Laccase-mediated biotransformation of phenolic compounds for the synthesis of new antioxidants

Submitted in fulfillment for the Masters of Applied Science (Biotechnology) degree in the Department of Biotechnology and Food Technology,

Durban University of Technology, Durban, South Africa

Bodine Mazibuko

21201007

MAppSci: Biotechnology

2020

Promoter/Supervisor: Professor Tukayi Kudanga

DECLARATION

I hereby declare that the work reported in this thesis and submitted at the Department of Biotechnology and Food Technology at Durban University of Technology for a Master's Degree is my original work. I confirm that it has not been previously submitted for a degree at any Higher Education Learning Institution.

Bodine Mazibuko Date

Student

As the candidate's supervisor I agree to the submission of this thesis

Prof Tukayi Kudanga Date

DEDICATION

The thesis is dedicated to my husband Fundiswa Lwazi Mazibuko and family, who have supported me throughout this journey.

ACKNOWLEDGEMENTS

I am grateful to the Lord Jesus for His sufficient grace and strength. I also acknowledge my supervisor Prof Kudanga for his assistance and guidance throughout the program. And thank you for believing in me and having so much patience with me throughout this journey. Special acknowledgements to the National Research Foundation (NRF) for their financial support. I also appreciate all the staff of the Department of Biotechnology and Food Technology. Everyone within the Enzyme Technology group is really appreciated for their contribution towards the success and completion of my work. These include my colleagues and friends; Sandile Ngubane, Blessing Nemadziva, Christiana Aruwa and Deepti Yadav. Many thanks to my husband for the support and being a pillar of strength. My siblings, my parents and family, thank you very much for believing in me. Lastly, I appreciate all those whose names have not been mentioned but contributed to the success of my program.

SUBMITTED MANUSCRIPT

Bodine Mazibuko, Neil Koorbanally, Tukayi Kudanga. 2020. Laccase-catalysed dimerization of eugenol improves antioxidant capacity, *Journal of Applied Microbiology and Biotechnology*, submitted.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ABTS: 2,2'-Azinobis 3-ethylbenzthiazoline-6-sulfonic acid

DPPH: 2,2-diphenyl-picryhydrazyl

FRAP: ferric-reducing antioxidant power

HPLC: high-performance liquid chromatography

LC−MS: liquid chromatography−mass spectrophotometry

NCDs: non-communicable diseases

NMR: nuclear magnetic resonance

TLC: thin-layer chromatography

ABSTRACT

The increased incidences, mortality rate and economic impact of noncommunicable diseases (e.g. high blood pressure and diabetes) associated with oxidative stress, have led to the higher demand for antioxidant supplements for their prevention. The use of naturally occurring antioxidants is becoming a more attractive option due to the health risks associated with synthetic antioxidants. Phenolic compounds from plants have been shown to have antioxidant properties with the potential to be used as substitutes to synthetic antioxidants. However, monomeric phenolic compounds have several short comings such as low bioavailability, poor solubility, and low antioxidant capacity while some have pro-oxidant properties at high concentrations. Hence there has been increasing research focused on the biotransformation of these phenolic antioxidants through enzymatic oligomerisation to higher molecular weight compounds with improved antioxidant capacity and stability.

Of the investigated enzymes, laccases have shown the most promise owing to their green catalytic properties. Their reaction mechanism involves the use of molecular oxygen as a co-substrate in oxidising phenolic compounds to corresponding radicals, with water as the only by-product. This study focused on the synthesis of antioxidants with enhanced antioxidant capacity using a laccase from *Trametes pubescens* as biocatalyst. To establish the potential of the phenolic compounds for use as substrates for the coupling reactions, a preliminary screening process was done. Guaiacol, caffeic acid, vanillic acid, eugenol, catechol, gallic acid, ferulic acid and quercetin hydrate were identified as suitable substrates for the laccase enzyme. However, only products from eugenol, coumaric acid and quercetin could be isolated, hence coupling reactions were carried out using these substrates in monophasic systems. Reaction products were monitored using thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Purification was carried out using preparative TLC and characterisation using liquid

chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR). The antioxidant capacities of reaction products were determined using ABTS (2,2'-Azinobis 3-ethylbenzthiazoline-6-sulfonic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (ferric-reducing antioxidant power) assays.

Quercetin hydrate oxidation produced one product which was purified and characterised. The product had an R_f of 0.68, t_R 13.567 and *m/z* 601 in negative mode, indicating that it was a dimeric form of quercetin. Oxidation of ρ-coumaric acid resulted in the production of two products designated P1 (R_f 0.47) and P2 (R_f 0.42). Further characterisation was done on product P2 since product P1 could not be successfully purified. P2 had a retention time of 11.295 and *m/z* 325, indicating that it was a dimer of ρ-coumaric. The ρ-coumaric dimer had an enhanced antioxidant capacity, approximately 2-fold, 3-fold and 6-fold higher compared to the substrate, as demonstrated by the ABTS, DPPH and FRAP assays, respectively.

A symmetrical 5-5 eugenol dimer (*m/z* 325, [M] =326), *bis*-eugenol, was produced from eugenol oxidation. Maximum product formation (50% yield) was obtained in a monophasic system with 40% v/v dioxane as co-solvent after incubation for 18 h. The bis- eugenol dimer had an improved antioxidant capacity of up to three and four times that of eugenol as demonstrated by the ABTS and DPPH assays, respectively.

In conclusion, two dimers with high antioxidant capacity were successfully produced, purified and characterised. The study has demonstrated the potential of the *T. pubescens* laccase as a catalyst for the synthesis of phenolic compounds with enhanced antioxidant capacity.

1. INTRODUCTION

The increase in oxidative stress and reduced antioxidant defences has been linked to the initiation and development of chronic diseases such as diabetes, cardiovascular disease, high blood pressure, and some forms of cancer (Pisoschi and Pop, 2015). It has also been established that, free radical cell damage due to oxidative stress, can contribute to several neurodegenerative conditions such as Parkinson, Huntington's disease and Alzheimer's disease (Lopez and Denicola, 2013). Oxidative stress is a result of the increase in reactive free radicals and the decrease in antioxidant defences in the human body.

Oxidative stress is as a result of the increase in reactive free radicals and decrease in antioxidant defences in the human body. Globally and in South Africa, non-communicable diseases (NCDs) are the principle causes of mortality (Pillay-van Wyk et al., 2016). In South Africa, diabetes and high blood pressure are the leading NCDs that have been associated with oxidative stress. According to the South African Demographic and Health Survey 2016, 46% of women and 44% of men from the age of 15 and above have high blood pressure. The national diabetes incidence in South Africa in 2016 was 2.5 cases per 1000 total population (National Department of Health, 2017). The economic impact of NCDs on the society, health system and individuals are very significant, hence the need to have national and locally suitable solutions to reduce their effect.

There has been therefore an increased demand for antioxidant supplements for the prevention of NCDs. However, in some cases antioxidant supplements at high dosages may be damaging. For example, the use of high-dose beta-carotene supplements which has been associated with increased risks of lung cancer in smokers (Tanvetyanon and Bepler, 2008). High doses of a vitamin E antioxidant supplement has been linked with the increase in possibility for haemorrhagic stroke as it interacts with anticoagulants and intensifies the risk of bleeding in

patients (National Institute of Health, 2013). Consequently, phenolics with high antioxidant potential have gained considerable interest in recent years.

Phenolic compounds from plants, proven to have antioxidant capacity can be considered as a decent alternative to synthetic antioxidants which are partaken under strict guidelines because they are a potential health hazard (Tripathi et al., 2007). However, most monomeric phenolic compounds have several disadvantages such as low antioxidant capacity, poor solubility and short half-life in the body, while some have pro-oxidant properties (Kurisawa et al., 2003). Compared to monomeric antioxidants, high molecular weight phenolic compounds have higher antioxidant activity, no pro-oxidant effects and a longer lifespan in vivo (Li and Xie, 2000). The use of these potent antioxidants can potentially reduce the need for dietary supplementary antioxidants.

Biotransformation of potent phenolic antioxidants can be conducted through enzymatic catalysis. Therefore, the use of enzymes for the synthesis of potent antioxidants from monomeric phenolic antioxidants and similar compounds, is attracting a lot of research interest. In this sense, the enzyme laccase has been of interest mainly because of its green catalytic properties. Laccases require only molecular oxygen as a co-substrate in the oxidation of phenolics and similar compounds to corresponding radicals, and water is the only by-product. Through subsequent coupling of the radicals, phenolic antioxidants with enhanced activity can theoretically be produced. Several studies have reported enhancement of antioxidant activity through laccase-mediated oligomerisation of monomeric phenols notably ferulic acid (Adelakun et al., 2012a), silybin (Gavezzotti et al., 2014), 2, 6 dimethoxyphenol (Adelakun et al., 2012b), hydroxytyrosol (Zwane et al., 2012) and rutin (Uzan et al., 2011). The enhanced antioxidant activity is based on the principle that oligomerisation supplements the product with hydroxyl groups as an outcome of the repeated monomeric units (Wolfe and Liu, 2008). Improvement in antioxidant activity can

also be due to the increase in electron donating groups after oligomerisation. A comprehensive review of compounds modified for improving bioactivity has been recently published (Kudanga et al., 2017). However, due to the wide structural variety of phenolics, there is scope for wider investigation of the potential of this approach on other phenolic substrates. In this study, the enzyme laccase was used to synthesise high molecular weight antioxidants using eugenol, quercetin hydrate and ρ-coumaric acid as substrates and the activities of the synthesised antioxidants were evaluated in comparison with their monomeric forms.

1.1. Hypothesis, Aim and Objectives

1.1.1. Hypothesis

Laccase-catalysed modification of monomeric phenolic compounds will result in the production of new antioxidants with enhanced antioxidant capacity in comparison to the original substrates.

Laccases are known to mediate the synthesis of homo-molecular and hetero-molecular coupling products (Kudanga et al., 2011). For example, laccase catalysis produced ferulic acid and 2,6 DMP dimers with enhanced antioxidant capacity compared to the monomeric building blocks (Kudanga et al., 2017).

1.1.2. Aim

To synthesis antioxidants with enhanced antioxidant capacity using laccase as a biocatalyst

1.1.3. Objectives

- To produce, partially purify and characterise the laccase enzyme from *T. pubescens*.

- To synthesise phenolic antioxidants through laccase-mediated homo-molecular coupling reactions.
- To purify and characterise the structures of the new antioxidants using LC-MS and NMR
- To determine the antioxidant capacity of the synthesised coupling products in comparison with their monomeric units, using standard assays such as DPPH, ABTS, and FRAP.

In Chapter 2 a literature review relevant to this study is provided. Chapters 3 and 4 are the experimental chapters focusing on laccase-mediated oxidation of selected phenolics and the dimerisation of eugenol, respectively. Chapter 5 is a general discussion of the experimental chapters. Chapter 6 concludes the thesis and also provides recommendations for further study.

2. LITERATURE REVIEW

2.1. Introduction

In this chapter, a review of literature relevant to this study is presented. The review focused on the global trends and incidences that have driven the upsurge in antioxidant research; antioxidants – concept, characterisation and therapeutic usage; phenolic compounds – characterisation, sources and applications; laccases – sources, catalytic mechanism and applications, focusing mainly on biocatalysis.

2.2. Oxidative stress – concept and global impact

Oxidative stress has been defined as an imbalance between the number of free radicals produced in the body and the body's ability to neutralize the free radicals through antioxidative protective mechanisms (Persson et al., 2014). Oxidative stress results from the over production of reactive oxygen species and nitrogen species or by the loss of antioxidant protective systems, which can be described as the decreased capacity of antioxidant protective systems to fight against oxidative cell damage. Thus, oxidative stress occurs from the over production of reactive oxygen species and nitrogen species or by the loss of antioxidant protective systems. This loss can be described as the decreased capacity of antioxidant protective systems to fight against oxidative cell damage. Relevant studies have shown that oxidative stress has been linked to over 100 diseases (Halliwell et al., 1992). The increase in oxidative stress in the body has been associated with the initiation of NCDs such as diabetes, cardiovascular diseases, cancer and the aging process (Lopez and Denicola, 2013). Additionally, it has been established that, free radical cell damage due to oxidative stress, can also contribute to several neurodegenerative conditions such as Parkinson, Huntington's disease and Alzheimer (Lopez and Denicola, 2013). Globally cardiovascular diseases (CVD) are the major NCDs that contribute to the high mortality rate associated with oxidative stress. Research has linked CVD to free radicals and the therapeutic

effect of antioxidant nutrients against the free radicals is now well established. For example, oxidative stress is a causative factor for atherosclerosis, which is a complication that leads to CVD (Ozkanlar et al., 2012). However, Stanner et al. (2004) reported the positive effect of antioxidants against reactive oxygen species in averting atherosclerosis, which is a complication that leads to CVD. Antioxidants assist in the prevention of atherosclerosis by inhibiting the oxidation of low-density lipoproteins combined with other defensive mechanisms (Maiolino et al., 2013).

Oxidative stress also plays a significant part in the pathogenesis of complications usually linked with diabetes. Endothelial dysfunction in diabetes mellitus has been reported to be caused by the production of superoxide free radical (Guzik et al., 2002). In the same study, the roles of NAD(P)H oxidases, protein kinase C and nitric oxide synthase as contributors to the production of vascular superoxide in diabetes mellitus were revealed (Guzik et al., 2002). A surge in expression levels of receptors for advanced glycated end products has also been linked to the increased levels of mitochondrial reactive oxygen species. Another study showed that oxidative stress is a major contributor to diabetic nephropathy (Kashihara et al., 2010). Antioxidants have been found to be beneficial against oxidative stress associated with diabetes and subsequently help to lower the hyperglycemic state in diabetes (Bajaj and Khan, 2012). Medicinal plants with high antioxidant content have been applied in the treatment of diabetic complications and thereby providing a low toxicity alternative to the currently available drugs for diabetes (Sankar et al., 2012). The use of a natural plant improved the endogenous antioxidant defense systems, and reduced the levels of reactive oxygen species in rat modules of type II diabetes (Sankar et al.,2012). Subsequently several organs were protected from the oxidative damage that occurs during diabetic conditions. In another study, the overexpression of catalase (an antioxidant enzyme) showed potential as a therapeutic agent against diabetic-induced cardiomyocyte dysfunction. The catalase enzyme had a positive effect in eliminating the high levels of reactive

oxygen species, averting apoptosis as well as reducing the diabetic-induced cellular responses associated with the transcription factor, silent information regulator, forkhead and Akt (Turdi et al., 2007). Kedziora-Kornatowska et al. (2003) reported the potential use of vitamin C and E as antioxidant vitamins. These vitamins were used to act against diabetic nephropathy (a diabetic complication that is directly associated with oxidative stress) in Wistar rat models. The results in the latter study showed that, the kidney glomeruli (where blood is filtered) had reduced levels of malondialdehyde formation and decreased levels of antioxidant enzymes. Therefore, the application of antioxidants for the treatment of oxidative stress associated diseases has been a success in experimental studies.

2.3. Free radicals - characterisation and reaction mechanisms

Free radicals have been defined as molecules or ions that have unpaired electrons on their outer orbitals. They are highly unstable and very reactive towards other molecules. Free radicals are generated from elements such as nitrogen, oxygen and sulphur resulting in reactive nitrogen species (RNS), reactive oxygen species (ROS) as well as reactive sulphur species (RSS). ROS comprises of radicals such as, superoxide anion (O_2), hydroxyl radical (OH), hydroperoxyl radical ($HO_2^{\cdot}$), and nitric oxide (NO). Reactive nitrogen species are a product of the reaction between nitric oxide and oxygen, thereby generating $ONOO^-$, whereas reactive sulphur species are produced by the reaction of reactive oxygen species with a thiol (Lü et al., 2010). The production of ROS is initiated by the breakdown of hydroperoxyl ion to generate the superoxide anion (Figure 2.1). The latter is followed by the interaction of the highly reactive superoxide anion with several molecules to form reactive oxygen species through direct reactions or enzymes or metal-catalysed reactions. Through the Haber-Weiss reaction, superoxide anion can be detoxified or neutralized to hydrogen peroxide by a dismutation reaction catalysed by the superoxide dismutase enzyme and to water by the catalase enzyme (Valko et al., 2006). The hydroxyl radical is formed when hydrogen peroxide reacts with an iron catalyst in a process

known as the Fenton reaction (Flora, 2009). The reaction mechanism for the production of RNS is $NO^{\cdot} + O_2^{-} \rightarrow ONOO^{-}$ (Squadrito and Pryor, 1998). With regards to RSS, these species are derived under an oxidative environment, to generate thiols from a disulphide. Upon further oxidation of the disulphide, intermediates such as disulphide-S-monoxide or dioxide are produced (Giles et al., 2001).

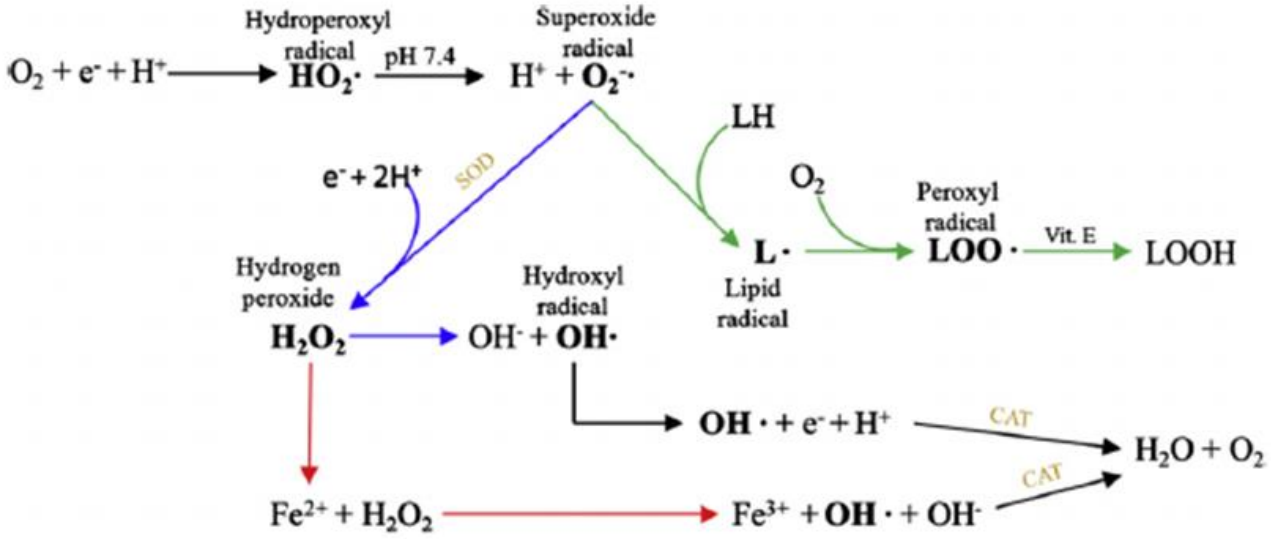


Figure 2.1: Outline of the reactions leading to the formation of reactive oxygen species (ROS) (Carocho and Ferreira, 2013)

2.4. Endogenous and exogenous sources of free radicals

Endogenous sources of free radicals are usually cellular compartments such as the endoplasmic reticulum, nuclei, plasma membranes, peroxisomes, the cytosol and mitochondria, or extracellular spaces (Balaban et al., 2005). Reactive oxygen species are mostly produced in the mitochondrial electron transportation chain of mammalian cells (Poyton et al., 2009b). In the mitochondria various enzymes are involved in the generation of reactive oxygen species. There are several external factors that can trigger oxidative stress through the over-production of free radicals. These external factors are drugs, certain foods, ionizing and nonionizing radiations, air

pollutants, tobacco smoke and xenobiotics (Figure 2.2) and the most common external sources of reactive oxygen species are heavy metals, organic solvents, quinones and pesticides (Yildirim et al., 2000).

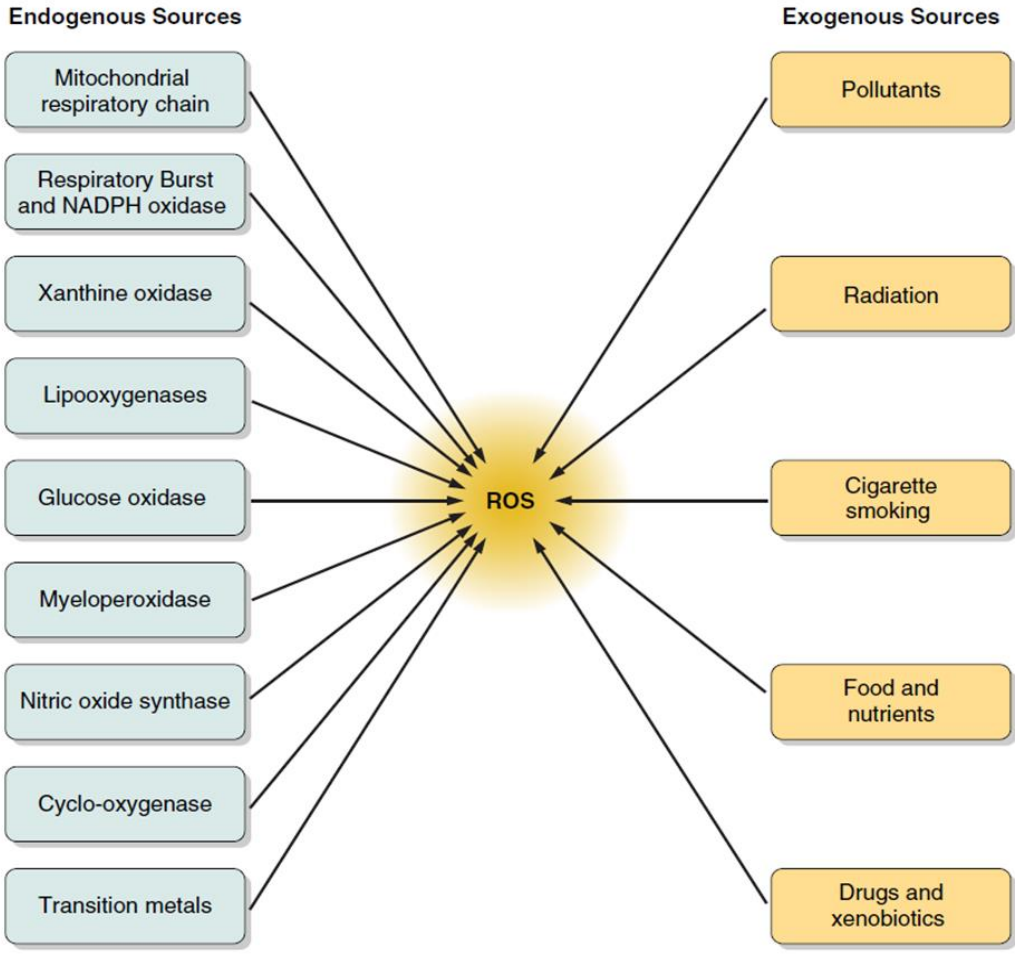


Figure 2.2: Endogenous and exogenous sources of reactive oxygen species production (Bhatacharya et al., 2014)

2.5. Oxidative cell damage

The lack of antioxidant defence mechanisms or the over-production of ROS results in oxidative stress in an organism. An increase in ROS production may modify DNA structure, resulting in the alteration of proteins and lipids.

The reaction between DNA bases or deoxyribose sugars with reactive oxygen species, particularly $OH^{\cdot}$ radicals, results in various products. An example is the addition of $OH^{\cdot}$ radical on to a guanine which results in the production of 8-hydroxy-2'-deoxyguanosine. The reaction between ROS and histone proteins leads to cross-linkages that hinder chromatin folding, transcription and DNA repair (Figure 2.3). The action of ROS on proteins can further lead to mutations or abnormal gene expression (Burton and Jaunlaux, 2011). Mitochondrial DNA is more susceptible to react with ROS, due to their proximity to the site where $O_2^{\cdot -}$ radical is produced in the electron transport chain. Other contributing factors include the absence of histone defense and the negligible restoration mechanisms that are available. Mitochondrial DNA damage can be very extensive, and also occurs under normal conditions (Burton and Jaunlaux, 2011). Oxidative damage to mitochondrial DNA, which encodes several proteins and enzymes of the electron transport chain, may lead to reduced energy production and the danger of extra electron leakage (Burton and Jaunlaux, 2011).

Lipid peroxidation usually occurs in the plasma membrane or other organelles that consist of significant amounts of polyunsaturated fatty acid side chains. Lipid peroxidation is predominately caused by the hydroxyl ion. The hydroxyl ion radical removes hydrogen from the hydro-carbon side chain of the polyunsaturated fatty acid to form a radical that is carbon centred ($C^{\cdot}$). In the presence of oxygen, the $C^{\cdot}$ radical reacts with oxygen to form a peroxyl radical (-C-O-$O^{\cdot}$). The peroxyl radical is then able to remove a hydrogen atom from an adjacent fatty acid, thereby causing a chain reaction (Figure 2.4). Lipid peroxidation increases tissue permeability

and inactivates receptors that are bound to the membrane (Giugliano et al., 1996). The by-products, such as malondialdehyde (MDA) and unsaturated aldehydes, which are produced during lipid peroxidation, can inactivate several proteins by generating protein cross-linkages. The by-products produced result in the induction of peroxide production, reduction of intracellular GSH (Uchida et al., 1999), and the induction of fibronectin generation (Tsukagoshi et al., 2002). To quench the effects of the peroxyl radicals, vitamin E, which is found in the interior of the lipid membrane behaves as the most significant chain breaker (Valko et al., 2006). Vitamin C reacts approximately four times faster with the peroxyl radicals than the adjacent polyunsaturated fatty acid side chains (Valko et al., 2006).

Reactive oxygen species also react with various amino acids intracellularly to produce several products such as altered and less active enzymes and denatured proteins (Butterfield et al., 1998). Reactive oxygen species cause peptide chain fragmentation as well as the increase of susceptibility to proteolysis due to the accumulation of cross-linked reaction products (Figure 2.4). Protein oxidation also results in an alteration of the protein electrical charge. Certain amino acids in a peptide chain are more susceptible to ROS oxidation than others, whereas numerous forms of ROS differ in their potential to oxidize the amino acids (Said et al., 2014). Amino acids that are most vulnerable to oxidation by ROS are cysteine and methionine. Oxidation of the methionine residues of proteins by ROS results in the degradation, unfolding and structural changes of proteins (Lyras et al., 1997). Inhibition of enzyme activity also occurs through metal-catalysed oxidation of enzymes that have metals at or near their active sites (Said et al., 2014).

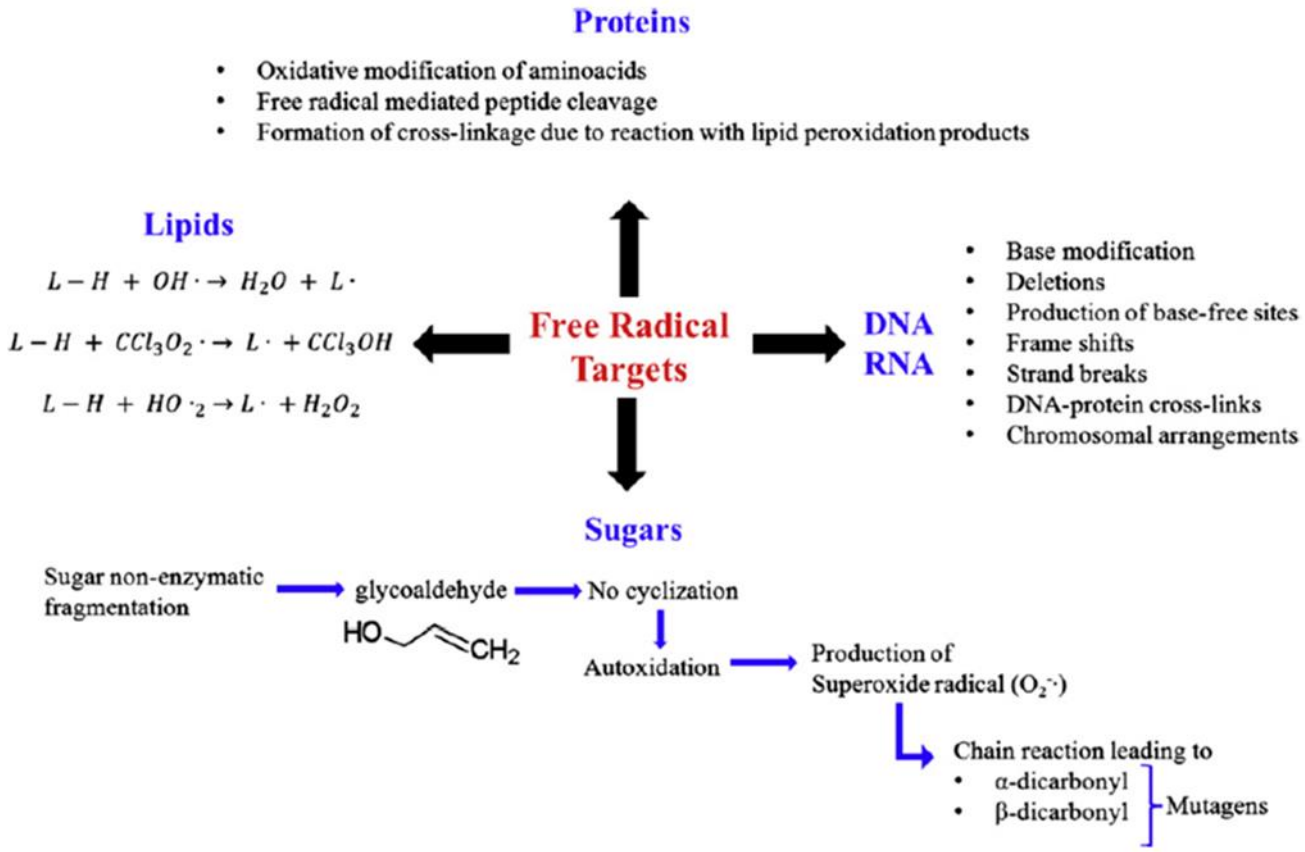


Figure 2.3: Target molecules of free radicals (Carocho and Ferreira, 2013).

2.6. Antioxidants - concept and characterisation

Organisms have established a wide range of defense mechanisms against the attack of free radicals (Cadenas, 1997). To overcome the effects of free radical-induced oxidative stress, categories of defence mechanisms have been recognized such as preventative mechanisms, physical defences, repair mechanisms and antioxidant defences. An antioxidant has been defined as any substance that is found in a low concentration compared to that of oxidizable substrate, with the ability to significantly inhibit the oxidation of the oxidizable substrate (Kurutas, 2016). An organism's antioxidant defence mechanisms can be categorized into enzymatic antioxidant defences, which consist of enzymes such as glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD). The other category is represented by non-enzymatic antioxidants

which include glutathione (GSH), ascorbic acid (Vitamin C), alpha-tocopherol (Vitamin E), flavonoids and carotenoids. Within ideal conditions in the body, there is an equilibrium between the generation of free radicals and the intracellular quantities of these antioxidants. The equilibrium between the formation of free radicals and antioxidant defense mechanism is vital for the prevention of oxidative stress (Kurutas, 2016).

2.6.1. Endogenous enzymatic antioxidants

Endogenous enzymatic antioxidants are the primary defense mechanism against ROSs. In most organisms, the key enzymatic antioxidants are glutathione peroxidase, glutathione-reductase, superoxide dismutase, and catalase. The enzyme superoxide reductase is an oxidoreductase which is only found in facultative and obligate anaerobic microorganisms (Pinto et al., 2010). Catalase and superoxide dismutase enzymes are responsible for the majority of antioxidant defence against ROS (Ighodaro and Akinloye, 2018).

Superoxide dismutases are enzymes that require a metal ion cofactor to catalyse the dismutation of the superoxide radical into oxygen and hydrogen peroxide. Within a human body, superoxide dismutases are found in three forms, which are the manganese requiring (Mn-SOD) found in the mitochondria, copper and zinc containing SOD (Cu-Zn-SOD) found in the cytosol and a copper and zinc containing SOD that is extracellular (Nozik et al., 2005). Other superoxide dismutases are the nickel containing superoxide dismutase which is only found in prokaryotes and the iron containing superoxide dismutase present in plants and bacteria. The Cu-Zn-SOD and Mn-SOD found in the inter-membrane space of the mitochondria and mitochondrial matrix respectively, dismutate the superoxide radical into hydrogen peroxide (Okado-Matsumoto and Fridovich, 2001). The resultant hydrogen peroxide is scavenged by a glutathione peroxidase that is found in the matrix of the mitochondria. Hydrogen peroxide permeates through the mitochondrial

membrane into the cytosol, whereby it is scavenged by the cytosolic Cu-Zn-SOD or catalase enzymes (Poyton et al., 2009a).

Glutathione peroxidase is a multifunctional enzyme which transforms glutathione (GSH) into oxidised glutathione (GSSG). Simultaneously, the enzyme reduces hydrogen peroxide into water, and lipid hydroperoxides to equivalent or corresponding stable alcohols. The glutathione peroxidase mode of action is coupled to glutathione reductase, which maintains the reduced glutathione (GSH) levels in an organism. Due to the inadequate levels of GSH in neurons, susceptibility to free radical damage is very high. Glutathione peroxidase plays a vital role in the defence of cells from the destructive effects of peroxide decomposition. Glutathione peroxidase isozymes are present in the mitochondrial, cytoplasmic and extracellular compartments (Toppo et al., 2008).

The enzyme glutathione reductase maintains the levels of glutathione (GSH) by the reduction of oxidised glutathione disulphide (GSSG) to glutathione (GSH). Glutathione reductase is a ubiquitous enzyme; however, it is not expressed in gram-negative bacteria, *Drosophila* and trypanosomes (Kanzok et al., 2001). Glutathione reductase is a homo-dimeric enzyme that belongs to the group of flavoprotein disulphide oxidoreductases. The two subunits of the enzyme contain four domains, which are the NADPH-binding domain, FAD-binding domain, an interface domain and a central domain. Glutathione reductase active site consists of the dimerised interface domains and catalytic activity is only expressed by the dimer (Bashir et al., 1995). The production of glutathione (GSH) from the reduction of glutathione disulphide (GSSG) by glutathione reductase defends red blood cells, haemoglobin and cell membranes from the effects of free radicals (Waggiallah and Alzohairy, 2011)

The catalase enzyme converts hydrogen peroxide into oxygen. This enzyme is mostly present in peroxisomes (Schrader and Fahimi, 2006). Catalases are iron-containing enzymes (heme enzymes), however a manganese catalase has also been reported in prokaryotes (Zamocky and Koller, 1999). In the human body, catalases are expressed in all the organs, but are significantly present in the erythrocytes, kidney and liver. Some pathogenic bacteria such as *Helicobacter hepaticus*, *Enterobacteriaceae* family bacteria, *Shigella* and *Salmonella* express catalases for the neutralisation of hydrogen peroxide to elude host response to the pathogen, thereby ensuring survival within the host (Bhatacharya et al., 2014).

2.6.2. Endogenous non-enzymatic antioxidants

A substantial amount of endogenous non-enzymatic antioxidants is present in an organism, which include enzyme cofactors (Q10), peptides such as glutathione, vitamins (A) and nitrogen compounds such as uric acid. Retinol, also known as vitamin A is a carotenoid that is fashioned in the liver, from the resultant breakdown of beta-carotene. Approximately a dozen forms of retinol (vitamin A) can be isolated from an organism. Vitamin A is known to have a positive or beneficial impact on endogenous organs, eyes and the skin. Vitamin A's antioxidant capacity can be attributed to its capability to couple with peroxyl radicals before they cause peroxidation of lipids (Jee et al., 2006). Coenzyme Q10 is ubiquitous in all cells and membranes. It is a vital component during cellular metabolism, and it plays a significant part in the respiratory chain. The mode of action of coenzyme Q10 is the direct prevention of the generation of lipid peroxyl radicals. The coenzyme is also responsible for neutralizing lipid peroxyl radicals after their generation. Coenzyme Q10 also plays a vital role in the regeneration of vitamin E (Turunen et al., 2004). During the metabolism of purine nucleotides, uric acid is produced as an end product. It has been observed that, 90% of uric acid is absorbed back into the body after being filtered through the kidneys. This conveys the importance of uric acid within the human body. Uric acid

is known to avert the mass production of oxo-hem oxidants that are a resultant of the reaction of peroxides with haemoglobin. Uric acid is also known to avert the lysis of erythrocytes by peroxidation. Uric acid is an effective scavenger of hydroxyl radicals and singlet oxygen (Kand'ár et al., 2006). Glutathione is a vital non-enzymatic antioxidant that is present in the body. It is ubiquitous in all eukaryotic cells and is usually found in its reduced form (GSH). Glutathione is a tripeptide that prevents the oxidation of cell components by free radicals through either hydrogen donation or electron donation. Glutathione system is formed from the ubiquitous expression of glutathione coupled with three enzymes, which are the glutathione S-transferases and glutathione reductase. This glutathione system behaves as an antioxidative defence barricade in the gut mucosa. Ingestion of high amounts of fruits and vegetables stimulates glutathione dependent enzymes (Hoensch et al., 2002).

2.6.3. Exogenous antioxidants

The endogenous antioxidant defence systems available in the body do not provide sufficient protection against free radicals. Therefore, there is need to boost the endogenous antioxidants system with exogenous antioxidants (Pietta, 2000). Amongst these exogenous antioxidants is vitamin C (ascorbic acid) and vitamin E (tocopherols). Ascorbic acid exists in two forms; L-dehydroascorbic acid and L-ascorbic acid. These compounds are taken up by the body through the gastrointestinal tract and subsequently enzymatically exchanged in vivo. Ascorbic acid is a potent scavenger of various free radicals, which include the hydroxyl radical, singlet oxygen, reactive nitrogen oxide, superoxide radical anion and hydrogen peroxide (Barros et al., 2011).

In a biological system, 8 isoforms of vitamin E are present, four of which are tocopherols while the other four are tocotrienols. The alpha-tocopherol is the most effective and copious isoform in biological systems. The structural characteristic that contributes to the antioxidant capacity of tocopherols is the chroman head group, whereas the phytyl tail has no effect on free radicals.

Lipid peroxidation is inhibited by vitamin E by the donation of its phenolic hydrogen to the peroxyl radical. The resultant tocopheroxyl radicals are harmless, unreactive and are incapable to propagate the oxidative chain reaction. Vitamin E protects the integrity of lipid structure (chiefly membranes), because it is the single key soluble lipid and chain breaking exogenous antioxidants found in plasma, red blood cells and tissues (Kurutas, 2016). Vitamin C and E show a synergistic relationship, whereby vitamin E is regenerated from tocopheroxyl radical through vitamin C (Kurutas, 2016).

Vitamin K is another group of exogenous antioxidants that are fat soluble compounds. These vitamins are vital for the post-translational transformation of glutamates (protein-bound) into carboxyglutamates in a variety of specific proteins. The structural component that contributes to their antioxidant capacity is the 1, 4-naphthoquinone structure.

Carotenoids are naturally occurring pigments that are only produced in microorganisms and plants. Carotenoids can be categorised into two large groups; carotenes and xanthophyls. Carotenes (carotenoid hydrocarbons) consist of end groups such as beta-carotene and lycopene while xanthophyls (oxygenated carotenoids) consist of groups such as lutein and zeaxanthin. Peroxyl radicals are known to be destructive towards carotenoids. Although carotenoids are fairly unreactive, they may decompose to produce stable compounds that may inhibit free radical damage by binding to the free radicals (Paiva and Russell, 1999).

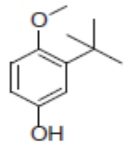
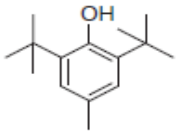
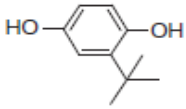
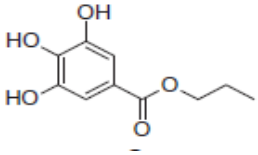
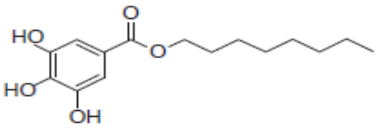
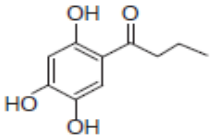
Minerals, which play a vital role in animal metabolism, are only present in minute quantities and are a minor proportion of dietary antioxidant. Selenium and zinc are regarded as vital minerals regarding antioxidant capacity. Selenium can be present in the body in its organic form such as; selenocysteine and selenomethionine and in its inorganic form such as selenite. Selenium does not affect free radicals directly; it is a significant co-factor for various enzymes, which includes

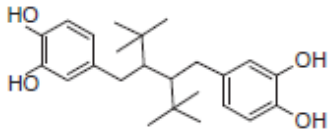
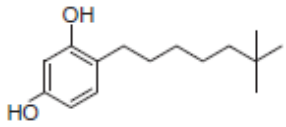
glutathione peroxidase, thioredoxin reductase and metalloenzymes, and these would not function without selenium (Tabassum et al., 2010). During metabolism, zinc is one mineral that is vital for a variety of pathways. Zinc does not directly act on free radicals, but it is vital in the inhibition of free radical formation. Zinc inhibits NADPH oxidases that catalyse the formation of singlet oxygen radical from oxygen with NADPH as an electron donor. Zinc is found in the enzyme superoxide dismutase, which is a vital antioxidant enzyme that converts the singlet oxygen into hydrogen peroxide. Metallothionein (hydroxyl radical scavenger) production is induced by zinc. Zinc competes with copper for binding onto the cell wall, therefore, minimising the production of hydroxyl radicals (Prasad et al., 2004).

2.6.4. Synthetic antioxidants

Synthetic antioxidants are synthesised for the standardisation of antioxidant capacity measurement system in comparison to natural antioxidants. They are also synthesised for incorporation into food products, to increase shelf life and the ability to withstand a variety of treatments and parameters. Synthetic antioxidants are mostly applied in fatty acid-rich foods to prevent food oxidation. They have been fortified in most processed foods. Although they have been deemed safe, some studies have contradicted the safety of synthetic antioxidants. The most frequently used synthetic antioxidants are butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Carocho and Ferreira, 2013). The European food safety authority conducted research on these two synthetic antioxidants and established revised acceptable daily intakes. The revised acceptable daily limit of BHA is 1.0 mg/kg bw/day and for BHT is 0.25 mg/kg bw/day, and these limits should not be exceeded in adults and children (EFSA, 2011, EFSA, 2012). Table 2.1 shows a variety of synthetic antioxidants applied in industry.

Table 2.1: Chemical structure and application of the most significant synthetic antioxidants (Carocho and Ferreira, 2013).

Compound name	Structure	Application	Reference
BHA (butylated hydroxyanisole)		Food antioxidants	Branen (1975)
BHT (butylated hydroxytoluene)			
TBHQ (tert-butylhydroquinone)		Animal processed food antioxidant	Gharavi and El-Kadi (2005)
PG (propylgallate)		Food antioxidant	Anton et al. (2004)
OG (octyl gallate)		Food and cosmetic antioxidant Antifungal properties	Kubo et al. (2001)
2,4,5-Trihydroxy butyrophenone		Food antioxidant	Astill et al. (1959)

NDGA (nordihydroguaiaretic acid)		Food antioxidant	Evan and Gardner (1979)
4-Hexylresorcinol		Prevention of food browning	Chen et al. (2004)

2.6.5. Therapeutic usage of antioxidants

Fruits and vegetables have been considered as decent sources of antioxidants; consuming a significant amount of these can help prevent cardiovascular diseases. Antioxidants also play a role in the possible treatment of neurodegenerative diseases such as Parkinson's disease, amylotrophic lateral sclerosis and Alzheimer's disease. Oxidative cell damage has been linked to several pathological diseases and disorders such as acquired immunodeficiency diseases, cardiovascular disorders, arthritis, rheumatoid and ulcerogenesis. Several studies have expounded on the vital role that antioxidants play during oxidative stress, thereby resulting in termination of various health conditions or diseases such as ischemic stroke, schizophrenia, rheumatoid arthritis, haemodialysis and post menopause of women and depression (Gautam et al., 2012). Antioxidants have been significantly applied in the treatment of male infertility, an example is the application of antioxidants in the treatment of idiopathic male infertility (Agarwal and Sekhon, 2010). A variety of antioxidants have been studied and linked to the inhibition of hyperoxaluria-mediated nephrolithiasis. In addition, it has been observed that antioxidants have significant potential for the treatment of urinary tract stone disease known as nephrolithiasis (Aggarwal et al., 2013). Some studies have reported the use of antioxidant supplements as an adjuvant therapy in patients that have stress-induced anxiety disorders and psychiatric disorders

(Gautam et al., 2012). Therapeutic application of antioxidants has been reported in several reviews (Seifried et al., 2007, El-Mowafy et al., 2010, Godic et al., 2014, Diaz-Gerevini et al., 2016, Sarangarajan et al., 2017)

2.7. Phenolic compounds – classification and application as antioxidants

Plant based phenolic compounds (polyphenols) can be described as compounds with a structural configuration of one or more aromatic rings with hydroxyl groups. Phenolic compounds are produced as secondary metabolites in plants (Parr and Bolwell, 2000). Plants produce between 1 and 3 mg/kg of polyphenols during their secondary metabolism. The quantity of polyphenols is dependent on the cultivar, the stage of the plant life cycle, plant part (leaves, stem, and roots), cultivation conditions as well as processing (extraction method) and storage conditions of the plant (Martinez et al., 2017). Phenolic compounds are categorised based on their structural configuration, such as their carbon skeletons, alignment of hydroxylations, occurrence of stereoisomers, oxidation state, acylation of heterocyclic rings with regards to phenolic acids and glycosylation with regards to flavonoids. Polyphenols have been categorised into 3 major groups which are: (1) phenolic acids (hydroxycinnamic acids and hydroxybenzoic acids), (2) flavonoids (flavones, isoflavones, flavonols, flavan-3-ols, flavanones and anthocyanins and anthocyanidins) and (3) other polyphenols such as tannins, lignins, xanthones, stilbenes, lignans, anthraquinones and chromones (Martinez et al., 2017).

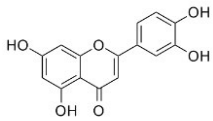
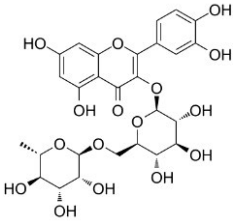
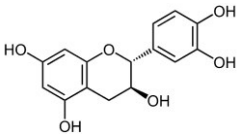
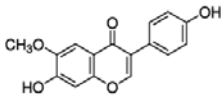
The structural configuration of hydroxybenzoic acids (C6-C1) are derived from benzoic acid. The disparity in the structure of hydroxybenzoic acids depends on the hydroxylations and methoxylations of the aromatic cycle (Murkovic, 2003). Hydroxybenzoic acids are commonly found in fruits, particularly berries. Vanillic acid, gallic acid, ellagic acid, salicylic acid, protocatechuic acid and syringic acid are abundant in grapes, grapefruit, blackberries, mangoes, cranberries, raspberries, strawberries, pomegranate, rhubarb, fresh fruit juices, red and white

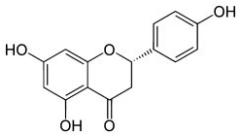
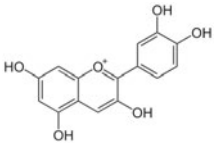
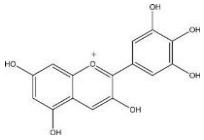
wines and tea (Costain, 2001, Selma et al., 2009). They can also be found in nuts (chestnuts, walnuts, peanuts, pecans), wheat and in some spices and herbs (Costain, 2001, Selma et al., 2009)

Hydroxycinnamic acids which are derived from cinnamic acid and are basically present as joint arrangements of four rudimentary molecules; p-coumaric acid, ferulic acid, cinnamic acid and sinapic acid. Common sources of hydroxycinnamic acids include cinnamon (cinnamic acid), coffee, ginger, lettuce, oranges, apples, cereal grains, bran, blueberries, cherries, pears, prunes, spinach, plums, potatoes, sunflower seeds and specific herbs (rosemary, thyme, sage, basal, oregano and marjoram) as well as turmeric powder. (Costain, 2001, Selma et al., 2009).

Flavonoids are the most abundant dietary polyphenols, with a 60% approximate representation in plants. Flavonoids share the same chemical structure (C_6-C_3-C_6) with a minimum of 15 carbons, 2 benzene rings and a heterocyclic ring. The classification of flavonoids is derived from the differences in the heterocyclic ring of the flavonoid (Table 2.2).

Table 2.2: Types of flavonoids and their sources

Flavonoid	Example	Source	Reference
Flavones	Luteolin 	thyme, beets, chamomile, artichokes, green and red chili peppers and parsley	Costain, 2001; Selma et al., 2009; Martinez et al., 2017
Flavonols	Rutin 	tomatoes, broccoli, onions, apples, leeks, red wine, kale, brussel sprouts and olives	Costain, 2001; Selma et al., 2009
Flavan-3-ols	Catechin 	spinach, coffee, white and red wine, cranberries	Selma et al., 2009; Costain, 2001
Isoflavones	Glycitein 	soy products and legumes.	Martinez et al., 2017

Flavanones	Naringenin 	prunes, citrus juice and fruits, cashew nuts	Selma et al., 2009; Costain, 2001;
Anthocyanidins and Anthocyanins	Cyaniding 	onions, currents, plums and other red fruits, red cabbage and radishes	Martinez et al., 2017
	Delphinidin 		

Stilbenes are characterised by a C_6-C_2-C_6 structure with 2 hydroxyl groups on one ring (A) and 1 on the other ring (B) (Figure 2.5). Stilbenes are produced in plants as glycosides that act as defence mechanism against mold and fungi. Phytoalexins such as resveratrol and piceatannol are examples of stilbenes. Resveratrol and piceatannol are found in red wine, the skin of grapes, mulberries, peanuts and pine nuts (Shrikanta, 2015).

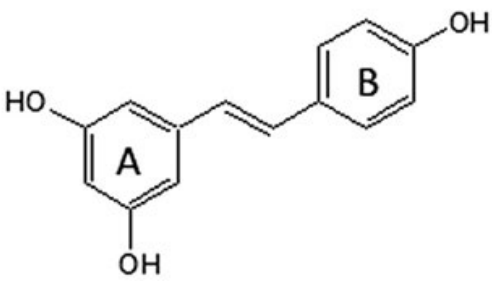


Figure 2.4: Structure of stilbene (resveratrol) (Shrikanta, 2015).

Tannins are split into two major groups; (1) nonhydrolysable and hydrolysable tannins and (2) procyanidins and proanthocyanidins. Nonhydrolysable and hydrolysable tannins are structurally composed of ornately hydroxylated oligomers or polymers of hydroxybenzoic acids (e.g. gallic acid) or flavan-3-ols (e.g. catechin). Nonhydrolysable tannins with high molecular weights can be composed of more than 50 flavan-3-ols subunits joined by C-C bonds. Gallo- and ellagitannins and tannic acid are examples of hydrolysable tannins. Gallotannins are found in grapes, pomegranate, berries and persimmons whereas ellagitannins are found in nuts, tea, fruits, coffee and berries (Selma et al., 2009, Costain, 2001,). Procyanidin A2 and B2, and proanthocyanidins are examples of nonhydrolysable tannins. These are composed of oligomers or polymers of flavan-3-ols catechin, gallocatechin and epicatechin. They are frequently found in cranberries, coffee, chocolate, lentils, chickpeas, black-eyed peas, fruits, red kidney beans, cocoa, nuts, grapes (red and green) and tea (Selma et al., 2009).

Lignans are phenylpropanoids that are structurally composed of C_6-C_3 structures and are formulated from phenylalanine with resultant C_6-C_3-C_3-C_6 structures. These include enterolactone, matairesinol, sesamol, secoisolariciresinol, enterodiol, pinoresinol, enterolactone and lariciresinol. They are mainly found in vegetables such as onion, carrots, broccoli, carrots and corn and fruits such as pears, cranberries and apples and legumes (Touillaud et al., 2007). Lignans can also be found in coffee, grains, tea and alcoholic drinks.

Naturally occurring antioxidants have been considered as a decent alternative to synthetic antioxidants due to their low cost, compliance with dietary intake and no damaging side effects in the human body. Naturally occurring antioxidants have been observed to be good free radical scavengers (Kuršvietienė et al., 2016). Fruits and vegetables have been shown to have good antioxidant potential (Kamiloglu et al., 2015, Villa-Rodriguez, 2015). Black and green tea also have good antioxidant capacity due to flavins present in black tea and the catechins present in

green tea (Lee et al., 2002). However, most monomeric phenolics have low antioxidant capacities (with some showing prooxidant properties), short half-life in the body, poor water solubility and are generally less stable (Kudanga et al., 2017). On the contrary, oligomeric or polymeric antioxidants are more stable, have higher antioxidant capacity and show no pro-oxidant properties (Kudanga et al., 2017). In addition, they are more soluble in water. Indeed, polyphenolic compounds, that are secondary plant metabolites found naturally in plants, are being investigated for potential use in inhibiting the promotion and progression stages of skin carcinogenesis (Perde-Schrepler et al., 2013). There is also increasing research activity in the use of oxidoreductase enzymes such as laccases to enhance the bioactivity of naturally occurring phenolic compounds (Kudanga et al., 2017). Therefore, the use of enzymes such as laccases for the synthesis of oligomeric or polymeric phenolics from monomeric forms could be a viable alternative to produce phenolics with enhanced physiological properties.

2.8. Laccases

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multi-copper oxidases that belong to the group of polyphenol oxidases that consists of copper atoms in their catalytic core. Laccases are glycoproteins which are ubiquitous in nature, but are mainly found in plants and various fungi (Riva, 2006). They use molecular oxygen to oxidize various aromatic and non-aromatic compounds through one-electron abstraction to form radicals (Claus, 2004).

2.8.1. Structure

Laccases from fungal origins frequently occur as isozymes that oligomerize to form multimeric complexes (Claus, 2004). The molecular mass of the laccase monomer varies from about 30 to 100 kDa. Laccases have an interesting characteristic of a covalently linked carbohydrate portion (10–45%), which is associated with their high stability (Claus, 2004). In order for this enzyme to catalyse a substrate, a minimum of four copper atoms is required (Figure 2.6). The Type 1

copper atom is paramagnetic and has an absorbance at 610 nm. Type 2 copper atom is non-blue and also paramagnetic. Type 3 copper consists of two copper atoms which are diamagnetic copper-copper with an absorbance at 330 nm (Claus, 2004).

Type 1 copper (Figure 2.6) has two histines and a cysteine ligand equatorially attached to the copper atom in a trigonal coordination. The fourth position usually varies with either methionine in bacterial laccases and leucine or phenylalanine in fungal laccases (Claus, 2004). The oxidation potential of the enzyme has been suggested to be influenced by the fourth axial position ligand. Type I copper gives the typical blue colour common to laccases. The blue colour is as a result of the intense electronic absorption caused by the covalent copper–cysteine bond. Oxidation of the various aromatic and non-aromatic compounds takes place at the type 1 copper atom due to its high redox potential. In the visible spectrum Type 2 copper (Figure 2.6) shows no absorption. Strategically the Type 2 copper is positioned close to the type 3 copper, a binuclear center which has an electron adsorption at 330 nm in its oxidized form. The type 2 and type 3 copper atoms form a trinuclear cluster where molecular oxygen is reduced, and water is released as the only by product. Six histidines match the one type 2 and two type 3 copper atoms. The two type 3 copper atoms are joined together by a hydroxyl bridge (Claus, 2004).

Laccases can be categorised into either two domain or three domain laccases. Most fungal laccases are commonly three domain laccases whereas bacteria laccases usually exist as two domain laccases. Bacterial laccases comprise of two cupredoxin domains. According to the position of their T1 centre (Figure 2.7), bacterial laccases are classified into three different types. Type A bacterial laccases have a T1 centre in each cupredoxin domain, in type B and C a single T1 centre is found in the first or second cupredoxin domain. Bacterial laccases have been found in various groups of bacteria, and this includes bacteria from the genus *Streptomyces* (Trubitsina et al., 2015).

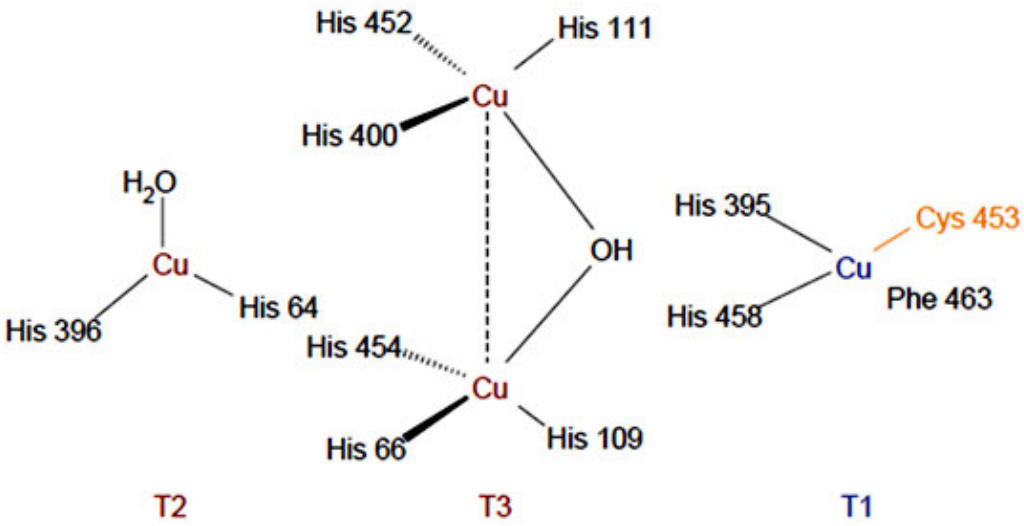


Figure 2.5: The trinuclear cluster of laccases showing the T1, T2 and T3 copper atoms (Riva, 2006).

2.8.2. Catalytic mechanism

Laccases functions in the presence of oxygen (O_2) to catalyze the monoelectronic oxidation of suitable substrates (usually anilines and phenolic compounds) and produce water as the only by-product, hence they are environmentally friendly (Riva, 2006). The monoelectronic oxidation of a suitable molecule produces reactive radicals that can undergo non-enzymatic reactions. The generated radicals react with each other to form dimers, oligomers or polymers covalently coupled by C–C, C–O and C–N bonds. Laccases participate in the metabolic breakdown of complex organic substances such as humeric matter or lignin and also participates in the organisms' morphogenesis, as well as its pathogenicity and immunity (Claus, 2004). The laccase redox reaction occurs with the involvement of four copper atoms that make up the catalytic center of the enzyme (Riva, 2006). The complete catalytic cycle is the reduction of one molecule of oxygen to two molecules of water and the simultaneous oxidation of four substrate molecules

to produce four radicals (Riva, 2006). The radicals are able to couple in coupling reactions to form dimers, oligomers and polymers (Kudanga et al., 2011)

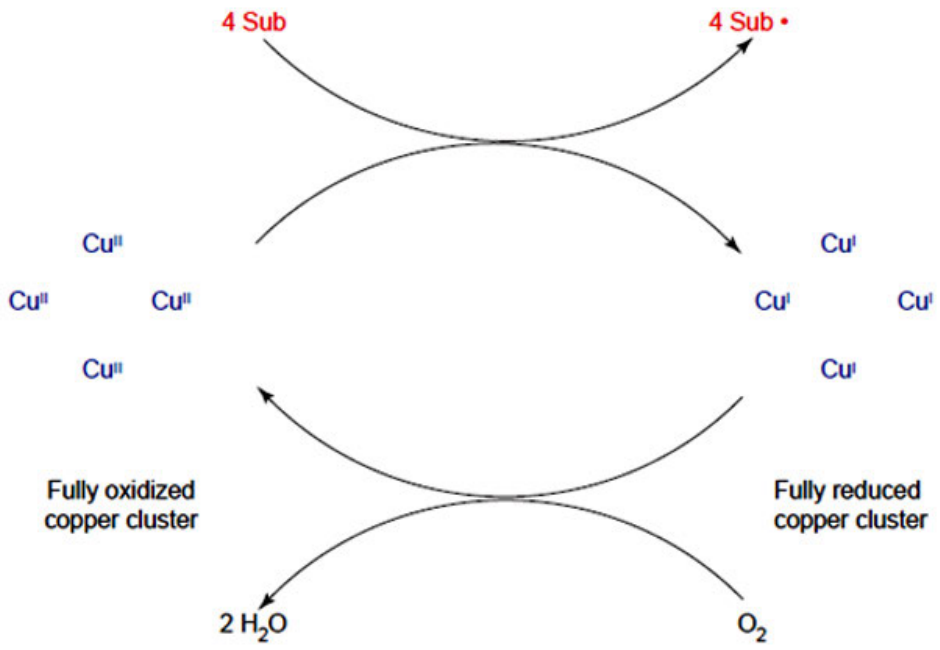


Figure 2.6: Schematic diagram of the catalytic mechanism of laccases (Riva, 2006).

2.8.3. Sources of laccases

Laccases were first described by Yoshida in 1883 as a constituent of the resin ducts of the lacquer tree *Rhus vernicifera* **(**Viswanath et al., 2014). Afterwards, laccases have been found to be ubiquitous in nature, and have been discovered in diverse organisms including plants, fungi and bacteria. Fungal sources of laccases include basidiomycetes, ascomycetes and deuteromycetes. The most effective lignin degraders and laccase producers are basidiomycetes (Singh-Arora and Sharma, 2010). Basidiomycetes produce laccases along with other ligninolytic enzymes such as lignin peroxidase, manganese peroxidase and versatile peroxidase which are useful for the fungi

to access nutrients (Singh-Arora and Sharma, 2010). Several laccase-producing basidiomycetes have been reported such as *Schizophyllum commune, Pycnoporus sanguineus, Pycnoporus cinnabarinus, Rigidoporus microporus, Polyporus brumalis, Fomes fomentarius, Cryptococcus neoformans, Coprinopsis cinereal, Cyathus bulleri, Ganoderma lucidum, Agaricus bisporus, Panus rudis, Cerrena unicolor, Phlebia radiata, Coriolopsis gallica, Pleurotus* species including *P. eryngii, P. florida, P. pulmonarius, and P. sajor-caju* and *Trametes* species including *T. hirsuta, T. pubescens, T. trogii, and T. villosa* (Baldrian, 2006, Singh-Arora and Sharma, 2010, Forootanfar and Faramarzi, 2015).

Laccases from *Trametes versicolor* and *Pleurotus ostreatus* are widely used in research. In plants and bacteria, laccases are used in diverse physiological activities such as lignin degradation (Dwivedi et al., 2011, Singh et al., 2011, Chandra and Chowdhary, 2015, Forootanfar and Faramarzi, 2015, Wang et al., 2015). Plants have been reported to have bigger laccase families compared to fungi, (e.g., there is a minimum of 22 laccase genes in rice) (Huang et al., 2016). Laccases from bacteria were only discovered much later compared to those from fungal and plant origin (Ausec et al., 2011). Bacterial laccases have been associated with several processes in bacteria such as metal oxidation, pigmentation, UV defence, sporulation and degradation of xenobiotics (Singh et al., 2011, Chandra and Chowdhary, 2015, Forootanfar and Faramarzi, 2015). Bacterial laccases have better physical properties compared to fungal laccases, such as higher thermostability (Santhanam et al., 2011, Singh et al., 2011, Chandra and Chowdhary, 2015, Martins et al., 2015). Although insect laccases have been reported (Dittmer and Kanost, 2010, Jeon et al., 2012, Ni and Tokuda, 2013), these laccases are the least characterised. Insect laccases play a vital role in the physiology of insects such as melanisation and cuticle sclerotization (Dittmer and Kanost, 2010, Jeon et al., 2012, Ni and Tokuda, 2013).

2.8.4. Applications of laccases

Laccase fall into the category of eco-friendly enzymes due to its catalytic mechanism; they use oxygen and produce water as the only by-product (Riva, 2006). The use of laccase in the industrial environments dates back to the 1990s (Pezzella et al., 2015). The oxidation reactions of laccases produce radical species which have a number of biotechnological applications (Table 2.3). In the textile industry laccases have been integrated into existing processes such as dyeing and fiber bleaching. Laccases have emerged as important green tools for the modification and improvement of textile properties. They are also used in the bioremediation of textile wastewaters (Pezzella et al., 2015).

Laccases are beneficial to the food industry in different aspects such as the improvement of the shelf-life of product by the modification of food sensory parameters and textures. Several beverages and foods contain various laccase substrates; e.g. phenols, unsaturated fatty acids and thiol-containing proteins, therefore laccases are used for the determination of compounds in foods and beverages (Pezzella et al., 2015). In environmental pollution control, laccase-catalysed coupling of molecules can be used in bioremediation. They are also used in the modification of lignocellulosic materials, either by grafting low molecular weight compounds onto lignocellulosic materials or by the in-situ cross-linking of lignin molecules. Laccase-mediated coupling reactions can also be used in dye removal. Studies have shown that a *T. villosa* laccase could degrade methyl orange and catalyse a coupling reaction between aromatic amines and catechol. In wastewater, laccase-mediated coupling reactions can form insoluble polymers of catechol which precipitate in the wastewater streams, and can be easily removed. Laccase-catalysed oxidative coupling to form polymers or oligomers of polyphenols has been used in the wine industry to stabilize wines. They can also be used in madeirization whereby the sensory characteristics of wine is altered through the oxidation of polyphenols. Coupling of molecules through laccase-mediated systems can give rise to the production of novel compounds with

enhanced properties (Kudanga et al., 2017). Therefore, laccases can be used in the synthesis of fine chemicals and to derive compounds that are biologically active e.g. cytostatics, amino acids, antioxidants and antibiotics (Kudanga et al., 2011).

Table 2.3: Industrial applications of laccases

Industry	Application	Reference
Food	Laccases selectively remove polyphenols from wine to avoid undesirable modifications of the wine's organoleptic properties. *Myceliophthora thermophila* laccase has been used commercially for treating wine cork stoppers. *T. versicolor* laccase was immobilised using coconut fibres that were activated with glutaraldehyde for juice clarification	Osma et al., 2010 Mate and Alcalde, 2016 Bezerra et al., 2015
Textiles	Bleaching of denim fabrics, and dying of wool and cotton fabrics through oxidative hetero-coupling. Grafting of wool fabrics with lauryl gallate.	Yavuz et al., 2014, Iracheta-Cardenas et al., 2016, Hadzhiyska et al., 2006, Dıaz Blanco et al., 2009, Hossain et al., 2009
Pulp and paper	Laccase-mediator system is being applied to improve paper quality and strength Laccases have also been applied in the removal of flexographic inks as an eco-friendly deinking alternative method. Laccase-mediated grafting of phenols onto flax fibres has been used for the production of paper.	Mate and Alcalde, 2015 Camarero et al., 2007, Fillat et al., 2010 Fillat et al., 2012, Aracri et al., 2010, Fillat et al., 2012
Biofuels	Laccase are vital for the biodegradation of lignin. They are also used as biocatalysts for the elimination of phenolic inhibitors during fermentation.	Kudanga and LeRoes-Hill, 2014, Gonzalez-Perez and Alcalde, 2014; Alcalde, 20
Organic synthesis	Laccases have a wide range of substrates and can catalyse a number of synthetic reactions such as the synthesis of new antioxidants	Piscitelli et al., 2012, Kudanga et al., 2017

Cosmetics	In the formulation of hair dyes, laccases can be substituted for H_2O_2 as an oxidising agent in the formulation of hair dyes. They have also been applied in skin lightening treatments.	Saito et al., 2012, Chen et al., 2013, Bhogal et al., 2013, Golz-Berner et al., 2004
Paint	In the preparation of resins that are used as binding agents in coatings, laccase mediator systems have been applied has an eco-friendly alternative to heavy-metal catalysts for the cross linking of alkyd resins.	Gooch, 2002, Greimel et al., 2013
Furniture	Laccase mediator system has been applied in the activation of lignin on wood fibre surfaces in the production of medium-density fibreboards.	Euring et al., 2011, Euring et al., 2015
Nanobiotechnology and biomedicine	Laccases are able to catalyse direct electron transfer. Thus, they have been used in the development of biosensors and biofuel cells	Mate and Alcalde, 2016
Enzymatic bioremediation	Laccases are able to breakdown hazardous pollutants such as phenols, organophosphorus insecticides and polycyclic aromatic hydrocarbons (PAHs).	Majcherczyk et al., 1998, Canas et al., 2007, Zeng et al., 2016

2.8.5. Laccase-mediated synthesis of new phenolic compounds

The synthesis and extraction of bioactive compounds in an economically feasible and eco-friendly process has become a topic of great interest. Bioactive compounds are secondary metabolites frequently found in minute amounts in sponges (Muller et al., 2004), plants (Kris-Etherton et al., 2002), bacteria, and fungi (Debbab et al., 2010) and have dietary benefits. The current methods for the synthesis and extraction of bioactive compounds are the supercritical fluid extraction method, heat reflux extraction method and accelerated solvent method. These methods are employed in harsh conditions with the use of radiation extraction and chemical synthesis processes (Martins et al., 2011). Furthermore, they are time and energy consuming, have low product yields and are not eco-friendly. Therefore, the search for novel methods that are eco-friendly and economically feasible has gained great interest due to the cumulative market value of bioactive compounds which is predicted to rise by 4.71% between 2013 and 2018 (Infiniti Research Limited, 2014). Thus, biocatalysis in the production of bioactive compounds has become a noteworthy alternative in organic synthesis, since it is environmentally friendly and less time consuming. In organic synthesis, laccases have shown great potential as biocatalysts. This potential is based on the broad substrate specificity of laccases with substrates ranging from phenolic compounds, aromatic and alkyl amines, polyamines, amino-phenols, anilines to benzenethiols (Kunamneni et al., 2008, Madhavi and Lele, 2009).

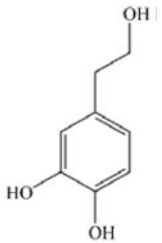
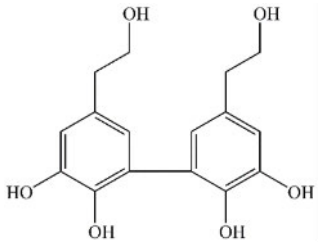
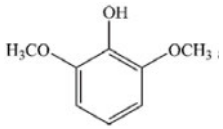
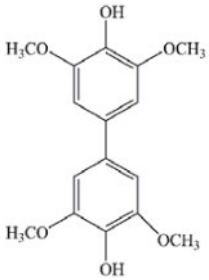
Phenolic compounds have been found to be the most beneficial in the synthesis of novel bioactive compounds because of their vast range of health benefits such as antioxidant, antimicrobial as well as anti-inflammatory properties (Balasundram et al., 2006, Pasha et al., 2013). Laccases behave as oxidizing agents through the oxidation of phenolic substrates into radicals. The resultant radicals are the building blocks for the synthesis of dimeric, oligomeric or polymeric phenolic compounds either through homo-molecular or hetero-molecular coupling (Kudanga et al., 2011). Homo-molecular coupling is the most frequently used mode of

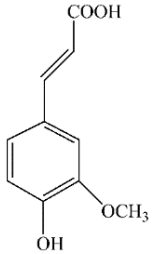
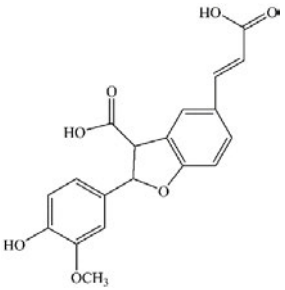
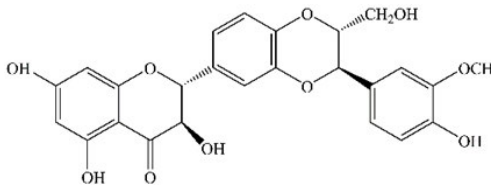
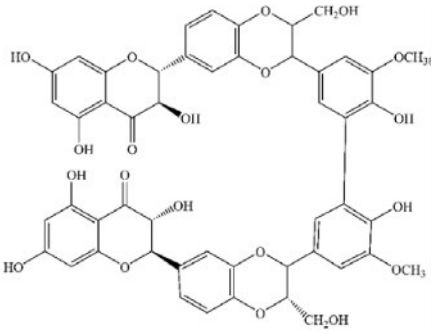
modification. Several studies have reported enhancement of antioxidant capacity through laccase-mediated oligomerisation of monomeric phenols notably ferulic acid (Adelakun et al., 2012a), silybin (Gavezzotti et al., 2014), 2,6 dimethoxyphenol (Adelakun et al., 2012b), hydroxytyrosol (Zwane et al., 2012), and rutin (Uzan et al., 2011). The synthesis of the enhanced antioxidant is based on the principle that oligomerisation increases the number of hydroxyl groups on a compound (Wolfe and Liu, 2008) which may also result in an increase in electron donating groups.

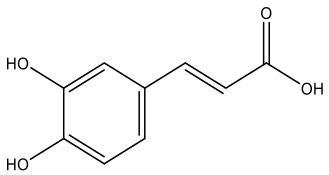
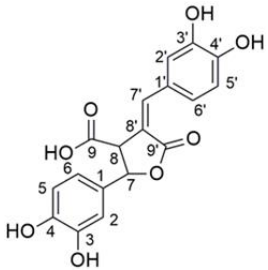
The potential free-radical scavenging capacity of a phenolic compound is determined by the ability of the compound to act as an electron or hydrogen donor (reduction potential); the stability of the resultant antioxidant radical, the reactive relationship with other antioxidants; the antioxidant's metal chelating potential (inhibition of transition metal catalysed free radical generation); the structural arrangements, e.g.: ortho 3',4'-dihydroxy moiety in quercetin and luteolin (Sajid et al., 2014), allyl group in eugenol (Ou et al., 2006), meta 5,7-dihydroxy arrangements in chrysin, kaempferol and apigenin (Sajid et al., 2014); the bond dissociation enthalpies i.e. antioxidants that possess active groups with lower bond dissociation enthalpies are better free radical scavengers because they effortlessly release electrons to radical species (Szymusiak and Zielinski, 2003); and the hydrophobicity, which permits the bioavailability of the antioxidant at sites where free radicals are produced (Ishige et al., 2001).

Therefore, structural modification has been applied to improve the antioxidant activity of some phenolic compounds. A comprehensive review of compounds modified for improving bioactivity has been recently published (Kudanga et al., 2017). Table 2.4 below provides examples of studies that were conducted to improve antioxidant capacity in the recent years.

Table 2.4: Effects of structural modification on phenolic compounds

Phenolic compound	Structural modification	Outcome of structural modification	Reference
 3-hydroxytyrosol	 3-hydroxytyrosol dimer	The increase in OH groups provided additional free radical scavenging sites, which leads to an increase in antioxidant activity.	(Burton and Davids, 2012, Zwane et al., 2012)
 2,6-DMP	 3,3′,5,5′-tetramethoxy biphenyl-4,4′-diol	The additional electron donating groups on the produced dimer lead to a decrease in bond dissociation enthalpies of the hydroxyl moieties. The latter leads to the formation of a stable radical due to the ease of electron delocalisation into the benzene ring.	(Adelakun et al., 2012a, Wan et al., 2008a, Wan et al., 2008b)

 Ferulic acid	 β–5 ferulic acid dimer	The increase in electron donating groups and the conserved C=C bond with its adjacent carboxylic acid group, provided extra free radical scavenging sites.	(Adelakun et al., 2012b, Constantin et al., 2012)
 Silybin A	 Silybin A dimer	The coupling of silybin monomers resulted in an increase in resonance. High resonance permits an antioxidant to form stable radicals when it has donated electrons.	(Gavezzotti et al., 2014)

 Caffeic acid	 β-β Caffeic acid dimer	The increase in hydroxyl groups and preservation of unsaturated C=C bond provided additional free radical scavenging sites.	(Nemadziva et al., 2018)

3. LACCASE-MEDIATED OXIDATION OF SELECTED PHENOLIC COMPOUNDS

3.1. Abstract

Laccases have recently gained attention as a viable approach for antioxidant synthesis. In this study, laccase with a fairly high activity (19.2 U/mg) and a molecular weight of 66 kDa was successfully produced from *Trametes pubescens*. The enzyme had a substrate dependent optimum pH between 4.0 and 5.0 and an optimum temperature of 60^{o}C. Laccase-catalysed coupling reactions were then carried out using ρ-coumaric acid and quercetin as substrates in a monophasic system. Quercetin hydrate oxidation produced a dimer of quercetin (*m/z* 601). Oxidation of *ρ*-coumaric acid resulted in the production of a *ρ*-coumaric acid dimer (*m/z* 325). The *ρ*-coumaric acid dimer exhibited a 2-fold, 3-fold and 6-fold increase in antioxidant capacity using the ABTS, DPPH and FRAP assays respectively. The products have potential for application in the pharmaceutical and cosmetic industries as alternative sources of antioxidants.

3.2. Introduction

Trametes pubescens is a non-edible fungus which belongs to the phylum Basidiomycota. It has a cap size of up to 6 cm and is less than 1 mm thick (Emberger, 2008). It is known to cause white rot on dead woods due to the production of lignin degrading enzymes such as laccases. Fungi are the main decomposers of lignocellulose biomass and the enzymes they secrete to break down lignocellulose may be useful in a number of industrial processes (Morgenstern et al., 2012). Recently, there has been growing interest in laccases due to their importance in several applications such as in bio-bleaching, delignification to produce pulp and paper, bioremediation and organic synthesis.

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are glycoproteins which belong to the group of polyphenol oxidases that consists of copper atoms in their catalytic core. Generally, laccases consist of four copper atoms in their catalytic site; a type-1, a type-2 and two type-3 copper atoms (Hakulinen and Rouvinen, 2015). They are ubiquitous in nature and are mostly found in plants and various fungi (Riva, 2006). Laccases have been applied in several biotechnological applications (Pezzella et al., 2015). In the textile industry laccases have been integrated into existing processes such as dyeing and fiber bleaching. Therefore, laccases have emerged as important green tools for the modification and improvement of textile properties. In addition, laccases are beneficial to the food industry in different aspects such as the improvement of the shelf-life of products by the modification of food sensory parameters and textures. Several beverages and foods contain various laccase substrates; e.g. phenols, unsaturated fatty acids and thiol- containing proteins. Therefore, laccases are used for the detection of compounds in foods and beverages (Pezzella et al., 2015).

Most biotechnological applications of laccases are achieved through its ability to catalyse coupling reactions. Coupling reactions occur when two organic molecules of similar size are linked together with the aid of a catalyst. Coupling reactions can be categorised into two main groups, namely hetero-coupling, in which two different molecules react to form one new molecule, and homo-coupling, where the reactant molecules are the same (Kudanga et al., 2011). Laccases vary in their ability to perform coupling reactions mainly because of different characteristics such as pH and temperature properties, substrate range (influenced mainly by redox potential) and their sensitivity to inhibitors and solvents which are encountered in reaction environments. Investigating the ideal reaction conditions is mandatory in order to obtain optimum enzyme activity in the reaction mixture. Therefore, the aim of the current study

was to partially characterise laccase from *T. pubescens* and apply the enzyme in oxidation reactions in a monophasic system.

3.3. Materials and Methods

3.3.1. Chemicals and enzyme

Phenolic compounds and other chemicals were purchased from Sigma–Aldrich, South Africa. The *T. pubescens* strain was obtained from the Institute of Applied Microbiology, University of Natural Resources and Life Sciences (Vienna, Austria), and is currently deposited in the stock culture collection at the Enzyme Research Group, at the Department of Biotechnology and Food Technology, Durban University of Technology.

3.3.2. Guaiacol plate screen

The guaiacol plate screen was used to confirm laccase activity through the oxidation of guaiacol. A liter of potatoe dextrose agar (PDA) supplemented with 100 µL of guaiacol was made and autoclaved at 121 ^{o}C for 20 min. Thereafter, 1 cm^2 pieces of *T. pubescens* were inoculated onto the plate. Plates were incubated at 28^{o}C for 24-48 hours. The formation of brown-reddish halo indicates oxidation of guaiacol.

3.3.3. Enzyme production (growth of *T. pubescens* in defined media)

A fresh culture of *T. pubescens* was inoculated on potato dextrose agar plates and incubated at 28˚C for 5 days. Three plugs of the fresh culture of *T. pubescens* were sub-cultured in 200 mL Trametes defined media supplemented with 1 g wheat bran and incubated at 28˚C for 5 days at 175 rpm. Laccase production was induced on the third day by the addition of 2.5 mM xylidine.

3.3.4. Laccase assay

Laccase activity was determined in triplicate at 25°C in 2 mL cuvettes, monitoring the increase in absorbance at 419 nm (ε=36000 $M^{-1}cm^{-1}$), using ABTS as substrate. The assay mixture contained 566 µL crude enzyme, 0.110 ml of 5 mM ABTS in 0.833 ml of 50 mM sodium acetate buffer pH 4.0. One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per minute at 25 °C (Adelakun et al., 2012a).

3.3.5. Partial purification of laccase

Ammonium sulphate (50-80%) was used to precipitate the laccase protein produced by *T. pubescens* after incubation. The precipitate was separated from the filtrate by centrifugation at 10 000 rpm for 15 min at 4°C. The precipitated protein was re-dissolved in sodium acetate buffer (50 mM, pH 4.6) and then dialyzed (Othman et al., 2014). The protein content was determined using the Lowry protein assay (Lowry, 1951), and the concentration was calculated using a standard curve, which showed a good correlation of R^2= 0.9935.

3.3.6. Electrophoretic analysis (Native-PAGE)

Native PAGE was used to determine the apparent molecular mass of the laccase enzyme. Native PAGE was performed with 12% polyacrylamide. Proteins were visualized by staining for 15 min with Coomassie Brilliant Blue-R250. The gel was destained with a mixture of acetic acid and ethanol (40:10). A native size marker was used as molecular weight marker (Othman et al., 2014).

3.3.7. Temperature optima and thermal stability

The effect of temperature on the activity of the enzyme was assessed at different incubation temperatures (30, 40, 50, 60, 70 and 80ºC) at pH 4.0. For thermal stability, the enzyme was

preincubated at different temperatures (30, 40, 50, 60, 70, 80 °C) and the enzyme activity monitored every 30 min for 2 hours at previously determined optimum conditions using ABTS as substrate (Othman et al., 2014).

3.3.8. pH optima and pH stability

The effect of pH on the activity of laccase was determined by monitoring oxidation of different substrates in different buffers (pH 2.5 – 8.0). pH stability was determined by preincubating the enzyme in different buffers (sodium acetate buffer (4.0–5.0); phosphate buffer (6.0-8.0) for different incubation periods. Identical aliquots of enzyme were removed at different time intervals and assayed for residual activity at the previously determined optimum pH and temperature using ABTS as substrate (Othman et al., 2014).

3.3.9. Substrate specificity

The activity of laccase was determined using different substrates (selected phenolic compounds) by incubating the partially purified enzyme with each individual substrate at final concentrations of 1.1 mM. The enzyme activity was assayed (Othman et al., 2014) using the selected phenolic compounds as substrates.

3.3.10. Effect of organic solvents on laccase activity

The enzyme was preincubated in different organic solvents (ethanol, methanol, acetone, ethyl acetate, dioxane, or acetone, 50% v/v) with buffer (pH 4.5) and the residual activity was monitored at optimal conditions every hour for 5 hours, using the ABTS assay as previously described (section 3.3.4.).

3.3.11. Oxidation of selected phenolic compounds

Oxidation of the selected phenolic compounds was conducted in a monophasic system comprising sodium acetate buffer (pH 4.5) and miscible solvents (dioxane, methanol, ethanol or acetone) as co-solvents. The miscible solvents were used at 70%, a concentration determined in preliminary reactions at which all the solvents produced the dimer with minimal polymerisation side reactions. Due to inactivation of the enzyme by solvents, the enzyme (0.632 U) was added to the reaction mixture every 6 hours. The reactions were carried out for 24 hours at 37^{o}C in a shaking incubator at 200 rpm and monitored by thin layer chromatography (TLC).

3.3.12. Chromatographic separation of reaction products

3.3.12.1. Thin Layer Chromatography

TLC analysis was performed on aluminum-backed silica gel 60 F254 plates (Merck South Africa) using toluene: dioxane: acetic acid (11:2.5:0.4, v/v/v) as the mobile phase. The compounds were then visualised by exposure to UV light at 254 nm.

3.3.12.2. High performance liquid chromatography (HPLC)

The enzyme was precipitated out of the reaction solution by the addition of an equal volume of ice-cold methanol and then the mixture was incubated at 0^{o}C for 20 min and centrifuged (14,000×g) at 0^{o}C for 15 min. The supernatant (1.5 mL aliquots) was filtered into clean vials and analysed by HPLC. HPLC analysis was carried out using a prominence HPLC system from Shimadzu (Shimadzu, Kyoto, Japan). Separation of the reaction products was carried out on a reversed phase Sunfire C18 5.0 µM, 4.6 mm×150 mm column (Waters, Warszawa, Poland). Gradient elution using 0.1% formic acid (solvent A) and acetonitrile (solvent B) was used to separate the compounds at a running time of 23 min. The gradient was 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 LabSolutions data manager software from Shimadzu.

3.3.13. Purification of reaction products

The reaction products were purified using preparative TLC. TLC analysis was performed on aluminum-backed silica gel 60 F254 plates (Merck South Africa) using toluene: dioxane: acetic acid (11:2.5:0.4, v/v/v) as the mobile phase. The compounds were then visualised by exposure to UV light at 254 nm. The reaction products were then scraped from TLC, dissolved in methanol and filtered to remove residual silica gel. The reaction products were further purified using TLC, using toluene: dioxane: acetic acid (11:2.5:0.4, v/v/v) as the mobile phase.

3.3.14. Characterisation of product

The purified reaction products were characterised by liquid chromatography-mass spectrometry (LC-MS).

3.3.14.1. Liquid chromatography–mass spectrometry (LC–MS)

LC–MS was performed using a prominence HPLC system from Shimadzu coupled to a mass spectrometer from Shimadzu (LCMS 2020) (Shimadzu, Kyoto, Japan). The products were separated using the same linear gradient of acetonitrile (solvent B) and 0.1% formic acid (solvent A) as previously described (Section 3.3.12.2.), at a flow rate of 1 mL/min, using an injection volume of 10 µL and an oven temperature of 30^{o} C. MS spectra were acquired in negative mode and electrospray voltage was set to +3500 V. Dry gas flow was set to 9 L/min with a temperature of 350^{o} C and nebulizer gas pressure was set to 35 psi.

3.3.15. Antioxidant activity

Antioxidant activity was determined using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-Azinobis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS) scavenging assay and the ferric reducing antioxidant power (FRAP) assay.

3.3.15.1. DPPH (2, 2-diphenyl-1-picrylhydrazyl) scavenging effect

The reaction mixture contained 3.9 mL of DPPH (0.025 mg/mL) dissolved in methanol and 0.1 mL sample (dissolved in methanol) at various concentrations. The mixture was homogenised, incubated at room temperature in the dark for 60 min, and the decrease in absorbance at 517 nm determined using a spectrometer. DPPH assay results are were expressed as IC_{50} values. The IC_{50} value is the concentration of an antioxidant compound required to have a 50% reduction in DPPH˙ concentration (Adelakun et al., 2012a).

3.3.15.2. ABTS (2, 2'-Azinobis (3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging effect

ABTS radical was generated by mixing a 2.0 mM ABTS solution with 7.0 mM $K_2S_2O_8$ and the mixture was incubated in the dark for 24 hours at room temperature. Before use, the ABTS radical solution was diluted in 95% methanol to get an absorbance of 0.7 at 734 nm. The diluted ABTS radical solution was added to the test samples to achieve a final concentration of 5, 10, 20, and 30 µM. The absorbance at 734 nm was recorded after 6.0 min.

3.3.15.3. FRAP (ferric reducing antioxidant power) assay

Ferric reducing antioxidant power (FRAP) of eugenol and the synthesised dimer were measured according to the method by Ahmed et al. (2015) with modifications. The working solution was prepared by mixing 25 mL acetate buffer (300 mM at pH 3.6), 2.5 mL TPTZ

solution (10 mM) and 2.5 mL ferric chloride solution (20 mM). The mixture was incubated for 15 min at 37 °C before use. Iron sulphate was used as a standard (5 µM to 100 µM). The reaction mixtures were incubated for 30 min in the dark, and the absorbances were measured at 593 nm. The results were reported as FRAP values in µM of Fe^{3+} converted to Fe^{2+}.

3.4. Results and Discussion

3.4.1. Enzyme production and partial purification

Due to several biotechnological applications, research on laccase production by different organisms and the optimisation of its production has increased (Niladevi and Prema, 2008). In this study, the laccase enzyme was produced from *T. pubescens* cultured on PDA plates and supplemented with guaiacol (laccase substrate). Guaiacol in the agar medium was oxidized to form a brownish-orange precipitate around the *T. pubescens* colony (Figure 3.1A) indicating laccase production. Subsequently *T. pubescens* was produced in liquid medium (Trametes defined media supplemented with wheat bran) followed by induction with xylidine. The presence of laccase was confirmed through qualitative tests on common laccase substrates such as ABTS, 2, 6-DMP and syringaldazine (Figure 3.1B, 3.1C and 3.1D, respectively) and quantitative analysis.

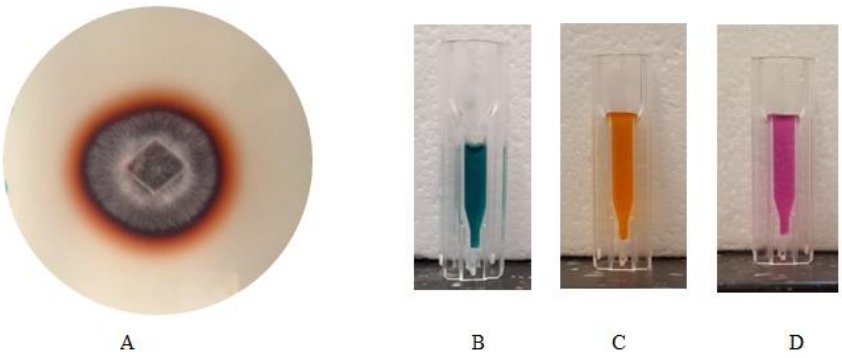


Figure 3.1: (A) Oxidation of guaiacol on potatoe dextrose agar (PDA) by *T. pubescens* laccase. Oxidation of 2,2'-Azinobis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) (B), 2, 6-DMP (C) and syringaldazine (D).

After enzyme production, purification of laccase involved a 2-step partial purification protocol, which included $(NH_4)_2SO_4$ precipitation and ultra-filtration, resulting in a 57.9 % yield (Table 3.1). The enzyme produced had an activity of 19.2 U/mg after partial purification. The enzyme activity (19.2 U/mg) was higher compared to that produced by Gonzalez et al. (2013). This could be attributed to the supplementation of the production media with wheat bran and induction of the enzyme with 2.5 xylidine.

Table 3.1: Purification table of laccase enzyme from *T. pubescens*

Fraction	Volume (ml)	Total protein (mg)	Activity (U/ml)	Total activity	Specific activity (Units/mg)	Purification fold	% Yield
Crude	3000	3515.31	0.16	480	0.14	1	100
Ammonium sulphate 80-90%	350	171.96	0.66	231	1.34	9.8	48.1
Ultra-Filtration	10	14.47	27.81	278.10	19.22	140.7	57.9

SDS-PAGE was used to determine the molecular weight of the laccase enzyme. The estimated weight was confirmed through native PAGE (L1 and L2) (Figure 3.2). The molecular weight of the enzyme was estimated to be 66 kDa (Figure 3.2A). Interestingly two bands were observed (Figure 3.2B), one band approximately146 kDa and the other approximately 66 kDa for both L1 and L2 (L1 0.3 mg/ml and L2 0.4 mg/ml enzyme concentration). Adelakun et al., (2012b) isolated a laccase from *T. pubescens* which had a molecular weight of 65 kDa. The other 146 kDa laccase band could perhaps be a dimer of the laccase enzyme (Thurston, 1994).

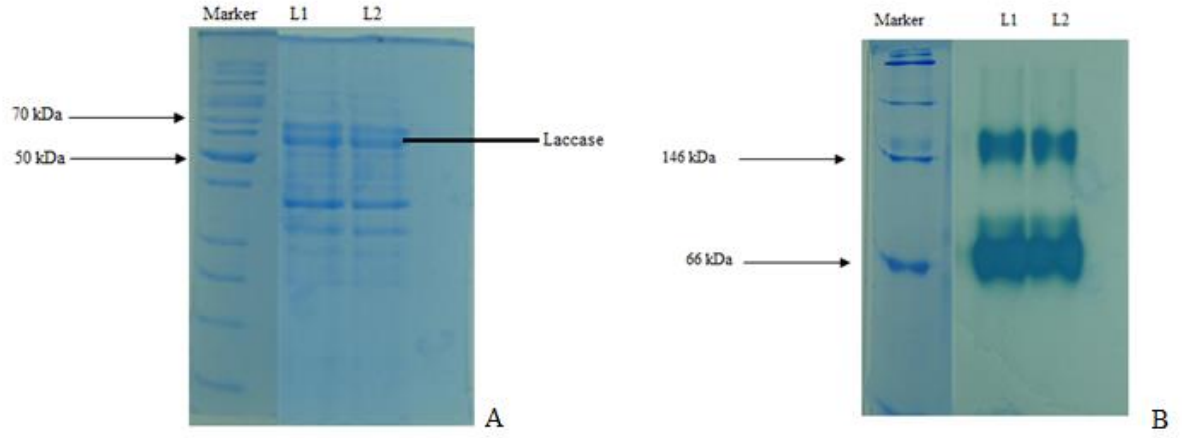


Figure 3.2: (A) SDS PAGE of *Trametes pubescens* laccase (B) Zymogram of laccase from *T. pubescens*.

3.4.2. Partial characterisation of *T. pubescens* laccase

The characterisation of *T. pubescens* laccase was performed in order to determine optimum reaction conditions. In this study, the focus was on pH, temperature and effect of co-solvent, which significantly impact enzyme function during coupling reactions.

3.4.2.1. Reaction conditions

The pH optima for the oxidation of selected substrates by laccase were substrate dependent and ranged between pH 4.0 and 5.0 (Figure 3.3). Ferulic acid and 2,6-dimethoxyphenol had a pH optimum of 4.5; guaiacol and caffeic acid pH 4.0, and syringaldazine was optimally oxidized at pH 5.0. Kudanga et al. (2009) also observed that pH optimum of a laccase from *Bacillus subtilis* spores was substrate dependent.

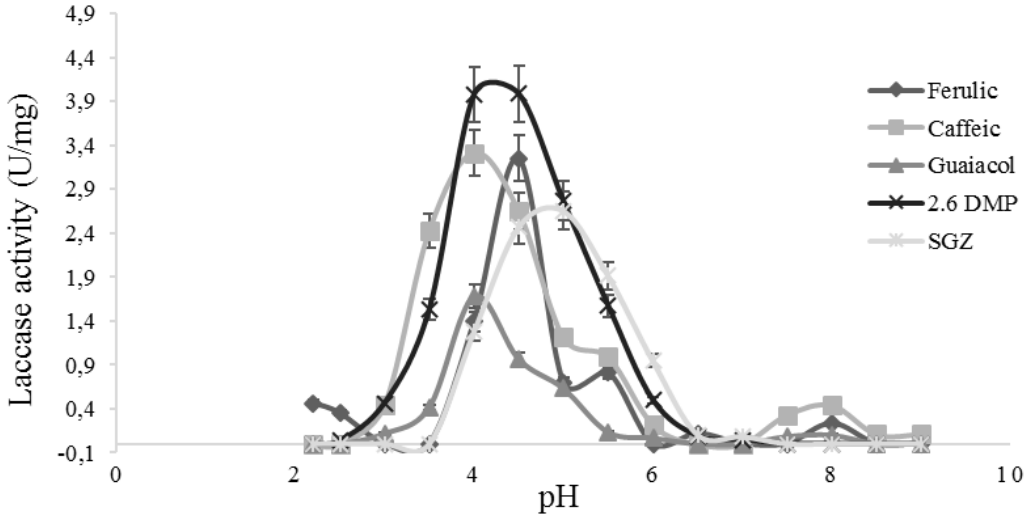


Figure 3.3: Effect of pH on enzyme activity. Results are presented as mean ± standard deviation of three replicate determinations.

The enzyme was more active in the acidic region. These results are consistent with the common observation that fungal laccases require acidic conditions whereas bacterial laccases are usually active in the neutral to alkaline conditions (Kudanga et al., 2009).

The enzyme retained 80% residual activity after 4 hours of incubation in the pH range 4.0-8.0 (Figure 3.4). This indicates that the enzyme can be utilised for the mediation of coupling reactions in both acidic and alkaline conditions without losing much activity.

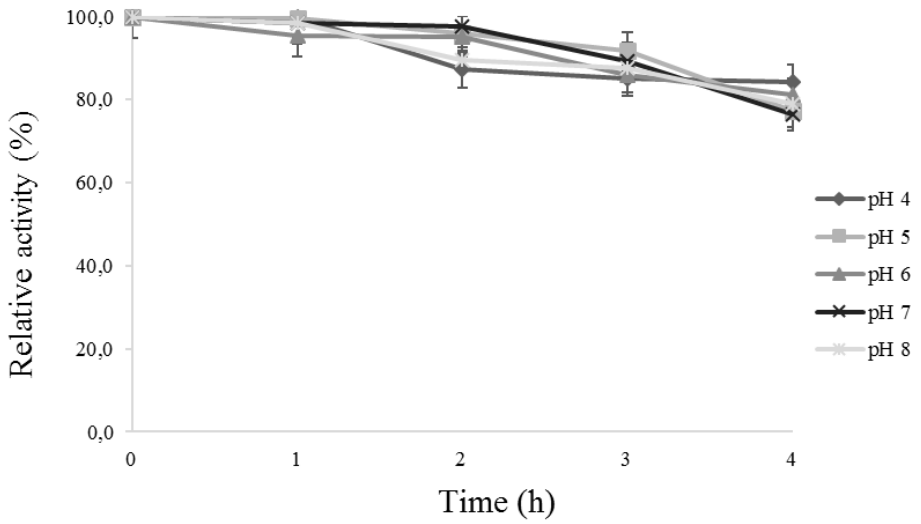


Figure 3.4: pH stability of *T. pubescens* laccase (activity at 100% 4.53 U/mg). Results are presented as mean ± standard deviation of three replicate determinations.

Temperature optimum and stability of the enzyme was assessed to determine the best reaction temperature for the enzyme. The enzyme had an optimum temperature of 60˚C (Figure 3.5) but was not stable at this temperature. The possible reason for the enzyme not being stable at its optimum temperature could be due to the assay reaction time, which only runs for 120 seconds whereas the enzyme was incubated for 3 hours when monitoring stability. Another factor could be that this enzyme is from a mesophilic fungus and not a thermostable fungus.

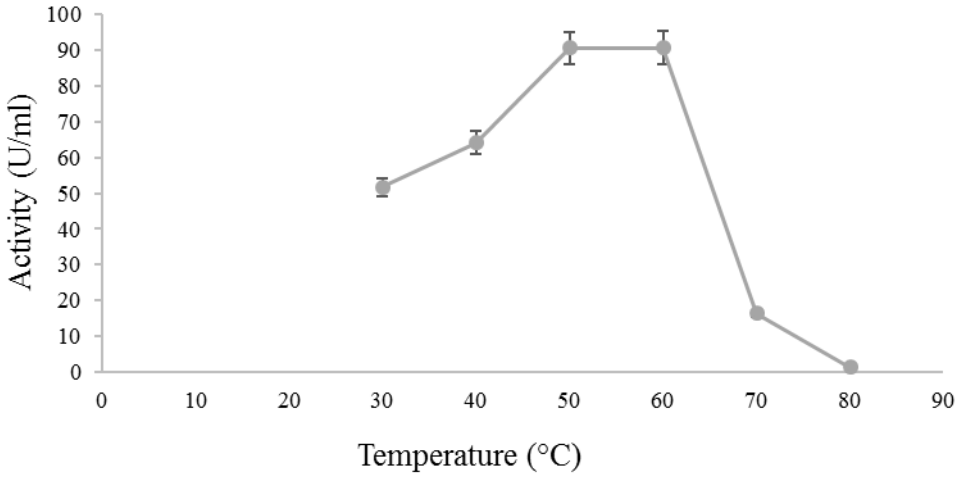


Figure 3.5: Effect of temperature on enzyme activity. Results are presented as mean ± standard deviation of three replicate determinations.

However, the enzyme retained over 95% activity at 40˚C for 2 hours (Figure 3.6) and the activity at 40˚C was not significantly lower than that at the optimum at 60˚C. Therefore, the coupling reactions were conducted at 40˚C to minimize loss of enzyme activity during the long reaction times required for the coupling reactions.

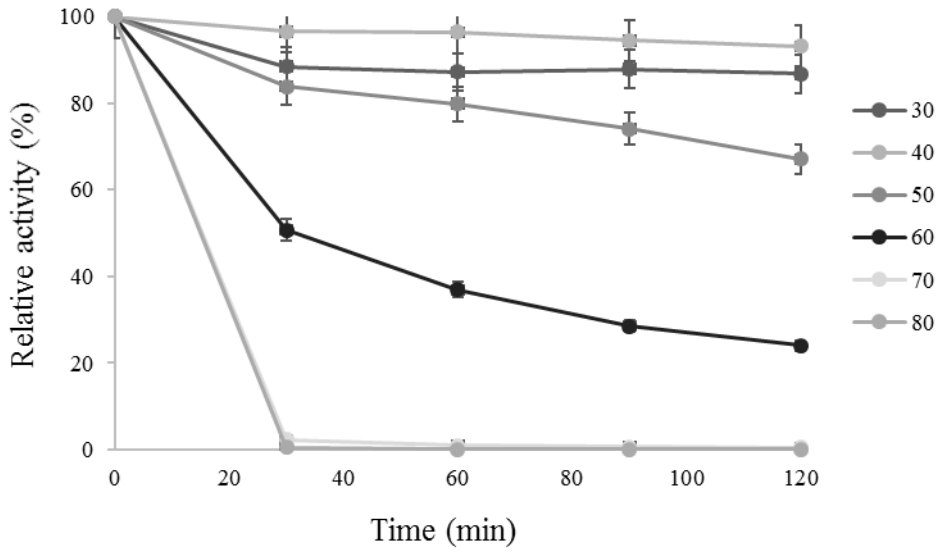


Figure 3.6: Temperature stability of laccase from *T. pubescens* (activity at 100% was 5.32 U/mg). Results are presented as mean ± standard deviation of three replicate determinations.

To establish which phenolic compounds could be used as substrates for the coupling reactions, the substrate specificity of the enzyme was established through the oxidation of various phenolic compounds. Enzyme activity is directly proportional to substrate specificity of the enzyme, therefore the higher the absorbance, the greater the substrate specificity. The highest activity was observed when guaiacol was used as a substrate (Figure 3.7). *ρ*-Coumaric acid and cinnamic acid were not easily oxidized. This may be attributed to carboxyl groups which are electron-withdrawing moieties and tend to make the molecules poor laccase substrates (Kudanga et al., 2009). Electron-donating methoxy groups seem to counteract the effect of electron withdrawing groups (Kudanga et al., 2009); this could be a possible reason for the slightly better activity on vanillic acid (Figure 3.7).

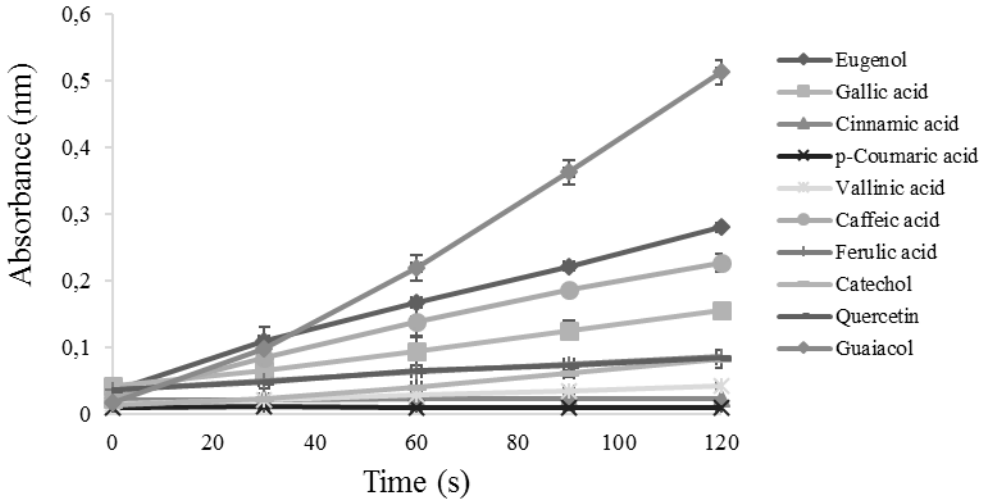


Figure 3.7: Substrate specificity of *T. pubescens* laccase. Results are presented as mean ± standard deviation of three replicate determinations.

Biocatalysis has been traditionally conducted in aqueous media. This was due to the perception that water played a major role in the activity, conformational flexibility and stability of the enzyme (Klibanov, 1989). According to Halling (1994), it was observed that it is the amount of water that is bound to the enzyme, due to water activity in the medium, which determine the activity of the enzyme. However, poor solubility of many organic compounds in aqueous media has led to the development of nonconventional media such as organic solvents for biocatalysis (Leon et al., 1998). Furthermore, the removal of water from large-scale reaction medium is laborious and expensive due to its high heat of evaporation and high boiling point is another challenge that has partly been solved using organic media in biocatalysis (Sellek and Chaudhuri, 1999). Biphasic systems can also increase the usefulness of enzymes in industry by providing an aqueous phase which maintains enzyme activity while the organic phase facilitates product recovery (Sellek and Chaudhuri, 1999). Therefore, in this study, the effect of organic solvents on enzyme activity was investigated to determine the most suitable organic solvent to be applied in the coupling reactions. Water-miscible and immiscible solvents were investigated. The enzyme retained up to 80% residual activity after 5 hours in DMSO, ethyl

acetate and dioxane, whereas the enzyme lost 60% activity after 1 hour in ethanol and methanol (Figure 3.8). The loss of enzyme activity in ethanol and methanol after only 1 hour could be due to the denaturing of the enzyme by the organic solvents. Methanol has been applied to precipitate proteins in coupling reactions (Adelakun et al., 2012b). Ethyl acetate is a water immiscible organic solvent and created a biphasic system, therefore the enzyme was not affected by the solvent since it was in the polar system whereas the solvent was non-polar.

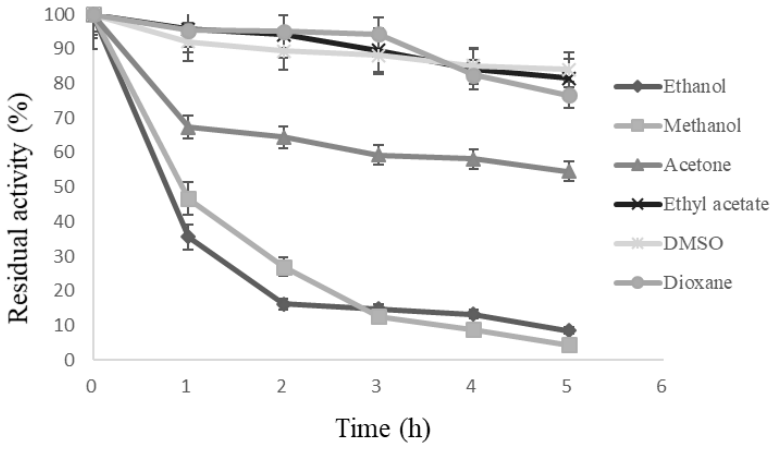


Figure 3.8: Effect of organic solvents on enzyme activity (activity at 100% was 4.76 U/mg). Results are presented as mean ± standard deviation of three replicate determinations.

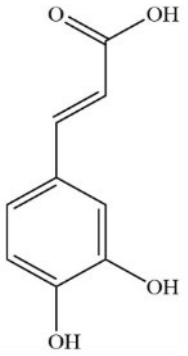
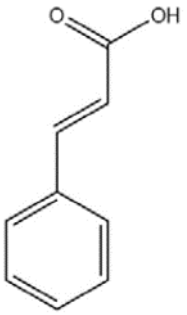
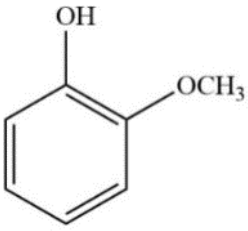
3.4.3. Preliminary coupling reactions

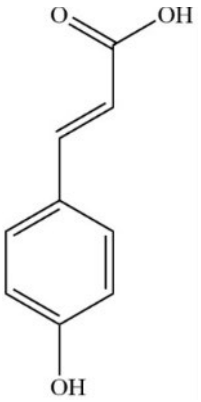
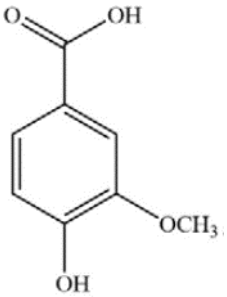
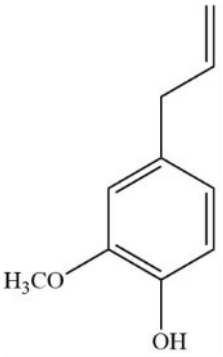
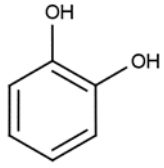
3.4.3.1. Substrate screening through oxidation reactions

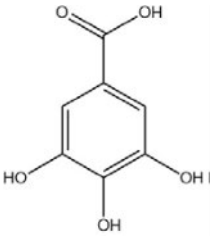
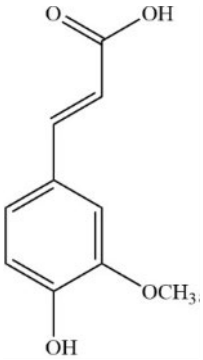
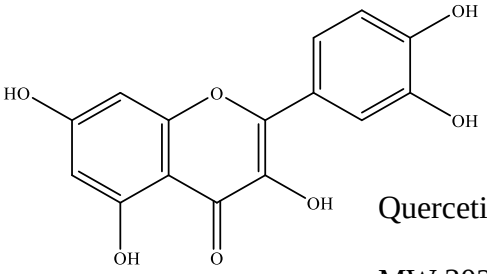
The application of bioactive compounds in various industries has led to a surge in the value of these compounds. The application of bioactive compounds in industries has brought about a demand for economically efficient methods to produce bioactive compounds, thus the escalation in the exploration of enzymatic approaches in biosynthesis (Kudanga et al., 2017). The wide substrate range of laccases, coupled with its eco-friendly catalytic mechanism,

provides a perfect enzyme that can be applied in biosynthesis. In this study, laccase from *T. pubescens* was investigated for potential oxidation of 10 phenolic substrates (Table 3.2).

Table 3.2: Phenolic substrates screened for oxidation by laccase from *T. pubescens*

Substrate	Reaction outcome	LC-MS products (*m/z*)	Remarks
 Caffeic acid MW 180	(+)	357 dimer	Substrate oxidized by laccase due to the structure configuration of caffeic acid, particularly the diphenolic structure.
 Cinnamic acid MW 148	(-)	293 dimer	Lack of phenolic groups or electron donating groups possibly makes it difficult to oxidise cinnamic acid.
 Guaiacol MW 124	(+)	245 dimer 367 trimer	The electron donating ortho methoxy group makes guaiacol easily oxidisable by laccases (Kudanga et al., 2017)

 *ρ*-Coumaric acid MW 164	(+)	325 dimer	*ρ*-coumaric acid was oxidized by laccase, this could be due to the unsaturated C-C between the carboxylic group and the phenolic ring.
 Vanillic acid MW 168	(+)	333 dimer	Vanillic acid was easily oxidized due to the electron donating methoxy group which counteracts the effects of the carboxylic group.
 Eugenol MW 164	(+)	325 dimer	Eugenol was easily oxidized by laccase because of the electron donating moieties such as methoxy group.
 Catechol MW 110	(+)	None	Catechol was easily oxidized, however, it easily polymerises and products precipitated out.

 Gallic acid MW 170	(+)	None	Gallic acid reacted in similar manner as catechol. Polymerisation occurred very quickly and products precipitated out.
 Ferulic acid MW 194	(+)	385 dimer	Ferulic acid was oxidized. This could be due to its electron donating moieties such as OH and OCH_3 groups.
 Quercetin MW 302	(+)	601 dimer	Quercetin was readily oxidized by laccase to produce a product that was detected by the LC-MS

(+) Positive reaction – Substrate oxidised by laccase
(-) Negative reaction – Substrate not oxidised by laccase

Positive reactions from Table 3.2 showing product formation on TLC were further investigated. However, only the products from eugenol, coumaric acid and quercetin could be isolated.

3.4.3.2. Purification and analysis of reaction products

For this study, TLC was used as the main separation method. In organic synthesis, TLC has remained the most cost-effective and efficient separating method in the purification of organic

compounds. The separation of compounds with TLC is based on the interaction between the mobile phase (running solvent) and stationary phase (silica gel). The silica gel matrix on the plates makes the stationary phase polar. Silica gel 60 F_{254} TLC plates were applied in this study. There are 3 aspects that need to be taken into consideration when using TLC as mode of separation. Firstly, the size and polarity of the organic molecule, the polarity of the stationary phase and lastly the polarity of the mobile phase. In this study, the polarity of the mobile phase was the only aspect that could be controlled and modified in order to obtain good separation, since the compounds of interest (the products) were new, and their polarity and molecular weight were unknown. Several solvent systems were used in attempt to separate the products from the substrates. The solvent system that was effective for the separation of products of quercetin hydrate and *ρ*-coumaric acid oxidation was toluene: ethyl acetate: formic acid (7:5:1, v/v/v) and for eugenol it was toluene: dioxane: acetic acid (11:2.5:0.4, v/v/v). The following sections of this chapter will discuss the results obtained from the oxidation reaction of quercetin hydrate and *ρ*-coumaric acid. Results on the oxidation reaction of eugenol will be reported in chapter 4.

3.4.3.3. Characterisation of quercetin hydrate oxidation product

Quercetin is an aglycone derived from plants. Quercetin has been applied as a dietary supplement and is considered to be beneficial in the fight against several diseases, such as cardiovascular diseases, diabetes, high blood pressure and cancer. It is also usually applied as an antioxidant. Quercetin is a yellow powder that is slightly soluble in hot aqueous medium, insoluble in cold aqueous medium but soluble in organic medium. Therefore, the oxidation of quercetin was conducted in organic medium. Organic solvents are commonly employed in synthetic chemistry because they provide kinetic and thermodynamic conditions that promote synthetic reactions to occur (Koskinen and Klibanov, 2012). A monophasic system comprising

of sodium acetate buffer at pH 4.5 and dioxane as the miscible co-solvent was used in the oxidation of quercetin. The oxidation of quercetin hydrate resulted in the formation of one distinct product with an R_f of 0.68 (Figure 3.9) on TLC and t_R of 13.567 (Figure 3.10) when analysed by HPLC.

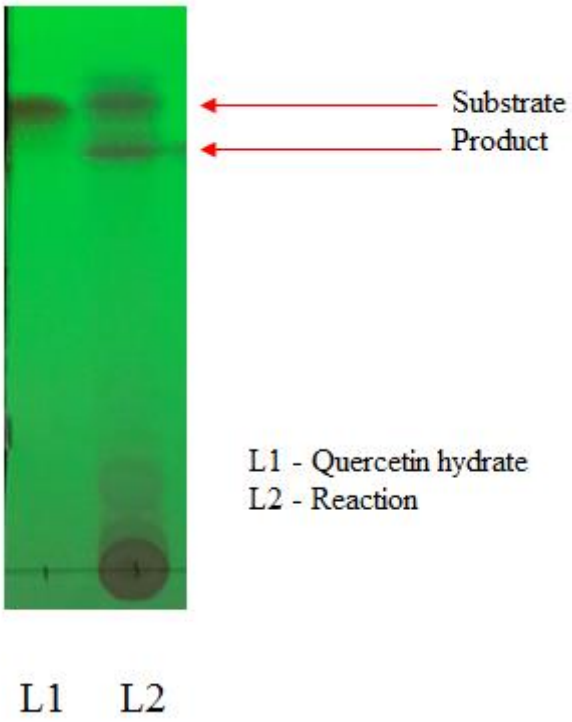


Figure 3.9: TLC plate of the oxidation reaction of quercetin.

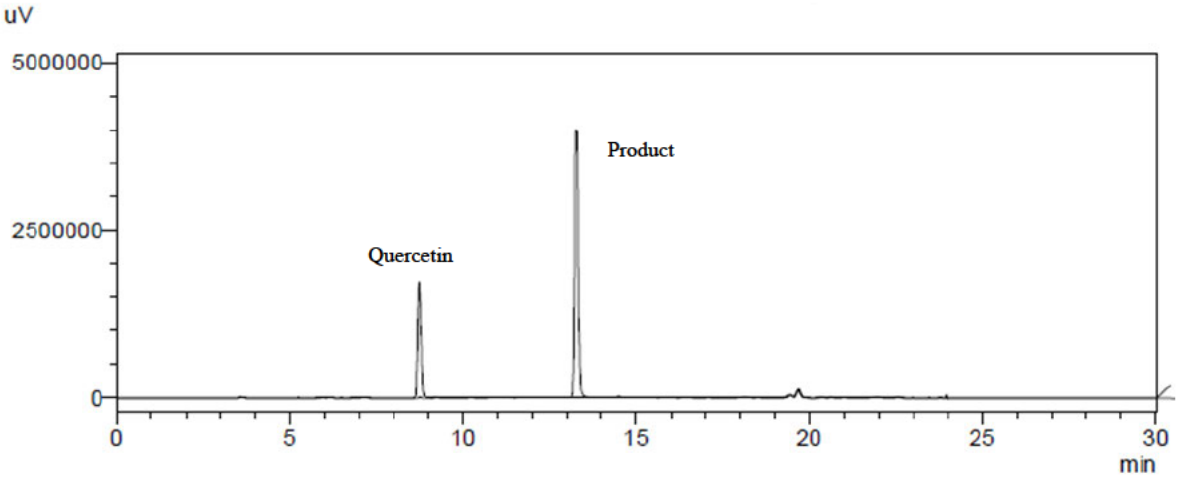


Figure 3.10: HPLC chromatogram of product formed during laccase-catalysed oxidation of quercetin.

LC-MS spectra of the quercetin oxidation product showed intensities at *m/z* 601 in the negative mode, suggesting that a dimeric form of quercetin was produced (Figure 3.11).

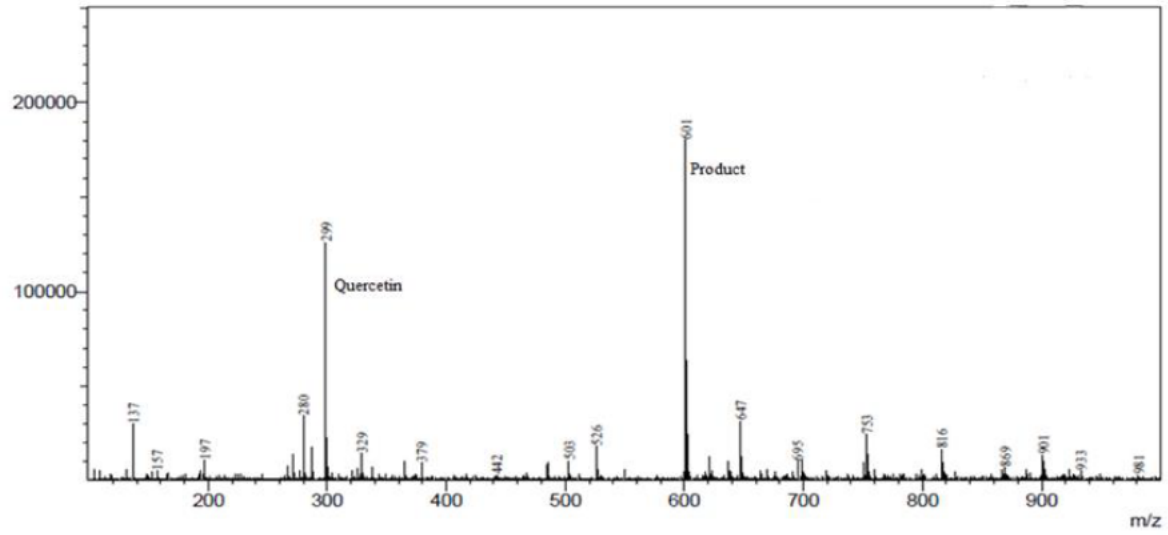


Figure 3.11: Mass spectrum of product formed during laccase-mediated oxidation of quercetin.

Based on the LC-MS results, the product structure was predicted (Figure 3.12). The LC-MS results assisted in the prediction of the possible structure of the produced product. The predicted structure of quercetin was suggested based on the hydroxyl groups at positions 3' and 4' that

are ortho directing, the steric hindrance at position 6 and the existence of a free 5' carbon. This suggests the formation of a C-C (5', 5') bond. From the predicted structure, a proposed reaction pathway was deduced (Figure 3.12). The laccase enzyme oxidises the quercetin para-hydroxyl group to produce a phenoxy radical. Through resonance stabilisation, various radicals can be produced when the unpaired electron occupies different positions on the radical. This would then be followed by a non-enzymatic reaction, whereby the produced radicals couple together and are stabilised to form dimers of quercetin.

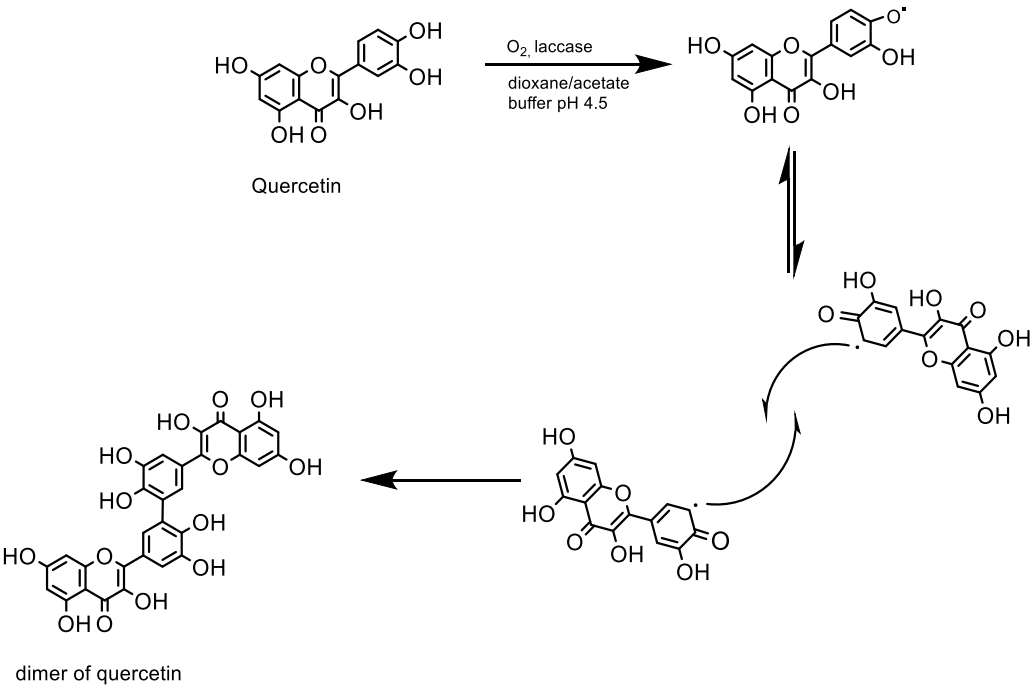


Figure 3.12: Proposed reaction pathway for the formation of the quercetin dimer.

3.4.3.4. Characterisation of ρ-coumaric acid oxidation product

ρ-Coumaric acid is a hydroxycinnamic acid that is abundant in fruits and vegetables. Together with ferulic acid and caffeic acid, the three compounds account for almost a third of the phenolic compounds in our dietary supplements. These hydroxycinnamic acids are potent

antioxidants that act as radical scavengers (Teixeira et al., 2013). Their scavenging ability stems from their electron or hydrogen donating potential, as well as their ability to delocalise the produced phenoxyl radical inside their structure (Teixeira et al., 2013). Oxidation of *ρ*-coumaric acid was conducted to observe if there would be an improvement in antioxidant capacity after coupling.

The oxidation of *ρ*-coumaric acid was conducted in a monophasic system, as previously described in 3.4.3.3. The oxidation of *ρ*-coumaric acid resulted in the production of two products (P1 and P2) with the R_f of 0.47 for P1 and R_f of 0.42 for P2 (Figure 3.13). The product P1 could not be successfully purified, therefore only further characterisation of P2 was conducted. P2 had a retention time of 11.295 (Figure 3.14).

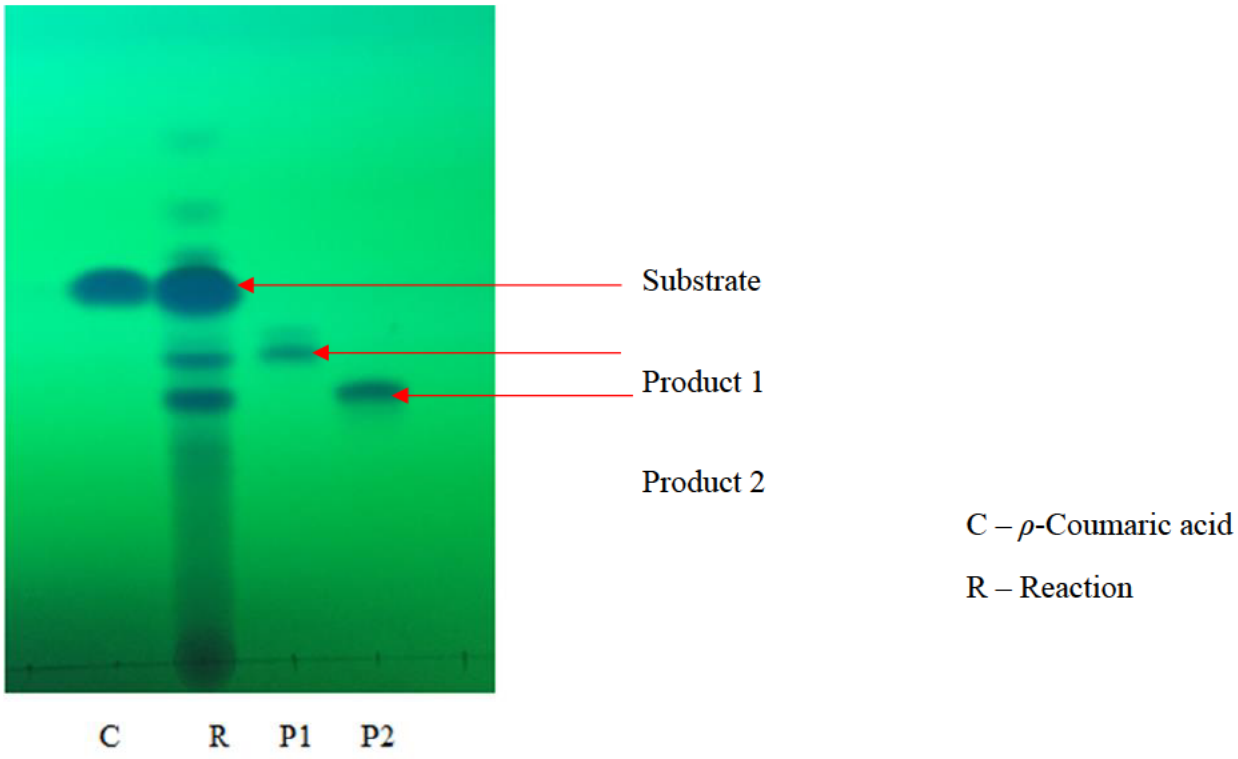


Figure 3.13: TLC plate of the oxidation products of *ρ*-coumaric acid.

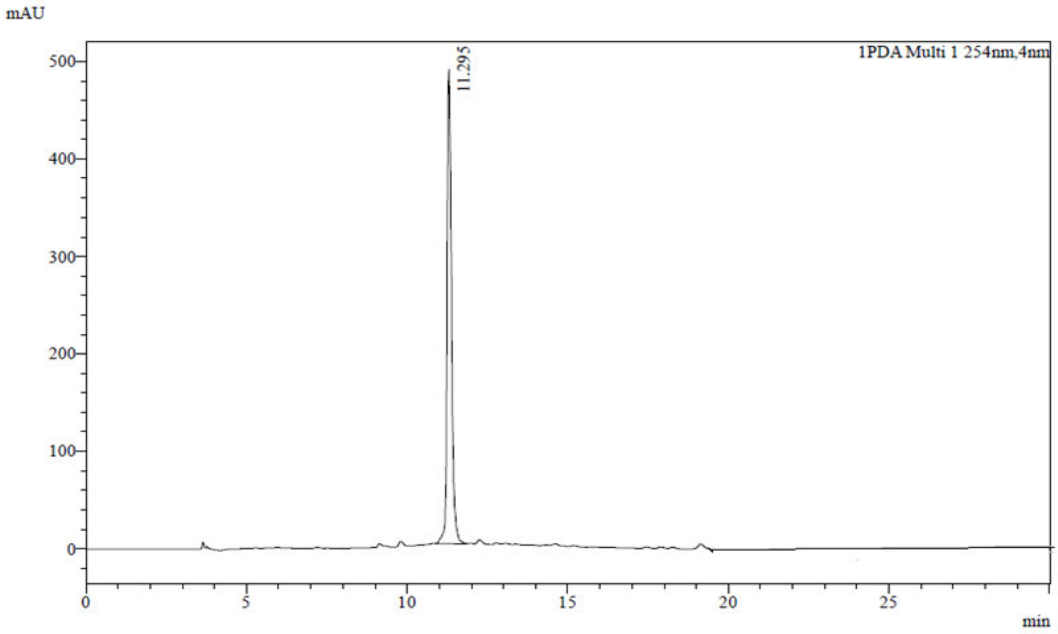


Figure 3.14: HPLC chromatogram of product formed during laccase-catalysed oxidation of *ρ*-coumaric acid.

LCMS analysis of P2 conducted in the negative mode showed *m/z* 325 (Figure 3.15), which indicated that the product was a dimer of *ρ*-coumaric acid.

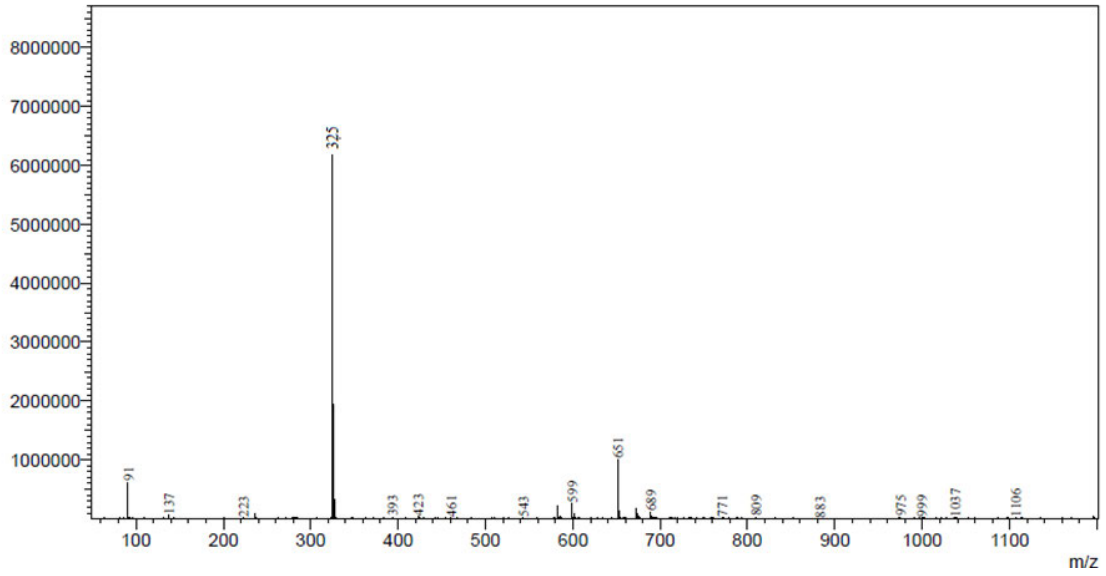


Figure 3.15: Mass spectrum of product formed during laccase-mediated oxidation of *p*-coumaric acid.

According to literature, the coupling of phenolic acids usually results in the formation of β-5 and β-β linkages due to the stability of C-C bonds and lower heat of formation of C-C linkages (Adelakun et al., 2012a, Nemadziva et al., 2018). Therefore, it can be predicted that the oxidation of *p*-coumaric acid could result in the formation of β-5 or β-β dimers. The laccase initiates the reaction by the removal of an electron from para-hydroxyl group to produce a radical. The unpaired electron can occupy different positions on the radical, through resonance stabilisation (Kudanga et al., 2011). The radicals are eventually stabilised through dimer formation, whereby an unpaired electron at the β position of one radical couples with another unpaired electron at the C5 of another radical to yield a β-5 linked dimer. With regards to the β-β linked dimers, covalent coupling of radicals with unpaired electrons at the β position will yield β-β linked dimers. Therefore, by using literature and the LC-MS results, the product structures and reaction pathways could be predicted (Figure 3.16).

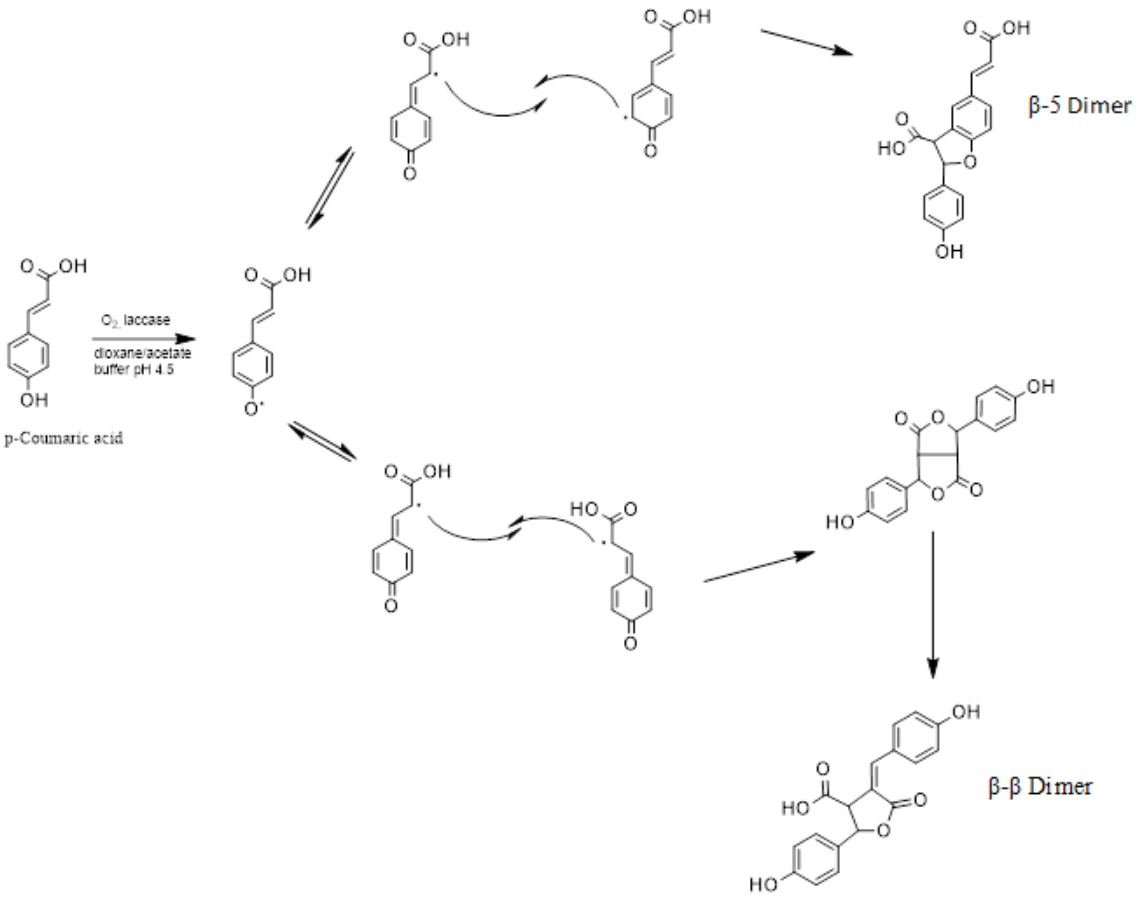


Figure 3.16: Proposed laccase-catalysed reaction pathway for the production of β-5 and β-β dimers.

3.4.3.5. Antioxidant properties

The antioxidant capacity of the *ρ*-coumaric acid oxidation product was compared to the monomeric form using ABTS, DPPH and FRAP assays. The ABTS and DPPH antioxidant assays are based on the radical scavenging potential of an antioxidant to reduce the ABTS•+ and the DPPH• radicals to their stable forms (Kilic and Yesiloglu, 2013). The principle of the Ferric reducing antioxidant power (FRAP) assay is based on the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) by an electron donating antioxidant or a reducing agent, which forms an iron-colorimetric probe complex (Fe^{2+}-TPTZ) with a distinct blue colour upon reduction and can be measured with a spectrophotometer at 593 nm (Gulcin et al., 2004). In comparison to the substrate, the product showed a 2-fold, 3-fold and 6-fold increase in antioxidant capacity

as measured by the ABTS, DPPH and FRAP assays, respectively (Table 3.3). The increase in antioxidant capacity could be attributed to the structure of the product, whereby there was an increase in the availability of electron donating groups attached to the aromatic ring as well as the formation of stable radicals (Kudanga et al., 2017). Therefore, the produced product could have been the β-β dimer (Figure 3.16), which has 1 extra OH group compared to the monomer.

Table 3.3: Antioxidant activity of *ρ*-Coumaric acid and its oxidation product (P2) determined using the DPPH, ABTS and FRAP assays. All results are means ± standard deviation of three replicate determinations

ABTS IC_{50} (µM) [a]		DPPH IC_{50} (µM) [a]		FRAP (µM) [b]	
ρ-Coumaric acid	Product	*ρ*-Coumaric acid	Product	*ρ*-Coumaric acid	Product
156.02 ± 0.050	95.84 ± 0.0134	1556.92 ± 0.024	529.06 ± 0.041	17 ± 0.036	97 ± 0.032

[a]Concentration (µM) of *ρ*-Coumaric acid or product required to reduce ABTS or DPPH radical by 50%. [b]The FRAP value of *ρ*-Coumaric acid and product in µM equivalent to the amount of Fe^{3+} converted to Fe^{2+} by 1000 µM of sample.

3.5. Conclusion

A 66 kDa laccase with relatively high activity was successfully produced from *T. pubescens*. The enzyme had optimum pH between 4 and 5 depending on the substrate. The optimum temperature was 60^{o}C and it was stable over a wide pH range at 40^{o}C. The enzyme showed high specificity for guaiacol and retained 80% activity after 5 hours of incubation in 50% v/v DMSO, ethyl acetate and dioxane reaction systems but was less stable in acetone, ethanol and methanol reaction systems. Laccase-catalysed coupling reactions in a monophasic system produced dimeric products when quercetin and *p*-coumaric acid were used as substrates. The purified *ρ*-coumaric acid oxidation product (*m/z 325*) showed a 2-fold, 3-fold and 6-fold increase in antioxidant capacity compared with the substrate, as measured by the ABTS, DPPH

and FRAP assays, respectively. The products have potential for application in the pharmaceutical, food and cosmetic industries as alternative sources of antioxidants.

4. LACCASE CATALYSED DIMERISATION OF EUGENOL IMPROVES ANTIOXIDANT ACTIVITY

4.1. Abstract

Enzymatic modification of naturally occurring phenolic compounds can potentially improve their bioactivity. In this study laccase-mediated modification of eugenol to improve its antioxidant capacity was investigated. In a monophasic system, a symmetrical 5-5 eugenol dimer (*m/z* 325; [M]=326), *bis*-Eugenol, was produced. Maximum product formation was obtained in a monophasic system with 40% v/v dioxane as co-solvent. Free radical scavenging activity of the dimer was four and three times higher than the monomeric form of eugenol when measured against DPPH radical and ABTS radical, respectively. The high antioxidant capacity of the eugenol dimer can be potentially exploited as an alternative to synthetic and low potency natural antioxidants in the pharmaceutical and cosmetic industries.

4.2. Introduction

Natural plant based phenolic compounds have gained considerable interest in recent studies due to their various biological properties such as antioxidant, anticancer, antibacterial and anti-inflammatory activities. One phenolic compound that has received wider recognition for its biological properties is eugenol.

Eugenol (4-allyl-2-methyoxyphenol) is commonly found in essential oils such as clove oil, camphorated oil, cinnamon leaf oil, and nutmeg oil and is slightly soluble in aqueous media but easily dissolved in organic media. It is a natural phenol with antipyretic, pain relieving, calming and sedative effects, antioxidant, antifungal, antibacterial, anticancer, as well as pest repellent properties (Kong et al., 2014). A dietary daily intake of 2.5 mg/kg per normal human

body weight has been deemed tolerable by both the World Health Organisation (WHO) and the Food and Agriculture Organisation (FAO) (Charan Raja, 2015). Eugenol has been acknowledged by the U.S. Food and Drug Administration (FDA) as a safe phenolic compound, without any mutagenic and carcinogenic properties. Therefore, it has been applied as a main component in flavouring agents, cosmetics and perfumes and in dentistry for treating toothaches (Bendre et al., 2016). However, at present, the main therapeutic potential of eugenol is its free radical scavenging activity. The free radical scavenging properties of eugenol is ascribed mainly to the allyl and phenolic groups in its molecular structure. Eugenol can prevent functional disorders of endothelial cells resulting from oxidized low-density lipoproteins by inhibiting the production of active oxygen species (Ou et al., 2006). Similarly, eugenol has been shown to reverse oxidative injury, protect against N-methyl-D-aspartate induced neurotoxicity (Wie et al. , 1997), as well as inhibit peroxidation of linoleic acid (Gulcin et al., 2004).

However, pro-oxidant properties have been observed through the formation of free radicals as the concentration of eugenol is increased (Charan Raja, 2015). These harmful phenoxyl radicals can lead to oxidative stress which has been linked to the initiation of chronic diseases such as diabetes, cardiovascular diseases and cancer (Lobo et al., 2010). The antioxidant and pro-oxidant activities of o-methoxyphenols are dependent on their metal-reducing potential, chelating behaviour, pH and solubility characteristics (Atsumi et al., 2000). However, enzymatic oligomerisation of these o-methoxyphenols has been shown to enhance their antioxidant capacity by (Adelakun et al., 2012a), which may negate the need to use them at high concentrations. Biotransformation of eugenol has been mainly conducted through chemical processes. The use of copper chloride, trimethylenediamine, iron chloride as shown in the study by Fujisawa et al. (2007) and ammonium hydroxide in another study by Guntero

et al. (2018) as catalysts for the biotransformation of eugenol has been reported. However, the application of inorganic reagents such as Zn, Fe, Mg, Na, chromium (VI), permanganate and manganese dioxide, in organic synthesis is one of the major causes of accumulation of environmental wastes (Kim Bong-Gyn et al., 2012). Acids that are applied in the downstream process (product recovery) are also another source of environmental pollutants (Kim Bong-Gyn et al., 2012). Consequently, green catalysis is now receiving wider interest as an alternative to chemical processes. For instance, plant cells have been used in the oxidation of eugenol, however, culturing of the plant cells is time consuming (Sheldon and Woodley, 2018). Processes that employ isolated enzymes in particular peroxidases have been applied in the oxidation of eugenol to form dimers (Thompson et al., 1989, Krawczyk et al., 1991, Anita et al., 2015). However, peroxidases require hydrogen peroxide as the co-factor which makes the process expensive and a potential health hazard (Pandey et al., 2017). Therefore, many researchers have shifted their attention to laccases as biocatalysts for the synthesis of bioactive compounds (Kudanga et al., 2017).

Unlike peroxidases, laccases (EC 1.10.3.2) are enzymes that oxidise phenols and related compounds to form radicals, without the need of a co-factor and produce water as the only by-product. They are therefore, generally regarded as eco-friendly enzymes (Kudanga et al., 2011). The coupling of the radicals formed can be used to generate oligomeric antioxidants. Several studies have reported enhancement of antioxidant capacity through laccase-mediated oligomerisation of monomeric phenols. A comprehensive review of compounds modified for improving bioactivity has been recently published (Kudanga et al., 2017). The synthesis of the enhanced antioxidant is based on the principle that oligomerisation supplements the product with additional hydroxyl groups and electron donating groups as an outcome of the repeated monomeric units (Wolfe and Liu, 2008). Therefore, the aim for this study was to apply the

Trametes pubescens laccase in the dimerisation of eugenol as a way of improving antioxidant capacity.

4.3. Materials and Methods

4.3.1. Chemicals and enzyme

Please refer to *Chapter 3 Section 3.3.1*

4.3.2. Enzyme activity

Laccase activity was determined in triplicate at 25 °C in 2 mL cuvettes, by monitoring the increase in absorbance at 419nm (ε=36000 $M^{-1}cm^{-1}$), using a UV-VIS spectrophotometer and 2,2'-Azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as substrate following method by Adelakun et al. (2012). The assay mixture contained 0.057 ml enzyme, 0.110 ml of 5 mM ABTS in 0.833 ml of 50 mM sodium acetate buffer pH 4.0. One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per minute at 25 °C.

4.3.3. Oxidation of eugenol

The oxidation reactions were conducted in a monophasic system comprising sodium acetate buffer pH 4.5 with miscible solvents (dioxane, methanol, ethanol or acetone) as co-solvents. The miscible solvents were used at 70%; the concentration at which all the solvents produced the dimer with minimal polymerisation side reactions. Due to inactivation of the enzyme by solvents, the enzyme (0.632 U) was added to the reaction mixture every 6 hours. The reactions were carried out for 24 hours at 37 ^{o}C in a shaking incubator at 200 rpm and monitored by Thin Layer Chromatography (TLC).

4.3.4. Effect of solvent concentration and reaction time

Having established the best solvent (dioxane), the effect of different percentages (20-90%, v/v) of the co-solvent was investigated. The reactions were carried out in sodium acetate buffer pH 4.5, at 37^{o}C, shaking at 200 rpm for 24 hours. Once the optimum organic solvent concentration was established, the reaction time which gave the maximum product yield was determined by monitoring the reaction every 6 hours over a 24-hour period. As in the previous section, enzyme (0.632 U) was added every 6 hours.

4.3.5. Chromatographic separation of reaction product

TLC analysis was performed on aluminium-backed silica gel 60 F254 (Merck) plates using toluene: dioxane: acetic acid (11:2.5:0.4, v/v/v) as the mobile phase. The compounds were then visualised by exposure to UV light at 254 nm.

4.3.6. High performance liquid chromatography (HPLC)

The enzyme was firstly precipitated out of the reaction solution by the addition of an equal volume of ice-cold methanol and then the mixture was incubated at 0^{o}C for 20 min and then centrifuged at 0^{o}C for 15 min at 14,000×g. The supernatant (1.5 mL aliquots) was filtered into clean vials and analysed by HPLC. HPLC analysis was carried out using a prominence HPLC system from Shimadzu (Shimadzu, Kyoto, Japan). Separation of the reaction products was carried out on a reverse phase Sunfire C18 5.0 µM, 4.6 mm×150 mm column (Waters, Warszawa, Poland). Gradient elution using 0.1% formic acid (solvent A) and acetonitrile (solvent B) was used to separate the compounds at a running time of 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 LabSolutions data manager software from Shimadzu.

4.3.7. Purification of reaction product

The reaction product was purified using preparative TLC. TLC analysis was performed on aluminium-backed silica gel 60 F254 (Merck) plates using toluene: dioxane: acetic acid (11:2.5:0.4, v/v/v) as the mobile phase. The compounds were then visualised by exposure to UV light at 254 nm. The reaction product was then scraped from TLC, dissolved in methanol and filtered to remove residual silica gel. The reaction product was further purified using TLC, applying the previously described steps.

4.3.8. Characterisation of product

The purified reaction product was characterised by mass spectrometry (LCMS) and nuclear magnetic resonance (NMR) analysis.

4.3.8.1. Liquid chromatography–mass spectrometry (LC–MS)

LC–MS was performed using a prominence HPLC system from Shimadzu coupled to a mass spectrometer from Shimadzu (LCMS 2020) (Shimadzu, Kyoto, Japan). The products were separated using the same linear gradient of acetonitrile (solvent B) and 0.1% formic acid (solvent A) as previously described, at a flow rate of 1 mL/min, using an injection volume of 10 µL and an oven temperature of 30^{o}C. MS spectra were acquired in negative mode and electrospray voltage was set to +3500 V. Dry gas flow was set to 9 L/min with a temperature of 350^{o}C and nebulizer gas pressure was set to 35 psi.

4.3.8.2. Nuclear magnetic resonance (NMR) analysis

For NMR analysis, 5-10 mg samples were dissolved in 0.5 mL CD_3OD. ^{1}H, ^{13}C and 2D NMR spectra were recorded on a Bruker Avance instrument operating at 400 MHz. Chemical shifts were reported as δ values (ppm) relative to the solvent line of CD_3OD (3.34 ppm for ^{1}H; 49.0 ppm for ^{13}C). *5,5'-diallyl-3,3'-dimethoxy-[1,1'-biphenyl]-2,2'-diol* **P2**, ^{1}H NMR (CD_3OD, 400

MHz): δ_H 6.01 (ddt, *J* = 17.0, 10.0, 7.0 Hz, H-8), 5.10 (dd, *J* = 17.0, 1.8 Hz, H-9 *trans*), 5.05 (dd, *J* = 10.0, 1.8 Hz, H-9 *cis*), 3.35 (t, *J* = 7.0 Hz, H-7), 6.76 (d, *J* = 2.1 Hz, H-2), 6.72 (d, *J* = 2.1 Hz, H-6), 3.88 (s, OCH_3). ^{13}C NMR (CD_3OD, 100 MHz): δ_C 150.1 (C-3), 144.5 (C-4), 139.6 (C-8), 131.7 (C-1), 128.3 (C-5), 124.2 (C-6), 115.5 (C-9), 111.8 (C-2), 56.5 ($O\underline{C}H_3$), 41.0 (C-7).

4.3.9. Antioxidant activity

Antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-Azinobis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS) scavenging assay and the ferric reducing antioxidant potential (FRAP) assay. Refer to *Chapter 3 Section 3.3.15*

4.4. Results and Discussion

4.4.1. Oxidation of eugenol in organic solvents

Due to the poor solubility of eugenol in aqueous media, the oxidation of eugenol was conducted in organic media. Other advantages of using organic media for the oxidation of eugenol, is that subsequent reactions are easily facilitated, which would be unlikely in an aqueous media due to kinetic restrictions. There is also ease of product recovery in organic media as compared to aqueous media. The oxidation reaction of eugenol in a monophasic system produced one distinct product (t_R 16.756) (Figure 4.1) code named EuP. The LCMS analysis of the product showed a dominant signal at *m/z* 325 in negative mode (Figure 4.2) which suggests that the product was a dimeric form of eugenol ([M] =326).

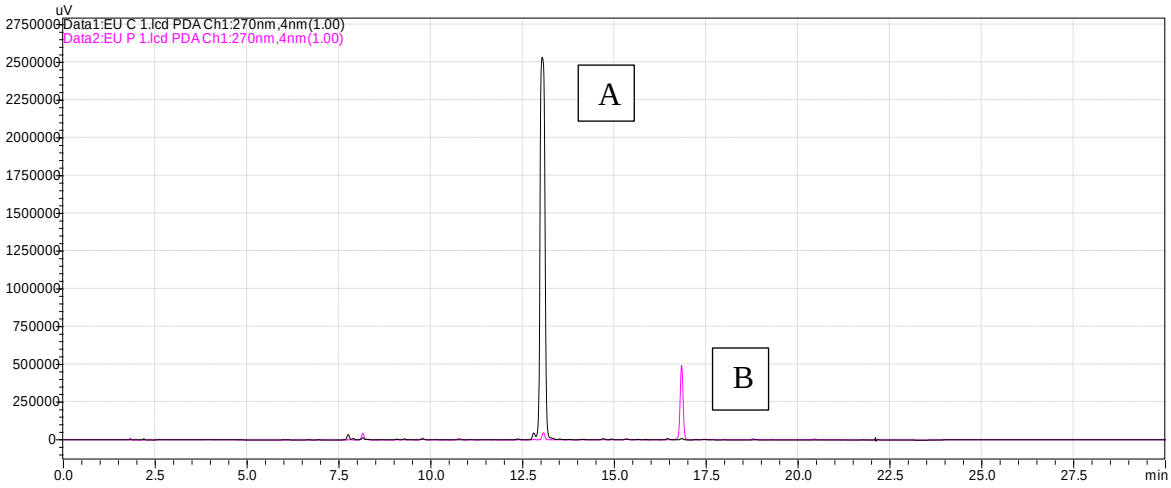


Figure 4.1: HPLC chromatogram of eugenol (A) and product (EuP) (B) formed during laccase-catalysed oxidation of eugenol.

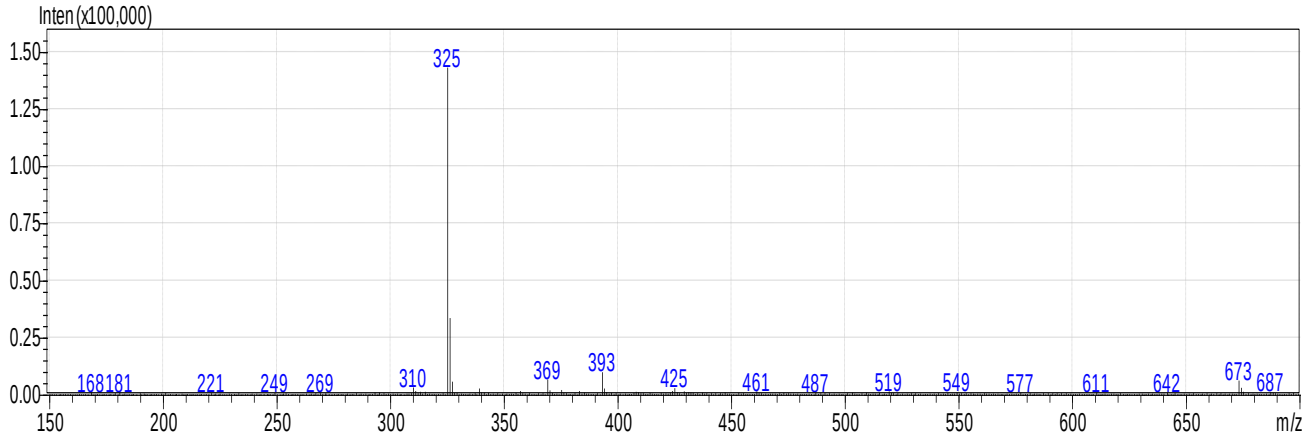


Figure 4.2: Mass spectrum of product (EuP) formed during laccase-mediated oxidation of eugenol.

4.4.2. Product characterization (NMR)

The ^{1}H NMR spectrum of EuP showed characteristic resonances for *bis*-eugenol, a symmetrical 5,5' dimer of eugenol (Figure 4.3A). The proton resonances of the allyl group could be seen distinctly at δ_H 6.01 as a ddt (H-8, J = 17.0, 10.0, 7.0 Hz), a double doublet at δ_H 5.10 (H-9 *trans*, J = 17.0, 1.8 Hz) and δ_H 5.05 (H-9 *cis*, J = 10.0, 1.8 Hz). H-7 could be seen as a triplet amidst the solvent peak at δ_H 3.35 (J = 7.0 Hz). H-2 and H-6 were seen *meta* coupled to each other as two doublets at δ_H 6.76 and 6.72 with J = 2.1 Hz. The methoxy resonance appeared

at δ_H 3.88 as a sharp singlet. Coupling between H-7 and H-8 and H-8 and H-9 in the COSY spectrum verified these assignments. The protonated carbon resonances of C-7, C-8, C-9, C-2 and C-6 were identified at δ_C 41.0, 139.6, 115.5, 111.8 and 124.2 by the HSQC spectrum. C-3 and C-4 were identified at δ_C 150.1 and δ_C 144.5 due to a HMBC correlation with H-2. C-3 was distinguished from C-4 due to a further HMBC correlation to the methoxy resonance at δ_H 3.88. C-5 was assigned to δ_C 128.3 due to a HMBC correlation with H-6 and the remaining singlet carbon resonance at δ_C 131.7 was assigned to C-1. Details of NMR analysis are shown in supplementary data (Appendix 1-7).

The formation of 5,5 dimer was not surprising. The hydroxyl group is ortho or para directing as reported by Li and Xie (2000), Jonas et al. (2000) and Suparno et al. (2005) and in the presence of a free C5 carbon, the formation of the more stable C-C (5,5) bond Schultz et al. (2001) is favoured. Similar results have been previously reported using peroxidase as enzyme (Krawczyk et al., 1991, Anita et al., 2015). Based on the results obtained from the LC-MS and NMR, a proposed reaction pathway could be deduced. As depicted in Figure 4.3B, the laccase enzyme oxidises the eugenol para-hydroxyl group to produce a phenoxy radical. Through resonance stabilisation various radicals can be produced when the unpaired electron occupies different positions on the radicalised molecule. This is followed by a non-enzymatic reaction, whereby the produced radicals couple together and are stabilised to form dimers (Thompson et al., 1989). The low heat of formation of C-C linkage compared to ether linkages as concluded by Del Rio and Gutierrez (2008) would favour the formation of the 5,5' dimer of eugenol, *bis*-eugenol (Figure 4.3B). Although peroxidase-catalysed dimerization of eugenol produced similar results reported by Krawczyk et al. (1991) and Anita et al. (2015), the requirement of hydrogen peroxide and its rapid degradation makes the process cumbersome, expensive and a potential health hazard (Valencia and Marinez, 2005, Burton, 2003). Therefore, it was not

surprising that only a 10% yield was produced by Anita et al. (2015) and 20% yield by Krawczyk et al. (1991) with peroxidase as enzyme compared to a 50% yield obtained in this study (Figure 4.6).

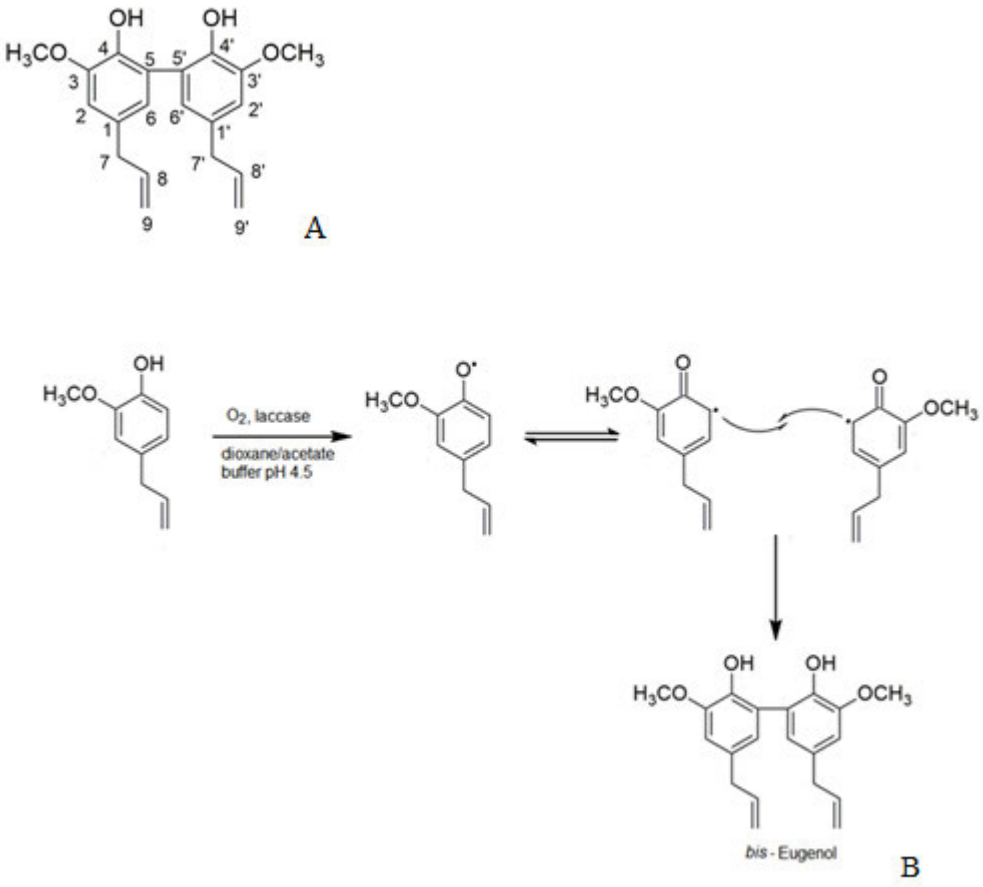


Figure 4.3: (A) 5,5' dimer of eugenol formed during laccase-mediated oxidation of eugenol and (B) Schematic diagram of laccase-mediated synthesis of 5,5' dimer of eugenol.

4.4.3. Effect of organic solvents and reaction time

Biocatalysis in organic solvents is usually favoured due to the increase in substrate solubility, inhibition of aqueous-dependent side reactions, exclusion of microbial contamination, reversal of the thermodynamic equilibrium of hydrolysis reactions and alternation of substrate specificity and enantioselectivity (Zaks and Klibanov, 1985, Klibanov et al., 1986, Kharazipour

et al., 1998). However, a major limitation of organic solvents is that, water is fundamentally required by enzymes for conformational flexibility and catalysis. Therefore, the loss of this essential water that is bound to the surface of the enzyme in organic solvent systems results in reduced enzyme activity and conformational flexibility (Doukyu and Ogino, 2010). Enzymatic activity is exceedingly reliant on the energy of substrate binding to the enzyme; the dissociation of solvent at the active site is required for substrate binding to occur (Zaks and Klibanov, 1985). In an aqueous solvent, hydrophobic substrates tend to be energetically enticed by the hydrophobic active sites found on most enzymes. The replacement of water with an organic solvent thermodynamically stabilises the grand state of the hydrophobic substrate as highlighted by Zaks and Klibanov (1985), this leads to the ease of substrate binding to the enzyme active site which is hydrophobic. Laccase is known to have a hydrophobic pocket near the T1 copper (Klibanov, 2003). Laccase-mediated synthesis of the eugenol dimer was carried out in a monophasic system. The organic co-solvents that were investigated were methanol, ethanol, dioxane and acetone. The order of maximum product formation was dioxane, methanol, followed by ethanol and then acetone (Figure 4.4). Dioxane as a co-solvent produced maximum product formation at 40% v/v (Figure 4.5). Laane et al. (1987) and Thorum et al. (2010), have suggested that several parameters such as the dielectric constant, dipole moment, hydrogen binding, polarisability and logarithm of partition coefficient (log P), can be correlated to biocatalysis in organic solvents. Organic solvents are far less able to engage in multiple hydrogen bonding as compared to water. Therefore, organic solvents hold less electric charge, due to their lower dielectric constants (Laane et al., 1987). These chemical properties can be attributed to dioxane having better product formation compared to the other organic solvents. The dielectric constant of dioxane, acetone, methanol and ethanol are 2.25, 20.7, 32.70, and 24.3 respectively. The low dielectric constant of dioxane enables slow production of the product with less chance of polymerisation by holding less electrical charge thereby slowing

the resultant non-enzymatic coupling reactions of the produced radicals. The polarity of dioxane could have also possibly contributed to the high product formation. Dioxane has a polarity index of 4.8 as compared to methanol, acetone and ethanol, with polarity indexes of 5.1, 5.6, and 5.2 respectively. The low polarity index of dioxane enables the more non-polar product to be easily formed (Figure 4.1).

Product formation increased with time (Figure 4.6). Maximum product formation was observed at 40% v/v dioxane at 18-hour incubation period with a yield of 50%. A higher yield was not obtained possibly due to incomplete substrate conversion after 18 hours of incubation. At 24 hours, polymerisation began to occur, although complete substrate conversion was achieved after 30 hours of incubation, less product was obtained due to polymerisation of the produced dimers.

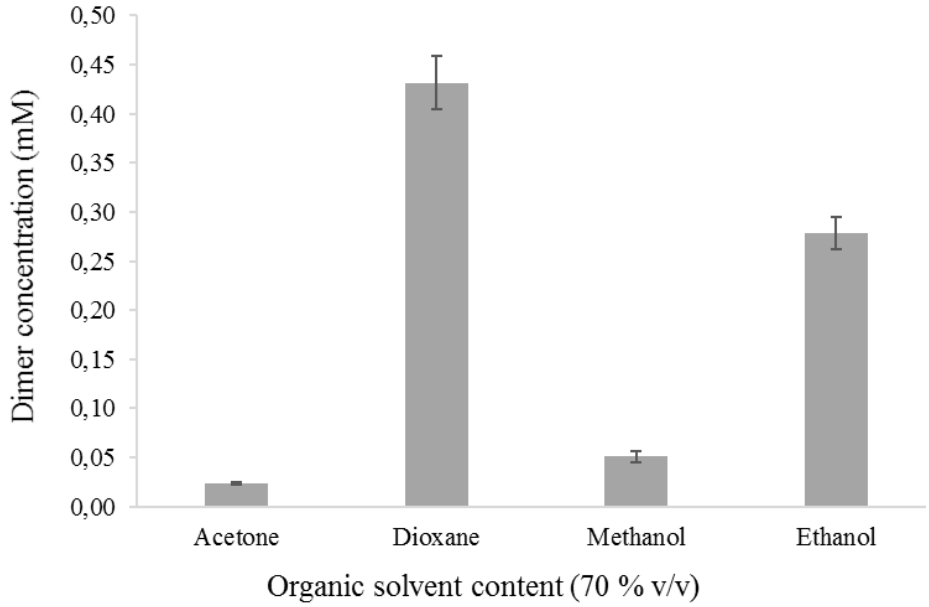


Figure 4.4: Effect of organic co-solvent on laccase-catalysed oxidation of eugenol to form EuP product. All results are means ± standard deviations of three replicate determinations.

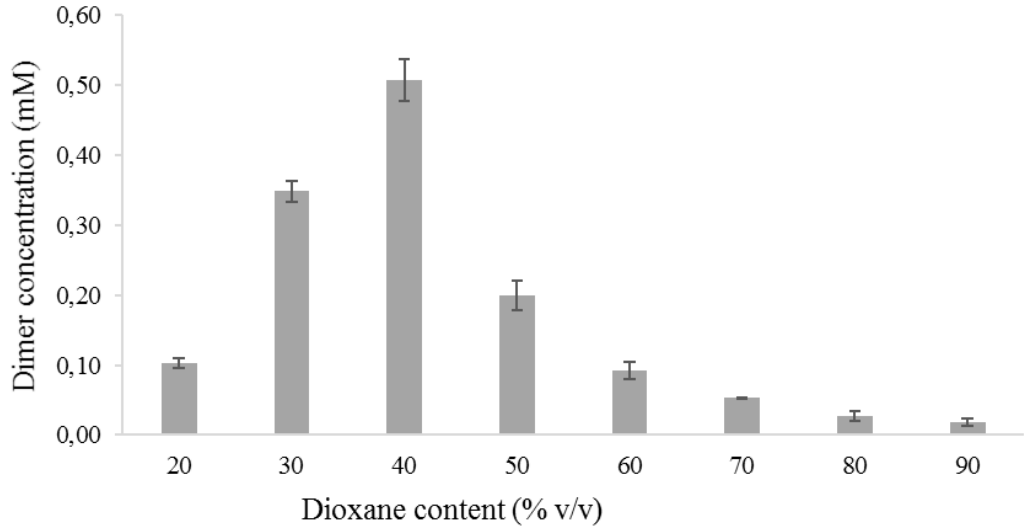


Figure 4.5: Effect of organic co-solvent % on laccase-catalysed oxidation of eugenol to form EuP product. All results are means ± standard deviations of three replicate determinations.

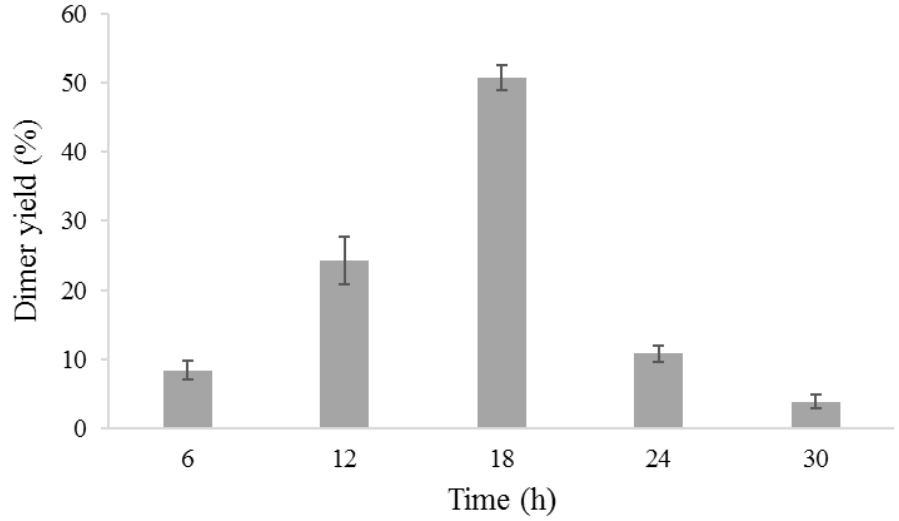


Figure 4.6: Effect of reaction time on laccase-catalysed oxidation of eugenol to form EuP product. All results are means ± standard deviations of duplicate determinations.

4.4.4. Antioxidant activity determination of EuP in relation to eugenol

The radical scavenging ability of eugenol and the dimer were measured from the reduction of DPPH or ABTS radical as well as the reduction of Fe^{3+} to Fe^{2+} (FRAP assay). When an electron

or hydrogen atom was donated to the DPPH radical (purple), the absorbance at 517 nm proportionally decreased, with the increase in the formation of DPPH-H (yellow). In the ABTS radical assay, transfer of electrons from the antioxidant causes the prepared sea-weed green ABTS cation to be decolourised (monitored at 734 nm) (Klibanov, 1997). The principle of the Ferric reducing antioxidant potential (FRAP) assay is based on the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) by an electron donating antioxidant or a reducing agent, which forms an iron-colorimetric probe complex (Fe^{2+}-TPTZ) with a distinct blue colour upon reduction and can be measured spectrophotometrically at 593 nm (Gulcin, 2011).

Plant phenolic compounds are known to be reducing agents and reactive oxygen species scavengers. Eugenol, a main constituent of clove oil, has demonstrated numerous biological properties, one of which is its radical scavenging ability (Ahmed et al., 2015). Radical scavenging activities of the eugenol dimer, *bis*-Eugenol, was compared to the monomeric form of eugenol. As hypothesised, the dimeric form of eugenol showed a higher scavenging effect as compared to its monomeric form (Table 1). The DPPH radical scavenging activity of the product was approximately 3 times higher than that of the eugenol monomer. A similar result was observed for the ABTS radical scavenging assay; however, the dimer was approximately 4 times more active than eugenol. In the FRAP assay, *bis*-Eugenol showed an increase in reducing power compared to its monomeric form (Table 4.1). Through dimerization, the ferric reducing power of eugenol was approximately doubled. The radical scavenging ability of phenolic compounds has been attributed to their structural characteristics that support hydrogen atom donation and stable resultant phenoxyl radicals (Ahmed et al., 2015). The free radical scavenging property of eugenol is owed to the existence of an allyl group in its molecular structure as shown by Ou et al. (2006) as well as the phenolic component and the electron donating methoxy group (which helps to stabilise the resultant radical) (Kudanga et al., 2017).

Therefore, through laccase-mediated dimerization of eugenol, these properties were improved resulting in the enhancement of the radical scavenging ability and increased stability of the resultant phenoxyl radicals. The structure of the 5,5' dimer of eugenol formed during laccase-mediated oxidation of eugenol vastly contributed to the increase in radical scavenging properties, by the addition of another allyl group, as well as the doubling up of the number of H atoms available for donation (Figure 4.3). The doubling up of the electron donating methoxy groups also contributes to the stability of the resultant radical. The improvement in the stability of the resultant phenoxyl radicals coupled with the synergistic improvement in antioxidant capacity, can potentially contribute to the reduction of the prooxidant properties of eugenol that usually occur at higher concentrations of the compound (Charan Raja, 2015). Similar findings have been reported during laccase-mediated dimerization of ferulic acid (Adelakun et al., 2012a), 3-hydroxytyrosol (Zwane et al., 2012), 2,6-dimethoxyphenol (Adelakun et al., 2012b) and caffeic acid (Nemadziva et al., 2018).

Table 4.1: Antioxidant activity of eugenol and its oxidation product (EuP) determined using the DPPH, ABTS and FRAP assays. All results are means ± standard deviation (SD) of three replicate determinations

ABTS IC_{50} µM[a]		DPPH IC_{50} µM[a]		FRAP Value µM[b]	
Eugenol	EuP	Eugenol	EuP	Eugenol	EuP
9.9 ± 0.017	2.4 ± 0.015	28.6 ± 0.025	9.2 ± 0.015	26 ± 0.002	54 ± 0.008

[a]Concentration (µM) of eugenol or EuP required to reduce ABTS or DPPH radical by 50%.
[b]The FRAP value of eugenol and EuP in µM equivalent to the amount of Fe^{3+} converted to Fe^{2+} by 10 µM of sample.

4.5. Conclusion

In conclusion, a symmetrical 5-5’ dimer of eugenol, bis-Eugenol, was successfully enzymatically synthesised by laccase in a monophasic system containing sodium acetate buffer and dioxane (40% v/v) as co-solvent. Dimerisation of eugenol significantly enhanced (up to 4x) antioxidant activities as demonstrated by standard assays (ABTS, DPPH radical scavenging activity and FRAP assay). Therefore, laccases can be an attractive alternative to inorganic catalysts and peroxidases in the biotransformation of eugenol for the improvement of antioxidant activity.

5. GENERAL DISCUSSION

Laccases have become important both in industrial catalysis and nature, due to their wide substrate range. In nature the catalytic and biochemical properties of laccases is grounded on their biological role. An example is the use of laccases to mineralise complex polymers by laccase-producing fungi. Fungal laccases have a high redox potential and are therefore able to depolymerise intricate natural polymeric compounds such as lignin (Kudanga and Le Roes-Hill, 2014).

In this study, the laccase enzyme was produced from *T. pubescens* cultured on PDA plates supplemented with guaiacol. Subsequently the *T. pubescens* was cultured in liquid medium (Trametes defined media supplemented with wheat bran) for laccase production. The enzyme produced had an activity of 19.2 U/mg (protein concentration was 1.44 mg/ml) after partial purification. The laccase activity (19.2 U/mg) produced in liquid medium was higher compared to that produced by Gonzalez et al. (2013). This could be attributed to the supplementation of the media with wheat bran and the subsequent induction of laccase production with xylidine. The purification of laccase involved a 2-step partial purification protocol; $(NH_4)_2SO_4$ precipitation and ultra-filtration, which resulted in a 57.9 % yield. The molecular weight of the enzyme was estimated to be 66 kDa, as observed from the native page. This was similar to the results obtained by Adelakun et al. (2012b). However, a second band observed at ≈ 146 kDa suggests a dimeric isoenzyme of the laccase enzyme. Isoenzymes are dimeric, oligomeric or polymeric forms of an enzyme. Lysozymes catalyse analogous reactions, however they differ to some extent in their chemical and kinetic properties. Laccase isoenzymes have been previously reported (Salas et al., 1995; Galhaup et al., 2002; Moldes et al., 2004).

The enzyme oxidised different substrates at a pH range of 4.0-5.0. However, optimum pH of fungal laccases usually ranges from 3.0 to 5.0 (Chefetz et al., 1998). The optimum pH of the enzyme is substrate dependent. Kudanga et al. (2009) reported that the optimum pH of a laccase from *Bacillus subtilis* was substrate dependent and was between pH 2.5 and 8.5. Temperature optimum and stability of the enzyme was assessed to determine the best reaction temperature for the enzyme. The optimal temperature range for fungal laccase activity is usually between 30 and 60°C (Sunil et al., 2011). In this study, the enzyme had an optimum temperature of 60˚C but was not stable at this temperature. The lack of stability at this optimum temperature could be due to the fact that the enzyme was from a mesophilic fungus (Chauhan et al., 2017). The reaction time for the stability and optimum temperature assays also plays a role. During a reaction the collisions that occur between molecules increases with increase in temperature. The increase in velocity coupled with the decrease in time between collisions, causes more molecules to reach activation energy, with resultant increase in reaction rate. Therefore, there is an increase in collisions between enzymes and substrates (Santhosh 2019). As depicted in Fig 5.1, reaction velocity increases with temperature up to an optimum level, followed by an abrupt decrease in reaction rate with the further increase in temperature, depending on the stability of the enzyme (Daniel and Danson, 2013).

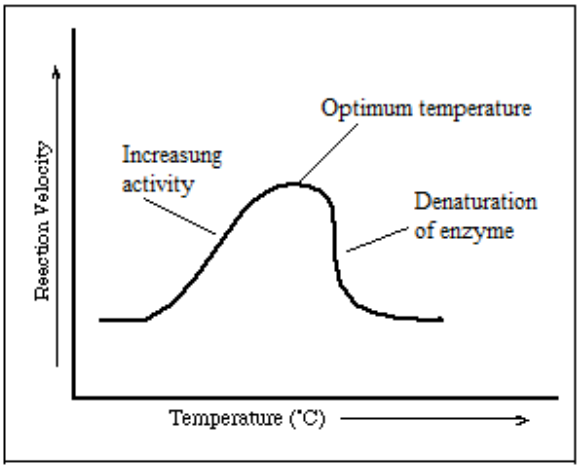


Figure 5.1. Effect of temperature on enzyme activity (http://www.worthington-biochem.com/introBiochem/tempEffects.html).

The application of bioactive compounds in various industries has led to a surge in the value of these compounds. This has brought about a demand for economically efficient methods to produce bioactive compounds, thus the increase in the exploration of enzymatic approaches in biosynthesis (Kudanga et al., 2017). The wide substrate range of laccases, coupled with its eco-friendly catalytic mechanism, provides a perfect enzyme that can be applied in biosynthesis.

Laccase from *T. pubescens* was able to oxidise a wide range of substrates such as guaiacol, caffeic acid, vanillic acid, eugenol, coumaric, catechol, gallic acid, ferulic acid and quercetin hydrate. This was similar to reports from previous studies (Adelakun et al., 2012a; Nemadziva et al., 2018; Kudanga et al., 2017). However, no products were detected on LC-MS for catechol and gallic acid due to the rapid polymerisation of the products; the polymeric products were filtered out before analysis. Therefore, in this study, oxidation products of eugenol, p-coumaric acid and quercetin were further investigated. The product produced from oxidation of quercetin hydrate was a dimeric form of quercetin suggested to be linked through a C-C (5, 5) bond. Oxidation of *ρ*-coumaric acid resulted in the production of two products. A dimer of *ρ*-coumaric acid (*m/z* 325) was successfully purified and characterised. Laccase-catalysed oxidation results

in the formation of radicals. Through resonance stabilisation, various radicals can be produced when the unpaired electron occupies different positions on the radicalised molecule. Thereafter, a non-enzymatic reaction occurs, whereby the produced radicals couple together and are stabilised to form dimers. The favoured product is the one with the lowest heat of formation, also taking into account steric hindrance and that hydroxyl groups are ortho or para directing (Kudanga et al., 2017). This was the basis for the suggested C-C (5',5') dimer of quercetin. A similar phenomenon occurs in the oxidation of *ρ*-coumaric acid. However, the coupling of phenolic acids usually results in the formation of β-5 and β-β linkages due to the stability of C-C bonds and the low heat that is required in the formation of β-5 linkages (Adelakun et al., 2012b, Nemadziva et al., 2018).

Hydroxycinnamic acids have been identified to be potent antioxidants that act as radical scavengers. Their scavenging ability is the result of their electron or hydrogen donating potential, as well as their ability to delocalise the produced phenoxyl radical inside their structure (Teixeira et al., 2013). Therefore, oxidation of *ρ*-coumaric acid was conducted to observe if there would be an improvement in antioxidant capacity after coupling. The antioxidant capacity of the *ρ*-coumaric acid oxidation product (*ρ*-coumaric acid dimer) was compared to the monomeric form using ABTS, DPPH and FRAP assays. The dimer showed significant increase in antioxidant capacity, approximately 2-fold, 3-fold and 6-fold increase in the ABTS, DPPH and FRAP assays, respectively. The improved antioxidant capacity can be attributed to the extra electron donating groups attached to the aromatic ring, as well as the formation of more stable resultant radicals (Kudanga et al., 2017).

The oxidation of eugenol was conducted in organic media because of poor solubility of the compound in aqueous media. Furthermore, using organic media allows for the facilitation of

subsequent reactions which is may not be possible in aqueous media due to kinetic restrictions. There is also easier product recovery in organic media as compared to aqueous media. Essentially, biocatalysis in organic solvents is usually favoured due to the increase in substrate solubility, inhibition of aqueous-dependent side reactions, exclusion of microbial contamination, and reversal of the thermodynamic equilibrium of hydrolysis reactions (Kharazipour et al., 1998; Zaks and Klibanov 1985). However, a major limitation of organic solvents is that, water is fundamentally required by enzymes for conformational flexibility and catalysis. Therefore, the loss of this essential water that is bound to the surface of the enzyme in organic solvent systems results in reduced enzyme activity and conformational flexibility (Doukyu and Ogino 2010.

The low heat of formation of C-C linkage compared to ether linkages (Kudanga et al. 2017) favoured the formation of the 5,5' dimer of eugenol, *bis*-eugenol. Although peroxidase-catalysed dimerization of eugenol produced similar results (Krawczyk et al. 1991; Anita et al. 2015), the requirement of hydrogen peroxide and its rapid degradation makes the process cumbersome, expensive and a potential health hazard (Burton 2003). Therefore, it was not surprising that only a 10% yield was produced by Anita et al. (2015) and 20% yield by Krawczyk et al. (1991) with peroxidase as enzyme compared to the 50% yield obtained in this study. The polarity of dioxane could also have possibly contributed to the high product formation. Dioxane has a polarity index of 4.8 as compared to methanol, acetone and ethanol, with polarity indexes of 5.1, 5.6 and 5.2 respectively. The low polarity index of dioxane enables the more non-polar product to be easily formed.

The antioxidant capacity of *bis*-eugenol was then compared to the monomeric form of eugenol. The dimer showed improved antioxidant capacity. The structure of the 5', 5' dimer of eugenol

formed, with the added allyl group as well as the doubling up of the number of OH groups available for proton donation, immensely contributed to the increase in radical scavenging properties. The addition of another electron donating methoxy group also contributed to the stability of the resultant radical. The improvement in the stability of the phenoxyl radicals coupled with the synergistic improvement in antioxidant capacity, can potentially contribute to the reduction of the prooxidant properties of eugenol that usually occur at higher concentrations of the compound (Charan raja et al. 2015). These results were consistent with findings reported during laccase-mediated dimerisation of ferulic acid (Adelakun et al., 2012a), 3-hydroxytyrosol (Zwane et al., 2012), 2, 6-dimethoxyphenol (Adelakun et al., 2012b) and caffeic acid (Nemadziva et al., 2018). This study generally highlighted the potential of laccase from *T. pubescens* as a green catalyst for the synthesis of antioxidant compounds with enhanced properties.

6. CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

Laccase catalysis remains a promising approach for the biotransformation of natural phenolic antioxidants. Higher molecular weight compounds with improved antioxidant capacity and stability are usually produced. Interest in laccase as biocatalyst for organic synthesis usually stems from its green catalytic properties. The key findings from this study can be summarized as follows:

- Laccase with a relatively high activity was successfully produced from *T. pubescens*. The molecular weight of the enzyme was estimated to be 66 kDa. The enzyme had optimum pH between pH 4.0 and 5.0 depending on the substrate. The enzyme had an optimum temperature of 60^{o}C and was stable over a wide pH range at 40^{o}C.
- The enzyme oxidised selected phenolic compounds: guaiacol, caffeic acid, vanillic acid, eugenol, catechol, gallic acid, ferulic acid and quercetin hydrate.
- Laccase-catalysed oxidation of eugenol, *ρ*-coumaric acid and quercetin in a monophasic system produced the corresponding dimers.
- The antioxidant capacity of the *ρ*-coumaric acid dimer showed improved antioxidant capacity of approximately 2-fold, 3-fold and 6-fold that of the monomer, when measured using the ABTS, DPPH and FRAP assays, respectively.
- Eugenol oxidation in a monophasic system produced a symmetrical 5-5 eugenol dimer, *bis*-eugenol as determined by LC-MS and NMR. Maximum product formation was obtained in the monophasic system with 40% v/v dioxane as co-solvent. Free radical scavenging activity of the dimer, against DPPH and ABTS radical, was four and three times higher than the monomeric form, respectively.

Overall, this study has demonstrated the potential of the *T. pubescens* laccase to synthesise antioxidants with enhanced antioxidant capacity. Three antioxidant compounds; dimer of quercetin, dimer of *p*-coumaric acid and *bis*-eugenol were produced. The products have potential for application as alternative sources of antioxidants in the pharmaceutical industry.

6.2. Recommendations

The produced products can be further investigated to determine their solubility and bioavailability compared to their monomeric forms. Since oxidative stress has been linked in the pathogenesis of complications usually linked with diabetes and other NCDs (Guzik et al., 2002), other pharmacological properties relevant to NCDs such as antidiabetic and anti-inflammatory properties can also be investigated to determine the best possible application of the produced phenolic compounds.

Laccases utilization as biocatalysts can be economically feasible for the synthesis of bioactive compounds. However, several factors need to be considered for the improvement of laccase into an economically viable industrial catalyst. Important factors that need to be considered in the development of a robust enzyme engineered to suit specific industrial processes include improved enzyme activity and production, improved thermostability and capacity to withstand different pH extremes, and solvents encountered during organic synthesis (Kudanga and Le Roes-Hill, 2014).

Biocatalysts have been proven to be green and sustainable alternatives to chemical catalysts. Enzymatic catalysis has been deemed to have great potential in diverse industrial applications (Rehman et al., 2017). However, the industrial requirements for an efficient catalyst such as high catalytic capacity, stability and reusability of the enzymes are rarely met by wild type

enzymes. To overcome these short falls, biotechnologists and microbiologists have established strategies for designing enzymes with specific catalytic properties for specific industrial applications. These strategies include protein engineering using direct evolution and site directed mutagenesis and chemical methods using immobilisation and chemical modification (Bornscheuer et al., 2012). These strategies can be explored for the improvement of the *T. pubescens* laccase. However, protein engineering offers a direct method to improve enzyme activity by modifying the structure of the amino acid residues at the catalytic site of the enzyme (Mate and Alcalde, 2016). Protein engineering strategies such as directed evolution and rational design have been applied in engineering of oxidoreductases. The main requirement for all protein engineering strategies is the availability of a suitable expression host that can produce the enzyme with enhanced properties. *Saccharomyces cerevisiae, Escherichia coli* and other expression systems have been applied for the heterologous expression of oxidoreductases genes (aryl-alcoholoxidase, ligninolytic peroxidases, unspecific peroxygenases, vanillyl-alcoholoxidase and dye-decolorizing peroxidase) (Garcia-Ruiz et al., 2014, Gygli et al., 2017, Vi˜na-Gonzalez et al., 2015). Similarly, the *T. pubescens* laccase can be engineered to improve its catalytic properties and stability for industrial applications.

REFERENCES

ADELAKUN, O. E., KUDANGA, T., GREEN, I. R., LE ROES-HILL, M. & BURTON, S. G. 2012a. Enzymatic modification of 2,6-dimethoxyphenol for the synthesis of dimers with high antioxidant capacity. *Process Biochemistry*, 47, 1926-1932.

ADELAKUN, O. E., KUDANGA, T., PARKER, A., GREEN, I. R., LE ROES-HILL, M. & BURTON, S. G. 2012b. Laccase-catalyzed dimerization of ferulic acid amplifies antioxidant activity. *Journal of Molecular Catalysis B: Enzymatic*, 74, 29-35.

AHMED, D., KHAN, M. M. & SAEED, R. 2015. Comparative analysis of phenolics, flavonoids, and antioxidant and antibacterial potential of methanolic, hexanic and aqueous extracts from adiantum caudatum leaves. *Antioxidants*, 4, 394-409.

ALCALDE, M. 2015. Engineering the ligninolytic consortium. *Trends in Biotechnology*, 33, 155–162.

ALU'DATT, M. H., RABABAH, T., ALHAMAD, M. N., AL-MAHASNEH, M. A., ALMAJWAL, A., GAMMOH, S., EREIFEJ, K., JOHARGY, A. & ALLI, I. 2017. A review of phenolic compounds in oil-bearing plants: Distribution, identification and occurrence of phenolic compounds. *Food Chemistry*, 218, 99-106.

AGARWAL, A. & SEKHON, L. H. 2010. The role of antioxidant therapy in the treatment of male infertility. *Human Fertility*, 13, 217-225.

AGGARWAL, D., SHARMA, M. & SINGLA, S. K. 2013. The role of natural antioxidants as potential therapeutic agent in Nephrolithiasis. *Asian Journal of Pharmaceutical Clinical Research*, 6, 48-53.

ANITA, Y., SUNDOWO, A., DEWI, N. L. P., FILAILLA, E., MULYANI, H., RISDIAN, C., BANJARNAHOR, S., HANAFI, M. & ISTYASTONO, E. P. 2015. Biotransformation

of eugenol to dehydroeugenol catalyzed by brassica juncea peroxidase and its cytotoxicity activities. *Procedia Chemistry*, 16, 265-271.

ARACRI, E., FILLAT, A., COLOM, J. F., GUTIÉRREZ, A., DEL RÍO, J. C., MARTÍNEZ, Á. T. & VIDAL T. 2010. Enzymatic grafting of simple phenols on flax and sisal pulp fibres using laccases. *Bioresource Technology*, 101, 8211–8216.

ATSUMI, T., FUJISAWA, S., SATOH, K., SAKAGAMI, H., IWAKURA, I., UEHA, T., SUGITA, Y. & YOKOE, I. 2000**.** Cytotoxicity and radical intensity of eugenol, isoeugenol or related dimers. *Anticancer Research*, 20, 2519–2524.

AUSEC, L., ZAKRZEWSKI, M., GOESMANN, A., SCHLÜTER, A. & MANDIC-MULEC, I. 2011. Bioinformatic analysis reveals high diversity of bacterial genes for laccase-like enzymes. *PLoS ONE*, 6, 25724.

BAJAJ, S. & KHAN, A. 2012. Antioxidants and diabetes. *Indian Journal of Endocrinology and Metabolism*, 16, S267-S271.

BALABAN, R. S., NEMOTO, S. & FINKEL, T. 2005. Mitochondria, oxidants, aging. *Cell*, 120, 483–495.

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

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

BARROS, A. I. R. N. A., NUNES, F. M., GONÇALVES, B., BENNETT, R. N. & SILVA, A.P. 2011. Effect of cooking on total vitamin C contents and antioxidant activity of sweet chestnuts (*Castanea sativa Mill*.). *Food Chemistry*, 128, 165–172.

BASHIR, A., PERHAM, R. N., SCRUTTON, N. S. & BERRY, A. 1995. Altering kinetic mechanism and enzyme stability by mutagenesis of the dimer interface of glutathione reductase. *Biochemical Journal*, 312, 527–533.

BENDRE, R. S., RAJPUT, J. D., BAGUL, S. D. & PRAVIN, S. K. 2016. Outlooks on medicinal properties of eugenol and its synthetic derivatives. *Natural Products Chemistry and Research*, 4, 2-2.

BEZERRA, T. M. D. S., BASSAN, J. C., SANTOS, V. T. D. O., FERRAZ, A. & MONTI, R. 2015. Covalent immobilization of laccase in green coconut fiber and use in clarification of apple juice. *Process Biochemistry*, 50, 417–423.

BHATACHARYA, A., CHATOPADHYAY, R., MITRA, S. & CROWE, S. E. 2014. Oxidative Stress: An essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiological Reviews*, 94, 329.

BHOGAL, R. K., CASEY, J., GANGULI, S., HUNTER, K. J., KOEK, J. H. & REDFERN, S. P. 2013. Hair colouring composition. WO2013189966A2.

BORNSCHEUER, U. T. & KAZLAUSKAS, R. J. 1999. Hydrolases in Organic Synthesis: Regio-and Stereoselective Biotransformations; John Wiley & Sons Inc.: *Chichester*.

BURTON, S. G. 2003. Oxidizing enzymes as biocatalysts. *Trends in Biotechnology*, 21, 543-549.

BURTON, S. G. & DAVIDS, L. M. 2012. Hydroxytyrosol compounds. Google Patents

BURTON, G. J. & JAUNIAUX, E. 2011. Oxidative stress. Best practice and research. *Clinical Obstetrics and Gynecology*, 25, 287-299.

BUTTERFIELD, D. A., HOWARD, B., SUBRAMANIAM, R., HALL, N., HENSLEY, K. & YATIN, S. 1998. Structural and functional changes in proteins induced by free radical

mediated oxidative stress and protective action of the antioxidants N-tert-butyl-alpha-phenylnitrone and vitamin E. *Annals of the New York Academy of Sciences*, 854, 448–62.

CADENAS, E. (1997). Basic mechanisms of antioxidant activity. *Biofactors*, 6, 391–397.

CAÑAS, A. I., ALCALDE, M., PLOU, F., MARTINEZ, M. J., MARTINEZ, A. T. & CAMARERO, S. 2007. Transformation of polycyclic aromatic hydrocarbons by laccase is strongly enhanced by phenolic compounds present in soil. *Environmental Science & Technology*, 41, 2964–2971.

CAMARERO, S., IBARRA, D., MARTÍNEZ, M. J. & MARTÍNEZ, A. T. 2002. Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. *Applied and Environmental Microbiology*, 71, 1775–1784.

CAROCHO, M. & FERREIRA, I. C. F. R. 2013. A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives. *Food and Chemical Toxicology*, 51, 15-25.

CHANDRA, R. & CHOWDHARY, P. 2015. Properties of bacterial laccases and their application in bioremediation of industrial wastes. *Environmental Science: Processes and Impacts*, 17, 326-42.

CHANG, J. C., VAN DER HOEVEN, L. H. & HADDOX, C. H. 1978. Glutathione reductase in the red blood cells. *Annals of Clinical and Laboratory Science*, 8, 23–29.

CHARAN RAJA, M. 2015. Versatile and synergistic potential of eugenol: A review. *International Journal of Pharmacy and Pharmaceutical Sciences*, 7, 35-40.

CHAUHAN P, S., GORADIA, B. & SAXENA, A. 2017. Bacterial laccase: recent update on production, properties and industrial applications. *3 Biotechnology*, 7, 323. doi:10.1007/s13205-017-0955-7

CHEFETZ, B., CHEN, Y. & HADAR, Y. 1998. Purification and characterization of laccase from *Chaetomium thermophilium*; and its role in humification. *Applied and Environmental Microbiology*, 64, 3175.

CHEN, C. Y., HUANG, Y. C., WEI, C. M., MENG, M., LIU, W. H. & YANG, C. H. 2013. Properties of the newly isolated extracellular thermo-alkali-stable laccase from thermophilic actinomycetes *Thermobifida fusca* and its application in dye intermediates oxidation. *AMB Express*, 3, 49.

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

COSTAIN, L. 2001. Super Nutrients Handbook. *Dorling Kindersley Books*, New York, NY.

CONSTANTIN, M. A., CONRAD, J. & BEIFUSS, U. 2012. Laccase-catalyzed oxidative phenolic coupling of vanillidene derivatives. *Green Chemistry*, 14, 2375-2379.

DANIEL, R. M. & DANSON, M. J. 2013. Temperature and the catalytic activity of enzymes: A fresh understanding. *FEBS Letters*, 587, 2738-2743.

DEBBAB, A., ALY, A. H., LIN, W. H. & PROKSCH, P. 2010. Bioactive compounds from marine bacteria and fungi. *Microbial Biotechnology*, 3, 544-563.

DEL RÍO, J. C. & GUTIÉRREZ, A. 2008. Rapid assessment of the lignin composition of lignocellulosic materials commonly used for paper pulp manufacturing by analytical pyrolysis. *Proceedings of the 18th International Symposium on Analytical and Applied Pyrolysis*; Lanzarote, Spain, May 18−23, 99.

DÍAZ BLANCO, C., GONZÁLEZ, M. D., MONMANY, J. M. D. & TZANOV, T. 2009. Dyeing properties, synthesis, isolation and characterization of an in situ generated phenolic pigment, covalently bound to cotton. *Enzyme and Microbial Technology*, 44, 380–385.

DIAZ-GEREVINI, G. T., REPOSSI, G., DAIN, A., TARRES, M. C., DAS, U. N. & EYNARD, A. R. 2016. Beneficial action of resveratrol: How and why? *Nutrition*, 32, 174-178.

DOUKYU, N. & OGINO, H. 2010. Organic solvent-tolerant enzymes. *Biochemical Engineering Journal*, 48, 270-282.

DITTMER, N. T. & KANOST, M. R. 2010. Insect multicopper oxidases: diversity, properties, and physiological roles. *Insect Biochemistry and Molecular. Biology*, 40, 179–188.

DWIVEDI, U. N., SINGH, P., PANDEY, V. P. & KUMAR, A. 2011. Structure–function relationship among bacterial, fungal and plant laccases. *Journal of Molecular Catalysis B: Enzymatic*, 68, 117-128.

EFSA PANEL ON FOOD ADDITIVES AND NUTRIENT SOURCES ADDED TO FOOD (ANS). 2011. Scientific opinion on the re-evaluation of butylated hydroxyanisole–BHA (E 320) as a food additive. *EFSA Journal, 9,2392.*

EFSA PANEL ON FOOD ADDITIVES AND NUTRIENT SOURCES ADDED TO FOOD (ANS). 2012. Scientific opinion on the reevaluation of butylated hydroxytoluene BHT (E 321) as a food additive. *EFSA Journal, 10, 2588.*

EL-MOWAFY, A. M., AL-GAYYAR, M. M., SALEM, H. A., EL-MESERY, M. E. & DARWEISH, M. M. 2010. Novel chemotherapeutic and renal protective effects for the green tea (EGCG): Role of oxidative stress and inflammatory-cytokine signaling. *Phytomedicine*, 17, 1067-1075.

EMBERGER, G. 2008. Messiah College [Online]. Available: http://www.messiah.edu/oakes/fungi_on_wood/poroid%20fungi/species%20pages/Trametes%20pubescens.htm. Accessed 24 July 2018

EURING, M., KIRSCH, A. & KHARAZIPOUR, A. 2015. Hot-air/hot-steam process for the production of laccase-mediator-system bound wood fiber insulation boards. *Bioresources*, 10, 3541–3552.

EURING, M., RÜHL, M., RITTER, N., KÜES, U. & KHARAZIPOUR, A. 2011. Laccase mediator systems for eco-friendly production of medium-density fiberboard (MDF) on a pilot scale: physicochemical analysis of the reaction mechanism. *Biotechnology Journal*, 6, 1253–1261.

FILLAT, A., COLOM, J. F. & VIDAL, T. 2010. A new approach to the biobleaching of flax pulp with laccase using natural mediators. *Bioresource Technology*, 101, 4104–4110.

FILLAT, U., PRIETO, A., CAMARERO, S., MARTÍNEZ, Á. T. & MARTÍNEZ, M. J. 2012. Biodeinking of flexographic inks by fungal laccases using synthetic and natural mediators. *Biochemical Engineering Journal*, 67, 97–103.

FLORA, S. J. S. 2009. Structural, chemical and biological aspects of antioxidants for strategies against metal and mettaloid exposure. *Oxidative Medicine and Cellular Longevity*, 2, 191–206.

FOROOTANFAR, H. & FARAMARZI, M. A. 2015. Insights into laccase producing organisms, fermentation states, purification strategies, and biotechnological applications. *Biotechnology Progress*, 31, 1443-1463.

FUJISAWA, S., ISHIHARA, M., MURAKAMI, Y., ATSUMI, T., KADOMA, Y. & YOKOE, I. 2007. Predicting the biological activities of 2-methoxyphenol antioxidants: effects of dimers. *In Vivo*, 21, 181-8.

GALHAUP, C. GOLLER, S. PETERBAUER, C. K. STRAUSS, J. HALTRICH, D. 2002. Characterization of the major laccase isoenzyme from *Trametes pubescens* and regulation of its synthesis by metal ions. *Microbiology*, 148, 2159-2169.

GARCIA-RUIZ, E., MATE, D.M., GONZALEZ-PEREZ, D., MOLINA-ESPEJA, P., CAMARERO, S., MARTÍNEZ, A.T., BALLESTEROS, A.O. AND ALCALDE, M. 2014. Directed evolution of ligninolytic oxidoreductases: from functional expression to stabilization and beyond. *In Cascade Biocatalysis*. S. Riva and W.D. Fessner (ed). Weinheim (Germany): Wiley-VCH Verlag GmbH & Co. KGaA.

GAUTAM, M., AGRAWAL, M., GAUTAM, M., SHARMA, P., GAUTAM, A. S. & GAUTAM, S. 2012. Role of antioxidants in generalised anxiety disorder and depression. *Indian Journal of Psychiatry*, 54, 244-247.

GAVEZZOTTI, P., VAVŘÍKOVÁ, E., VALENTOVÁ, K., FRONZA, G., KUDANGA, T., KUZMA, M., RIVA, S., BIEDERMANN, D. & KŘEN, V. 2014. Enzymatic oxidative dimerization of silymarin flavonolignans. *Journal of Molecular Catalysis B: Enzymatic*, 109, 24-30

GILES, G. I., TASKER, K. M., JACOB, C. 2001. Hypothesis: the role of reactive sulfur species in oxidative stress. *Free Radical Biology and Medicine,* 31, 1279–1283.

GIUGLIANO, D., CERIOLLO, A. & PAOLISSO, G. 1996. Oxidative stress and diabetic vascular complications. *Diabetes Care*, 19, 257-67.

GODIC, A., POLJŠAK, B., ADAMIC, M. & DAHMANE, R. 2014. The role of antioxidants in skin cancer prevention and treatment. *Oxidative Medicine and Cellular Longevity*, 2014, 860479.

GOLZ-BERNER, K., WALZEL, B., ZASTROW, L. & DOUCET, O. 2004. Cosmetic or dermatological preparation with skin-lightening proteins. WO2004017931A1.

GONZALEZ, J. C., MEDINA, S. C., RODRIGUEZ, A., OSMA, J. F., ALMÉCIGA-DÍAZ, C. J. AND SÁNCHEZ, O. F. 2013. Production of *Trametes pubescens* laccase under

submerged and semi-solid culture conditions on agro-industrial wastes. *PLoS ONE*, 8 (9), 73721.

GONZALEZ-PEREZ, D. & ALCALDE, M. 2014. Assembly of evolved ligninolytic genes in *Saccharomyces cerevisiae*. *Bioengineered*, 5, 254–263.

GOOCH, J. W. 2002. Emulsification and Polymerization of Alkyd Resins. *Dordrecht: Springe*r *Science and Business Media.*

GREIMEL, K. J., PERZ, V., KOREN, K., FEOLA, R., TEMEL, A. & SOHAR, C. 2013. Banning toxic heavy-metal catalysts from paints: enzymatic cross-linking of alkyd resins. *Green Chemistry*, 15, 381–388.

GÜLÇIN, I. 2011. Antioxidant activity of eugenol: A structure-activity relationship study. *Journal of Medicinal Food*, 14, 975-85.

GULCIN, I. G. BEYDEMIR, S. ELMASTAS M & KUFREVIOGLU O. I. 2004. Comparison of antioxidant activity of clove (*Eugenia caryophylata* Thunb) buds and lavender (*Lavandula stoechas* L.). *Food Chemistry*, 87, 393–400.

GUNTERO, V., CRISTIÁN, A., FERRETTI, P., MANCINI, M. & MARÍA N. KNEETEMAN. 2018. Synthesis and encapsulation of bis-eugenol in a mesoporous solid material: enhancement of the antioxidant activity of a natural compound from clove oil. *Chemical Science International Journal*, 22, 2456-706X.

GUZIK, T. J., MUSSA, S., GASTALDI, D., SADOWSKI, J., RATNATUNGA, C., PILLAI, R. & CHANNON, K. M. 2002. Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. *Circulation*, 105, 1656-62.

GYGLI, G., & VAN BERKEL, W. J. 2017. Communication. vanillyl alcohol oxidases produced in Komagataella phaffii contain a highly stable noncovalently bound anionic FAD semiquinone. *Biocatalysis*, *3*, 17-26.

HADZHIYSKA, H., CALAFELL, M., GIBERT J. M., DAGÀ, J. M. & TZANOV, T. 2006. Laccase-assisted dyeing of cotton. *Biotechnology Letters*, 28, 755–759.

HAKULINEN, N. & ROUVINEN, J. 2015. Three-dimensional structures of laccases. *Cellular and Molecular Life Sciences*, 72, 857-868.

HALLING, P. J. 1994.Thermodynamic predictions for biocatalysis in nonconventional media –theory, tests, and recommendations for experimental-design and analysis. *Enzyme and Microbial Technology*, 16, 178-206.

HALLIWELL, B., GUTTERIDGE, J. M. C. & CROSS, C. E. 1992. Free radicals, antioxidants, and human disease: where are we now? *Journal Laboratory and Clinical. Medicine*, 119, 598-620.

HOENSCH, H., MORGENSTERN, I., PETEREIT, G., SIEPMANN, M., PETERS, W. H., ROELOFS, H. M. & KIRCH, W. 2002. Influence of clinical factors, diet, and drugs on the human upper gastrointestinal glutathione system. *Gut*, 50, 235–240.

HOSSAIN, K., GONZÁLEZ, M. D., LOZANO, G. R. & TZANOV, T. 2009. Multifunctional modification of wool using an enzymatic process in aqueous-organic media. *Journal of Biotechnology*, 141, 58–63.

HUANG, M. T., LU, Y. C., ZHANG, S., LUO, F. & YANG, H. 2016. Rice (*Oryza sativa*) laccases involved in modification and detoxification of herbicides atrazine and isoproturon residues in plants. *Journal of Agricultural and Food Chemistry*, 64, 6397–6406.

INFINITI RESEARCH LIMITED. 2014. Global antioxidants market 2014–2018. Sandler Research. http://www.sandlerresearch.org/global-antioxidants-market-2014-2018.html.2014. Accessed 06 November 2018

ISHIGE, K., SCHUBERT, D. & SAGARA, Y. 2001. Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. *Free Radical Biology and Medicine*, 30, 433-446.

IRACHETA-CÁRDENAS, M. M., ROCHA-PEÑA, M. A., GALÁN-WONG, L. J., ARÉVALO-NIÑO K. & TOVAR-HERRERA, O. E. 2016. A *Pycnoporus sanguineus* laccase for denim bleaching and its comparison with an enzymatic commercial formulation. *Journal of Environmental Management*, 177, 93–100.

IGHODARO, O. M. & O. A. AKINLOYE 2018. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid, *Alexandria Journal of Medicine*, 54, 287-293.

JEE, J., LIM, S., PARK, J. & KIM, C. 2006. Stabilization of all-trans retinol by loading lipophilic antioxidants in solid lipid nanoparticles. *European Journal of Pharmaceutics and Biopharmaceutics*, 63, 134–139.

JEON, J. R., BALDRIAN, P., MURUGESAN, K. & CHANG, Y. S. 2012. Laccase catalysed oxidations of naturally occurring phenols: from in vivo biosynthetic pathways to green synthetic applications. *Microbial Biotechnology*, 5, 318–332.

JONAS, U., HAMMER, E., HAUPT, E. & SCHAUER, F. 2000. Characterisation of coupling products formed by biotransformation of biphenyl and diphenyl ether by the white rot fungus *Pycnoporus cinnabarinus*. *Archives of Microbiology*, 174, 393−398.

KAMILOGLU, S., TOYDEMIR, G., BOYACIOGLU, D., BEEKWILDER, J., HALL, R. & CAPANOGLU, E. 2015. A review on the effect of drying on antioxidant potential of fruits and vegetables. *Critical Reviews in Food Science and Nutrition*, 56, S110-S129.

KAND'ÁR, R., ZÁKOVÁ, P. & MUZÁKOVÁ, V. 2006. Monitoring of antioxidant properties of uric acid in humans for a consideration measuring of levels of allantoin in plasma by liquid chromatography. *Clinica Chimica Acta*, 365, 249–256.

KANZOK, S. M., FECHNER, A., BAUER, H., ULSCHMID, J. K., MULLER, H. M., BOTELLA-MUNOZ, J., SCHNEUWLY, S., SCHIRMER, R. & BECKER K. 2001. Substitution of the thioredoxin system for glutathione reductase in *Drosophila melanogaster*. *Science*, 291, 643–646.

KASHIHARA, N., HARUNA, Y., KONDETI, V. K. & KANWAR, Y. S. 2010. Oxidative stress in diabetic nephropathy. *Current Medicinal Chemistry*, 17, 4256-4269.

KEDZIORA-KORNATOWSKA, K., SZRAM, S., KORNATOWSKI, T., SZADUJKIS-SZADURSKI, L., KEDZIORA, J. & BARTOSZ, G. 2003. Effect of vitamin E and vitamin C supplementation on antioxidative state and renal glomerular basement membrane thickness in diabetic kidney. *Nephron Experimental Nephrology*, 95, 134-143.

KHARAZIPOUR, A., MAI, C. AND HÜTTERMANN, A. 1998. Polyphenols for compounded materials. *Polymer Degradation and Stability*, 59, 237-243.

KILIÇ, I. AND Y. YEŞILOĞLU. 2013. Spectroscopic studies on the antioxidant activity of *p*-coumaric acid. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 115, 719-724.

KIM BONG-GYU, K., JAE YOUNG, Y. I., YONGSUB, LIM, & YOONGHO. 2012. Biotransformation of eugenol to bis-eugenol by *Kalopanax pictus* cell culture. *Journal of the Korean Society for Applied Biological Chemistry*, 55, 677-680.

KLIBANOV, A. M. 2003. Asymmetric enzymatic oxidoreductions in organic solvents. *Current Opinion in Biotechnology*, 14, 427-431.

KLIBANOV, A. M. 1997. Why are enzymes less active in organic solvents than in water? *Trends Biotechnology*, 15, 97-101.

KLIBANOV, A. M. 1989. Enzymatic catalysis in an hydrous organic solvents. *Trends in Biochemical Sciences*, 14, 141-144.

KLIBANOV, A. M. 1986. Enzymes that work in organic solvents. *Chemtech*, 16, 354-359.

KONG, X. J., LIU, X. W., LI, J. Y. & YANG, Y. J. 2014. Advances in pharmacological research of eugenol. *Current Opinion in Complement Alternative Medicine*, 1, 8-11.

KOSKINEN, A. AND A. KLIBANOV. 2012. Enzymatic Reactions in Organic Media, *Springer Netherlands*.

KRAWCZYK A, LIPKOWSKA E, WRÓBEL TJ. 1991. Horseradish peroxidase-mediated preparation of dimers from eugenol and isoeugenol. *Collection of Czechoslovak Chemical Communications*, 56, 1147-1150.

KRIS-ETHERTON, P. M., HECKER, K. D., BONANOME, A., COVAL, S. M., BINKOSKI, A. E., HILPERT, K. F., GRIEL, A. E. & ETHERTON, T. D. 2002. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *The American Journal of Medicine*, 113, 71-88.

KUDANGA, T. & LE ROES-HILL, M. 2014. Laccase applications in biofuels production: current status and future prospects. *Applied Microbiology and Biotechnology*, 98, 6525–6542.

KUDANGA, T., NYANHONGO, G. S., GUEBITZ, G. M. & BURTON, S. 2011. Potential applications of laccase-mediated coupling and grafting reactions: A review. *Enzyme and Microbial Technology*, 48, 195-208.

KUDANGA, T., NEMADZIVA, B. & LE ROES-HILL, M. 2017. Laccase catalysis for the synthesis of bioactive compounds. *Applied Microbiology and Biotechnology*, 101, 13-33.

KUDANGA, T., NUGROHO PRASETYO, E., SIPILÄ, J., EBERL, A., NYANHONGO, G. S. & GUEBITZ, G. M. 2009. Coupling of aromatic amines onto syringylglycerol β-guaiacylether using *Bacillus SF* spore laccase: A model for functionalization of lignin-based materials. *Journal of Molecular Catalysis B: Enzymatic*, 61, 143-149.

KUNAMNENI, A., CAMARERO, S., GARCÍA-BURGOS, C., PLOU, F. J., BALLESTEROS, A. & ALCALDE, M. 2008. Engineering and applications of fungal laccases for organic synthesis. *Microbial Cell Factories*, 7, 32.

KURISAWA, M CHUNG, J. E., UYAMA, H. & KOBAYASHI, S. 2003. Laccase-catalyzed synthesis and antioxidant property of poly(catechin). *Macromolecular Bioscience*, 3, 758–764.

KURŠVIETIENĖ, L., STANEVIČIENĖ, I., MONGIRDIENĖ, A. & BERNATONIENĖ, J. 2016. Multiplicity of effects and health benefits of resveratrol. *Medicina*, 52, 148-155.

KURUTAS, E. B. 2016. The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state, *Nutrition Journal***,** 15, 71-71.

LAANE, C., BOEREN, S., VOS, K. & VEEGER, C. 1987. Rules for optimization of biocatalysis in organic solvents. *Biotechnology and Bioengineering*, 30, 81-87.

LEE, K. W., LEE, H. J. & LEE, C. Y. 2002. Antioxidant activity of black tea vs. green tea. *The Journal of Nutrition*, 132, 785-785.

LEON, R., FERNANDES, P., PINHEIRO, H. M. AND CABRAL, J. M. S. 1998. Whole-cell biocatalysis in organic media. *Enzyme and Microbial Technology*, 23, 483–500.

LI, C. & XIE, B. 2000. Evaluation of the antioxidant and pro-oxidant effects of tea catechin oxypolymers. *Journal of Agricultural and Food Chemistry*, 48, 6362-1666.

LOBO, V., PATIL, A., PHATAK, A. & CHANDRA, N. 2010. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy Reviews*, 4, 118–126.

LOPEZ-ALARCONA, C. & DENICOLA, A. 2013. Evaluating the antioxidant capacity of natural products: a review on chemical and cellular-based assays. *Analytica Chimica Acta*, 763, 1-10.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. 1951. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, 193, 265-275.

LÜ, J., LIN, P.H., YAO, Q., CHEN, C. 2010. Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. *Journal of Cellular and Molecular Medicine*, 14, 840–860.

LYRAS, L., CAIRNS, N. J., JENNER, A., JENNER, P. & HALLIWELL, B. 1997. An assessment of oxidative damage to proteins, lipids, and DNA in brain from patients with Alzheimer's disease. *Journal of Neurochemistry*, 68, 2061–2069.

MADHAVI, V. & LELE, S. S. 2009. Laccase: Properties and applications. *Bioresources*, 4, 1694-1717.

MAIOLINO, G., ROSSITTO, G., CAIELLI, P., BISOGNI, V., ROSSI, G. P. & CALO, L. A. 2013. The role of oxidized low-density lipoproteins in atherosclerosis: the myths and the facts. *Mediators of Inflammation*, 2013, 714653.

MAJCHERCZYK, A., JOHANNES, C. & HÜTTERMANN, A. 1998. Oxidation of polycyclic aromatic hydrocarbons (PAH) by laccase of *Trametes versicolor*. *Enzyme Microbial Technology*, 22, 335–341.

MARTINEZ, K. B., MACKERT, J. D. & MCINTOSH, M. K. 2017. Chapter 18 - polyphenols and intestinal health. In: Nutrition and functional foods for healthy aging. Watson, R. R. (ed.). United States of America: Academic Press, 1, 191-210

MARTINS, L. O., DURAO, P., BRISSOS, V. & LINDLEY, P. F. 2015. Laccases of prokaryotic origin: enzymes at the interface of protein science and protein technology. *Cellular and Molecular Life Sciences*, 72, 911–922.

MARTINS, S., MUSSATTO, S. I., MARTÍNEZ-AVILA, G., MONTAÑEZ-SAENZ, J., AGUILAR, C. N. & TEIXEIRA, J. 2011. Bioactive phenolic compounds: production and extraction by solid-state fermentation. A review, *Biotechnology Advances*, 29, 365–73.

MATE, D. M. & ALCALDE, M. 2015. Laccase engineering: From rational design to directed evolution. *Biotechnology Advances*, 33, 25-40.

MATE, D. M. & ALCALDE, M. 2016. Laccase: a multi-purpose biocatalyst at the forefront of biotechnology. *Microbial Biotechnology*, 10, 1457-1467.

MOLDES D., LORENZO M., SANROMÁN M. A. 2004. Different proportions of laccase isoenzymes produced by submerged cultures of Trametes versicolor grown on lignocellulosic wastes. *Biotechnology Letters*, 26, 327-330.

MORGENSTERN, I., POWLOWSKI, J., ISHMAEL, N., DARMOND, C., MARQUETEAU, S., MOISAN, M.-C., QUENNEVILLE, G. & TSANG, A. 2012. A molecular phylogeny of thermophilic fungi. *Fungal Biology*, 116, 489-502.

MÜLLER, W. E. G., SCHRÖDER, H. C., WIENS, M., PEROVIĆ-OTTSTADT, S., BATEL, R. & MÜLLER, I. M. 2004. Traditional and modern biomedical prospecting: part ii-the benefits: approaches for a sustainable exploitation of biodiversity (secondary metabolites and biomaterials from sponges). *Evidence-Based Complementary and Alternative Medicine*, 1, 133-144.

MURKOVIC, M. 2003. Phenolic compounds. Encyclopedia of Food Sciences and Nutrition (Second Edition). Oxford: Academic Press.

NATIONAL DEPARTMENT OF HEALTH (NDoH), STATISTICS SOUTH AFRICA (STATS SA), SOUTH AFRICAN MEDICAL RESEARCH COUNCIL (SAMRC) & ICF. 2017. *South Africa Demographic and Health Survey 2016: Key Indicators* Pretoria, South Africa and Rockville, Maryland, USA: NDoH, Stats SA, SAMRC and ICF.

NATIONAL INSTITUTE OF HEALTH (NIH). 2013. Vitamin E fact sheet for health professionals. Available at: http://ods.od.nih.gov/factsheets/VitaminE-HealthProfessional/. Accessed 15 October 2017.

NEMADZIVA, B., LE ROES-HILL, M., KOORBANALLY, N. & KUDANGA, T. 2018. Small laccase-catalyzed synthesis of a caffeic acid dimer with high antioxidant capacity. *Process Biochemistry*, 69, 99-105.

NI, J. & TOKUDA, G. 2013. Lignocellulose-degrading enzymes from termites and their symbiotic microbiota. *Biotechnology Advances*, 31, 838–850.

NILADEVI, K. N. & PREMA, P. 2008. Effect of inducers and process parameters on laccase production by *Streptomyces psammoticus* and its application in dye decolourization. *Bioresources Technology*, 99, 4583–4589.

NOZIK-GRAYCK, E., SULIMAN, H. B. & PIANTADOSI, C. A. 2005. Extracellular superoxide dismutase. *International Journal Biochemistry and Cell Biology*, 37, 2466–2471.

OKADO-MATSUMOTO, A. & FRIDOVICH, I. 2001. Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu,Zn-SOD in mitochondria. *Journal of Biological Chemistry*, 276, 38388–38393.

OSMA, J. F., TOCA-HERRERA, J. L. & RODRÍGUEZ-COUTO, S. 2010. Uses of laccases in the food industry. *Enzyme Research*, 8, 918761-918761

OTHMAN, A. M., ELSHAFEI, A. M., HASSAN, M. M., HAROUN, B. M., ELSAYED, M. A. & FARRAG, A. A. 2014. Purification, biochemical characterization and applications of *Pleurotus ostreatus* ARC280 laccase. *British Microbiology Research Journal*, 4, 1418-1439.

OU, H. C., CHOU, F. I. & LIN, T. M. 2006 Protective effects of eugenol against oxidized Ldl induced cytotoxicity and adhesion molecule expression in endothelial cell. *Food and Chemical Toxicology*, 44, 1485-595.

OZKANLAR, S. & AKCAY, F. 2012. Antioxidant vitamins in atherosclerosis animal experiments and clinical studies. *Advances in Clinical and Experimental Medicine*, 21, 115-123.

PAIVA, S. A. R., RUSSELL, R. M. 1999. *b*-Carotene and other carotenoids as antioxidants. *Journal of American College of Nutrition*, 18, 426–433.

PANDEY VEDA, P, M. A., SWATI, S., SAMEEKSHA, T. & UPENDRA, N DWIVEDI. 2017. A comprehensive review on function and application of plant peroxidases. *Biochemistry & Analytical Biochemistry*, 6, 308.

PARR, A. J. & BOLWELL, G. P. 2000. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content profile. *Journal of Agricultural and Food Chemistry*, 80, 985–1012.

PASHA, I., SAEED, F., WAQAS, K., ANJUM, F. M. & ARSHAD, M. U. 2013. Nutraceutical and functional scenario of wheat straw. *Critical Reviews in Food Science and Nutrition*, 53, 287-95.

PERDE-SCHREPLER, M., CHERECHES, **G., BRIE, I., TATOMIR, C., POSTESCU, I. D., SORAN, L. & FILIP, A.** 2013. Grape seed extract as photochemopreventive agent against UVB-induced skin cancer. *Journal of Photochemistry and Photobiology B: Biology*, 118, 16-21.

PERSSON, T., POPESCU, B. O. & CEDAZO-MINGUEZ, A. 2014. Oxidative stress in Alzheimer's disease: why did antioxidant therapy fail? *Oxidative Medicine and Cellular Longevity*,11, 427318-427318.

PEZZELLA, C., GUARINO, L. & PISCITELLI, A. 2015. How to enjoy laccases. *Cellular and Molecular Life Sciences*, 72, 923-940.

PIETTA, P. 2000. Flavonoids as antioxidants. *Journal of. Natural. Products*, 63, 1035–1042.

PILLAY-VAN WYK, V., MSEMBURI, W., LAUBSCHER, R., DORRINGTON, R. E., GROENEWALD, P., GLASS, T., NOJILANA, B., JOUBERT, J. D., MATZOPOULOS, R., PRINSLOO, M., NANNAN, N., GWEBUSHE, N., VOS, T., SOMDYALA, N., SITHOLE, N., NEETHLING, I., NICOL, E., ROSSOUW, A. & BRADSHAW, D. 2016. Mortality trends and differentials in South Africa from 1997 to 2012: second national burden of disease study. *Lancet Global Health*, 4, 642-653.

PINTO, A. F., RODRIGUES, J.V. & TEIXEIRA, M. 2010. Reductive elimination of superoxide: structure and mechanism of superoxide reductases. *Biochimica et Biophysica Acta*, 1804, 285–297.

PISCITELLI, A., AMORE, A. & FARACO, V. 2012. Last advances in synthesis of added value compounds and materials by laccase-mediated biocatalysis. *Current Organic Chemistry*, 16, 2508–2524.

PISOSCHI, A. M. & POP, A. 2015**.** The role of antioxidants in the chemistry of oxidative stress: A review. *European Journal of Medicinal Chemistry*, 97, 55-74.

POYTON, R. O., CASTELLO, P. R., BALL, K. A., WOO, D. K. & PAN, N. 2009a. Mitochondria and hypoxic signaling: a new view. *Annals of the New York Academy of Sciences*, 1177, 48–56.

POYTON, R. O., BALL, K. A. & CASTELLO, P. R. 2009b. Mitochondrial generation of free radicals and hypoxic signaling. *Trends in Endocrinology and Metabolism*, 20, 332–340.

PRASAD, A. S., BAO, B., BECK, F. W. J., KUCK, O., & SARKAR, F. H. 2004. Antioxidant effect of zinc in humans. *Free Radical Biology and Medicine*, 37, 1182–1190.

REHMANN, L., IVANOVA, E., GUNARATNE, H. Q. N., SEDDON, K. R. & STEPHENS, G. 2014. Enhanced laccase stability through mediator partitioning into hydrophobic ionic liquids. *Green Chemistry*, 16, 1462−1469.

RICHTER, C., PARK, J. W. & AMES, B. N. 1988. Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proceedings of the National Academy of Sciences of the United States of America*, 85, 6465–6467.

RIVA, S. 2006. Laccases: blue enzymes for green chemistry. *Trends in Biotechnology*, 24, 219-226.

RODRÍGUEZ COUTO, S. & TOCA HERRERA, J. L. 2006. Industrial and biotechnological applications of laccases: A review. *Biotechnology Advances*, 24, 500-513.

SAJID, M., SOOTTAWAT, B., AISHA, A. & ASIFA, A. 2014. Phenolic compounds and plant phenolic extracts as natural antioxidants in prevention of lipid oxidation in seafood: a detailed review. *Comprehensive Reviews in Food Science and Food Safety*, 13, 1125-1140.

SAID, M. & AL-DALAEN, A. I. A. Q. 2014. Review Article: Oxidative stress versus antioxidants. *American Journal of Bioscience and Bioengineering*, 2, 60-71.

SAITO, K., IKEDA, R., ENDO, K., TSUJINO, Y., TAKAGI, M. & TAMIYA, E. 2012. Isolation of a novel alkaline-induced laccase from *Flammulina velutipes* and its application for hair coloring. *Journal of Bioscience and Bioengineering*, 113, 575–579.

SALAS, C. L., SERGIO; LARRAIN, JUAN; SALAS, LORETO; CULLEN, DANIEL; VICUNA, RAFAEL. (1995). Properties of laccase isoenzymes produced by the basidiomycete, *Ceriporiopsis subvermispora*. *Biotechnology and Applied Biochemistry*, 21, 323-333.

SANKAR, P., SUBHASHREE, S. & SUDHARANI, S. 2012. Effect of *Trigonella foenumgraecum* seed powder on the antioxidant levels of high fat diet and low dose streptozotocin induced type II diabetic rats. *European Review for Medical and Pharmacological Sciences*, 16, 10-17.

SANTHANAM, N., VIVANCO, J, M., DECKER, S. R. & REARDON, K. F. 2011. Expression of industrially relevant laccases: prokaryotic style. *Trends Biotechnology*, 29, 480–489.

SANTHOSH, L. 2019. The effects of temperature on enzyme activity and biology. Available at https://sciencing.com/effects-temperature-enzyme-activity-biology-6049.html. Accessed 17 July 2019.

SARANGARAJAN, R., MEERA, S., RUKKUMANI, R., SANKAR, P. & ANURADHA, G. 2017. Antioxidants: Friend or foe? *Asian Pacific Journal of Tropical Medicine*, 10, 1111-1116.

SCHRADER, M. & FAHIMI, H. D. 2006. Peroxisomes and oxidative stress. *Biochimica et Biophysica Acta*, 1763, 1755–1766.

SCHULTZ, A., JONAS, U., HAMMER, E. & SCHAUER, F. 2001. Dehalogenation of chlorinated hydroxybiphenyls by fungal laccase. *Applied Environmental. Microbiology*, 67, 4377−4381.

SEIFRIED, H. E., ANDERSON, D. E., FISHER, E. I. & MILNER, J. A. 2007. A review of the interaction among dietary antioxidants and reactive oxygen species. *Journal of Nutritional Biochemistry*, 18, 567-79.

SELLEK, G. A. & CHAUDHURI, J. B. 1999. Biocatalysis in organic media using enzymes from extremophiles. *Enzyme and Microbial Technology*, 25, 471–482.

SELMA, M. V., ESPÍN, J. C. & TOMÁS-BARBERÁN, F. A. 2009. Interaction between phenolics and gut microbiota: role in human health. *Journal of Agricultural and Food Chemistry*, 57, 6485–6501.

SHELDON, R. A. & WOODLEY, J. M. 2018. Role of biocatalysis in sustainable chemistry. *Chemical Reviews*, 118, 801-838.

SHRIKANTA, A., KUMAR, A., & GOVINDASWAMY, V. 2015. Resveratrol content and antioxidant properties of underutilized fruits. *Journal of Food Science and Technology*, 52, 383-390.

SINGH ARORA, D. & KUMAR SHARMA, R. 2010. Ligninolytic fungal laccases and their biotechnological applications. *Applied Biochemistry and Biotechnology*, 160, 1760-1788.

SINGH, G., BHALLA, A., KAUR, P., CAPALASH, N., AND SHARMA, P. 2011. Laccase from prokaryotes: a new source for an old enzyme. *Reviews in Environmental. Science and Biotechnology*, 10, 309–326.

SQUADRITO, G. L. & PRYOR, W. A. 1998. Oxidative chemistry of nitric oxide: the roles of superoxide, peroxynitite and carbon dioxide. *Free Radical Biology and Medicine*, 25, 392–403.

STANNER, S. A., HUGHES, J., KELLY, C. N. & BUTTRISS, J. 2004. A review of the epidemiological evidence for the 'antioxidant hypothesis'. *Public Health and Nutrition*, 7, 407-22.

SUNIL, S., MORE, RENUKA, P. S., PRUTHVI, K., SWETHA M., S. MALINI, AND VEENA S. M. 2011. Isolation, purification, and characterization of fungal laccase from *Pleurotus* sp. *Enzyme Research*, 2011, 248735-248735

SUPARNO, O., COVINGTON, A., PHILLIPS, P. & EVANS, C. 2005. An innovative new application for waste phenolic compounds: Use of kraft lignin and naphthols in leather tanning. *Resources, Conservation and Recycling*, 45, 114−127.

SZYMUSIAK, H. & ZIELINSKI, R. 2003. Bond dissociation enthalpy of phenolic antioxidants. *Polish Journal of Food and Nutrition Sciences*, 53, 129-135.

TABASSUM, A., BRISTOW, R.G., VENKATESWARAN, V. 2010. Ingestion of selenium and other antioxidants during prostate cancer radiotherapy: a good thing? *Cancer Treatment. Rev*iews, 36, 230–234.

TANVETYANON, T. & BEPLER, G. 2008. Beta-carotene in multivitamins and the possible risk of lung cancer among smokers versus former smokers: a meta-analysis and evaluation of national brands. *Cancer*, 113, 150-7.

TEIXEIRA, J., GASPAR, A., GARRIDO, E. M., GARRIDO, J. & BORGES, F. 2013. Hydroxycinnamic acid antioxidants: an electrochemical overview. *BioMed Research International*, 11, 251754- 251765.

THOMPSON, D., NORBECK, K., OLSSON, L. I., CONSTANTIN-TEODOSIU, D., VAN DER ZEE, J. & MOLDEUS, P. 1989. Peroxidase-catalyzed oxidation of eugenol: formation of a cytotoxic metabolite(s). *Journal of Biological Chemistry*, 264, 1016-1021.

THORUM, M. S., ANDERSON, C. A., HATCH, J. J., CAMPBELL, A. S., MARSHALL, N. M., ZIMMERMAN, S. C., LU, Y. & GEWIRTH, A. A. 2010. Direct, electrocatalytic oxygen reduction by laccase on anthracene-2-methanethiol modified gold. *The Journal of Physical Chemistry Letters*, 1, 2251-2254.

THURSTON, C. F. 1994. The structure and function of fungal laccases, *Microbiology*, 140, 19-26.

TOPPO. S., VANIN, S., BOSELLO, V. & TOSATTO, S. C. 2008. Evolutionary and structural insights into the multifaceted glutathione peroxidase (Gpx) superfamily. *Antioxidants and Redox Signaling*, 10, 1501–1514.

TOUILLAUD, M., THIEBAUT, A., FOURNIER, A., NIRAVONG, M., BOUTRON-RUAULT, M. &CHAVEL-CHAPELON, F. 2007. Dietary lignin intake and postmenopausal

breast cancer risk by estrogen and progesterone receptor status. *Journal of the National Cancer Institute*, 99, 475–486.

TRIPATHI, R., MOHAN, H. & KAMAT, J. P. 2007. Modulation of oxidative damage by natural products. *Food Chemistry*, 100, 81–90.

TRUBITSINA, L. I., TISHCHENKO, S. V., GABDULKHAKOV, A. G., LISOV, A. V., ZAKHAROVA, M. V. & LEONTIEVSKY, A. A. 2015. Structural and functional characterization of two-domain laccase from *Streptomyces viridochromogenes*. *Biochimie*, 112, 151-159.

TSUKAGOSHI, H., KAWATA, T., SHIMIZU, Y., ISHIZUKA, T., DOBASHI, K. & MORI M. 2002. 4-Hydroxy-2-nonenal enhances fibronectin production by IMR-90 human lung fibroblasts partly via activation of epidermal growth factor receptor-linked extracellular signal-regulated kinase p44/42 pathway. *Toxicology and Applied Pharmacology*, 184, 127–135.

TURDI, S., LI, Q., LOPEZ, F, L. R. & REN, J. 2007. Catalase alleviates cardiomyocyte dysfunction in diabetes: role of Akt, forkhead transcriptional factor and silent information regulator 2. *Life Sciences*, 81, 895-905.

TURUNEN, M., OLSSON, J. & DALLNER, G. 2004. Metabolism and function of coenzyme Q. *Biochimica et Biophysica Acta*, 1660, 171–199.

UCHIDA, K., SHIRAISHI, M., NAITO, Y., TORII, Y., NAKAMURA, Y. & OSAWA, T. 1999. Activation of stress signaling pathways by the end product of lipid peroxidation. 4-hydroxy-2-nonenal is a potential inducer of intracellular peroxide production. *Journal of Biological Chemistry*, 274, 2234–2242.

UZAN, E., PORTET, B., LUBRANO, C., MILESI, S., FAVEL, A., LESAGE-MEESSEN, L. & LOMASCOLO, A. 2011. Pycnoporus laccase-mediated bioconversion of rutin to

oligomers suitable for biotechnology applications. *Applied Microbiology and Biotechnology*, 90, 97-105.

VALENCIA, P. & MARINEZ, L. 2005. Lignin in the restoration of the cultural heritage. *Revista Colombiana De Física*, 37, 106−109.

VALKO, M., RHODES, C. J., MONCOL, J., IZAKOVIC, M. & MAZUR, M. 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions*, 160, 1–40.

VILLA-RODRIGUEZ, J. A., PALAFOX-CARLOS, H., YAHIA, E. M., AYALA-ZAVALA, J. F. & GONZALEZ-AGUILAR, G. A. 2015. Maintaining antioxidant potential of fresh fruits and vegetables after harvest. *Food Science and Nutrition*, 55, 806-22.

VIÑA-GONZALEZ, J., GONZALEZ-PEREZ, D., FERREIRA, P., MARTINEZ, A. T., & ALCALDE, M. 2015. Focused directed evolution of aryl-alcohol oxidase in Saccharomyces cerevisiae by using chimeric signal peptides. *Applied Environmental Microbiology*, *81*, 6451-6462.

VISWANATH, B., RAJESH, B., JANARDHAN, A., KUMAR, A. P. & NARASIMHA, G. 2014. Fungal laccases and their applications in bioremediation. *Enzyme Research*, 2014, 163242-163242.

WAN, Y., DU, Y. & MIYAKOSHI, T. 2008a. Enzymatic catalysis of 2,6-dimethoxyphenol by laccases and products characterization in organic solutions. *Science China Chemistry*, 51, 669-676.

WAN, Y. Y., DU, Y. M. & MIYAKOSHI, T. 2008b. Rhus laccase catalysis and product characterization of 1,2-dimethoxyphenol in organic solutions. *Chinese Chemical Letters*, 19, 333-336.

WAGGIALLAH, H. AND M. ALZOHAIRY. 2011. The effect of oxidative stress on human red cells glutathione peroxidase, glutathione reductase level, and prevalence of anemia among diabetics. *North American Journal of Medical Sciences*, 3, 344-347.

WANG, J., FENG, J., JIA, W., CHANG, S., LI, S., & LI, Y. 2015. Lignin engineering through laccase modification: a promising field for energy plant improvement. *Biotechnology Biofuels*, 8, 145.

WIE M, WON M, LEE K, SHIN J, LEE J, SUH H, SONG D, KIM Y. 1997. Eugenol protects neuronal cells from excitotoxic and oxidative injury in primary cortical cultures. *Neuroscience Letters*, 225, 93–96

WOLFE, K. & LIU R.H. 2008. Structure–activity relationships of flavonoids in the cellular antioxidant activity assay. *Journal of Agricultural and Food Chemistry*, 56, 8404–8411.

YAVUZ, M., KAYA, G. & AYTEKIN, C. 2014. Using *Ceriporiopsis subvermispora* CZ-3 laccase for indigo carmine decolourization and denim bleaching. *International Biodeterioration and Biodegradation*, 88, 199–205.

YILDIRIM, A., MAVI, A., OKTAY, M., KARA, A. A., ALGUR, O. F. & BILALOGLU, V. 2000. Comparison of antioxidant and antimicrobial activities of *Tilia* (*Tilia argentea Desf ex DC*), sage (*Salvia trilobal*), and black tea (*Camellia sinensis*) extracts. *Journal of Agricultural and Food Chemistry*, 48, 5030–5034.

ZAKS, A. & KLIBANOV, A. M. 1985. Enzyme-catalyzed processes in organic solvents. *Proceedings of the National Academy of Sciences of the USA*, 82, 3192–3196.

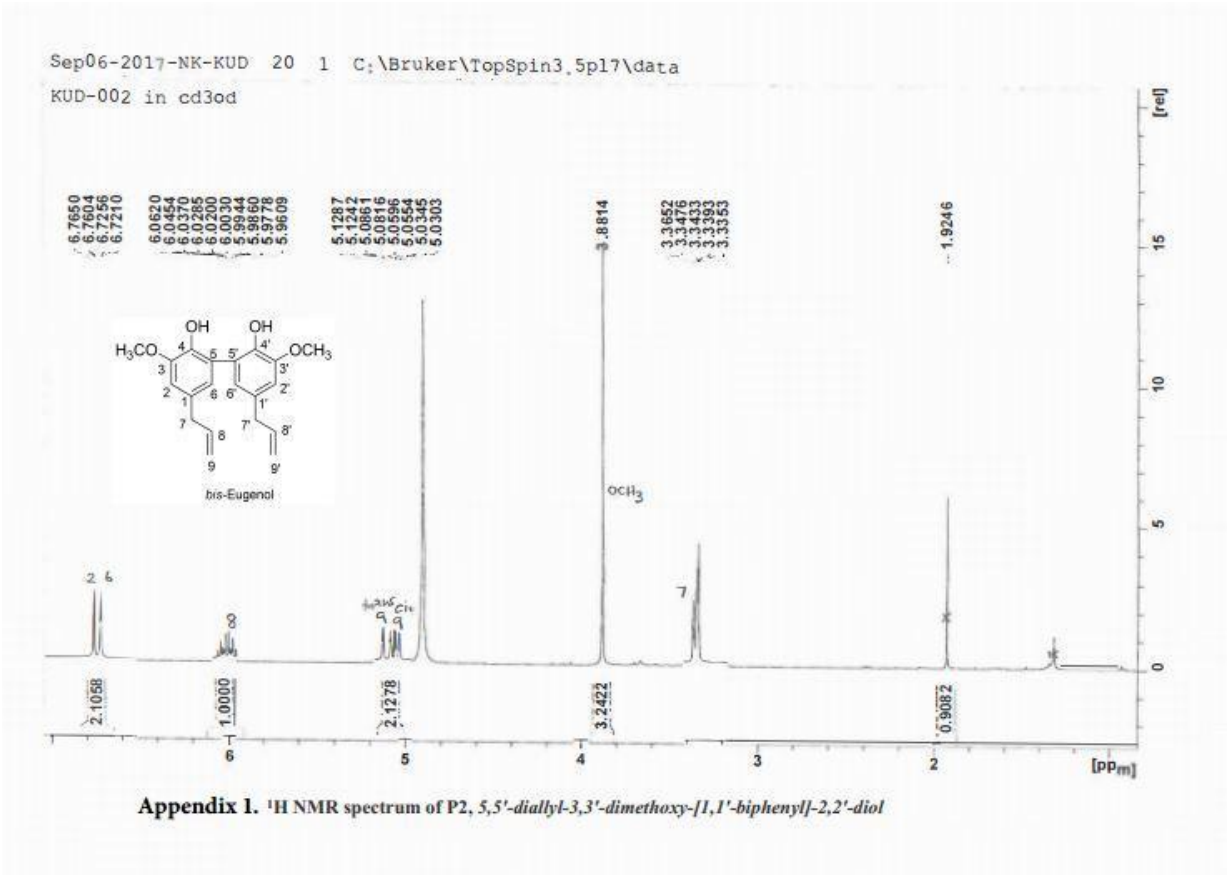
ZAMOCKY, M. & KOLLER, F. 1999. Understanding the structure and function of catalases: clues from molecular evolution and in vitro mutagenesis. *Progress in Biophysics and Molecular Biology*, 72, 19–66.

ZENG, J., ZHU, Q., WU, Y. & LIN, X. 2016. Oxidation of polycyclic aromatic hydrocarbons using *Bacillus subtilis* CotA with high laccase activity and copper independence. *Chemosphere*, 148, 1–7.

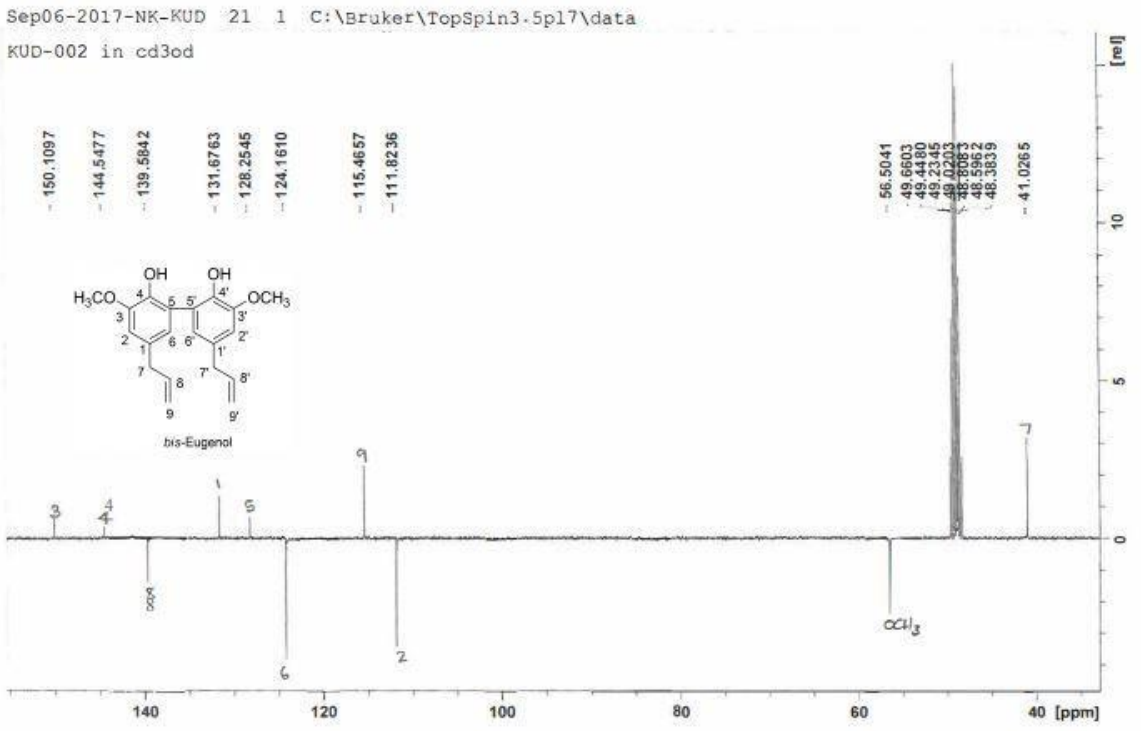
ZUMÁRRAGA, M., PLOU, F. J., GARCÍA-ARELLANO, H., BALLESTEROS, A. & ALCALDE, M. 2007. Bioremediation of polycyclic aromatic hydrocarbons by fungal laccases engineered by directed evolution. *Biocatalysis and Biotransformation*, 25, 219–228.

ZWANE, R. E., PARKER, A., KUDANGA, T., DAVIDS, L. M. & BURTON, S. G. 2012. Novel, biocatalytically produced hydroxytyrosol dimer protects against ultraviolet-induced cell death in human immortalized keratinocytes. *Journal of Agricultural and Food Chemistry*, 60, 11509-11517.

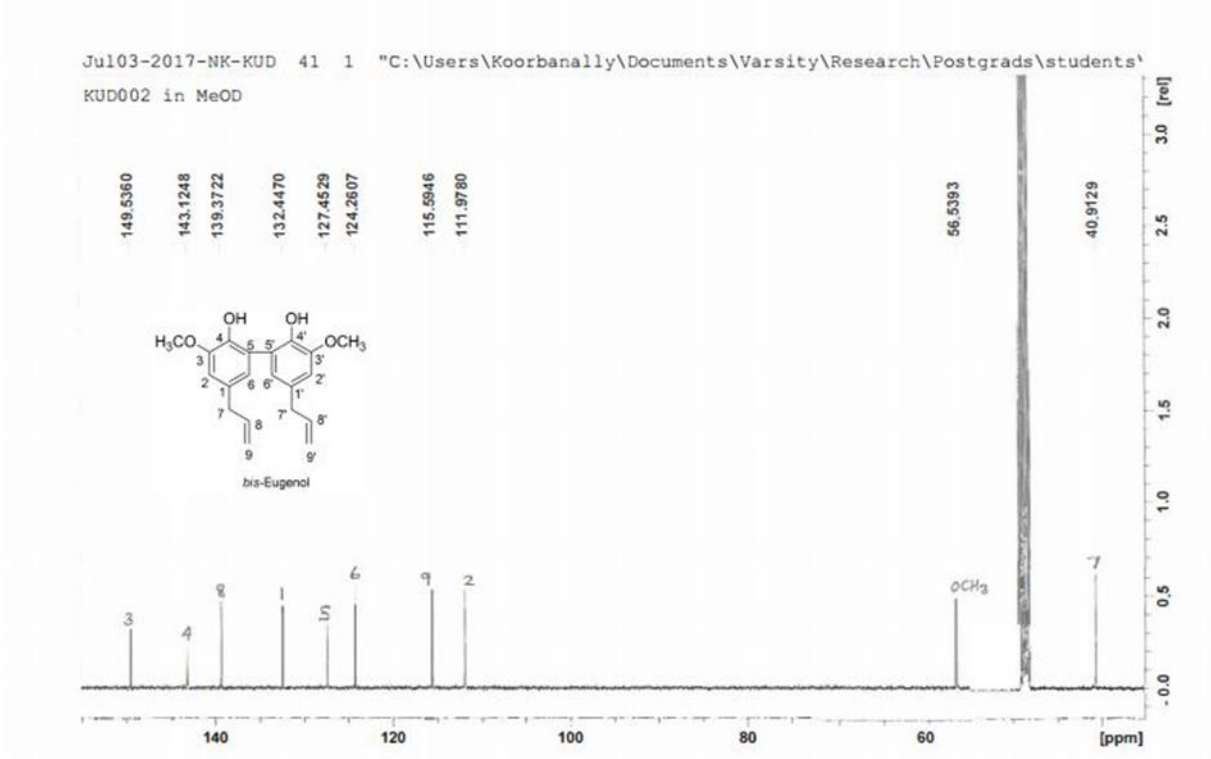
APPENDICES



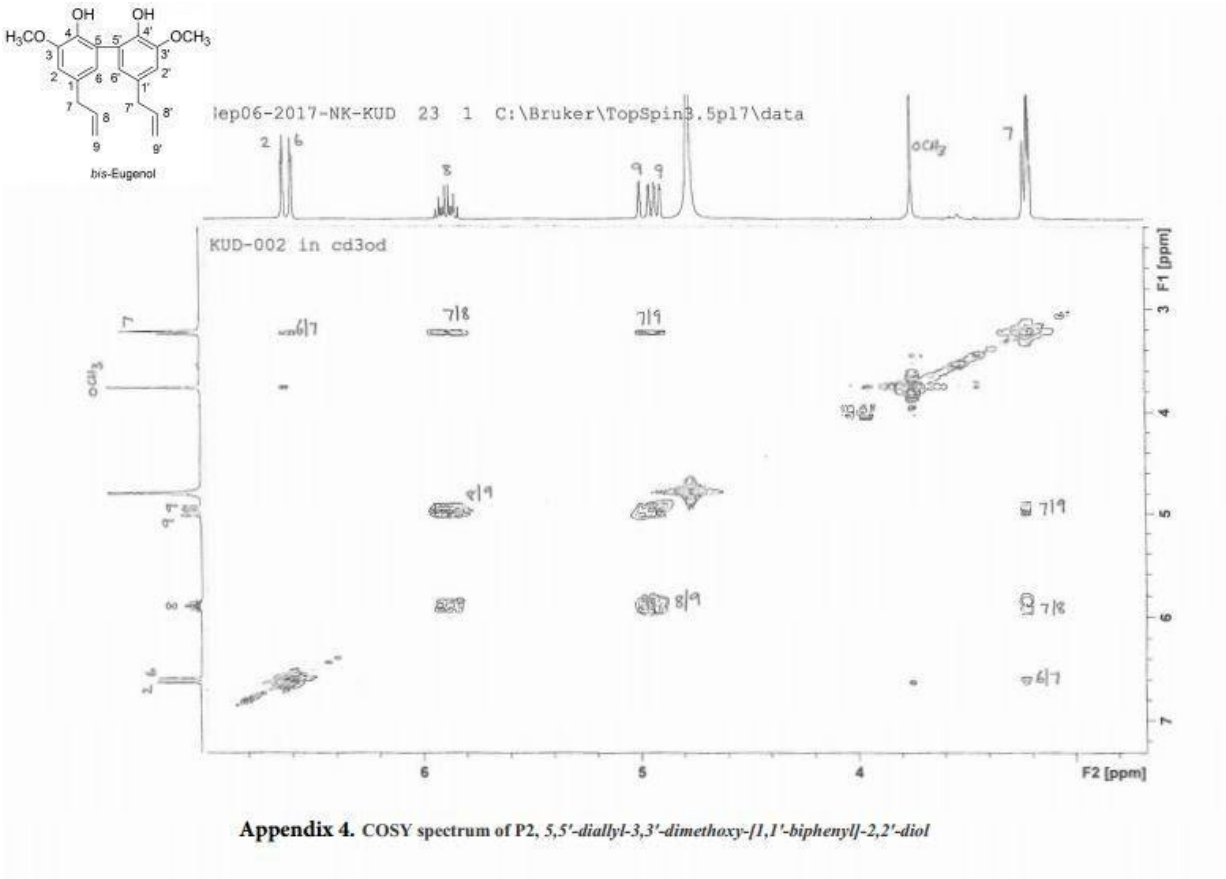
Appendix 1. ^{1}H NMR spectrum of P2, *5,5'-diallyl-3,3'-dimethoxy-[1,1'-biphenyl]-2,2'-diol*



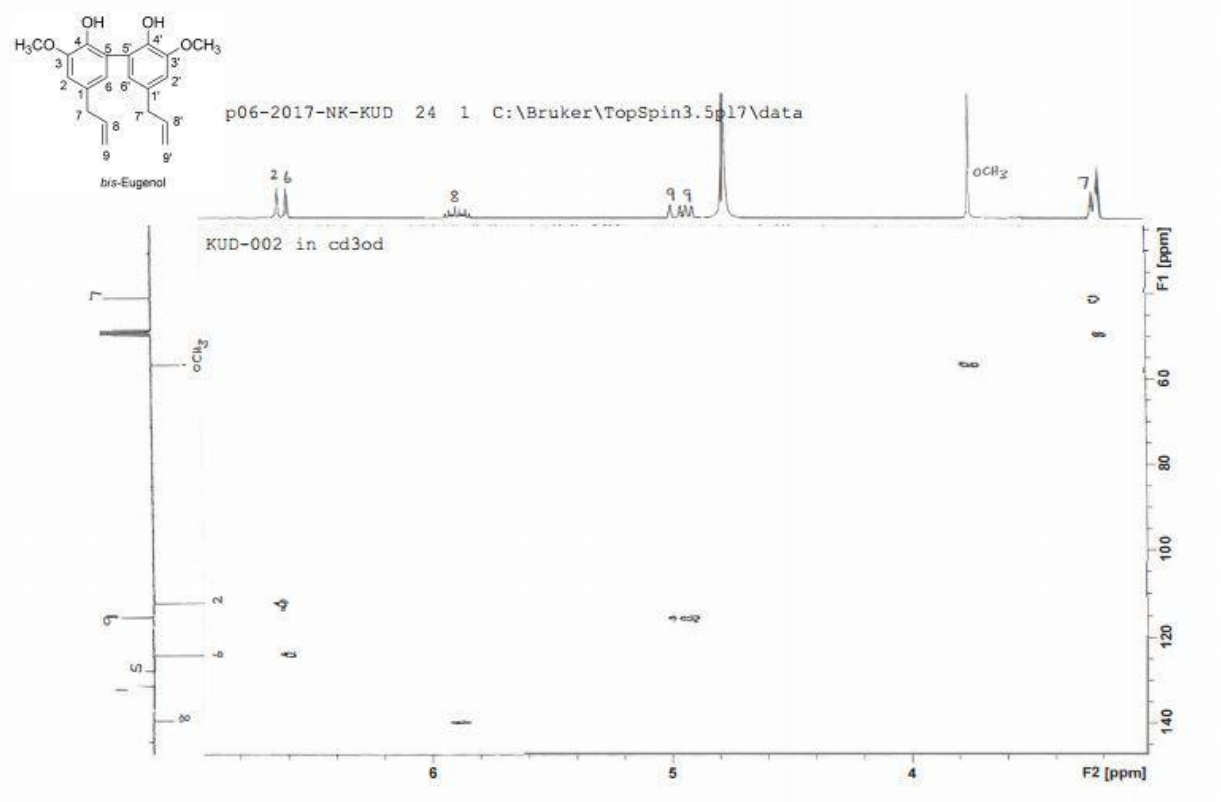
Appendix 2. ^{13}C (APT) NMR spectrum of P2, *5,5'-diallyl-3,3'-dimethoxy-[1,1'-biphenyl]-2,2'-diol*



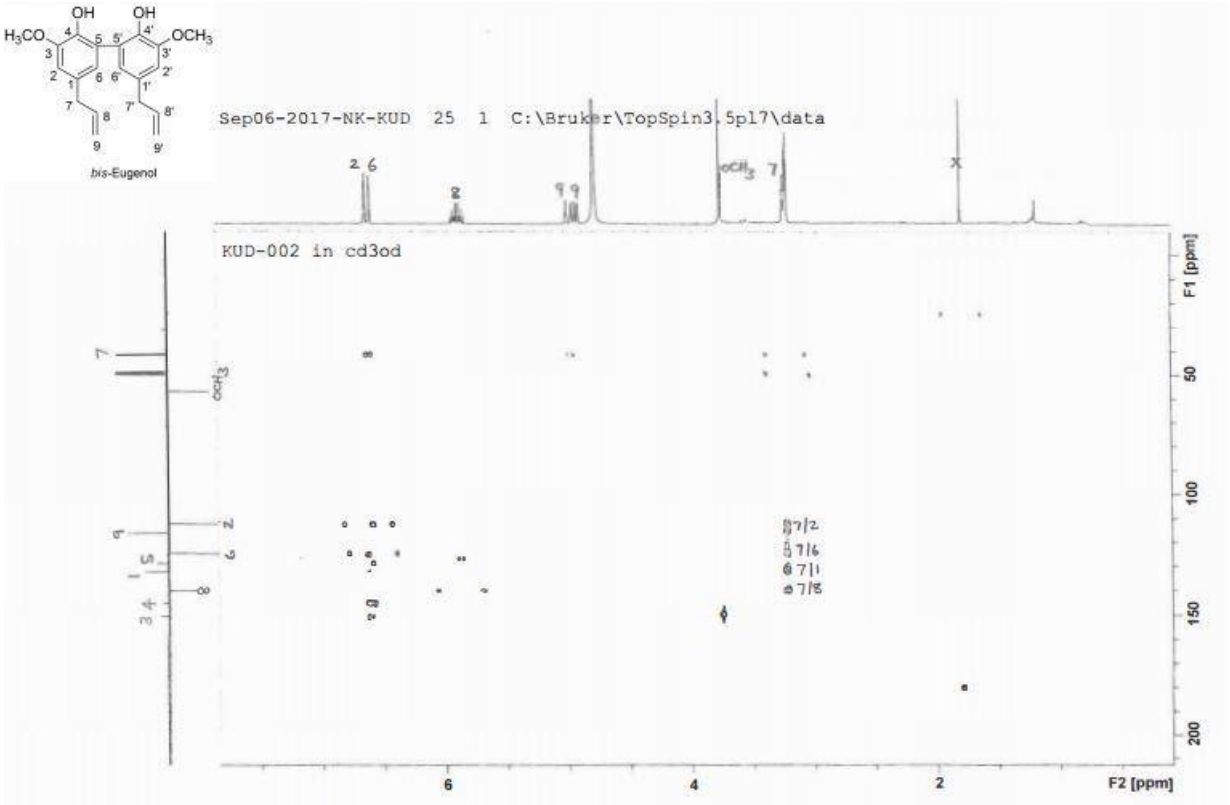
Appendix 3. ^{13}C NMR spectrum of P2, *5,5'-diallyl-3,3'-dimethoxy-[1,1'-biphenyl]-2,2'-diol*



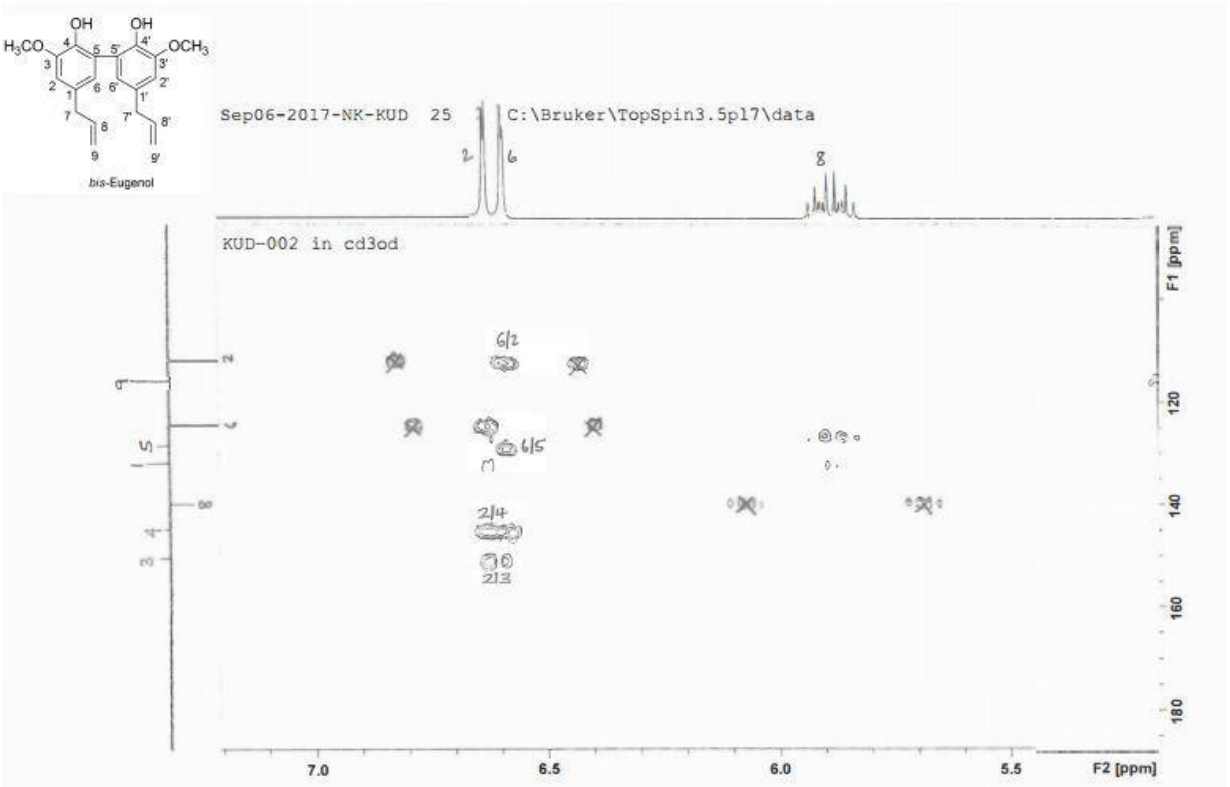
Appendix 4. COSY spectrum of P2, *5,5'-diallyl-3,3'-dimethoxy-[1,1'-biphenyl]-2,2'-diol*



Appendix 5. HSQC spectrum of P2, *5,5'-diallyl-3,3'-dimethoxy-[1,1'-biphenyl]-2,2'-diol*



Appendix 6. HMBC spectrum of P2, *5,5'-diallyl-3,3'-dimethoxy-[1,1'-biphenyl]-2,2'-diol*



Appendix 7. Expanded HMBC spectrum of P2, *5,5'-diallyl-3,3'-dimethoxy-[1,1'-biphenyl]-2,2'-diol*