | | 62 (totarol) | 63 (C-C dimer) | 64 (C-O dimer) | |
| Acetone | 96.3 | 3.7 | 85.0 | 3.3 | 3.7:100 |
| Acetonitrile | 71.3 | 28.7 | 60.3 | 3.6 | 5.6:100 |
| Methanol | 62.6 | 37.4 | 49.1 | 6.0 | 10.9:100 |

**Table 4.4 Conversions of totarol by laccase in biphasic reaction medium systems as detected by HPLC for each reaction after 4 d.**

| Organic solvent | Conversion % | % | | | C-O dimer/C-C dimer ratio |
|---|---|---|---|---|---|
| | | totarol | C-C dimer | C-O dimer | |
| Ethyl acetate | 6.8 | 93.2 | 1.0 | 0.4 | / |
| Chloroform | 7.2 | 92.8 | 0.3 | 0 | / |
| Methyl *t*-butyl ether | 0 | 100 | 0 | 0 | / |
| *t*-Amyl alcholol | 56.1 | 43.9 | 38.6 | 9.9 | 20 : 100 |
| Toluene | 7.7 | 92.3 | 0.7 | 0.7 | / |



### 4.3.4 Optimization of totarol reactions: effects of buffer concentration, pH temperature and organic solvents

In this study, an attempt was made to optimize the reactions conditions for higher conversions of totarol to its dimers. Therefore, the effects of buffer concentration in the bioconversion of totarol were investigated, based on the previous reports that these parameters would affect enzyme stability and activity (Kim and Nicell, 2006). No significant improvement was achieved in bioconversion as a result of using either lower or higher salt concentration (results not shown).

However, the pH of the reaction mixture did affect the bioconversion of totarol, the best performances being achieved at pH 4.5-5. These results are consistent with various literature reports which state that laccase enzyme is more stable around pH 5 (Nikitina *et al.*, 2005).

Also investigated was the effect of temperature on the bioconversion of totarol. The conversion of totarol at various temperatures is shown in Figure 4.5. The data shows that the conversion of totarol achieved increased with temperature up to an optimum temperature 30 °C, and beyond this temperature conversion decreased. Under these optimized reaction conditions a 62.6 % conversion of totarol was observed after 24 h, as demonstrated by HPLC.

The incomplete biotransformation in reactions that used methanol as cosolvent, for instance, was mainly due to substrate precipitation even at low concentration used (1 mg/ml). In order to improve products yields, several other water miscible cosolvents were considered and, among them, acetonitrile and acetone proved to be the most suitable ones. Reactions were performed on a semi-preparative scale (100 mg of totarol) in the presence of 50 % v/v of these solvents as well as of methanol (for the sake of comparison). As reported in Table 4.3, the best results were obtained in the presence of acetone, which allowed an almost quantitative conversion of totarol (96.3 %), as dectected by the HPLC, and the C-C totarol dimer, product **63**, was the major product of this optimized reaction, constituting 85 % of the total product. The increased conversion of totarol by laccase in the reaction medium containing acetone could be attributed to the increased solulibity of totarol under these reaction conditions. Thus, the availability of totarol for laccase oxidation could be dependent on the solubility of totarol itself in the reaction medium. This result is consistent with generally known concept that catalytic activity of the enzymes strongly depends on their ability to bind the substrate (Rodakiewicz-Nowak and Jarosz-Wilkołazka, 2007). Although the stability of laccase obtained from *T. pubescens* in acetone containing medium was not evaluated in this study, higher conversion of totarol suggests that laccase retained its native conformation and a high catalytic activity. The results obtained in this study demonstrate that totarol dimer synthesis catalysed by laccase requires only mild conditions, an important factor for development of environmentally benign processes.

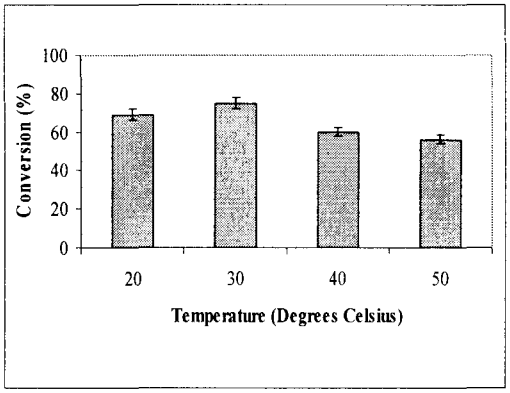



**Figure 4.5 Effects of temperature on the bioconversion of totarol by laccase. The reaction mixture contained 1 mg totarol in 1 ml methanol and 10 mg (6 U) *T. pubescens* laccase dissolved in 1 ml 20 mM sodium acetate buffer at pH 5. The samples were analysed after 4 h of incubation, using HPLC.**

### 4.3.5 Biological activities of C-C totarol dimer produced from totarol-laccase reactions

#### 4.3.5.1 Antioxidant activity

The antioxidant activity of totarol and the C-C totarol dimer, **63**, were measured using the DPPH radical method. This part of the study reports for the first time the antioxidant activity of totarol and the novel totarol C-C dimer. Totarol and the C-C totarol dimer were dissolved in methanol at initial concentration of 0.7 mM. Both of these compounds reacted slowly with DPPH radical. Totarol had quenched 23 % of the DPPH radical after 100 min while C-C totarol dimer quenched 7 % after 100 min (Figure 4.6) Increasing the concentration of totarol to 1.4 mM resulted in an increase of the amount of DPPH radical quenched (i.e. 49 % was quenched after 100 min as shown in Figure 4.6).

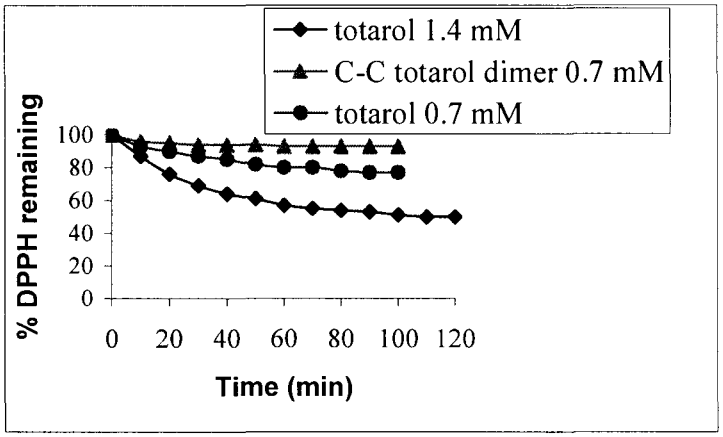



**Figure 4.6 DPPH assay on totarol and C-C totarol dimer (63), the product of totarol-laccase reaction.**



These results suggest that totarol has higher antioxidant activity than its dimer. These results are not consistent with those in Chapter 3 where it is reported that poly (8-hydroxyquinoline) and tyrosol dimer (Chapter 2), the products of laccase reactions, had higher antioxidant activity than that of parent compounds. Furthermore, the results are not consistent with the common suggestion that increased numbers of hydroxyl groups in a compound result in a higher antioxidant activity. For instance, the high antioxidant activity of gallates is explained by the presence of galloyl moiety attached at the flavan-3-ol at the 3 position, adding three more hydroxyl groups. Soobrattee *et al.* (2005) reported that among reports they studied, the greatest antioxidant activity was observed in the dimeric procyanidin B1 and B2, and the high antioxidant activity of the procyanidin dimers is attributed to their hydroxyl functions that are potent hydrogen donators. However, in the present study, the increased number of hydroxyl groups in a totarol dimer did not result in an increase of antioxidant activity. The decreased antioxidant activity may be attributable to the bulky structure and conformation of dimer **63** which could result into less availability of hydroxyl groups able to donate the hydrogens required to quench the radicals. These results are in also agreement with the report by Ting (2004) who suggested that the decreased antioxidant of activity of an isoeugenol dimer, as compared to its monomer, was due to steric hindrance. The results are also consistent with the report that higher molecular weight procyanidins, (although not dimers), showed least activity in the epicatechnin series,

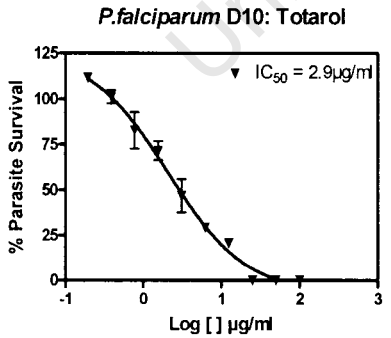
possibly due to increasing crowding and less availability of those affected hydroxyls to readily donate hydrogen (Lu and Foo, 2000).

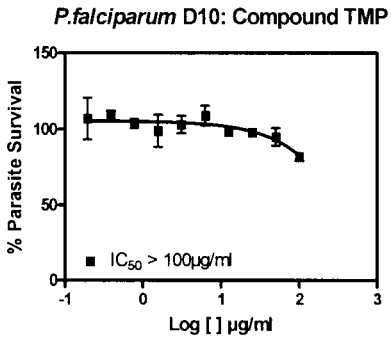
### 4.3.5.2 Antimalarial activity of totarol and C-C totarol dimer obtained from totarol-laccase reaction

Values of $IC_{50}$ against *P. falciparum* were determined as described previously (see Section 4.2.8.2). The 50 % inhibitory concentration ($IC_{50}$) values for the compounds were obtained from the dose-response curves, using non-linear dose-response curve fitting analyses with GraphPad Prism v.3.00 software. The $IC_{50}$ value of C-C totarol dimer, **63**, was greater than 100 µg/ml. Thus these results show that a C-C totarol dimer has no *in vitro* antiplasmodial activity against the sensitive strain of the parasite (Table 4.5 and Figure 4.7), while the $IC_{50}$ value for totarol was comparable to those obtained by Clarkson (2003).

**Table 4.5 *In vitro* antiplasmodial activity against *P.falciparum* CQS D10 strain**

| Compound | Mass (mg) | $IC_{50}$ (µg/ml) |
|---|---|---|
| **C-C Totarol dimer 63** | 2.2 | >100 |
| **Totarol 62** | 2.5 | 2.9 |
| **CQ (ng/ml)** | | 19.2 |



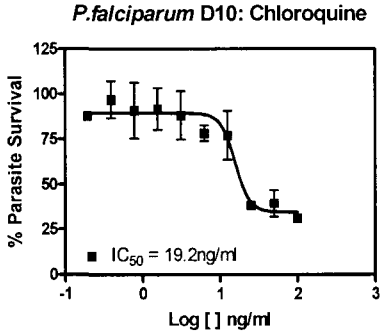



**Figure 4.7 Dose-response curves of compounds (totarol, chloroquine, and C-C totarol dimer 63 (compound TMP) against CQS D10 strain of *P.falciparum*.**

From the *in vitro* assay results presented in Table 4.4 and Figure 4.7, it is apparent that dimerisation of totarol by laccase did not result in improved activity but instead, the activity was lost. Although the C-C totarol dimer, **63**, was the only compound tested in this study, the preliminary inference that can be drawn from these results is that hydroxyl group in the aromatic region of the totarol plays an important role in the antimalarial activity. Thus introduction of a new, larger moiety adjacent to this hydroxyl group might lead to high steric hindrance and hence the decreased effect of this group in antimalarial activity. The loss of antimalarial activity could also be due to fact that the new product does not comply with Lipinki's Rule which suggests that the success of compounds as oral drugs depends on absorption, distribution, metabolism, excretion and toxicity (ADMET) properties.

From the structure-activity relationship (SAR) data obtained in this study, and with some few comparisons from literature, it can be generalized that dimerisation reduces the biological activity of the parent compound, and further affirms that oxidation by laccase alters the activity of the parent compound.

## 4.4 Conclusion

In an investigation to extend our understanding of laccase biotransformation of phenolic compounds, totarol was oxidized by laccase obtained from *T. pubescens*, to yield C-C and C-O totarol dimers (compounds **63** and **64** respectively). No polymeric product was observed in this reaction when the products were analyzed using MALDI-TOF spectrometry. Therefore the results suggested that the reaction produced bulky products which could not react further with other radicals in the medium. This phenomenon is particularly desirable if the target product is a dimeric or trimeric product.

The effect of organic solvents on the nature of these dimers was evaluated. It was discovered that the C-C linked dimer was a dominant product in most of the organic solvents tested. However, production of C-O linked dimer was increased in a reaction mixture that contained *t*-amyl alcohol. Thus, from this study, it is also apparent that reaction selectivity is controllable by manipulation of the reaction medium. These results are consistent with those reported in Chapter 2 where it was found that hydroxytyrosol-laccase reaction can either produce dimer or trimer depending on the type of organic solvents used, and with results reported in Chapter 3 where it was found that the polymeric products of 8-hydroxyquinoline differ, especially with the respect to the particle size, as a result of change of organic solvent. In essence, the results obtained in this study confirm that although laccase is responsible for the production of radicals, it is not necessarily directly involved in the structures of the final products. As a result, manipulation of the reaction medium provides a potential route to control of the reactions and formation of specifically selected products

The results obtained in this part of this study also serve to advance the potential application of biocatalysts, particularly laccase, in organic synthesis. It is clear that C-C or C-O bond formation, which are important chemical reactions, are achievable with enzymatic synthesis. This is especially useful where complex natural compounds such as totarol are involved. Thus, the methods obtained in this study can be explored further for other natural compounds of

pharmaceutical importance. The reaction conditions applied in this study were generally mild, and therefore this study has also demonstrated the potential of developing new environmentally friendly oxidation reactions, that representing an important field in green chemistry, and supporting the development of sustainable processes.

Further, this part of the study sought to elucidate the relationship between the structure of laccase-totarol reaction products and their biological activity. It was observed that antioxidant activity and antimalarial activity of the C-C totarol dimeric product were lower than totarol itself. This was attributed to steric hindrance preventing the further reaction of this dimeric product with other molecules. Although, the biological activity of C-O totarol dimer was not evaluated in this study, we would expect, on the basis of the C-C dimer properties, that both its antioxidant and antimalarial activities would be lower than that of C-C totarol dimer. The reason for this would be the fact that C-C totarol dimer contained two hydroxyl groups whereas C-O totarol dimer had only one hydroxyl group, and as it is generally accepted that increased numbers of hydroxyl groups in a compound result in a compound with higher antioxidant activity.

The results obtained in this study, and also those reported in literature, suggest that there is close relationship between the structure of the compound and its antioxidant activity. However, this kind of relationship cannot be regarded as "one size fit all". Increasing the number of hydroxyl groups by dimerisation or polymerization of phenolic structures does not necessarily lead to higher antioxidant activity. Nevertheless, antioxidant activity is a phenomenon which can also be linked to structural configuration. The ideal structure of a compound that could lead to higher antioxidant activity should have low steric hindrance, for instance, to ensure the availability of hydroxyl groups that can donate hydrogens. It is thus apparent that for this to be achieved, oxidation of phenolic compounds by laccase needs to be controlled and rationalized. For, instance, the 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 delocalization, a characteristic among others that may lead to higher antioxidant activity.

# Chapter 5

## General discussion and conclusions

The broad aim of this study was to investigate the reactions of *T. pubescens* laccase with phenolic compounds of low and high molecular weight. In the elucidation of these reactions the emphasis was placed on the effect of steric properties and the influence of organic solvents on the nature of the products. The target substrates included tyrosol, hydroxytyrosol, 8-hydroxyquinoline and totarol. All of these compounds have recognised biological activity, both as antioxidants and antimicrobials. The products of the laccase-catalysed reactions were expected to have biological activities different from those of the starting materials, and in particular, the antioxidant activity of these compounds was considered to be of importance.

The tyrosol and hydroxytyrosol, in particular, are known to be found in wastewaters. At higher concentrations these compounds are thought to have a negative impact on the living organisms in water, and therefore removal of these compounds from water could be an important step towards a clean environment and sustainable development.

Therefore the specific objectives of this study were:

- to develop new laccase reactions for production of new compounds
- to investigate the biological activity and potential value of the reaction products and
- to investigate the potential application of these reactions in waste removal bioprocesses.

These objectives were accomplished, and the major findings of this work can be summarised as follows:

**i)** The novel enzymatic process to synthesize poly (8-hydroxyquinoline) using laccase obtained from *T. pubescens* in acetone, methanol or ethyl acetate-sodium acetate buffer medium, was achieved. From this study, we established that the degree of

polymerisation of 8-hydroxyquinoline was controllable by using acetone at various concentrations. Furthermore, it was established that various organic solvents influence the polymer particle size, with water and aqueous methanol favouring formation of small particles while acetone and ethyl acetate favoured formation of larger particles. Although the activity of laccase was lost during the process as a result of the acidic reaction medium, and possibly also due to accumulation of the product, 80 % conversion of 8-hydroxyquinoline was achieved at 40 °C. The polymeric product, with average molecular weight 780 m/z, was tested for antioxidant activity. This polymeric product was found to have antioxidant activity which was 10 % higher than that of its parent compound.

**ii)** Tyrosol was oxidised by laccase obtained from *T. pubescens* in sodium acetate buffer medium containing various cosolvents such as methanol, acetone or ethyl acetate. The reaction products were largely polymeric, and the polymeric product was observed in the reaction medium within 3 hours. The higher polymerisation reactivity of this compound was due to its hydroxyl group at the *para* position. The studies by Gianfreda *et al.* (2003) had reported that *meta*-tyrosol was less reactive in reactions catalysed by laccase. This was a confirmation that laccase reactions are also dependent on the nature of substrate structure or position of the functional groups. One of the reaction products was characterised by LC-MS and $^{1}$H-NMR. This product, a tyrosol dimer, was similar as that produced using laccase from *Lentinus edodes* by Vinciguerra *et al.* (1996). The antioxidant activity of this product was evaluated and found that to have higher antioxidant activity (45 %) as compared to tyrosol (3 %), the parent compound. This improved antioxidant activity was thought to be attributable to enhanced *pi*-electron delocalization in the dimeric product.

**iii)** In a comparative study, hydroxytyrosol was oxidised by laccase obtained from *T. pubescens*. Since hydroxytyrosol is not commercially available, it was necessary to develop the production of this compound, through work based on that of Espín *et al.* (2001). The conversion of tyrosol to hydroxytyrosol, as mediated by tyrosinase, was highest under the conditions where the reaction medium was supplemented with 2

mM SDS, the pH was at 7, and the temperature was 40 °C. It was also established that the use of immobilised tyrosinase could potentially lead to higher yields, using a continuous packed-bed reactor. The hydroxytyrosol was oxidised by laccase in presence of various organic solvents. The reaction products were characterised by HPLC and LC-MS. The mixture of products (comprising polymeric products) was obtained when the reaction was performed at 20 % methanol. The dimeric product was the main product in a reaction mixture that contained 50 % acetone, and the product **59** as main product of the reaction in a reaction mixture containing 50 % methanol, indicating that organic solvents can be used to control polymerisation of hydroxytyrosol. Since separation of these products was problematic, the polymeric, product **59** and dimeric products was initially assayed as a mixture, for antioxidant activity, and compared with that of pure hydroxytyrosol. It was found that the reaction of this mixture with DPPH radical was very slow as compared with pure hydroxytyrosol. However, 100 % reduction of DPPH radicals by this mixture was achieved after 4 hours. Furthermore, product **59,** once purified, was found to have less antioxidant activity as compared with the mixture of products. From these results, it was concluded that the product mixture had improved antioxidant activity due to some some synergistic mechanisms, consistent of the recognised fact that mixtures of phenolic compounds may have higher total antioxidant activity than isolated compounds.

**iv)** Besides oxidation by laccase of low molecular weight compounds, this study also reports for the first time the dimerisation of the relatively complex natural compound totarol by laccase from *T. pubescens*. The reaction was conducted both in monophasic organic medium and biphasic organic medium, and products were analysed by MALD-TOF analysis. This system was characterised by the absence of polymeric products, in contrast to the results for smaller substrates summarised above. The explanation of these results was based on the fact that the totarol molecule is large, and therefore, addition of a second molecule increases the steric hindrance even further, and changes the molecular conformation to the point where further polymerisation is not favoured. The products obtained were also characterised by

HPLC, ESI-MS and NMR, and they were found to include a C-C linked dimer and a C-O linked dimer. Again, organic solvents were used to influence the selectivity of the reaction. The dominant product in terms of the percentage yield was the C-C dimer. The ratio of C-C to C-O dimer was found to increase with the use of polar organic solvents such as acetone and acetonitrile. However, the C-O dimer production was increased in a reaction that contained *t*-amyl alcohol. It was suggested that the nature of the reaction medium had a profound effect on the final product. The C-C dimer was assayed for both antioxidant and antimalarial activity and both activities were reduced as compared to the parent compound. This reduction in activity was attributed to increased steric hindrance, and hence it was concluded that the biological activity of the compound was dependent on its structural configuration.



## 5.1. Implications of the research carried out in this study

Biotransformation of 8-hydroxyquinoline, tyrosol, hydroxytyrosol and totarol by *T. pubescens,* and the investigation of the biological activity of the products of these reactions, culminated in findings that have implications in various aspects of biocatalysis and medicinal chemistry. The implications of this study can be considered in terms of potential application of the product or process in organic synthesis, in new information regarding structure-biological activity relationships, and in bioremediation.

At the fundamental level, this study provides new evidence on the type of substrate that laccase can oxidise and the possible products. It is becoming clear that laccases can react with both higher molecular weight substrates (totarol) and low molecular weight substrates (tyrosol, hydroxytyrosol and 8-hydroxyquinoline). From the results obtained from this study it was evident that the nature of laccase reaction products was also dependent on the structural configuration of the substrate. Polymeric products were obtained when low molecular weight compounds with no steric hindrance on the reactive hydroxyl groups where the reaction with laccase is initiated. Conversely, lower molecular weight oligomers (dimers or trimers) may be obtained when a high molecular weight compound with some degree of steric hindrance is used as a substrate for

laccase, as in the case of totarol. The nature of products isolated from laccase reactions are summarised in Table 5.1. When tyrosol-laccase and hydroxytyrosol-laccase reactions are compared, it was observed that polymerisation of hydroxytyrosol was readily controllable. This was thought be a result of the fact that another hydroxyl group in hydroxytyrosol could have altered the delocalization of electrons within the molecule. The radical would then be stabilized and hence become less reactive. This phenomenon could lead to lower molecular weight products. Although not evaluated in this study, the shape of the substrate molecules could also have an effect on the reactivity the radicals generated from these molecules. The planarity of totarol dimers, for instance, may increase electron delocalization, and thus result in a stable compound or less reactive radicals that could not couple further to form polymeric products. Thus, in this study the preliminary assertion made was that the structure of the substrate could have an influence on the nature of the laccase reaction products. However, computational molecular orbital modeling will need to be perfomed in order to elucidate the effect of planarity, ionization potential, electron affinity and charge distribution on chemical reactivity of all four substrates (tyrosol, hydroxytyrosol, 8-hydroxyquinoline and totarol) and their intermediate radicals.

**Table 5.1 Summary of laccase reaction products**

| Starting material | Nature of the products |
|---|---|
| tyrosol | The major product was a polymer with molecular weight of 962 m/z (the linkages of monomers was not determined).<br>The minor product was a dimeric tetracylic ketone. The reactions could not be controlled using organic solvent to favour a dimeric product. |
| hydroxytyrosol | The major products were:<br>Polymeric products (1202 m/z) obtained from reaction media containing 20 % methanol,<br>C-C dimeric products (306 m/z) from reaction medium containing 50 % acetone and<br>Thus, the reaction was readily controllable using organic solvents |
| 8-hydroxyquinoline | Polymeric products (780 m/z) in a reaction mixture that contained 8 % acetone. |
| totarol | Dimeric products (571 m/z) linked by C-C or C-O bonds. |



The nature of the compounds produced in this study was also dependent on the reaction medium, particularly, the use of media containing organic solvents. The degree of polymerisation of hydroxytyrosol and 8-hydroxyquinoline, in particular, was controllable in media containing acetone or methanol. The degree of polymerisation of the above mentioned compounds decreased with an increase in acetone or methanol concentration in the reaction medium, and this was attributed to the decreased hydrophobic interactions of radicals that would normally couple to form dimeric or oligomeric to polymeric products. It has been suggested that, among other factors, hydrophobic interactions of molecules is enhanced in aqueous reaction medium as

opposed to organic solvent containing medium. The aqueous reaction medium favours the interaction of molecules, and hence the degree of polymerisation and self-aggregating of molecules is increased. The effect of organic solvents on the nature of laccase reactions products was also observed in reactions involving totarol. In these reactions, the ratio of C-C/C-O linked totarol dimers was influenced by nature of organic solvents used. Polar organic solvents such as acetone, acetonitrile or methanol contained in a reaction medium favoured the formation of C-C linked totarol dimer. In non-polar solvents such as *t*-amyl alcohol, the production of C-O-C linked totarol dimer was improved. This was an indication that radicals may interact differently with each other, and that was dependent on the nature of organic solvents used. The mechanism whereby the organic solvents have an effect is not apparent, and it would require further investigation. The ability to control reactions is desirable particularly if the compound produced is to be used for pharmaceutical purposes where biologically active isomers would need to be produced in large quantities.

The products were evaluated for biological activity with an aim of understanding the relationship between the structure of the compounds and their biological activity. Generally, these compounds showed different biological activities as compared with their parent compounds. The biological activities were either increased or decreased, dependent on the structural configuration. The increased antioxidant activity of poly (tyrosol) and poly (8-hydroxyquinoline), for instance, as compared to their monomers, was thought to be due to increased number of *ortho* or *para*-hydroxyl groups in these polymers or increased delocalisation of electrons due to double bonds conjugation. Hydroxyl groups, in particular, are regarded as reactive functional groups which are important for many biological activities, and in the case of antioxidant activity, hydroxyl groups is the source for hydrogen that is donated to the radical, and hence confering antiradical or antioxidant activity. Furthermore, the increased antioxidant activity could also be due to the stability of the new antioxidant radical formed after hydrogen abstraction, and this stability could be confered by the presence of C-C linkage of monomers which may allow more electron delocalisation within the whole molecule resulting in stable antioxidant radical. The stability of the antioxidant or phenoxy radical would allow the situation whereby more energy would be required to reverse the hydrogen-abstraction reaction, and therefore the forward reaction, leading to scavenging of DPPH radical, would be favoured. The stability of the antioxidant radical could

also depend on the structure of the antioxidants itself. It has been suggested that molecular planarity of the compound is also important for radical-scavenging ability of the antioxidant since it also allows more delocolisation of the electrons within the whole molecule (Fukuhara, 2006). In this study, the dimerisation of totarol by laccase, for instance, might have altered this compound to a non-planar dimeric compound resulting in less stable antioxidant, and hence decreased potential to scaveng more free radicals. Furthermore, the decreased antioxidant activity of C-C totarol dimer could be attributed to the steric effects. The bulky compound may result in steric hindrance, and thus the hydroxyl groups in C-C totarol may not be readily available for donation of hydrogen that quenches DPPH radicals. Again, molecular mechanics methods may be used to obtain an intermolecular distance scan of the intereaction energy between the antioxidant molecule and the radical molecule. A variety of molecular configurations could be generated using molecular dynamics methods to ensure that the optimum conformations are considered.

The implication of this study is that laccase reactions need to be controlled in order to obtain the desired products. The laccase reactions products would need to be modelled such that important requirements for antioxidant activity such as increased number of *para* or *ortho*-hydroxyl groups, steric effects and stability of the antioxidant radical are considered during the process of synthesis. Furthermore, the linkages of monomers (whether it is C-C or C-O linkage) would need to be controlled, as C-O linkage in particular may result to reduction of hydroxyl groups, and hence destruction of some moiety playing a significant role in antioxidant activity.

The laccase reactions developed in this study have potential application in bioremediation processes. The 8-hydroxyquinoline-laccase and tyrosol-laccase reactions were characterised by synthesis of the polymeric compounds that precipitated in solution, and the precipitate recovered physically by filtration. In these reactions, 8-hydroxyquinoline and tyrosol were completely removed from solution (converted to polymers). We envisage that this system can be used in the treatment of wastewaters contaminated with phenols. The polymeric products recovered in large quantities from these reactions could provide new products with novel properties. The polymeric products could be used as new materials for preventing auto oxidation in various polymers.

Polymers, due to the nature of their structure, make the stable antioxidants which are particularly desired for polymeric materials which readily undergo auto oxidation reaction.

In cause of this study, tyrosol was hydroxylated by tyrosinase to hydroxytyrosol which was further oxidised by laccase to yield hydroxytyrosol dimer, trimer or polymer depending on the reaction conditions. The advantage of this system is that each reaction step could be optimised for high yields of the desired product. The development of this dual enzyme reaction system, itself represents a significant and a useful process that could be applied in pharmaceutical industries for production of biological active compounds.

### 5.2 Future work

In addition to recommendations made in previous chapters, the biocatalytic processes developed in this study to synthesize dimeric and polymeric compounds presented some important issues which should be investigated further. It was observed that the activity of the laccase was lost particularly in the process of synthesizing poly (8-hydroxyquionoline). Thus future investigation should be focused on minimizing the lost of enzyme activity, and this could be achieved by utilization of immobilised laccase in the oxidation of compounds such as those used. Immobilization assists in retaining the activity of enzymes under adverse reaction conditions brought about the accumulation of products or the content of co-solvents in the medium. The effects of the immobilisation of laccase on the product yields and selectivity would need to be investigated.

From the characterisation of the laccase reaction products, it was apparent that the structure of dimeric compounds could be characterised with high degree of confidence using techniques such as NMR, HPLC and LC-MS. However, polymeric compounds proved to be more complicated, especially where a mixture of compounds existed in a sample. Thus more techniques should be explored in order to fully understand the structures of the polymers especially with respect to type of linkages of monomers (i.e. C-C or C-O linkage). Such information will further help in understanding the structure-activity properties of the products.

The long term goal of the study was to investigate the potential application of laccases in the development of biotransformation processes resulting to chemical compounds of economic importance, and therefore it is appropriate that the products (such as tyrosol, hydroxytyrosol and 8-hydroxyquinoline polymers) be investigated for other novel properties. For instance, future work could examine the advantages of producing smaller particles of polymer from biocatalytic reactions, as compared to larger particles. Also, potential application of the polymers in nanotechnology applications would need to be explored.

# Appendices

## Appendix A

### A.1 Solutions for protein SDS-PAGE

#### A.1.1 PMSF

Prepare a 100 mM solution in isopropanol

Aliquot and store at –20 $^{0}$C.

#### A.1.2 Separating gel (20 %)

| Constituent | Volume |
|---|---|
| Bisacrylamide (40 % acrylamide, 0.2 % bisacrylamide | 12.5 ml |
| 1.125 M Tris-HCl pH 8.8 | 8.35 ml |
| SDS (10 %) (w/v) | 0.250 ml |
| AMPS (10 %) (w/v) | 0.250 ml |
| $dH_2O$ | 3.65 ml |
| TEMED | 25 µl |

#### Stacking gel (6.5 %)

| Constituent | Volume |
|---|---|
| Bisacrylamide (40 % acrylamide, 0.2 % bisacrylamide | 2 ml |
| 0.375 M Tris-HCl pH 6.8 | 4 ml |
| SDS (10 %) (w/v) | 0.125 ml |
| AMPS (10 %) (w/v) | 0.300 ml |
| $dH_2O$ | 5.65 ml |
| TEMED | 20 µl |

**AMPS (10 %) (w/v)**

AMPS (5 g)

Make up to 50 ml with distilled water.

Kept at 4 $^{0}$C

Make fresh every 2 weeks.

**SDS (10 %) (w/v)**

SDS (10 g)

Make up to 100 ml with distilled water.

**1 X Running buffer**

Tris (3 g)

Glycine (30 g)

SDS (1 g)

Make up to 1000 ml with sterile distilled water.

**Sample application buffer**

125 mM Tris-HCl pH 6.8

SDS 4 % (w/v)

Glycerol (20 %) (v/v)

Make up to 10 ml with sterile distilled water.



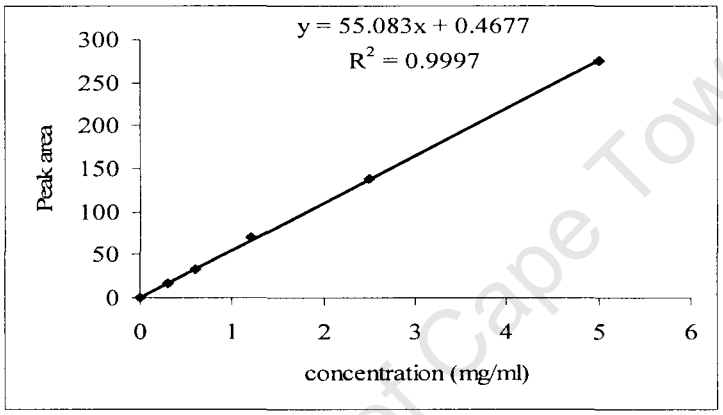
1 2 3 4

**SDS-PAGE analysis of partially purified laccase: lane 1, standard protein (molecular weight marker); Lane 2, laccase (not heated); lane 3, laccase (not heated); lane 4, laccase (heated for 5 min). A band with mass of 65 kDa appeared in lane 1 to lane 3.**

## Appendix B

## Quantification of hydroxytyrosol

Hydroxytyrosol was prepared in methanol at various concentrations and used to construct a hydroxytyrosol standard curve. In a calibration curve, the HPLC peak area was plotted against the known concentration of hydroxytyrosol. The concentrations of hydroxytyrosol fractions were calculated from equation of this curve.

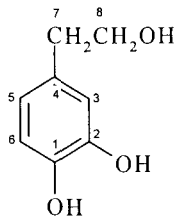



**HPLC calibration standard curve for hydroxytyrosol**

# Appendix C

## $^1$H-NMR spectral data of hydroxytyrosol obtained from biotransformation of tyrosol by tyrosinase

$^1$H-NMR ($CD_3OD$) *ä* 2.67 (*t*, 2H, $J_{7,8}$ = 7.2 Hz, H-7), 3.68 (*t*, 2H, $J_{1,2}$ = 7.2 Hz, H-8), 6.53 (*dd*, 1H, $J_{3,5}$ = 2.0 Hz, $J_{5,6}$ = 8.0 Hz, H-5), 6.66 (*d*, 1H, *J* = 2.0 Hz, H-3), 6.68 (*d*, 1H, *J*= 8.0 Hz, H-6) ( Capasso *et al.*, 1999).




**product 25**

**Hydroxytyrosol structure**

# Appendix D

## Calculation of amount of hydroxytyrosol produced from fixed-packed bed reactor

Flow rate = Q = 2.5 ml/min or 0.0025 l/min

Differentiation of the area under the curve in Figure 3.9 (Mathcad 2001 programme was used)

$Y = Ax/1 + Bx,$

Where A = 1.73186E+07 and B = 1.11269E + 08

The total hydroxytyrosol produced in a packed-bed reactor was calculated using the area of the curve was found to be 35 mg over 240 min.

# Appendix E

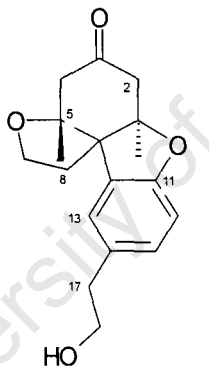
## TLC plate was stained by Pancaldi solution

The progress of the reaction was monitored by TLC (eluent, petroleum ether: ethyl acetate at a ratio of 19: 1 and the visualisation of spots was done by staining TLC plate with PANCALDI solution (21 g ammonium molybdate tetrahydrate, 7 g cerium (IV) sulphate-4-hydrate and 31 ml sulphuric acid concentrated) and the plate dried in the oven (Gerber-Lemaire *et al.*, 2006)

## Appendix F

**$^1$H-NMR of tyrosol dimer 50, the product obtained from the reaction of laccase with tyrosol (coupling constant *J* expressed Hz)**

$^1$H-NMR (400 MHz, $CDCl_3$): δ: 2.75 (1H,dd, J =18, 35) 2.32 (1 H, dd, J = 18.25, H-2); 3.92 (1H,br t, J =3.5, H-3); 4.76 (1H,br t, J = 3, H-5); 3.04 (1H,dd, J =18, 3) 2.94 (1H,dd, J =18, 3, H-6), 4.04 (1H,dt, J =23, 85) 3.94 (1H,dt, J =22.5, 3, H-8); 2.6 (1H,ddd, J =13.5, 9.5, 6.5) 2.0 (1H,ddd, J =13.5, 8.5, 6.5, H-9); 7.34 (1H,d, J =2, H-13); 7.2 (1H,dd, J =8,2, H-15), 6.92 (1H,d, J =8, H-16); 3.08 (1H,t, J =7, H-17); 4.14 t (1H,dd, J =7, H-18) ( Vinciguerra *et al.*, 1997)




**product 50**

## Appendix G

### NMR spectral data of totarol dimers (63 and 64) obtained from biotransformation of totarol by laccase

**Totarol (62) TLC, $R_f$: 0.40; HPLC, $R_t$: 7 min**

$^1$H-NMR (400 MHz, $CDCl_3$): δ: 7.03 (1 H, d, $J$ = 8.5 Hz, H-11); 6.54 (1 H, d, $J$ = 8.5 Hz, H-12); 3.31 (1 H, sept, $J$ = 7.0 Hz, H-15); 2.96 (1 H, dd, $J_1$ = 17.1 Hz, $J_2$ = 6.3 Hz, H-$7_{eq}$); 2.78 (1 H, ddd, $J_1$ =17.1 Hz, $J_2$ = 11.5 Hz, $J_3$ = 7.8 Hz, H-$7_{ax}$); 2.25 (1 H, dt, $J_1$ = 12.7 Hz, $J_2$ = 3.7 Hz, H-$1_{eq}$) and 1.36 (1 H, td, $J_1$ = 12.8 Hz, $J_2$ = 3.8 Hz, H-$1_{ax}$); 1.94 (1 H, ddt, $J_1$ = 13.3 Hz, $J_2$ =7.8 Hz, $J_3$ = 1.8 Hz, H-$6_{eq}$) and 1.67 (1 H, m, H-$6_{ax}$); 1.75 (1 H, qt, $J_1$ = 13.7 Hz, $J_2$ = 3.4 Hz, H-$2_{ax}$) and 1.61 (1 H,d quint, $J_1$ =13.8 Hz, $J_2$ = 3.7 Hz, H-$2_{eq}$); 1.49 (1 H, ddt, $J_1$ = 13.2 Hz, $J_2$ = 3.7 Hz, $J_3$ = 1.4 Hz, H-$3_{eq}$) and 1.22 (1 H, td, $J_1$ = 13.4 Hz, $J_2$ = 4.1 Hz, H-$3_{ax}$); 1.29 (1 H, dd, $J_1$ = 12.7 Hz, $J_2$ =2.2 Hz, H-5); 1.38 and 1.36 (3 H each, d each, J = 7.1 Hz, $CH_3$-16 and $CH_3$-17); 1.20 (3 H, s, $CH_3$-20); 0.97 (3 H, s, CH3-18); 0.94 (3H, s, $CH_3$-19).

$^{13}$C-NMR (100 MHz, $CDCl_3$): δ: 152.6 (C-13); 144.0 (C-9); 134.7 (C-8); 131.7 (C-14); 123.7 (C-11); 115.0 (C-12); 50.3 (C-5); 42.3 (C-3); 40.4 (C-1); 38.4 (C-10); 33.9 (C-18); 29.4 (C-7); 27.8 (C-15); 25.8 (C-20); 22.3 (C-19); 21.1 (C-16 and C-17); 20.2 (C-2); 20.1 (C-6).

**Totarol-laccase reactionproduct (63, symmetric C-C dimer) TLC, $R_f$: 0.90; HPLC, $R_t$: 12 min**

$^1$H-NMR (400 MHz, $CDCl_3$): δ: 7.03 (2 H, s, H-11 and H-11'); 3.35 (2 H, m, H-15 and H-15'); 3.02 (2 H, dd, $J_1$ = 17.2 Hz, $J_2$ = 6.4 Hz, H-$7_{eq}$ and H-7'$_{eq}$ ); 2.82 (2 H, m, H-$7_{ax}$ and H-7'$_{ax}$); 2.25 (2 H, br d, $J$ = 12.6 Hz, H-$1_{eq}$ and H-1'$_{eq}$); 1.97 (2 H, br dd, $J_1$ = 13.2 Hz, $J_2$ = 7.9 Hz, H-$6_{eq}$ and H-6'$_{eq}$); 1.72 (4H, m, H-$6_{ax}$, H-6'$_{ax}$, H-$2_{ax}$, H-2'$_{ax}$); 1.60 (2 H, br d quint, $J_1$ = 13.9 Hz, $J_2$ = 3.4 Hz, H-$2_{eq}$ and H-2'$_{eq}$); 1.49 (2 H, br d, $J$ = 13.1 Hz, H-$3_{eq}$ and H-3'$_{eq}$); 1.40-1.20 (6 H , m, H-$1_{ax}$, H-1'$_{ax}$, H-$3_{ax}$, H-3'$_{ax}$, H-5, H-5'); 1.40 and 1.38 (6 H each, d each, $J$ = 7.1 Hz, $CH_3$-16 and $CH_3$-16', $CH_3$-17 and $CH_3$-17'); 1.22 (6 H, s, $CH_3$-20 and $CH_3$-20'); 0.99 (6 H, s, $CH_3$-18 and $CH_3$-18'); 0.95 (6H, s, $CH_3$-19 and $CH_3$-19').

$^{13}$C-NMR (100 MHz, $CDCl_3$): δ: 150.4 (C-13 and C-13'); 144.0 (C-9 and C-9'); 135.3 (C-8 and C-8'); 132.7 (C-14 and C-14'); 125.1 (C-11 and C-11'); 121.5 (C-12 and C-12'); 50.3 (C-5 and

C-5'); 42.3 (C-3 and C-3'); 40.3 (C-1 and C-1'); 38.5 (C-10 and C-10'); 34,0 (C-4 and C-4'); 33.9 (C-18 and C-18'); 29.5 (C-7 and C-7'); 28.4 (C-15 and C-15'); 26.0 (C-20 and C-20'); 22.3 (C-19 and C-19'); 20.9 (C-16 and C-16', C-17 and C-17'); 20.2 (C-2 and C-2'); 20.1 (C-6 and C-6').

ESI-MS, positive mode: 593,43389 $[M + Na]^+$ (theoretic: 593.43290).

**Totarol-laccase reaction product (64, C-O dimer) TLC, $R_f$: 0.60; HPLC, $R_t$: 15.0 min**

$^1$H-NMR (400 MHz, $CDCl_3$): selected data δ: 7.04 (1H, d, $J$ = 8.8 Hz, H-11); 6.67 (1 H, s, H-11'); 6.56 (1H, d, $J$ = 8.8 Hz, H-12); 3.38 and 3.30 (1 H each, m, H-15 and H-15'); 3.02 and 2.95 (1 H each, dd each, $J_1$ = 16.9 Hz, $J_2$ = 6.1 Hz, H-$7_{eq}$ and H-$7'_{eq}$ ); 2.84 and 2.75 (1 H each, ddd each, $J_1$ =16.9 Hz, $J_2$ = 9.1 Hz, $J_3$ = 5.3 Hz,, H-$7_{ax}$ and H-$7'_{ax}$); 1.42 and 1.41, 1.40 and 1.38 (3 H each, d each, $J$ = 7.1 Hz, $CH_3$-16 and $CH_3$-16', $CH_3$-17 and $CH_3$-17'); 1.22 and 1.14 (3 H each, s, $CH_3$-20 and $CH_3$-20'); 0.99 and 0.96 (3 H each, s, $CH_3$-18 and $CH_3$-18'); 0.95 and 0.91 (3H each, s, $CH_3$-19 and $CH_3$-19').

$^{13}$C-NMR (100 MHz, $CDCl_3$): δ: 148.2 and 146.1 (C-13 and C-13'); 145.0 and 143.0 (C-9 and C-9'); 135.7 and 134.8 (C-8 and C-8'); 132.4 (C-14); 128,9 (C-14'), 123.9 (C-11); 115.7 (C-12); 113.3 (C-11'); 50.5 and 50.3 (C-5 and C-5'); 42.3 (C-3 and C-3'); 40.3 (C-1 and C-1'); 38.6 (C-10 and C-10'); 34.0 (C-4 and C-4'); 33.9 (C-18 and C-18'); 29.5 and 29.1 (C-7 and C-7'); 28.5 and 28.2 (C-15 and C-15'); 25.8 (C-20 and C-20'); 22.3 (C-19 and C-19'); 21.8 and 20.9 (C-16 and C-16', C-17 and C-17'); 20.1 (C-2 and C-2', C-6 and C-6').

ESI-MS, positive mode: 593,43130 $[M + Na]^+$ (theoretic: 593.43290).

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