BIOCATALYTIC PRODUCTION OF NEW ANTIOXIDANT COMPOUNDS AND THE CHARACTERIZATION OF THEIR ANTIOXIDANT EFFECTS

by

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DECLARATION

I, Oluyemisi Elizabeth Adelakun, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

	30 July 2012
Signed	**Date**

ABSTRACT

Antioxidants are an important class of compounds that quench reactive free radical intermediates formed during oxidative reactions. They prevent oxidative reactions in food and protect biological tissues against oxidative damage. Plant phenols and phenolic acids are increasingly becoming a subject of intensive research due to their bioactive properties such as antioxidant, anti-mutagenic, anti-viral and anti-inflammatory activity. Modification of the structure of natural phenolic compounds can be achieved through the use of enzymes in biocatalysis reactions with the potential to enhance the antioxidant capacity of these natural phenolic compounds. The work reported here employed the oxidative enzyme, laccase from *Trametes pubescens*, in the modification of the antioxidant phenolic molecules, ferulic acid and 2,6-dimethoxyphenol (2,6-DMP) as a way of enhancing their antioxidant capacity. In addition, various phenolic compounds were focused upon for coupling reactions, with the aim to increase the antioxidant capacity of the compounds.

The *T. pubescens* strain was cultured in a 4L airlift reactor and extracellular laccase production was monitored using the standard ABTS assay. The enzyme was isolated and purified from the culture filtrate once optimal enzyme production was detected. The enzyme was purified using standard ammonium sulphate precipitation and dialysis. Enzymatic modification of phenolic compounds with laccase was carried out in a biphasic system (solvent is immiscible with water but the enzyme is solubilized in the water phase) or in a monophasic system (solvent is miscible with water and the enzyme is in suspension in the miscible liquid). Products were separated by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC), purified by flash chromatography, and characterized by liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR). Antioxidant activity was determined by 2,2'-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical-scavenging activity.

Preliminary small scale reactions of coupling ferulic acid monomers, 2,6-DMP monomers and the possibility of coupling quercetin or catechin, a known and active antioxidant onto other phenolic compounds (gallic acid, vanillic acid, caffeic acid, guaiacol, 2,6-DMP, pyrogallol, vanillin, eugenol, isoeugenol, protocatechuic acid and catechol) with laccase were performed with the aim of increasing the antioxidant activity over and above that of the starting materials. Coupling of catechin or quercetin onto other phenolic molecules was successful as observed by LC-MS, but the final yield of the products was very low for most of the experiments. The only coupling reaction that produced a significant amount of pure product was quercetin onto catechol. The coupling product showed higher antioxidant activity than

catechol but slightly lower activity than quercetin. Similarly, reactions using ferulic acid and 2,6-DMP yielded sufficient amounts of product to allow for the optimization of scale-up reactions.

Biocatalysis reactions with ferulic acid yielded two dimeric products (*m/z* 385.1) which were purified and characterized as β-5 and β-β dimers. In the monophasic system, the type of organic co-solvent used influenced the dimer formed: the β-5 preferentially formed in dioxane while β-β formation was enriched in ethanol. In the biphasic system, there was an increase in the formation of the dimers as the concentration of ethyl acetate was increased from 80 to 95% (v/v). The β-5 dimer showed higher antioxidant capacity than the ferulic acid monomer. In the reaction of 2,6-DMP, however, only one dimeric product (*m/z* 305.1) was purified and characterized: 3,3',5,5'-tetramethoxydiphenylquinone. In the monophasic system, the dimer was preferentially formed in acetone as co-solvent while in the biphasic system, formation of the dimer increased as the concentration of ethyl acetate was increased from 50 to 90% (v/v). The dimer showed higher antioxidant capacity than the monomer (2,6-DMP) as demonstrated by standard antioxidant assays (DPPH, FRAP and TEAC). From these results it is clear that alteration of the reaction conditions may influence laccase-mediated oxidation of phenolic compounds to form dimers with higher antioxidant capacity than the monomers. All products obtained from this study could find useful application in the health and cosmetic industries, although the biocatalytic processes require time (mostly 24hrs reaction for dimeric products and 5hrs reaction for coupling products), and observation of modest increases in antioxidant activity of the products as compared to the starting materials, but, the exploration of "green" ways of enhancing these bioactive properties over chemical treatment will be benefitial to health.

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DEDICATION

This project is dedicated to Almighty God, who gave me the strength throughout the study, and to the memory of my Late Father figure, Dr Olatunbosun Adefolaju whose death made me to be more curious about antioxidants.

LIST OF PUBLICATIONS

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

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GLOSSARY

Terms/Acronyms/Abbreviations	Definition/Explanation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
TLC	thin layer chromatography
HPLC	high performance liquid chromatography
LC-MS	liquid chromatography mass spectrometry
NMR	nuclear magnetic resonance
UV light	ultraviolet light
TEAC	Trolox equivalent antioxidant activity
FRAP	Ferric reducing antioxidant power
DPPH	2,2’-Diphenyl-1-picrylhydrazyl

CHAPTER ONE

INTRODUCTION

1.1 Introduction

Over the past few years there has been an increased interest in natural phenolic compounds, mostly due to their antioxidant capacity and potential health benefits (Penarrieta *et al.*, 2011). Phenolic compounds are part of a number of secondary metabolites in most plants which aid in the protection against insects, infections, pathogen attack and UV radiation (Del Mar Verde Mendez *et al.*, 2004; Manach, 2004). Generally, antioxidants are an important class of compounds that when present at low concentrations relative to an oxidizable substrate, significantly delay, retard or inhibits oxidation of that substrate (Halliwell and Gutteridge, 1999). Phenolic antioxidants in particular are compounds that act as terminators for free radicals (da Silva Oliveira *et al.*, 2011).

Free radicals have extremely high chemical reactivity due to the presence of unpaired electrons, which explains why and how they inflict damage in cells (Mohajeri and Asemani, 2009). Free radicals have been reported to be the cause of several diseases such as liver cirrhosis, atherosclerosis, cancer and diabetes and compounds that can scavenge free radicals therefore have great potential in ameliorating these disease processes (Behera *et al.*, 2006; Gerber *et al.*, 2002; Kris-Etherton *et al.*, 2002; Serafini *et al.*, 2002). Thus, antioxidants can play an important role to protect the human body against damage by reactive oxygen species (Tutour, 1990).

Plant foods serve as sources of a wide variety of dietary antioxidants, such as vitamins C and E, carotenoids, flavonoids and other phenolic compounds. Presently, natural antioxidants such as vitamin C and E are used as a means of enhancing biological function and improving the stability of lipid and lipid-containing products. While synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propylgallate (PG) are also very effective, they are used under strict regulation because of potential health hazards (Tripathi *et al.*, 2007). Therefore, researchers have embarked on the continuous search for natural antioxidants as alternatives to synthetic ones.

Biocatalysis, which involves applications of chemical reactions catalyzed by enzymes, can be used to synthesize natural antioxidants. The aim of this project is to employ an oxidative biocatalyst to produce new and better antioxidants from simple plant derived

compounds, and to investigate their antioxidant activities compared with the starting substrate and other natural antioxidants.

1.2 Background to the Research Problem

The scavenging activity of an antioxidant is documented to be the first line of defence against free radicals, particularly reactive oxygen species (ROS) which contribute towards the development and progression of several chronic and degenerative diseases such as inflammation, cardiovascular diseases, neurodegenerative diseases, cancer and age-related disorders (Locatelli *et al.*, 2009). Reports also show that oxidation in food can induce rancidity and/or deterioration of nutritional quality, organoleptic properties (colour, flavour, texture) and consequently is a safety concern (Antolovich *et al.*, 2002).

Examples of ROS include oxygen species which contain unpaired electrons (e.g. $O_2^{\bullet -}$, $OH^{\bullet}$) or ones with the ability to extract electrons from other molecules (e.g. H_2O_2) and these species can initiate chain reactions which can result in extensive damage of cell structures or damage biomolecules directly causing oxidative stress (Kulys and Bratkovskaja, 2007). Currently, the use of natural antioxidants to protect against oxidative stress due to their scavenging activity against ROS is being promoted because of concerns regarding the safety of synthetic antioxidants (Locatelli *et al.*, 2009). New antioxidants can be synthesised using biocatalysts which offer a number of advantages. Biocatalysts function under mild reaction conditions (which are environmentally friendly) and are stable in solution, thereby encouraging their re-use (unlike chemical catalysts which are consumed during the reaction; Hahn *et al.*, 2009).

Plant phenols are reported to have numerous biological effects such as antioxidant, anti-mutagenic, anti-viral and anti-inflammatory activity (Bordini *et al.*, 2002; Nakagawa, 1999; Jakun *et al.,* 1997). Laccases (EC 1.10.3.2) are enzymes that are capable of catalyzing the oxidation of phenols and produce water as the only by-product thereby making them an ideal, ‘green’ enzyme (Michizoe *et al.*, 2005). The use of laccases in catalytic reactions such as oxidative cross-coupling and polymerization of phenolic compounds, thereby amplifying their antioxidative capability, has been reported (Ncanana and Burton, 2007; Kurisawa *et al.*, 2003a, b). The increase in antioxidant activity is a result of the modification of the structure of the natural phenolic compounds as a result of the biocatalysis reaction catalyzed.

1.3 Statement of Research Problem

Various synthetic or natural antioxidants can be used in order to prevent oxidative reactions in food products and to protect biological tissues against damage to molecular targets such as proteins, lipids, carbohydrates and DNA (Locatelli *et al.*, 2009). However, because of the potential health hazard associated with synthetic antioxidants, the focus of this study was to employ an oxidative biocatalyst (laccase) to produce value-added antioxidants from simple plant-derived phenolic compounds.

1.4 Hypothesis

Plant phenolic compounds are known to have antioxidant capacity. Biocatalysis reactions employing the oxidative enzyme, laccase, will result in the production of dimeric and polymeric products, thereby increasing the antioxidant capacity of the original substrate.

1.5 Objectives of the Research

The aim of this research is to produce value-added compounds with high antioxidant activity from plant–derived substrates, through the use of biocatalysis. This was achieved with the following specific objectives:

- Production of laccase by the white-rot fungus, *Trametes pubescens*.
- Purification of laccase and formulation of the enzyme as a stable, active biocatalyst.
- Investigate the potential of laccase to catalyze the conversion and coupling of various plant–derived substrates.
- The analysis and purification of the products using techniques such as high performance liquid chromatography (HPLC), thin layer chromatography (TLC), liquid chromatography-mass spectroscopy (LC-MS) and nuclear magnetic resonance (NMR).
- Determination of the antioxidant activities of products using selected spectrophotometric or biochemical methods, and to compare them with the antioxidant activities of the starting substrates.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction to biocatalysis

Biocatalysis are defined as chemical reactions catalyzed by free or immobilized isolated enzymes. Biocatalysis reactions were observed by humans long before they were deduced as having an underlying microbial cause, for example the rotting of food and the microbial fermentation of grape juice in the production of wine. The principles and properties of biocatalysts only became generally appreciated as a result of studies conducted in the early 1900s (Parales *et al.*, 2002) involving the industrial-scale fermentation of acetone in order to meet the needs of Great Britain during World War I. Applications of biocatalysis exploit the unique properties of biocatalysts, namely their stereo- and regiospecificity and their ability to carry out reactions at neutral pH values and ambient temperatures, and they may be used to carry out specific conversions of simple or complex substrates.

Novel products or known products can be produced more efficiently by employing the use of biocatalysis (Giri *et al.*, 2001) than chemical synthesis (Hahn *et al.*, 2009). Various chemical compounds can be modified through biocatalysis reactions (Franssen and Walton, 1999); these include aromatics, steroids, alkaloids, coumarins, terpenoids and lignans. For example, plant cell cultures exhibit a vast biochemical potential for the production of a range of specific secondary metabolites through the biotransformation of exogenous substrates. The compounds are not necessarily natural intermediates in plant metabolism, but can also be of synthetic origin (Pras *et al.*, 1995). Thus, through biocatalysis, cheap and abundant natural and/or synthetic substances, such as industrial by-products, can be transformed into rare and expensive, value-added products.

2.1.1 Enzymes as biocatalysts

Enzymes are capable of catalysing a vast number of chemical reactions that are essential for maintaining the life functions of the cell, including growth and reproduction (Leuenberger, 1990). Biocatalysts are favourably differentiated from common chemical catalysts by their high specificity with respect to the reaction they catalyze, the structure and the stereochemistry of the substrates they accept and also the products they form. Furthermore, biocatalysts have the ability to function under mild conditions and generate high reaction rates due to a significant decrease of the activation energy required in the enzyme/substrate complex (Leuenberger, 1990). Biocatalysts are environmentally friendly and because of their inherent advantages,

industries have developed ways to apply them for specific transformations in the production of metabolites, pharmaceuticals and agrochemicals (Preisig and Byng, 2001).

2.1.2 Biocatalysis in organic media

Aqueous media are the traditional media for biocatalysis. This is because the role of water was envisaged to be crucial in influencing their activity, stability and conformational flexibility (Klibanov, 1989). It was, however, later observed that it is the amount of water bound to the enzyme, which is a direct function of the water activity in the medium that primarily determines enzyme activity (Halling, 1994). Biocatalysis in organic media was developed due to the poor solubility in aqueous media of many organic compounds of commercial interest that can potentially be transformed by enzymes (Leon *et al*., 1998). It is now possible to enhance the usefulness of enzymes through the use of organic solvents rather than water since large scale removal of water from reaction medium is tedious and expensive due to its high boiling point and high heat of evaporation. The advantages of using organic solvents include increased solubility of hydrophobic substrates; ability to carry out new reactions which are impossible in water because of kinetic or thermodynamic restrictions in order to generate desired products; greater stability of enzymes; reduced incidence of the side reactions found in water, reduced microbial contamination, relative ease of product recovery from organic solvents as compared to water, and the insolubility of enzymes in organic media, which permits their easy recovery and reuse thus eliminating the need for immobilization (Sellek and Chaudhuri, 1999; Leon *et al*., 1998; Klibanov, 1986; Zaks and Klibanov, 1985).

2.1.2.1 Types of organic solvent systems

Organic solvent systems can be classified into three main types based on the miscibility of an organic solvent with water and the relative proportion of the solvent and water in the medium. These are:

(1) Water-water-miscible (hydrophilic) organic solvent system (organic co-solvent system),

(2) Water-water-immiscible (hydrophobic) organic solvent system (two phase system or biphasic system), and

(3) a nearly anhydrous organic solvent system (Ogino and Ishikawa, 2001).

These three systems are summarized below.

2.1.2.1.1. Water-water-miscible organic solvent system

Water–water-miscible organic solvent system is a monophasic system that has no separate aqueous phase (Ogino and Ishikawa, 2001). It is produced when water-miscible co-solvents such as methanol, ethanol or acetone, are introduced to the medium in order to improve the solubility of compounds which are insoluble in water (Khmelnitsky *et al.*, 1988). In this type of solvent system, concentration of substrates and products can easily be controlled leading to prevention of high substrate and product concentrations around the enzyme. Although this system can lead to severe distortion of the enzyme structure, rapid denaturation and inactivation of the enzyme due to direct contact with organic solvent, there is a reduction in the mass-transfer limitations, leading to more rapid reactions rates for hydrophobic compounds. Furthermore, this reaction system has advantages for the synthesis of esters and peptides because the thermodynamic equilibria created would favour synthesis over hydrolysis (Doukyu and Ogino, 2010; Ogino and Ishikawa, 2001).

2.1.2.1.2. Water-water-immiscible organic solvent system

Water–water-immiscible organic solvent system is a biphasic system (e.g ethyl acetate or chloroform in water) which consists of the aqueous phase where the enzyme is dissolved and the phase of an immiscible organic solvent (Ogino and Ishikawa, 2001). A separate layer is formed between the aqueous phase and the organic solvent through the interfacial area. In this type of system, biocatalysis proceeds in the aqueous phase containing enzyme (Doukyu and Ogino, 2010) and products are partitioned into the organic phase. The substrate is converted by the enzyme, while the product is extracted into the organic solvent phase. This makes separation of the product from the solvent simple and enzyme regeneration easy. Because direct contact of the enzyme with an organic solvent is minimized in the biphasic system, the enzyme is relatively stable. Generally, the rate of enzyme inactivation in a two-phase system is lower than those in a monophasic system. However, reaction rates are relatively low due to a low rate of mass-transfer across the interface, but this hindrance can be eliminated by intensive stirring (Doukyu and Ogino, 2010).

2.1.2.1.3. Nearly anhydrous organic solvent system

In a nearly anhydrous organic solvent system (0.01% water), native enzymes are insoluble. Therefore, it is necessary to solubilise enzymes in this organic solvent, either by lyophilization, immobilization or modifications with amphipathic compounds. This system has been shown to be useful in several enzymatic processes, such as the synthesis and transesterification of esters, peptide synthesis, and transformation

of various hydrophobic compounds (Doukyu and Ogino, 2010; Khmelnitsky *et al.*, 1988).

2.1.2.2 Solvent engineering

Although biocatalysis in organic solvents have numerous advantages, the application of enzymes in these media is restricted because most enzymes are less active and stable in the presence of organic solvents. Methods such as the immobilization of enzymes on insoluble support matrices, chemical modification of enzymes, physical modification of enzymes with lipids or surfactants, entrapment of enzymes in reversed micelles, and the molecular engineering of enzymes, are the various methods which have been developed to improve the activity and/or stability of enzymes to be used in the presence of organic solvents (Doukyu and Ogino, 2010; Castro and Knubovets, 2003; Khmelnitsky *et al.*, 1988). Nevertheless, an organic-solvent-tolerant bacterium, which secretes an organic-solvent-stable lipolytic enzyme and an organic solvent tolerant bacterium, which secretes an organic solvent-stable proteolytic enzyme have been reported by Ogino *et al.* (1995; 1994). Their work was the first to identify natural enzymes that are tolerant to organic solvents and could find useful applications without having to modify the enzymes in order to stabilize them. After these, various organic solvent-tolerant enzymes have been reported (Doukyu and Ogino, 2010).

In addition to the fact that catalytic activities in organic media may be lower than those in aqueous solution despite the inherent advantages of these organic solvents, by careful selection of the type of enzyme preparation to use and the reaction conditions, it is often possible to achieve catalytic activities in the same order of magnitude as in water. Important factors to consider are the solvent, the water content and enzyme ionization effects (Adlercreutz, 2008). For instance, the solvent can influence an enzymatic reaction both by direct interaction with the enzyme (which can influence its stability as well as activity) and by influencing the solvation of the substrates and products in the reaction medium.

Enzyme activity in organic solvents is highly dependent on the nature of the solvent while enzyme stability depends on its direct interaction with solvent. For example, non-polar hydrophobic solvents often provide higher reaction rates than more polar, hydrophilic solvents (Adlercreutz, 2008). In kinetic studies, the K_m values of enzymes for certain substrates in the presence of organic solvents are often observed to be higher than those in water for the corresponding reactions. The effects are normally as a result of the effective solvation of the substrate in the organic solvent which reduces its free energy and that causes the free energy of activation of the enzymatic

reaction to increase, resulting in a lower reaction rate. It is possible to increase the rate of reaction by increasing the substrate concentration but when the substrate concentration is fixed at a moderate level, a solvent which can dissolve higher substrate concentrations should be avoided or else the reaction rate will be low due to an increase in the apparent K_m value (Adlercreutz, 2008).

When biocatalysis reactions are to be performed in the presence of an organic solvent, there are often so many solvents to select from. The selection of an appropriate organic solvent as a medium for biocatalysis depends on various factors which include; solvent hydrophobicity, density, surface tension, viscocity, toxicity, flammability, waste disposal and cost (Dordick, 1989). By combining data from the literature together with the logarithm of the partition coefficient (log *P*) as a quantitative measure of solvent polarity, the rules for the optimization of different biocatalytic systems in various types of media containing organic solvents are obtained (Laane *et al.,* 1987). Biocatalysis in organic solvents is low in polar solvents having a log $P < 2$, because they distort the essential water layer that stabilizes the biocatalysts. It is equally moderate in solvents having a log *P* between 2 and 4, and is high in a polar solvents having a log $P > 4$ as they do not interfere with the essential water coat surrounding the enzyme molecule in its active state (Kermasha *et al*., 2001; Laane *et al*., 1987)

Biocatalysis in organic media had been reported by many researchers and by careful selection of different organic solvents and also by varying the percentage co-solvent, optimization of their reactions were obtained. Tranchimand *et al*. (2006) reported the use of biphasic media for laccase-catalysed transformation of sinapinic acid and ferulic acid with the aim of improving the yield through adaptation of the reaction medium. They observed higher yield, higher product selectivity and higher product stability by using ethyl acetate as added non-miscible co-solvent as compared to buffer alone. Moreover, the use of this organic solvent made product separation easier. Laccase-catalyzed oxidation of phenolic compounds in organic media was also reported by Ma *et al.* (2009). These researchers investigated the use of laccase from *Rhus vernificera* to catalyze the oxidation of phenolic compounds (catechin, epicatechin and catechol) in selected organic solvents in order to search for the favourable reaction medium.Their investigation on reaction parameters showed that optimal laccase activity was obtained in hexane at 30°C, pH 7.75 for the oxidation of catechin as well as for epicatechin, and in toluene at 35°C, pH 7.25 for the oxidation of catechol. Kermasha *et al*. (2001) also investigated the enzymatic activity of mushroom tyrosinase using catechin as a substrate in the presence of selected

organic solvents (heptane, toluene, dichloromethane and dichloroethane). Their results indicated that the presence of 12.5% acetone in the reaction medium of dichloromethane and 22.0% in those of toluene and dichloroethane produced a maximal increase of 42.6%, 92.1% and 71.8% respectively. But additional increases in acetone concentration resulted in an inhibition of tyrosinase activity.

2.2 Oxidoreductases (oxidases)

An oxidase is an enzyme that catalyzes an oxidation/reduction reaction involving molecular oxygen (O_2) as the electron acceptor. In these reactions, oxygen is reduced to water (H_2O) or hydrogen peroxide (Burton and Le Roes-Hill, 2008). Different classes relevant to biocalytic systems are described below.

2.2.1 Monooxygenases

Monooxygenases require two electrons and two protons to reductively cleave oxygen (O_2) and generate a single molecule of water per molecule of substrate. The second oxygen atom is generally incorporated into the substrate resulting in an oxy-functionalized product. Monooxygenases generally require a co-factor such as NADH or NADPH to provide reducing potential for the supply of electrons to the substrate (Burton and Le Roes-Hill, 2008):

$$\text{Substrate} + \text{Donor} - \text{H} + O_2 + H^+ \rightarrow \text{Substrate} - \text{O} + \text{Donor} + H_2O$$

Monooxygenases can be classified into different types of families based on the type of co-factor they require for catalysis. Some of these are: heme-dependent monooxygenases, flavin-dependent monooxygenases, copper-dependent monooxygenases and non-heme iron-dependent monooxygenases. Although most of monooxygenases usually require an organic or metallic co-factor for their catalytic activity, some have been identified that do not require any co-factors, majority of these enzymes are mainly present in streptomycetes. Generally, in biocatalysis, monooxygenases find applications in co-enzyme recycling. This is achieved either chemically, electrochemically, photochemically or enzymatic (Torres Pazmino *et al.,* 2010).

2.2.2 Dioxygenases

Dioxygenases catalyze the incorporation of two atoms of oxygen into substrates (Wackett, 2002) and most dioxygenase enzymes require a metal co-factor, which is most often iron(II) or iron(III). Since dioxygenases catalyze the incorporation of two

atoms of oxygen from dioxygen into the products of the reaction, a common strategy is to form one C–O bond with the substrate first, before O–O bond cleavage, in the form of an alkyl hydroperoxide intermediate (Bugg, 2003).

A common example is bacterial aromatic hydrocarbon dioxygenases. These are multicomponent enzyme systems that add dioxygen to the aromatic nucleus to form arene *cis*-diols. Figure 2.1 shows the oxidation of benzene to *cis*-1,2-dihydroxycyclohexa-3,5-diene (benzene *cis*-diol) by toluene dioxygenase (Gibson and Parales, 2000). Bacterial aromatic hydrocarbon dioxygenases can function in the development of bioremediation technology especially in the removal of aromatic hydrocarbons which are common contaminants of soils and groundwater and can also fulfill the 'green chemistry' requirement in the synthesis of useful chemicals (Gibson and Parales, 2000).

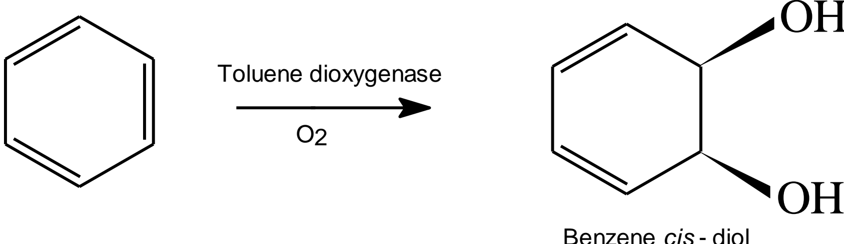


Fig. 2.1: Oxidation of benzene to benzene *cis*-diol by toluene dioxygenase (Gibson and Parales, 2000).

2.2.3 Peroxidases

Peroxidases are a group of enzymes especially common in plants that can use organic or hydrogen peroxides to oxidize phenols [Eq. (1)] and perform *N*-demethylation, releasing formaldehyde [Eq. (2)] (Holland, 1992).

$$2A - H + ROOH \longrightarrow A - A + ROH + H_2O \qquad (1)$$

$$R_2NCH_3 \longrightarrow R_2NH + CH_2O \qquad (2)$$

Generally, peroxidases can be grouped into haem-containing and non-haem-containing enzymes. They are widely distributed in nature and have been isolated and described in animals, plants and microorganisms (le Roes-Hill *et al.*, 2011). Peroxidases can be involved in enzymatic browning, since diphenols may function as reducing substrates in the peroxidase reaction (Robinson, 1991). Other application of

peroxidases include: degradation of pollutants, degradation of lignin and lignocelluloses and dye degradation (le Roes-Hill *et al.*, 2011)

2.2.4 Typical examples of oxidases applied in biocatalysis reactions

Glucose oxidases, monoamine oxidases, cytochrome P450s, NADPH-dependent oxidases, xanthine oxidases and tyrosinases, are typical examples of oxidases that are commonly used in biocatalysis reactions and are briefly described below, while laccases (employed in this study) are discussed in more detail.

2.2.4.1 Glucose oxidase

Glucose oxidase (GOX) (EC 1.1.3.4) binds to beta-D-glucopyranose (a hemiacetal form of the six-carbon sugar glucose) and aids in breaking the sugar down into smaller metabolites. GOX is a dimeric protein that catalyses the oxidation of beta-D-glucose into D-glucono-1,5-lactone, which then hydrolyzes to gluconic acid by utilizing molecular oxygen as an electron acceptor, with simultaneous production of hydrogen peroxide (H_2O_2) (Hatzinikolaou and Macris, 1995).

GOX is an important enzyme which has numerous applications in the food industry and clinical fields (Buck, 1983; Richter, 1983), including: glucose removal from dried egg; improvement of colour, flavour, and shelf life of food materials; oxygen removal from fruit juices and canned beverages; and prevention of rancidity in food. It has also been used in an automatic glucose assay kit in conjunction with catalase (Hatzinikolaou and Macris, 1995), particularly in biosensors for the detection and estimation of glucose in industrial solutions and in body fluids such as blood and urine (Petruccioli *et al.,* 1999). Implantable glucose sensors have also found applications for diabetes patients (Gerritsen *et al.*, 2001). GOX has also been used as an ingredient of toothpaste (Petruccioli *et al.*, 1999), for the production of gluconic acid, and as a food preservative (Pluschkell *et al.*, 1996).

2.2.4.2 Monoamine oxidase

Monoamine oxidase (MAO, EC 1.4.3.4) catalyzes the oxidation of monoamines. MAOs are found bound to the outer membrane of mitochondria in most cell types in the body. They catalyze the oxidative deamination of many biogenic amines, and play a vital role in the regulation of neurotransmitters in the mammalian central nervous system (Abell and Kwan, 2001; Berry *et al.*, 1994; Weyler *et al.*, 1990). MAOs contain the covalently bound co-factor FAD (flavin adenosine dinucleotide) and therefore, are classified as flavoproteins (Binda *et al.*, 2002; Abell and Kwan, 2001). The reaction catalyzed by MAO requires molecular oxygen to oxidize the amine substrate to its

aldehyde intermediate, producing hydrogen peroxide as a by-product (Fig. 2.2). The aldehyde intermediate is then rapidly oxidized by aldehyde dehydrogenase or aldehyde reductase.

$$R\text{-}CH_2N\text{-}RR + H_2O + O_2 \xrightarrow[FAD \rightarrow FADH]{} RCH{=}O + NRR + H_2O_2$$

$$R = H \text{ or } CH_3$$

Fig. 2.2: The reaction catalyzed by monoamine oxidase. A monoamine substrate is oxidatively deaminated to form an aldehyde product with hydrogen peroxide as a by-product (Lewis *et al.*, 2007).

2.2.4.3 Cytochrome P450

Cytochrome P450s are a very large and diverse superfamily of hemoproteins found in all domains of life. The most common reaction catalyzed by cytochrome P450 is a monooxygenase reaction, e.g insertion of one atom of oxygen into an organic substrate (RH) while the other oxygen atom is reduced to water:

$$RH + O_2 + 2H^+ + 2e^- \longrightarrow ROH + H_2O$$

Cytochrome P450 is reported to take part in many physiological processes (Durst, 1991) including xenobiotic degradation (Khatisashvili *et al.*, 1997; Gordeziani *et al.*, 1987) which is of importance for phytoremediation (Salt *et al.*, 1998).

2.2.4.4 NADPH oxidase

NADPH oxidase [Nicotinamide adenine dinucleotide phosphate – oxidase (Nox)] is a membrane-bound enzyme complex. Apart from simple organisms such as slime molds and fungi, Nox genes are widely distributed in plants and animals. In higher plants, Nox homologues were first identified in rice (Groom *et al.*, 1996) and then in various plant species including *Arabidopsis* (Keller *et al.,* 1998), potato (Yoshioka *et al.*, 2001), tomato and a pondweed (Amicucci *et al.*, 1999), and in a close relative of tobacco (Yoshioka *et al.*, 2003). Moreover, a distinct Nox isoform, NoxD, has been identified in two species of algae (Kawahara and Imanishi, 2007). The enzyme catalyses oxidative burst activity in response to pathogen infection (Wojtaszek, 1997), and has also been shown to mediate plant growth (Rodríguez *et al.*, 2002). NADPH oxidase is found in the plasma membrane of mammalian cells which produces reactive oxygen species (ROS) and these ROSs activate an enzyme that makes

macrophages adhere to artery walls causing atherosclerosis. However, this process is counter balanced by Nox inhibitors and by antioxidants (Park *et al.*, 2004; Sun *et al.*, 2002).

2.2.4.5 Xanthine Oxidase

Xanthine oxidase (XO, a form of xanthine oxidoreductase that generates reactive oxygen species) catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid. Although XO has broad specificity for a wide variety of substrates, its physiological role is believed to be in purine catabolism where it catalyzes the oxidation of hypoxanthine to xanthine and then xanthine to uric acid, with concomitant reduction of molecular oxygen (Hille and Nishino, 1995). The following chemical reactions are catalyzed by xanthine oxidase (Fig. 2.3).

$$*\text{Hypoxanthine} + H_2O + O_2 \longrightarrow \text{Xanthine} + H_2O_2$$

$$*\text{Xanthine} + H_2O + O_2 \longrightarrow \text{Uric acid} + H_2O_2$$

Hypoxanthine
(One oxygen atom)

Xanthine
(Two oxygen)

Uric acid
(Three oxygen)

Fig. 2.3: Chemical reactions catalyzed by xanthine oxidase (Hille and Nishino, 1995).

In humans, XO is normally found in the liver and not free in the blood. During severe liver damage, XOis released into the blood, and thus a blood assay for XO is a way to determine if liver damage has occurred (Sanhueza *et al.*, 1992).

2.2.4.6 Tyrosinase

Tyrosinase (EC 1.14.18.1), also called polyphenol oxidase, is a copper-containing enzyme (Bubacco *et al.*, 1999) widely distributed throughout the phylogenetic scale (Van Gelder and Flurkey, 1997). Tyrosinase catalyses the hydroxylation of monophenols to *o*-diphenols using molecular oxygen, followed by the oxidation of *o*-diphenols to *o*-quinones (Solomon *et al.,* 1996). The enzyme is responsible for skin,

eye, inner ear and hair melanisation and browning in fruits and vegetables (Rodriguez-Lopez *et al.*, 1992). In its first reaction, called monooxygenase (cresolase) activity, tyrosinase hydroxylates a phenolic substrate at an *ortho* position to the hydroxyl group. In its second reaction, oxidase (catecholase) activity, the *o*-dihydroxy compound is oxidized to its *o*-quinone derivative (Fig. 2.4). *o*-Quinones, in turn, take part in a series of chemical and further enzymatic reactions and finally polymerize to form brown pigments, called melanins (Garcia-Canovas *et al.*, 1992).

OH
R
Tyrosinase + O_2
Cresolase reaction
OH
OH
R
Tyrosinase + O_2
Catecholase reaction
O
O
R
Intermediates
Macromolecules

Fig. 2.4: The enzymatic oxidation of phenolic compounds by tyrosinase to form natural macromolecules such as lignin and melanin. The "R" group could be different in different cells or experiments (Haghbeen and Wue Tan, 2003).

2.3 Laccases

Laccase (*p*-diphenol: oxygen oxidoreductase, EC1.10.3.2), one of the most important members of ligninolytic systems, is a multicopper enzyme belonging to the group of blue-copper proteins (Minussi *et al.,* 2007; Aramayo and Timberlake, 1993; Nun *et al.,* 1988). The copper ions present in the enzyme's active site are classified as type 1, type 2 and type 3 because of their spectroscopic properties. The assembly of type 2 and 3 copper ions is called a trinuclear cluster. Laccase is found in higher plants such as the varnish tree *Rhus vernicifera* (Morozova *et al.*, 2007) but the majority of laccases are found in white-rot causing polypores, geophilous saprophytic fungi, as well as some insects and bacteria (Sadhasivam *et al.*, 2008).

2.3.1 Structure and Catalytic Mechanism of Laccase

Structuraly, laccases can either be monomeric or multimeric glycoproteins, that may exhibit additional heterogeneity because of their variable carbohydrate content or differences in copper content (Woods, 1980). These enzymes have active sites consisting of a metallic cluster which contains four copper atoms, all of them being involved in the redox process via a radical cyclic mechanism in which one oxygen molecule is reduced to give two water molecules, while four substrate molecules (usually phenols or aliphatic/aromatic amines) are oxidized to the corresponding reactive radicals (Riva, 2006).

Fungal laccases usually occur as isoenzymes that oligomerize to form multimeric complexes. The molecular mass of the monomer ranges from about 50 to 100 kDa. An important feature is a covalently linked carbohydrate moiety (10–45%), which may contribute to the high stability of the enzymes. For the catalytic activity a minimum of four copper atoms per active protein unit is needed: Type 1: paramagnetic 'blue' copper, absorbance at 610 nm (ox.); Type 2: paramagnetic 'non-blue' copper; and Type 3: diamagnetic spin-coupled copper-copper pair, absorbance at 330 nm (ox.). The type I copper confers the typical blue colour to multicopper proteins, which results from the intense electronic absorption caused by the covalent copper–cysteine bond. Type 1 copper is the site where substrate oxidation takes place due to its high redox potential. Type 2 and type 3 copper form a trinuclear cluster, where reduction of molecular oxygen and the release of water take place. The type 2 copper is coordinated by two histidine residues and type 3 copper atoms by six histidines. The strong anti-ferromagnetical coupling between the two type 3 copper atoms, is maintained by a hydroxyl bridge (Fig. 2.5).

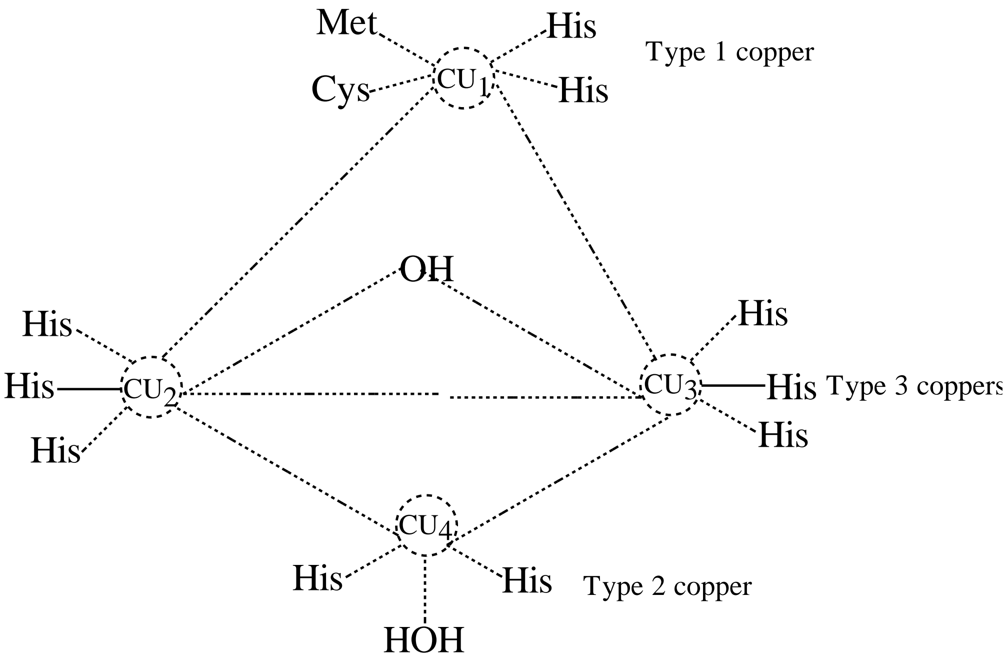


Fig. 2.5: Structure of the laccase active site (adapted from Witayakran and Ragauskas, 2009).

The catalytic properties of laccase are attributed to the following three major steps as recently reported by (Witayakran and Ragauskas, 2009):

(i) Type 1 copper is reduced by accepting electrons from the reducing substrate.

(ii) Electrons are transferred from the type 1 copper to the trinuclear T2/T3 cluster.

(iii) Molecular oxygen is activated and reduced to water at the trinuclear T2/T3 cluster.

Laccase catalyzes one-electron oxidation of four reducing-substrate molecules and reducing the four-electron of molecular oxygen to water by its various copper centres which drive electrons from a reducing substrate to molecular oxygen without releasing toxic peroxide intermediates in the process (Claus, 2004). This makes laccase an ideal green enzyme (Niladevi *et al.*, 2009). The three steps highlighted above makes this possible while oxidation of substrates during this process creates reactive radicals that can undergo non-enzymatic reactions (Witayakran and Ragauskas, 2009). Laccases oxidize a wide range of substrates but phenolic compounds are their preferred substrates (Claus, 2004).

Oxidation of phenolic substrates, by laccase results in formation of an aryloxyradical which is an active species that, in turn, is converted to a quinone in the second stage of the oxidation. The quinone intermediates quickly react with each other to form soluble or insoluble coloured oligomers, depending on the substrate reaction conditions. Laccase can also decarboxylate phenolic and methoxyphenolic acids,

attack methoxyl groups through demethylation and dehalogenation of substituents located in the *ortho* or *para* position in the case of substituted compounds (Majeau *et al.,* 2010).

2.3.2 General Application of Laccase

Laccase catalyzes the oxidation of a variety of organic compounds including phenols, methoxy-substituted phenols, aminophenols and diamines (Morozova *et al*., 2007), with reduction of molecular oxygen to water (Minussi *et al*., 2007). Some of the general applications of laccase are presented in Table 2.1.

Table 2.1: General applications of laccase

Uses	References
laccases function in lignin degradation, pathogenesis, detoxification, and fungal development and morphogenesis	Baldrian, 2006; Leonowicz, 2001
Delignification of wood fibers for preparation of pulp.	Mayer and Staple, 2002
Detoxification of industrial effluents, mostly from the paper and pulp	Kuhad *et al*., 1997
Application in textile and petrochemical industries	Abadulla *et al*., 2000; Blánquez *et al*., 2004; Hou *et al*., 2004; Rodríguez Couto *et al*., 2005
Use of laccase expressing yeast strains for improved production of ethanol from lignocellulose	Larsson *et al*., 2001
Wine clarification	Servili *et al*., 2000
Used as a tool for medical diagnostics	Roy *et al.,* 2005
As a bioremediation agent to clean up herbicides, pesticides and certain explosives in soil	Pointing, 2001
Laccases are also employed as cleaning agents for certain water purification systems	Couto and Herrera, 2006
Act as catalysts for the manufacture of anti-cancer drugs and even as ingredients in cosmetics	Golz-Berner *et al*., 2004
Has the capacity to remove xenobiotic substances and produce polymeric products and as such are useful tool for bioremediation purposes in food industry	Minussi *et al*., 2002

Further, laccase-oxidised syringaldazine was recently reported to be a reliable tool for measuring antioxidant activity of vitamin C and vitamin E (Nugroho Prasetyo *et al*., 2010). The performance of laccase-oxidised syringaldazine was comparable to the commercially available 2,2-diphenyl-l-picrylhydrazyl (DPPH) radical. Syringaldazine, a

yellowish compound which is converted to tetramethoxy azobismethylene quinone (TMAMQ), has a maximum absorbance at 530 nm (deep purple colour) (Holm *et al.*, 1998; Harkin *et al.*, 1974) and is a known laccase substrate which can be used to detect peroxidase activities. The reaction of laccase with syringaldazine first generates a free radical and the loss of the second electron can either proceed enzymatically or by disproportionation, forming the deep purple coloured quinone (TMAMQ – Fig. 2.6) which is not prone to polymerisation under appropriate conditions (Kuznetsova and Romakh, 1996; Thurston, 1994).

H3C-O H H O-CH3 HO C-N-N-C OH + Laccase O2 H2O H3C-O O-CH3

Syringaldazine

H3C-O H H O-CH3 O C-N-N-C O H3C-O O-CH3

Tetramethoxy azobismethylene quinone

Fig. 2.6: Laccase-catalysed oxidation of syringaldazine (Nugroho Prasetyo *et al.*, 2010)

2.3.3 Application of laccase in phenolic compound coupling

Phenolic compounds form an important class of compounds which serve to inhibit the oxidation of material of both commercial and biological importance (Nikolic, 2006). Due to the presence of hydroxyl and carboxyl groups, phenolic compounds have a high tendency to chelate metals. The antioxidant efficiency of phenolic acids has also been related to the number of hydroxyl groups in the molecule and also to their hydrogen radical donating abilities (Miller and Rice-Evans, 1997). It is envisaged that when phenolic compounds are linked together (coupling), their hydrogen donating capability is enhanced.

Coupling in itself is a reaction where two organic molecules are linked together with the aid of a catalyst. Coupling can be homo-coupling where two molecules react to form one molecule or cross-coupling in which two different molecules react to form one new molecule. Metal catalysts such as Cu, Na, Ni and Pb are the traditional catalyst medium where coupling takes place (Kudanga *et al.,* 2011). However, with the increasing search for more environmental friendly and mild reaction conditions, enzymes such as laccases are being employed due to their numerous inherent advantages. Generally, laccases can oxidize a wide range of substrates but phenolic compounds are their preferred substrates (Claus, 2004). They have been shown to catalyze the polymerization of many phenolic compounds and as such many natural

or artificial natural products have been synthesized. A typical reaction of laccase is as shown in Fig. 2.7.

During the coupling of monomeric compounds in the presence of a biocatalyst, phenoxyradicals are generated, two of which are coupled together to form quinone methide intermediates which further react with suitable nucleophiles in intra- or intermolecular reactions. Other hydrolytic reactions, eliminations and/or rearrangement will then follow to yield a stable end product.

Fig. 2.7: The oxidation of different phenolic compounds - a typical reaction of laccase (Minussi *et al.*, 2002).

How the two phenoxy radicals couple, selection of reaction route and ratio of possible dimeric products are dependent on the stereo-electronic effects relating to the structure of the phenoxy radicals. Reaction conditions and the catalyst will also affect the coupling. The possible coupling positions which can lead to different types of C-C and C-O bonds are as shown in Fig. 2.8.

Fig. 2.8: Possible combinations of resonance stabilized phenoxy radicals generated from 4-hydroxycinnamics (Adapted from Setala, 2008).

A+C coupling (4-O-5) is only possible if there is no substituent in the C-5 (and/or C-3) position of the aromatic ring, and most times this coupling occurs when there is no β-coupling possibility in another phenoxy radical forming compound. 5-5' (C+C) is also possible if the C-5 (and/or C-3) position has no substituent and is likely to occur if there is no β-radical coupling possibility in either phenoxy radical forming compound. 8-1' (β-1) (B+E) coupling is possible if there is no β-radical coupling possibility in another phenoxy radical forming compound and more likely if at the same time the C-3 and (or) C-5 positions in the aromatic ring are blocked (Setala, 2008).

The use of laccase in coupling reactions has been studied by many researchers. Recently, laccase mediated cross-linking of β-lactoglobulin (BLG) in the presence of sour cherry phenolics was reported (Tantoush *et al.*, 2011). BLG is an important nutrient of dairy products, but poses a serious health risk in patients who are allergic to milk. The cross-linking potential of laccase on the purified form of BLG in the presence of a sour cherry extract as a source of phenolic mediator was investigated.

The impact of what the enzymatic action will have in the presence of sour cherry phenolic on the allergenicity and digestibility of cross-linked BLG was also studied. Their study showed that processing of new and safer foods with improved functional properties was possible.

Another study reported the enzymatic enrichment of naringenin with hydroxylated and/or methoxylated phenolic compounds (Prasetyo *et al.*, 2011). The investigation was performed in order to determine the possibility of coupling simple phenolic molecules that are rich in hydroxyl groups and/or methoxyl groups onto naringenin by using laccases with the view of enhancing its antioxidant activity. The success was achieved as evidenced by LC–MS analysis reported.

2.3.4 Laccase-catalysed transformation of phenolic compounds

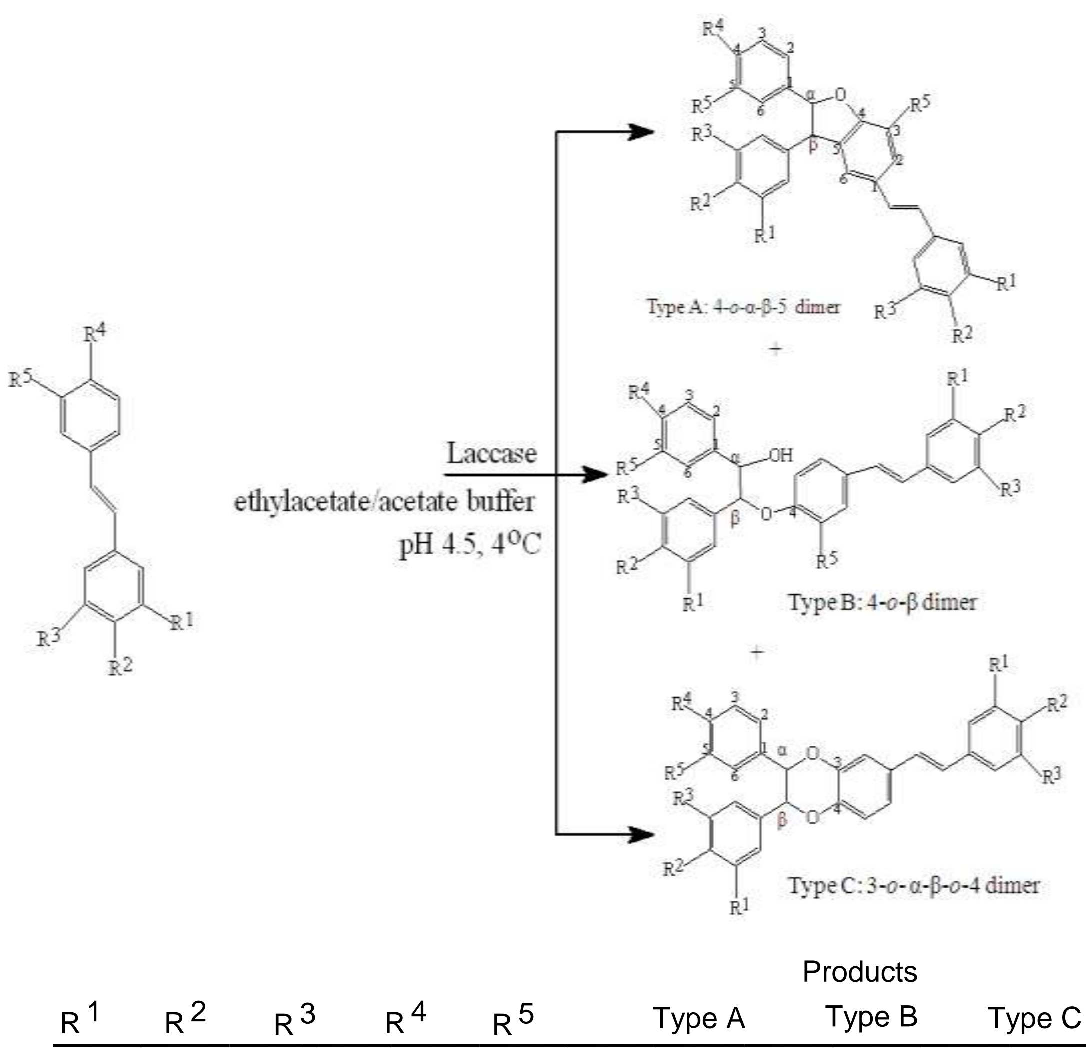
Laccase is able to catalyze the oxidation of a variety of compounds, such as *ortho*- and *para*-benzenediols, polyphenols, aminophenols, polyamines and lignin. This is achieved by abstracting an electron from the substrate to produce a free radical, and reduces oxygen to water. Fig. 2.9 shows the simplified form of laccase-catalyze oxidation with hydroquinone (Witayakran and Ragauskas, 2009).

Fig. 2.9: Oxidation of hydroquinone by laccase (Witayakran and Ragauskas, 2009)

Nicotra *et al.*, (2004), for the first time, utilized the laccase from *Myceliophtora thermophyla* and from *Trametes pubescens* for the synthesis of the resveratrol dimer which showed antioxidant properties, in good yields and under very mild reaction conditions (atmospheric air, enzyme, solvent) (Fig. 2.10). Ponzoni *et al.* (2007) also reported the oxidation of a series of hydroxystilbenes, analogues of phytoalexin resveratrol, by lacccase from *T. pubescens* in an ethyl acetate/acetate buffer system (Fig. 2.11).

M. thermophyla laccase
n-butanol-phosphate buffer
pH 6.5, 45 °C, 4 days
trans-resveratrol
dehydrodimer product
31%

Fig. 2.10: The transformation of trans-resveratrol (3,5,4' –trihydroxystilbene) by the *M. thermophyla* laccase (Nicotra *et al.,* 2004),



					Products		
R^1	R^2	R^3	R^4	R^5	Type A	Type B	Type C
OMe	H	OMe	OH	H	59%	6%	-
H	OMe	H	OH	OH	14%	-	14%

Fig. 2.11: The oxidation of hydroxystilbenes by the *T. pubescens* laccase (Ponzoni *et al*., 2007).

Chigorimbo-Murefu (2007) studied the production of bio-active compounds from ferulic acid using biocatalysis and reported that the synthesis of vanillin and vanillic acid from ferulic acid and laccase under the condition of study was not successful. Instead, ferulic acid polymers were formed. However, these were shown to have higher antioxidant activity than the precursor. Production of vanillin and vanillic acid from biotransformation of ferulic acid by the white rot fungus *Pycnoporus cinnabarinus* was recently reviewed by Witayakran and Ragauskas (2009) (Fig 2.12).

COOH

P. cinnabarinus I-937
Laccase
6 days

COOH + CHO

OCH_3 OCH_3 OCH_3

OH OH OH

Ferulic acid Vanillic acid Vanillin (27.5%)

Fig. 2.12: Biotransformation of ferulic acid by the *P. cinnabarinus* laccase (Witayakran and Ragauskas, 2009).

Kurisawa *et al.* (2003a) studied the enzymatic synthesis and antioxidant properties of poly(rutin). In the study, poly(rutin) was synthesised by the enzyme-catalyzed oxidative coupling using laccase as a biocatalyst to amplify the antioxidant activity of rutin. The polymer showed higher superoxide scavenging activity compared with the rutin monomer, and also a more potent protector from low density lipoprotein (LDL) oxidation than the monomer. The authors also used laccase derived from *Myceliophthora* to catalyze the oxidative polymerization of catechin in aqueous organic solvents and reported that a polymer with a molecular weight of several thousands was obtained in high yields. This polymer exhibited amplified superoxide scavenging activity and xanthine oxidase (XO) inhibitory activity as compared to the monomeric catechin (Kurisawa *et al.*, 2003b).

Oxidation of 8-hydroxyquinoline catalyzed by laccase from *T. pubescens* was demonstrated by Ncanana and Burton (2007). To the authors' knowledge, it was the first report of the production of poly-(8-hydroxyquinoline), using laccase from a *T. pubescens* strain. The polymerisation was catalyzed by the laccase in acetone–

sodium acetate buffer at ambient temperature and pressure which yielded an antioxidant polymer (Fig. 2.13).

Fig. 2.13: Structure of the poly (8-hydroxyquinoline) product generated during a laccase-catalyzed reaction (Ncanana and Burton 2007).

Ncanana *et al.* (2007) also worked on laccase-mediated oxidation of totarol. They reported on the synthesis of novel dimers of totarol that were biologically active. The two dimeric products were linked either by carbon-carbon (1a) or by carbon-oxygen bonds (1b) and the ratio of products obtained were affected by the nature of solvent used (Fig. 2.14).

Fig. 2.14: Structure of synthesized dimeric products (1a and 1b) obtained from laccase-catalysed oxidation of totarol (adapted from Ncanana *et al.*, 2007).

2.4 Antioxidants and Free Radicals

Cornelli (2009) described an antioxidant as a product that inhibits oxidation *in vitro* and reduces oxidative stress *in vivo.* Antioxidant scavenging activity is the first line of defence against reactive oxygen species (ROS) that are generated through physiological processes and can also protect against these ROS by their chain-breaking properties. In these types of protection, antioxidants react with the reactive species. The rate of the reaction of an antioxidant with the reactive species determines the effectiveness of antioxidant (Balk *et al.,* 2009).

The adverse effects of ROS are represented by oxidative stress (OS) that can arise due to a lack of antioxidant defense or by an increase of oxidative processes in the body. OS occurs when there is an imbalance between the generation of ROS and antioxidant defence systems in the body, so that the latter become overwhelmed (Katsiki and Manes, 2009). Many different illnesses (such as cardiovascular disease, cancer, and neurological and endocrinological disorders) have been related to OS, which can be either a cause or a consequence of the disease (Cornelli, 2009). Fat-containing food materials can also deteriorate from oxidation thereby reducing their nutritional quality; synthetic or natural antioxidants can be employed in order to prevent such deterioration (Locatelli *et al.*, 2009).

Many researchers have demonstrated other benefits of antioxidants, which have been reviewed by Wootton-Beard and Ryan (2011), Yoshihara *et al.* (2010) and Moure *et al.* (2001). Antioxidants can prevent loss of pigments and improve the stability of pigments from red beet juice in the food industry, protect and stabilize aroma compounds in food and they can also be applied in oral pharmaceutical adjuncts to limit infertility (Visioli and Hagen, 2011) and cosmetic compositions.

2.4.1 Types of Antioxidants

Antioxidants can be classified as liposoluble or hydrosoluble antioxidants. Liposoluble antioxidants are located mainly on membranes or are associated with lipoproteins, while hydrosoluble antioxidants circulate more freely in the blood. Vitamin E, which is highly liposoluble, has a particular affinity for lipoproteins, whereas vitamin C, which is highly hydrosoluble, circulates freely with minimal protein binding (Cornelli, 2009). Functionally, antioxidants can be grouped according to their preferential localization and Cornelli (2009) reported this for the formulation of antioxidant combinations. This classification identifies antioxidants as follows:

- Membrane antioxidants: these are represented by vitamin E, β-carotene, vitamin A, and are known also as lipophilic antioxidants. They have an affinity for membranes of cells and lipoproteins (low-density lipoprotein, very low density lipoprotein, high-density lipoprotein).
- Circulating antioxidants: these consist of vitamin C, amino acids, and polyphenols, which are also known as hydrophilic antioxidants. They are not heavily bound to proteins and may circulate freely in body fluids.
- Cytosol antioxidants: these are produced by cells. Members of this class are lipoic acid, squalene, coenzyme Q10. They are intermediates for the synthesis of endogenous molecules or macromolecules (cytochromes).
- System antioxidants: these are trace metals (such as Se and Zn) or amino acids (such as L-cysteine) (Cornelli, 2009).

Another group of antioxidants is classified based on the direct or indirect activity of these compounds. Direct activity refers to the capacity of a molecule to become a chain breaker or quencher, whereas indirect activity may interfere with processes that stimulate the production of reactive species (RS). Steroid, nonsteroidal anti-inflammatory drugs, statins, and some antihypertensive drugs (such as angiotensin-converting enzyme inhibitors) are examples of indirect antioxidants (Cornelli, 2009).

2.4.2 Sources of Antioxidants

2.4.2.1 Introduction

Plant foods provide a wide variety of dietary antioxidants, such as vitamins C and E, carotenoids, flavonoids and other phenolic compounds. Natural antioxidants such as vitamin C and E are used as a means of enhancing biological functions and improving the stability of lipid and lipid-containing products. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ) and propylgallate (PG) are also used but are under strict regulation because of potential health hazards (Hettiarachchy and Qi, 1998). Due to potential health hazards of synthetic antioxidants, researchers have embarked on a continuous search for natural antioxidants as alternatives to synthetic ones for application in food products and cosmetics.

2.4.2.2 Phenolic Compounds

Phenolic compounds are part of the important group of secondary metabolites produced by plants. They are characterized by at least one aromatic ring (C6) that bears one or more hydroxyl groups (Michalak, 2006). Biosynthesis of phenolic compounds in plants through aromatic amino acids commences via the shikimate

pathway. The significance of this pathway is demonstrated by the fact that, under normal growth conditions, 20% of the carbon fixed by plants flows through this route. The aromatic amino acids, phenylalanine, tyrosine and tryptophan, are formed via this pathway; which are later utilized for protein synthesis or transformed via phenylpropanoid metabolism to secondary metabolites such as phenolic compounds (Diaz *et al.,* 2001) (Fig. 2.15).

Phenolic compounds are divided into several groups and are based on the number of constitutive carbon atoms in conjuction with the structure of the basic phenolic skeleton (Michalak, 2006). Figures 2.16 to 2.20, show the structures of some of the major groups of phenolic compounds.

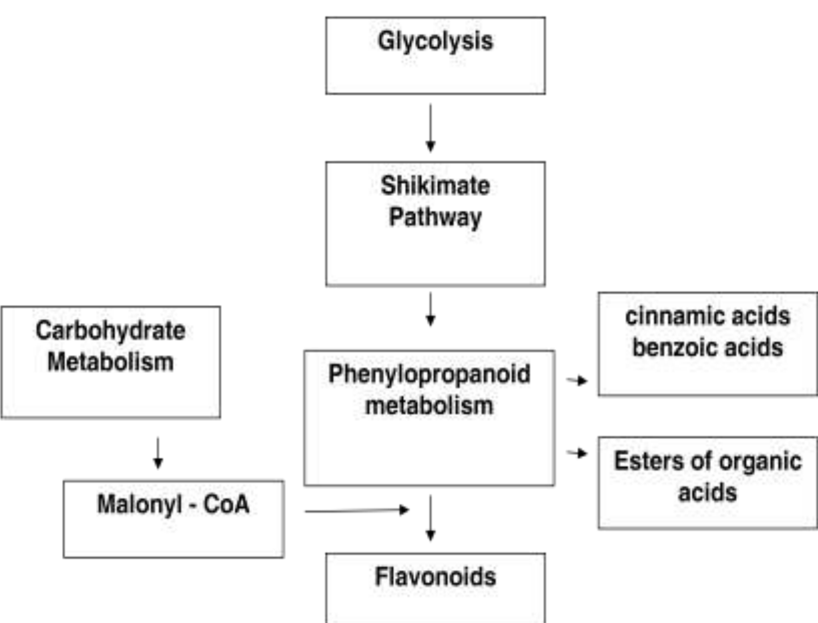


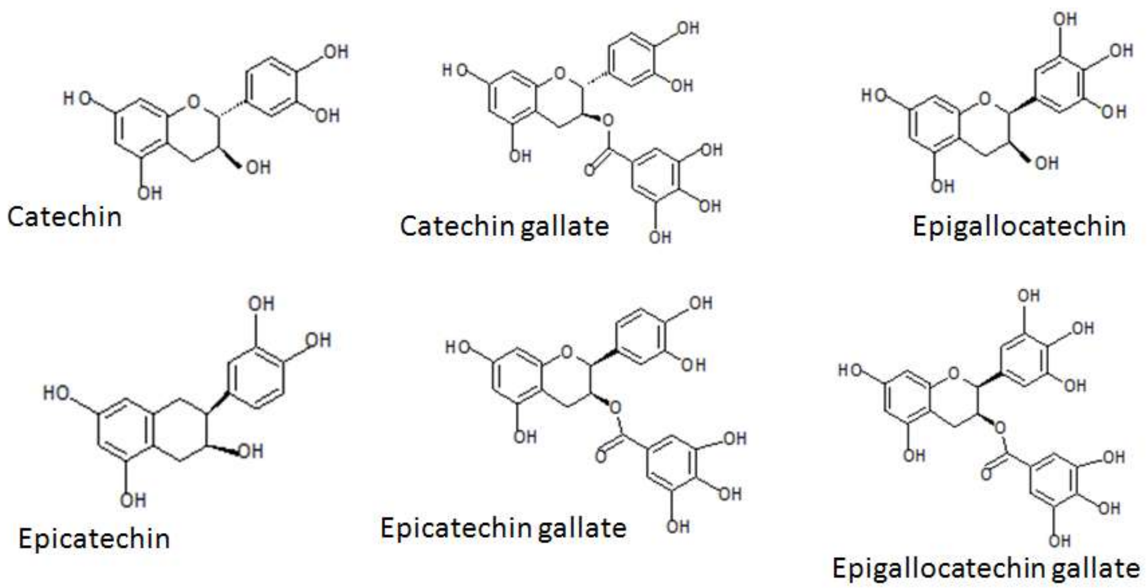
Fig. 2.15: Biosynthesis pathways leading to formation of main groups of phenolic compounds (Michalak, 2006).

Daidzein Daidzin Glycitein

Glycitin Genistein Genistin

ISOFLAVONES

Fig. 2.16: Examples of naturally occurring flavonoids - Isoflavones.



FLAVANOLS

Fig. 2.17: Examples of naturally occurring flavonoids – Flavanols.

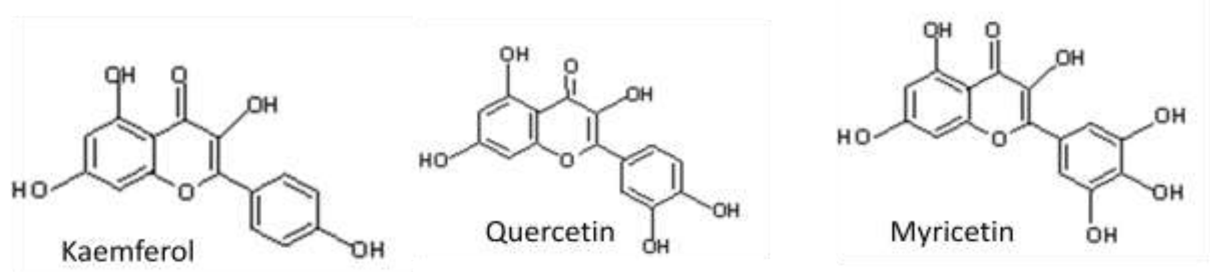
Chrysin

Rutin

Apegenin

Luteoin

FLAVONES



FLAVONOLS

Fig. 2.18: Examples of naturally occurring flavonoids – Flavones and Flavonols.

FLAVANONES

Cyanidin

Malvidin

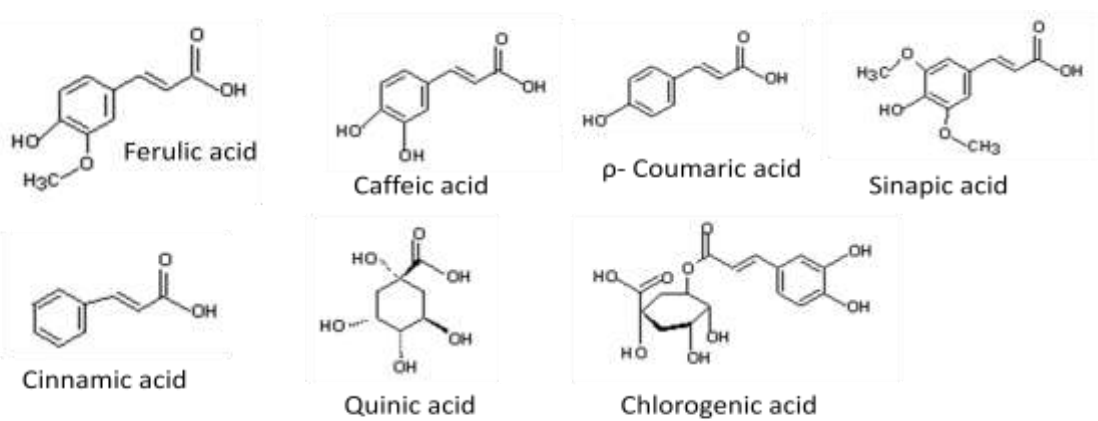
ANTHOCYANIDINS

Fig. 2.19: Examples of naturally occuring flavonoids – Flavanones and Anthocyanidins.

Gallic acid Protocatechuic acid Vanillic acid ρ -Hydroxybenzoic acid

Ellagic acid Syringic acid Gentisic acid Salicylic acid

HYDROXYBENZOIC ACIDS



HYDROXYCINNAMIC ACIDS

Fig. 2.20: Examples of naturally occurring phenolic acids – Hydroxybenzoic and Hydroxycinnamic acids.

Plants provide a large variety of bioactive compounds with substantial application in the area of health and food and are seen to be good sources of phenolic compounds with very interesting nutritional and therapeutic applications. There has been a strong correlation between antioxidant activity and the total phenolic content in the plants which suggests that phenolic compounds could be the major contributor of their antioxidant capacity. Phenolic compounds are widely distributed in plants, usually with a higher concentration in leaves and green steams. These compounds are considered natural defense substances, and their concentration in each plant may be influenced by several factors including physiological variations, environmental conditions, geographic variation, genetic factors and evolution (Matins *et al.*, 2011).

Phenolic compounds such as phenolic acids and some vitamins are well known as powerful antioxidants. They form an important class of compounds which serve to inhibit the oxidation of material of both commercial and biological importance (Nikolic, 2006). Phenolic compounds do not have intrinsic antioxidant activity unless substitution at either the *ortho*- or *para*-position increases the electron density at the hydroxyl group and lowers the O–H bond energy. The antioxidant efficiency of phenolic acids has been related to the number of hydroxyl groups in the molecule and also to their hydrogen radical donating abilities (Miller and Rice-Evans, 1997). For instance in the hydroxycinnamic acids group given above (Fig. 2.20); the presence of electron donating groups on the benzene ring of ferulic acid (3-methoxy and 4-hydroxyl) contributes to its property of terminating free radical chain reactions.

There are three proposed mechanisms by which phenolic antioxidants (ArOH) can have an antioxidant effect (Zhang and Ji, 2006; Zhang *et al.*, 2003). The first one involves a direct hydrogen atom transfer (HAT) (Mayer and Rhile, 2004) from the antioxidant to the radical ($ROO^{\cdot}$), Eq. (1). The second mechanism, Eq. (2), involves single electron transfer (SET) (Rojano *et al.*, 2008) from the antioxidant to the radical leading to indirect H-abstraction. The third mechanism, Eq. (3), has been termed sequential proton loss-electron transfer (SPLET) (Estevez and Mosquera, 2008) and takes place once the anion (ArO^-) has been formed. All three mechanisms may occur in parallel, but at a different rate.

$$RO^{\cdot}_2 + ArOH \longrightarrow ROOH + ArO^{\cdot} \qquad (1)$$

$$RO^{\cdot}_2 + ArOH \longrightarrow RO^-_2 + ArOH^{+} \longrightarrow ROOH + ArO^{\cdot} \qquad (2)$$

$$ArOH \longrightarrow ArO^- + H^+;\ ArO^- + RO^{\cdot}_2 \longrightarrow RO^-_2 + ArO^{\cdot};\ RO^-_2 + H^+ \longrightarrow ROOH \qquad (3)$$

The first mechanism (HAT) is governed by the O–H bond dissociation enthalpy (BDE) of ArOH, while in the second one (SET) the ionization potential (IP) and the reactivity of $ArOH^{\cdot+}$ are the most significant factors for scavenging activity evaluation. Finally, the O–H heterolytic bond dissociation enthalpy is involved in mechanism three (SPLET), where the ionization potential of $ArO^{\cdot}$ is a controlling parameter.

Antioxidants can act by the following mechanisms in lipid peroxidation: (i) decreasing localized oxygen concentrations; (ii) preventing chain initiation by scavenging initiating radicals; (iii) binding catalysts, such as metal ions, to prevent initiating radical generation; (iv) decomposing peroxides so they cannot be reconverted to initiating

radicals; and (v) chain-breaking to prevent continued abstraction by active radicals (Tepe *et al*., 2006; Dorman *et al*., 2003).

2.4.3 Specific examples of plant-derived antioxidants

Plant-derived compounds serve as good sources of antioxidants and various phenolic compounds have shown many beneficial attributes some of these are presented in Table 2.2.

Table 2.2: Specific examples of plant derived antioxidants

Plant derived antioxidant	Uses and source	References
Hydroxytyrosol	Prevent atherosclerosis through inhibition of low density lipoprotein (LDL) oxidation. Decrease of LDL uptake by macrophage polyphenol derived from olive oil.	Marrugat *et al*., 2004 Tripoli *et al*., 2005
trans-Resveratrol	Presence in wine could be responsible for the decrease in coronary heart disease observed among wine drinkers plays a role in the prevention of carcinogenesis. Found in legumes, grapes, grape juice and red wine.	Frankel *et al.,* 1993 Latruffe *et al*., 2002 Siemann and Creasy, 1992
Vanillin	Is one of the most important and widely used flavour compounds in the food industry chemically produced from guaiacol or lignin. Natural vanillin is obtained from *Vanilla planifolia.*	Priefert *et al*., 2001 Ramachandra and Ravishankar, 2000; Van den Heuvel *et al.,* 2001

Ferulic acid is the major phenolic compound investigated in this study and is therefore described in detail below.

2.4.3.1 Ferulic acid

Ferulic acid [3-(4-hydroxy-3-methoxy-phenyl)-acrylic acid], is one of the major phenolic lignin monomers found in woods, grasses, corn hulls, cereal brans and sugar beet pulp (Ferreira *et al*., 2007). It serves as a renewable aromatic feed stock for conversion into other useful and value-added chemicals such as guaiacol, vanillin, vanillic acid and protocatechuic acid (Prasad *et al*., 2006). Ferulic acid is said to be one of the effective components in many Chinese traditional medicines, which play indispensable roles in the prevention and treatment of diseases in the East and it shows many physiological functions, including antioxidant, anti-microbial, anti-inflammatory, anti-thrombosis, and anti-cancer activities. It can protect against angina, hypertensive diseases, and coronary disease, it also lowers cholesterol in serum and the liver, and increases sperm viability (Wang *et al.,* 2008).

2.4.3.2 Sources and Occurrences of Ferulic Acid

Ferulic acid is found abundantly in cereal grains as a free acid and as low molecular weight conjugates. It also occurs mostly as an ester-linked substituent of cell-wall heteroxylans, especially in brans. Ferulic acid arises from the metabolism of phenylalanine and tyrosine via the Shiikimate pathway in plants. The chemical structure and synthesis of ferulic acid and related compounds in plants are shown in Fig. 2.21 (Zhao and Moghadasian, 2008).

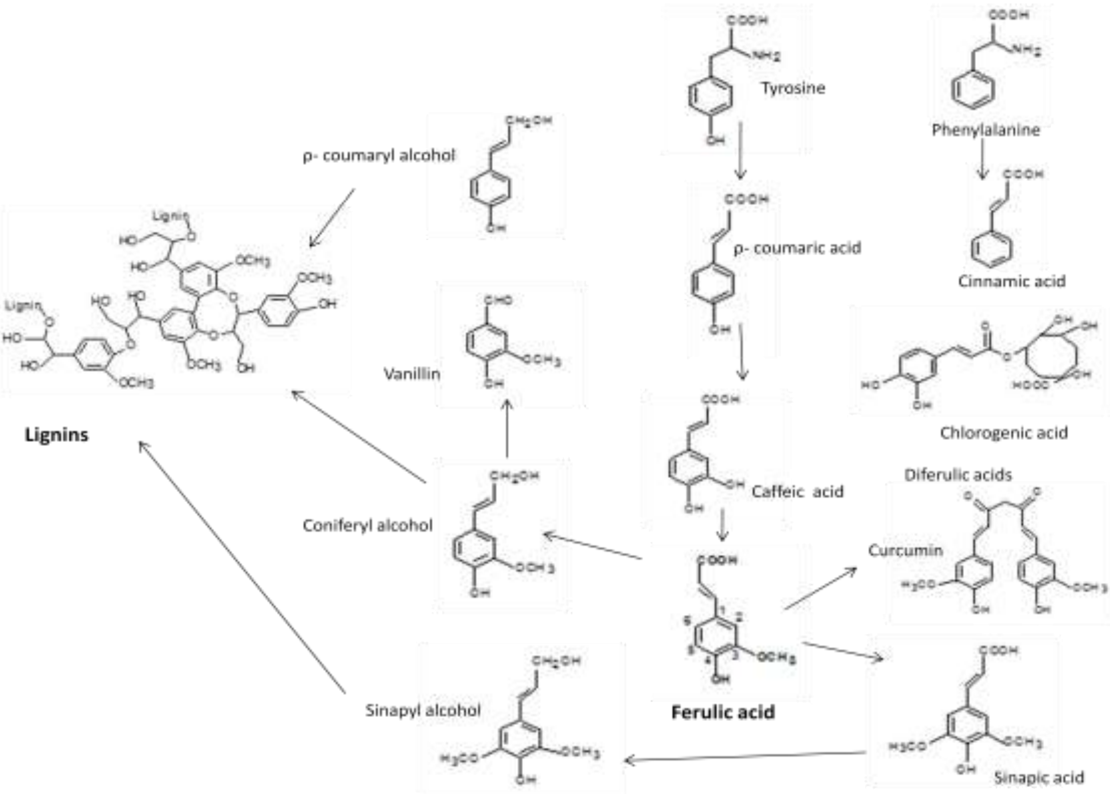


Fig. 2.21: Chemical structure and synthesis of ferulic acid and related compounds in plants. Adapted from Zhao and Moghadasian (2008).

Ferulic acid is a highly versatile phytochemical and its controlled release from plant material, particularly from agro-food by-products, is of potential economic interest to some industries. Because ferulic acid is an important constituent of the cell wall of many plants, (cell adhesion and covalently linkage by ester bonds to polysaccharides); it can survive certain agri-food processing activities and appears as bound ferulic acid in by-products of several plants.

Ferreira *et al.* (2007) worked on the release of ferulic acid and feruloylated oligosaccharides from sugar beet pulp by *Streptomyces tendae.* Their study was designed to identify actinomycete strains which are capable of releasing high levels of

ferulic acid from sugar beet pulp. Out of the 47 strains tested, 37% were found to release free ferulic acid from the growth substrate. The *S. tendae* strain was able to release 80% of the ferulic acid ester-linked to the pectin in the sugar beet pulp after only 5 days of growth. Their results suggested that some actinomycetes are capable of releasing ferulic acid and feruloylated oligosaccharides from sugar beet pulp and that during the growth of *Streptomyces* species on the pulp, solubilization and release of feruloylated oligosaccharides by specific carbohydrase activities occurred; before de-esterification and release of free ferulic acid. Saulnier *et al.* (2001) also studied the thermal and enzymatic treatments for the release of free ferulic acid from maize bran. They reported that autoclaving treatment of the bran with the subsequent action of a feruloyl esterase is the most efficient treatment to produce free ferulic acid from maize bran.

Several dehydrodimers of ferulic acids have also been isolated from the cell walls of plants as they result from oxidative coupling of ferulate esters and represent mainly products of 8-5, 8-O-4, and 5-5 radical coupling (Fig. 2.22; Ralph *et al.,* 1994).

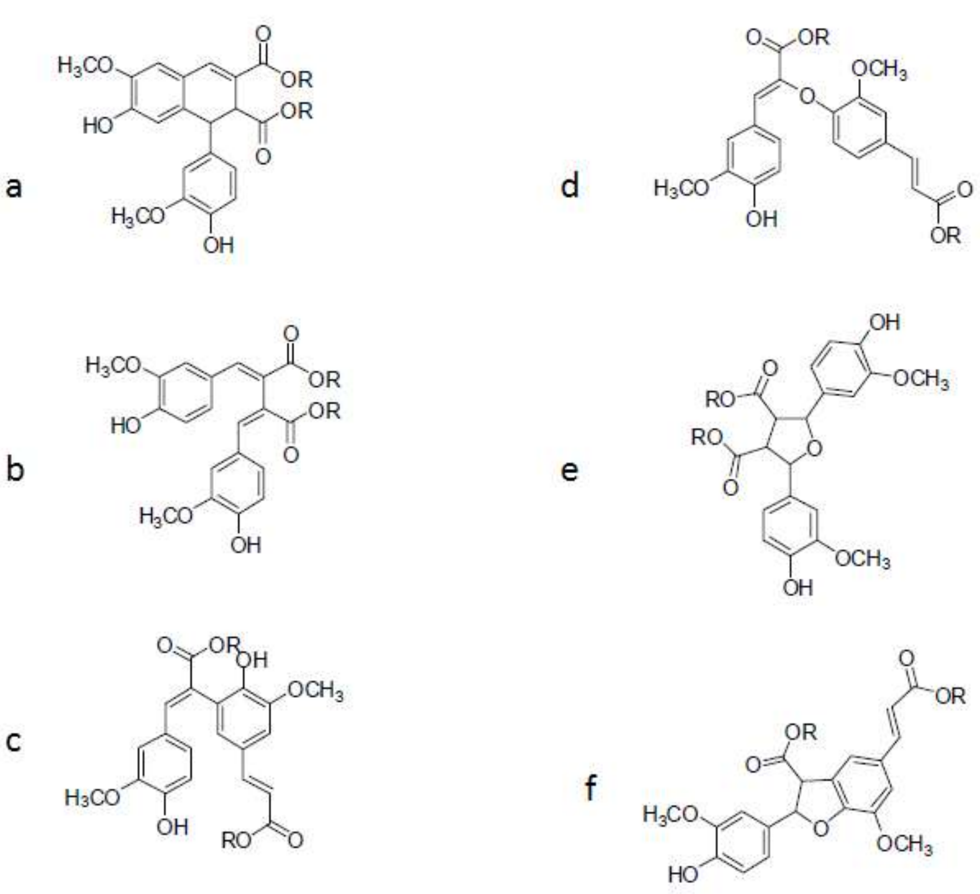


Fig. 2.22: Main dimeric structure of dehydrodiferulic acids in plants: a – β-β-Diferulate, cyclic form; b – β-β-Diferulate, open chain form; c – β-5-Diferulate; d – β-*O*-4-Diferulate; e – β-β-Diferulate, furan type; f – β-5-Diferulate, dihydrobenzofuran type (Ralph *et al.,* 1994).

Rouau *et al.* (2003), while analyzing the phenolic compounds of maize bran, reported the isolation and the structural characterization of a new compound which is a dehydrotrimer of ferulic acid (Fig. 2.23). This new phenolic acid trimer was detected by coupled liquid chromatography/mass spectroscopy in alkali extracts and was purified by preparative silica gel chromatography.

Fig. 2.23: A dehydrotrimer of ferulic acid isolated from maize bran by Rouau *et al.* (2003).

2.4.3.3 Ferulic acid as an antioxidant

Ferulic acid was first isolated from a commercial resin in 1866 and chemically synthesized in 1925, but its biological effects were only identified in the 1970s when Japanese researchers discovered the antioxidant properties of ferulic acid steryl esters extracted from rice oil (Yagi and Ohishi, 1979).

One of the best documented biological activities of ferulic acid is its antioxidant properties, which is due to its phenolic nucleus and an extended side chain (Fig. 2.24). As a result of its phenolic nucleus and an extended side chain conjugation, it readily forms a resonance stabilized phenoxy radical which accounts for its potent antioxidant potential. It possesses three distinctive structural motifs that can possibly contribute to its free radical scavenging capability. The presence of electron donating groups on the benzene ring (3-methoxy and more importantly 4-hydroxyl) of ferulic acid gives the additional property of terminating free radical chain reactions. The next functionality, the carboxylic acid group in ferulic acid with an adjacent unsaturated C–C double bond, can provide additional attack sites for free radicals and thus prevent them from attacking the membrane. In addition, this carboxylic acid group also acts

as an anchor of ferulic acid, by which it binds to the lipid bilayer, providing some protection against lipid peroxidation (Kanaski *et al.*, 2002).

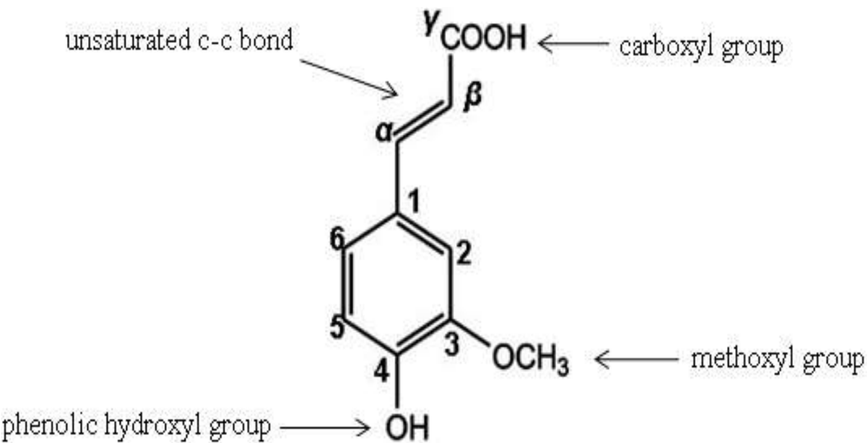


Fig. 2.24: Chemical structure of ferulic acid and the distinctive structural motifs that account for its antioxidant properties.

Kikuzaki *et al.* (2002) studied the antioxidant activity of 24 ferulic acid-related compounds together with 6 gallic acid-related compounds with the use of several different physical systems. The radical scavenging activity of these compounds was also studied. They reported that ferulic acid showed the most effective antioxidant activity among the tested hydroxycinnamic acids when tested against the auto-oxidation of linoleic acid in an ethanol-buffer system. In addition, a study of theantioxidant and antiradical activity of ferulates was performed by Karamac *et al.* (2005). The antioxidant performance of the tested compounds in a β-carotene-linoleate model system exhibited antioxidant properties to varying degrees which were in the order of methyl ferulate > ferulic acid > coniferyl aldehyde > isoferulic acid.

Prasad *et al.* (2006) reported the radioprotective effect of ferulic acid on γ-radiation-induced cytogenetic and biochemical damages in human lymphocytes treated *in vitro*. The protection was concentration dependent (ranging from 1 to 10 μg/ml) and the effect eliminated when ferulic acid was absent, indicating that it has to be taken up by the cells to exert its radioprotective effect.

Ferulic acid has several potential industrial and medical applications including its use as a topical protective agent against UV-radiation-induced skin damage (Saija *et al.,*

1999). UV absorption by ferulic acid also catalyzes stable phenoxy radical formation making its ability to terminate free radical chain reactions posible. By virtue of effectively scavenging deleterious radicals and suppressing radiation-induced oxidative reactions, ferulic acid may serve an important antioxidant function in preserving physiological integrity of cells exposed to both air and impinging UV radiation. Incorporation of ferulic acid in cosmetic lotions can give the skin photo-protection. Its addition to foods inhibits lipid peroxidation and prevents subsequent oxidative spoilage. By the same mechanism ferulic acid may protect against various inflammatory diseases. A number of other industrial applications are based on the antioxidant potential of ferulic acid (Graf, 1992), such as its use as a food preservative, or as a photo protectant in the textile industry (Graf, 1992).

2.4.3.4 Ferulic acid and biotransformation

Biotransformation of ferulic acid to value added product has been reported in literature. Some of these are as shown in Table 2.3.

Table 2.3: Ferulic acid and biotransformation

Biotransformation	Organism strain	Products	Reference
Esterification of ferulic acid with polyols	ferulic acid esterase from *Aspergillus niger*	1- glyceryl ferulate with higher antioxidant than butyl hydroxytoluene	Tsuchiyama *et al.*, 2006
Bioconversion of ferulic acid	thermophilic fungus *Sporotrichum thermophile*	vanillic acid	Topakas *et al.*, 2003
Decarboxylation of ferulic acid	whole cells of *Bacillus pumilus*	vinylguaiacol	Lee *et al.*, 1998
Degradation of ferulic acid	strain of *Bacillus coagulans*	4-vinylguaiacol, vanillin, vanillic acid, protocatechuic acid	Karmaka *et al.*, 2000

2.4.4 Biocatalytic production of antioxidants

Several researchers have reported that antioxidants can be made by the process of biocatalysis using simple plant-derived compounds as the starting materials. Some examples are discussed below.

Polyphenol oxidase (PPO) can be used in the conversion of phenolic substrates to catechols and/or quinones. It is readily available as extracts with high activity from inexpensive sources, and does not require extensive purification. The use of PPO,

obtained from *Agaricus bisporus*, in hydroxylating a range of phenolic substrates to yield catechols was reported by Burton *et al.* (1998) and is shown in Fig. 2.25.

Phenol Catechol *o*-Quinone

Fig. 2.25: Hydroxylation of phenols and oxidation of catechols catalysed by polyphenol oxidase (Burton *et al.,* 1998).

Mushroom tyrosinase (PPO) can be used in the synthesis of value-added products such as L-DOPA, the anti-Parkinson's disease drug (Carvalho, 2000; Pialis and Saville, 1998). Additionally, reports have suggested its application in the production of the antioxidant and anti-microbial agent hydroxytyrosol [2-(3,4-dihydroxyphenyl) ethanol] from tyrosol [2-(4-hydroxyphenyl) ethanol] (Miro-Casas *et al.*, 2003; Tuck and Hayball, 2002; Espin *et al.*, 2001; Ramos-Cormenzana *et al.*,1996).

Hydroxytyrosol has also been reported to be produced from the tyrosinase–catalysed transformation of tyrosol by whole cells of *Pseudomonas aeruginosa* (Allouche *et al.*, 2004). Brooks *et al.* (2006) studied the ability of immobilised cell extracts of *Pseudomonas putida* F6 to transform tyrosol to hydroxytyrosol. *P. putida* F6 was previously isolated from soil and shown to possess a tyrosinase capable of transforming *p*-hydroxyphenylacetic acid to 3,4-dihydroxyphenylacetic acid (O'Connor, 2001) as well as a range of halogenated phenols to the corresponding catechols (Brooks *et al.*, 2004). This catalytic ability, like that of mushroom tyrosinase, does not require the exogenous addition of co-factors (O'Connor, 2001). It was reported that a bacterial tyrosinase may offer a cheaper and more accessible source of tyrosinase activity than mushroom tyrosinase. Optimisation of tyrosol transformation by an immobilized bacterial tyrosinase activity resulted in complete substrate removal and an overall yield of 77% of hydroxytyrosol in the presence of ascorbic acid (Brook *et al.*, 2006).

2.4.5 Determination of antioxidant activity

Antioxidants are essential because they help in scavenging the free radicals that tend to damage human cells under 'oxidative stress' conditions. Free radicals have been

identified to cause various disturbances in cell metabolism because they posses unpaired electrons which look for ways of stabilizing themselves. Different sources of free radicals include metabolism by-products, neutrophiles, UV radiation, air and water pollutants, fatty foods, hazardous chemicals, and cigarette smoke (Ozyurt *et al.*, 2007). So far, synthetic and natural antioxidants have been employed in combating these free radicals. However, natural sources are preferred because of potential health risk envisaged from synthetic sources.

In literature, different antioxidant activity assay methods are employed. Prior *et al.*, (2005) reported that a meeting of the First International Congress on Antioxidant Methods was held in June 2004 at Orlando, FL. Deliberations were made on how to deal with analytical issues relative to assessing antioxidant capacity (AOC) in food, botanicals, nutraceuticals, and other dietary supplements and it was suggested that one or more analytical methods should be standardized for routine assessment of AOC. Standardized test methods will allow for (1) guidance for appropriate application of assays, (2) meaningful comparisons of food or commercial products, (3) a means to control variation within or between products, and (4) provision of quality standards for regulatory issues and health claims (Prior *et al.*, 2005). In the standardization, the assay to be employed should be of analytical range, repeatable, possess good recovery, and also be able to recognize interfering substances.

2.4.5.1 Methods of antioxidant activity determination

Antioxidants have the capacity to reduce or prevent oxidation of a substrate. Antolovich *et al.* (2002) reported that antioxidant activity may not be measured *per se,* but the effect an antioxidant has in inhibiting oxidation of a substance can be measured. Therefore, antioxidant activities are measured in order to determine the inhibitory power with respect to the oxidation of the target compounds.

Ivekovic *et al.* (2005) reported that two types of analytical methods are used for the evaluation of antioxidant activity, which are:

(i) inhibition methods, in which the inhibition of oxidative damage of the target molecule is measured in the presence of antioxidants and related to a known standard, and

(ii) the direct measurement of the scavenging of stable free radicals by antioxidants present in the sample.

Furthermore, Huang *et al.* (2005) and Prior *et al.* (2005) discussed that antioxidant capacity assays can be divided into two different categories based on their chemical

reactions or reaction mechanisms which are Hydrogen Atom Transfer (HAT) based assay and Single Electron Transfer (SET) based assay. The end result for these two reactions is the same, but kinetics and potential for side reactions differ.

HAT-based methods measure the ability of an antioxidant to quench free radicals by hydrogen donation (AH) = any H donor); Eq. 1.

$$X^{\cdot} + AH \longrightarrow XH + A^{\cdot} \qquad (1)$$

The HAT-based methods are generally composed of a synthetic free radical generator, an oxidizable molecular probe and an antioxidant (Huang *et al.*, 2005). Most of the HAT-based assays apply a competitive scheme, whereby the antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo-compounds (Zulueta *et al.*, 2009).

SET-based methods on the other hand detect the ability of a potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls, and radicals (Eq. 1 - 4).

$$X^{\cdot} + AH \longrightarrow X^{-} + AH^{\cdot+} \qquad (1)$$

$$AH^{\cdot+} \xleftrightarrow{H_2O} A^{\cdot} + H_3O^{+} \qquad (2)$$

$$X^{-} + H_3O^{+} \longrightarrow XH + H_2O \qquad (3)$$

$$M(III) + AH \longrightarrow AH^{+} + M(II) \qquad (4)$$

The SET–based assay methods involve one redox reaction with the oxidant (also as a probe for monitoring the reaction) as an indicator of the reaction endpoint. These assays analyse the ability of an antioxidant when during the reduction of an oxidant, changes colour when reduced. The degree of the colour change is correlated with the sample's antioxidant concentrations (Zulueta *et al.*, 2009).

Different assays utilize different reaction mechanisms some of which are highlighted below (Huang *et al.*, 2005):

Assays that utilize Hydrogen Atom Transfer (HAT)

1. ORAC (Oxygen Radical Absorbance Capacity)
2. TRAP (Total Radical Trapping Antioxidant Parameter)
3. Crocin bleaching assay
4. IOU (Inhibited Oxygen Uptake)
5. Inhibition of linoleic acid oxidation
6. Inhibition of low density lipoprotein (LDL) oxidation

Assays that utilize Single Electron Transfer (SET)

1. TEAC (Trolox Equivalent Antioxidant Paramenter)
2. FRAP (Ferric ion Reducing Antioxidant Parameter)
3. DPPH (diphenyl-1-picrylhydrazyl)
4. Copper(II) reduction capacity
5. Total phenols assay by Folin-Ciocalteu reagent

2.4.5.1.1 ORAC assay

The ORAC method (oxygen radical absorbance capacity) determines the antioxidant scavenging activity against peroxyl radicals induced by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) at 37°C. Since it measures the antioxidant inhibition of peroxyl radical induced oxidations, it's therefore a reflection of a classical radical chain breaking antioxidant activity by H atom transfer (Prior *et al.*, 2005). The ORAC assay gives an outstanding and complete assessment whereby the inhibition time and inhibition degree can be measured as the reaction goes to completion (Ou *et al.,* 2001).

Ou *et al.* (2001) developed and validated an improved ORAC assay using fluorescein as the fluorescent probe. This was necessary due to the shortcomings observed in the method of Cao *et al.* (1993). In the method of Cao *et al.* (1993), β-phycoerythrin (β-PE), a protein isolated from *Porphyridium cruentum*, was employed as the fluorescent probe. However, the β-PE gives inconsistency from batch to batch; it can be photo bleached after exposure to excitation light for a certain time and is therefore not photostable, and lastly, β-PE was observed to interact with polyphenols due to the non-specific protein binding. In their study, Ou *et al.* (2001) observed that the introduction of (3',6'-dihydroxyspiro[isobenzofuran-1[3*H*],9'[9*H*]-xanthen]-3-one) as the fluorescent probe resulted in a direct measurement of hydrophilic chain-breaking antioxidant capacity against peroxyl radicals.

The ORAC assay gives a controlled source of peroxyl radicals which model reactions of antioxidants with lipids in both food and physiological systems. By altering the solvent and radical sources, the ORAC assay method can identify both hydrophilic and hydrophobic antioxidants. Prior *et al.* (2003) worked on the assays for hydrophilic and lipophilic antioxidant capacity ($ORAC_{FL}$) of plasma and other biological and food samples. This study focused on the extraction and analysis of hydrophilic and lipophilic antioxidants, employing the modifications of the oxygen radical absorbing capacity ($ORAC_{FL}$) procedure. Their methods for the first time gave the capability to obtain a measure of “total antioxidant capacity” in the protein-free plasma, through the use of the same peroxyl radical generator for both lipophilic and hydrophilic antioxidants.

2.4.5.1.2 TRAP assay

The total radical trapping parameter (TRAP) assay employs the peroxyl radicals generated from 2,2’-azobis(2-amidinopropane) dihydrochloride (AAPH) and a peroxidizable substance that is present in plasma or other biological fluids. This method, which has been the most widely used for evaluating plasma antioxidant capacity, was introduced by Wayner *et al.* (1985). The peroxyl radicals generated, have sufficient energy to abstract hydrogen from a (lipid) substrate, thereafter initiating a (lipid) peroxidation chain which is sensitive to all known chain breaking antioxidants. The quenching reaction is measured in the presence of antioxidants, the potential of which is evaluated by measuring the delay in decolouration. This method is time-consuming; requiring about 2 hours per sample which means only a limited number of samples can be handled daily; it is also relatively complex and requires a high degree of expertise and experience (Ghiselli *et al.*, 2000).

2.4.5.1.3 Crocin bleaching assay

Crocin bleaching assay is an assay that measures the inhibition capacity of antioxidants by preventing the bleaching of crocin, which is a naturally occurring carotenoid derivative, by the free radical generator AAPH (Huang *et al.*, 2005). Crocin is a method where the antioxidant effect on it is displayed by hydrogen transfer (Bortolomeazzi *et al.,* 2007). It has straightforward reactions and bleaches only by the radical oxidation pathway (Eq 1 - 2).

$$\text{crocin-H (orange)} + ROO^{\cdot} \longrightarrow \text{crocin}^{\cdot} \text{ (Bleached)} + ROOH \quad (1)$$

$$\text{crocin-H (orange)} + ROO^{\cdot} + AH \longrightarrow \text{crocin} + ROOH + A^{\cdot} \quad (2)$$

The colour loss is monitored optically at 443 nm (89000 M^{-1} cm^{-1} in phosphate buffer, pH 7.4), which means that the reaction does not require any special instrumentation. However, its limitations are that crocin is not available commercially and must be extracted. It is a mixture of natural pigments that is extracted from saffron, and hence is subject to batch-to-batch variability (Huang *et al.*, 2005). This somewhat limits its industrial application in a quantitative procedure as there are no standard formats for expressing results; every study has a different method for calculating the inhibition kinetics (Prior e*t al.*, 2005).

Several spectrophotometric assays are currently employed to measure the antioxidant capacity of biological samples, the most popular are 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, and the FRAP (ferric reducing antioxidant power) assay (Floegel *et al.,* 2011). These assays are quick and do not require sophisticated equipment, like fluorescence detector or GC-MS (gas chromatograph-mass spectrometer), which make them suitable for analyses of multiple tissue samples. Antioxidant activity of the synthesised products and their substrates in this study was evaluated using these three methods.These methods are described in sections 2.4.5.1.4, 2.4.5.1.5 and 2.4.5.1.6.

2.4.5.1.4 TEAC (Trolox Equivalent Antioxidant Capacity) assay

The Trolox equivalent antioxidant capacity (TEAC) or ABTS assay is based on the scavenging of the radical cation of ABTS by the antioxidants present in a sample (Zulueta *et al.*, 2009). The $ABTS^{\cdot+}$ radical normally has a bluish-green colour which has a characteristic long wavelength absorption spectrum showing maxima at 660, 734, and 820 nm (Prior e*t al.*, 2005). When there are antioxidant compounds in the reaction medium, they capture the free radical, this is revealed as a loss of colour and a reduced absorbance is observed which corresponds quantitatively to the concentration of antioxidants present. The ABTS radical cation is formed by the interaction of ABTS with the ferrylmyoglobin radical species, generated by the activation of metmyoglobin with H_2O_2. This original TEAC assay measures the ability of a compound in the reducing ABTS radical.

Typically, the $ABTS^{\cdot+}$ is prepared either through the use of peroxidase or myoglobin (Villano *et al.*, 2005; Yu, 1999), electrochemically (Ivekovic *et al.*, 2005) or chemically (Re *et al.*, 1999). This assay for total antioxidant activity (TAA), or TEAC, measures the concentration of the Trolox solution with an equivalent antioxidant potential to a standard concentration of the compounds which is being investigated. The TEAC

reflects the ability of hydrogen-donating antioxidants to scavenge the $ABTS^{•+}$ radical cation compared to that of Trolox which is a water-soluble vitamin E analogue. Antioxidants suppress the absorbance at 734 nm to an extent and on a time scale dependent on the antioxidant activity. Thus, for the TEAC assay, the results can be defined as the concentration of the Trolox solution with equivalent antioxidant potential to that of a 1mM concentration of the compound under investigation (Rice-Evans and Miller, 1997).

2.4.5.1.5 FRAP (Ferric Ion Reducing Antioxidant Power Assay) assay

The FRAP assay takes advantage of electron-transfer reactions. The antioxidant activity of the tested sample is estimated by measuring the increase in absorbance caused by the formation of ferrous ions from the FRAP reagent containing TPTZ (2,4,6-tri-pyridyl-s-triazine) and $FeCl_3$.6H2O. The principle behind this is that, at low pH, TPTZ complex to the ferrous form (which has an intense blue colour) and can be monitored by measuring the change in absorbance at 593 nm. The reaction is non-specific, in that any half reaction that has lower redox potential, under reaction conditions, than that of ferric ferrous half reaction, will drive the ferrous (Fe III to Fe II) ion formation. The change in absorbance is therefore directly related to the combined or “total” reducing power of the electron donating antioxidants present in the reaction mixture (Huang *et al.*, 2005; Benzie and Strain, 1999 and 1996).

2.4.5.1.6 DPPH (1, 1 diphenyl 2, picryl hydrazyl) assay

DPPH is the most widely reported method used in testing for antioxidant activity of many food products, botanicals, nutraceuticals, and other dietary supplements. This method is based on the reduction of methanolic solution of the coloured free radical DPPH by a free radical scavenger. Any substance with the ability to donate a hydrogen atom (antioxidant) to $DPPH^{•}$, reduce the stable free radical and change the colour of the solution from violet to pale yellow (Milardovic *et al.*, 2006). The procedure involves the measurement of the decrease in absorbance of DPPH at its absorption maxima of 517 nm, which is proportional to the concentration of free radical scavenger added to the DPPH solution. The activity is expressed as efficiency concentration (EC_{50}), which is the concentration needed to reduce the initial free radical DPPH by 50%.

2.4.5.1.7 Other assay methods

i. The total antioxidant activity determination by ferric thiocyanate methodmeasures the amount of peroxide that is the primary product of oxidation produced during the initial stages of oxidation (Ak and Gulcin, 2008).

ii. Ferrous ion (Fe^{2+}) chelating activity: here the Fe^{2+} - chelating ability of the sample is monitored by the absorbance of the ferrous iron-ferrozine complex at 562nm (Ak and Gulcin, 2008).

Hydrogen peroxide scavenging activity; the principle behind the method is that there will be a decrease in absorbance of H_2O_2 upon oxidation of H_2O_2 (Ak and Gulcin, 2008).

Many researchers have also reported other novel methods which can be employed to measure the antioxidant activity of plants, food and nutraceutic materials, some of which are shown in Table 2.4.

Table 2.4: Some novel methods for antioxidant capacity determination

Method	Reference
Spectrophotometric method based on reducing capacity of Ce(IV)	Ozyurt *et al*., 2007
Potential use of laccase for the detection of antioxidant compounds	Kulys and Bratkovskaja, 2007
An antioxidant activity assay based on laccase-generated radicals	Nugroho Prasetyo *et al*., 2009
The voltammetric method (electrochemical method)	Medeiros *et al.,* 2010; Diaz *et al*., 2004; Diaz *et al*., 1998; Ceballos and Fernandez, 2000; Agüí *et al.,* 1995

The current study

This project investigated the production and purification of the oxidoreductase, laccase produced by the white-rot fungus, *Trametes pubescens*. Preliminary small scale reactions of ferulic acid, 2,6-DMP and the coupling of quercetin or catechin, known and active antioxidants onto other phenolic compounds (gallic acid, vanillic acid, caffeic acid, guaiacol, 2,6-DMP, pyrogallol, vanillin, eugenol, isoeugenol, protocatechuic acid and catechol) with laccase were performed with the view of increasing the antioxidant activity of the starting materials. Optimization and scale-up production of these reactions were performed in monophasic and biphasic organic solvents. Products were separated by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC), purified by flash chromatography, and characterized by liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR). Antioxidant activity was determined through the use of the 2, 2' -Diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical-scavenging activity assays.

CHAPTER THREE

ENZYME PRODUCTION AND PRELIMINARY SMALL SCALE BIOCATALYSIS REACTIONS

3.1 Introduction

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are the most widely studied among the multicopper oxidase group of enzymes. They catalyze the one-electron oxidation of four reducing-substrate molecules and at the same time reduce molecular oxygen to water (Piontek *et al.,* 2002). These enzymes are known to catalyze the oxidation of a wide range of phenolic compounds and aromatic amines. While many sources exist, such as bacteria (Claus, 2003; Martins *et al.*, 2002; Berrocal *et al.*, 1997), higher plants such as the Chinese lacquer tree *Rhus vernicifera* and insects (Sadhasivam *et al.*, 2008), the white-rot fungi, e.g. *Trametes versicolor* and *Trametes pubescens* are the best-known laccase producers (Galhaup and Haltrich, 2001; Thurston, 1994).

Generally, laccases can oxidize a wide range of substrates but phenolic compounds are their preferred substrates (Claus, 2004). They have been shown to catalyze the polymerization of many phenolic compounds and as such many natural or “artificial” natural products have been synthesized. One quality of phenolic compounds which make them good antioxidants is their ability to inhibit oxidation (Nikolic, 2006). Because the antioxidant efficiency of phenolic acids have been related to the number of hydroxyl groups in the molecule and also to the hydrogen radical donating abilities (Miller and Rice-Evans, 1997), recent research efforts have focused on oligomerisation of antioxidants to higher molecular weight compounds with increased stability and physiolological properties (Adelakun *et al.*, 2012; Nugroho Prasetyo *et al.*, 2011; Ncanana and Burton, 2007; Kurisawa *et al.*, 2003a,b).

Large scale production of laccase was carried out in this study in an airlift reactor. An airlift reactor was used to produce laccase by free cells of *T. pubescens* because of its simplicity in design, reliability and low cost. It provides a low shear environment for enzyme production by free mycelial pellets and does not require a mechanical stirrer. The risk of contamination and energy demand is relatively lessened when compared to stirred-tank reactors (Trager *et al.*, 1989). Scale-up for laccase production in our research group (Biocatalysis and Technical Biology Research Group) has previously been optimised for production in an airlift reactor (Ryan *et al.*, 2005) as this would result in high yields, thereby ensuring the availability of laccase which could be used further for biocatalysis reactions.

This chapter describes the production of laccase using a strain of *T. pubescens*, isolation and purification of the enzyme and the application of the enzyme in some preliminary small scale biocatalysis reactions.

3.2 Materials and Methods

All chemicals and solvents used were of analytical grade and purchased from Merck and Sigma Aldrich South Africa. *T. pubescens* (CBS 696.94) was obtained from the BOKU, the University of Natural Resources and Life Sciences, Vienna, in Austria.

3.2.1 Production of laccase

3.2.1.2 Preservation of the *T. pubescens* strain

A culture of *T. pubescens* was maintained on a 3% (w/v) malt extract agar plate at 4°C and sub-cultured every 60 days to maintain viability.

3.2.1.3 Growth of *T. pubescens* in flasks for laccase production

A fresh culture of *T. pubescens* was grown on a 3% (w/v) malt extract agar plate and incubated at 28°C for 5 days. Two portions of autoclaved 200 ml of *Trametes* Defined Media (TDM; Appendix A) were aseptically inoculated with homogenised (autoclaved blender) extract of *T. pubescens* grown on solid media. These were incubated for 4 days in 1000 ml Erlenmeyer flasks at 28°C with agitation at 175 rpm (Chigorimbo-Murefu, 2007).

3.2.1.4 Large scale production of laccase in airlift bioreactor

The large scale production of laccase was performed according to the method of Ryan *et al.* (2005). *T. pubescens* was cultured in 4 L airlift bioreactors. Each portion of 200 ml starter cultures in 1000 ml Erlenmeyer flask was aseptically inoculated into 3.3 L previously autoclaved TDM inside two separate 4 L airlift bioreactors to start the biomass production. The culture was induced on the 6th day with 0.234 g phenol + 0.084 g *p*-cresol dissolved in 30 ml water (filter-sterilized) or 0.234 g phenol + 0.084 g p-cresol + 81.6 l *m*-cresol + 239 µl *o*-cresol + 1 gram of glucose dissolved in sterile water. Samples were collected daily, and analysed for laccase activity (section 3.2.3) in triplicate. The values in the Fig. 3.1 and Fig. 3.2 correspond to mean values of replicate experiments with a standard deviation.

After the 10th day, the enzyme was harvested. The medium was centrifuged at 15000 rpm for 10 minutes to remove fungal biomass and the supernatant fluid used for the purification of the laccase.

3.2.2 Laccase purification

3.2.2.1 Purification of laccase by acetone precipitation

After the termination of the growth in the airlift reactor, biomass was removed by centrifugation (see above). An aliquot was filtered and equal volumes of laccase containing medium and cold acetone were mixed gently and kept stirring at 4°C for 1 h. The mixture was centrifuged at 10000 rpm for 10 minutes and the pellets washed with distilled water and resuspended in 0.1 x the initial volume in 0.1 M sodium acetate buffer, pH 5.0.

3.2.2.2 Purification of laccase by ammonium sulphate precipitation

Medium containing laccase was treated using ammonium sulphate. Successive amounts of ammonium sulphate (50, 60, 70, 80 or 50-80% saturation of ammonium sulphate, respectively) were added in order to precipitate the protein. This was left stirring at 4°C for 1 h and the mixture was centrifuged at 10000 rpm for 15 minutes. The pellets were resuspended in 0.1 x the initial volume in 0.1 M sodium acetate buffer (pH 5.0) and the collected sample dialysed against 0.1 M sodium acetate buffer pH 5.0 at 4°C for 24 hours.

3.2.3 Enzyme activity determination by laccase assay

Laccase activity was determined spectrophotometrically by monitoring the oxidation of 2,2,'-Azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, ε_{420} = 36,000 $M^{-1}cm^{-1}$) as substrate at 420 nm. The reaction mixture contained 0.330 ml ABTS (5 mM, prepared in distilled water), 2.5 ml 0.1 M sodium acetate buffer (pH 5.0) and 0.17 ml laccase. Oxidation of ABTS was monitored in the time scan mode for 80 seconds. One unit of laccase activity was defined as the amount of enzyme required to oxidise 1 µmol of ABTS per minute at 25°C.

3.2.4 Protein quantification of enzyme samples

Protein quantification of enzyme samples was performed using the Bradford assay (Bradford, 1976). 1 mg/ml of bovine serum albumin (BSA) solution was prepared by dissolving 10 mg BSA in 10 ml distilled water. Using the stock, various concentrations (between 0.1 and 1.0 mg/ml) of BSA standards were prepared. In 1 ml cuvettes, 1 ml of Bradford reagent was added and then 20 µl of the BSA standard or enzyme samples was added and mixed gently by inversion. These were left to incubate at room temperature for 5 min. Thereafter the absorbance was measured at 595 nm using a spectrophotometer (Rayleigh, model UV – 9200, China) and a standard curve was prepared (Appendix B).

3.2.5 SDS-PAGE analysis

The molecular weight of the purified laccase was determined by SDS-PAGE (sodium dodecylsulphate-polyacrylamide gel electrophoresis) as per the method of Laemmli (1970). SDS-PAGE analysis was performed to separate and visualise the purified proteins. Running gel buffer pH 8.8, stacking gel pH 6.8, 10 X running buffer pH 8.3, 30% acrylamide were prepared as shown in Appendix C. Just before being subjected to SDS–PAGE, laccase samples (approximately 20 µg proteins) were mixed with an equal volume of sample buffer containing 25 mM Tris–HCl buffer, pH 8.0, 2% SDS, 2% dithiothreitol, 20% glycerol and 0.2% bromophenol blue. After having been heated at 100°C for 5 min, they were loaded onto the gel. The samples were electrophoresed at 55 V at room temperature and stopped when the tracking dye front had reached the bottom of the gel. The gels were stained using coomassie brilliant blue R250, and destained with distilled water. PageRuler™ Prestained Protein Ladder (FE SM0671) was used as standard protein marker.

3.3 Initial small scale biocatalysis reactions – Oligomerization and coupling reactions

3.3.1 Dimerization reactions of phenolic compounds with laccase

Selected phenolic compounds (ferulic acid, 2,6-DMP and protocatechuic acid) reactions with laccase were performed on a small scale. The reaction mixture contained phenolic compound (10 mM), laccase (10 U) in 100 mM sodium acetate buffer, pH 5.0, and ethyl acetate (90%, previously determined as the optimum in this study for product yield and reduced side reactions). The reactions were carried out for 24 hours at 28°C with shaking at 180 rpm. The reactions were monitored by TLC and HPLC (see section 3.4).

3.3.2 Coupling reactions of phenolic compounds with laccase

Catechin (10 mM) or quercetin (5 mM) was coupled onto various phenolic compounds (gallic acid, vanillic acid, caffeic acid, guaiacol, 2,6-DMP, pyrogallol, vanillin, eugenol, isoeugenol, protocatechuic acid and catechol) (10 mM) in 25 mM ammonium acetate buffer, pH 4.5. Reactions were carried out at 28°C while shaking at 180 rpm. Catechin or quercetin was initially incubated with laccase (3.25 U) and ammonium acetate buffer (pH 4.5) for 1 h; after which the test phenolic compound was added and the reaction continued for another 4 hours or until a pronounced colour change was observed. All reactions were incubated on ice and an equal volume of ice-cold methanol (500 µl) was added and incubated for a further 20 minutes on ice. The

reaction was centrifuged at 14 000 *g*, at 4°C for 15 min and 700 µl aliquots of the supernatant were transferred into clean vials for LC-MS analysis.

3.4 Chromatographic separation of reaction products

3.4.1 Thin Layer Chromatography (TLC)

TLC analysis was performed on aluminium–backed silica gel 60 F_{254} (Merck) plates using toluene: dioxane: acetic acid (10:2.5:0.2; v/v/v) or ethyl acetate: dioxane: acetic acid (6:0.2:0.05; v/v/v) or chloroform: methanol: acetic acid (7:1:0.05; v/v/v) as mobile phase. Different solvent systems were tested in order to get the best mobile phase for separation of the product of interest, and after careful analysis, these different mobile phases were employed for the separation of different products studied. The compounds were visualized by exposure of the TLC plates to UV light at 254 nm.

3.4.2 High Performance Liquid chromatography (HPLC)

HPLC analysis of the samples was carried out using a Hitachi LaChrom HPLC system from Merck (Merck, Hitachi, Germany). Separation of the reaction products was carried out on a reversed phase LUNA 5µ PFP(2) 100A, 250×4.60mm column under isocratic conditions using acetonitrile: water: acetic acid (25:75:0.1; v/v/v) as mobile phase at a flow rate of 1 ml/min, running time (1 h), and the products were detected at 270 nm. Alternatively, a gradient elution using 0.1% formic acid (solvent A) and acetonitrile (solvent B) was used in order to reduce running time to 23 min. The gradient set up was as follows: 98% A to 0% A (20 min); 0% A to 98% A (20-21 min); 98% A (21-23 min). Peaks were analysed using HPLC Manager, Merck Hitachi model D 700 data software.

3.4.3 Liquid chromatography-mass spectrometry (LC-MS)

LC-MS was performed on a Dionex HPLC system equipped with a binary solvent manager and autosampler coupled to a Bruker ESI Q-TOF mass spectrometer. The products were separated using the same linear gradient of acetonitrile (solvent B) and 0.1% formic acid (solvent A) as described above, at a flow rate of 1 ml/min, using an injection volume of 10 µl and an oven temperature of 30°C. MS spectra were acquired in negative or positive mode and electrospray voltage was set to +3500 V. Dry gas flow was set to 9 L/min with a temperature of 350°C and nebulizer gas pressure was set to 35 psi. Selected structures were verified by nuclear magnetic resonance (NMR) – service provided by the University of the Western Cape.

3.5 Results and discussion

3.5.1 Production of laccase

Due to various biotechnological applications of laccase, studies on different organisms producing this enzyme and the optimization of its production from different microorganisms by several researchers have lately been intensified (Niladevi and Prema, 2008). In this study production of laccase to be used for the biocatalysis was performed either in a flask or on a large scale, in an airlift reactor. The airlift reactor is a 4 L glass tube with internal stainless loop which allows circulation of air to allow for free cells of *T. pubescens* to grow as pellets. *T. pubescens* is a white-rot fungus that has been routinely used in our research group because it is a good laccase producer. Since laccase is an extracellular enzyme, it was secreted into the medium by the fungal strain used. Before employing the airlift reactor, 2 L flasks were initially used to produce laccase, but only 1.08 ± 0.01 U/ml of laccase activity was observed.

Inducers such as aromatic and phenolic compounds have been established to enhance laccase production in fungi especially in white-rot fungi (Terron *et al.,* 2004). At the scale up level in the airlift reactor, two types of inducer mixtures were compared. These were 0.234 g phenol + 0.084 g *p*-cresol + 81.6 µl *m*-cresol + 239 µl *o*-cresol + 1 g of glucose dissolved in sterile water (reactor 1) or 0.234 g phenol + 0.084 g *p*-cresol dissolved in 30 ml water and filter-sterilized (reactor 2) (Zwane, 2009; Chigorimbo-Murefu, 2007). The results indicated that both inducers were capable of enhancing laccase production by *T. pubescens* (Fig. 3.1). The inducer mixture in reactor 2 gave the highest laccase activity and this was further employed in the course of this study

The laccase assay was performed on a daily basis and the culture medium containing the enzyme was harvested on the 9th day. The laccase activity increased to 5.3 U/ml as shown in the Fig. 3.2. The harvested crude laccase was stored at -20°C and purified prior to further use. It was, however, observed that the final activity of the enzyme from samples with the same inducer in both Fig 3.1 (2.73 U) and Fig. 3.2 (5.3 U) differ significantly; the starter culture activity difference (0.09 U and 0.5 U respectively) was most probably responsible for the difference.

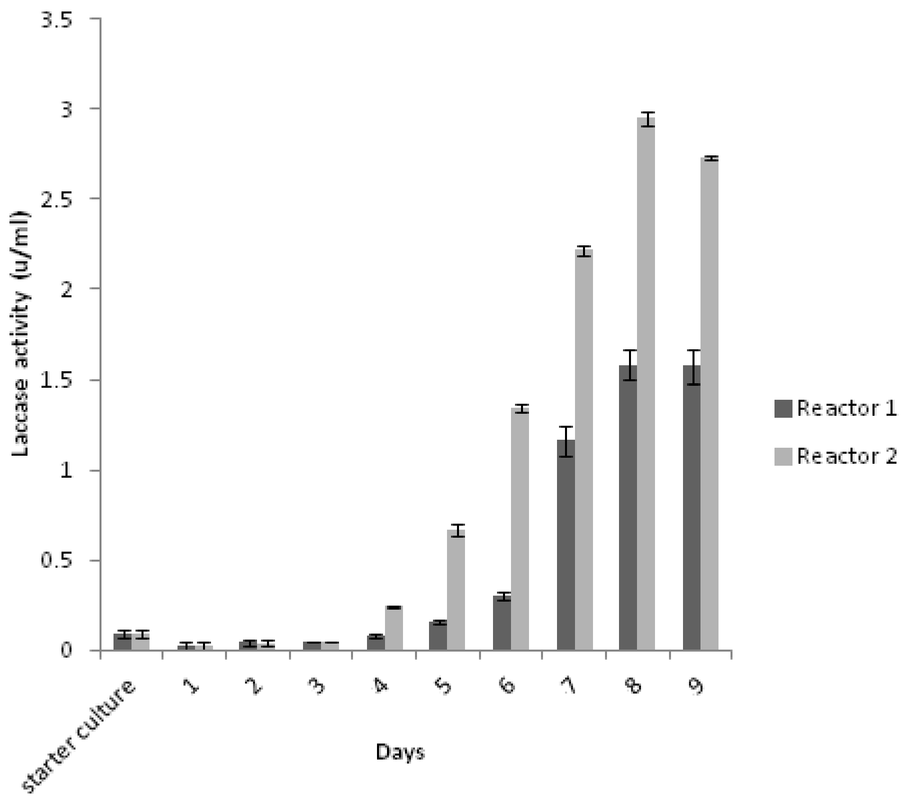


Fig. 3.1: Influence of different inducers on the production of laccase in an airlift reactor. Reactor 1 (0.234 g phenol + 0.084 g *p*-cresol + 81.6 µl *m*-cresol + 239 µl *o*-cresol + 1 g of glucose dissolved in 30ml of sterile water); Reactor 2 (0.234 g phenol + 0.084 g *p*-cresol dissolved in 30ml water and filter-sterilized).

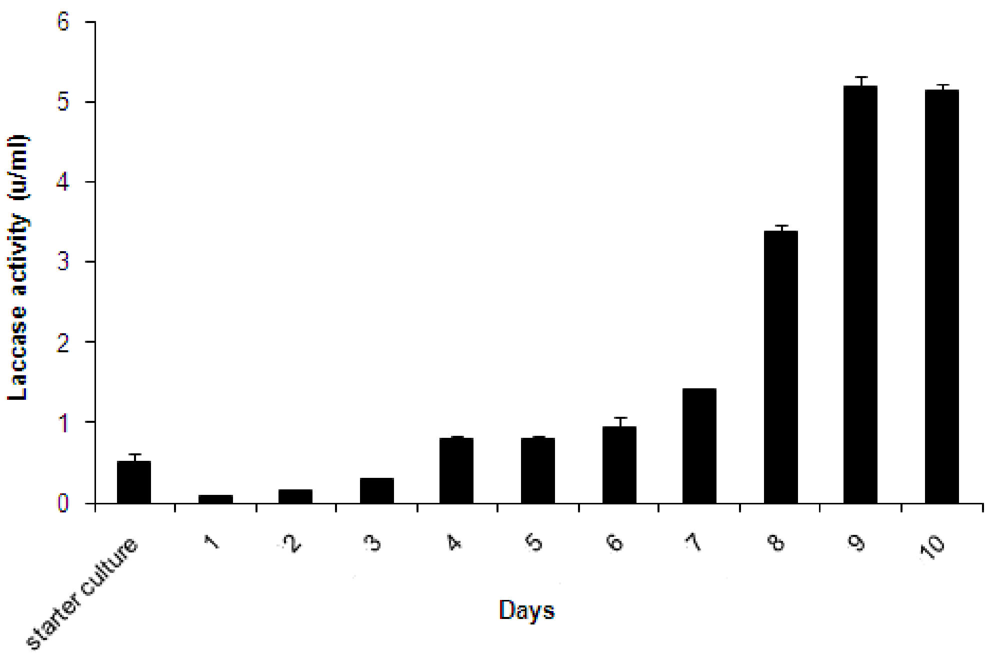


Fig. 3.2: Production of laccase by *T. pubescens* in an air lift reactor.
Medium: *Trametes* Defined Medium; cultivation period: 10 days; temperature: 28°C.

3.5.2 Purification of laccase

The crude laccase was partially purified by either acetone or ammonium sulphate precipitation after removal of the fungal cell mass and medium precipitate. This was analysed for laccase activity and protein content and the specific activity was calculated. A standard curve for protein determination showed good correlation (R^2 = 0.9992) (Appendix B) and protein content for the samples were determined using this standard curve.

The summary of the purification is as shown in Table 3.1. The laccase employed for the biocatalysis reactions in this study was purified by ammonium sulphate precipitation as this gave a better yield (Table 3.1). As shown in the table, a recovery yield of only 6.33 or 4.67% was obtained when 60 or 70% cold acetone purification was performed, respectively. Although a yield of 77.39% was observed for 70% ammonium sulphate purification, the laccase activity and specific activity (139.3 U; 48.71 U/mg) was low when compared with the 50-80% purification method which gave a total activity of 286.15 U and a specific activity of 112 U/mg (Table 3.1). The partially purified enzyme gave a single dominant protein band when analysed by SDS-PAGE with an apparent molecular weight of 65 kDa (Fig. 3.3). This is similar to the 70-80 kDa molecular masses recorded for fungal laccases (Sadhasivam *et al.*, 2008).

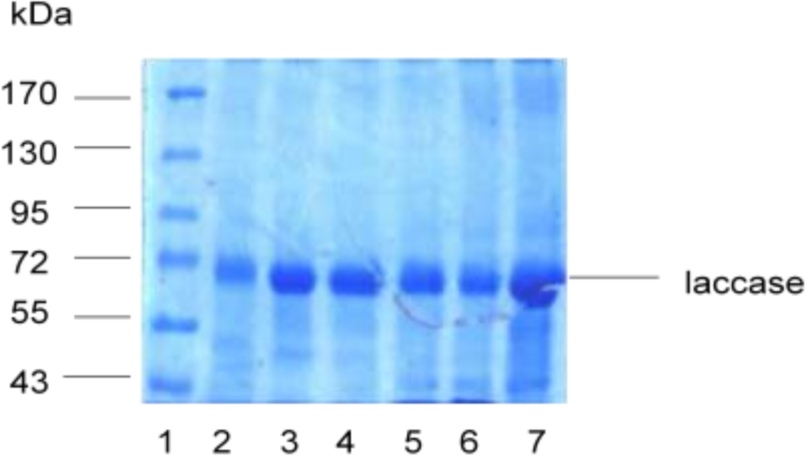


Fig. 3.3: SDS-PAGE: Lane 1: 20 µg protein marker; Lane 2: 20 µg crude laccase; Lanes 3-7: 20 µg partially purified laccase.

Table 3.1: Partial purification of the extracellular laccase from the culture filtrate of *Trametes pubescens*

Purification method	Volume (ml)	Start activity (U)	Start Protein (mg)	Pellet activity (U)	Pellet Protein (mg)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Culture filtrate 1	1000	1.8	0.093	-	-	1800	9.3	19.35	100	1
Culture filtrate 2	1000	3.91	0.121	-	-	3910	12.1	32.31	100	1
Culture filtrate 3	1000	5.3	0.097	-	-	5300	9.7	54.64	100	1
60% acetone (Culture filtrate 1)	4	1.8	0.093	1.14	0.119	4.56	0.476	9.58	6.33	0.5
70% acetone (Culture filtrate 1)	3	1.8	0.093	0.84	0.102	2.52	0.306	8.24	4.67	0.43
50% Amm. Sulphate (Culture filtrate 3)	10	5.3	0.097	4.18	0.214	41.80	2.14	19.53	7.89	0.36
60% Amm. Sulphate (Culture filtrate 1)	10	1.8	0.093	5.4	0.218	54.0	2.18	24.77	30.00	1.28
70% Amm. Sulphate (Culture filtrate 1)	10	1.8	0.093	13.93	0.286	139.3	2.86	48.71	77.39	2.52
80% Amm. Sulphate (Culture filtrate 3)	10	5.3	0.097	30.24	0.353	302.4	3.53	85.67	57.06	1.56
50-80% Amm. Sulphate (Culture filtrate 2)	10	3.91	0.121	28.62	0.256	286.2	2.55	112.23	73.20	3.47

3.5.3 Dimerization reactions of phenolic compounds with laccase

Laccase-catalysed dimerization reactions of different phenolic compounds namely ferulic acid, 2,6-DMP and protocatechuic acid reactions were performed on a small scale. This section describes the preliminary results obtained with HPLC and LC-MS analyses.

3.5.3.1 Oxidation of ferulic acid

From literature, transformation of ferulic acid by laccase was reported to lead to the formation of semi-quinone feruloyl radicals that may later dimerise non-enzymatically (Mustafa *et al*., 2005). Carunchio *et al*. (2001) reported the formation of a ferulic acid dimer using laccase from *Pyricularia oryzae* in reaction media of acetate buffer (pH 6) containing 45% ethanol. Mustafa *et al.* (2005) also worked on phenolic colourants obtained by enzymatic synthesis using a fungal laccase in a hydro-organic biphasic system. The oxidation of ferulic acid by the laccase from *Myceliophthora thermophila* in a biphasic hydro-organic system consisting of ethyl acetate and sodium–phosphate buffer resulted in intermediate stable yellow products. Results achieved in their study showed that the enzymatic activity and the stability of the products varied depending on the nature of the reaction medium. Furthermore, Tranchimand *et al*. (2006) reported transformation of ferulic acid by laccase in a biphasic reaction solvent system. Higher yield for bis-lactone lignans synthesis and higher product stability was achieved using ethyl acetate added as the non-miscible co-solvent compared to buffer alone.

Although oxidation of ferulic acid by laccase had been reported in literature (Tranchimand *et al*., 2006; Mustafa *et al*., 2005; Carunchio *et al*., 2001) and possible structures of the oxidation products proposed (Carunchio *et al*., 2001), none of these researchers conclusively elucidated the structures nor reported the antioxidant capacity of the products. This current study investigated the modification of ferulic acid with laccase produced from *T. pubescens* as a way of enhancing the antioxidant capacity.

3.5.3.1.1 HPLC analysis of products of ferulic acid oxidation

Preliminary oxidation of ferulic acid with laccase was performed for four days and samples were taken out of the reaction mixture every 24 hours and analysed by HPLC. HPLC analysis of the biocatalytic products and phenolics standards were performed using methanol: water: acetic acid (20:80:2.5, v/v/v) as mobile phase. The standards chosen were either phenolics or possible reaction products (vanillin, vanillic acid, gallic acid, coumaric acid, catechin, and catechol). Out of all the standards

analysed, only vanillin had a retention time which correlated to one of the reaction products of ferulic acid (21 minutes) when methanol: water: acetic (20:80:2.5, v/v/v) was used as mobile phase (Table 3.2).

Extending the reaction time of ferulic acid with laccase above 24 to 72 hours revealed that one of the biocatalytic products of ferulic acid oxidation had the same retention time as vanillin, therefore, a spiking experiment was performed to confirm this assertion. Briefly, 0.125 g of vanillin was dissolve in 250 ml of ethyl acetate to give 500 ppm concentration (Ting, 2004); 1350 µl ferulic acid reaction + 150 µl vanillin standard (which is the spiked sample) was analysed by HPLC using methanol: water: acetic (20:80:2.5, v/v/v) as mobile phase (1 - ferulic acid standard; 2 – vanillin standard; 3 – ferulic acid reaction only; 4 – ferulic acid + spiked vanillin) Fig. 3.4.

Table 3.2: HPLC retention times of standards and reaction products. Mobile phase used was water:methanol:acetic acid (80:20:2.5, v/v/v).

Samples	Products	Retention time (Minutes)	Intensity (AU)
Ferulic acid standard	-	39.28	4.00
24 hr reaction	Product 1	4.11	0.005
	Product 2	8.43	0.005
	Product 3	21.49	0.001
	Product 4	30.20	0.001
	Product 5	41.10	0.006
48 hr reaction	Product 1	4.12	0.003
	Product 2	8.38	0.021
	Product 3	21.45	0.005
	Product 4	30.16	0.002
	Product 5	41.19	0.001
72 hr reaction	Product 1	8.45	0.025
	Product 2	21.57	0.030
84 hr reaction	Product 1	1.99	0.003
	Product 2	8.54	0.025
	Product 3	21.23	0.005
	Product 4	29.67	0.002
Vanillin standard	-	21.25	-
Vanillic acid standard	-	15.20	-
Gallic acid standard	-	5.76	-
Coumaric acid standard	-	28.75	-
Catechin standard	-	7.80	-
Catechol	-	8.60	-

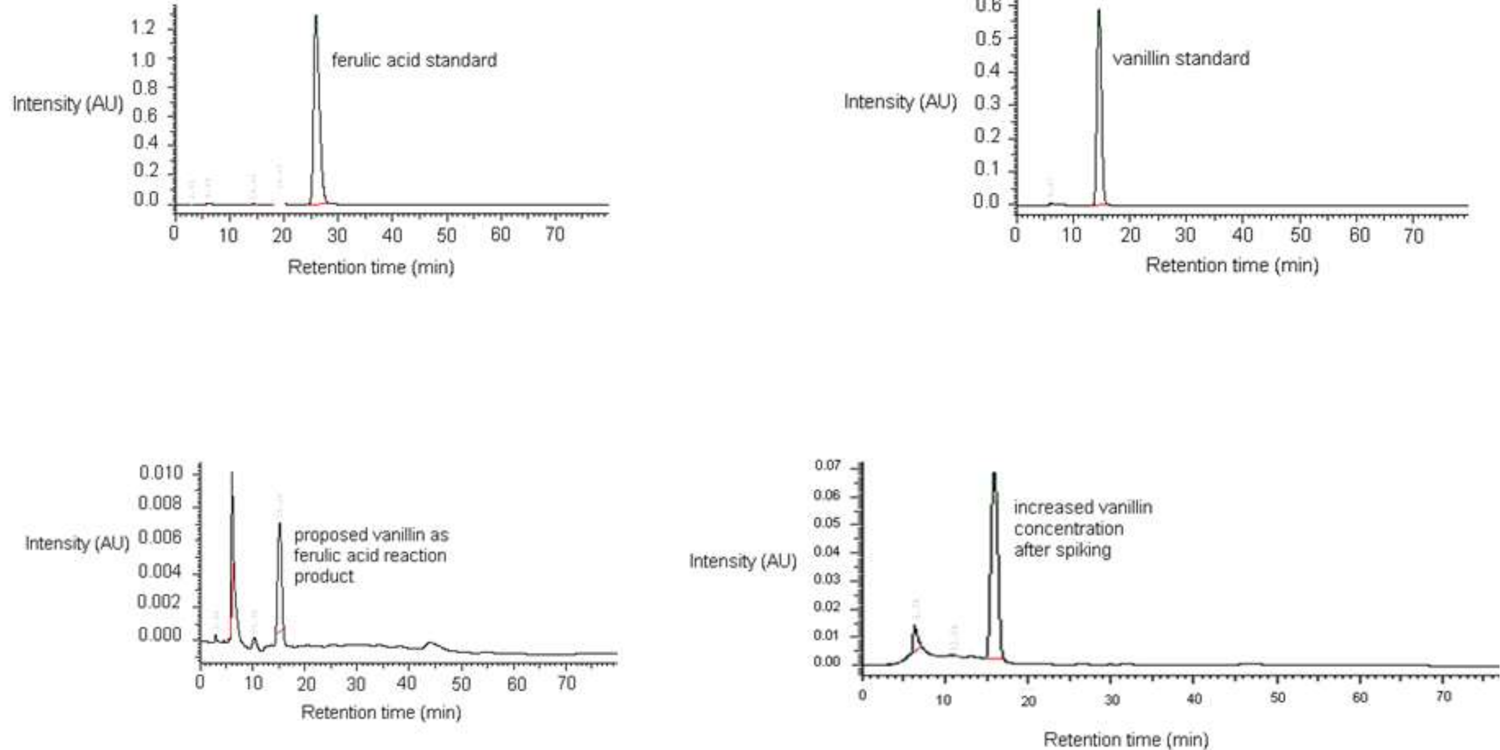


Fig. 3.4: HPLC chromatograms of the ferulic acid, vanillin, ferulic acid product and spiked samples.

The HPLC results (Fig. 3.4) showed that vanillin might have been produced in the ferulic acid oxidation by laccase especially if the reaction time is extended to 72 hours. The increase in the vanillin concentration in the reaction sample after spiking with vanillin proved this ascertion. Further analysis of this sample was performed by LC-MS (Fig. 3.5).

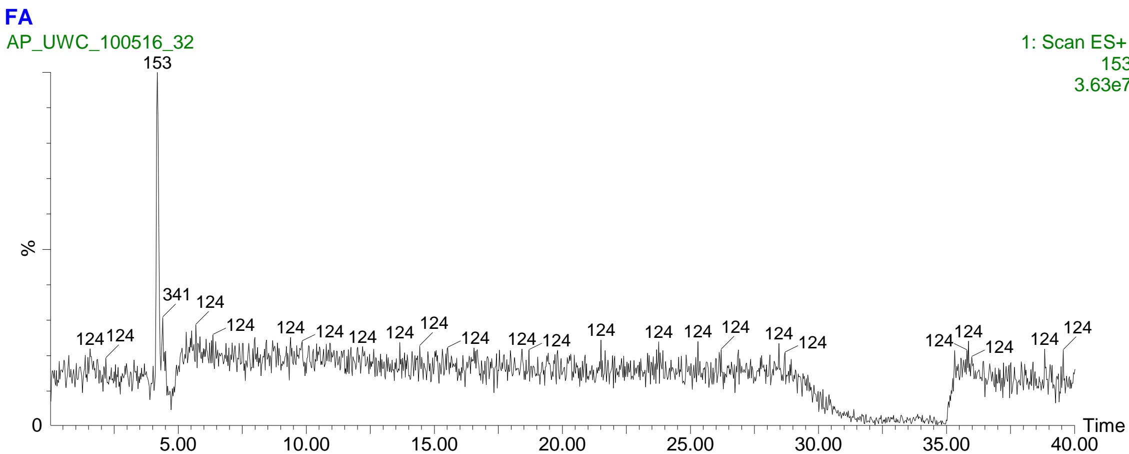


Fig. 3.5: Mass spectrum of the vanillin produced by laccase-catalysed oxidation of ferulic acid; observed mass [M+H] – 153.

Reseachers have reported the production of vanillin from ferulic acid by microbial biotransformation. Ferulic acid metabolism in cultures of the white-rot fungus *Pycnoporus cinnabarinus* I-937 was reported by Falconnier *et al.* (1994). After 6 days of growth, during the secondary metabolism of the fungus, vanillin concentration in the culture medium had reached a maximum of 64 mg/l, corresponding to a molar yield of 27.5% (Falconnier *et al.*, 1994). Mathew *et al.* (2007) worked on the rapid conversion of ferulic acid to 4-vinyl guaiacol and vanillin metabolites by *Debaryomyces hansenii.* Their focus was on the microbial transformation of ferulic acid, a renewable material, as a means of generating higher value-added products. The production of vanillin was observed to reach a maximum of 169 mg/l at the fifth hour. Although production of vanillin is not the focus of this study, it was observed here that if the reaction time is extended above 24 hours, vanillin, which is one of the degradation products of ferulic acid, could be obtained. This is due to decarboxylation of ferulic acid firstly to 4-vinyl guaiacol and its further oxidation to vanillin (Mathew *et al.,* 2007; Karmakar *et al.*, 2000) (Fig. 3.6).

Fig. 3.6: Proposed pathway for the synthesis of vanillin from ferulic acid by *T. pubescens* laccase.

3.5.3.1.2 Thin Layer Chromatography (TLC) of the reaction products of ferulic acid

Thin layer chromatography (TLC) can be applied to separate compounds of interest. However, this can be achieved only if the right mobile phase is identified. The separation of compounds by TLC is advantageous because a variety of media can be used and also corrosive reagents can even be employed for better detection. Furthermore, TLC-based methods are very fast: separation of compounds can be completed in less than an hour (Sharma *et al.,* 1998).

From the solvent systems listed below, solvent systems 7, 11 and 14 were suitable for the TLC analysis of ferulic acid and its products. Solvent systems 11 and 14 had inherent disadvantages. Due to the toxicity of benzene (solvent system 11), a solvent with similar polarity (toluene) was opted for solvent system 14. However, it was observed that a longer time was required to dry the purified samples after flash chromatography due to the high boiling point of toluene (110.6°C). Therefore, solvent system 7 was chosen to be the most suitable for the resolution of ferulic acid and the products after biocatalysis. The solvent systems tested were:

1. Toluene : acetic acid (4:1, v/v)
2. Ethyl acetate : dioxane : acetic acid (5:3:0.2, v/v/v)
3. Ethyl acetate : dioxane : acetic acid (4:3:0.2, v/v/v)
4. Ethyl acetate : dioxane : acetic acid (6:2:0.2, v/v/v)
5. Ethyl acetate : dioxane : acetic acid (6:0.2:0.2, v/v/v)
6. Ethyl acetate : dioxane : acetic acid (6:0.2: 0.1, v/v/v)

7. Ethyl acetate : dioxane : acetic acid (6:0.2:0.05, v/v/v)
8. Cyclohexane : ethyl acetate (3:0.1, v/v)
9. Heptane : ethyl acetate (3:0.1, v/v)
10. Iso-octane : ethyl acetate (3:0.1, v/v)
11. Benzene : diozane : acetic acid (9:2.5:0.2, v/v/v)
12. Toluene : diozane : acetic acid (9:2.5:0.2, v/v/v)
13. Toluene : diozane : acetic acid (9:2.5:0.4, v/v/v)
14. Toluene : diozane : acetic acid) (10:2.5:0.2, v/v/v)

Four distinct spots were observed under UV light at 254 nm. Ferulic acid (Rf – 0.78) was visualised as a pink spot, product 1 (Rf – 0.83) as a yellow spot, while dimers 1 (Rf – 0.22) and 2 (Rf - 0.43) were seen as dark spots under UV (Fig. 3.7).

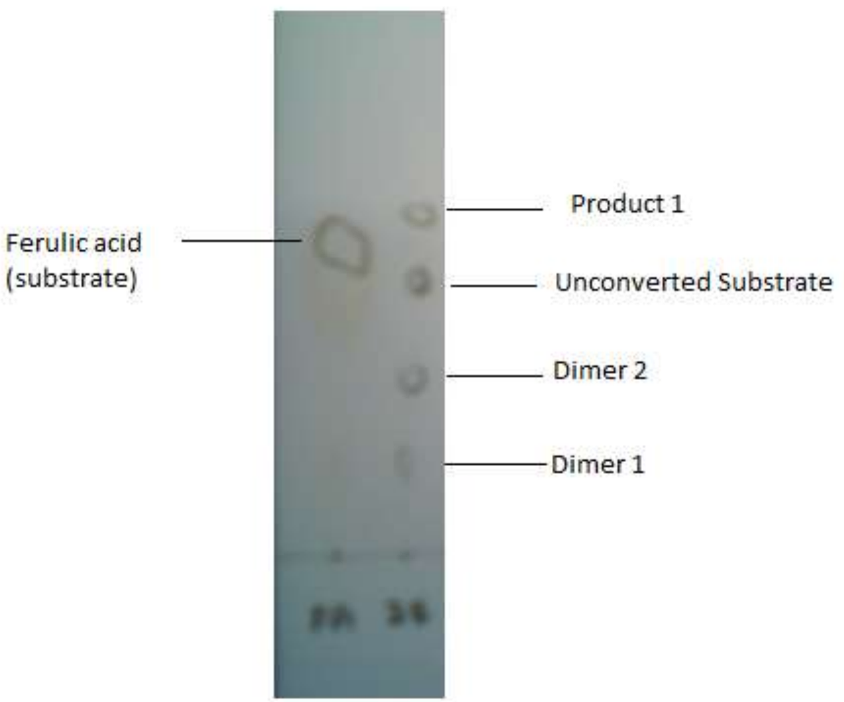


Fig. 3.7: Thin layer chromatography of the products of ferulic acid reaction with laccase. Mobile phase - ethyl acetate : dioxane : acetic acid (6:0.2:0.05, v/v/v).

3.5.3.2 Analysis of biocatalytic products of selected phenolic compounds with laccase

Analysis of the products of oxidation of 2,6-DMP, protocatechuic acid or ferulic acid with laccase by LC–MS showed that dimers and trimers of the initial substrates were obtained (Fig. 3.8; 3.9; 3.10). Scale-up production, optimization, purification and application of these reaction products in terms of antioxidant capacity are further explained in subsequent chapters.

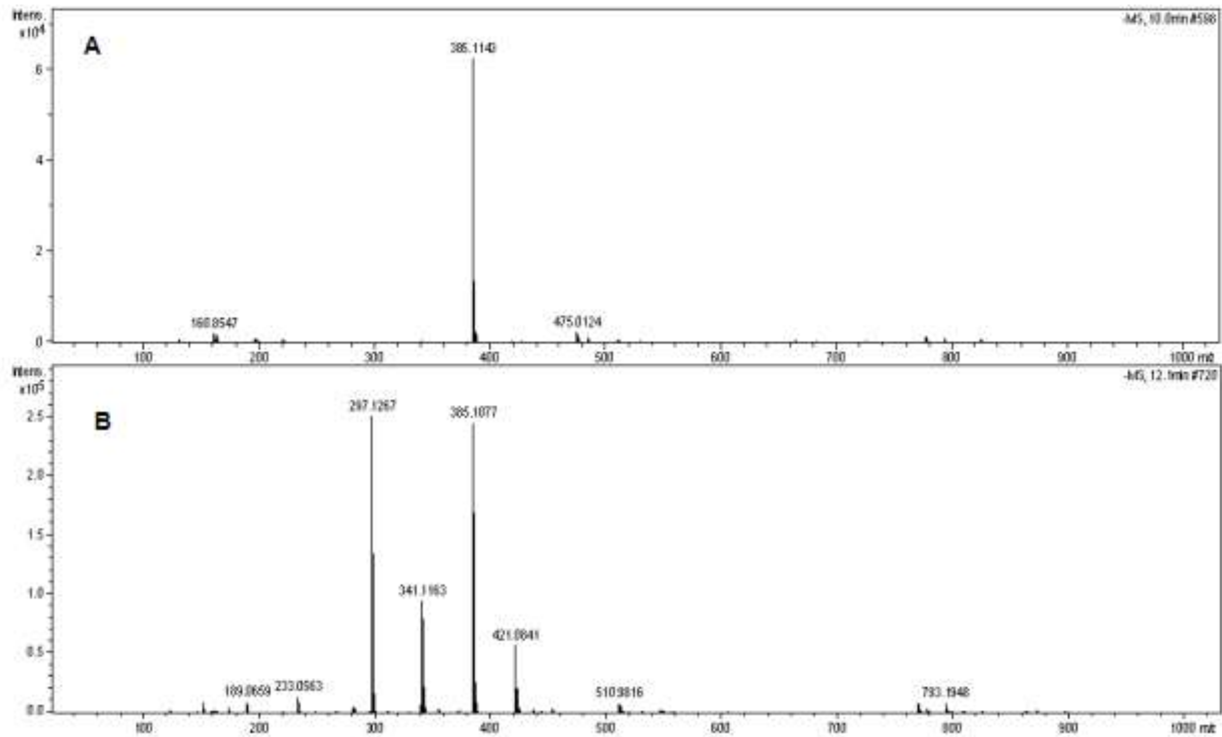


Fig. 3.8: Mass spectrum of dimers formed during laccase-mediated oxidation of ferulic acid (m/z 385.1). – A: *β*-5 dimer; B: *β*-*β* dimer

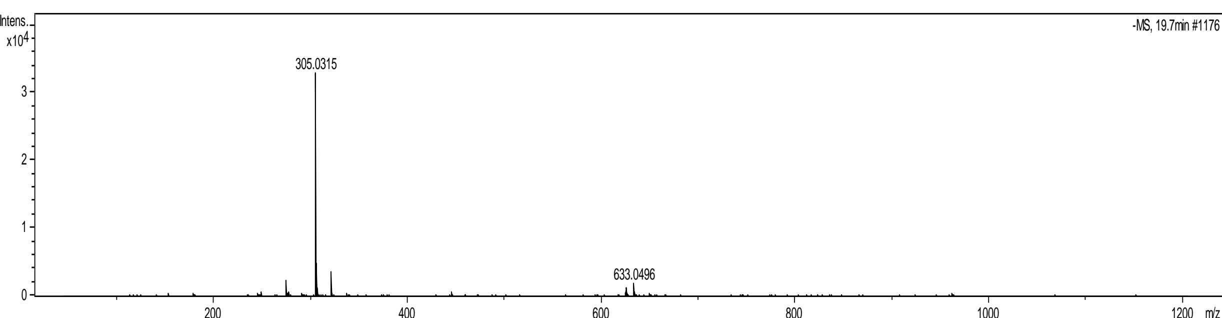


Fig. 3.9: Mass spectrum of a dimer formed during laccase-mediated oxidation of 2,6-DMP (m/z 305.03).

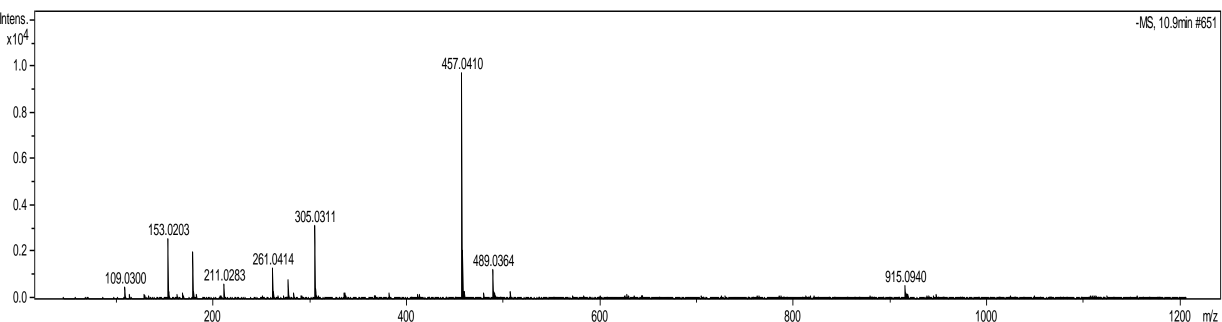


Fig. 3.10: Mass spectrum of a trimer formed during laccase-mediated oxidation of protocatechuic acid (m/z 457.04).

3.5.4 Use of laccase from *T. pubescens* in phenolic acid coupling

Coupling is a reaction where two organic molecules are linked together with the aid of a catalyst. This can be homo-coupling where two identical molecules react to form one molecule or cross-coupling in which two different molecules react to form one new molecule. It is achieved traditionally with the aid of metal catalysts such as Cu, Na, Ni and Pb (Kudanga *et al.,* 2011). However, with the increasing search for more environmentally friendly and mild reaction conditions for catalysis reactions, enzymes such as laccases are being employed due to their numerous inherent advantages. More recently, the enzymatic enrichment of naringenin with hydroxylated and/or methoxylated phenolic compounds were studied (Nugroho Prasetyo *et al*., 2011). They reported an increase in hydroxyl and/or methoxyl groups which may positively influence the antioxidant activities. In the next section of this study, the coupling of catechin or quercetin with various monomeric phenolic compounds together with the antioxidant activity of some of the products from the coupling reactions is reported.

3.5.4.1 Coupling products between catechin or quercetin with other phenolic compounds

Coupling of various phenolic molecules - gallic acid, vanillic acid, ferulic acid, guaiacol, pyrogallol, 2,6-DMP, catechol, vanillin, eugenol, isoeugenol, protocatechuic, naringin, phloridzin onto catechin or quercetin (Fig. 3.11) by laccase is envisaged to be initiated by abstracting one electron from the hydroxyl group of catechin or quercetin and the phenolic compound (Nugroho Prasetyo *et al.,* 2011). Therefore, we first pre-oxidised catechin or quercetin to form radicals before the addition of the phenolic compound.

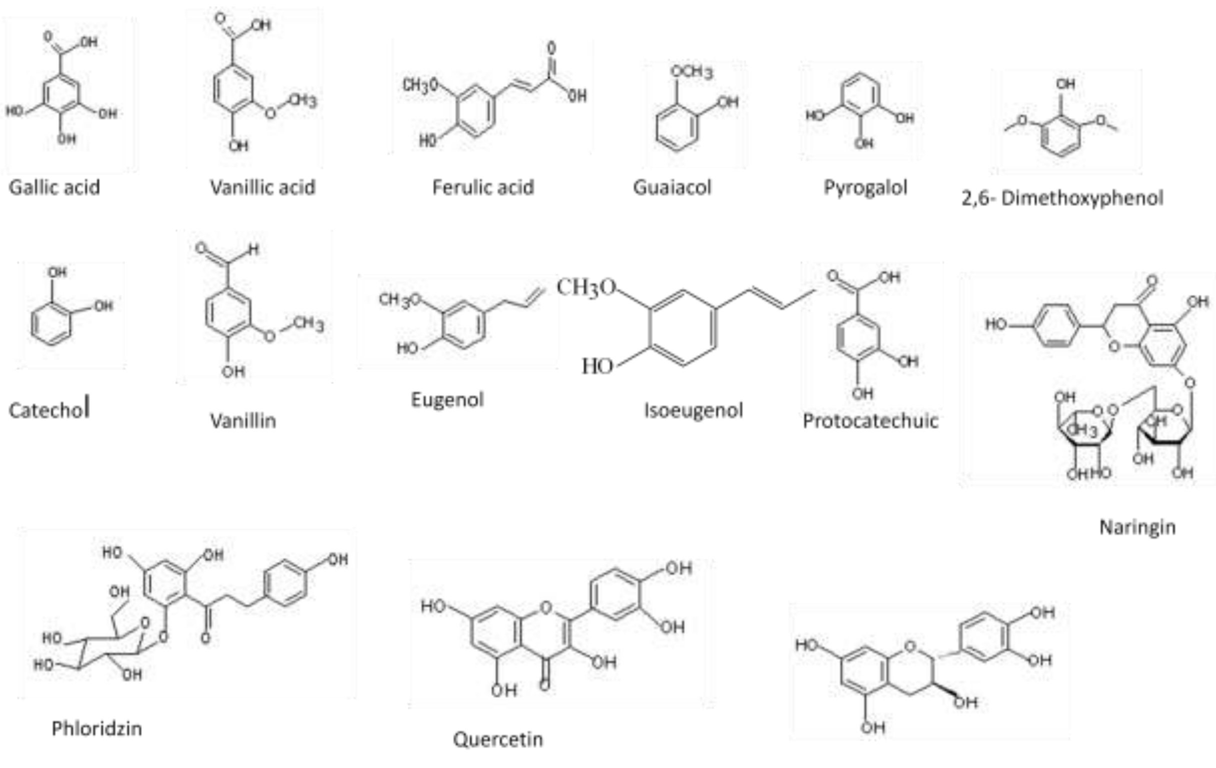


Fig. 3.11: Quercetin, catechin and other phenolic compounds used in coupling reactions.

LC-MS analysis showed that the laccase-catalysed coupling of catechin or quercetin with many of the phenolic compounds used was successful (Fig. 3.12 - 3.13). As observed from the LC-MS spectra, laccase-mediated coupling products of phenolic monomers to catechin showed dominant signals at *m/z* 577.2 t_R = 4.3 min (catechin); *m/z* 457.1 t_R = 2.1 min (gallic acid); *m/z* 455.1 t_R = 14.0 min (vanillic acid); *m/z* 467.1 t_R = 1.8 min (caffeic acid); *m/z* 411.1 t_R = 9.3 min (guaiacol); *m/z* 441.1 t_R = 6.1 min (2,6-DMP); *m/z* 397.1 t_R = 9.0 min (catechol); *m/z* 451.1 t_R = 13.6 min (eugenol); *m/z* 451.1 t_R = 14.6 min (isoeugenol); *m/z* 441.1 t_R = 2.2 min (protocatechuic acid). These molecular weights suggest 1:1 coupling products. The structures were elucidated based on the rationale used by Widsten *et al.* (2010). According to existing knowledge, the hydroxyl group on the benzene ring is *ortho* or *para* directing and molecules with a free C-5 position usually cross-couple through 5–5 linkages due to stability of C–C bonds (Schultz *et al.*, 2001; Jonas *et al.,* 2000). Since quercetin and catechin have their 5th position free on the B-ring this should therefore promote direct coupling, establishing C–C coupling products. On this basis the proposed structures of coupling products were established (Fig. 3.12 - 3.13).

Similarly, laccase-mediated coupling products of phenolic monomers to quercetin showed dominant signals at *m/z* 467.0 t_R = 14.8 min (gallic acid); *m/z* 467.1 t_R = 14.9 min (vanillic acid); *m/z* 479.1.0 t_R = 13.0 min (caffeic acid); *m/z* 423.1 t_R = 11.4 min

(guaiacol); *m/z* 453.1 t_R = 11.9 min (2,6-DMP); *m/z* 425.1 t_R = 13.0 min (pyrogallol); *m/z* 451.0 t_R = 11.0 min (vanillin); *m/z* 463.1 t_R = 16.5 min (eugenol); *m/z* 463.1 t_R = 13.9 min (isoeugenol); *m/z* 453.1 t_R = 12.0 min (protocatechuic acid) which suggests a 1:1 coupling. In addition, *m/z* 753.1 (t_R = 14.8 min) was observed between quercetin and protocatechuic suggesting coupling of two quercetin and one protocatechuic acid (Fig. 3.14 - 1). Three dominant signals of coupling products between quercetin and catechol were observed at *m/z* 409.1, *m/z* 517.1 and *m/z* 709.1 (Fig. 3.14 – 2,3). This suggests that coupling occurred between one catechol and one quercetin (*m/z* 409.1), two catechol molecules and one quercetin (*m/z* 517.1) and two quercetin molecules and one catechol (*m/z* 709.1). This shows that quercetin can easily be manipulated as a substrate for the synthesis of hybrid molecules of interest. From a structural point of view, this should lead to an increase in the antioxidant activity of quercetin/catechin due to increased hydroxyl groups and/or methoxyl groups. We further performed the scale-up of this interesting reaction which will further be explained later in this chapter.

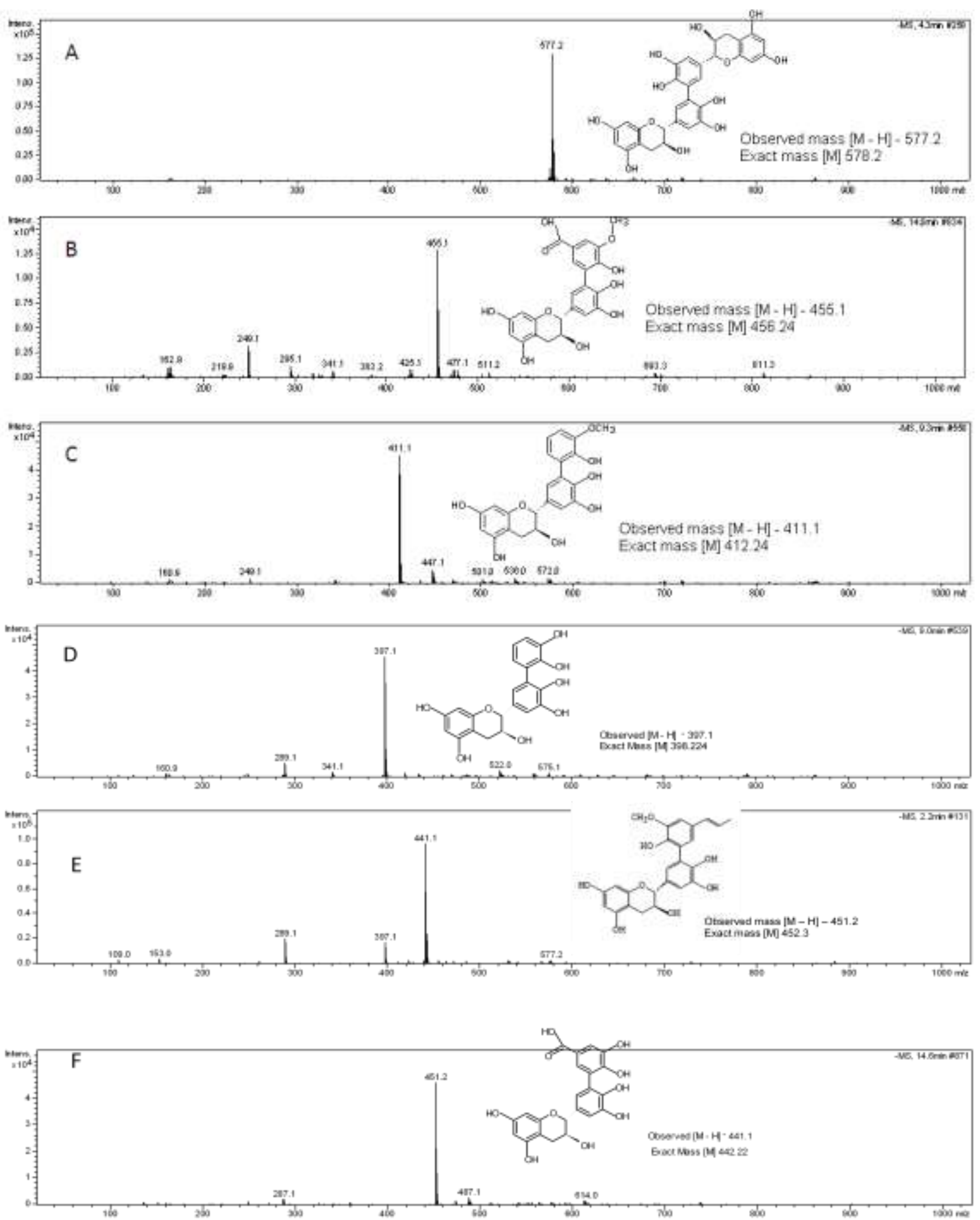


Fig. 3.12: Mass spectra of laccase-mediated coupling products between catechin with various phenolic compounds of A, B, C, D, E & F (catechin, vanillic acid, guaiacol, catechol, isoeugenol and protocatechuic acid, respectively) and the proposed chemical structures.

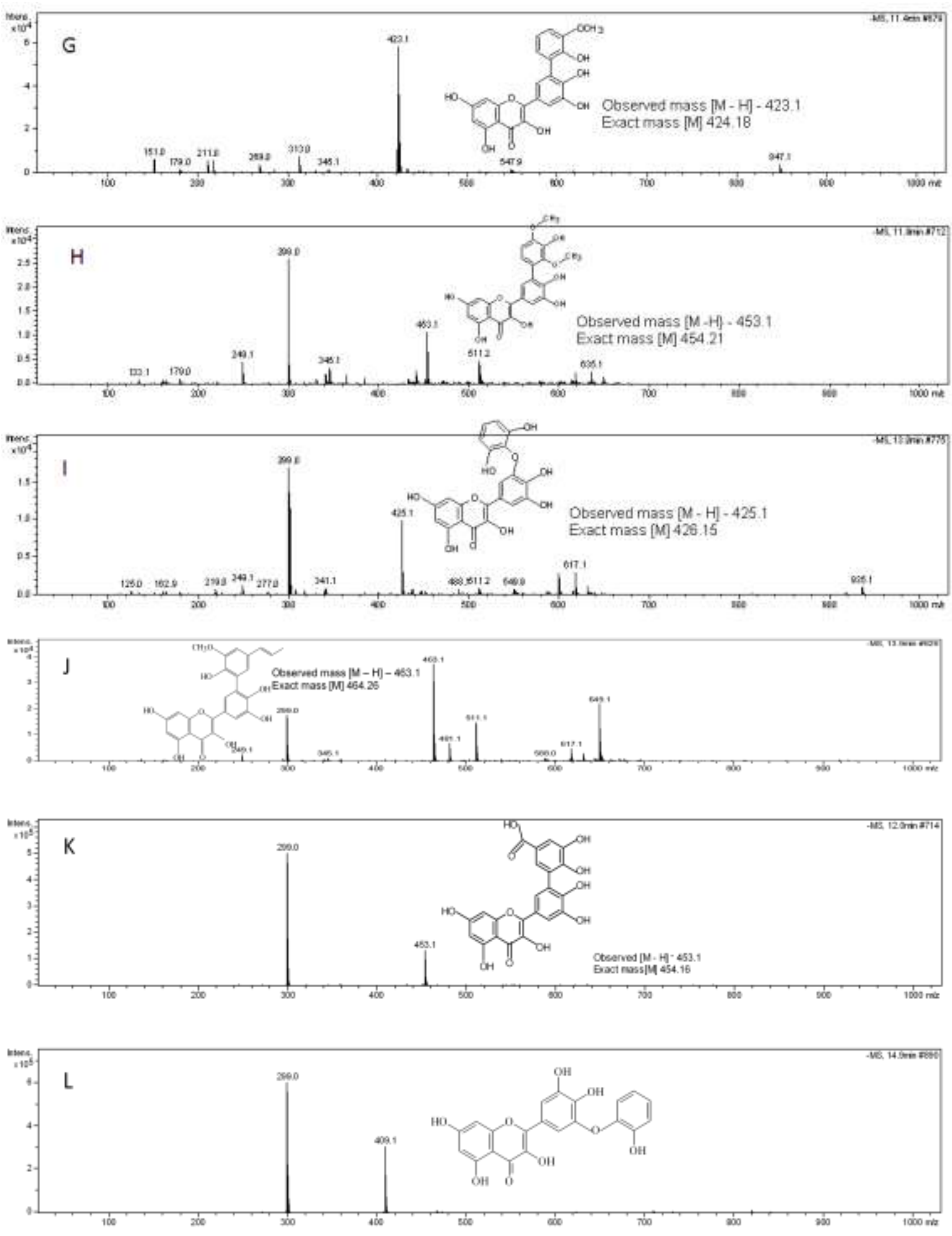


Fig. 3.13: Mass spectra of laccase-mediated coupling products between quercetin with various phenolic compounds G, H, **I**, J, K & L (guaiacol, 2,6-DMP, pyrogallol, isoegenol, protocatechuic acid and catechol, respectively) and the proposed chemical structures.

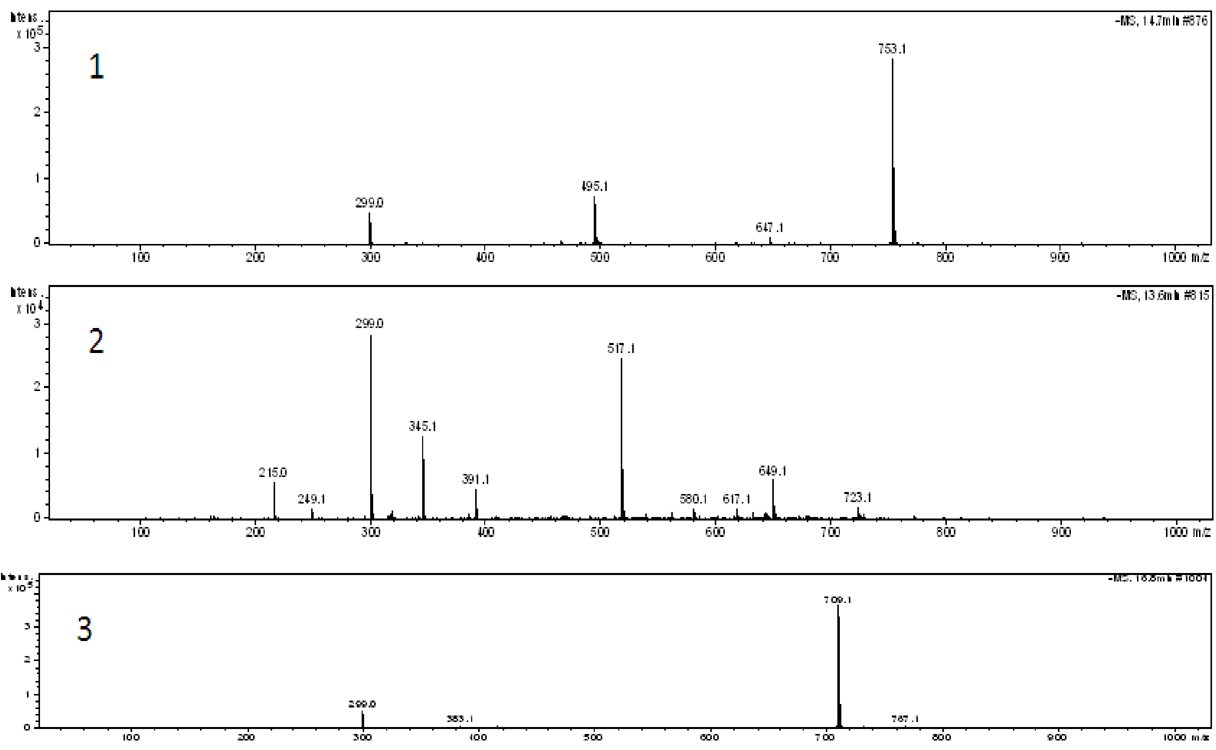


Fig. 3.14: Mass spectra of laccase-mediated quercetin multiple coupling products 1 (two quercetin + protocatechuic acid *m/z* 753); 2 (quercetin + two catechol *m/z* 517); 3 (two quercetin + catechol *m/z* 709).

3.5.4.2 Scale – up production and purification of coupling products between quercetin and catechol and their application as antioxidants

Laccase-mediated coupling products between quercetin and catechol as analysed by LC–MS revealed that one or two molecules of the catechol were coupled onto one or more quercetin molecules (Fig. 3.14). From a structural point of view, this should lead to an increase in the antioxidant activity of catechol. Scale–up reactions of this interesting reaction was further performed and purified, but only the coupling product with *m/z* 409.1 was recovered after purification with a yield of 6.27%. The product was characterized by NMR and the antioxidant activity (DPPH and TEAC) determined.

NMR analysis (Table 3.3; Appendices D,E) of the main product (*m/z* 409.1) showed that the coupling occurred via an ether linkage (Fig. 3.15). Signals in the ^{1}H NMR spectrum were due to aromatic hydrogens only. A very close pair of 1-proton *meta* coupled doublets at δ 5.83 and 5.84 (J = 2.2 Hz) were assigned to H-6 and H-8 respectively since these are in the typical region the ring A protons appear for flavonoids. A doublet of doublets at δ 6.59 (J = 8.4 and 1.2 Hz) is assigned to H-6'' since the signal shows both *ortho* and *meta* coupling to H-5'' and H-4'' respectively. Not unexpectedly, H-3'', H-4'' and H-5'' appeared as a 3-proton multiplet centred at δ

6.82. Finally the pair of *meta* coupled protons H-6’ and H-2’ appeared as two doublets at δ 6.94 and 7.13 with J = 2.4 Hz.

Due to the very low concentration of sample, the DCl in the $CDCl_3$ was able to exchange all the OH groups and these were thus not observed.

Table 3.3: NMR spectra data for the coupling product (*m/z* 409.1) between quercetin and catechol.

^{1}H-NMR and ^{13}C-NMR spectra data	
δH1	δC13
5.83(1H, d, J = 2.2 Hz, H-6)	96.5(C8)
5.84 (1H, d, J = 2.2 Hz, H-8)	97.4 (C6)
6.59 (1H, dd, J = 8.4 and 2.2 Hz, H-6’’)	104.5 (C4a)
6.82 (3H, m, H-3’’, H-4’’ and H-5’’)	114.3 (C2’)
6.94 (1H, d, J = 2.4 Hz, H-6’)	115.1 (C6’’)
7.13 (1H, d, J = 2.4 Hz, H-2’).	117.0 (C6’),
	117.2 (C1’)
	120.0 (C3’’)
	122.3 (C4’’)
	122.5 (C5’’)
	138.5 (C3)
	140.3(C4’)
	140.7 (C3’)
	142.0 (C1’’)
	143.9 (C2)
	146.4 (C2’’)
	150.5 (C5’),
	154.0 (C8a)
	159.8 (C5),
	161.3 (C7)
	168.1 (C4-C=O).

Fig. 3.15: Structure of the product formed formed during laccase-catalyzed coupling of catechol onto quercetin.

Based on the LC-MS and NMR results, the scheme shown in Fig. 3.16 is proposed as the possible pathway for the coupling of catechol onto quercetin. As shown in Fig. 3.16, laccase initiates the reaction by abstracting an electron from the quercetin

resulting in a radical which forms a resonance structure. As the catechol is introduced to the reaction, it is oxidized by laccase and attacked the quercetin radical on the position 3' thereby establishing an ether linkage (Kudanga *et al.*, 2010).

Fig. 3.16: The proposed reaction pathway for laccase-mediated coupling of catechol onto quercetin. [O] = laccase-catalysed oxidation.

During the scale-up process, five organic solvents namely acetone, ethyl acetate, ethanol, dioxane and methanol were employed in order to get the best solvent system for the production of the coupling product. Ethanol was observed to yield the highest amount of product (Fig. 3.17). Coupling reactions conducted in the water-miscible solvents (ethanol, methanol, acethone, dioxane) produced higher coupling product as compared to the reactions carried out in the water-immiscible solvent (ethyl acetate). In general, solvents with a higher polarity index favour the production of the coupling product: ethanol (5.2) > methanol (5.1) > acetone (5.1) > dioxane (4.8) > ethyl acetate (4.4). Although, enhancement of catechol-laccase catalysis in less polar organic solvents as compared with highly polar media (due to the hydrophobicity of the end product), has been reported by Ma *et al.* (2009); significant and unexpected solvent influence on the selectivity of laccase-catalyzed coupling reactions has been documented (Intra *et al.*, 2005).

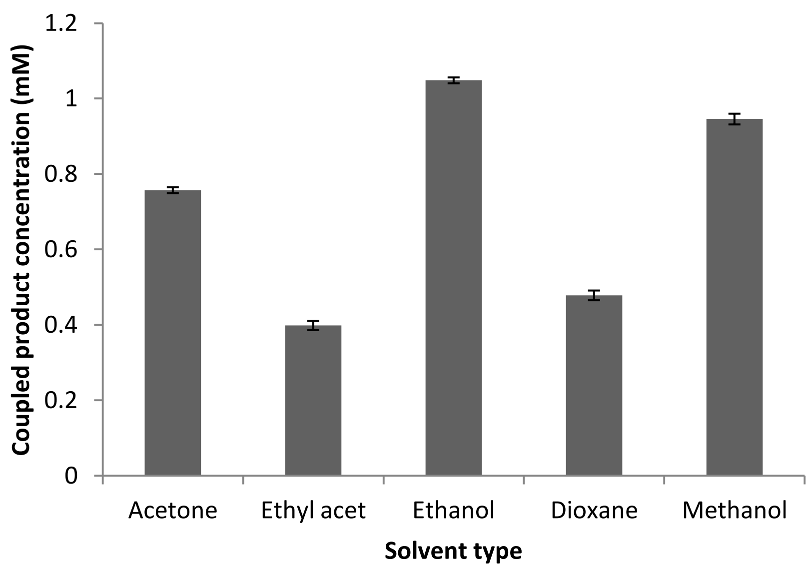


Fig. 3.17: Effect of organic co-solvent on the formation of the quercetin/catechol coupled product. All results are means ± SD of three replicate determinations.

3.5.4.3 Antioxidant activity

The 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays were employed to determine the antioxidant activity of the purified coupling product and compared with the antioxidant activity of the starting substrates (quercetin and catechol). The ABTS assay is based on the generation of a blue/green $ABTS^{\cdot+}$ that can be reduced by antioxidants and the DPPH assay is based on the reduction of the purple $DPPH^{\cdot}$ to 1,1-diphenyl-2-picryl hydrazine. The concentration of the antioxidant required to decrease the initial susbtrate concentration by 50% is a parameter that is widely employed to measure the antioxidant capacity. The higher the EC_{50}, the lower the antioxidant capacity and vice-versa. Table 3.4 shows a summary of the molecular weight, structure and antioxidant activity of quercetin, catechol and the coupling product.

The coupling product exhibited higher antioxidant capacity than catechol but lower antioxidant capacity than quercetin. However, we observed that the solubility of the newly coupled product is greatly improved (visual observation as the new product dissolved instantly in ethanol while quercetin takes a longer time or required an elevated temperature, usually 70°C, to dissolve). It has been reported that orally administered quercetin is poorly absorbed, and its bioavailability administered in capsule form to human beings is less than 1% (Erlund, 2004). Conjugation of quercetin with glucose has been suggested to enhance quercetin absorption in the small intestine (Wach *et al.*, 2007; Chang *et al.*, 2005; Cornard *et al.*, 1999) and several formulation optimization trials have been conducted to increase the

bioavailability of quercetin (Wang *et al.*, 2011). The improved solubility of the coupling product over that of quercetin, suggests that its absorption might be improved. This study offers an alternative approach to increasing the antioxidant capacity of flavonoid compounds, thereby reducing daily dosages recommended for these antioxidants.

Table 3.4: Summary of the molecular weight, structure and antioxidant activity of quercetin, catechol and the coupling product.

Sample	Molecular weight	Structure	EC_{50} for DPPH[a] (mM)	TEAC[b] value (mM TEAC eqv)
Quercetin	302.23		0.248 ± 0.0016	0.711 ± 0.016
Catechol	110.1		0.483 ± 0.0008	1.009 ± 0.019
Quercetin/ Catechol Coupling product	410.1		0.301 ± 0.0037	0.886 ± 0.013

[a]EC_{50} parameter is defined as the concentration of substrate that brings about 50% loss of the DPPH• (Yu *et al.*, 2002)

[b]The Trolox equivalent antioxidant activity, (TEAC) of the antioxidant is defined as the "concentration of antioxidant giving the same percentage inhibition of absorbance of the radical cation ($ABTS^+$) at 734 nm as 1 mM of Trolox, at specific points" calculated relative to the decrease in absorbance of Trolox at a specific time point (Re *et al.*, 1999).

3.5.4.4 Conclusions

In conclusion, this study clearly shows that laccase can be used to couple simple phenolics onto quercetin/catechin resulting in an increase in hydroxyl groups which ultimately could potentially lead to an increase in the antioxidant activity of the product. Coupling may also improve other physiological properties as evidenced by the increase in solubility which might enhance bioavailability.

CHAPTER FOUR

LACCASE-CATALYZED DIMERIZATION OF FERULIC ACID AMPLIFIES ANTIOXIDANT ACTIVITY

4.1 Introduction

Plant phenols and phenolic acids are increasingly becoming a subject of intensive research due to their bioactive properties which include antioxidant, anti-mutagenic, anti-viral and anti-inflammatory activities (Bordoni *et al.*, 2002; Middleton *et al.*, 2000; Nakagawa *et al.*, 1999; Jankun *et al.*, 1997). Among the phenolic acids, ferulic acid (3-(4-hydroxy-3-methoxy-phenyl)-acrylic acid) is the most abundant hydroxycinnamic acid in the plant world (Mathew and Abraham, 2004), constituting 5 g/kg in wheat bran, 9 g/kg in sugar beet pulp (Bunzel *et al.*, 2005; Kroon *et al.*, 1997;), 15−28 g/kg of rice bran oil (Zhang and Xu, 1997) and 25 g/kg in corn kernel (Buranov and Mazza, 2009). It is one of the major phenolic lignin monomers found in woods and grasses, and is widely distributed in cereals, fruits and vegetables (Ferreira *et al.*, 2007; Balasubashini *et al.*, 2003). Some of these sources are used to provide ferulic acid as a substrate for conversion into value added chemicals such as guaiacol, vanillin, vanillic acid and protocatechuic acid (Prasad *et al.*, 2006).

Ferulic acid is found as the free acid, low molecular weight conjugates, esters with cell wall heteroxylans, or are covalently bound to lignin and other biopolymers. Ferulic acid is one of the active ingredients of many Chinese traditional medicines used in the prevention and treatment of various diseases (Lu *et al.*, 2005; Hou *et al.*, 2004; Liu and Fang, 2001). It shows many physiological functions, including antioxidant, antimicrobial, anti-inflammatory, anti-thrombosis, and anti-cancer activities (Fazary and Ju, 2007; Ou *et al.*, 2007) and can be easily absorbed and metabolized in the human body (Hou *et al.*, 2004). It has been reported to also increase sperm viability, lower cholesterol in serum and in the liver, and protect against angina, hypertensive and coronary diseases (Wang *et al.*, 2008). However, it has been most widely investigated for its antioxidant properties which are important in the prevention of lipid oxidation in food and also in the putative prevention of free-radical-induced diseases such as cancer and atherosclerosis or aging caused by oxidative tissue degeneration (Srinivasan *et al.*, 2007; Ou and Kwok, 2004). The antioxidant properties are ascribed to its structure (Fig. 4.1) and specifically to its phenolic nucleus and an extended side chain conjugation which facilitate the formation of a resonance-stabilized phenoxy radical (Graf, 1992). Other important structural properties include electron-donating groups on the benzene ring (3-methoxyl and, more importantly, 4-hydroxyl) which give the additional property of terminating free radical chain reactions; the carboxylic

acid group with an adjacent unsaturated C-C double bond which can provide additional attack sites for free radicals thus preventing them from attacking membranes; and the carboxylic acid group which acts as an anchor group which binds it to the lipid bilayer, providing some protection against lipid peroxidation (Srinivasan *et al*., 2007). Despite having these attractive properties, the antioxidant capacity of ferulic acid is generally low when compared to conventional antioxidants and other hydroxyl-cinnamic acids (Kikuzaki *et al*., 2002; Chen and Ho, 1997). Sánchez-Moreno *et al*. (1999) also reported that ferulic acid has a low free radical scavenging activity, but showed higher activity in the inhibition of lipid oxidation and in the protection against hydroxyl and peroxyl radical oxidation in synaptosomal and neuronal cell culture systems *in vitro* (Kanski *et al*., 2002). Recently there have been a number of attempts to enzymatically modify phenolic molecules as a way of improving antioxidant properties. Laccases are among the enzymes that are currently being investigated.

Laccases (EC 1.10.3.2) are enzymes that are capable of catalyzing the one-electron oxidation of phenols to produce phenoxy radicals and concomitantly reduce molecular oxygen to water (Kudanga *et al*., 2011). Oligomerization or polymerisation of the radicals to form oligomers or polymers with higher antioxidant properties than the starting materials has been reported (Gogoi *et al*., 2009; Ncanana and Burton, 2007; Kurisawa *et al*., 2003a, b). Consequently, research interest in enzymatically transforming ferulic acid is now increasing with some researchers having reported the transformation of ferulic acid by laccase (Tranchimand *et al*., 2006; Mustafa *et al.,* 2005; Carunchio *et al*., 2001). Although, possible structures of the oxidation products have been proposed (Carunchio *et al*., 2001), none of the researchers conclusively elucidated the structures nor reported the antioxidant capacity of the products. This prompted us to investigate the modification of ferulic acid by laccase produced from *Trametes pubescens* (strain CBS 696.94) as a way of enhancing the antioxidant capacity of ferulic acid. To the best of our knowledge, our work for the first time conclusively elucidates the structure of two dimers formed from laccase-mediated oxidation of ferulic acid in organic media, one of which has higher antioxidant capacity than the substrate.

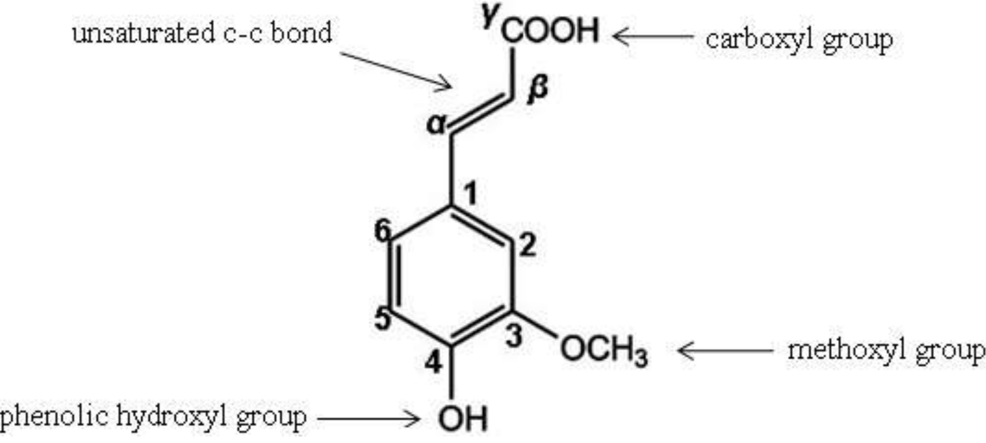


Fig. 4.1: Structure of ferulic acid showing the important features for antioxidant activity.

4.2. Materials and methods

4.2.1 Chemicals and enzyme

Ferulic acid and other chemicals were purchased from Sigma–Aldrich, South Africa. An airlift reactor was used to grow *T. pubescens* (strain CBS 696.94) for the production of laccase (Ncanana and Burton, 2007) which was purified according to the method of Ryan *et al.* (2005) (see Chapter 3).

4.2.2 Enzyme activity

Laccase activity was determined spectrophotometrically by monitoring the oxidation of 2,2,'-Azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, ε_{420} = 36,000 $M^{-1}cm^{-1}$) as the substrate (Childs and Bardsley, 1975) (see section 3.2.3).

4.2.3 Oxidation of ferulic acid

The oxidation reactions were carried out either in a biphasic system comprising of buffer with ethyl acetate as co-solvent or in a monophasic system with water-miscible solvents (dioxane, methanol, ethanol or acetone) as co-solvents. For the biphasic system the reaction mixture contained ferulic acid (10 mM), laccase (10 U) in 100 mM sodium acetate buffer (pH 5.0) and ethyl acetate at various concentrations (80, 85, 90, 95 and 96%, v/v). For the monophasic system the water-miscible solvents were used at 80% (previously determined as the optimum for product yield and reduced side reactions). The reactions were carried out for 24 hours at 28°C with shaking on an orbital shaker at 180 rpm. The reactions were monitored by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) as described in sections 4.2.4 and 4.2.5.

4.2.4 Chromatographic separation of reaction products

TLC analysis was performed on aluminium – backed silica gel 60 F_{254} (Merck) plates using toluene: dioxane: acetic acid (10:2.5:0.2, v/v/v) or ethyl acetate: dioxane: acetic acid (6:0.2:0.05, v/v/v) as the mobile phase. The compounds were visualized by exposure of the TLC plate to UV light at 254 nm.

4.2.5 High Performance Liquid Chromatography (HPLC)

Prior to HPLC analysis, the enzyme was precipitated out of the reaction solution by the addition of an equal volume of ice-cold methanol (only when water-miscible solvents were used; in the biphasic system, the enzyme was readily separated from the product). The mixture was incubated at 0°C for 20 minutes and centrifuged at 4°C for 15 minutes at 14000 *g*. The supernatant (1.5 ml aliquots) was transferred into clean vials and analyzed by HPLC. HPLC analysis was carried out using a Hitachi LaChrom HPLC system from Merck (Merck, Hitachi, Germany). Separation of the reaction products was carried out on a reversed phase LUNA 5µ PFP(2) 100A, 250×4.60mm column under isocratic conditions or gradient elution as described in section 3.4.2.

4.2.6 Purification of reaction products

The reaction products were purified by flash chromatography. The water-miscible solvents containing products were evaporated using a rotary evaporator and the product extracted with ethyl acetate followed by separation using a separation funnel. The aqueous phase was washed twice and monitored for the absence of product. The organic phase was dried using a rotary evaporator (Heidolph, Germany). For the biphasic system, the organic phase was separated using a separation funnel and the aqueous phase washed twice with ethyl acetate. The organic phase was evaporated under reduced pressure with a rotary evaporator and the crude residue purified by silica flash chromatography using ethyl acetate: dioxane: acetic acid (6:0.2:0.05, v/v/v) as mobile phase for purifying product 1 (**P1**) or toluene: dioxane: acetic acid (10:2.5:0.2, v/v/v) as mobile phase for purifying product 2 (**P2**). The pure fractions were dried using a rotary evaporator and the products sequentially washed with acetone, methanol and then acetone again to remove the acetic acid.

4.2.7 Characterization of products

The purified products (P1 and P2) were characterized by mass spectrometry and nuclear magnetic resonance (NMR) analysis.

4.2.7.1 Liquid chromatography-mass spectrometry (LC-MS)

LC-MS was performed on a Dionex HPLC system equipped with a binary solvent manager and autosampler coupled to a Bruker ESI Q-TOF mass spectrometer (see section 3.4.3).

4.2.7.2 Nuclear Magnetic Resonance (NMR) analysis

NMR spectra were recorded using a VARIAN 200 spectrometer (^{1}H, 200MHz; ^{13}C, 50MHz). The spectra were determined at ambient temperature in deuterated chloroform ($CDCl_3$) and methanol solutions, with $CHCl_3$ at d 7.26 for ^{1}H NMR spectra and chloroform (d 77.00) for ^{13}C-NMR spectra as internal standards. In the NMR spectra, assignments of signals with the same superscripts are interchangeable. Splitting patterns are designated as “s”, “d”, “t”, “q”, “m” and “bs”. These symbols indicate “singlet”, “doublet”, “triplet”, “quartet”, “multiplet” and “broad singlet”. The analysis was provided as a service by the University of the Western Cape.

4.2.8 Antioxidant activity determination

4.2.8.1 DPPH (2, 2’ -Diphenyl-1-picrylhydrazyl) scavenging effect

Antioxidant capacity was determined by measuring DPPH radical-scavenging activity (Yu *et al.*, 2002). Briefly, 3.9 ml of DPPH dissolved in methanol (0.025 mg/ml) was added to 0.1 ml sample (dissolved in methanol) at various concentrations. The mixture was shaken vigorously and incubated at room temperature in the dark for 60 min, and the decrease in absorbance at 517 nm determined using a spectrophotometer. The remaining concentration of DPPH in the reaction medium was then calculated from a calibration curve obtained with DPPH at 517 nm.

The percentage of remaining DPPH ($DPPH_R$) was calculated as follows:

$$\% DPPH_R = [(DPPH)_T/(DPPH)_{T=0}] \times 100$$

where $(DPPH)_T$ is the concentration of DPPH at the time of 60 min and $(DPPH)_{T=0}$ is the concentration of DPPH at time zero (initial concentration). The percentage of remaining DPPH against the sample/standard concentration was plotted to obtain the amount of antioxidant (mM) necessary to decrease the initial concentration of DPPH by 50% (EC_{50}).

4.2.8.2 TEAC (Trolox equivalent antioxidant capacity) assay

The ABTS radical scavenging activity of ferulic acid and the two products were determined according to the method described by Re *et al.* (1999). The TEAC

method is based on the ability of antioxidant molecules to quench $ABTS^{\cdot+}$, a blue-green chromophore with characteristic absorption at 734 nm, compared with that of Trolox, a water soluble vitamin E analog. The addition of antioxidants to the preformed radical cation decolourizes the $ABTS^{\cdot+}$ as it is reduced to ABTS. $ABTS^{+}$ solution was prepared 12-16 hours before use by mixing ABTS salt (7 mM) with potassium persulfate (2.45 mM) and then stored in the dark until the assay was performed. The $ABTS^{\cdot+}$ solution was diluted with methanol to give an absorbance of 0.70 ± 0.002 at 734 nm. Each sample (100 µl) prepared at different concentrations was mixed with 1100 µl $ABTS^{\cdot+}$ solution and the absorbance was read after a 30 min incubation at 25°C.

4.3. Results and Discussion

4.3.1 Oxidation of ferulic acid in organic solvents

Biocatalysis is conducted in organic media for a number of reasons, mainly poor solubility of some compounds in aqueous media; ability to carry out new reactions which are impossible in water because of kinetic or thermodynamic restrictions; relative ease of product recovery from organic solvents as compared to water; and the insolubility of enzymes in organic media, which permits their easy recovery and reuse (thus eliminating the need for immobilization) (Leon *et al.*, 1998; Zaks and Klibanov, 1985). Ferulic acid is not soluble in aqueous media, thus organic solvents were employed for this study in either a monophasic or biphasic system. Both the biphasic system and monophasic systems produced three main oxidation products which were designated as **P1** (t_R=10.535); P2 (t_R=12.202); P3 (t_R=13.446) (Fig. 4.2). LC-MS analysis of the oxidation products in negative mode showed dominant signals at *m/z* 385.1143 (**P1**) and *m/z* 385.1073 (**P2**) (Fig. 4.3), which suggested that these oxidation products were dimers of ferulic acid (exact mass [M] 386.1). When the mass spectrometer was set to run in positive mode $[M+H]^+$ ion signals were observed at *m/z* 387.1, while signals at m/z 409.1 indicating Na adducts, confirmed that the molecules were indeed dimers. LC–MS results of the product **P3** (m/z 579.2154) suggest oligomerization to form a trimer. In addition, a tetramer (m/z 769.1948; t_R = 13.7) was observed with the more sensitive MS detector (not observed with HPLC). After purification, the yields of P1 and P2 were 11.190% and 38.189%, respectively.

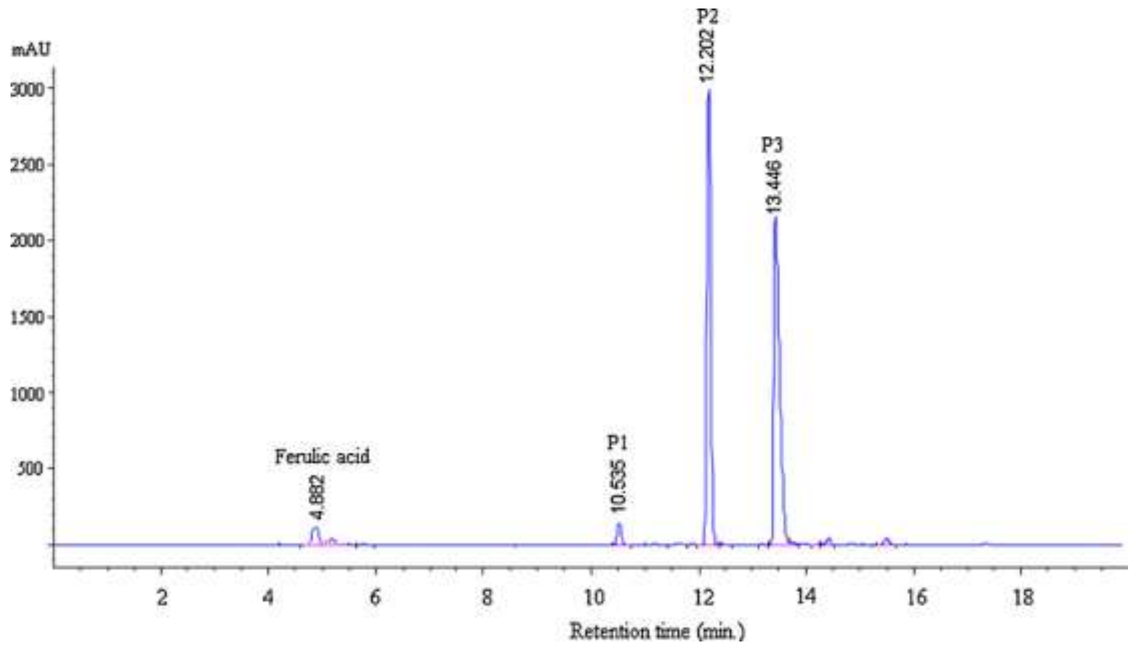


Fig. 4.2: HPLC chromatogram of products formed during laccase-catalyzed oxidation of ferulic acid. P1, P2 and P3 – oxidation products.

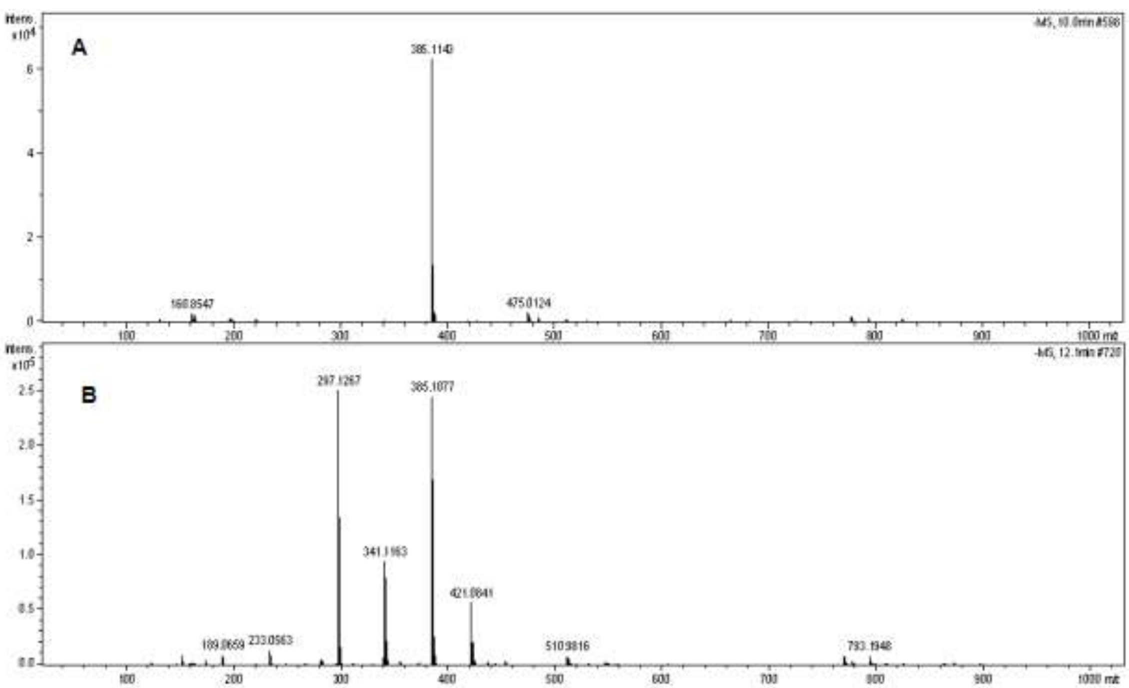


Fig. 4.3: Mass spectrum of Product P1 (A) and P2 (B) formed during laccase-mediated oxidation of ferulic acid.

The NMR results (Appendices F, G,H, I) indicated that P1 is a dimer of two ferulic acid monomers covalently bound through a *β*-5 linkage while the results for P2 suggest dimerization through a *β* – *β* linkage (Fig. 4.4). The symmetry of the molecule P2 made assignment easy due to the signals being clear and separate from each other. In the ^{1}H NMR spectrum 2-H appeared as a single peak at δ 3.56 while the

methoxy group appeared as a three proton singlet at δ 3.91. The phenolic OH appeared as a single peak at δ 5.86 in close proximity to 3-H which appeared as a single peak at δ 5.69. Coupling between 2-H and 3-H was not observed due to the dihedral angle being in the vicinity of ~90◦. The aromatic region displayed a typical pattern of a 1,3,4-tri-substituted system which is a 2-proton multiplet at δ 6.79 assigned to 2'-H and 6'-H while 5'-H appeared as an *ortho* coupled doublet at δ 6.93 with J = 8.8 Hz. In the ^{13}C NMR spectrum all signals could be assigned according to the structure with C2 at δ 48.5, the methoxy group at δ 56.3 and C3 at δ 82.0. Three strong signals at δ 107.6, 115.1 and 117.5 were assigned to C2', C5' and C6' respectively while C1' appeared at δ 130.0. The more de-shielded carbon signals of C3' and C4' appeared at δ 146.5 and 147.2. Finally, the lactone carbonyl appeared as expected at δ 175.0.

As expected the spectra were more complicated for P1 due to a lack of symmetry in the compound and the analysis was performed in deuteromethanol. The two methoxy groups appeared as 3-proton singlets at δ 3.83 and 3.91 while 3-H appeared as a sharp doublet at δ 4.01 (J = 2.6 Hz). A clear doublet at δ 5.61 (J = 2.6 Hz) was assigned to 2-H. A rather broad multiplet centred around δ 6.70 integrated for 5 protons and was assigned to 6-H, 5'-H, 1'-H, 2'-H and 4'-OH. A double doublet at δ 7.01 (J = 8.0 and 2.0 Hz) was assigned to 6'-H while the corresponding doublet at δ 7.07 (J = 2.0 Hz) was assigned to 2'-H. A downfield doublet at δ 7.57 (J = 1.8 Hz) was assigned to 4-H. The two -COOH hydrogens were not observed due to exchange with protons in the deuteromethanol solvent. The ^{13}C spectrum was assigned as follows: A broadish signal at δ 55.7 is due to the two methoxy carbons as well as C3. The signal at δ 81.2 is assigned to C2. Strong signals in the aromatic region have been assigned as follows: δ 108.3(C2'), 112.7(C8), 115.1(C5'), 118.0(C4), 118.7(C6'). The sp2 carbons of the ethylene side chain were assigned as follows: δ 115.2(C2') and 146.3(C1'). The ring junction carbons C3a and C7a were assigned as follows: δ 125.5 and 148.9 respectively, C5 and C1' were assigned to the signals at δ 125.9 and 131.2 respectively. The de-shielded carbon atoms attached to the oxygens were assigned as: C7(δ 140.8), C4'(δ 147.5) and C3' (δ 148.9). Finally, the two carbonyl signals at δ 169.2 and 172.6 were assigned to the two carboxylic acid groups.

Fig. 4.4: Dimers (P1 and P2) formed during laccase-catalyzed oxidation of ferulic acid.

Based on the results from LC-MS and NMR, the scheme shown in Fig. 4.5 is proposed as the possible reaction pathway for the dimerization of ferulic acid to form a β-5 and β-β linkage (Carunchio *et al.*, 2001). As depicted in Fig. 4.5, laccase initiates the reaction by oxidizing the para-hydroxyl group resulting in a radical. Through resonance stabilization, the unpaired electron can occupy different positions on the radicalized molecule (Kudanga *et al.*, 2011). The radicals are stabilized through coupling with other radicals to form dimers. Therefore coupling of an unpaired electron at the β position with another at position C5 of the next radical yields a β-5 linked dimer. Similarly covalent coupling of radicals both with unpaired electrons at the β position produces the β-β linked dimer. While there were several possibilities for coupling due to the large number of mesomeric forms, it appears that our reaction conditions favoured the formation of β-5 and β-β linkages due to the stability of C-C bonds (del Rio and Gutierrez, 2008) and the lower heat of formation of β-5 linkages (Valencia and Marinez, 2005). We have observed similar findings in which C-C bonds predominated in the coupling of functional molecules to lignin models, though mostly 5-5 linkages were formed due to the absence of unsaturated side chains (Kudanga *et al.*, 2010 a,b). In addition, recent reviews agree that in the dimerization of a similar molecule, coniferyl alcohol, the radicals favour coupling at their β positions, resulting essentially in only the β-β, β-*O*-4, and β-5 dimers (Vanholme *et al.*, 2010; Ralph, 1999;) although the β-O-4 are the predominant linkages in polymerization reactions during lignin synthesis (Kandanarachchi *et al.*, 2002).

Fig. 4.5: Laccase-catalyzed oxidation of ferulic acid to produce *β-β* and *β*-5 dimers.

4.3.2 Effect of organic solvents

The effect of the nature of the organic solvent and the concentration of the solvent was studied in order to obtain the best reaction conditions for product formation. In the biphasic system, there was an increase in the formation of P2 (*β–β* dimer) as the concentration of ethyl acetate was increased from 80% to 90% after which a decline in product formation was observed (Fig. 4.6). A similar pattern was observed during the formation of P1 (*β*-5 dimer) except that a decline was observed after the composition of the organic solvent was increased beyond 95% (Fig. 4.6).

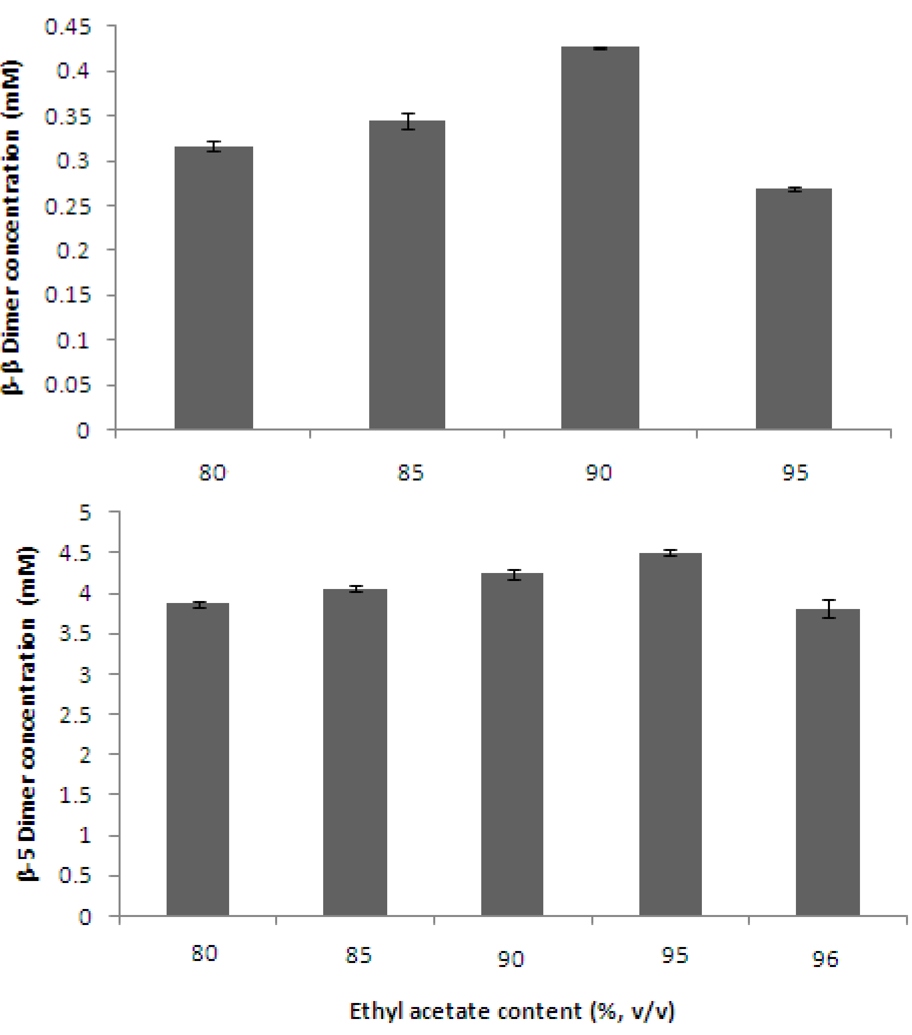


Fig. 4.6: Effect of ethyl acetate content on the formation of *β-β* and *β*-5 dimers in a biphasic system with sodium acetate buffer (pH 5.0) as co-solvent. All results are means ± standard deviation (SD) of three replicate determinations.

In the monophasic system, four co-solvents namely acetone, dioxane, ethanol and methanol were investigated. As shown in Fig. 4.7, most of the substrate was converted to products in the presence of ethyl acetate: a biphasic system. In the monophasic system, the order of substrate conversion was ethanol > acetone > dioxane > methanol. However, for product formation, *β-β* dimer formation was facilitated by the use of ethanol as co-solvent followed by methanol, ethyl acetate, dioxane and acetone. Ethyl acetate, which was the best solvent for combined product formation, promoted the formation of the *β*-5 dimer and product formation of this product decreased in the order of ethyl acetate > dioxane > methanol > ethanol > acetone. For the water-miscible organic solvents employed in this study, solvents with a higher polarity index favoured the formation of the *β-β* dimer: ethanol (5.2) > methanol (5.1) > dioxane (4.8). However, the reverse was observed for the *β*–5 dimer: dioxane (4.8) > methanol (5.1) > ethanol (5.2); only acetone did not follow a particular order for both dimers. The effect of different solvents on enzyme catalysis

allows ‘medium engineering’ in order to modulate and possibly improve, the regioselective outcome of reactions (Carrea *et al.*, 1995). However, some water-miscible solvents tended to inactivate the enzyme over time. For example, only 0.12% or 2.8% residual activity was observed after three hours when methanol or dioxane was used, respectively, which explains the poor substrate conversion in these solvents. Therefore, meticulous optimisation is required in order to convert the substrate quickly within the first few hours; the second phase of the reaction (resonance stabilisation and coupling) is non-enzymatic. In contrast, the enzyme was more stable in ethyl acetate which probably explains the higher conversion of substrate to products. In related work, Ma *et al.* (2009) reported the enhancement of catechol-laccase catalysis in less polar organic solvents as compared with highly polar media, due to the hydrophobicity of the end product. It should be noted that both dimers are less polar than the substrate (ferulic acid) as demonstrated by the retention times which were observed to be longer than that of ferulic acid (Fig. 4.2).

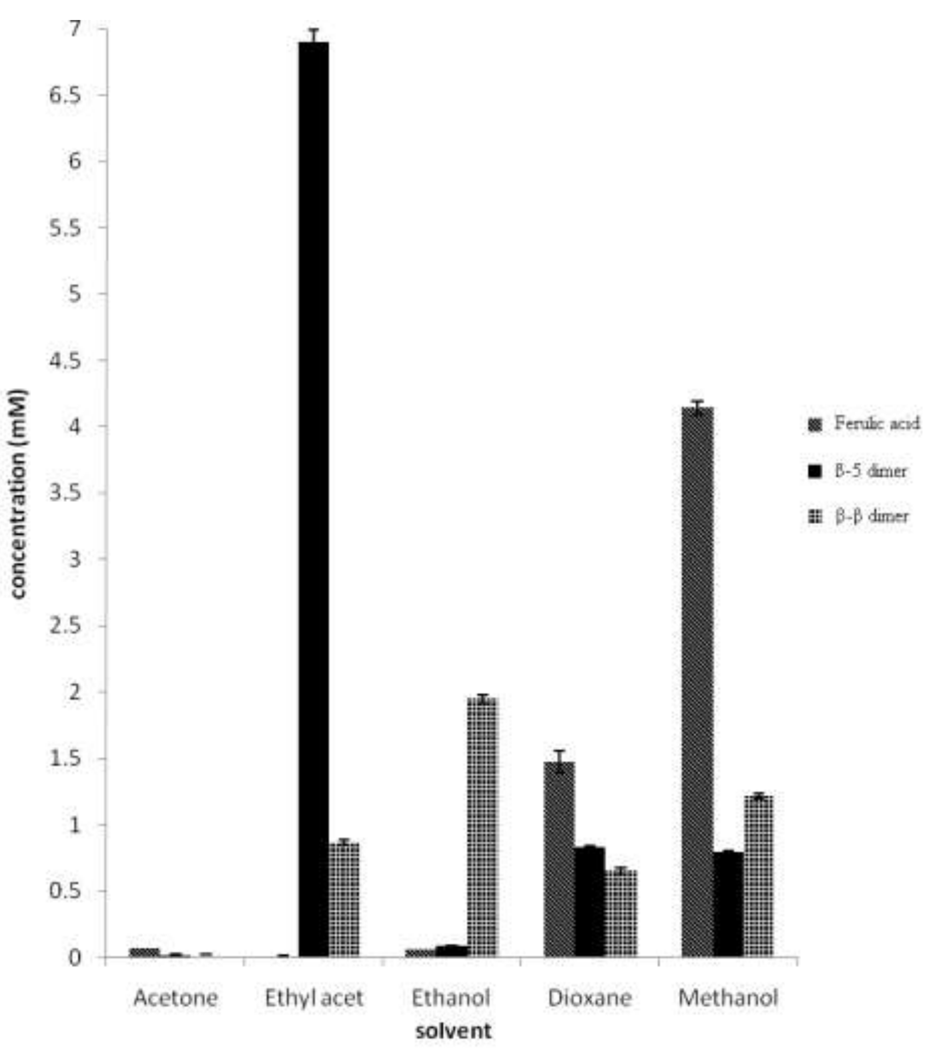


Fig. 4.7: Effect of organic co-solvent on laccase-catalyzed oxidation of ferulic acid to form *β*-5 and *β*-*β* dimers. All results are means ± SD of three replicate determinations.

4.3.3 Antioxidant activity determination

Antioxidant activities of the synthesised dimers were evaluated in relation to that of ferulic acid. Interestingly, the DPPH scavenging effect and Trolox equivalent antioxidant capacity (TEAC) of the products showed that the *β*-5 dimer exhibited higher antioxidant activity than ferulic acid (Table 4.1). However, the *β*-*β* dimer showed lower antioxidant activity than ferulic acid. The antioxidant activity of phenolic compounds depends on various structural features such as the O-H bound dissociation energy, resonance delocalisation of the phenoxyl radical and the steric hindrance due to bulky groups substituting hydrogen in the aromatic ring (Sanchez-Moreno *et al*., 1998). The increase in antioxidant capacity of the *β*-5 dimer could be attributed to an increase in electron donating groups after dimerization (Matsuura and Ohkatsu, 2000) and the carboxylic acid group with an adjacent unsaturated C-C double bond which can provide additional attack sites for free radicals (Srinivasan and Sudheer, 2007). In the *β*-*β* dimer, the unsaturated C-C bonds and carboxyl groups are lost during the dimerization process which could explain the reduction in antioxidant capacity.

Table 4.1: Antioxidant activity of ferulic acid and the dimers, P1 and P2 produced in this study.

Molecule	Molecular weight	EC_{50} DPPH[a] (mM)	TEAC[b] value (mM TEAC eqv)
Ferulic acid	194.18	1.837 ± 0.012	0.682 ± 0.005
P1(*β*-5 dimer)	386.11	1.310 ± 0.016	0.653 ± 0.006
P2 (*β*–*β* dimer)	386.11	2.320 ± 0.057	0.700 ± 0.013

[a]EC_{50} parameter is defined as the concentration (mM) of substrate that brings about 50 % loss of the DPPH∙ (Yu *et al*., 2002). Values are means ± SD of three replicate determinations.

[b]The Trolox equivalent antioxidant activity (TEAC) of the antioxidant is defined as the concentration of antioxidant giving the same percentage inhibition of absorbance of the radical cation ($ABTS^+$) at 734 nm, as 1 mM of Trolox, at specific points (Re *et al.,* 1999).

4.4 Conclusion

This study has, for the first time, conclusively isolated and elucidated the structures of two dimers formed during laccase-catalyzed oxidation of ferulic acid in organic media. Although, Carunchio (2001) reported that these two dimers were the products of ferulic acid/laccase reaction, their purification and isolation were however not reported. By altering the nature of the organic solvent used, it was possible to facilitate the formation of the *β*-5 dimer which is more potent than ferulic acid in terms of antioxidant capacity. This new product may find useful application in the health and cosmetic industries.

CHAPTER FIVE

LACCASE-MEDIATED OXIDATION OF 2,6-DIMETHOXYPHENOL YIELDS A DIMER WITH HIGHER ANTIOXIDANT PROPERTY

5.1 Introduction

Natural phenolic compounds are attracting much attention in recent years due to their antioxidant capacity and potential health benefits (Penarrieta, *et al.*, 2011). They are part of a number of secondary metabolites in most plants which aid in the protection against insects, infections, pathogen attack and UV radiation (Del Mar Verde Mendez *et al.*, 2004; Manach, 2004). Antioxidants generally, are an important class of compounds that when present at low concentrations relative to an oxidizable substrate, significantly delay, retard or inhibits oxidation of that substrate (Halliwell and Gutteridge, 1999). They prevent oxidative reactions in foods and protect biological tissues against damage which can lead to various diseases. As attention is being shifted from synthetic antioxidants due to potential health hazards, plant phenols and phenolic acids are increasingly becoming a subject of intensive research due to their bioactive properties. Phenolic antioxidants, in particular, are compounds that act as terminators for free radicals (da Silva Oliveira *et al.*, 2011).

2,6–Dimethoxyphenol (2,6-DMP) is the predominant smoke component of thermal degradation products from hardwood. It forms about 70-80% of total methoxyphenols in birchwood smoke which is of great importance for the smoke flavour due to the preserving antioxidant effect. As a major component of birchwood smoke, its antioxidant capacity is stronger than the 2-methoxyphenols that are present in lower amounts - 20-30% (Kjallstrand and Petersson, 2001). 2,6-DMP is widely documented as a substrate for the determination of laccase activity. Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multi-copper-containing enzymes which reduce molecular oxygen to water and simultaneously perform one-electron oxidation of various substrates such as diphenols, methoxy-substituted monophenols and aromatic and aliphatic amines (Kudanga *et al.*, 2011). Laccases have been reported to catalyze the polymerization of many phenolic compounds as a way of increasing antioxidant capacity (Ncanana and Burton, 2007; Kurisawa *et al.*, 2003a, b). The compound (2,6-DMP) has been used extensively to indicate the activity of the enzyme laccase and some of the products of laccase oxidation have been characterized (Wan *et al.*, 2008; Betts and King, 1991).

Although some products of laccase oxidation of 2,6-DMP have been characterised, their application as antioxidants has not been documented. As part of our attempts to

enzymatically modify phenolic molecules by laccase as a way of enhancing their bioactive properties (Adelakun *et al.*, 2012; Zwane, 2009; Chigorimbo-Murefu, 2007; Ncanana and Burton, 2007; Ting, 2004;), the potential of laccase produced by *Trametes pubescens* (strain CBS 696.94) to modify 2,6-DMP as a way of increasing antioxidant capacity, is investigated in this part of the study.

5.2 Materials and methods

5.2.1 Chemicals and enzyme

2,6-DMP and other chemicals were purchased from Sigma–Aldrich, South Africa. Extracellular laccase was produced by fermentation of the white-rot fungal strain *Trametes pubescens* (strain CBS 696.94) using an airlift reactor, and purified as described by Ryan *et al.* (2005) (see Chapter 3).

5.2.2 Enzyme activity

Laccase activity was determined spectrophotometrically by monitoring the oxidation of 2,2,'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, ε_{420} = 36,000 $M^{-1}cm^{-1}$) as the substrate (Childs and Bardsley, 1975). See section 3.2.3

5.2.3 Oxidation of 2,6-DMP

A biphasic system comprising buffer with ethyl acetate as co-solvent or monophasic system with miscible solvents (methanol, ethanol, toluene or acetone) as co-solvents was employed for the oxidation reactions. For the biphasic system the reaction mixture contained 2,6-DMP (10 mM), laccase (10 U) in 100 mM sodium acetate buffer (pH 5.0) and ethyl acetate at various concentrations (50, 60, 70, 80, 90, and 95%, v/v) and time [1 – 8 hours; time variation only done using 90% (v/v) ethyl acetate – previously determined as the optimum co-solvent concentration]. In the monophasic system the miscible solvents were used at 80% (previously determined as the optimum for product yield and reduced side reactions). The reactions were carried out for 24 hours at 28°C with shaking on an orbital shaker at 180 rpm. The reactions were monitored by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

5.2.4 Thin Layer Chromatography

TLC analysis was performed on aluminium – backed silica gel 60 F254 (Merck) plates using heptane: ethyl acetate: acetic acid (1:1:0.05, v/v/v) as the mobile phase. The compounds were visualized by exposure of the TLC plates to UV light at 254 nm.

5.2.5 High Performance Liquid chromatography (HPLC)

When water-miscible solvents were used, an equal volume of ice cold methanol was added to the reaction mixtures to precipitate out the protein. The mixture was allowed to stand on ice for 20 min before centrifuging at 4ºC for 15 min at 14000 *g*. In the biphasic system however, the enzyme was readily separated from the product. The supernatant (1.2 ml aliquots) was transferred into clean vials and analyzed by HPLC. HPLC analysis was carried out using a Hitachi LaChrom HPLC system from Merck (Merck, Hitachi, Germany) (See section 3.4.2). The products were however detected at 280 nm

5.2.6 Purification of reaction products

The reaction products were purified by flash chromatography. The water-miscible solvents containing products were evaporated using a rotary evaporator and the product extracted with ethyl acetate followed by separation using a separation funnel. The aqueous phase was washed twice and monitored for the absence of product. The organic phase was dried using a rotary evaporator (Heidolph, Germany). For the biphasic system, the organic phase was separated using a separation funnel and the aqueous phase washed twice with ethyl acetate. The organic phase was evaporated under reduced pressure with a rotary evaporator and the crude residue purified by silica flash chromatography using heptane: ethyl acetate: acetic acid (1:1:0.05, v/v/v) as mobile phase. The pure fractions were dried using a rotary evaporator and the products sequentially washed with acetone, methanol and then acetone again to remove the acetic acid.

5.2.7 Characterization of products

The purified product was characterized by mass spectrometry and nuclear magnetic resonance (NMR) analyses.

5.2.7.1 Liquid chromatography-mass spectrometry (LC-MS)

LC-MS was performed on a Dionex HPLC system equipped with a binary solvent manager and autosampler coupled to a Bruker ESI Q-TOF mass spectrometer as described in section 3.4.3.

5.2.7.2 Nuclear Magnetic Resonance (NMR) analysis

NMR spectra were recorded using a VARIAN 200 spectrometer (^{1}H, 200MHz; ^{13}C, 50MHz). (See section 4.2.7.2 for details).

5.2.8 Antioxidant activity determination

5.2.8.1 DPPH (2, 2' -Diphenyl-1-picrylhydrazyl) scavenging effect

Antioxidant capacity was determined by measuring DPPH radical-scavenging activity (Yu *et al*., 2002) as described in section 4.2.8.1.

5.2.8.2 TEAC (Trolox equivalent antioxidant capacity) assay

The ABTS radical scavenging activity of 2,6-DMP and its product were determined according to the method described by Re *et al*. (1999) as described in section 4.2.8.2.

5.2.8.3 FRAP (ferric reducing antioxidant power) analysis

Total antioxidant activity was measured by ferric reducing antioxidant power (FRAP) assay of Benzie and Strain (1999; 1996). The FRAP assay uses antioxidants as reductants in a redox-linked colourimetric method, employing an easily reduced oxidant system present in stoichiometric excess. The principle behind this is that, at low pH, reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to the ferrous form (which has an intense blue colour) can be monitored by measuring the change in absorbance at 593 nm. The reaction is non-specific, in that any half reaction that has lower redox potential, under reaction conditions, than that of ferric ferrous half reaction, will drive the ferrous (Fe III to Fe II) ion formation. The change in absorbance is therefore directly related to the combined or "total" reducing power of the electron donating antioxidants present in the reaction mixture.

The working FRAP reagent was produced by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution and 20 mM $FeCl_3.6H_2O$ in a 10:1:1 ratio just before use and heated to 37 ^oC. The 300 mM acetate buffer was prepared by mixing 3.1 g of sodium acetate trihydrate ($C_2H_3NaO_2$.3H2O) with 16 ml glacial acetic acid and brought to 1 L with distilled water. The TPTZ solution was prepared by making a solution of 10 mM TPTZ in 40 mM HCl. Sample (100 µl) was mixed with 3 ml of working FRAP reagent and absorbance (593 nm) is measured at 0 minute after vortexing. Thereafter, samples were placed at 37°C in water bath and absorbance was again measured after 4 minutes. Ascorbic acid standards (0.1 mM-1.0 mM) were processed in the same way. The change in absorbance after 4 min from the initial blank reading was compared to that of a standard. A standard curve was prepared by plotting the average FRAP value versus its concentration, while the FRAP values for the samples were determined using this standard curve.

5.3 Results and Discussion

5.3.1 Optimization of reaction and characterization of reaction product

Laccase-mediated oxidation of 2,6-DMP using *T. pubescens* laccase as analysed by HPLC revealed that one product was formed (Fig. 5.1). LC-MS analysis of the oxidation product in negative mode showed a dominant signal at *m/z* 305.1022 which suggest that this oxidation product is a dimer of 2,6-DMP (exact mass [M] 306.1 – Fig. 5.2).

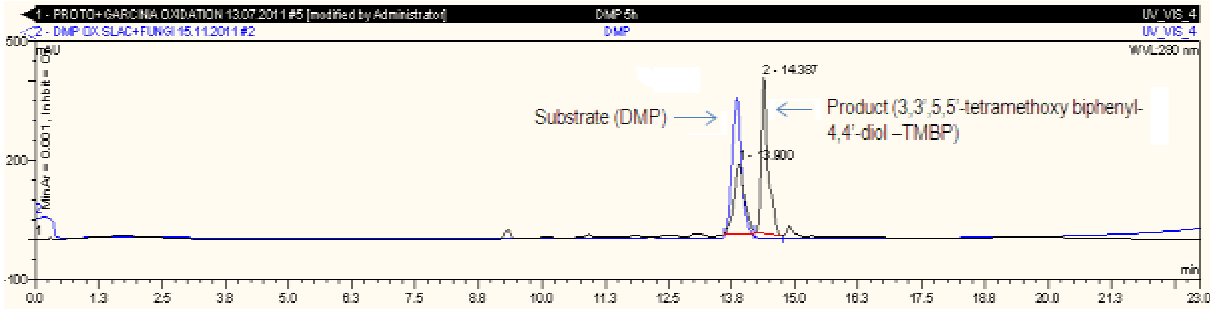


Fig. 5.1: Laccase-catalysed oxidation of 2,6-DMP to form 3,3',5,5'-tetramethoxy biphenyl-4,4'-diol (TMBP) as determined by HPLC.

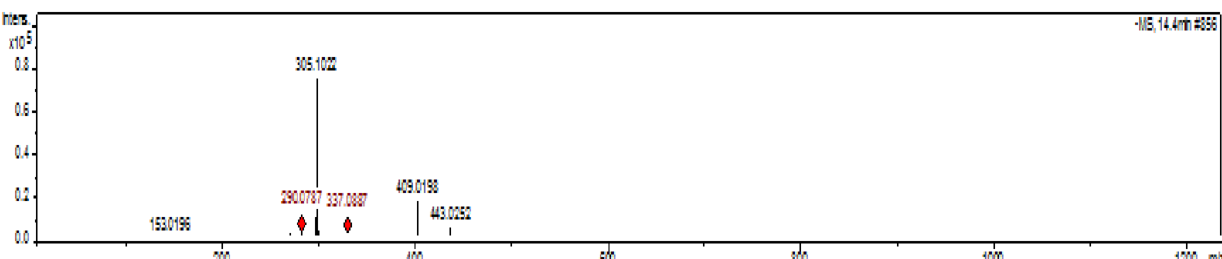


Fig.5.2: Mass spectrum of the product formed during laccase-mediated oxidation of 2,6-DMP (m/z 305.03).

Fig. 5.3 shows the ^{1}H NMR spectrum of the product formed during laccase-mediated oxidation of 2,6-DMP while Fig. 5.4 shows the structure of the dimer (3,3',5,5'-tetramethoxy biphenyl-4,4'-diol) formed during the oxidation.

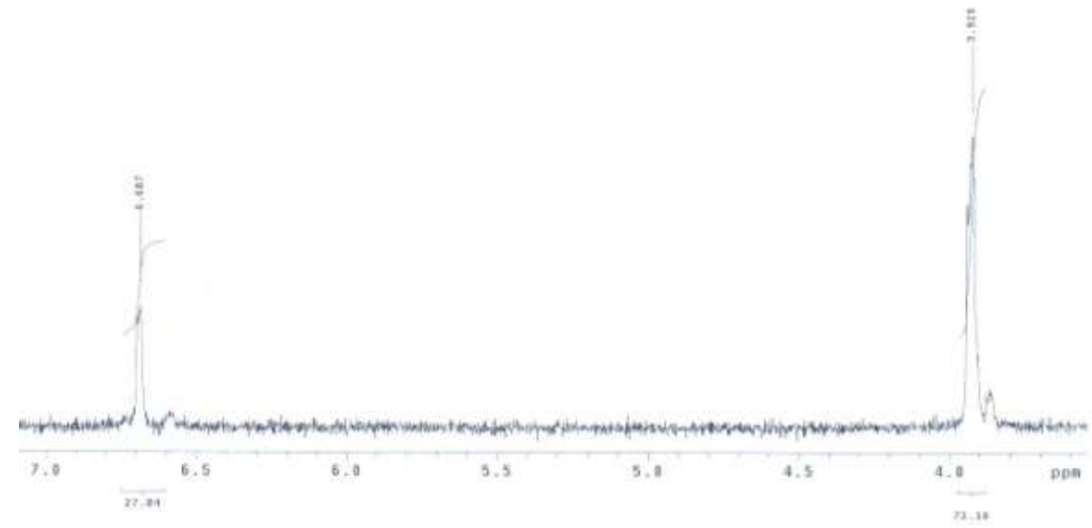


Fig. 5.3: ^{1}H NMR spectrum of Product formed during laccase-mediated oxidation of 2,6-DMP.

Fig. 5.4: The dimer (3,3',5,5'-tetramethoxy biphenyl-4,4'-diol) formed during laccase-catalyzed oxidation of 2,6-DMP.

Rationale for assignment of dimeric structure in Fig 5.4 was that, usually, spectra are greatly simplified in their interpretation when molecules are symmetric and this was the case for this dimer. A singlet at δ 3.96 integrating for 12 protons is assigned to 4 methoxy groups attached to an aryl ring. The fact that a 4-proton singlet was observed at δ 6.68 confirms the aromatic nature of these protons and hence the molecule as well. Finally a 2-proton broad single peak at δ 6.58 is typical for phenolic groups and thus the only structure which would fit the data is the dimeric one suggested in which the C4-C4' linkage between the two rings would provide the symmetry. The fact that one does not observe *meta*-coupling of 2 Hz between H-3 and H-5 might be due to the mixed solvent system used.

Only five signals were observed in the ^{13}C spectrum of which four were in the aromatic region and this again is indicative of a highly symmetrical molecule. A strong signal at δ 56.2 is assigned to the four methoxy groups while an equally strong signal at δ 102.2 in the aromatic region is assigned to the four aromatic hydrogens on C3, C5, C3’ and C5’ since the symmetry will be fully accounted for in such an arrangement. A weaker signal at δ 137.7 is assigned to the carbons linking the two rings viz., C4 and C4’ while the two downfield signals at δ 143.3 and 148.1 reflect the fact that the carbon atoms are attached to electron-withdrawing atoms viz., O and are assigned to C1/C1’ and C2/C6/C1’and C6’ respectively.

Fig. 5.5: The proposed mechanism for the laccase–catalyzed oxidation of 2,6–DMP to produce the dimer (3,3’,5,5’-tetramethoxy biphenyl-4,4’-diol).

Based on the results obtained from LC-MS and NMR analyses, the scheme shown in Fig. 5.5 is proposed as the possible pathway for the synthesis of the dimer. As shown in Fig. 5.5, the 2,6-DMP went through a single-electron-oxidation by *T. pubescens* laccase catalysis to produce 2,6-DMP radical species that resonance with corresponding para-radical species. A recombination of 2 moles of para-radical species then produced the product (Fig. 5.5). The fragmentation pattern revealed m/z of 290.078 and 153.019 indicating a loss of $-CH_3$ group and 2,6-DMP monomer respectively (Fig. 5.2). This observation is similar to the work of Wan *et al.* (2008) who had earlier characterized the oxidized product to be 3,3’,5,5’-tetramethoxy biphenyl-4,4’-diol (TMBP). Two dimeric products (m/z 304 and m/z 306) were earlier reported to be observed in crystal form when the extracellular enzyme activities of *T. versicolor* was investigated for their ability to oxidatively couple 2,6-DMP (Betts and King, 1991). Comparative experiments using different laccase substrates demonstrated that 2,6-DMP was the most suitable substrate for laccase assays as judged by a number of factors including the stability of its oxidized dimeric coloured product (3,3’,5,5’-tetramethoxydiphenylquinone) form, its high absorption molar coefficient, weak acidic optimal pH and oxidation efficiency for a number of blue multicopper enzymes

(Solano *et al.*, 2001). In related studies, laccase-catalysed coupling of 2,6-DMP (Me_2P) in acetone buffer mixture has been reported to produce 3,3'5,5'-tetramethoxydiphenolquinone via the C-C coupling in acidic condition and C-O coupling in basic condition producing 2,6-dimethyl-1,4-phenylene oxide (Kobayashi and Higashimura, 2003).

After purification, the yield of the 2,6-DMP dimer was 20.91%. This is slightly higher than the yield of 19% as reported by Wan *et al.* (2008) when 2,6-DMP was catalysed by *Rhus* laccases (RL). However, when catalyzed by fungal laccases, *Pycnoporus coccineus* laccases (PCL), 8% yield of 2,6-DMP dimer was observed by the same author.

2,6-DMP is frequently used as substrate of laccase oxidation assay and the enzyme determination is usually performed in aqueous solution. However, due to inherent advantages of using organic solvents in biocatalysis reactions (Leon *et al.*, 1998; Zaks and Klibanov, 1985), the effect of the nature of the organic solvent, the concentration of the solvent and time were studied in order to obtain the best reaction conditions for product formation. Biocatalysis in the presence of organic solvents already has proven to be capable of synthesizing novel compounds and opening up new synthetic pathways. Generally, organic solvents have a lot of advantages when employed in biocatalysis, such as higher solubility of hydrophobic species, reduction of water activity, reduction of microbial contamination and incidence of side reactions found in water, aids separations and improvement of yields (Zaks, 2001).

In the biphasic system, there was an increase in the formation of the product (dimer) as the concentration of ethyl acetate was increased from 50% to 90% after which a decline in product formation was observed (Fig. 5.6). However in toluene, no product of interest was formed. Production of the dimer in ethyl acetate as co-solvent was increased until 7 h of incubation after which there was a pronounced decline in product formation (Fig. 5.7).

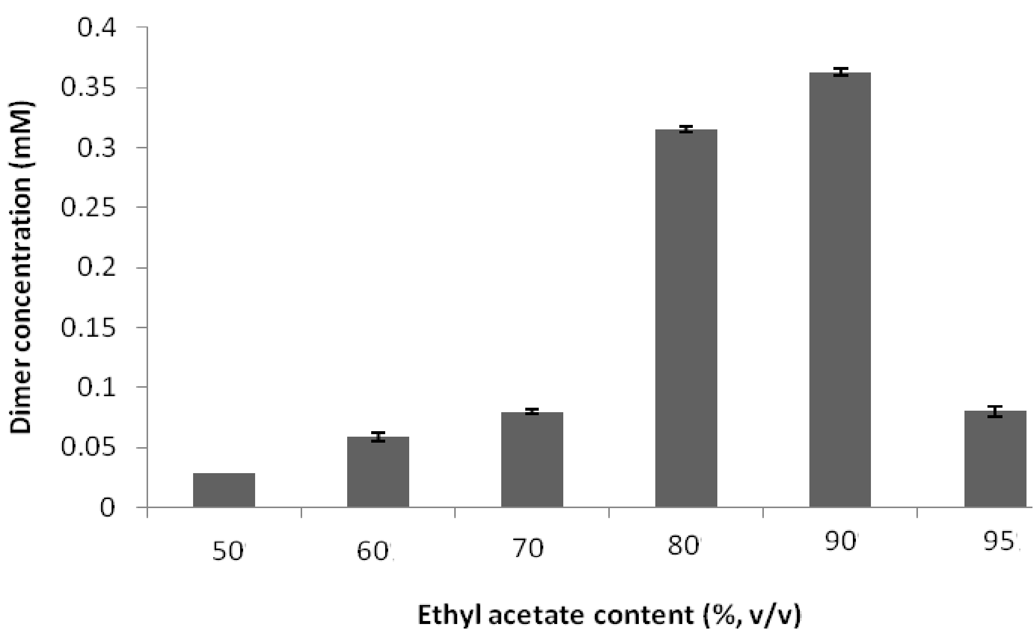


Fig. 5.6: Effect of ethyl acetate content on the formation of the 2,6-DMP dimer in a biphasic system with sodium acetate buffer (pH 5.0) as co-solvent. All results are means ± standard deviation (SD) of three replicate determinations.

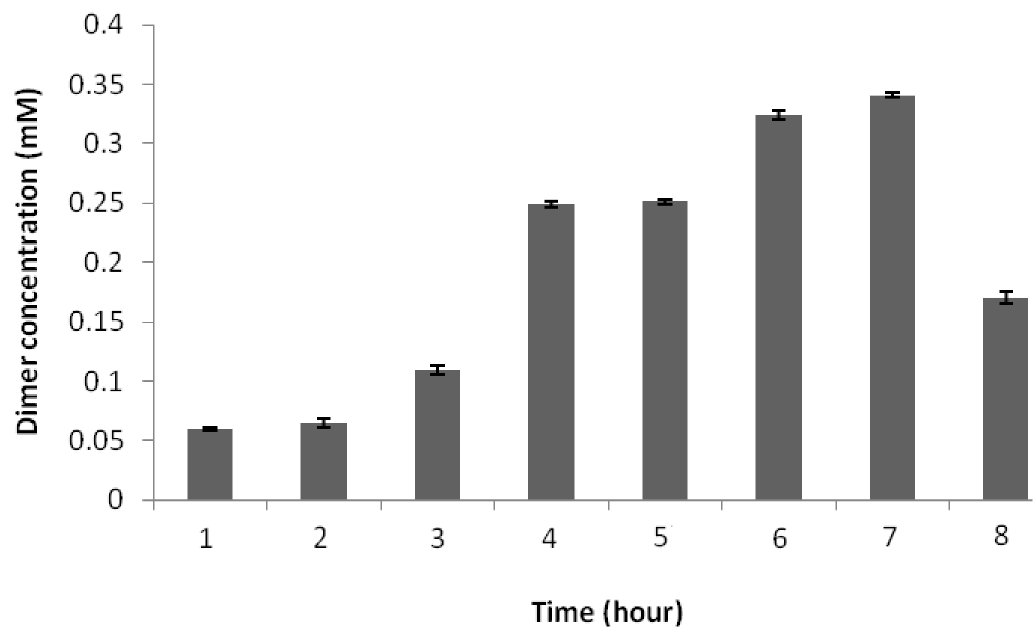


Fig. 5.7: Effect of reaction time on laccase-catalyzed oxidation of 2,6-DMP. All results are means ± SD of three replicate determinations.

In the monophasic system, solvents with lower value of relative polarity favoured the formation of the 2,6-DMP dimer (Fig. 5.8): acetone (0.355) > ethanol (0.654) > methanol (0.762). The nature of the solvent has earlier been discovered to affect enzyme activity in biocatalysis with non-polar hydrophobic solvents often providing higher reaction rates than more polar, hydrophilic solvents (Adlercreutz, 2008). In

related work, enhancement of catechol-laccase catalysis in less polar organic solvents as compared with highly polar media, due to the hydrophobicity of the end product, has been reported (Ma *et al.*, 2009). These results further show that the enzyme employed for this study has the ability to function in a solvent with lower polarity, where the essential water layer bound around the enzyme active site has not been stripped away (Kermasha *et al.*, 2001).

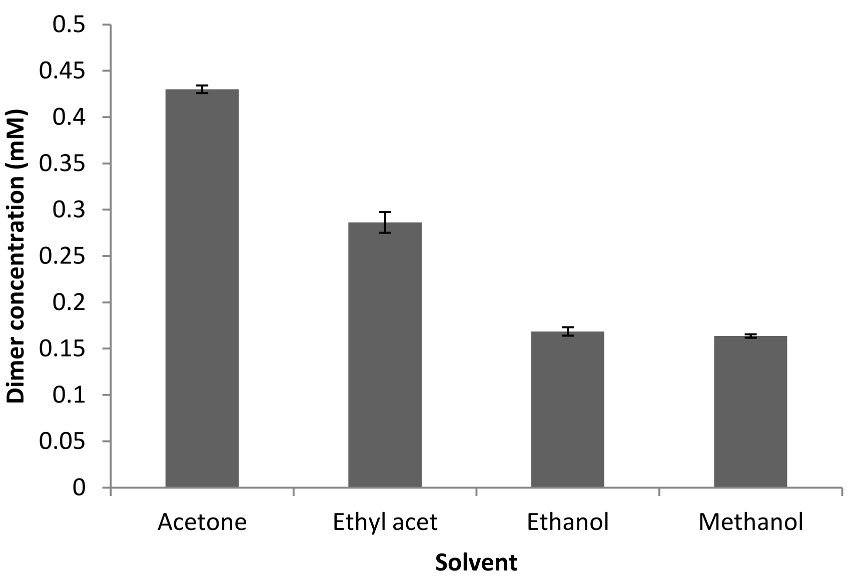


Fig. 5.8: Effect of organic co-solvent on laccase-catalyzed oxidation of 2,6-DMP to form a dimer. All results are means ± SD of three replicate determinations.

5.3.2 Antioxidant activity determination

Many spectrophotometric assays are currently employed to measure the antioxidant capacity of biological samples, the most popular are 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, and the FRAP (ferric reducing antioxidant power) assays (Floegel *et al.,* 2011). Specifically, the ABTS assay is based on the generation of a blue/green $ABTS^{\cdot+}$ that can be reduced by antioxidants; the DPPH assay is based on the reduction of the purple $DPPH^{\cdot}$ to 1,1-diphenyl-2-picryl hydrazine whereas, the FRAP assay is different from the the previous two as there are no free radicals involved, but rather involves the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). These assays are quick and do not require sophisticated equipment, such as fluorescence detectors or a GC-MS (gas chromatograph-mass spectrometer), which make these assays suitable for the analyses of multiple tissue samples. Therefore antioxidant activities of the synthesised product and the substrate were evaluated using these three methods.

Interestingly, the DPPH scavenging activity, TEAC and FRAP analyses of the products showed that the dimer exhibited higher antioxidant activity than the substrate (Table 5.1). The dimer showed 119.32, 53.15 and 93.25% increase in antioxidant activity for FRAP, TEAC and DPPH, respectively, when compared to the starting substrate. The calibration curve (Fig. 5.9) revealed a highly positive linear (R^2 = 0.9882) relation between the mean FRAP value and the concentration of ascorbic acid standards. This curve was used to estimate antioxidant potential of the test samples. The increase in antioxidant capacity of the dimer could be attributed to an increase in electron donating groups after dimerization (Matsuura and Ohkatsu, 2000) which tends to reduce the O-H bond dissociation energy and favour the resonance delocalisation of the phenoxyl radical (Sanchez-Moreno *et al.*, 1998)

Table 5.1: Antioxidant activity of 2,6-DMP and the dimer produced in this study.

Molecule	**Molecular weight**	**EC_{50} DPPH[a] (mM)**	**TEAC[b] value (mM TEAC eqv)**	**FRAP[c] value**
2,6-DMP	154.18	0.802 ± 0.005	1.095 ± 0.006	1.242 ± 0.005
2,6-DMP dimer	306.11	0.415 ± 0.012	1.677 ± 0.011	2.724 ± 0.045

[a]EC_{50} parameter is defined as the concentration (mM) of substrate that brings about 50 % loss of the DPPH• (Yu *et al.,* 2002). Values are means ± SD of three replicate determinations.
[b]The Trolox equivalent antioxidant activity (TEAC) of the antioxidant is defined as the concentration of Trolox solution (mM) with an equivalent antioxidant potential to 1.0 mM solution of the substance under investigation (Sanshez-Moreno, 2002).
[c]The FRAP (ferric reducing antioxidant power) of the sample is the concentration of ascorbic solution (mM) having the ability to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) with an equivalent antioxidant potential to 1.0 mM solution of the sample under investigation (Benzie and Strain, 1996).

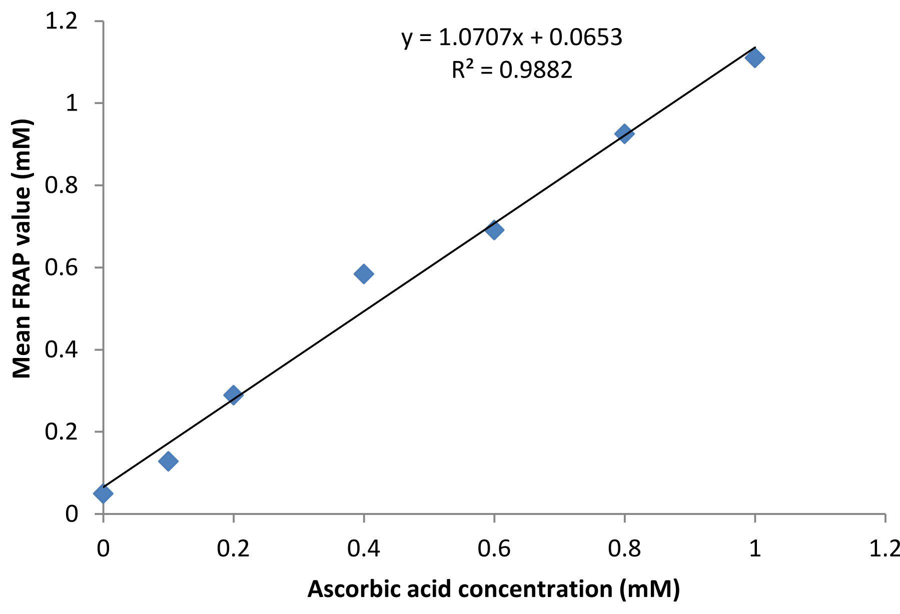


Fig. 5.9: A representative calibration curve for the determination of FRAP value using ascorbic acid standards (0.1, 0.2, 0.4, 0.6, 0.8, 1.0 mM). y = 1.0707x + 0.0653, R^2 = 0.9882.

5.4 Conclusion

This study characterized the product from laccase-mediated oxidation of 2,6-DMP and tested its antioxidant capacity. Our work showed that a dimeric compound (3,3’,5,5’-tetramethoxy biphenyl-4,4’-diol) was synthesized which has a higher antioxidant capacity than the substrate as demonstrated by standard antioxidant assays (DPPH, FRAP and TEAC). As antioxidants continue to have value as nutraceuticals and /or cosmeceuticals, this compound can find useful application in such fields.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 General conclusions

Prevention of oxidative reactions in foods and protection of biological tissues against damage to molecular targets such as proteins, lipids, carbohydrates and DNA can be achieved by various synthetic or natural antioxidants (Locatelli *et al.*, 2009). Due to the potential health hazard associated with the use of synthetic antioxidants, natural phenolic compounds are attracting much attention due to their antioxidant capacity and potential health benefits (Penarrieta *et al.*, 2011). The major target substrates in this study included ferulic acid, 2,6-dimethoxyphenol (2,6-DMP), quercetin and catechol. These compounds are reported to have antioxidant activity (Srinivasan *et al.*, 2007; Erlund, 2004; Kjallstrand and Petersson, 2001).

Ferulic acid in particular is widely distributed in cereals, fruits and vegetables (Ferreira *et al.*, 2007). Although it is high in antioxidant capacity due to its important structural properties (Srinivasan *et al.*, 2007), antioxidant activity is generally low when compared to conventional antioxidants and other hydroxyl-cinnamic acids (Kikuzaki *et al.*, 2002). Also, 2,6–DMP is the predominant smoke component of thermal degradation products from hardwood and is of great importance for the smoke flavour due to the preserving antioxidant effect. Although some products of laccase oxidation of 2,6-DMP have previously been characterised, their application as antioxidants has not been documented. Quercetin on the other hand, is regularly consumed by humans: it is found in various fruits, vegetables or herbal medicines, e.g. apples, onions and sophora flowers (Wang *et al.*, 2011). When orally administered, quercetin is poorly absorbed, and its bioavailability when administered in capsule form is reported to be less than 1% (Erlund, 2004). Several trials have been conducted to increase the bioavailability of quercetin by optimizing its formulation (Wang *et al.*, 2011) and therefore, modification of this compound via coupling with other phenolic compounds by the use of laccase could be an important step to improve its bioavailability.

This project, however, further took advantage of the oxidative catalytic ability of the enzyme, laccase, in generating dimers and polymers during the biotransformation of phenolic substrates to produce value-added antioxidants from simple plant-derived phenolic compounds.

The specific objectives of this study were:

- Production of laccase by microbial strain of *Trametes pubescens*.
- Purification of laccase and formulation of the enzyme as a stable, active biocatalyst.
- Investigate the potential of laccase to catalyze the conversion of various plant–derived substrates, including ferulic acid.
- An analysis of the products using techniques such as High Performance Liquid Chromatography (HPLC), Thin Layer Chromatography (TLC), Liquid Chromatography-Mass Spectroscopy (LC-MS) and Nuclear Magnetic Resonance (NMR).
- Determination of the antioxidant activities of products using selected spectrophotometric methods, and to compare their antioxidant activities to that of the substrates.

These objectives were accomplished. The synthesis of compounds with high bioactive activities, from selected phenolic compounds with the use of laccase as a biocatalyst were explained in this study and the major findings of this work are summarized as follows:

i. Production of laccase from free cells of *T. pubescens* was achieved using an airlift reactor, reaching an average activity of 5.3 U/ml on the ninth day of cultivation.

ii. Two dimeric products (*m/z* 385.1) of laccase–catalyzed oxidation of ferulic acid were purified and characterized as β-5 and β-β dimers. In the monophasic system, four co-solvents namely acetone, dioxane, ethanol and methanol were investigated and the type of organic co-solvent used was found to influence the dimer formed. The β-5 dimer was preferentially formed in dioxane while the β-β dimer formation was enriched in ethanol. In the biphasic system, there was an increase in the formation of the dimers as the concentration of ethyl acetate was increased from 80 to 95%. Inactivation of the enzyme in some of the miscible solvents was observed over time. For example, only 0.12% or 2.8% residual activity was observed after three hours when methanol or dioxane was used, respectively, which explains the poor substrate conversion in these solvents. In contrast, stability of the enzyme was observed in ethyl acetate which probably explains the higher conversion of substrate to products. The β-5 dimer produced from the oxidation of ferulic acid, when tested for antioxidant activity, was found

to have higher activity than the *β-β* dimer and also higher antioxidant activity than that of its parent compound.

iii. When laccase from *T. pubescens* was used to catalyze the modification of 2,6-DMP in a monophasic or biphasic system, as a way of enhancing its antioxidant capacity, one dimeric product (*m/z* 305.1) was purified and characterized as 3,3'5,5'-tetramethoxydiphenolquinone. In the monophasic system, the dimer was preferentially formed in acetone as co-solvent while in the biphasic system, formation of the dimer increased as the concentration of ethyl acetate was increased from 50 to 90%. This dimer of 2,6-DMP (3,3'5,5'-tetramethoxydiphenolquinone) was observed to show higher antioxidant capacity than the starting substrate.

iv. The possibility of coupling quercetin or catechin (both known and active antioxidants) with other phenolic compounds as a way of increasing antioxidant activity was investigated. Coupling of catechin or quercetin with other phenolic acids was successful although in very small quantities. Purification of a coupling product between quercetin and catechol (m/z 409.1) was, however, achieved. It was also established from this study that alteration of reaction conditions influence the formation of the quercetin/catechol coupling product. The quercetin/catechol coupling product showed a higher antioxidant capacity than catechol but a slightly lower capacity than quercetin. However, this coupling product showed an increased solubility when compared to quercetin and may offer an alternative delivery system for this antioxidant.

v. All products obtained from this study which show high antioxidant capacity could find useful application in the health and cosmetic industries. Although the biocatalytic processes employed in this study require time (mostly 24 hours reaction for dimeric products and 5 hours reaction for coupling products), and modest increases in antioxidant activity of the products as compared to the starting materials was observed, but, the exploration of "green" ways of enhancing these bioactive properties over chemical treatment will be benefial to health.

6.2 Major findings and general discussions

In this study, selected phenolic compounds were subjected to small scale oxidation reactions (through the use of a fungal laccase) and the products analyzed by TLC, HPLC and LC–MS. Scaled-up production, optimization, purification and application of

products of interest (dimers and coupling products) in terms of antioxidant capacity were further investigated and explained in the course of this study. The nature of the products characterized from laccase-mediated reactions is summarized in Table 6.1.

Table 6.1: Summary of laccase reaction products produced in this study

Substrates	Products
Ferulic acid	The major product was a *β* – *β* dimer of two ferulic acid monomers with molecular weight of 385.1 *m/z*. The minor product was a *β*-5 dimeric monomer of ferulic acid with molecular weight of 385.1 *m/z*. Another product with molecular weight of 579.2 *m/z* suggest oligomerization to form a trimer
2,6-dimethoxyphenol (2,6-DMP)	One dimeric product (*m/z* 305.1) was purified and characterized as 3,3'5,5'-tetramethoxy biphenyl-4,4'-diol
Quercetin, catechol	Coupling product between quercetin and catechol (m/z 409.1) was purified and identified as an ether linked coupling product.
Quercetin with selected phenolic compounds	LC-MS of selected phenolic compound onto quercetin showed dominant signals at *m/z* 467.0 (gallic acid); *m/z* 467.1 (vanillic acid); *m/z* 479.1.0 (caffeic acid); *m/z* 423.1 (guaiacol); *m/z* 453.1 (2,6-dimethoxyphenol); *m/z* 425.1 (pyrogallol); *m/z* 451.0 (vanillin); *m/z* 463.1 (eugenol); *m/z* 463.1 (isoeugenol); *m/z* 453.1 (protocatechuic acid)
Catechin with selected phenolic compounds	LC-MS of selected phenolic compound onto catechin showed dominant signals at *m/z* 577.2 (catechin); *m/z* 457.1 (gallic acid); *m/z* 455.1 (vanillic acid); *m/z* 467.1 (caffeic acid); *m/z* 411.1 (guaiacol); *m/z* 441.1 (2,6-dimethoxyphenol); *m/z* 397.1 (catechol); *m/z* 451.1 (eugenol)

The two products that were isolated and characterized from the reaction of ferulic acid with laccase are the *β*-5 and *β–β* dimers of ferulic acid; the yield of which were 11.190 and 38.189%, respectively after purification. The possible reaction pathway for the dimerization of ferulic acid to form a *β*-5 and *β*-*β* linkage according to Carunchio *et al.* (2001) is that laccase initiates the reaction by oxidizing the para-hydroxyl group resulting in the formation of a radical. These radicals are then stabilized through coupling with other radicals to form dimers.

Similarly, in the reaction of 2,6-DMP with laccase, it went through a single-electron-oxidation by *T. pubescens* laccase catalysis to produce 2,6-DMP radical species that

resonance with corresponding para-radical species. A recombination of 2 moles of this para-radical species then produced the product (3,3'5,5'-tetramethoxydiphenolquinone) which is a dimeric product of 2,6-DMP (*m/z* 305.1).

The coupling of catechin or quercetin with various monomeric phenolic compounds together with the antioxidant activity of some of the products from the coupling reactions, is reported in this study. Coupling of various phenolic molecules (gallic acid, vanillic acid, ferulic acid, guaiacol, pyrogallol, 2,6-dimethoxyphenol, catechol, vanillin, eugenol, isoeugenol, protocatechuic, naringin, phloridzin) onto catechin or quercetin by laccase is envisaged to be initiated by abstracting one electron from the hydroxyl group of catechin or quercetin and the phenolic compound after forming radicals (Nugroho Prasetyo *et al.,* 2011). Although, the laccase-catalysed coupling of catechin or quercetin with many of the phenolic compounds used was successfully in most cases (as revealed by LC-MS analysis), only the product of catechol onto quercetin with *m/z* 409.1 was recovered after purification during scale–up reactions and this was shown to couple via the ether linkage when analysed by NMR. The structures of other coupling products were elucidated based on the report of Widsten *et al*. (2010) which suggested that, since quercetin and catechin have their 5th position free on the B-ring and according to existing knowledge, the hydroxyl group on the benzene ring is *ortho* or *para* directing and molecules with a free C-5 position usually cross-couple through 5–5 linkages due to stability of C–C bonds (Schultz *et al*., 2001; Jonas *et al.,* 2000), this should promote direct coupling, establishing C–C coupling products. On this basis the proposed structures of coupling products were established.

It was also established in this study that the reaction medium had effect on the reaction products especially the use of organic solvents as co-solvents. In the biphasic system (ethyl acetate as co-solvent) most of the substrate was converted to products and there was an increase in the formation of the *β–β* dimer of ferulic acid as the concentration of ethyl acetate was increased from 80 to 90% after which a decline in product formation was observed. Also, the formation of the *β*-5 dimer was enhanced by increase concentration of organic solvent and a decline was observed after the composition of the organic solvent was increased beyond 95%. In the monophasic system, four co-solvents namely acetone, dioxane, ethanol and methanol were investigated and it was revealed that the order of substrate conversion was ethanol > acetone > dioxane > methanol. However, for product formation, the *β-β* dimer formation was facilitated by the use of ethanol as co-solvent. Furthermore, in the production of the dimer from 2,6-DMP oxidation, there was an increase in the

formation of the product as the concentration of ethyl acetate was increased from 50 to 90% after which a decline in product formation was observed. Coupling reactions conducted in the water-miscible solvents (ethanol, methanol, acetone, dioxane) produced higher concentrations of coupling product as compared to the reactions carried out in the water-immiscible solvents (e.g. ethyl acetate). Biocatalysis in the presence of organic solvents already has proven to be capable of synthesizing novel compounds and opening up new synthetic pathways. Generally, organic solvents have a lot of advantages when employed in biocatalysis, such as higher solubility of hydrophobic species, reduction of water activity, reduction of microbial contamination and incidence of side reactions found in water, aids separations and improvement of yields (Zaks, 2001).

Antioxidant activities of the synthesised products were evaluated in relation to that of the substrates. The DPPH scavenging effect and Trolox equivalent antioxidant capacity (TEAC) of the products showed that the β-5 dimer exhibited higher antioxidant activity than ferulic acid, however, the β-β dimer showed lower antioxidant activity than ferulic acid. The increase in antioxidant capacity of the β-5 dimer could be attributed to an increase in electron donating groups after dimerization (Matsuura and Ohkatsu, 2000) and the carboxylic acid group with an adjacent unsaturated C-C double bond, which can provide additional attack sites for free radicals (Srinivasan and Sudheer, 2007). In the β-β dimer, the unsaturated C-C bonds and carboxyl groups are lost during the dimerization process which could explain the reduction in antioxidant capacity. The dimer of 2,6-DMP on the other hand, showed a 93.25, 34.70 and 54.41% increase in antioxidant activity for FRAP, TEAC and DPPH, respectively, when compared to the starting substrate. On the contrary, the coupling product of quercetin and catechol exhibited lower antioxidant capacity than quercetin but higher antioxidant capacity than catechol. However, it was observed that the solubility of the newly coupled product is greatly improved. Coupling may also improve other physiological properties as evidenced by the increase in solubility, which might enhance bioavailability.

6.3 Recommendations

This study has demonstrated the biocatalytic production of novel products with potentially higher antioxidant capacity than the starting materials. Future work should include investigating the practical applications of these products in cosmetics, the health industry and as potential nutraceuticals. In addition to this, the biocatalytic processes developed in this study to synthesize dimeric and coupling products should be investigated further. It was observed that the inactivation of laccase activity in the

process of synthesizing β-β and β-5 dimers of ferulic acid occurred. Future investigation should be focused on minimizing the loss of enzyme activity especially during the formation of the β-5 dimer due to its importance in terms of higher antioxidant capacity. Immobilization, which assists in retaining the activity of enzymes under adverse reaction conditions, can be explored.

In addition, future studies should also focus on resolving racemic mixtures of products of dimerisation of ferulic acid into optically pure compounds since diastereomers have different biological activity.

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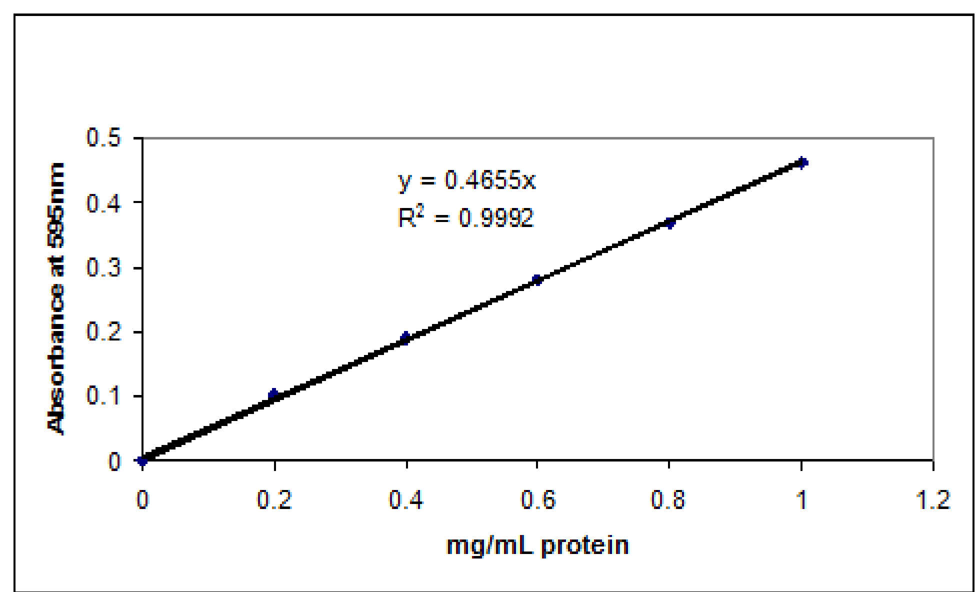
APPENDICES

APPENDIX A: *Trametes* Defined Medium

2 L *Trametes* defined medium (TDM)		500 ml 100X Trace Elements	
Glucose	20 g	Iron sulphate	0.28 g
Peptone	10.46 g	Copper sulphate	0.016 g
KH_2PO_4	4 g	Zinc chloride	0.034 g
$MgSO_4$	1.0 g	Manganese sulphate	0.169 g
$CaCl_2$	0.2 g	Cobalt chloride	0.095 g
NaCl	0.58 g	Nickel chloride	0.0012 g
Trace elements	20 ml	Ammonium molybdate	0.309 g

APPENDIX B: Standard curve for protein determination



Standard curve for protein determination.

APPENDIX C: SDS-PAGE Buffers and SDS-PAGE components

Stacking gel buffer (6.8)

9.08 g Tris

0.6 g SDS

Water (200 ml)

Running gel buffer (pH 8.8)

27.25 g Tris

0.6 g SDS

Water (200 ml)

10x Running gel (pH 8.3)

15.14 g Tris

72.06 g Glycine

5 g SDS

Water (500 ml)

Acrylamide

30 g acrylamide

0.8 g bis-acrylamide

Water (100 ml)

Cover with foil and store at 4°C.

5X Sample buffer (15 ml)

1.5 g SDS

3.75 ml 1M Tris HCl pH 6.8

0.015 g bromophenol blue

0.5 ml 2-mercaptoethanol or 1.16 g DTT

7.5 ml glycerol

1M Tris buffer

1.2114 g in 10 ml

pH with HCl to 6.8

Ammonium persulphate (Amps) – 0.1 g in 1 ml make fresh on day of use

Running gel

4.2 ml water

3.3 ml running gel buffer

2.5 ml Acrylamide

7 µl Temed

100 µl Amps

Stacking Gel

5.27 ml

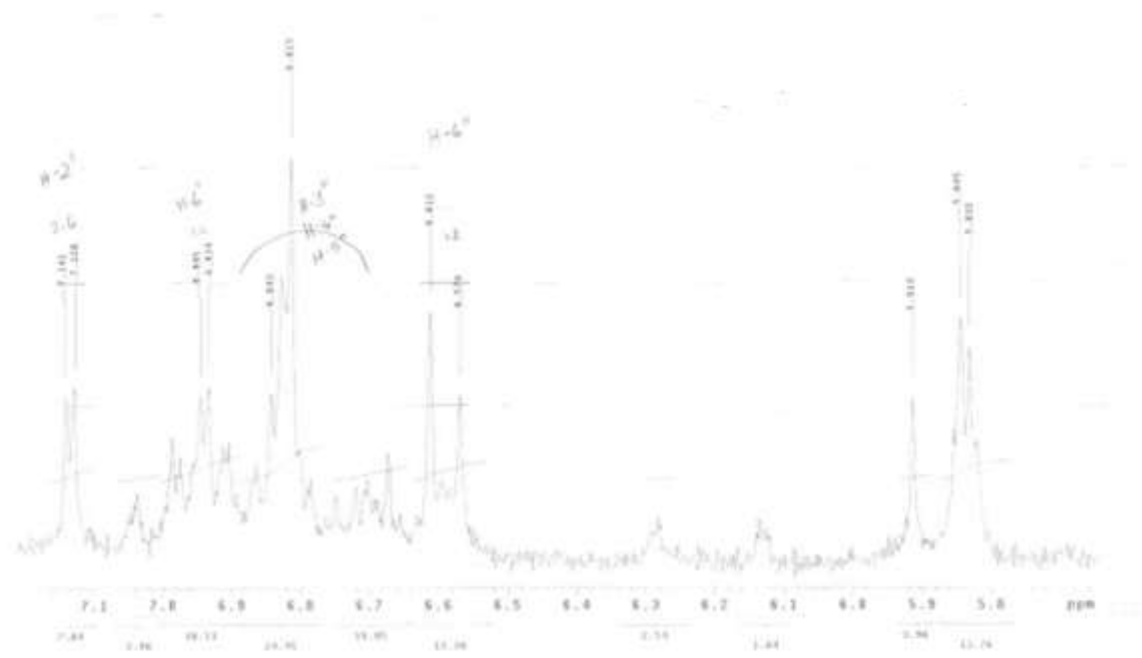
3.3 ml stacking gel buffer

1.1 ml acrylamide

20 µl Temed

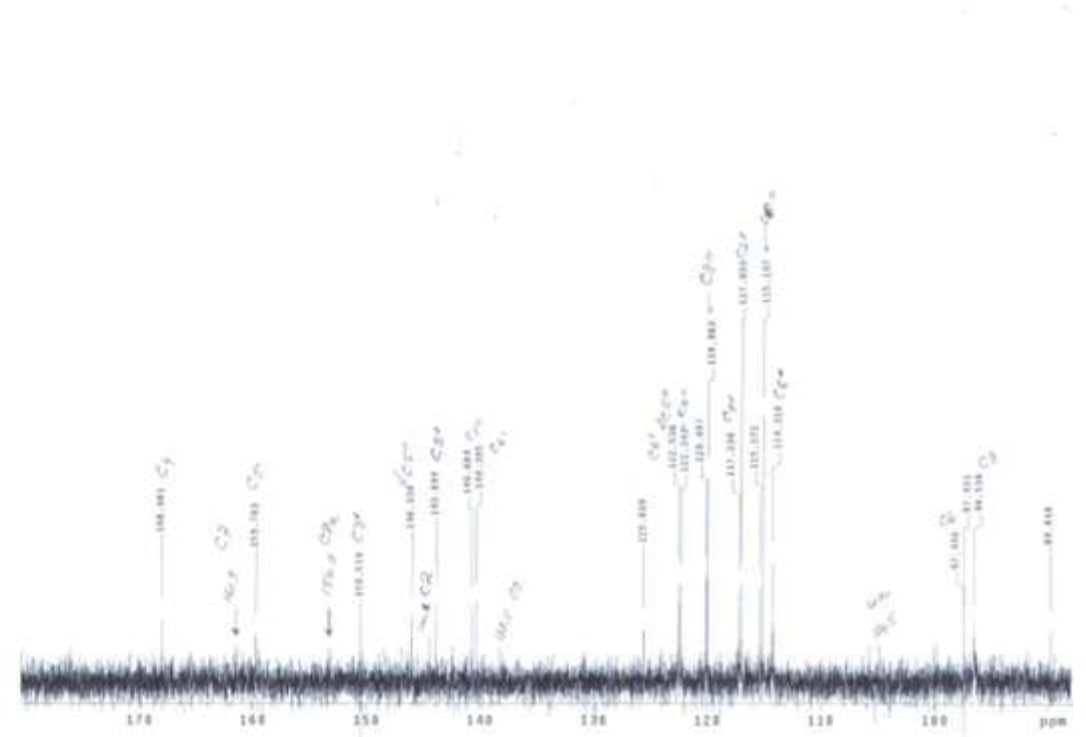
300 µl Amps

APPENDIX D: ^{1}H NMR of the coupling product between catechol and quercetin



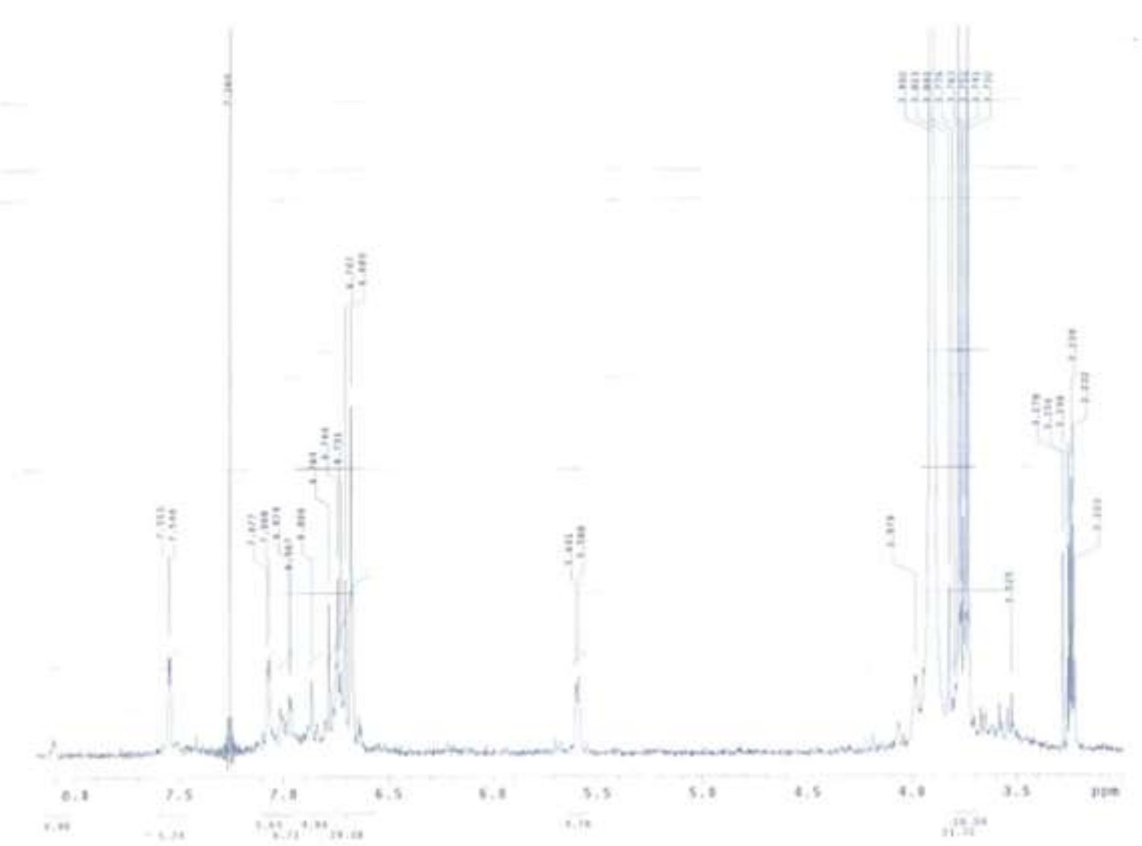
^{1}H NMR spectrum of the product formed during laccase-mediated coupling of catechol onto quercetin.

APPENDIX E: ^{13}C NMR of the coupling product between catechol and quercetin



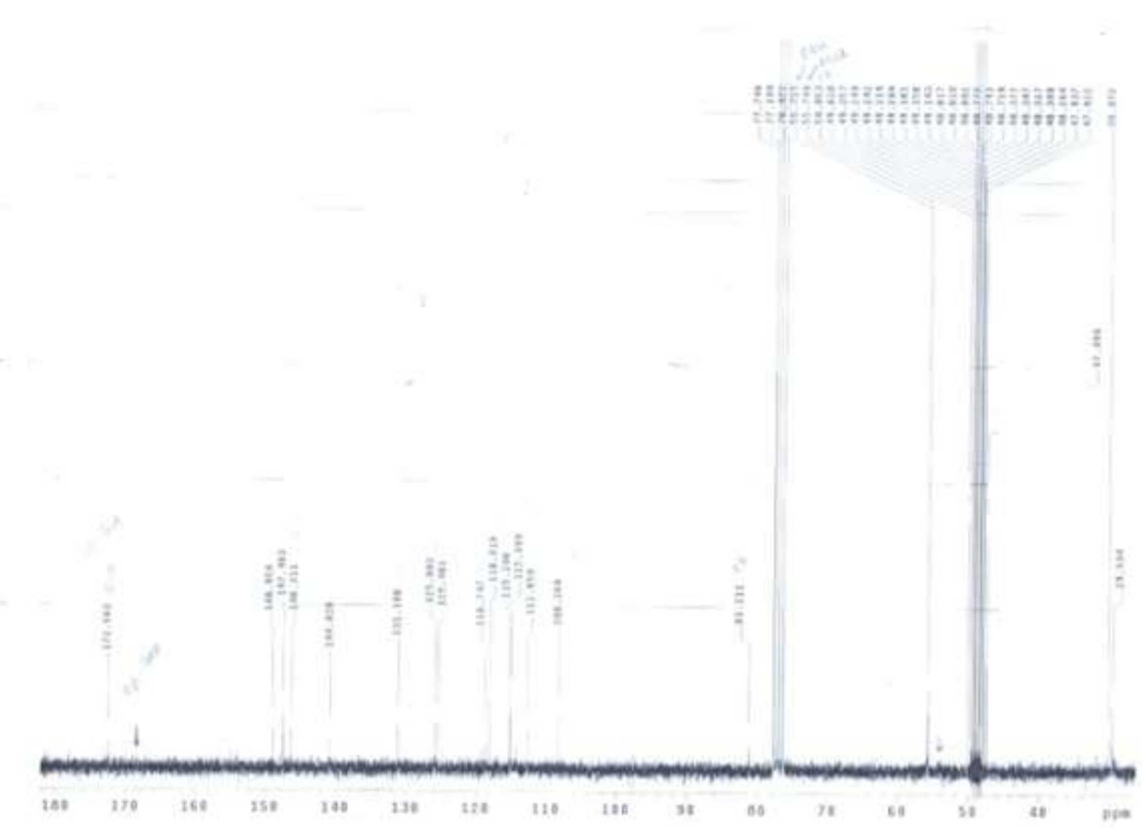
^{13}C NMR spectrum of the product formed during laccase-mediated coupling of catechol onto quercetin.

APPENDIX F: ^{1}H NMR of ferulic acid *β*-5 dimer



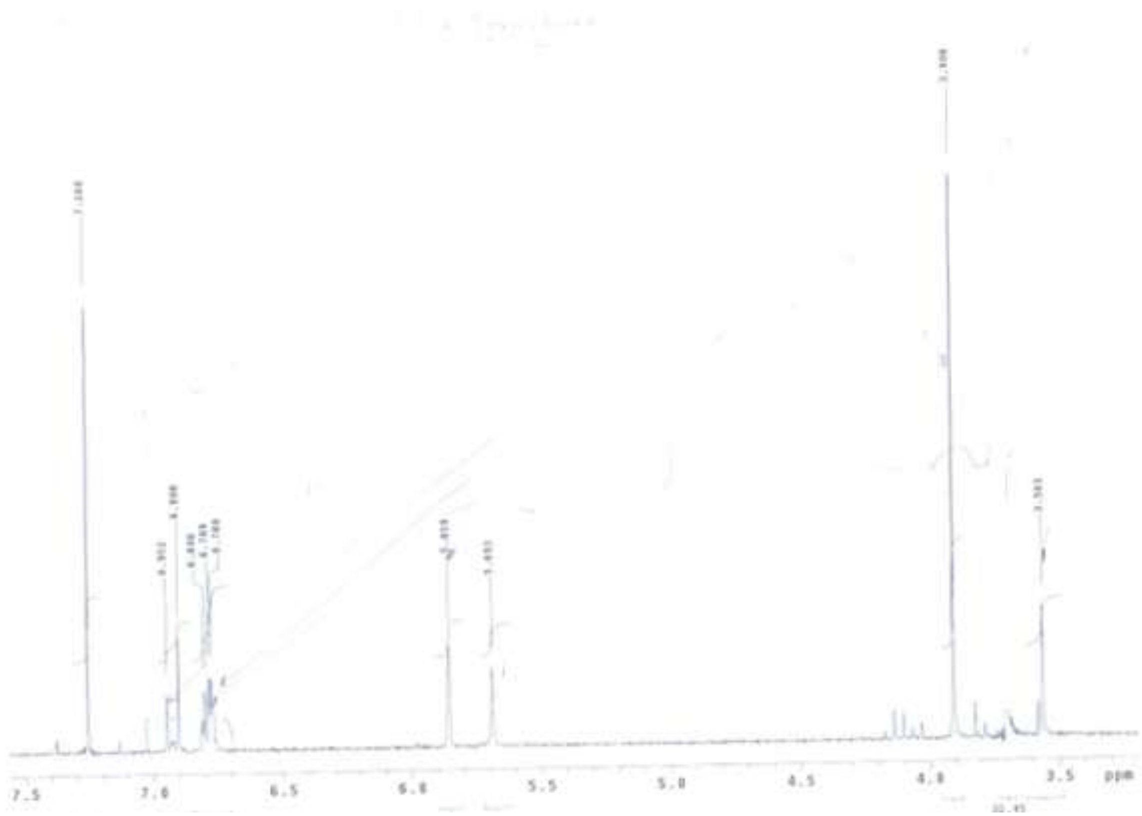
^{1}H NMR spectrum of the product (*β*-5 dimer) formed during laccase-mediated oxidation of ferulic acid

APPENDIX G: ^{13}C NMR of ferulic acid *β*-5 dimer



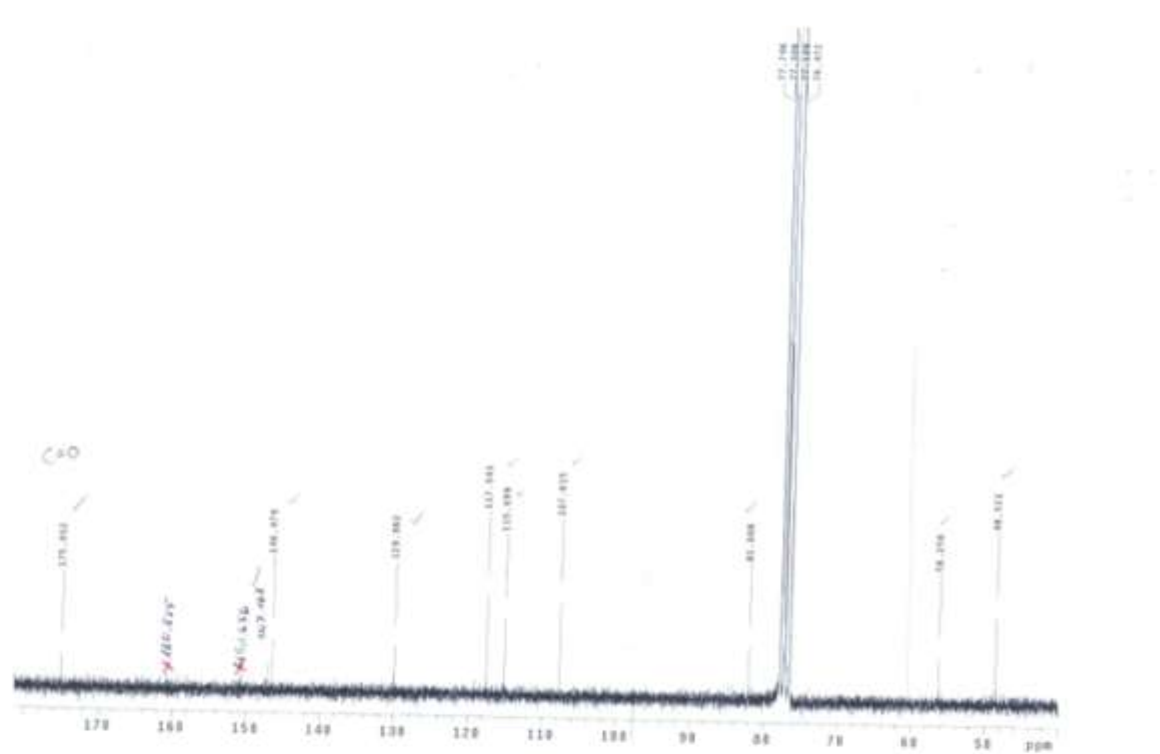
^{13}C NMR spectrum of the product (*β*-5 dimer) formed during laccase-mediated oxidation of ferulic acid

APPENDIX H: ^{1}H NMR of ferulic acid *β*- *β* dimer



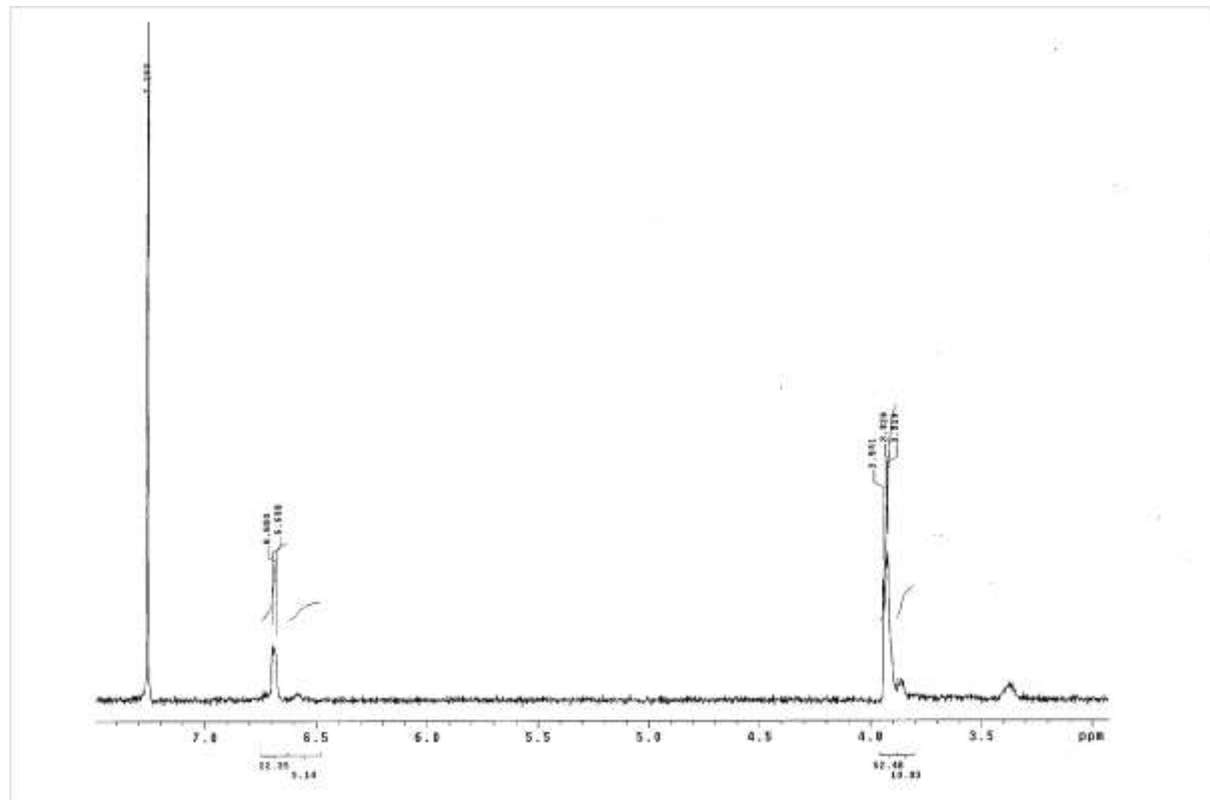
^{1}H NMR spectrum of the product (*β*-*β* dimer) formed during laccase-mediated oxidation of ferulic acid.

APPENDIX I: ^{13}C NMR of ferulic acid *β*- *β* dimer



^{13}C NMR spectrum of the product (*β*- *β* dimer) formed during laccase-mediated oxidation of ferulic acid

APPENDIX J: ^{1}H NMR of the 2,6-dimethoxyphenol dimer



^{1}H NMR spectrum of the product formed during laccase-mediated oxidation of 2,6-dimethoxyphenol.