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LACCASES AS BIOCATALYSTS FOR THE BIOSYNTHESIS OF HYBRID ANTIOXIDANTS

Submitted in fulfilment for the Master of Applied Sciences (Biotechnology) degree in the Department of Biotechnology and Food Science, Durban University of Technology, Durban, South Africa

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REFERENCE DECLARATION

I, Mr S. Ngubane – 21556750 and Prof Tukayi Kudanga (full name of promoter/ supervisor) do hereby declare that in respect of the following dissertation:

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AUTHORS DECLARATION

This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Science, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Prof Tukayi Kudanga** and **Prof Kugen Permaul**.

Student's signature

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PREFACE

This dissertation is organised into seven major sections. The ABSTRACT provides the reader with a summary of the critical experiments and their outcomes. The INTRODUCTION provides a brief background on the relationship between oxidative stress, non-communicable diseases, and synthetic and natural phenolic antioxidants. This section also suggests laccases as the suitable enzymes for synthesising novel antioxidants from environmentally derived phenolic compounds. Furthermore, a hypothesis is postulated, and the section closes off by highlighting the objectives that need to be fulfilled to test the hypothesis.

The LITERATURE REVIEW provides an overview of the causes of oxidative stress, natural versus synthetic antioxidants, and the different enzymes that have been used in the synthesis of novel phenolic compounds. The suitability of laccases for the same task is reviewed by introducing the reader to vital laccase-catalysed synthesis reactions conducted over the past two decades. Furthermore, the different purification and analysis techniques employed in organic synthesis experiments are reviewed. CHAPTER 3 focusses on the production, purification, and characterisation of laccase. The characterised laccase was then used to synthesise novel phenolic compounds (CHAPTER 4). CHAPTER 5 provides a general discussion of the results obtained in the preceding two chapters, and then CHAPTER 6 wraps up the whole dissertation by providing conclusions and recommendations for the future advancement of this work.

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ABBREVIATIONS USED

AAPH	2,2'-Azobis (2-amidinopropane) dihydrochloride
ABAP	2,2'-Azobis (2-amidinopropane)
ABTS	2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
BHA	Butylated hydroxyl anisole
BHT	Butylated hydroxyl toluene
CAT	Catalase
CL	Chemiluminescence
DMPD	N, N-dimethyl-p-phenylenediamine
DMP	2,6-dimethoxy phenol
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC	Enzyme Commission
FRAP	Ferric reducing ability of plasma
G6PD	Glucose-6-phosphate dehydrogenase
GR	Glutathione reductase
GSH	Glutathione
GSH-Px	Glutathione peroxidase
GSSG	Glutathione disulphide
GST	Glutathione-S-transferase
HAT	Hydrogen atom transfer
HBT	1-hydroxy benzotriazole
HPLC	High performance liquid chromatography
LC-MS	Liquid chromatography – mass spectrometry
LMS	Laccase-mediator systems
MSR	Methionine sulphoxide reductase
NADH	Nicotinamide adenine dinucleotide + hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NCDs	Non-communicable diseases

NDGA	Nordihydroguaiaretic acid
NMR	Nuclear magnetic resonance
ORAC	Oxygen radical absorbance capacity
PCL	Photochemiluminescence
PG	Propyl gallate
PRXs	Peroxiredoxins
PUFA	Polyunsaturated fatty acids
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAA	Sulphur amino acids
SH	Sulphydryl proteins
SLAC	Small laccases
SOD	Superoxide dismutase
*t*BHQ	tertiary butyl hydroquinone
TEAC	Trolox equivalent antioxidant capacity
TLC	Thin layer chromatography
TPTZ	Tripyridyltriazine
TRAP	Total radical trapping antioxidant parameter
TRXs	Thioredoxins
USD	United States Dollars
WHO	World Health Organization

ABSTRACT

The past century has seen an overwhelming upsurge in research interest concerning natural antioxidants, primarily due to rising awareness and knowledge regarding the carcinogenicity of the previously used synthetic antioxidants. Owing to their functional role in numerous redox systems, natural phenolic antioxidants can be applied in diverse areas such as pharmaceuticals, food products, dietary supplements, cosmetics and many other products. However, some natural antioxidants have been shown to exhibit undesirable properties such as low solubility (leads to instability in certain solvents), low bioavailability, low heat stability, low antioxidant capacity and pro-oxidant activity when present at high concentrations alongside transition metal ions such as Cu^{2+} and Fe^{3+}. Structural modification of these natural compounds is accomplished by chemical or enzymatic means. Biocatalysis has attracted notable attention as a viable way to modify and synthesise bioactive compounds. Laccases are better suited for this function since they can be applied in a plethora of environmentally benign organic synthesis mechanisms through bond formation reactions such as oxidative decomposition, nuclear amination, thio bond formation, oxidative coupling, and C-C bond forming reactions.

In this study, the biotransformation of natural phenolic compounds using laccases from *Trametes pubescens* CBS 696.94 was investigated. Before its application, laccase was biochemically characterised and had its thermodynamic parameters determined. Catechol, gallic acid, quercetin and nordihydroguaiaretic acid were identified as promising substrates and were used in subsequent hetero-coupling studies. Hetero-coupling reactions were carried out in a mixture of a water-miscible and a buffer. Products were monitored using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), purified using preparative TLC and column chromatography, and their molecular weight determined using liquid chromatography-mass spectrometry (LCMS). The antioxidant activities of the products were determined by using the ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) scavenging assays. The antibacterial activities of the products were assessed against selected American Type Culture Collection (ATCC) bacteria, and their minimum inhibitory concentrations (MICs) were determined.

T. pubescens CBS 696.94 produced high titres of extracellular laccase (2330 ± 50 U/l). The enzyme (~58 kDa) had an optimum activity at 60°C while optimum pH varied with the substrate used. The activity was shown to drop drastically at temperatures above 60°C, while the enzyme was most stable between pH 4.5 and 5.0. Enzyme activity was enhanced when the enzyme was pre-incubated in 20 mM $CuSO_4$. The kinetic constants (K_m) values for ABTS, syringaldazine (SGZ), 2,6-dimethoxy phenol (2,6-DMP) and guaiacol were 198 µM, 211 µM, 168 µM, and 102 µM, respectively. The k_{cat} values were 103 s^{-1}, 32 s^{-1}, 12 s^{-1}, and 13 s^{-1} with corresponding catalytic efficiency values (k_{cat}/ K_m) of 5.2×10^5 s^{-1} M^{-1}, 5.8×10^4 s^{-1} M^{-1}, 1.9×10^5 s^{-1} M^{-1} and 1.2×10^5 s^{-1} M^{-1}, respectively. The $t_{1/2}$ values of the *T. pubescens* CBS 696.94 laccase at 50°C, 60°C and 70°C were 7.8 h, 3.8 h, and 0.72 h, respectively. The enzyme deactivation energy (E_d) was 109.362 kJ/mol while ΔG, ΔH, and ΔS for thermal inactivation of the *T. pubescens* CBS 696.94 laccase were all positive. The enzyme was susceptible to non-competitive (in the presence of sodium azide and sodium dodecyl sulphate) or uncompetitive modes of inhibition (in the presence of L-cysteine, hydrogen peroxide and dithiothreitol). Three heterodimers (catechol + quercetin, quercetin + nordihydroguaiaretic acid (NDGA) and gallic acid + nordihydroguaiaretic acid) and a single heterotrimer (2× quercetin and 1× catechol) were successfully produced, purified and partially characterised.

The large scale catechol/quercetin coupling reaction yielded 15.6 ± 1.26% and 9.8 ± 1.12% of the heterodimer and heterotrimer, respectively. The best yields of the catechol/quercetin product were achieved in a monophasic system consisting of 50% dioxane and sodium acetate buffer pH 5.0, with shaking at 200 rpm, temperature 37°C and reaction time 6 h. The products heterodimer showed inferior antioxidant activity, while the heterotrimer displayed enhanced antimicrobial activity against *Listeria monocytogenes* and *Staphylococcus aureus* at minimum inhibitory concentrations of 200 and 150 µg/ml, respectively.

Large scale reaction of the quercetin/NDGA coupling reaction yielded 14.71 ± 0.59% of the heterodimer. The optimum yield was achieved in a monophasic system consisting of 50% dioxane and sodium acetate buffer pH 5.0, with shaking at 200 rpm, temperature 37°C and reaction time 6 h. The heterodimer showed superior antioxidant activity, exhibiting 1.3 and 1.9-fold increases in the ABTS radical scavenging capacity, 1.3- and 2.0-fold increases in DPPH radical scavenging activity, and 1.14- and 1.6-fold increases in FRAP units when

compared to quercetin and nordihydroguaiaretic acid, respectively. It also showed enhanced antimicrobial activity against *L. monocytogenes*, *S. aureus*, *Escherichia coli* and *Enterobacter cloacae* at minimum inhibitory concentrations of 200, 100 and 50 μg/ml.

The large scale reaction of the gallic acid/NDGA coupling reaction yielded 14.12 ± 0.53% of the heterodimer. The optimum yield was achieved in a monophasic system consisting of 60% dioxane and sodium acetate buffer pH 5.0, with shaking at 200 rpm, temperature 37°C and reaction time 6 h. The heterodimer displayed superior antioxidant activity, exhibiting 1.7- and 2.2-fold increases in the ABTS radical scavenging capacity, 2.1- and 3.0-fold increases in DPPH radical scavenging activity, and 1.4- and 1.8-fold increases in FRAP units when compared to nordihydroguaiaretic acid and gallic acid, respectively.

In conclusion, two antioxidant and antibacterial compounds were successfully produced, purified and characterised. Overall, this study has demonstrated that laccases from *T. pubescens* CBS 696.94 can facilitate the cross-coupling of phenolic compounds to form hybrid compounds with enhanced antioxidant and antibacterial activity.

1. INTRODUCTION

Non-communicable diseases (NCDs) such as cardiovascular diseases, diabetes, cancers and chronic respiratory diseases account for approximately 71% of deaths worldwide, and they are projected to exceed communicable, maternal, neonatal, and nutritional diseases as the most common causes of death (Bigna and Noubiap 2019). This has prompted the World Health Organization (WHO) to recommend interventions to combat the scourge of NCDs (WHO 2018). The United Nations General Assembly (UNGA) high-level meeting of 2018 also assigned high priority to the prevention and control of NCDs in all parts of the world (Collins *et al.* 2019). However, NCDs associated deaths continue to increase, with 50% of the countries projected to miss the WHO and UNGA's 2030 target (Bakhtiari *et al.* 2020). Substantial evidence links NCDs to oxidative stress caused by an imbalance between the production of reactive oxygen species (ROS) and the biological systems inability to readily detoxify the ROS or to quickly repair the resulting damage (Nediani and Giovannelli 2020). Hence, the increased human dependency on dietary and supplementary antioxidants (Grosso 2018).

Antioxidants are defined as any substance whose presence at low concentrations compared to that of an oxidisable substrate, significantly postpones or impedes oxidation of that substrate by being oxidised itself (Gulcin 2020). The compound should have the ability, after the detoxification of the ROS to form a new ROS that is stable through either intramolecular hydrogen bonding or unpaired electron delocalisation for it to be considered as an antioxidant (Santos-Sánchez *et al.* 2019). Propyl gallate (PG), *tert*-butyl hydroquinone (*t*BHQ), butylated Hydroxytoluene (BHT), and butylated hydroxyl anisole (BHA) are some of the common synthetic antioxidants used as food preservatives and therefore are consumed by humans in appreciable amounts (Neha *et al.* 2019). However, the use of these antioxidants has been associated with health risks due to the toxicity problems which may arise from their use (Anwar, Hussain and Mustafa 2018). For example, BHT causes lesion formation in the forestomach of rats, and at high dosages, it has been shown to cause internal and external haemorrhaging severe enough to cause death in some strains of mice and guinea pigs (Lorenzo *et al.* 2018; Neha *et al.* 2019).

There has been an increasing interest in natural phenolic antioxidants simply because they are presumed safe and non-toxic (Fernandes *et al.* 2018; Lorenzo *et al.* 2018). Another reason for the rising interest in natural phenolic antioxidants is the universality of their action in various redox systems and, consequently, broad spectra of possible applications, i.e., as ingredients for pharmaceuticals, functional foods, dietary supplements, cosmetics and other products (Ramana *et al.* 2018; Neha *et al.* 2019). However, some natural antioxidants have been reported to have undesirable properties such as low solubility, poor oxidative and thermal stability, poor bioavailability, and low antioxidant capacity (Faridi Esfanjani and Mahdi Jafari 2017).

Chemical methods have been successfully employed to modify the physical properties of natural antioxidants such as the esterification of polyphenols with functionalised aliphatic molecules (fatty acid or alcohols) by reaction with acid chlorides and acid anhydrides (Kalla, Kim and Kim 2018; Wang *et al.* 2020). However, these chemical methods are generally used under very harsh conditions involving corrosive acids, such as sulfuric acid and hydrogen fluoride followed by re-esterification steps (Su *et al.* 2018). In this regard, there are concerns over adverse environmental impacts, safety, and waste of the chemical modification methods. On the contrary, the enzymatic modification (biocatalysis) of natural antioxidants can be done without or/ with minimal use of hazardous chemicals. Waste generation can be prevented by performing catalytic reactions with high stereo- and regio-selectivity, thus increasing the atom economy by avoiding the numerous protection and deprotection steps. Enzymatic modifications can be done with high energy efficiency and safe chemistry because they can be conducted at near-neutral pH, ambient temperatures, and atmospheric pressure (Li *et al.* 2018; Su *et al.* 2018).

Enzymes such as laccases, catalases and peroxidases have been used for the enzymatic modification of natural antioxidants (Kudanga *et al*. 2017). However, Laccases are superior as they do not require additional co-factors to catalyse the reaction and have broad substrate specificity, including phenols, polyphenols, anilines, aryl diamines, methoxy-substituted phenols, hydroxyindoles, benzo thiols, inorganic and organic metal compounds (Su *et al.* 2018). Laccases are generally described as multi-copper oxidase enzymes that belong to the blue copper family of enzymes. During oxidation of compounds, free radicals are formed and laccases employ molecular oxygen as the final electron acceptor with the concomitant production of water as the only by-product (Yu *et al.* 2020). Subsequently, the reactive free

radicals can undergo spontaneous (non-enzymatic) cross-coupling reactions to produce polymeric compounds via C-C, C-O, C-N, or C-S covalent coupling mechanisms (Qingzhu, Kai and Youbin 2021).

Laccase-catalysed modification of natural antioxidants has been reported to enhance their antioxidant capacity (Jeon *et al.* 2012). The reactions mainly involve homo-molecular coupling. Even though laccases have been shown to possess hetero-molecular coupling capabilities (Zerva *et al.* 2019), the studies mainly focused on the laccase catalysed synthesis of fine chemicals and the derivatisation of some biologically active compounds such as antibiotics, amino acids and cytostatics (Su *et al.* 2018). However, to the best of our knowledge, there is limited information on the application of laccases as biocatalysts for the synthesis of hybrid antioxidants. Therefore, this research investigated the prospective ability of *Trametes pubescens* laccase to catalyse the synthesis of hybrid antioxidants from natural phenolic antioxidants. In addition, the antioxidant capacities and antimicrobial activities of the coupling products were compared with the monomeric units.

Hypothesis

Laccase catalysed hetero-coupling of plant-derived natural phenolic antioxidants will result in the production of novel hybrid antioxidants.

Aim: To synthesise hybrid antioxidants from plant-derived phenolic antioxidants using *Trametes pubescens* CBS 696.94 laccase as a catalyst.

Objectives:

- To produce, purify, and characterise *Trametes pubescens* CBS 696.94 laccase.
- To screen phenolic molecules for oxidation with laccase.
- To synthesise phenolic antioxidants through laccase-mediated hetero-coupling reactions.
- To purify the products and characterise their structure and in-vitro antioxidant and antimicrobial capacities.

2. LITERATURE REVIEW

2.1. Introduction

This chapter presents a review of the literature relevant to this study. Some of the areas covered in this review include the sources, structure and catalytic activities of the various laccases; the methods used for biochemical characterisation and determination of thermodynamic parameters of enzymes; application of laccases, especially in the organic synthesis of bioactive compounds; medium engineering and reaction engineering in laccase facilitated organic synthesis of bioactive compounds, e.g. antioxidants; the global insurgency of oxidative stress-related diseases as a primary driver for research in the organic synthesis of novel antioxidants; an overview of previous studies on laccase-facilitated production of novel antioxidants, and the methods used in antioxidant activity analysis, and analytical methods used in the characterisation of antioxidants. The literature review concludes with the scope of this study as a way of linking the literature reviewed and the current study.

2.2. The global insurgency of oxidative stress-related diseases

Non-communicable diseases such as mental health conditions, chronic obstructive pulmonary disease, diabetes, cardiovascular disease and cancer are the leading cause of death globally (responsible for seven out of ten deaths worldwide), and their scourge profoundly affects the low- and middle-income regions (Allen and Feigl 2017; Bennett *et al.* 2018). Non-communicable diseases continue to push scores of people into poverty and are estimated to cost the global economy USD 47 trillion in the next 20 years (Allen and Feigl 2017). There is mounting evidence showing oxidative stress and generation of free radicals (as either a primary or secondary event) as one of the significant pathogenesis of the most non-communicable disease (Liguori *et al.* 2018). Thus, oxidative stress is acknowledged as a critical pathophysiological mechanism in different frequent non-communicable diseases, i.e. rheumatoid arthritis, cardiovascular disease, diabetes, cancers, or neurological diseases such as Alzheimer or Parkinson disease (Tan *et al.* 2018). Oxidative stress is defined as the production of free radicals (reactive oxygen and nitrogen species) that overwhelms the body's antioxidant systems ability to detoxify the formed reactive intermediates readily or to repair their damage (Ganguly *et al.* 2019). Oxidative stress can lead to the oxidative modification of proteins and lipids, impairment in the expression of genes due to DNA damage and subsequent cellular deterioration (Gutteridge and Halliwell 2018).

2.2.1. Reactive oxygen and nitrogen species

Four endogenous processes have been identified as the sources of cellular reactive oxygen species (ROS): (i) The oxidative burst of cells phagocytic cells, i.e. eosinophils, macrophages, and neutrophils, can produce ROS, (ii) Oxidative phosphorylation in the mitochondria results in the concomitant production of ROS as by-products of electron transfer reactions, (iii) the peroxisomes' production of H_2O_2 under physiological conditions, and (iv) the oxidation of unsaturated fatty acids and the concomitant reduction of molecular oxygen to superoxide radicals and H_2O_2 (Capdevila *et al.* 1981; Inoue *et al.* 2003; Valko *et al.* 2004). The mitochondria are the primary source of oxygen radicals, such as singlet oxygen ($^1O_2$), hydroxyl radical ($OH^{\cdot}$), superoxide anion ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2) (Buttke and Sandstrom 1994; Morel and Barouki 1999). The precursor of most ROS and a mediator in oxidation chain reactions, superoxide anion ($O_2^{-\cdot}$), is a product of a one-electron reduction of oxygen. The dismutation of the superoxide anion (spontaneously or through a superoxide dismutase catalysed reaction) yields hydrogen peroxide (H_2O_2). This, in turn, can be reduced entirely to water or partially reduced to the hydroxyl radical ($OH^{\cdot}$), one of the most potent oxidants in nature. $OH^{\cdot}$ is realised through catalysis by reduced transition metals, which in turn may be reduced back to their initial state by $O_2^{-\cdot}$ (Figure 2.1) (Liochev and Fridovich 1999). Peroxisomes, cytochrome P_{450} metabolism, and inflammatory cell activation are some of the well-known endogenous sources of ROS (Valko *et al.* 2006).

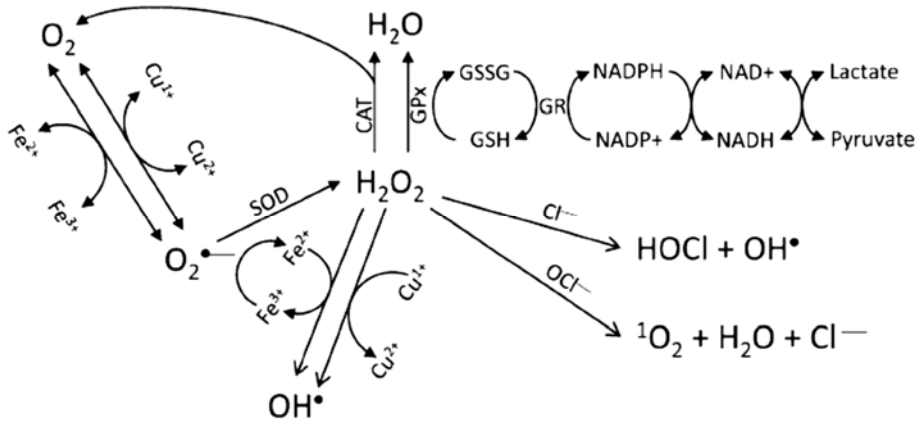


Figure 2.1: A diagrammatical representation of the formation of ROS from the mitochondria (Ramalingam and Kim 2012).

Also, $O_2^{-\bullet}$ may react with other radicals, including nitric oxide ($ON^{\bullet}$) in a reaction driven by the rate of diffusion of both radicals, thus forming peroxynitrite, another potent oxidant (Beckman and Koppenol 1996; Radi *et al.* 2002). Nitric oxide and its derivatives, i.e. nitroyl ($ON^{\bullet}$), peroxynitrile ($ONOO^{-}$), nitrogen dioxide (NO_2) and nitric oxide (NO), are generally referred to as reactive nitrogen species (RNS) (Figure 2.2) (Turrens 2003; Poon *et al.* 2004). These ROSs/RNs can also come from the surrounding environment as well as unhealthy habits such as smoking (Ames, Shigenaga and Hagen 1993; Zhao and Hopke 2012; Blanco and Blanco 2017).

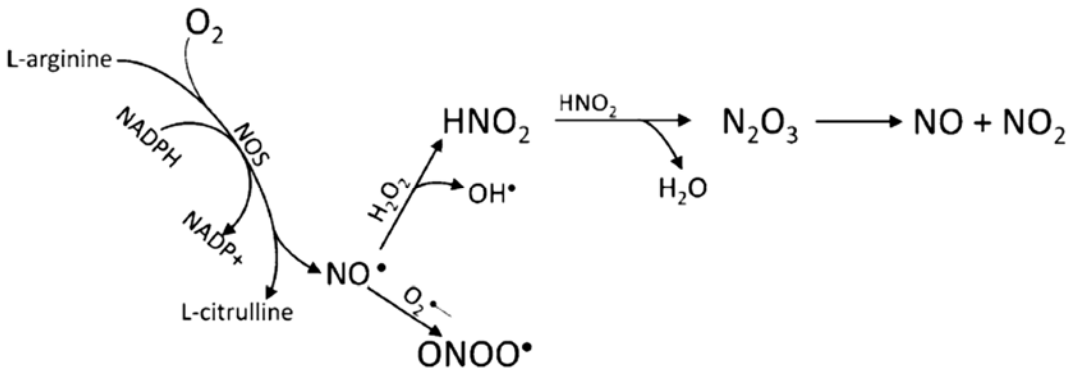


Figure 2.2: A diagrammatical representation of the formation of reactive nitrogen species (RNS) from the mitochondria (Ramalingam and Kim 2012).

The human body has endogenous, as well as exogenous, antioxidant systems that exist to scavenge free radicals and to maintain a sufficient balance between free radical production and removal (Fang *et al.* 2002; Horton 2003).

2.3. Antioxidants and their role in the quenching of free radicals

Any substance whose presence at low concentrations, in comparison to those of an oxidisable substrate, significantly delays or inhibits oxidation of that substrate by being oxidised itself is called an antioxidant (Carocho and Ferreira 2013; Maisarah *et al.* 2013; Rafieian-Kopaei *et al.* 2013). The substance's ability, after it has been oxidised, to form a new reactive oxygen species that are stable through either intramolecular hydrogen bonding or unpaired electron delocalisation (chain-breaking function) on further oxidation is another significant criterion by which it can be considered an antioxidant (RiceEvans *et al.* 1997; Carocho and Ferreira 2013).

There are numerous ways through which antioxidant activity can be useful: (i) as preventative oxidants where they inhibit free lipid radical formation through inhibition of free radical oxidation reactions; (ii) as chain-breaking antioxidants where they interrupt autoxidation chain reaction propagation; (iii) as quenchers of singlet oxygen (through coaction with other antioxidants); (iv) they can function as reducing agents where they convert hydroperoxides into chemically stable compounds; (v) as metal chelators that transform pro-oxidant metals into chemically stable products; and (vi) as pro-oxidative enzyme inhibitors, e.g. lipoxygenases (Darmanyan *et al.* 1998; Heim, Tagliaferro and Bobilya 2002; Min and Boff 2002; Pokorný 2007; Kancheva 2009).

$$\mathbf{R + AH \rightarrow RH + A} \qquad \mathbf{RO + AH \rightarrow ROH + A}$$

$$\mathbf{ROO + AH \rightarrow ROOH + A} \qquad \mathbf{R + A \rightarrow RA}$$

$$\mathbf{RO + A \rightarrow ROA} \qquad \mathbf{ROO + A \rightarrow ROOH}$$

$$\mathbf{Antioxidant + O_2 \rightarrow Oxidized\ antioxidant}$$

Figure 2.3: The general mechanism of action of antioxidants [Adapted from Mehta and Gowder (2015)].

Antioxidants are broadly classified as either enzymatic or non-enzymatic antioxidants, and the aforementioned fundamental definition of an antioxidant is inclusive of both enzymatic and non-enzymatic compounds (Sies 1997; Mehta and Gowder 2015). The antioxidants in the two major classes may be further grouped into three categories: primary, secondary and tertiary antioxidants (Singh *et al.* 2003). Primary antioxidants play a role in inhibiting oxidant formation; secondary antioxidants function as scavengers of ROS/RNS, and tertiary antioxidants (typically dietary or consecutive antioxidants) are essential in repairing oxidative damage (Mehta and Gowder 2015). Non-enzymatic antioxidants have less specificity than that of enzymatic ones. However, they (non-enzymatic antioxidants) occupy the first line of antioxidative defence, in a synergistic relationship with enzymatic antioxidants (Fang *et al*. 2002; Augustyniak *et al.* 2010; Blanco and Blanco 2017; Čolak *et al.* 2017).

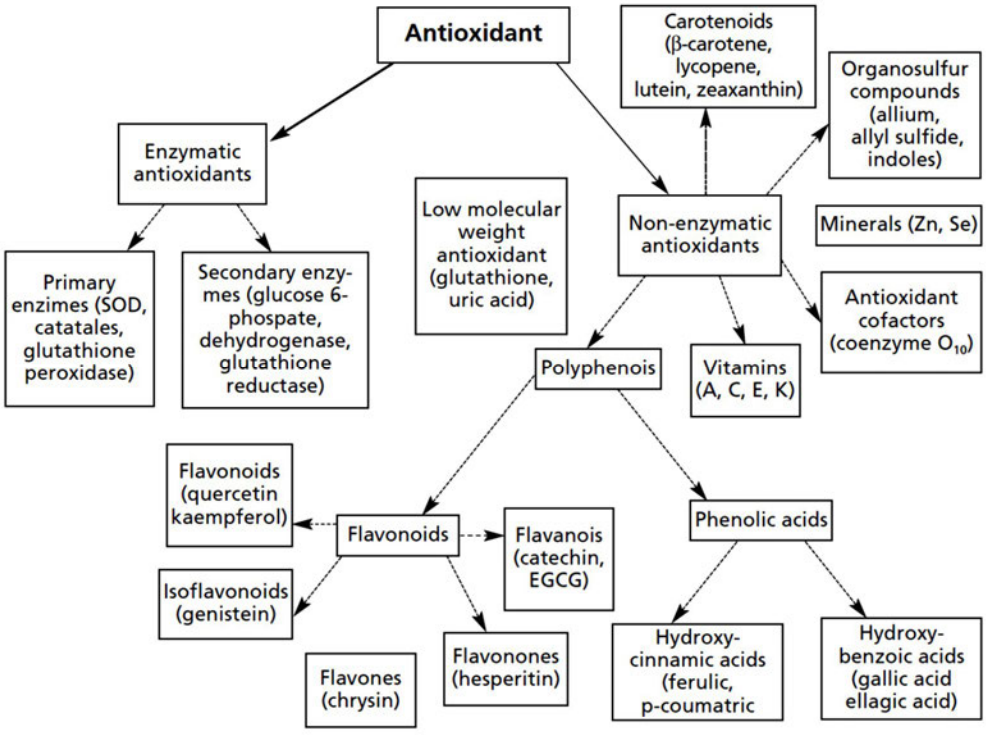


Figure 2.4: Antioxidant classification (Iannitti and Palmieri 2009).

2.3.1. The endogenous enzymatic antioxidant system of the human body

The enzymatic antioxidants cohort is composed of primary and secondary enzymatic antioxidant defences. The primary enzymatic antioxidant defence encompasses three enzymes that are essential in preventing the formation of free radicals and their neutralisation; catalase (CAT, EC 1.11.1.6), which transforms hydrogen peroxide into water and molecular oxygen; glutathione peroxidase (GSH-Px, EC 1.11.1.9), which reduces peroxides to selenoles by donating two electrons and hinders the Fenton reaction through the elimination of peroxides as potential substrates; and superoxide dismutase (SOD, EC 1.15.1.11) transforms superoxide ions into hydrogen peroxide as a substrate for catalase (Rahman 2007; Ighodaro and Akinloye 2018). Moreover, other redox proteins such as peroxiredoxins (PRXs, EC 1.11.1.15), thioredoxins (TRXs, EC 1.8.4.10), glutaredoxins, and antioxidants like hemeioxygenase-1 (EC 1.14.99.3) have crucial functions in the antioxidant defences of the pulmonary system (Birben *et al.* 2012).

Glucose-6-phosphate dehydrogenase (G6PD or G6PDH, EC 1.1.1.49) and glutathione reductase (GR, EC1.8.1.7) are constituents of the secondary enzymatic defence. Glucose-6-phosphate dehydrogenase creates a reducing environment by generating one of the coenzymes of anabolic reactions, nicotinamide adenine dinucleotide phosphate (NADPH). On the other hand, glutathione reductase recycles glutathione (antioxidant) from its oxidised to its reduced state, thus allowing it to continue free radical neutralisation (Gamble and Burke 1984; Ratnam *et al.* 2006). Glucose-6-phosphate and glutathione reductase play a supporting role to the other endogenous antioxidants (Carocho and Ferreira 2013).

2.3.1.1. Non-enzymatic endogenous antioxidants

Vitamins A, peptides (glutathione), nitrogen compounds (uric acid), and enzyme cofactors Q_{10} (Ubiquinol) make up the group of non-enzymatic endogenous antioxidants. The carotenoid, retinol or vitamin A, is a breakdown product of β-carotene that is made in the liver and has a reputation for its beneficial impact on internal organs, eyes and skin. Its propensity to combine with peroxyl radicals before lipid peroxidation confers its antioxidant activity (Palace *et al.* 1999; Jee *et al.* 2006; Ighodaro and Akinloye 2018). The endogenous tripeptide, glutathione, donates an electron or hydrogen to prevent free radical facilitated cellular damage and is essential in the re-propagation of antioxidants such as ascorbate (Curello *et al.* 1985; Steenvoorden and van Henegouwen 1997).

Coenzyme Q_{10} is found in all cells and plays a crucial role in cellular metabolism (particularly in the electron transport chain). This coenzyme works by preventing lipid peroxyl radical formation and can re-propagate vitamin E (Turunen, Olsson and Dallner 2004; Masella *et al.* 2005). Vitamin E is a lipid-soluble compound that is the primary defence against the deleterious effects of oxidants on cell membranes (White, Shannon and Patterson 1997). Uric acid, the purine nucleotide metabolism product, forestalls the excess production of haemoglobin peroxidation products (Oxo-hem oxidants) and per oxidative lysis of erythrocytes. This non-enzymatic endogenous antioxidant is a potent scavenger of hydroxyl and singlet oxygen radicals (Kand'ár, Žáková and Mužáková 2006). Albeit very efficient, the endogenous antioxidant system cannot keep up with the constant influx of ROS and RNS radicals. Humans are, therefore, dependant on the consumption of dietary antioxidants to effectively maintain low levels of free radical concentrations (Pietta 2000).

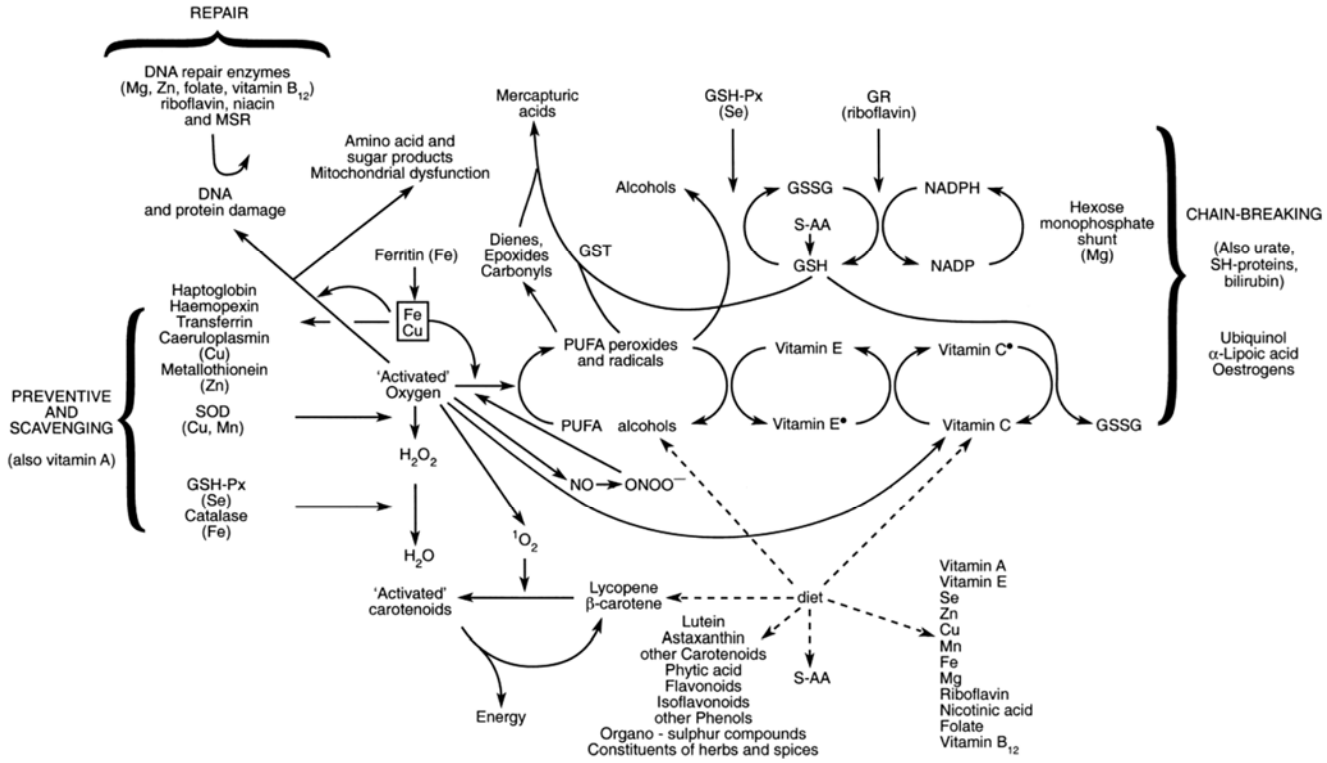
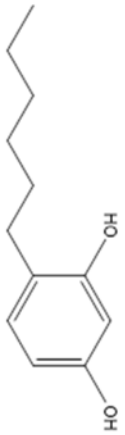


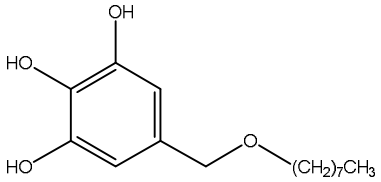
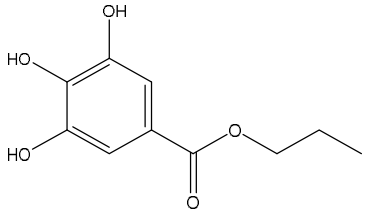
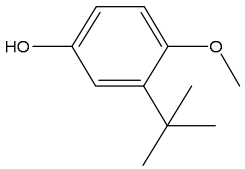
Figure 2.5: The antioxidant defence system of the human body showing the roles of some major contributors, i.e. glutathione reductase, glutathione peroxidase, glutathione-s-transferase, methionine sulphoxide reductase and superoxide dismutase (Benzie 2000).

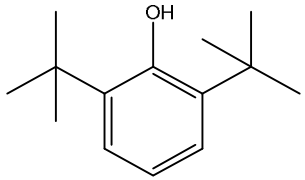
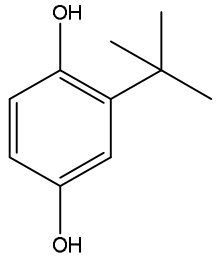
2.3.2. Synthetic non-enzymatic exogenous antioxidants

Non-enzymatic exogenous antioxidants can be divided into two collections called "Natural Antioxidants" and "Synthetic Antioxidants" (RiceEvans, Miller and Paganga 1997; Blokhina, Virolainen and Fagerstedt 2003; Carocho and Ferreira 2013). Synthetic antioxidants were primarily developed to serve as standards in antioxidant activity measurement systems in comparison with natural antioxidants, and for incorporation into foods. The addition of these pure compounds prolongs food shelf life and gives it the ability to withstand various treatments and conditions. Therefore, almost all processed foods contain synthetic antioxidants. Compounds such as nordihydroguaiaretic acid (NDGA), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (*t*BHQ), and propyl gallate (PG) are widely used. They mainly prolong shelf life through the prevention of fatty acid oxidation. Out of the mentioned antioxidants, BHT and BHA are the most preferred. *TBHQ* is used in animal food products for stabilisation and preservation of freshness, nutritive value, colour and flavour (Carocho and Ferreira 2013).

Table 2.1: Chemical structures and applications of prevalent synthetic antioxidants

Name of the compound	Chemical structure	Application	References
2,4,5-Trihydroxybutyrophenone		Food Antioxidant.	Astill, Fassett and Roudabush (1959)
4-Hexylresorcinol		Prevention of food browning.	Chen *et al.* (2004) Monsalve-Gonzalez *et al.* (1993)
		Induction of the activity of the antioxidant enzymes in human lymphocytes.	Yen, Duh and Lin (2003)
		Development of bactericidal, antiseptic, and anaesthetic properties in addition to vermifuges activity.	Ahrenfeldt (1953) Musto, Sane and Warner (1979) Nickel (2005)
		Additive in anti-ageing, skin whitening & lightening creams and hair dyes.	Pel, Bordin and Rodriguez (1998) Johnson *et al.* (2011)

Octyl Gallate (OG)		Cosmetic and food antioxidant. Antifungal properties. Antiviral activity against HSV-1, RNA viruses, vesicular stomatitis virus and poliovirus.	Kubo, Xiao and Fujita (2001) Uozaki *et al.* (2007)
Propyl Gallate (PG)		Food antioxidant Antimicrobial properties. Additive in some cosmetics formulations. Anticancer activity.	Anton *et al.* (2004) Soares, Andreazza and Salvador (2003) Chung *et al.* (1993) Wang *et al.* (2013) Jo, Park and Kim (2016) O'Brien (1994)
Butylated Hydroxyanisole (BHA)		Food antioxidant. Additive in some cosmetics formulations.	Branen (1975) Wang *et al.* (2013)

Butylated Hydroxytoluene (BHT)		Food antioxidant.	Botterweck *et al.* (2000) Aguillar *et al.* (2011) Aguillar *et al.* (2012)
***tert*-butylhydroquinone (*t*BHQ)**		Animal processed food antioxidant. Anti-carcinogenic effect.	Gharavi and El-Kadi (2005)

* A modified adaptation from Carocho and Ferreira (2013).

However, the utilisation of synthetic antioxidants is under strict regulation due to their associated health hazards. The health hazards associated with synthetic antioxidants have prompted researchers to embark on a search for natural antioxidants to substitute for the synthetic ones in food products, supplements and cosmetics (Evan and Gardner Jr 1979; McCarthy *et al.* 2001; Adelakun 2012; Shweta U. Tavasalkar 2012; Carocho and Ferreira 2013).

2.3.3. Natural non-enzymatic exogenous antioxidants

Natural antioxidants are synthesised as secondary metabolites by thousands of plant species like berries, fruits, vegetables, medicinal plants, aromatic plants and many other botanicals. They comprise vitamin C (ascorbic acid), Vitamin E (tocopherols), vitamin K, flavonoids, phenolic acids, carotenoids and minerals (Agnieszka Augustyniak 2010; Carocho and Ferreira 2013). Below is an in-depth description of each group of natural antioxidants, as mentioned above.

2.3.3.1. Some antioxidative vitamins

Ascorbic acid (widely known as vitamin C) is a naturally occurring antioxidant in nature, and most plants and animals can synthesise it *in vivo* from glucose (Traikovich 1999; Alam, Gladstone and Tung 2009). However, humans and other invertebrates are unable to synthesise it *in vivo* owing to the absence of the enzyme L-gulono-γ-lactone oxidase (EC 1.1.3.8) which is essential for this process. Hence, they acquire it from dietary sources, e.g. citrus fruits, strawberries, green leafy vegetables, papaya and broccoli (Farris 2011). Ascorbic acid comprises two compounds exhibiting antioxidant activity, i.e., L-ascorbic acid and L-dehydroascorbic acid. This antioxidant is effective in scavenging the superoxide radical anions, hydroxyl radicals, reactive nitrogen oxide, hydrogen peroxide, and singlet oxygen (Blokhina, Virolainen and Fagerstedt 2003; Brewer 2011; Carocho and Ferreira 2013).

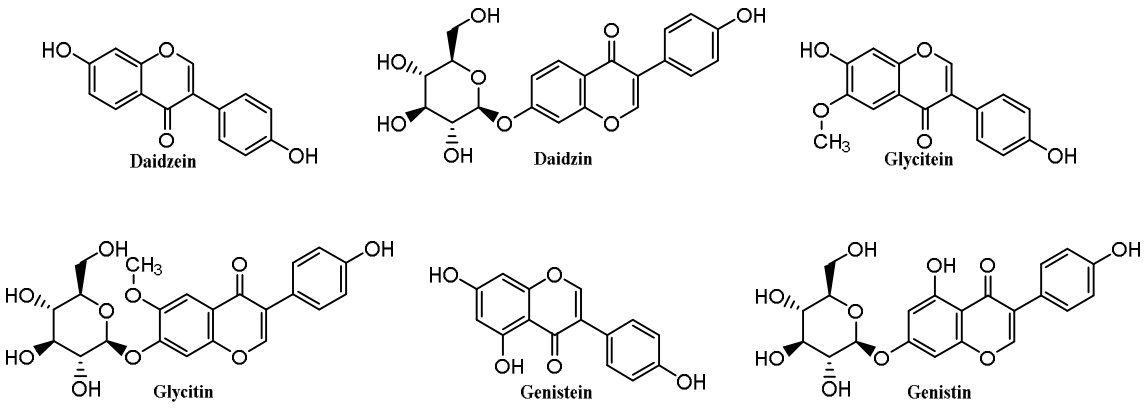
Vitamin E is a lipid-soluble, chain-breaking antioxidant composed of eight isoforms, four tocopherols (α, β, ɣ and δ -tocopherol) and four tocotrienols (α, β, ɣ and δ-tocotrienols) with α-tocopherols being the most biologically abundant isoform and having the best antioxidant capacity. Tocopherols high antioxidant activity is attributed to the chroman head group, which donates its phenolic hydrogen to peroxyl radicals. The process promotes the formation of tocopherol radicals which are unreactive, thus inhibiting further lipid peroxidation. Ascorbic acid and tocopherols have been reported to have a synergistic relationship in which tocopherols is

regenerated through ascorbic acid from the tocopherol radical, thus re-establishing its former antioxidant capacity (Brewer 2011; Shweta U. Tavasalkar 2012; Carocho and Ferreira 2013).

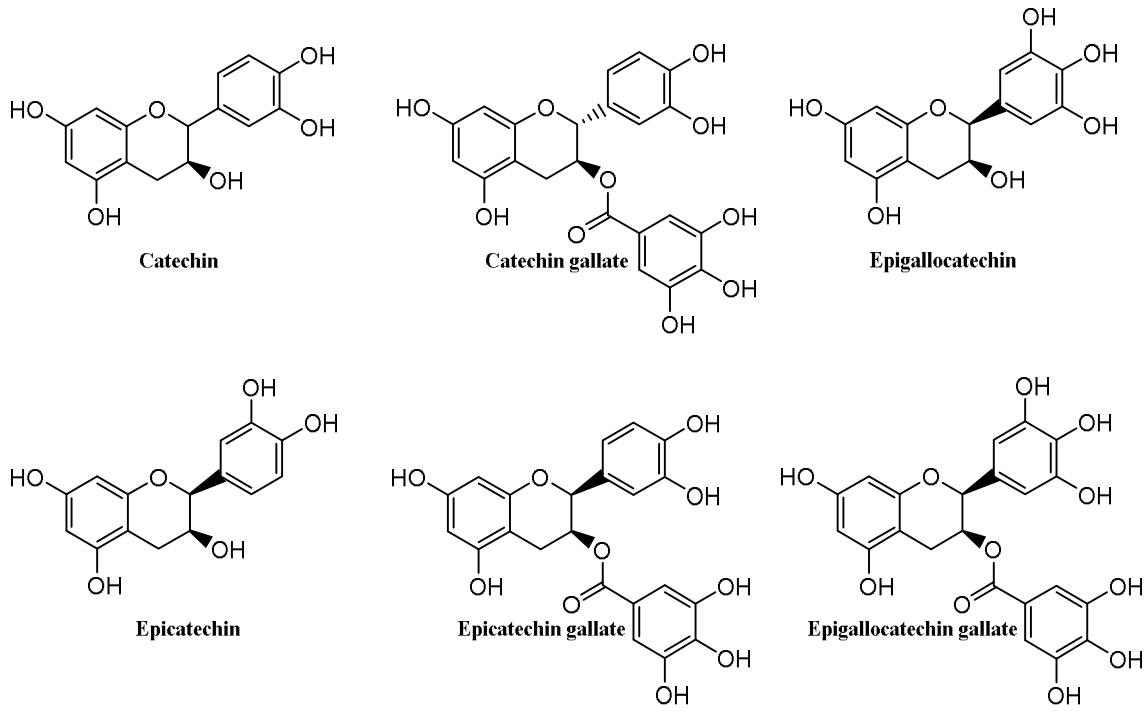
Vitamin K is a collection of fat-soluble compounds comprising of two natural isoforms (i.e. K_1 and K_2), and they are responsible for post-translational conversion of protein-bound glutamates into ɣ-carboxyglutamates in various target proteins. These vitamins owe their antioxidant activity to their 1,4-naphthoquinone structure (RiceEvans, Miller and Paganga 1997; Agnieszka Augustyniak 2010; Carocho and Ferreira 2013).

2.3.3.2. Flavonoids as antioxidants

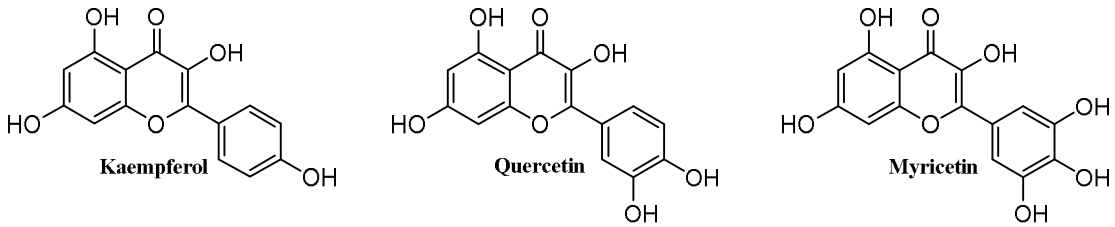
Flavonoids are a group of compounds abundant in plants that consist of the same diphenyl propane ($C_6C_3C_6$) skeleton, and they comprise flavonols, flavanols, anthocyanins, isoflavonoids, flavanones and flavones. A specific enzyme connects flavanones and flavones, and therefore, are typically found in the same fruits. Flavones and flavanols do not exhibit the same relationship and are rarely found together. The absence of anthocyanins in flavanone-rich plants has also been reported. The phenolic hydroxyl groups that are covalently bound to the ring structures are responsible for the antioxidant activity of flavonoids. These structures can act as reducing agents, singlet oxygen quenchers, superoxide radical scavengers, hydrogen donators and even as metal chelators. They also have been reported to activate antioxidant enzymes, reduce tocopheroxyls, inhibit oxidases, alleviate nitrosative stress, and increase levels of uric acids and low molecular weight molecules. Catechin, catechin-gallate, quercetin and kaempferol are some of the most important flavonoids. Flavonoids have also been reported by some researchers to inhibit activities of enzymes such as lipoxygenase, cyclooxygenase, monooxygenase, protein kinases and phospholipase A_2 (Cao, Sofic and Prior 1997; Heim, Tagliaferro and Bobilya 2002; Blokhina, Virolainen and Fagerstedt 2003; Carocho and Ferreira 2013). Phenolic acids are made up of hydroxyl cinnamic and hydroxyl benzoic acids; they are abundant in plant material and can sometimes be present as esters and glycosides. They possess antioxidant activity as chelators and free radical scavengers with a unique impact on hydroxyl and peroxyl radicals, superoxide anions and peroxy nitrites. Gallic acid, which is also a precursor of many types of tannin, is one of the most studied antioxidants in the hydroxybenzoic group (Larson 1988; RiceEvans, Miller and Paganga 1997; Carocho and Ferreira 2013).



ISOFLAVONES

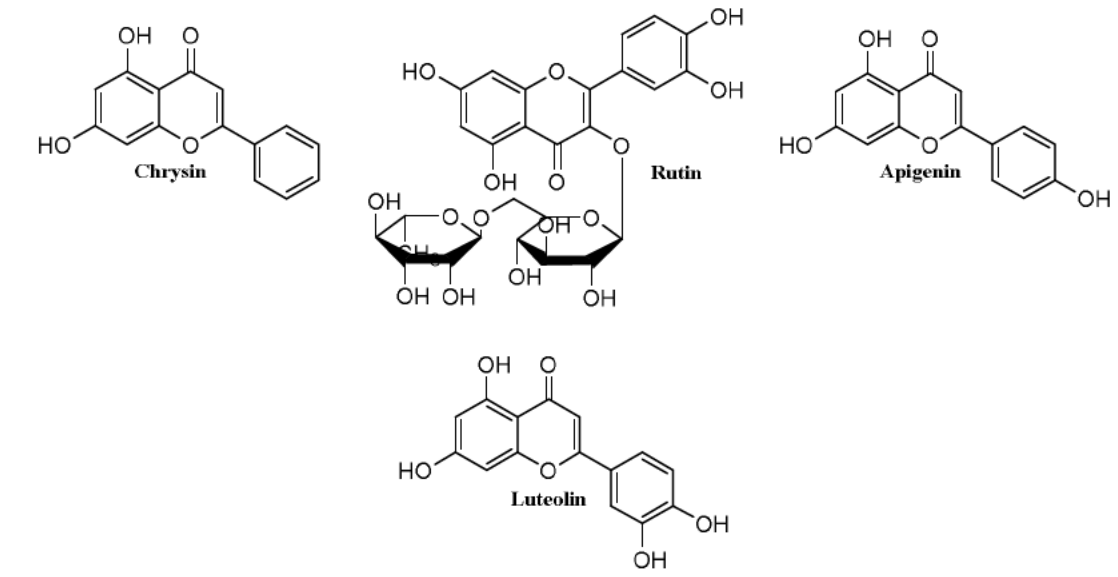


FLAVANOLS

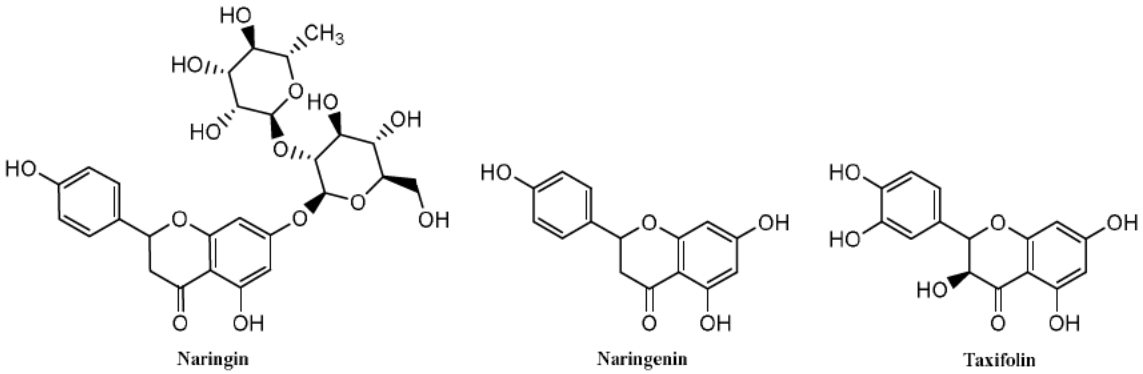


FLAVONOLS

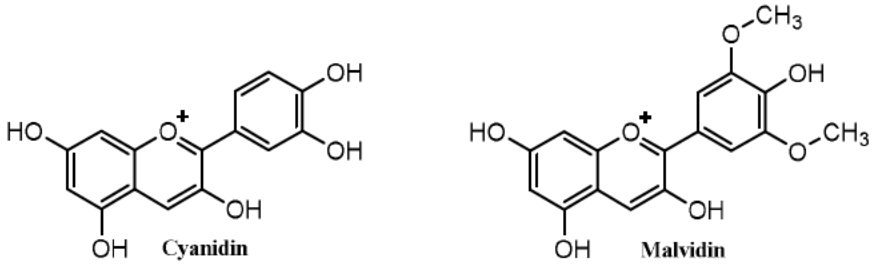
Figure 2.6: Examples of naturally occurring Flavonoids (i.e. Isoflavones, Flavanols and Flavonols).



FLAVONES



FLAVANONES



ANTHOCYANIDINS

Figure 2.7: Examples of naturally occurring Flavonoids (i.e. Flavones, Flavanones and Anthocyanidines).

2.3.3.3. Natural pigments of microorganisms and plants

Plants and microorganisms synthesise a group of natural pigments that are called carotenoids; they comprise two major groups: the carotenoid hydrocarbons known as the carotenes which contain β-carotene and lycopene; and the oxygenated carotenoids known as xanthophylls, like zeaxanthin and lutein. The carotenoids' ability to quench singlet oxygen species results in excited carotenoids that scatter the freshly acquired energy through a succession of rotational and vibrational interactions with the solvent, thus returning to an unexcited state and enabling them to quench more radical species. This can take place while the carotenoids have intra-molecular conjugated double bonds. However, carotenoids are destroyed by free peroxyl radicals and are relatively unreactive but are capable of decaying to form non-radical compounds that may terminate free radical attacks by binding to the radicals (Brewer 2011; Carocho and Ferreira 2013; Shebis *et al.* 2013).

Minerals are found in trace levels in animals, and they comprise a small proportion of the dietary antioxidants, their importance in metabolism is vast. Concerning antioxidant activity, selenium and zinc are essential minerals. In the human body, selenium can be found exhibiting both organic (selenocysteine and selenomethionine) and inorganic (selenite) forms. Selenium does not directly scavenge free radicals but is central to the functioning of antioxidant enzymes such as metalloenzyme, glutathione peroxidase and thioredoxin reductase. Zinc is very pivotal to a vast array of metabolic pathways. However, just like selenium, zinc does not directly interact with free radicals but is very significant in preventing their formation. Zinc can also inhibit NADPH oxidases which are responsible for the formation of singlet oxygen, thus halting singlet oxygen formation. It is present in superoxide dismutase, an important antioxidant enzyme that converts the singlet oxygen radicals into hydrogen peroxide. Zinc also induces the production of metallothionein that is responsible for scavenging hydroxyl radicals (Carocho and Ferreira 2013).

2.3.4. Antioxidant measuring methods

High consumption of secondary metabolites, vastly distributed in plants, has been emphasised by a catalogue of epidemiological research as crucial in reducing the incidence of oxidative stress-related diseases due to their high antioxidant activity (Antolovich *et al.* 2002; Schlesier *et al.* 2002; Lo *et al.* 2011). Over the foregoing years, numerous methods for the determination of antioxidant activity in a range of milieus (e.g. fruits, beverages, vegetable, and plasma) have been developed (Wang, Cao and Prior 1996; Maxwell 1997; Wiseman, Balentine and Frei 1997; Velioglu *et al.* 1998). These methods use different oxidants (e.g. radicals and metal ions) to measure the antioxidant's ability to reduce pro-oxidants (Schlesier *et al.* 2002; Lo *et al.* 2011). Extreme diversity is exhibited by the methods currently in use. Some methods have no clear peculiarity between the various steps in the procedure, while others follow discrete oxidation steps trailed by measurement of the result. The key participants of any of these oxidation reactions are a substrate, an oxidant, an initiator, intermediates and the subsequent products. Antioxidant activity can be assessed by measurement of any one of the aforementioned participants, it is often in comparison with compounds of known antioxidant activities such as ascorbic acid, citric acid and butylated hydroxyanisole (Clarkson 1995; Antolovich *et al.* 2002; Lo *et al.* 2011). Albeit related, the methods to measure the antioxidant activity should be distinguished from the processes of measuring an antioxidants concentration. The pertinent characteristics of antioxidant-measuring methods are mechanistic intervention, rate of scavenging, concentration effectiveness, and synergistic effect with other antioxidants (Antolovich *et al.* 2002). Below, the mechanism of each of the more common methods is described.

2.3.4.1. 2,2-Diphenyl-1-picrylhydrazyl assay

The DPPH assay is a fast, non-complex, and vastly employed method for the determination of the antioxidant activity of an extract or compound. This assay is known for its sensitivity towards phenolic and polyphenolic compounds (Frezzini *et al.* 2019). 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet+}$) is a stable free radical, owing to the delocalisation of its unpaired electron over the molecule, and accepts hydrogen radicals or electrons from donor molecules (Pisoschi and Negulescu 2011; Sahu, Kar and Routray 2013; Sridhar and Charles 2019). Therefore, $DPPH^{\bullet+}$ is widely used for the evaluation of the free radical scavenging ability of antioxidants. The DPPH assay is based on the reduction of the methanoic $DPPH^{\bullet+}$ (a free radical with an absorption

maximum at 517 nm) in the presence of a hydrogen donating antioxidant (Schlesier *et al.* 2002; Kedare and Singh 2011; Lo *et al.* 2011).

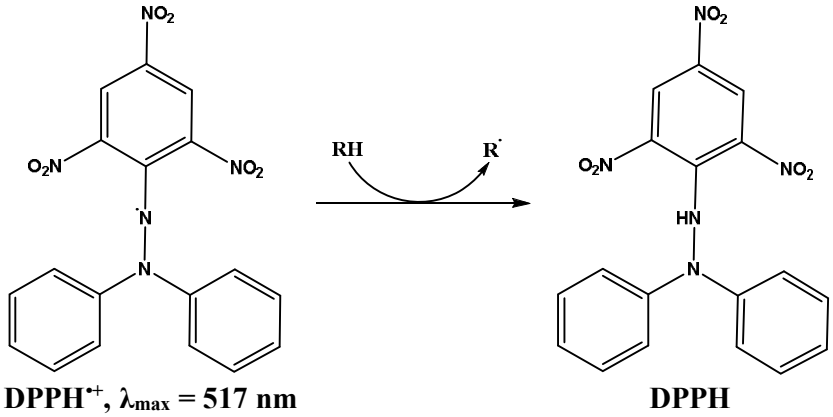


Figure 2.8: A depiction of the structure of 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet+}$) and subsequent transformation upon its reduction by an antioxidant [Adapted from Hamlaoui *et al.* (2018)].

The reduction rate is measured spectrophotometrically at intervals (usually 5 minutes) until a change of ~ 0.003 min^{-1} in the extinction (ΔE) is achieved. To calculate the antioxidant activity, a decrease in the absorbance (extinction) of methanoic $DPPH^{\bullet+}$ is determined using the following equation (Brand-Williams, Cuvelier and Berset 1995; Hasan *et al.* 2009; Lo *et al.* 2011):

$$\text{Scavenging Ability (\%)} = \left(\frac{(E_{DPPH^{\bullet+}} - E_{Sample})}{E_{DPPH^{\bullet+}}}\right) \times 100\%$$

The antioxidant activity is reported as efficiency concentration (EC_{50}), which is the concentration of an antioxidant required to reduce that of the initial $DPPH^{\bullet+}$ by 50%.

2.3.4.2. Ferric reducing ability of plasma assay

The ferric reducing ability of plasma (FRAP) assay is a simple, rapid, inexpensive, and robust antioxidant activity determination method that can be carried out via automated, semi-automated, or manual techniques (Benzie and Strain 1999; Antolovich *et al.* 2002; Prior, Wu and Schaich 2005; Badarinath *et al.* 2010). The method was conceived for application in plasma, but its utilisation has been broadened to include foods, plant extracts and other biological fluids (Benzie and Strain 1999; Gardner *et al.* 2000). This colorimetric assay uses an electron transfer

reaction to evaluate the reducing aptitude of a given antioxidant (Powell 2016). It is based on the reduction of the Fe^{3+} complex of the Fe(III)-tripyridyltriazine ($[Fe(III)(TPTZ)_2]^{3+}$) to the intensely blue coloured Fe^{2+} complex ($[Fe(II)(TPTZ)_2]^{2+}$) by antioxidants under acidic conditions (Antolovich *et al.* 2002; Powell 2016). The reaction is considered non-specific because any half-reaction that exhibits a lower redox potential than that of the $[Fe(III)(TPTZ)_2]^{3+}/[Fe(II)(TPTZ)_2]^{2+}$ reaction under the FRAP assay conditions will shift the reaction rate towards the formation of the $[Fe(II)(TPTZ)_2]^{2+}$ complex, thus indicating falsely high values (Karadag, Ozcelik and Saner 2009; Adelakun 2012).

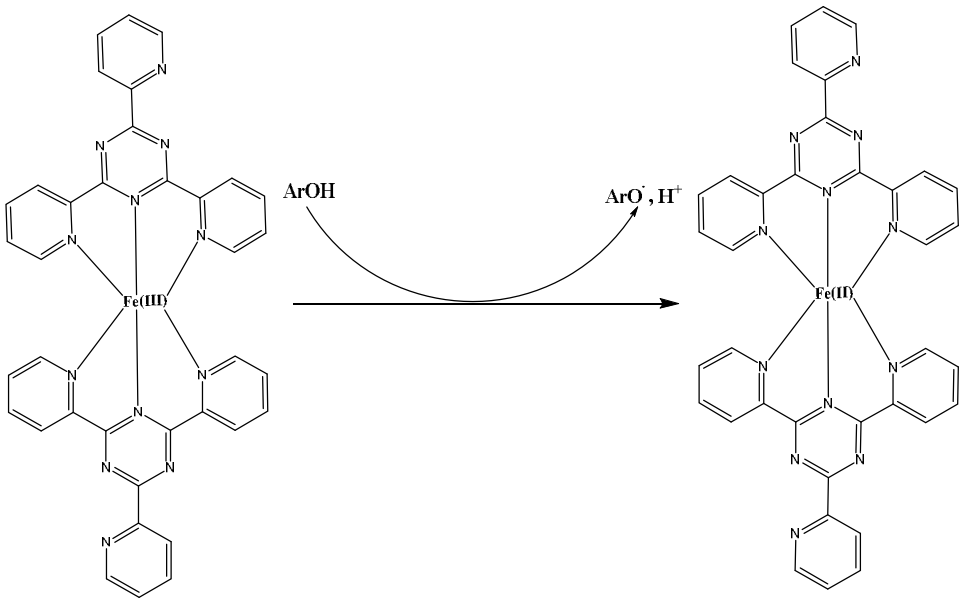


Figure 2.9: A depiction of the structure of ferric 2,4,6-Tris(2-pyridyl)-s-triazine ($[Fe(III)(TPTZ)_2]^{3+}$**)** and subsequent transformation upon its reduction by an antioxidant [Adapted from Gupta (2015) and Powell (2016)].

The increasing concentration of $[Fe(II)(TPTZ)_2]^{2+}$ in an acidic solution harbouring a stoichiometric excess of the $[Fe(III)(TPTZ)_2]^{3+}$ complex and increasing antioxidant concentration is monitored spectrophotometrically at 593 nm (Antolovich *et al.* 2002; Gupta 2015; Powell 2016). The change in absorbance is subsequently compared to a standard curve of known concentrations of the $[Fe(II)(TPTZ)_2]^{2+}$ complex, thus enabling the determination of the $[Fe(II)(TPTZ)_2]^{2+}$ complex produced against specific antioxidant concentrations (Powell 2016). The antioxidant activity is reported as micro molar Fe^{2+} (μM Fe^{2+}) equivalents or related to an antioxidant standard (Antolovich *et al.* 2002; Bolanos de la Torre *et al.* 2015). In other literature

"FRAP units" are used as a measure of antioxidant activity. One FRAP unit is defined as the reduction of 1 mole of the $[Fe(III)(TPTZ)_2]^{3+}$ complex to the $[Fe(II)(TPTZ)_2]^{2+}$ complex (Huang, Ou and Prior 2005). There is no relationship between the number of electrons an antioxidant can donate and the FRAP value (MacDonald-Wicks, Wood and Garg 2006). For example, even though bilirubin and ascorbic acid are both two-electron reductants, bilirubin exhibits a FRAP value that is one-fold higher than that of ascorbic acid (Karadag, Ozcelik and Saner 2009).

2.3.4.3. Trolox equivalent antioxidant capacity assay

The Trolox® equivalent antioxidant capacity (TEAC) assay is based on the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to its blue-green radical cation ($ABTS^{\bullet+}$), which exhibits maximum absorption at 734 nm. The $ABTS^{\bullet+}$ cation solution is subsequently exposed to an antioxidant and the rate of its reduction is evaluated spectrophotometrically by measuring the decrease in absorbance at 734 nm (Schlesier *et al.* 2002). This is compared with a reference antioxidant standard (Trolox®) (Zulueta, Esteve and Frígola 2009).

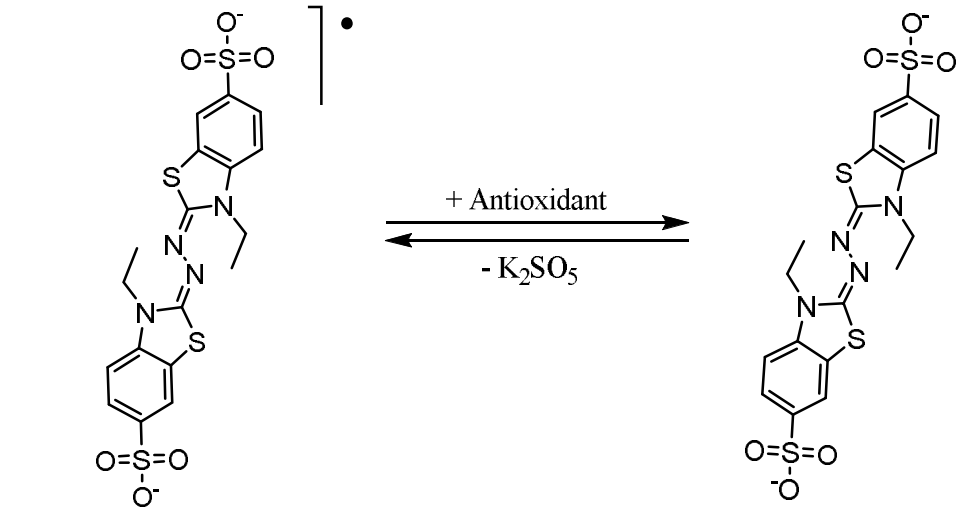


Figure 2.10: A schematic representation of the reaction of the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical in the presence of an antioxidant during the Trolox® equivalent antioxidant capacity (TEAC) assay (Zulueta, Esteve and Frígola 2009).

There are mainly three procedures through which ABTS can be oxidised before the introduction of an antioxidant. The first procedure (TEAC-1) would be to utilise H_2O_2 and metmyoglobin as

facilitators of ABTS oxidation, in which case the antioxidant activity is determined by measuring the length of the lag phase between the introduction of an antioxidant and an increase in absorbance at varying concentrations of the antioxidant (Miller *et al.* 1993). The second procedure (TEAC-2) involves filtering the solution of ABTS through a layer of manganese dioxide (MnO_2) powder (i.e. an oxidising agent) (Miller *et al.* 1996). In the third procedure (TEAC-3) $ABTS^{\bullet+}$ is generated by mixing ABTS with potassium persulfate ($K_2O_8S_2$), an oxidising agent (Re *et al.* 1999). The antioxidant activity, for both the second and third procedure, is determined by measuring the decrease in absorbance at varying antioxidant concentrations by using the equation below (Miller *et al.* 1996; Re *et al.* 1999; Schlesier *et al.* 2002):

$$\%\ \text{Antioxidant Activity} = \left(\frac{(E_{ABTS^{\bullet+}} - E_{Sample})}{E_{ABTS^{\bullet+}}}\right) \times 100\%$$

These ABTS oxidation procedures and their pertinent antioxidant capacity determination methods are easily distinguishable from each other. TEAC-1 quantifies the ability of an antioxidant to delay the radical formation, and that of scavenging the radical. On the other hand, TEAC-2 and TEAC-3 only determine the scavenging ability of an antioxidant (Schlesier *et al.* 2002).

2.3.4.4. Oxygen radical absorbance capacity assay

The oxygen radical absorbance capacity (ORAC) assay, a modified procedure of Cao *et al.*, (1993) and Ou *et al.*, (2001), is one of the widely employed standard methods for determining antioxidant activity (Cao, Alessio and Cutler 1993; Ou, Hampsch-Woodill and Prior 2001; Gupta 2015). This assay is valued in evaluating the antioxidant capacity of a variety of biological samples, pure compounds, and complex matrices (Alvarez-Suarez *et al.* 2009). The method is based on the reticence of a peroxyl radical-induced oxidation of a fluorescent probe instigated by the thermal decay of an azo compound such as 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) (Cao, Alessio and Cutler 1993; Litescu *et al.* 2014). In the original method, β-phycoerythrin was the preferred fluorescent probe due to its high sensitivity to ROS stemming from its aptitude to absorb visible light and give off a fluorescence yield (Litescu *et al.* 2014). However, owing to the unpredictability of β-phycoerythrin's fluorescent emission and its interaction with phenol and polyphenol harbouring samples, new fluorescent probes were recommended. These recommended probes exhibited characteristics such as high molecular

absorption coefficients, photochemical stabilities, and substantial fluorescent yields. Fluorescein and 6-carboxyfluorescein are amongst the most frequently used (Naguib 2000; Ou, Hampsch-Woodill and Prior 2001; Huang *et al.* 2002). The peroxyl-induced oxidation of fluorescein yields a non-fluorescence product. The loss of fluorescein's fluorescence caused by the peroxyl radical that is propagated from the thermal decay of AAPH (at 37°C) is measured fluorometrically. An application of excitation at 485 nm and monitoring emission at 538 nm, enables the determination of the fluorescence intensity. When an antioxidant is introduced to this reaction it suppresses fluorescein oxidation via a hydrogen transfer mechanism. The antioxidant activity is determined by the deceleration of the loss in fluorescence (Gupta 2015). During the cause of the assay, the intensity of the fluorescence signal is always directly proportional to the concentration of the antioxidant and is evaluated by relating the net area under the curve of the antioxidant with that of a known antioxidant standard such as Trolox® (Zulueta, Esteve and Frígola 2009).

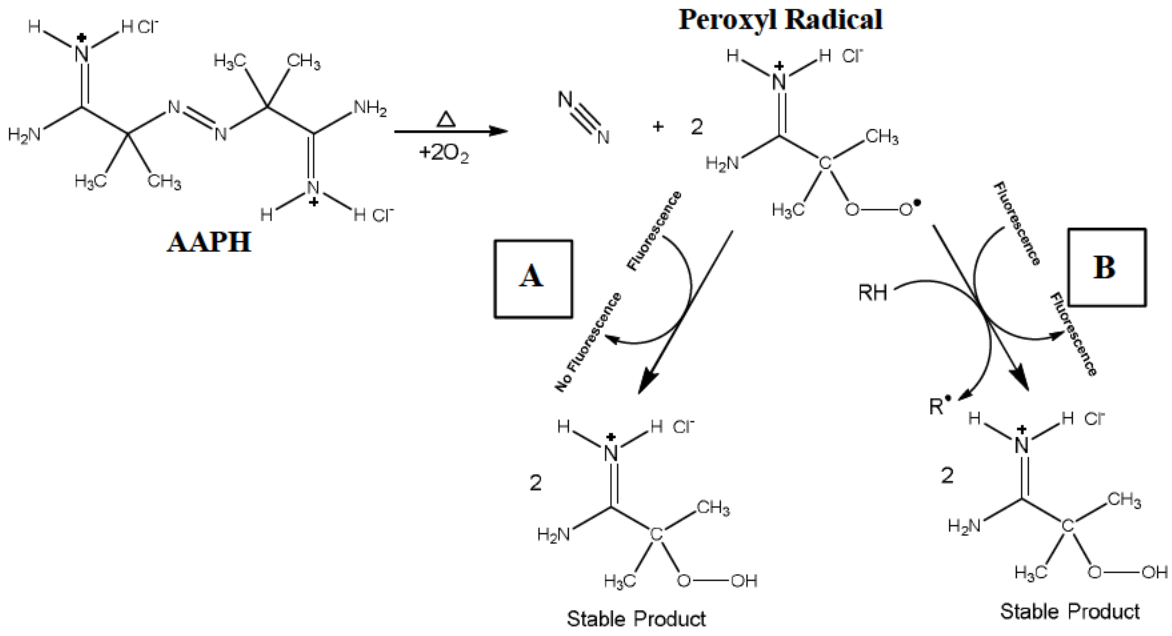


Figure 2.11: A schematic illustration depicting the interaction of fluorescein with the 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) derived peroxyl radical in the absence (A) and presence (B) of an antioxidant during the progression of the Oxygen Radical Absorbance Capacity (ORAC) assay. [Adapted from Zulueta, Esteve and Frígola (2009); Gupta (2015); and Apak *et al.* (2016)].

The antioxidant activity for the ORAC assay is commonly expressed as micro molar Trolox® equivalents (μM TE), and is calculated by applying the following formula (Zulueta, Esteve and Frígola 2009):

$$\text{ORAC (μM TE)} = \frac{C_{\text{Trolox}} \cdot \left(AUC_{\text{Sample}} - AUC_{\text{Blank}}\right) \cdot k}{\left(AUC_{\text{Trolox}} - AUC_{\text{Blank}}\right)}$$

Where C_{Trolox} is the Trolox® concentration, k is the dilution factor of the sample, and AUC is the area below the fluorescence decay curve of the blank, sample, and Trolox respectively.

2.3.4.5. Total radical-trapping antioxidant parameter assay

The total radical-trapping antioxidant parameter (TRAP) assay, originally conceived by Wayner *et al.*, (1985), is a widely used method for total antioxidant activity determination. The method was initially based on evaluating oxygen depletion during a controlled lipid oxidation reaction instigated by the thermal decay of AAPH (Wayner *et al.* 1985; Antolovich *etoal.* 2002). This involved monitoring oxygen depletion in a thermostable oxygen electrode cell for the duration of the free radical facilitated oxidation of linoleate (a lipid). However, due to the oxygen electrode's instability, the assay was later amended to use luminol-enriched chemiluminescence (CL) as the end-point, thus boosting precision and enabling superior automation (Rice-Evans and Miller 1994; Luukkainen *et al.* 1999; Antolovich *et al.* 2002). In this amended version of the assay, the peroxyl radicals (AAPH or ABAP [2,2'-azobis-amidinopropane]) heighten the rate of the CL reaction. Introducing an antioxidant snuffs the CL reaction for a duration proportional to the antioxidants radical trapping ability. The differences in stoichiometric factors of pure antioxidants require that they are taken into account when calculating molar concentrations from TRAP values (Rice-Evans and Miller 1994; Antolovich *et al.* 2002; Schlesier *et al.* 2002; Pisoschi and Negulescu 2011; Alam, Bristi and Rafiquzzaman 2013). R-phycoerythrin (R-PE) is generally utilised as a fluorescent probe of choice (known to harbour one of the phycobilins chromophores called phycoerythrobilin), and its reaction with AAPH is monitored fluorometrically [excitation at 495 nm and emission at 575 nm] (Ficner and Huber 1993; Karadag, Ozcelik and Saner 2009; Gupta 2015). The length of the lag phase due to the antioxidant in comparison to that of a known standard, such as Trolox, is used to calculate the TRAP value (Gupta 2015).

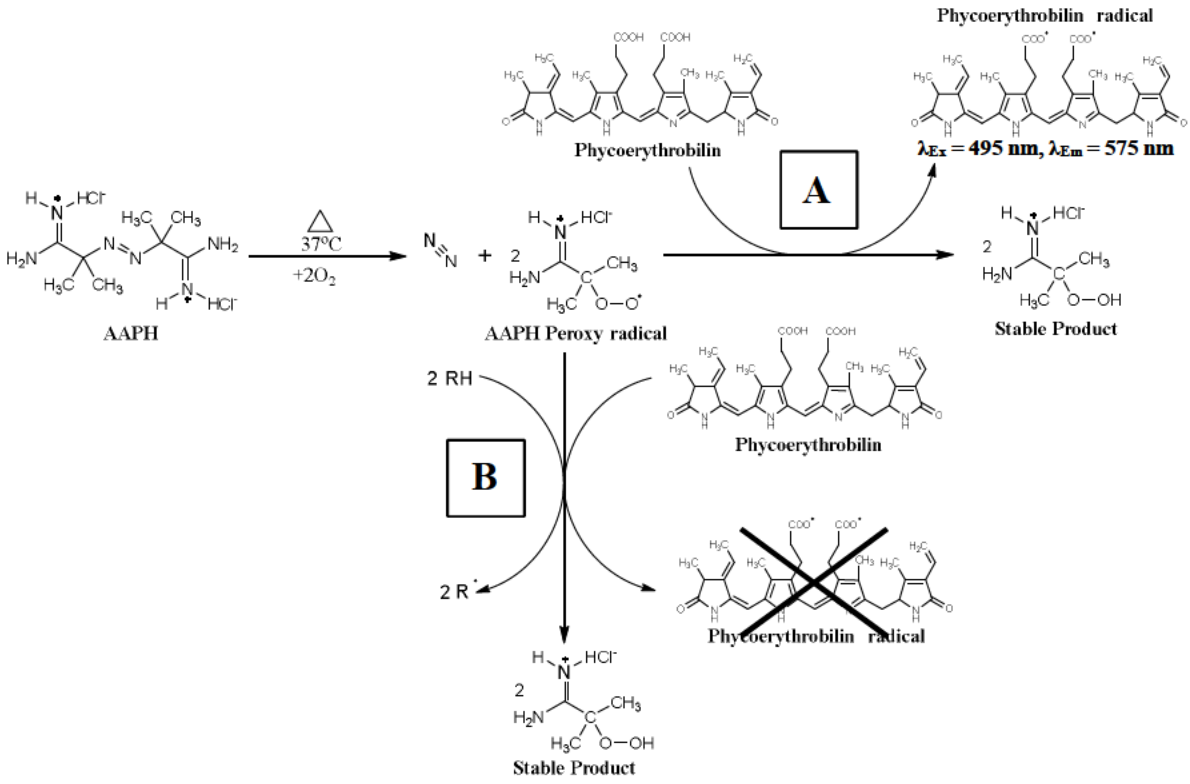


Figure 2.12: A schematic illustration depicting the interaction of phycoerythrobilin, a chromophore found in the R-phycoerythrin protein, with the 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) derived peroxyl radical in the absence (A) and presence (B) of an antioxidant during the progression of the Total peroxyl Radical-trapping Potential (TRAP) assay [Adapted from Dammeyer and Frankenberg-Dinkel (2006); Zulueta, Esteve and Frígola (2009); Gupta (2015); and Apak *et al.* (2016)].

2.3.4.6. N, N-Dimethyl-P-phenylenediamine assay

The N, N-dimethyl-p-phenylenediamine (DMPD) assay is one of the methods that have been used for the determination of antioxidant activity in biological and food samples. This assay is based on the ferric chloride ($FeCl_3$)/ potassium persulphate ($K_2O_8S_2$)-induced oxidation of DMPD to its purple coloured radical, $DMPD^{\bullet+}$, which exhibits maximum absorption at 505 nm. The $DMPD^{\bullet+}$ radical is subsequently reduced by an antioxidant via hydrogen donation, and the rate of its reduction is evaluated spectrophotometrically by measuring the decrease in absorbance at 505 nm (Fogliano *et al.* 1999; Asghar *et al.* 2007; Alam, Bristi and Rafiquzzaman 2013; Gupta 2015). This is normally carried out in comparison with a known standard such as Trolox® (Gupta 2015).

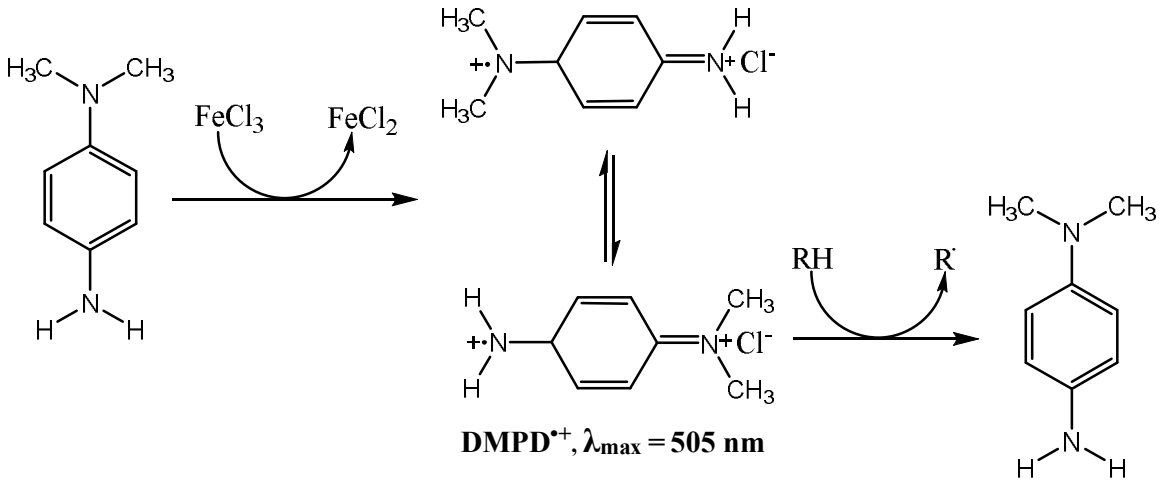


Figure 2.13: A schematic illustration depicting the interaction of the N,N-dimethyl-p-phenylendiamine radical ($DMPD^{\bullet+}$) with a hydrogen-donor antioxidant in the presence of $FeCl_3$ during the progression of the N,N-dimethyl-p-phenylendiamine assay (DMPD) [Adapted from Kamer *et al.* (2019)].

To calculate the antioxidant activity, a decrease in the absorbance of $DMPD^{\bullet+}$ is determined using the following equation (Schlesier *et al.* 2002):

$$\text{Scavenging Ability (\%)} = \left(\frac{(E_{DMPD^{\bullet+}} - E_{Sample})}{E_{DMPD^{\bullet+}}}\right) \times 100\%$$

The antioxidant activity is reported as efficiency concentration (EC_{50}), which is the concentration of an antioxidant required to reduce that of the initial $DMPD^{\bullet+}$ by 50% (Sánchez-Moreno, Larrauri and Saura-Calixto 1998). Using an appropriate calibration curve, the antioxidant activity can also be expressed in millimolar Trolox equivalents (mM TE) (Alam, Bristi and Rafiquzzaman 2013).

2.3.4.7. Photochemiluminescence assay

The photochemiluminescence (PCL) assay, originally described by Popov and Lewin (1999), has been employed meritoriously in several antioxidant activity characterisation investigations. This is due to its positive attributes such as not requiring oxidising agents for the generation of free radicals, and not being limited to a specific temperature range and pH value. The assay is based on the optical excitation induced propagation of $O_2^{\bullet -}$ radicals (in the presence of oxygen) by a photosensitiser such as Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), which also acts as a chemiluminescence reagent for monitoring free radicals (Schlesier *et al.* 2002; Karadag, Ozcelik and Saner 2009; Bauerfeind *et al.* 2014; Gupta 2015).

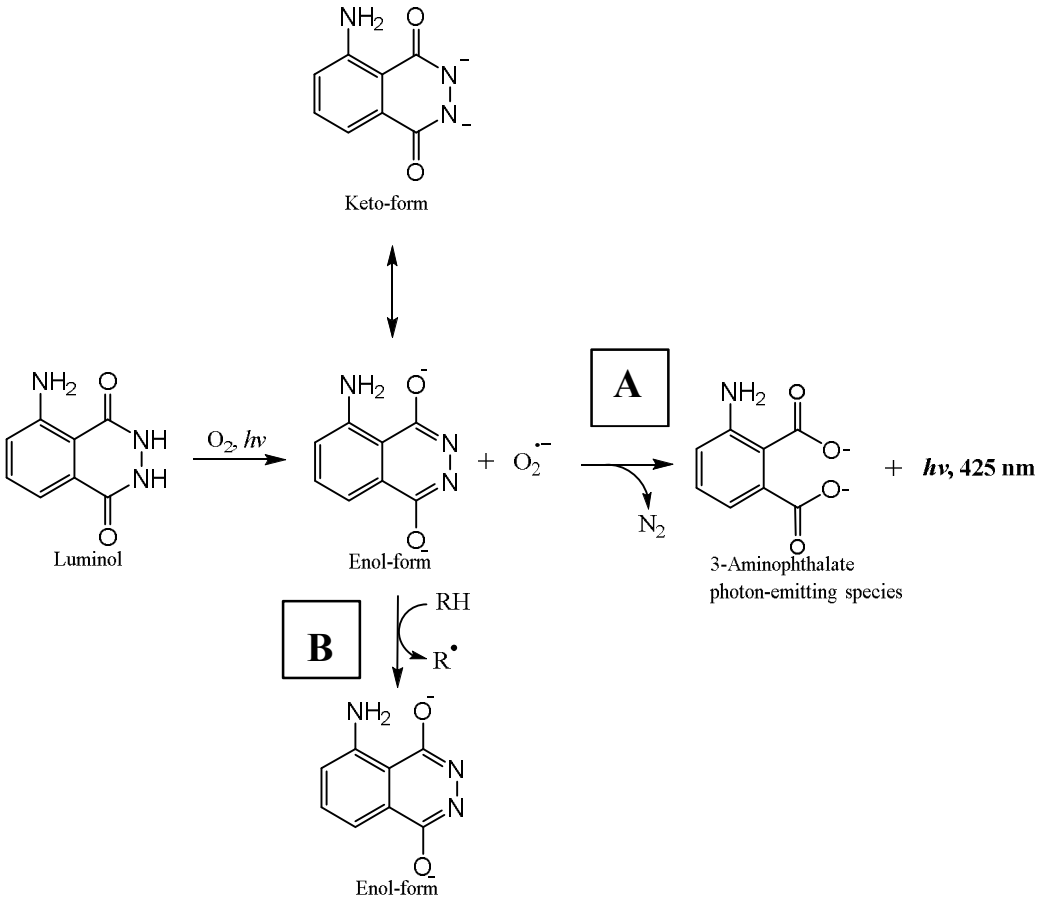


Figure 2.14: A supposed schematic illustration depicting the interaction of the enol-form of luminol with a superoxide radical to yield the photon-emitting 3-Aminophthalate, in (A) the presence and (B) the absence of an antioxidant [Adapted from Deng, Xu and Chen (2014), and Wint *et al.* (2019)].

2.3.5. The upsurge of research into natural antioxidants

The past century has seen an overwhelming upsurge of researchers' curiosity concerning natural antioxidants, primarily due to rising awareness and knowledge regarding the carcinogenicity of the previously used synthetic antioxidants (Buisman *et al.* 1998; McCarthy *et al.* 2001; Adelakun 2012). Natural antioxidants, especially the phenolic ones, exhibit functional universality in numerous redox systems that is desirable to a broad spectrum of researchers, and they have a broad spectrum of possible applications in pharmaceuticals, food products, dietary supplements, cosmetics and many other products (Agnieszka Augustyniak 2010). However, some natural antioxidants have been shown to exhibit undesirable properties such as low solubility (leads to instability in certain solvents), low bioavailability, low heat stability, low antioxidant capacity and pro-oxidant activity when present at low concentrations in existence with some transition metals (Rice-Evans, Miller and Paganga 1996; Cao, Sofic and Prior 1997; Torres *et al.* 2008; Carocho and Ferreira 2013). For example, the hydrophilic character of phenolic acids reduces their antioxidant effectiveness in stabilising fats and oils and has been reported as a severe disadvantage if an aqueous phase is also present (Stamatis, Sereti and Kolisis 2001). Most flavonoids have been reported to have low solubility and poor stability in both polar and non-polar media, which actively hampers their incorporation in many formulations (Wang *et al.* 2010). The improvement of solubility, bioavailability, heat stability, antioxidant capacity while eliminating pro-oxidant activity is accomplished by chemical or enzymatic structural modification (Stamatis, Sereti and Kolisis 2001; Torres *et al.* 2008; Wang *et al.* 2010).

2.3.6. The chemical modification of natural antioxidants

Chemical modification of natural antioxidants often involves cross-coupling (i.e. reaction of two different molecules to form one new molecule), homo-coupling (i.e. reaction of the same reactant molecules to form one new molecule) and grafting reactions. These are traditionally conducted with the aid of metal catalysts such as Na, Cu, Pd or Ni. However, these metal catalysed reactions are very intolerant of water or oxygen, especially oxygen, as many of the metal catalysed coupling reactions can be easily disrupted in the presence of oxygen owing to their occurrence through unsaturated metal complexes lacking eight valence electrons as per octet rule. Furthermore, the high temperatures under which these reactions are carried out may lead to irreversible destruction of the desired sub-structure and the formation of unwanted by-products (Kudanga *et al.* 2011b).

Chemical modification of natural antioxidants can also be carried out via esterification with an aliphatic molecule such as fatty acids and alcohols. The esterification reactions involve preparation steps in which the antioxidant is reacted with the strongly corrosive acids such as sulphuric acid or hydrogen fluoride followed by a re-esterification with fatty acids or alcohols, e.g. esterification of ascorbic acid to form ascorbyl palmitate. The esterification of phenols is usually carried out via reaction with an acid chloride or acid anhydrides under reflux (Buisman *et al.* 1998; Torres *et al.* 2008). These antioxidant modification methods have their disadvantages, including the use of less biocompatible chemicals and solvents, the formation of a mixture of products, and low yields due to low regio-selectivity (Mellou *et al.* 2005; Karmee 2009; Kidwai *et al.* 2009; Ziaullah *et al.* 2013). Therefore, further purification is often required using methods that consume large amounts of volatile organic solvents (Hancock and Viola 2002; Karmee 2009).

Chemical methods are of concern due to adverse environmental impact, safety, waste and high energy consumption. Also, chemical methods require numerous protection and deprotection steps to obtain selective modifications, and they often do not meet the requirements necessary for food applications (Stamatis, Sereti and Kolisis 1999; Wang *et al.* 2010; Yang 2011; Sun *et al.* 2013). As much as some of the compounds used in these methods can be recycled, strict environmental control is required, resulting in significant waste disposal costs (Hancock and Viola 2002). On the contrary, the enzymatic approach can be used for both simple and complex modification without the tiresome protection and deprotection steps that are common in enantio- and regio-selective chemical modification methods. The high selectivity gives efficient reactions with very limited by-products. With enzymatic modification, there is an ability to produce enantiomer specific products and operate at near-neutral pH, ambient temperature and atmospheric pressures (Stamatis, Sereti and Kolisis 1999; Chebil *et al.* 2006; Ran *et al.* 2008; Wang *et al.* 2010; Ziaullah *et al.* 2013).

2.3.7. The enzymatic modification of natural antioxidants

In this regard, enzymes such as peroxidases, catalases, tyrosinases, toluene dioxygenases, and laccases have been used for the enzymatic modification of natural antioxidants (Mayer and Staples 2002; Xu 2005; Mikolasch and Schauer 2009a; Adelakun 2012; Jeon *et al.* 2012; Polak and Jarosz-Wilkolazka 2012; Mohammed Sherif *et al.* 2013; Aljawish *et al.* 2014; Kudanga and Le Roes-Hill 2014).

2.3.7.1. Peroxidases

Peroxidases (EC 1.11.1.7) are haem- or non-haem-harbouring enzymes that are responsible for the oxidation of an assortment of xenobiotics and facilitate a two-electron reduction of peroxides, such as hydrogen peroxide, to form corresponding alcohol (Saunders 1973; O'Brien 2000; Hamid 2009; le Roes-Hill, Khan and Burton 2011; Chanwun *et al.* 2013). The innate peroxidases usually contain a ferriprotoporphyrin IX haem group consisting of four pyrrole nitrogens bound to the Fe (III). The sixth coordination position the innate peroxidases is normally vacant whilst on the proximal side of the haem, the fifth coordination position is occupied by a histidine residue's imidazole side chain (Welinder *et al.* 1993; O'Brien 2000). Peroxidases play a significant part in diverse physiological roles such as suberisation, disease resistance, lignin synthesis, fruit ripening, wound healing and auxin metabolism (Bernards *et al.* 1999; Veitch 2004; Huang *et al.* 2007; Kumar *et al.* 2007; Gross 2008). The mechanism of action of peroxidases is essentially instigated by the interaction of H_2O_2 with the enzyme, yielding a peroxidase intermediate with a concomitant reduction of H_2O_2 to water. Subsequently, two cycles of one-electron oxidation of phenolic substrates revert the unstable peroxidase intermediate to the stable Fe (III) state (Hamid 2009; Bansal and Kanwar 2013).

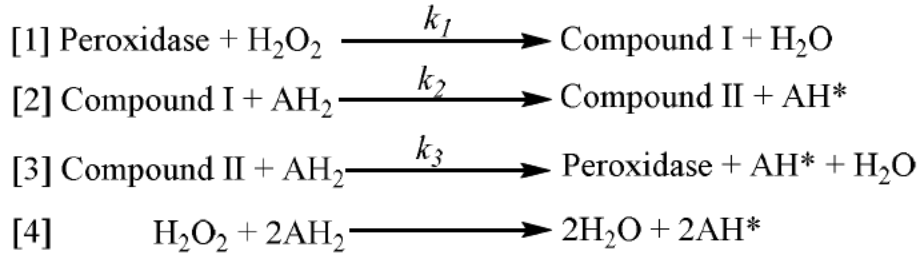


Figure 2.15: The general mechanism of action of peroxidases (Travascio, Sen and Bennet 2006).

Plant peroxidases can oxidise a plethora of phenolic compounds, such as chlorogenic acid, *o*-dianisidine, pyrogallol, catechin, guaiacol and catechol (Passardi *et al.* 2005). There are many applications of peroxidases in biocatalysis, particularly in reactions that lead to carbon-carbon bond formation (coupling reactions) such as the formation of polymerisation products from phenols (Uyama and Kobayashi 2002; Kobayashi and Higashimura 2003). The peroxidase catalysed synthesis of *ortho*-directed poly-anilines and polyphenols, such as the benzylic oxidation of toluene derivatives to yield benzaldehyde products, is one such reaction (Lim and Yoo 2000; Russ, Zelinski and Anke 2002; Uyama and Kobayashi 2002).

2.3.7.2. Catalases

Catalases (EC 1.11.1.6) constitute a sub-group of metalloenzymes within the oxidoreductases family with an ability to catalyse the disintegration of hydrogen peroxide into water and molecular oxygen, and hydrogen donors such as methanol, ethanol, formic acid, and phenols (Figure 2.16). These metalloenzymes can be separated into four classes; minor catalases, mono-functional haem-containing catalases, manganese catalases, and catalase peroxidases (Susmitha *et al.* 2015).

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

$$ROOH + AH_2 \xrightarrow{\text{catalase}} H_2O + ROH + A$$

Figure 2.16: The general catalytic action of catalases against hydrogen peroxide and hydrogen donors e.g. methanol, ethanol, formic acid, or phenols (Susmitha *et al.* 2015).

Catalases are ubiquitous, they have been shown to exist in all aerobic organisms that have been studied to date, and many anaerobic organisms (Switala and Loewen 2002; Susmitha *et al.* 2015). The enzyme is found in multiple forms in many plants, such as tobacco, saffron, cotton, mustard, castor beans, sunflower, wheat, maize, spinach, and pepper (Havir and McHale 1987). The native structure of catalase consists of four identical 60 kDa units that are tetrahedrally arranged, and each subunit harbours a single ferriprotoporphyrin group (Aebi 1980; Dunford 2010). The mechanism of action of catalases is generally initiated by the oxidation of the haem to an oxyferryl species by hydrogen peroxide. In this first step, one electron is removed from iron and the porphyrin ring to yield a porphyrin cation. The second molecule of hydrogen peroxide is reduced by the intermediate catalase, thus regenerating the stable or resting state of the enzyme (Switala and Loewen 2002). Albeit not essential for all cells in customary conditions, catalase is essential in the procurement of oxidative stress tolerance for the cells adaptive response (Aebi 1980).

There are several industrial applications of catalases, such as textile or food processing for the eradication of hydrogen peroxide used during bleaching or sterilisation, and catalysis of such oxidation reactions as epoxidation and hydroxylation (Robertson, Sanyal and Adhikary 1999; Liu and Kokare 2017). Di Gennaro *et al.* (2014) reported the successful use of the one-electron

reduced form of catalase for the polymerisation of simple phenols to produce oligomers. Moreover, a catalase from *Scytalidium thermophilum* has been reported to possess phenol oxidase activity toward (+)-catechin, catechol, caffeic acid, and chlorogenic acid. Product analysis exposed the formation of both hydrophilic and hydrophobic dimers, trimers, tetramers and oligomers of (+)-catechin, catechol, caffeic acid and chlorogenic acid (Avci *et al.* 2013; Lončar and Fraaije 2015).

2.3.7.3. Tyrosinases

Tyrosinases (EC 1.14.18.1) is a copper-containing group of enzymes that are vastly distributed in lives domains (Claus and Decker 2006). They have been identified in several prokaryotes, fungi, plants, mammals, and arthropods (Sánchez-Ferrer *et al.* 1995; Faccio *et al.* 2012). In mammals, they are responsible for melanin formation in hair and skin colour, while they are implicated in the cell damage-related browning of fruits and vegetables (Claus and Decker 2006; Faccio *et al.* 2012; Fairhead and Thöny-Meyer 2012). In many invertebrates, sponges, and plants, tyrosinases play a vital role in primary immune responses and wound healing (Claus and Decker 2006). In arthropods, they are responsible for sclerotization, and they protect DNA from UV radiation in bacteria (Faccio *et al.* 2012). The general structure of native tyrosinases consists of three domains; central, N-terminal, and C-terminal domain (Van Gelder, Flurkey and Wichers 1997). The central domain harbours six histidine residues and contains Cu-A and Cu-B ions. Whilst the central is the most conserved among tyrosinases, the Cu-B site demonstrates higher conservation than the Cu-A site (Van Gelder, Flurkey and Wichers 1997; Schweikardt *et al.* 2007; Tran, Taylor and Constabel 2012). The biochemical studies of tyrosinases from different reveals the importance of the conserved histidine residues in copper binding (Nakamura *et al.* 2000; Olivares, García-Borrón and Solano 2002; Schweikardt *et al.* 2007; Kaintz *et al.* 2015).

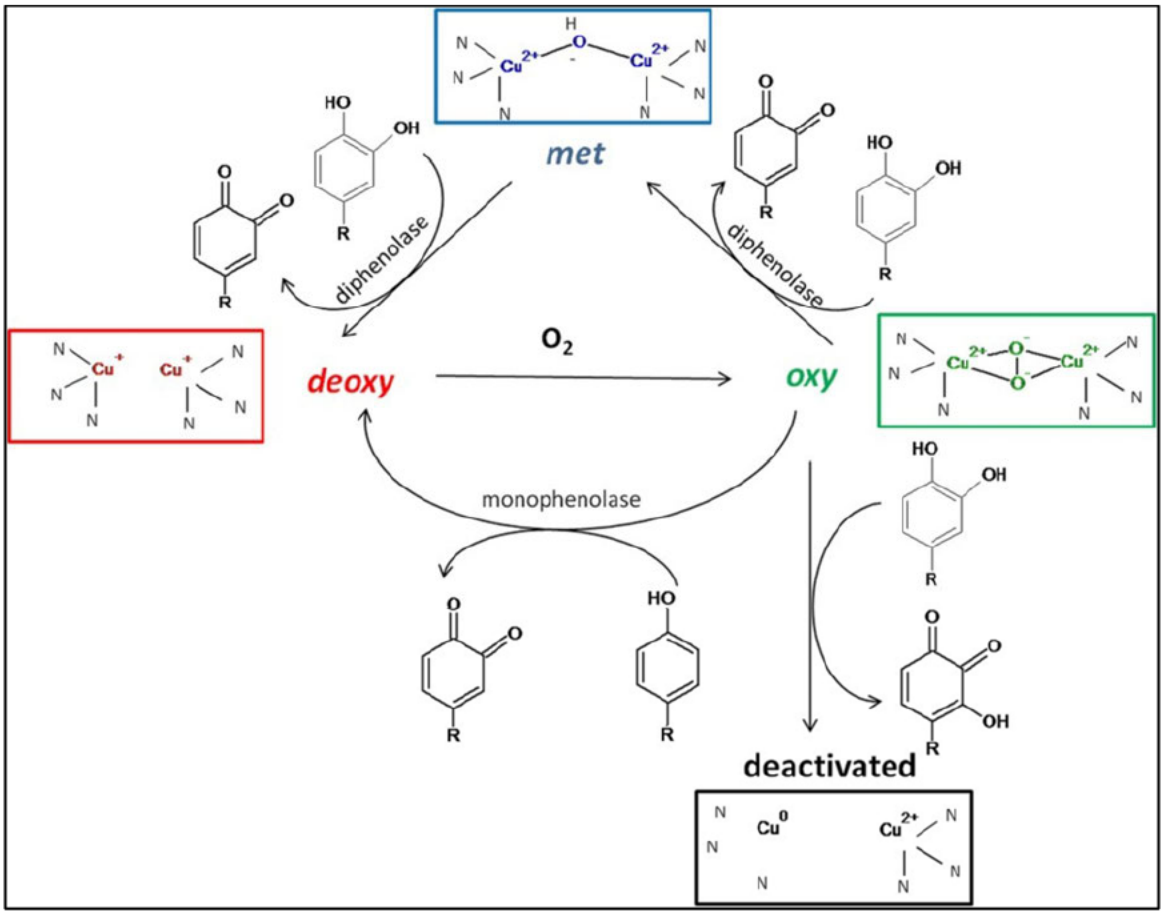


Figure 2.17: The four discrete oxidation states of tyrosinases (Kanteev, Goldfeder and Fishman 2015).

The four oxidation states of the active site (Figure 2.17) determine the properties of tyrosinases (Ramsden and Riley 2014). The *met* state, in which two Cu^{2+} are ligated to a hydroxyl ion, is the most common for the native tyrosinase. Catechol is one of the compounds that can be oxidised by the *met* state resulting in the reduction of tyrosinase to the *deoxy* state, whose subsequent consequence is the reduction of both copper ions to Cu^{1+}. The *deoxy* state is converted to the *oxy* state through the ligation of two oxygen molecules by two Cu^{2+}. Oxidation of catechols and phenols to *o*-quinones is possible with *deoxy* tyrosinase, albeit via different oxidative mechanisms (Sánchez-Ferrer *et al.* 1995; Ramsden, Stratford and Riley 2009; Muñoz-Muñoz *et al.* 2010; Ramsden and Riley 2014). Tyrosinases' ability to facilitate the conversion of monophenols to diphenols encouraged investigations on the production of antioxidant *ortho*-diphenols with advantageous attributes as pharmaceutical drugs and food additives (Halaouli *et al.* 2006). Satô (1969) was the first to report tyrosinase catalysed production of caffeic acid from *p*-coumaric acid. Furthermore, tyrosinase is highly effective in converting *p*-tyrosol (an agro-

industrial by-product) to hydroxytyrosol, a potent antioxidant (Espín, Soler-Rivas and Wichers 2000; Espín *et al.* 2001; Lesage-Meessen *et al.* 2001; Halaouli *et al.* 2005).

2.3.7.4. Toluene dioxygenases

Toluene dioxygenases (EC 1.14.12.11) catalyses the formation of *cis*-hydrodiols from aromatic substrates such as toluene, chlorobenzene, *p*-fluorotoluene, ethylbenzene, *p*-chlorotoluene, *p*-bromotoluene and *p*-xylene. The *cis*-hydroxylation of the substrates occurs through the incorporation of both atoms of molecular oxygen onto the substrate's aromatic nucleus in the presence of NADH as a coenzyme (Yeh, Gibson and Liu 1977; Subramanian *et al.* 1979; Subramanian *et al.* 1985; Wackett, Kwart and Gibson 1988; Butler and Mason 1996). These enzymes are treasured in the enantio-controlled synthesis of compounds due to their ability to convert substrates into stereo- and regio-specific products (Hudlicky *et al.* 1994). Toluene dioxygenases are also known to catalyse the formation of *cis*-1,2-indandiol through the addition of dioxygen to the non-aromatic double bond of indene. The reactions (more especially the oxidation of toluene to *cis*-toluene dihydrodiol) are catalysed by a multicomponent enzyme system (Figure 2.18) (Gibson *et al.* 1970; Kobal *et al.* 1973; Ziffer *et al.* 1973; Wackett, Kwart and Gibson 1988).

The enzyme system is comprised of three components; a flavoprotein reductase, a ferredoxin (responsible for transferring electrons from NADH), and the iron-sulphur protein (ISP_{TOL}). ISP_{TOL} is comprised of the α- and β-subunits; the Rieske-type [2Fe-2S] cluster, a mononuclear non-haem iron oxygen activation centre and substrate-binding site, is contained in the α-subunit of ISP_{TOL} (Zamanian and Mason 1987; Butler and Mason 1996; Jiang *et al.* 1996; Kauppi *et al.* 1998; Jiang, Parales and Gibson 1999). While it may have been previously speculated that the β-subunit might be involved in the $ferredoxin_{TOL}$ docking and electron transfer, the work by Jiang, Parales and Gibson (1999) demonstrated that the α-subunit accepts electrons from the reduced $ferredoxin_{TOL}$ in a reaction that is independent of the β-subunit. However, in the absence of the β-subunit, the reduced α-subunit failed to oxidize toluene to *cis*-toluene dihydrodiol. (Butler and Mason 1996; Jiang, Parales and Gibson 1999). Albeit essential for catalysis, the β-subunit mainly functions in the structural capacity to maintain the contact between adjacent α-subunits which play a major in the enzyme's substrate specificity (Erickson and Mondello 1993; Tan and Cheong 1994; Kimura *et al.* 1997; Mondello *et al.* 1997; Parales *et al.* 1998; Jiang, Parales and Gibson 1999).

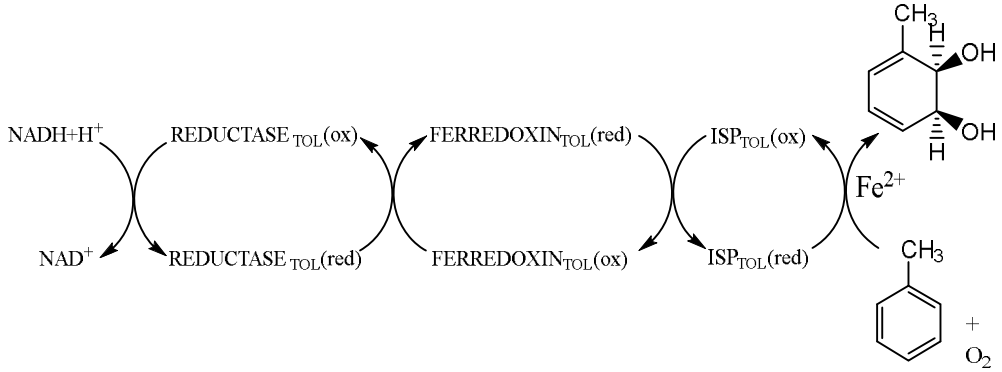


Figure 2.18: The general toluene dioxygenase components and their organisation [Adapted from Subramanian *et al.* (1979); and Gibson *et al.* (1982)].

Electrons are reassigned from NADH through the reduction of a flavoprotein ($REDUCTASE_{TOL}$) and a diferric-disulphide protein ($FERRODOXIN_{TOL}$) to a terminal iron-sulphur protein (ISP_{TOL}) (i.e. a reduced form of the terminal oxygenase component) that facilitates the oxidative formation of *cis*-toluene dihydrodiol (Subramanian *et al.* 1979; Subramanian *et al.* 1981; Gibson *et al.* 1982). Arenes' toluene dioxygenase catalysed dihydroxylation makes way for a vast variety of reactions and products, where the hydroxylation positions are dependent on the substrates substituents (Boyd, Sharma and Allen 2001). These enantio- and regio-selective toluene dioxygenase biocatalysis reactions produce essential synthetic building blocks such as catechols and hydroquinones (Suske *et al.* 1997; Burton 2003b; Boyd *et al.* 2015). There has been sustained attention on the application of these synthetic precursors of chiral ligands, natural products, and compounds of value in medicinal chemistry (Lee, Brand and Gibson 1995; Boyd *et al.* 2004; Dasseux and Oniciu 2004; Boyd *et al.* 2015). Furthermore, toluene dioxygenases are some of the enzymes that have been implicated in the biodegradation of trichloroethylene, a chlorinated solvent with adverse human health effects (Wackett, Kwart and Gibson 1988; Wackett and Householder 1989; Chiu *et al.* 2013).

However, Laccases are superior and have been subject to an intense investigation because they have broad substrate specificity, e.g. phenols, polyphenols, anilines, aryl diamines, methoxy-substituted phenols, hydroxyindoles, benzothiazoles, inorganic and organic metal compounds, and they do not require additional co-factors to catalyse the reaction, i.e. they use molecular oxygen as the final electron acceptor with the concomitant production of water as the only by-product (Jeon *et al.* 2012; Mohammed Sherif *et al.* 2013; Kudanga and Le Roes-Hill 2014).

2.4. Laccases

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multi-copper oxidase enzymes belonging to the blue copper family of enzymes. They catalyse the oxidation of various non-phenolic and phenolic compounds to produce radicals with concomitant four-electron reduction of molecular oxygen to water (Cohen, Persky and Hadar 2002; Dwivedi *et al.* 2011; Santhanam *et al.* 2011; Riva 2013). The name "Laccase" stems from the enzyme's original identification in the exudates of the Japanese lacquer tree, *Rhus vernicifera* (Harvey 1997; Polaina and MacCabe 2007). The enzyme was first characterised as a metal-containing oxidase by Bertrand in 1985 (Mayer and Staples 2002). Laccases have been found in plants, arthropods, bacteria but are predominant in fungi, i.e., their presence has been reported in essentially every fungus that has been tested for them (Gavnholt and Larsen 2002; Mayer and Staples 2002; Polaina and MacCabe 2007). Physiological functions amongst individual organisms differ, but they all involve catalysis of polymerisation or depolymerisation processes (Mohammed Sherif *et al.* 2013; Riva 2013).

2.4.1. Sources of laccases

Multiple forms of laccases have been reported from a variety of plants such as mango, mung bean, peach, pine, prune, and sycamore (Madhavi and Lele 2009). They share some of their properties and characteristics with fungal laccases except for size, optimal pH, and that they are mostly secreted, with a few intra-cellular variants predicted to be found in mitochondria (Wang *et al.* 2015). Plant laccases have an isoelectric point (pI) range of pH 3 – 9 and an optimal pH range of pH 6.8 – 7.4 (Benfield *et al.* 1964). They are normally made of 500 – 600 amino acid residues and have an approximate molecular weight of 60 – 130 kDa (Giardina *et al.* 2010; Solomon *et al.* 2014). Although the reaction mechanism and molecular architecture of their active site are the same as those of bacterial or fungal laccases, they have a lower redox potential due to leucine or methionine (rather than phenylalanine) bound to their T1 site (Morozova *et al.* 2007a). They manifest greater molecular weights due to their high extent of glycosylation (contributes 10 – 50% of their molecular weight), which has been reported to be accountable for enzyme stability, enzyme activity, and copper retention (Harvey 1997; Madhavi and Lele 2009; Dwivedi *et al.* 2011; Babu, Pinnamaneni and Koona 2012b; Wang *et al.* 2015). Plant laccases have been implicated, by many studies, in the biosynthesis of the plant's lignin (Caparrós-Ruiz *et al.* 2006; He *et al.* 2019; Li *et al.* 2019). Berthet *et al.* (2011) found that when they knock-out prominent laccase genes (i.e. AtLAC4 and AtLAC17) in the *Arabidopsis thaliana* plant, using

T-DNA insertional mutagenesis, the xylem was distorted. On the other hand, they observed that when they knocked out the AtLAC4, AtLAC11, and AtLAC17 genes of the same plant it resulted in physiological changes, such as growth retardation, constricted stems, and absence of lignified vascular bundle (Li *et al.* 2019). Thus, suggesting that the laccase coded for by the AtLAC11 gene may be involved in lignin polymerisation (Gavnholt and Larsen 2002). Some plant laccases, such as those expressed in non-woody tissues, participate in the oxidation of flavonoids (Caparrós-Ruiz *et al.* 2006; Pourcel *et al.* 2007; Turlapati *et al.* 2011). Turlapati *et al.* (2011) also reported the involvement of laccase in the plant's stress response. Plant laccases also play a role in biological processes such as the preservation of the cell wall structure and integrity, wound healing, iron metabolism, and the biosynthesis of secondary metabolites (Caparrós-Ruiz *et al.* 2006; Wang *et al.* 2015; Li *et al.* 2019).

Laccases are found in a variety of fungi such as white-rot fungi (e.g., *Pleurotus*), yeasts (e.g., *Cryptococcus*), mushrooms (e.g., *Agaricus*), and moulds (e.g., *Penicillium*) (Mikolasch and Schauer 2009a). With more than 125 basidiomycetous laccase genes having been identified and described, ligninolytic basidiomycetes (*Nematoloma*, *Phanerochaete*, *Stropharia*, *Trametes*, *Sporotrichum*, etc.) undoubtedly harbour a vast array of laccases (Hoegger *et al.* 2006). Also, there is a lot of literature documenting the production of laccase by several *Ascomycetes* such as *Podospora anserine*, *Melanocarpus albomyces*, *Magnaporthe grisea*, *Mauginella*, *Neurospora crassa*, and *Gaeumannomyces graminis*; and *Deuteromycetes* (Esser and Minuth 1970; Froehner and Eriksson 1974; Thakker, Evans and Rao 1992; Binz and Canevascini 1997; Edens *et al.* 1999; Kiiskinen, Viikari and Kruus 2002; Iyer and Chattoo 2003; Palonen *et al.* 2003; Chaurasia, Bharati and Singh 2013). Fungal laccases are either intra- or extra-cellular monomeric, homo-dimeric (i.e., composed of two identical subunits with molecular weight typical of monomeric laccases) or tetrameric glycoproteins with an approximate molecular weight of between 60-100 kDa, and they are the best characterised amongst all laccases (Baldrian 2006; Hildén, Hakala and Lundell 2009). Fungal laccases are implicated in several cellular processes such as plant pathogenesis, delignification, the formation of the fruiting body, sporulation, and pigmentation (Thurston 1994; Yaver *et al.* 2001). However, literature demonstrating experimental data about some of the aforementioned functions is scarce (Kunamneni *et al.* 2007a). A majority of fungal laccases are known to operate as phenol oxidases under mildly acidic conditions (pH 4-6) and within the optimal temperature range of 30-55°C. Some fungal laccases have been reported to exhibit catalytic activity in temperatures as high as 80°C. However, for most fungal laccases, the catalytic activity decreases at temperatures above 60°C

(Baldrian 2006; Hildén, Hakala and Lundell 2009; Santhanam *et al.* 2011). Several fungal species have been reported to exhibit the production of more than one isoenzyme, typically with an isoelectric point in the range of pH 4-6 (Baldrian 2006).

The prokaryotes are the main source of the uppermost thermos-stable laccases whose incidence is mostly restricted in species within the genera *Aquifex*, *Azospirillum*, *Bacillus*, *Marinomonas*, *Pyrobaculum*, *Ralstonia*, or *Streptomyces* (Claus 2003; Hildén, Hakala and Lundell 2009; Mikolasch and Schauer 2009a). The first bacterial laccase was discovered in 1993 in non-motile strains of *Azospirillum lipoferum* isolated from the rhizosphere of rice. The use of computational methods to mine genome sequence databases led to the discovery of a large number of laccase genes in the genomes of a diverse set of bacteria, predominantly in the streptomycetes, bacilli, and pseudomonads (Santhanam *et al.* 2011). Laccases found in *Azospirillum lipoferum* were found to be involved in the formation of melanin, and plant cultivations that were inoculated with this bacteria displayed substantial growth improvements (Faure, Bouillant and Bally 1995). A thermostable laccase from *Bacillus subtilis* was implicated in the production of brown spore pigment in the endospore coat, and this has been said to protect the spore coat from ultra-violet radiation and hydrogen peroxide (Martins *et al.* 2002; Dwivedi *et al.* 2011). Bacterial laccases are either intra- or extra-cellular but are mostly intracellular such as those of *Marinomonas Mediterranea*, *Bacillus subtilis*, and *Azospirillum lipoferum* (Diamantidis *et al.* 2000; Martins *et al.* 2002). These laccases are monomeric, dimeric or tetrameric glycoproteins with a wide range of molecular weights varying between 28 and 180 kDa. They have been reported to exhibit higher thermal and alkaline pH stability when compared to fungal laccases, i.e. they operate within a temperatures range of 30-92°C, pH range of pH 3-8, as well as high concentrations of chloride and copper ions (Held *et al.* 2005; Rosconi *et al.* 2005; McMahon *et al.* 2007; Dwivedi *et al.* 2011; Santhanam *et al.* 2011; Mohammed Sherif *et al.* 2013). Therefore, bacterial laccases may represent particularly robust industrial biocatalysts, particularly the immobilised spore laccases which are more compatible with almost all industrial processes (Dwivedi *et al.* 2011; Mohammed Sherif *et al.* 2013).

Laccases have also been found in the insect of genera such as *Tenebrio*, *Bombyx*, *Schistocerca*, *Calliphora*, *Sarcophaga*, *Lucilla*, *Oryctes*, *Diploptera*, *Manduca*, *Rhodnius*, *Drosophila*, *Papillo*, *Musca*, and *Phormia*. They catalyse the oxidative coupling of catechols and proteins which may be an important precursor to cuticle sclerotization (Babu, Pinnamaneni and Koona 2012a).

2.4.2. General molecular structure of laccases

Laccases are dimeric or tetrameric glycoproteins, usually containing four copper atoms per monomer (Claus 2004; Dwivedi *et al.* 2011). The molecular mass of the monomers ranges between 50 and 100 kDa; the covalently bound carbohydrate moiety is an essential feature as it may contribute to the high stability of the enzymes (Claus 2004; Polak and Jarosz-Wilkolazka 2012). To perform catalytic functions, laccases are dependent on the copper atoms that are distributed at the three copper centres. The copper centres are categorised into three groups, i.e., type 1 (T1) or blue copper centre, type 2 (T2) or normal copper and type 3 (T3) or coupled binuclear copper centre (Claus 2004; Dwivedi *et al.* 2011). T1 copper is liganded by two histidines and one cysteine residue in the formation of a distorted trigonal geometry. This copper type absorbs intensely at 600 nm and is responsible for the blue colour of the enzyme due to the intense electronic absorption caused by the covalent copper-cysteine bond. T2 copper is coordinated by two histidines, but shows no absorption in the visible spectrum and proves to have paramagnetic properties. Six histidine residues coordinate T3 copper; these copper atoms absorb distinctively at 330 nm and form a tri-nuclear cluster with the T2 copper atom. A hydroxide bridge maintains the strong anti-ferromagnetic coupling between the two T3 copper atoms (Leontievsky *et al.* 1997; Claus 2004; Santhanam *et al.* 2011; Chaurasia, Bharati and Singh 2013).

The three interconnected, cupredoxin domains are twisted together to form a globular structure with the T1 site typically located in domain three and the T2/T3 tri-nuclear cluster located between the first and third domains. Disulphide bridges link domains two and three, the amino acid residues of domains two and three form the substrate-binding pocket. Regardless of low sequence homology between fungal and bacterial laccases, the overall geometry of their active sites is similar, comprising of highly conserved features and an active site containing four specifically structured copper ions with an exception to the *Coprinus cinereus* laccase that is missing T2 copper and small laccases (SLAC) from *Streptomyces coelicolor* which consists of only two domains, with the tri-nuclear cluster positioned between domains one and two. The second domain in fungal laccases does not constitute an active site but contributes to the stability of the three-domain monomer. In SLAC, the stability of the trimeric unit is ensured by the symmetric arrangement of the two-domain monomers (Santhanam *et al.* 2011; Polak and Jarosz-Wilkolazka 2012).

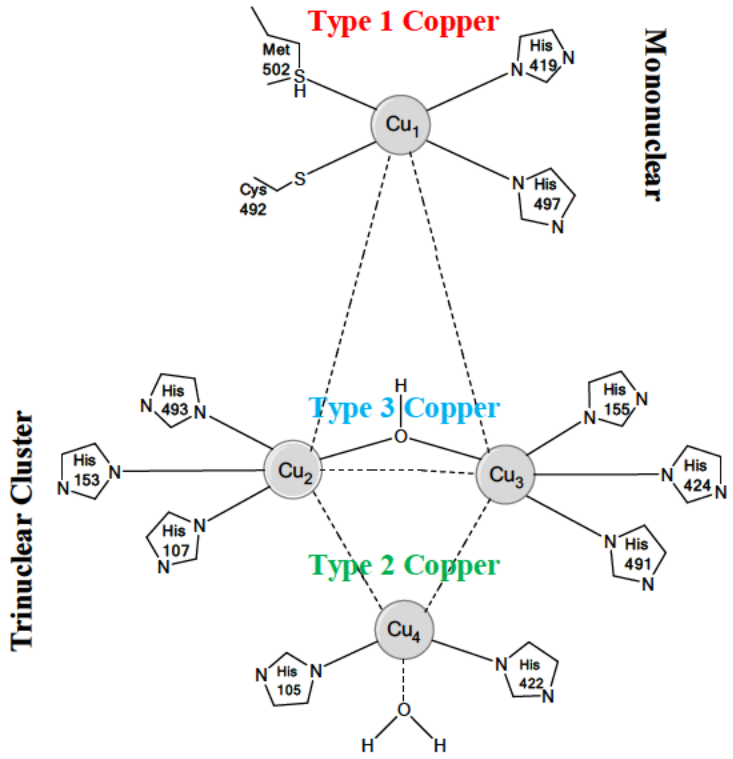


Figure 2.19: A diagram of the general arrangement of the four conserved copper atoms found in the catalytic sites of most laccases [Adapted from Dwivedi *et al.* (2011)].

Apart from blue laccases, as mentioned above, there are also yellow laccases that are similar to blue laccases except for two differences: (1) yellow laccases lack the absorption band around 610 nm (i.e., absence of T1 copper) which is always found in blue laccases, (2) they oxidise non-phenolic substrates in the absence of mediator molecules, i.e., low molecular weight compounds with a high redox potential (above 900 mV) which can enhance the range of laccase substrates, which are always required in the case of blue laccases (Baldrian 2006; Chaurasia, Bharati and Singh 2013). Leontievsky *et al.*, 1997, reported that the fungal strains that secrete blue laccases secrete yellow laccases when grown on solid lignin containing substrates (Leontievsky *et al.* 1997)

2.4.3. The reaction mechanism of laccases

The four copper atoms found within the three interconnected cupredoxin domains work in unison to permit the coupling of four one-electron oxidations of an appropriate substrate to the four-electron reduction of the molecular oxygen to water. Due to the T1 site's high redox potential of between 0.5 and 0.8 V, laccases are thought to initiate their catalytic cycle there. Laccases utilise the T1 site to execute the oxidation of the substrate molecule through four one-electron transfers in a stepwise process. The electrons are subsequently transferred to the T3 copper pair which carries out the re-oxidation of laccase through transferring four electrons in two-electron steps to molecular oxygen, thus reducing it to water. The free radicals that are a product of substrate oxidation can undergo oxidative coupling or degradation, which can lead to either non-enzymatic coupling reactions or depolymerisation reactions (Claus 2003; Claus 2004; Mikolasch and Schauer 2009a; Goodwin 2010; Santhanam *et al.* 2011; Polak and Jarosz-Wilkolazka 2012). Bacterial laccases have low redox potentialsoatotheoT1 site, withovaluesousuallyobelowo0.5 V, thus limiting the range of substrates that they can oxidise (Santhanam *et al.* 2011).

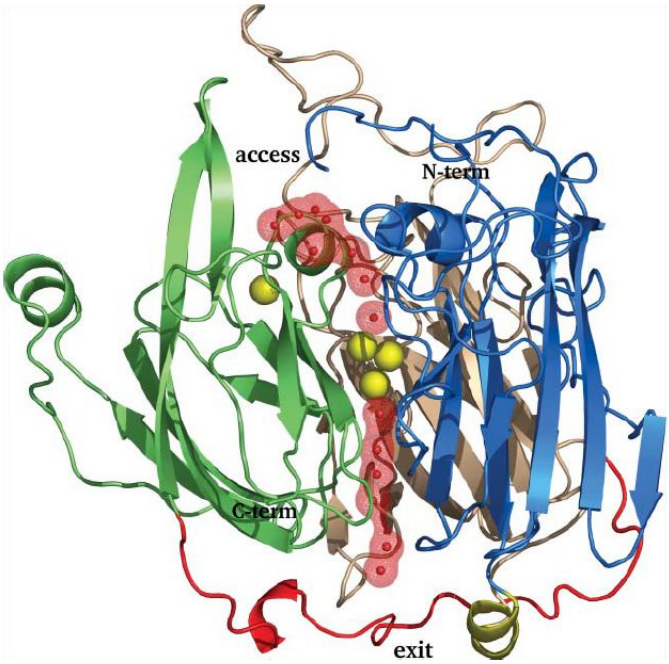
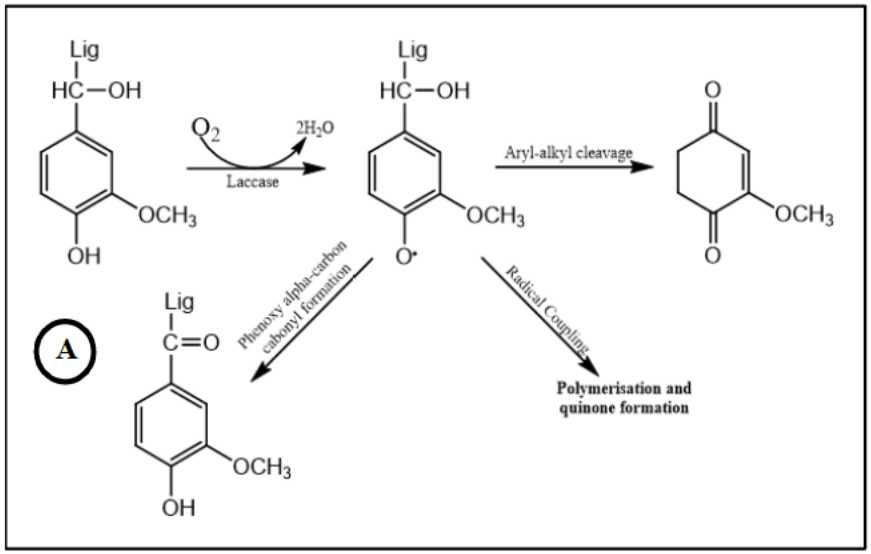


Figure 2.20: A protein ribbon structure of *Bacillus subtilis*' endospore coat laccase (CotA) showing the channels that permit molecular oxygen into the trinuclear centre and allows the departure of water molecules ensuing from the reduction of molecular oxygen (Bento *et al.* 2005).

2.4.4. Laccase mediator systems

Attributable to the random polymer nature of lignin and laccases' lower redox potential compared to other ligninolytic enzymes, laccases can only oxidise phenolic fragments of lignin. Low molecular weight compounds with high redox potential than laccase, known as mediators, are employed to avoid the steric hindrance problems encountered by the enzymes active site on bulky or non-phenolic substrates. The mediator molecule behaves as sort of an electron shuttle, in that it is oxidised by the enzyme to yield a robust oxidising intermediate. The intermediate low molecular weight compound can rapidly diffuse out of the enzyme active site, and oxidise compounds of high molecular weight or with high ionisation potentials. For instance, lignin and synthetic dyes are not directly oxidised by laccases but rather by laccase-mediator systems (LMS). The intermediate low molecular weight compound could utilise an oxidation mechanism that is not available to the enzyme, thus widening the substrate range. Previous studies suggest that when carrying out laccase-dependant oxidation of non-phenolic substrates having a low oxidation potential, an electron transfer mechanism with a mediator such as ABTS would be optimal. However, if a substrate harbours weak C-H bonds, an N-OH type mediator may be utilised to facilitate substrate oxidation through a radical hydrogen atom transfer (HAT) route (Desai and Nityanand 2011).

These low molecular weight compounds have been used in a variety of applications such as organic synthesis, polycyclic aromatic hydrocarbon degradation, pulp delignification, insecticide degradation, and textile dye bleaching (Desai and Nityanand 2011). The use of synthetic mediators such as ABTS and 1-hydroxy benzotriazole (HBT) with fungal laccases has been extensively studied. Synthetic mediators have significant drawbacks, such as high cost and the formation of toxic compounds. Recently, several studies have focused on the search for natural mediators as cheaper and environmentally friendly alternatives. Fungal metabolites such as 3-hydroxyanthranilic acid and natural substituted phenols related to lignin such as syringaldehyde and acetosyringone are reported to be efficient mediators (Kunamneni *et al.* 2007b). Bacterial laccases from *Streptomyces psammoticus* and *Streptomyces coelicolor* have been reported to decolourise synthetic dyes in the presence of the synthetic mediator HBT and the natural mediator syringaldehyde, respectively (Cohen, Persky and Hadar 2002; Dwivedi *et al.* 2011; Santhanam *et al.* 2011; Mohammed Sherif *et al.* 2013).



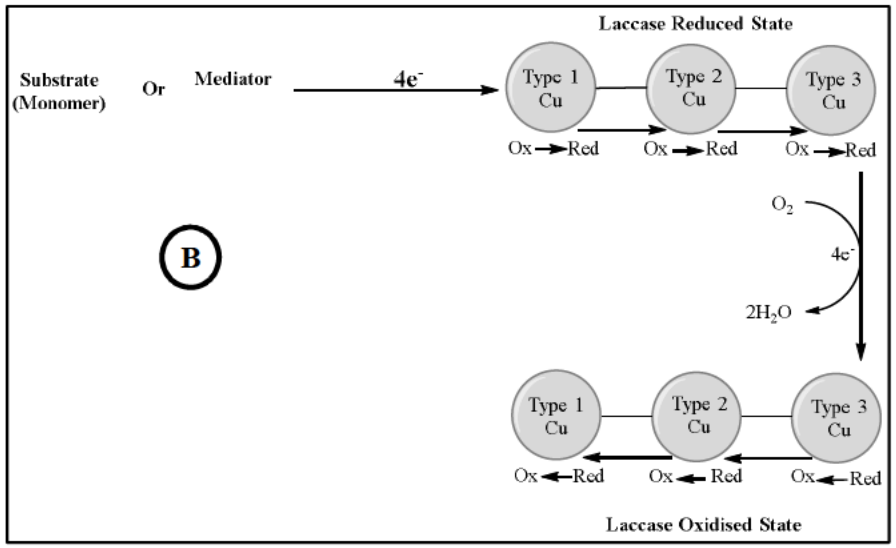
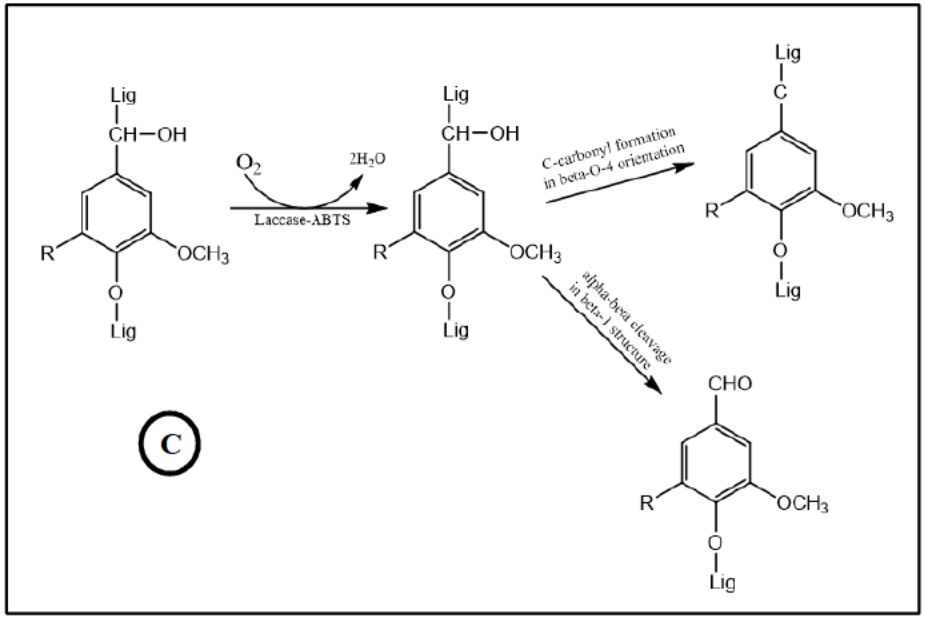


Figure 2.21: A schematic depiction of (A) the reaction of laccases with phenolic substrates in the presence of molecular oxygen to yield water as a by-product and a variety of compounds (Kunamneni *et al*. 2007);(B) the abstraction of electrons from either a substrate or mediator molecule, and their subsequent shuttling between T1 copper and T3 copper (Goodwin 2010); and (C) a mediator (in this case ABTS) assisted reaction of laccases with non-phenolic substrates in the presence of molecular oxygen to yield water as a by-product, and β-O-4 orientated carbonyl structures or α-β cleavage of the β-1 structures (Goodwin 2010).



2.4.5. Putative substrates of laccases

Laccases catalyse the one-electron oxidation of *ortho*- and *para*-diphenols and aromatic amines by removing an electron and a proton from a hydroxyl group to form free radicals. The enzyme facilitates the cleavage of alkyl-phenyl and C_α-C_β bonds of phenolic lignin dimers and catalyses demethoxylation of several lignin model compounds (Cohen, Persky and Hadar 2002). Furthermore, laccases oxidise anilines, aryl diamines, hydroxyindols, benzenethiols, and some inorganic ions such as $[W(CN)_8]^{4-}$, $[Os(CN)_6]^{4-}$, $[Mo(CN)_8]^{4-}$, and $[Fe(CN)_6]^{4-}$ (Dwivedi *et al.* 2011; Santhanam *et al.* 2011).

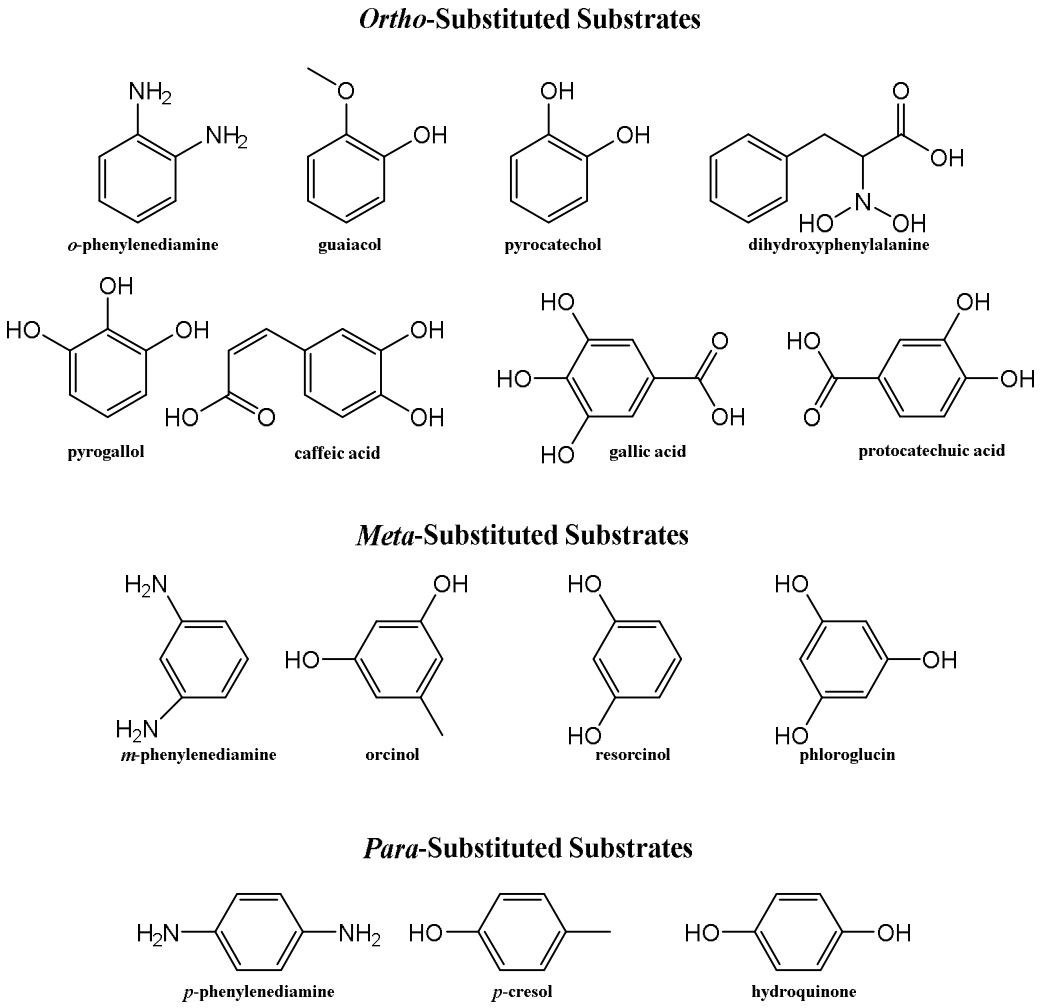


Figure 2.22: *Ortho*-, *Meta*- and *Para*-substituted putative substrates of laccases (Wellington 2012).

Lacking the need for cofactors such as NAD(P)H, contrary to other oxidoreductases, is of utmost importance to laccases as biocatalysts. In contrast to peroxidases, laccases do not produce toxic peroxide intermediates (Santhanam *et al.* 2011). The synthetic substrates commonly used to

assay for laccase activity are 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) and syringaldazine (SGZ). Alternative naturally occurring and plant-derived compounds such as furilic acid, *p*-coumaric acid, 2,6 dimethoxy phenol (DMP) and guaiacol can also be used in some cases (Cohen, Persky and Hadar 2002; Dwivedi *et al.* 2011; Santhanam *et al.* 2011; Mohammed Sherif *et al.* 2013).

2.4.6. Putative inhibitors of laccases

Laccases can be very strongly inhibited by various reagents such as small anions like azide, halide, cyanide, thiocyanide, fluoride, and hydroxides (Harvey 1997; Baldrian 2006; Madhavi and Lele 2009). They bind to the type 2 and type 3 copper resulting in the interruption of internal electron transfer and inhibition of activity. Other inhibitors include metal ions (e.g., Hg^{2+}), fatty acids, sulfhydryl reagents (e.g., diethyldithiocarbamate, thioglycolic acid, dithiothreitol, and cysteine), hydroxyglycine, kojic acid, desferal and cationic quaternary ammonium detergents the reaction with which may involve amino acid residue modifications, conformational changes or copper chelation (Harvey 1997; Baldrian 2006; Polaina and MacCabe 2007).

2.4.7. Applications of laccases

Laccases are an ideal biocatalyst for polymer synthesis due to their capability to generate radicals; hence, they are utilised to synthesise polyphenolic polymers from monomeric phenols such as catechol, quercetin and other plant flavonoids (Xu 2005; Mohammed Sherif *et al*. 2013). The reactions are referred to as “oxidative coupling,” “oxidative condensation,” and “phenolic oxidative coupling,” respectively (Mikolasch and Schauer 2009a). Laccases are also used to synthesise several intricate medicinal agents, including triazole (benzo) cycloalkyl thiadiazole, vinblastine, penicillin X dimer, cephalosporin antibiotics, and dimerised indole. Functional organic compounds such as polymers with specific mechanical or electrical or optical properties, textile dyes, cosmetic pigments, flavour agents, and pesticides are amongst various organic compounds that can be synthesised via laccase-catalysed reactions (Xu 2005). Apart from homo-molecular coupling activities, laccases are also able to couple a laccase substrate and a non-laccase substrate to create new hetero-molecular hybrid molecules such as phenolic structures (type III dimers), quinonoid structures (type IV dimers), or quinonoid structures (type V dimers). If the non-laccase substrate consists of a free amino group, the quinonoid or the quinonoid structure can be generated by oxidation coupled with nuclear amination (Mikolasch and Schauer 2009a).

A continuously expanding body of research in the use of laccases for a number of industrial applications has led to them playing a pivotal role in critical applications such as (1) Lignocellulosic transformation, which is fundamental in the production of such composites as particle boards and liner boards; (2) Pulp bleaching/ Textile dye-bleaching of finished dyed cotton fabric, Application of laccase in this manner eliminates the need to use conventional chemical oxidants such as hypochlorite; (3) Food applications in which laccase is used in certain processing to enhance or modify the colour appearance of food or beverage (e.g., fruit juice and fermented products); (4) Environmental protection in which laccase can be used to oxidise lipids such as trilinolein and methyl linoleate to yield products such as hydro-peroxides and peroxides, transformation of various xenobiotics and other pollutants found in industrial waste; (5) Biosensors and Bio-reporters in which laccase can be used in the detection of O_2 reduction and to assay various enzymes (e.g., amylase, alanine-specific aminopeptidases); and (6) Medical and personal care to develop new cosmetic pigments, hair dyeing materials, deodorants, tooth pastes, mouth washes, and many other useful products (Cohen, Persky and Hadar 2002; Xu 2005; Mikolasch and Schauer 2009a; Dwivedi *et al.* 2011; Polak and Jarosz-Wilkolazka 2012; Wellington 2012; Mohammed Sherif *et al.* 2013).

2.5. Laccase reaction engineering for the synthesis of bioactive compounds

Since time immemorial, the environment has been an unrivalled source of organic compounds with an assortment of structural, functional diversities, and bioactive properties (Sharma 2011). These plant-derived organic compounds are called secondary metabolites; and they are loosely categorised into phenolic compounds, antibiotics, alkaloids, mycotoxins, food-grade pigments, and growth factors (Martins *et al.* 2011). An immense number of these bioactive compounds have found application in the pharmaceutical industry, chemical industry, food industry, agrochemical, cosmetics, geo-medicine, and nano-bioscience (Martins *et al.* 2011; Joana Gil-Chávez *et al.* 2013; Guaadaoui *et al.* 2014; Molina *et al.* 2015). However, there is still a large gap that exists between the demand and supply of such natural compounds due to their insufficient yields from natural resources (Sharma 2011). Therefore, the synthesis of rare bioactive compounds has become crucial to meet the expanding demand, and chemical modification of abundant secondary metabolites has proven useful in the synthesis of bioactive compounds and their analogues (Nicolaou and Snyder 2004).

Usually, these bioactive compounds are extracted and produced using techniques such as pressurized-liquid processes, subcritical and supercritical methods, microwave and ultrasound-assisted methods, and chemical methods (Wang and Weller 2006; Martins *et al.* 2011; Joana Gil-Chávez *et al.* 2013). Conventional physicochemical processes are generally considered long, energy-intensive, low yielding with the concomitant production of large amounts of waste that harms the environment (Kudanga *et al*. 2017). Using the concept of atom economy, the inefficiency of the conventional methods has been reported to be very high. Thus highlighting the challenges of conventional organic syntheses in resource conservation, environmental impact, and health concerns associated with the production of chemical waste. Therefore, the search for innovative solutions for the reduction of chemical steps, wastes, and energy has intensified due to the deleterious environmental impact of some conventional chemical processes (Li and Trost 2008).

A growing interest in the use of enzyme-based processes, attributed to substantial evidence showing their viability as alternatives for conventional chemical processes, has prompted an upsurge in the search for suitable biocatalysts for organic synthesis (Burton, Cowan and Woodley 2002; Kudanga *et al.* 2011b). The enzyme-based processes are ruminated to be more efficient, cost-effective, environmentally friendly and sustainable; due to their significantly reduced dependence on non-renewable resources, the selectivity of the catalysts, potential reusability of the enzymes and their biodegradability (Rozzell 1999; Liu and Yu 2010; Kudanga and Le Roes-Hill 2014). Through enzyme-based processes, a specific product of interest can be produced in a single catalysed step, whereas synthesis of the same product through conventional chemical processes may require numerous steps leading to the production of a mixture of unwanted isomeric, epimeric, or rearranged products (Glazer and Nikaido 2007). The production of the fundamental raw material for cephalosporin antibiotics and semi-synthetic penicillin, 6-aminopenicillanic acid (6-APA), through the hydrolysis of penicillin G is one of the reactions which demonstrates the benefits of supplanting conventional organic chemistry with biocatalysis (Bruggink, Roos and de Vroom 1998; Wegman *et al.* 2001).

These biotransformation reactions are very crucial due to their ability to yield structurally complex compounds with essential biological activities and have proven to be central in the generation and optimisation of an ever-expanding array of compounds for drug discovery and development (Venisetty and Ciddi 2003). Furthermore, the introduction of punitive action and legislation prompted by the need to improve human health and environmental protection,

pressure from environmental protection activists and conscious proactive efforts, have forced industries to consider enzymatic approaches for the development of environmentally benign organic synthesis processes (Kudanga and Le Roes-Hill 2014). There are some well-known industrially competent enzymes such as microbial lipases which have been employed in the cost-effective synthesise of agrochemicals, biopolymers, pharmaceuticals, and biodiesel from renewable waste resources; β-glycosidases which have been used in plant biomass saccharification; and fungal oxidoreductases which are prospective tools in the conversion of biomass into a renewable starting material for the synthesis of biodegradable products (Ben Bacha, Moubayed and Al-Assaf 2016; Martinez *et al.* 2017; Matsuzawa, Watanabe and Yaoi 2017). Laccases are amongst the groups of enzymes that have shown great potential as biocatalysts in organic synthesis.

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multi-copper oxidase enzymes belonging to the blue copper family of enzymes. They catalyse the oxidation of various phenolic and non-phenolic compounds (e.g. phenols, diphenols, *ortho*- and *para*-diphenols, methoxy substituted phenols, anilines, aryl and alkyl amines , hydroxyindoles, benzene thiols, and some inorganic ions such as $[Mo(CN)_8]^{4-}$, $[Fe(CN)_6]^{4-}$, $[Os(CN)_6]^{4-}$ and $[W(CN)_8]^{4-}$) to produce radicals with concomitant four-electron reduction of molecular oxygen to water (Cohen, Persky and Hadar 2002; Dwivedi *et al.* 2011; Santhanam *et al.* 2011; Riva 2013). Their ability to oxidise a myriad of compounds to equivalent free radicals and subsequently produce water as the only by-product has led to them being regarded as “green catalyst”, and has presented them as a useful group of enzymes for diverse biotechnological applications (Kudanga *et al.* 2011a; Polak and Jarosz-Wilkolazka 2012; Mate and Alcalde 2017). The catalytic activity of laccases involves the confiscation of a single electron from substrates to produce reactive free radicals, which are significant intermediates that can undergo coupling reactions to produce dimeric, oligomeric, polymeric or cross-coupling products (Figure 2.23).

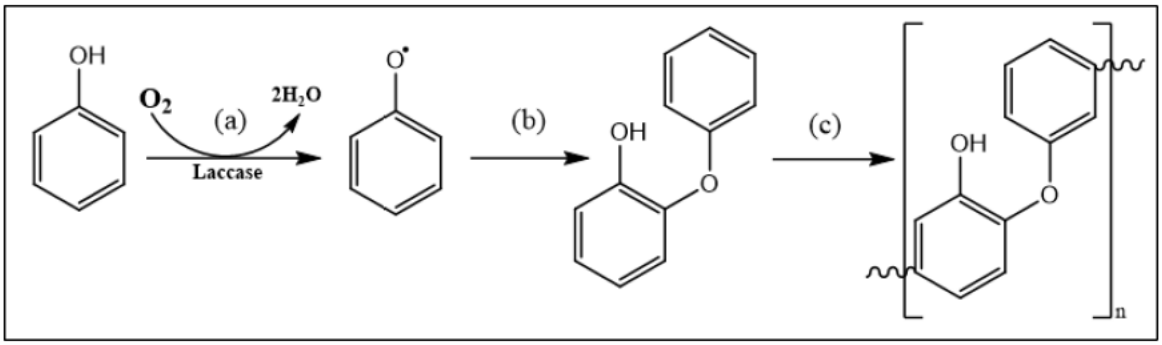


Figure 2.23: The catalytic activity of laccase showing (a) laccase catalysed oxidation of a phenolic substrate to form radicals of the substrate; (b) radicals undergo oxidative coupling to produce dimers and (c) further coupling results in polymerization (Kudanga *et al*. 2017).

As a result, their utilization has resulted in the organic synthesis of novel bioactive compounds mostly derived from aromatic substrates (Milstein, Nicklas and Hüttermann 1989; Cañas *et al.* 2007; Kudanga *etoal*. 2017). Ferulic acid is one of the phenolic compounds that have been exposed to a laccase catalysed transformation to oligomers of semiquinone feruloyl radicals (Carunchio *etoal.* 2001; Mustafa*oeoal*.o2005; Adelakun *etoal.* 2012). However, with free-radical processes such as in laccase-catalysed reactions, many radical forms of the oxidized molecule may be produced. This results in the formation of a wide range of racemic mixtures of products at low concentrations (Kudanga *et al*. 2017). Furthermore, several other challenges need to be addressed to widen the natural enzymes' scope; such as the enzymes instability in vitro, low selectivity, product substrate inhibition, and low reaction yield in non-aqueous solvents (Glazer and Nikaido 2007; Kaul and Asano 2012). The utilisation of high concentrations of organic solvents to abate the production of free radicals, reduce polymerisation reactions, and therefore increase yield, but drastically reduces enzyme activity (Kudanga *et al*. 2017). Additive approach, chemical modification, enzyme immobilisation, and protein engineering are usually the general approaches used to address the above-mentioned challenges, with a focus on the alteration of the physicochemical properties of media, enzymatic surface residue, and support material for enzyme stability [with a lesser focus on protein engineering] (Ó'Fágáin 2003; Minteer 2017).

Therefore, reaction engineering to increase product yields (without compromising enzyme activity) remains a significant challenge in the laccase-mediated synthesis of bioactive compounds. A broad number of reviews have been published discussing the structure and catalytic activity of laccases (Mayer and Staples 2002; Claus 2004; Morozova *et al.* 2007a;

Madhavi and Lele 2009), and their potential for industrial utilisation (Riva 2006a; Rodríguez Couto and Toca Herrera 2006; Mikolasch and Schauer 2009b; Witayakran and Ragauskas 2009; Cañas and Camarero 2010; Kudanga *et al.* 2011b; Jeon *et al.* 2012; Kudanga and Le Roes-Hill 2014). However, there has not been any recent comprehensive reviews on the reaction engineering of laccase catalysed organic synthesis reactions. Therefore, the next subsections will review the work that has been covered with regards to the development of reaction engineering for the laccase catalysed synthesis of bioactive compounds.

2.5.1. The effect of organic solvents on laccase activity

A significant number of substrates for laccase-catalysed reactions have low solubility in aqueous solvents, thus limiting the biotechnological application of laccase in their conversion. Organic solvents have been used as suitable co-solvents in several successful laccase-catalysed reactions owing to their inherent biocatalytic advantages (Burton 2003a; Riva 2006a; Mohtashami *et al.* 2019). These include optimisation of substrate specificity and enantio-selectivity, solubilisation of non-polar substrates, a reversal of the thermodynamic equilibrium of hydrolysis reactions, removal of water-dependent side reactions, and elimination of microbial contaminations (Kudanga *et al*. 2017). Different solvents exhibit varying abilities for substrate solvation and may dictate its thermodynamic activity, and partitioning coefficient (and that of the product). Thus, solvent selection is heavily dependent on the substrate and catalyst type and is a crucial aspect of ensuring an efficacious application of the enzyme in organic synthesis (Gogoi *et al.* 2009). For instance, employing the laccase from *Bacillus licheniformis* LS04 in organic synthesis would proceed smoothly with ethanol, methanol, acetonitrile, acetone and DMSO as the organic solvents of choice, owing to the enzyme's high tolerance for these solvents (Lu *et al.* 2012).

Generally, in numerous cases, the addition of an organic solvent diminishes the reaction rate. This may be attributed to the organic solvents ability to directly interact with the enzyme, leading to the exchange of the solvent's molecules with those of the water in the active centre, and subsequently, the protein structure is distorted and may result in the irreversible inactivation of the enzyme (Dordick 1989; Zheng and Ornstein 1996; Bell, Janssen and Halling 1997). Also, physicochemical properties of the enzyme reaction media, such as pH, hydrophobicity, dielectric constant, the free energy of substrate binding by the enzyme, and the chemical potential of the reactants are significantly affected by the addition of the organic solvent (Gogoi *et al.* 2009). The organic solvents' propensity to affect laccases' catalytic activity and stability rest on the

enzyme's source and purification processes, and the chemical properties of the organic solvent (Rogalski *et al.* 1995; Rogalski *et al.* 1999; Rodakiewicz-Nowak *et al.* 2000).

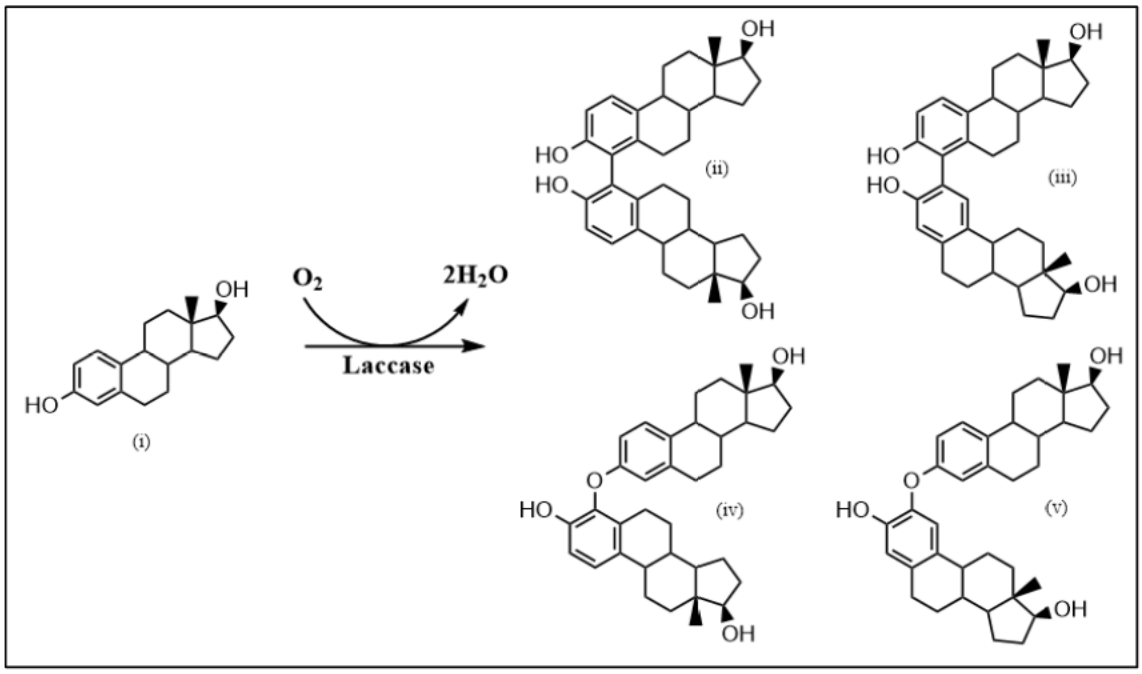


Figure 2.24: The catalytic activity of laccase showing the laccase catalysed production of dimeric products (ii – v) from the oxidation of β-estradiol (i) (Riva 2013).

This is well demonstrated in a reaction involving the oxidation of syringaldazine and 2,6-dimethoxyphenol in the presence of acetone, dimethylsulphoxide (DMSO) and acetonitrile in which acetonitrile was found not to affect the reaction rates. On the other hand, DMSO was found to cause a rapid loss of laccase activity (Doukyu and Ogino 2010). This is a common occurrence in many laccase-catalysed reactions (Lacki and Duvnjak 1998; Srebotnik and Hammel 2000). The non-native nature of organic solvents presents a problem for enzymatic function, in that the folding of the enzymes is generally defined by their function in aqueous solutions and the interaction of their side residues with water molecules. Therefore, in the presence of organic solvents enzyme inactivation and instability are inexorable (Stepankova *et al.* 2013). However, utilisation of optimum concentrations of some organic solvents can facilitate product recovery (Adelakun *et al.* 2012), as illustrated by Xu *et al.* (1997), where 30% acetonitrile expedited product recovery in the synthesis of 2-(4-hydroxyphenyl) coumarans. Another such reaction was published by where they describe the utilisation of a water-solvent biphasic system (ethyl acetate/ acetate buffer) in the oxidation of a steroid molecule (i.e. β-estradiol) by laccases from *Polyporus versicolor* (Figure 2.24).

The reaction depicted in Figure 2.24 does not only serve as evidence of the beneficial characteristics of organic solvents in laccase–catalysed biotransformations, but it also emphasises a common trend in this form of organic synthesis which involves the utilisation of water/ aqueous–organic solvent biphasic systems. Several previous studies describing the synthetic application of laccases reported using considerable amounts of water-miscible organic cosolvents (Riva 2006b). The work published by Barreca *et al.* (2003) reports the laccase-catalysed synthesis of Alderone from the oxidation of a known non-phenolic lignin model, Alderol in a mixed solvent system of a buffer, water and dioxane (Figure 25 a). Potthast *et al.* (1995a) and Baiocco *et al.* (2003) also described the laccase-catalysed oxidation of some benzyl alcohols (e.g. toluene and 3,4-dimethoxytoluene) to the corresponding benzaldehydes (e.g. benzaldehyde and 3,4-dimethoxybenzaldehyde) in a buffer/ tetrahydrofuran or dioxane or acetonitrile cosolvent systems (Figure 2.25 b). This use of cosolvent systems was demonstrated further by d'Acunzo, Barreca and Galli (2004) when they reported the laccase-catalysed oxidation of 4-methoxybenzyl alcohol to 4-methoxyacetophenone in buffer/ dioxane or isopropanol or ethylene glycol or acetonitrile cosolvent systems.

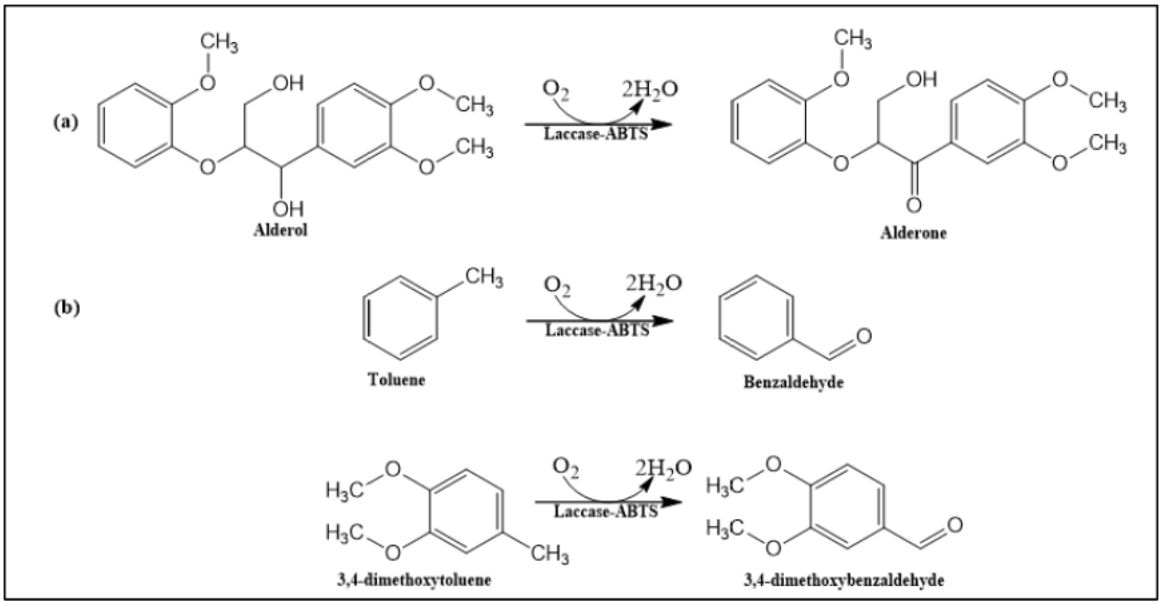


Figure 2.25: The laccase-catalysed synthesis of (a) Alderone from the oxidation of a non-phenolic lignin model, Alderol, in a mixed solvent system of a buffer, water and dioxane (Barreca *et al.* 2003), and (b) selected benzaldehydes from the oxidation of the corresponding substituted benzyl alcohols in a buffer/ tetrahydrofuran or dioxane or acetonitrile cosolvent systems (Potthast *et al.* 1995a).

The extent to which activities and stabilities of different laccases are affected by a myriad of water-miscible cosolvents has been explored by different authors (Rodakiewicz-Nowak 2000). They generally concluded that the enzyme's reaction rate and order of magnitude were maintained in aqueous solutions, reverse micelle systems, and in the presence of 20 – 30% (v/v) of numerous water-miscible solvents. Albeit, with a negative impact on enzyme stability and a substantial decrease in enzyme activity when the enzyme came into contact with cosolvents such as dioxane, isopropanol or acetonitrile (Mozhaev *et al.* 1989; d'Acunzo, Barreca and Galli 2004). The complexation of laccases with alkylated amphiphilic polymers has enabled its efficacious use in low water-containing apolar solvents (Vakurov *et al.* 1994). Intra *et al.* (2005) were one of the first authors to show that the nature of the organic solvent has a significant influence on the ratio of dimers produced from the laccase-catalysed oxidation of tetrahydro-2-naphthol (Figure 2.26).

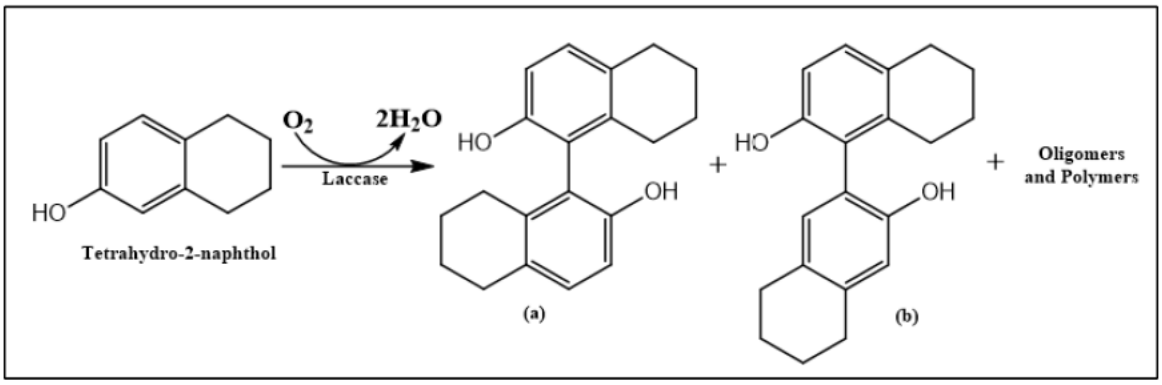


Figure 2.26: The laccase-catalysed oxidation of Tetrahydro-2-naphthol in a TRIS buffer/ benzene or (toluene, chloroform, ethyl acetate, methyl *tert*-butyl ether, *tert*-amyl alcohol, 1,2-dichlorobenzene, nitrobenzene, chlorobenzene, propylbenzene, *tert*-butylbenzene) cosolvent system. Dimers (a) and (b) were produced in a racemic mixture of enantiomeric atropisomers, and higher yields of (a) were observed with the TRIS buffer/ benzene or toluene cosolvent systems (Intra *et al.* 2005).

2.5.2. The effect of monophasic versus biphasic solvent systems on laccase reactivity

Based on the organic solvents' ability to form a homogeneous mixture with water and the relative ratios of the solvent and water in the medium, organic solvent systems are generally classified into three principal types, (1) water-water-miscible/ monophasic organic solvent system; (2) water-water-immiscible/ biphasic organic solvent system (Adelakun 2012); and (3) near anhydrous organic solvent system (Ogino and Ishikawa 2001). The miscibility can be quantitatively predicted using the magnitude of the partitioning coefficient (*P*) of the cosolvent in a water/octanol biphasic system. This water/octanol biphasic system is generally defined as the proportion of the equilibrium concentrations of the cosolvent in the water and the octanol phase of the biphasic system (Mozhaev *et al.* 1989):

$$P = \frac{[Cosolvent]_{octanol}}{[Cosolvent]_{water}}.$$

The above relationship unmistakeably indicates that higher values of the partitioning coefficient correspond to water-immiscible cosolvents and a lower affinity of the cosolvent with water. Good cosolvents are generally considered to be those that enable the reduction of water content while maintaining the activity and stability of the enzyme (Mozhaev *et al.* 1989).

2.5.2.1. Monophasic solvent systems

Monophasic organic systems lack a separation of organic and aqueous phases and result from the introduction of water-miscible organic solvents such as 1,4-dioxane, acetone, ethanol, t-butyl alcohol and methanol, in an effort to ameliorate the solubility of hydrophobic compounds or those with a low polarity index (Khmelnitsky *et al.* 1988; Ogino and Ishikawa 2001). This type of a solvent system has been reported to offer high regulation capabilities in so far as the substrate, and product concentrations are concerned, thus deterring the accumulation of excess substrate and product around the enzyme (Adelakun 2012). Albeit deleterious to enzyme structure and activity, monophasic solvent systems are beneficial for hydrophobic substrate conversion, and they foster thermodynamic equilibria that support synthesis over hydrolysis, thus making possible the synthesis of compounds such as esters and proteins (Ogino and Ishikawa 2001; Doukyu and Ogino 2010). The positioning of the organic substrate-binding site close to the surface of most laccases make it easily accessible to water-miscible solvents and is an essential factor in the enzymes' catalysis (Rodakiewicz-Nowak *et al.* 2000). Water miscible

organic solvents with low log P values generally make for better cosolvents in monophasic solvent systems. However, it should be stressed that this determination can only be valid when comparing cosolvents of the same functionality (Mozhaev *et al.* 1989).

Milstein, Nicklas and Hüttermann (1989) were amongst the first researchers to investigate the effect of monophasic solvent systems on laccase activity. They reported syringaldazine oxidation rates comparable to those in a buffer when using monophasic solvent systems that contained either 60% (v/v) acetonitrile, 55% (v/v) acetone, 50% (v/v) 1,4-dioxane or 60% (v/v) ethanol in water, albeit non-sustainable. Furthermore, in monophasic solvent systems that comprised of at least 3.5% (v/v) of water, the reaction rates of the laccase-catalysed syringaldazine oxidation were highest with acetonitrile and lowest with methanol. Rodakiewicz-Nowak *et al.* (2000) investigated the effect of organic solvents such as ethanol, methanol, acetone and acetonitrile on the activity of *P. radiata*, *C. versicolor*, *P. oryzae* and blue laccases during the oxidation of either 2,6-dimethoxyphenol or syringaldazine. The tested organic solvents were shown to have an inhibitory effect, with the mode of inhibition (i.e. weak competitive or mixed inhibition) depending on the organic solvent, substrate and laccase source. 2,6-dimethoxyphenol oxidation by *P. radiata* laccase was hindered via mixed inhibition in the presence of either ethanol or methanol, whereas acetone and acetonitrile resulted in simple competitive inhibition. The oxidation of 2,6-dimethoxyphenol and syringaldazine by *C. versicolor* and blue laccase in ethanol was hindered via mixed inhibition (Rodakiewicz-Nowak *et al.* 2000).

Table 2.2: The effect of different monophasic solvent systems on the activity of laccases from various bacterial and fungal sources

Laccase Source	Substrate(s)	Organic Solvent	Buffer	Organic Solvent Concentration (%)	Reaction Conditions and Duration	Relative Activity (%)	Reference
T. versicolor	2,6-DMP & Syringaldazine	Acetonitrile, Acetone, Ethanol, 1,4-Dioxane, Tetrahydrofuran & Methanol.	0.1M Potassium-acetate buffer at pH 4.5	93 – 95.5	30°C	~0.5 – 20.7	Milstein, Nicklas and Hüttermann (1989)
	4HB	DMSO, 2-Methoxyethanol, Acetonitrile, 1,4-Dioxane, DMF & 1-Propanol.	0.01M Phosphate buffer at pH 5.0	30	60°C	*np	Tominaga *et al.* (2004)
	ABTS	Ethanol, *n*-Propanol, DMSO, Acetone & DMF.	*np	20	20°C	10 – 27	
	Organosolv lignin	1,4-Dioxane	0.1M Phosphate-citrate buffer at pH 7.0	70 – 95	30°C	27 – 95	
B. licheniformis	Syringaldazine	Acetone, Acetonitrile, Ethanol, DMSO & Methanol.	0.1M Citrate-Phosphate buffer at pH 6.6	10 – 50	30°C	~27.3 – 141.9	Lu *et al.* (2012)

P. versicolor	Pyrocatechol	Formamide, Ethylene glycol, Methanol, DMF, Acetonitrile, 1-Propanol, 2-Propanol, Acetone, HMPA & 1,4-Dioxane.	0.05M mM Tris-acetate buffer at pH 7.0	4.5 – 93.1	25°C	50	Khmelnitsky *et al.* (1991), Mozhaev *et al.* (1989)
C. hirsutus	Syringaldazine	Methanol, Acetonitrile, 2-Propanol, Acetone, 1,4-Dioxane, Tetrahydrofuran & Ethanol.	0.1 M Potassium citrate buffer at pH 4	96	*np	~0.1 – 41	van ERP, Kamenskaya and Khmelnitsky (1991)
P. radiata	Syringaldazine & 2,6-DMP	Ethanol, Methanol, Acetone & 1,4-Dioxane.	0.1M Citrate-Phosphate buffer at pH 4.5	0 – 80	*np	0 – 100	Rogalski *et al.* (1995)
C. unicolor	Syringaldazine	Ethanol, 1,4-Dioxane, Ethyl glycol, Acetone & Formamide.	0.1M Citrate-Phosphate buffer at pH 4.5	0 – 90	30°C	0 – 100	Rogalski *et al.* (1999)
		Dimethyl sulfoxide, Ethylene glycol, Methanol, Formamide, DMF, 1,4-Dioxane & 2-Methoxy ethanol.	0.1M Citrate-Phosphate buffer at pH 5.6	0 – 60	20°C	6 – 100	

P. coccineous	Syringic acid	Acetone, Acetonitrile, 1,4-Dioxane, Ethanol & Tetrahydrofuran.	0.1M Acetate buffer at pH 5.0	40-50	25°C	*np	Ikeda, Uyama and Kobayashi (1996)
M. albomyces	2,6-DMP	Acetone, Ethanol, DGME & Propylene glycol	0.025M Sodium-citrate buffer at pH 5.5	0 – 70	22°C	10 – 122	Mattinen *et al.* (2011)
Cerrena sp.	ABTS, 2,6-DMP & Guaiacol.	Acetone, Methanol, Ethanol, DMSO, DMF, 1-Propanol, Acetonitrile & Formaldehyde	0.08M Citric-Phosphate buffer at pH 3.0 **[for ABTS]** & 0.06M Citric-Phosphate buffer at pH 4.0 **[for 2,6-DMP & Guaiacol]**	30	30°C	*np	Wu *et al.* (2019)
R. vernicifera	2,6-DMP or DMPD	Methanol, Ethanol, 1-Propanol, 2-Propanediol, 1,2-Propanediol, 1,3-Propanediol, 1,3-Butanediol, 1,4-Butanediol, glycerol, Acetone, 1,4-Dioxane & Acetonitrile.	0.2M Sodium-phosphate buffer at pH 7.0	10 – 100	25°C	*np	

4HB = 4-Hydroxybiphenyl, DMSO = Dimethylsulphoxide, DMF = *N,N*-Dimethyl formamide, HMPA = Hexamethyl phosphoramide, DGME = Diethyl glycol monomethyl ether, DMPD = *N,N*-dimethyl-p-phenylenediamine, *np = not provided.

Tominaga *et al.* (2004) achieved the highest conversion of 4-Hydroxybiphenol (53% yield) with 30% of DMSO, i.e. 30% DMSO was the most suitable for retaining the functional conformation of the active site. Increasing the concentration of DMSO to 70% reduces the conversion rate from 53% to 0%. Furthermore, the aqueous phase pH was varied between 3 and 7 in the presence of 30% [b]DMSO, and the authors deduced that DMSO did not influence the pH dependency of the laccase's catalytic activity. Liu *et al.* (2019) reported a reduction in the relative activity of between 66 and 90%, with 20% Methanol being the most tolerable organic solvent (at a relative activity of 34%) and *N, N*-dimethyl formamide being the least tolerable organic solvent (at 10%). The results also show an inverse proportionality between DMSO concentration and laccase activity. The immobilised laccase's ability to convert organosolv lignin in the presence of either 1,4-dioxane, tetrahydrofuran or acetonitrile was investigated by Milstein *et al.* (1993). They found that the incubation of laccase for a prolonged time (i.e. 6 days) in 20% and 30% of tetrahydrofuran and 1,4-dioxane, respectively, resulted in the reduction of activity by ~50%. Contrarily, the enzyme almost lost all of its activity when subjected to prolonged (i.e. 5 days) incubation in the potassium citrate buffer. The amount of water in these monophasic solvent systems was the critical determinant of the rate of laccase activity diminution. Thus, suggesting the suitability of water-miscible solvents in laccase-catalysed reactions, albeit in the presence of an adequate amount of water.

During the biochemical characterisation of *B. licheniformis* spore laccase conducted by Lu *et al.* (2012), the tolerance of the laccase to methanol and ethanol at 30% (v/v) was reported. Higher concentrations of methanol and ethanol (i.e. 50%) resulted in the reduction of relative activity by ~56 and 40%, respectively. Acetone and DMSO had a positive influence on the enzyme at 10 and 20%, respectively. Increasing the concentration of acetone and DMSO to 30% had an inhibitory effect on the enzyme (i.e. activity reduced by ~40%). Furthermore, the laccase was strongly inhibited by higher concentrations (i.e. 50%) of acetone, acetonitrile and DMSO. Using immobilised laccase from *C. hirsutus* van ERP, Kamenskaya and Khmelnitsky (1991) investigated the effect of various organic solvents on its catalytic behaviour [i.e. changes in maximal velocity (V_{max}) and Michaelis constant (K_m)] with syringaldazine as the substrate. The V_{max} and K_m exhibited a significant dependence on the water content of the solvent system, i.e. enzyme activity increased with an increase in water content, albeit, remaining significantly below the values obtained with the aqueous solution. Moreover, the authors observed that the higher the hydrophilicity of the solvent, the great was its ability to remove water from the hydration shell of the enzyme.

Rogalski *et al.* (1995) investigated the ability of the *P. radiate* laccase (immobilised and native) to oxidise 2,6-DMP in the presence of selected water-miscible solvents. DMSO and 1,4-dioxane were found to exhibit the most potent inhibitory effect; replacement of the buffer with 20% of the solvent dramatically reduced the activity to 10% of the initial activity. The native laccase exhibited the best stability in 20% and 40% of methanol and ethanol, respectively. The immobilised laccase demonstrated quadruple the activity of native laccase in 20% acetone. Furthermore, the organic solvents remarkably increase the K_m values for the oxidation of 2,6-DMP. Comparable results were obtained by Rogalski *et al.* (1999) with laccase from *C. unicolor*, 1,4-dioxane and formamide the most robust inhibitory effect for the native form. Replacing the buffer with 20% of dioxane or formamide resulted in the enzyme losing ~70% of its initial activity. In all cases, the immobilised laccase performed better, albeit depending on the immobilisation support material. The proclivity of *C. unicolor* laccase's oxidative activity to be affected by organic solvent was also investigated by Luterek *et al.* (1998). They found that their selected solvents exhibited an inhibitory effect comparable to that reported by Milstein, Nicklas and Hüttermann (1989). Laccase was more tolerant of ethylene glycol and methoxy ethanol.

In more recent studies, Wan *et al.* (2010) investigated the effect of a range of water-miscible organic solvents on *R. vernicifera* laccases' catalytic activity against 2,6-DMP. The results obtained led to the authors concluding that a threshold of water content is necessary to initiate activity, below which there is little or no activity; the activity significantly depended on the type of alcohol or non-alcohol used as a solvent. Mattinen *et al.* (2011) spectrophotometrically assessed the behaviour of *M. albomyces* laccase in various water-miscible organic solvent/ buffer cosolvent systems using 2,6-DMP, matairesinol and 7-hydroxymatairesinol as substrates. The enzyme displayed diminished activity in increasing concentrations of organic solvents; the best rates of 2,6-DMP oxidation were observed with diethyl glycol monomethyl ether and propylene glycol containing solvent systems, with even a slight increase in 20 - 30% of the former. A significant decrease in the oxidation rate was observed at concentrations above 40%. The enzyme exhibited continued conversion of matairesinol and 7-hydroxymatairesinol even in the highest of propylene glycol concentrations. In all of the work discussed here so far, with regards to monophasic cosolvent systems, eludes to the propensity of water-miscible organic solvents to increase the availability of hydrophobic or near-hydrophobic substrates in laccase-catalysed reactions [i.e. increased K_m values], albeit significantly encumbering the enzymes' activity [i.e. reduced V_{max} values] (van ERP, Kamenskaya and Khmelnitsky 1991; Milstein *et al.* 1993; Rodakiewicz-Nowak *et al.* 2000). A notable, and one-of-a-kind, study by Wu *et al.* (2019)

showed that pre-incubation of laccase (from *Cerrena sp.*) in selected water-miscible solvents reversibly enhanced its activity. All laccases studied exhibited non-substrate-specific improvement in activity when pre-incubated in DMSO, acetone, ethanol, N, N-dimethyl formamide and methanol. Further studies for the elucidation of enzyme structural changes in response to the organic solvents showed that the laccase structure remained undisrupted and stable in high concentrations of organic solvent.

2.5.2.2. Biphasic solvent systems

The biphasic solvent systems generally consist of the enzyme-harbouring aqueous constituent and the substrate-harbouring hydrophobic organic solvent constituent (Adelakun 2012). The enzyme converts the substrate in the aqueous phase, and the product is deposited into the organic phase. This arrangement facilitates efficient product separation and effortless enzyme regeneration due to its infinitesimal interaction with the organic solvent. Although biphasic systems offer significantly lower rates of enzyme inhibition when compared to monophasic systems, they are prone to low reaction rates owing to the inherently low mass-transfer rates between the two phases; exhaustive mixing of the reaction media may be sufficient to remove this limitation (Doukyu and Ogino 2010). Some of the hydrophobic organic solvents have been shown to exhibit a form of selectivity of one product isomer in laccase-catalysed reactions. One such case was communicated by Intra *et al.* (2005) where a strong solvent influence on the dimerization of phenols by immobilised *M. thermophyla* laccase, and comparably higher yields than other chemical or enzymatic oxidations were reported. This suggests a form of interaction between hydrophobic solvents and the enzyme similar to that postulated by Ducros *et al.* (1998).

Lugaro *et al.* (1973) conducted one of the earliest studies purporting to uncover the influence of biphasic solvent systems in *P. versicolor* laccase-catalysed oxidation reactions. They concluded that the best organic solvents for the laccase-catalysed oxidative conversion of steroid molecules were hydrophobic or near hydrophobic. The influence of selected hydrophilic and hydrophobic organic solvent on the oxidation of 2,6-DMP by laccase from *T. versicolor* was investigated by Milstein, Nicklas and Hüttermann (1989). An appreciable oxidation rate was observed with selected hydrophobic solvents, albeit requiring pre-saturation with water. The kinetic parameters of the enzyme in the studied organic solvents demonstrated a reduction in the enzymes' affinity for both syringaldazine and 2,6-DMP, which might be a contributing factor to the drastically reduced activity, i.e. 10% to 20% of initial activity.

Table 3.3: The effect of different biphasic solvent systems on the activity of laccases from various bacterial and fungal sources

Source	Substrate (s)	Organic Solvent	Buffer	Solvent % (v/v)	Temperature & Duration	Major findings	Reference
P. versicolor	Estradiol	*n*-hexane, isooctane, cyclohexane, carbon tetrachloride, toluene, benzene, trichloroethylene, ethylene dichloride, chlorobenzene, chloroform, methylene chloride, diethyl ether, butyl acetate, methyl ethyl ketone, and ethyl acetate.	0.1M Acetate buffer at pH 5.4	50	25°C, ≥ 12hrs	Ethyl acetate, diethyl ether, butyl acetate and methyl ethyl ketone were the best organic solvents for the conversion of estradiol.	Lugaro *et al.* (1973)
T. versicolor	2,6-DMP & Syringaldazine	Ethyl acetate, toluene, benzene, ether, isooctane, n-hexane, cyclohexane, chloroform and dichloroethane.	0.1M Potassium-citrate buffer at pH 4.5	93	30°C, ≤ 24hrs	An appreciable oxidation rate was observed with selected hydrophobic solvents, albeit requiring presaturation with water. The kinetic parameters of the enzyme in the studied organic solvents demonstrated a reduction in the enzymes' affinity for both syringaldazine and 2,6-DMP, which might be a contributing factor to the	Milstein, Nicklas and Hüttermann (1989)

						drastically reduced activity, i.e. 10% to 20% of initial activity.	
	ABTS	Hexane	0.1M Citrate buffer at pH 4.5	50	25 & 40°C, ~3.25hrs	The enzyme was found to be more stable in hexane and exhibited better activity, than in the other comparative solvents. Moreover, hexane was shown to be the least disruptive co-solvent out of the tested solvents.	Jafari *et al.* (2020)
M. thermophyla	5,6,7,8-tetrahydronaphthalen-2-ol	Benzene, toluene, choroform, ethyl acetate, methyl tert-butyl ether, tert-amyl alcohol, 1,2-dichlorobenzene, nitrobenzene, chlorobenzene, propyl benzene, and tert-butyl benzene.	0.05M TRIS-HCl buffer at pH 6.5	25 - 100	45°C, ≤ 48 hrs	Strong solvent influence on the dimerization tetrahydronaphthalenyl derivative and comparably higher yields than other chemical or enzymatic oxidations were reported. This suggests a form of interaction between hydrophobic solvents and the enzyme similar to that postulated by Ducros *et al*. (1998). The best solvents in terms of yield and selectivity of isomer product 1 over 2 were arranged as follows: benzene > toluene > chloroform > methyl tert-butyl ether > tert-amyl alcohol.	Intra *et al.* (2005), Ducros *et al*. (1998)
R. vernicifera	2,6-DMP	Ethyl acetate, benzene, toluene and chloroform.	0.2M Na-Phosphate	97 – 99.9	25°C, ~ 5 min	Partial precipitation of the enzyme was observed with 99% ethyl acetate.	Wan *et al.* (2010)

			buffer at pH 7.0			The enzyme activity increased with an increase in water content and reached a peak at 97% ethyl acetate. A similar trend was observed for benzene, toluene and chloroform; maximum enzyme activity was achieved at a water content of 0.5, 0.15 and 0.5 %, respectively.	
Trametes sp.	2,6-DMP	Cyclohexane, *n*-hexane, isooctane, and *n*-decane	0.01M Phosphate Buffer at pH 5.0	*np	27°C, ~ 5 min	The polarity of solvents was shown to have direct proportionality with the enzyme activity. The influence of each organic solvent on activity followed the trend; Cyclohexane > *n*-hexane > isooctane > n-decane.	Mohidem and Mat (2012)
T. pubescens	17β-estradiol	Ethyl acetate	0.02M Acetate buffer at pH 4.5	50	25°C, ~ 48hrs	The reaction with ethyl acetate facilitated the production of one form C-C dimer while toluene facilitated a less specific reaction, i.e. equimolecular C-C and C-O dimers were formed at a 1:3 ratio.	Nicotra *et al.* (2004b)
Myceliopthora		Toluene	0.05M Tris buffer at pH 6.5	75	45°C, ~ 9 days		
M. thermophila	2,6-DMP	Ethyl acetate	0.05M Na-phosphate buffer at pH 7.5	80	30°C	The biphasic system was reported to stabilise the intermediate products (i.e. before the formation of dimers). Furthermore, the biphasic media	Mustafa *et al.* (2005)

						facilitated an 85% conversion to the corresponding dimer.	
T. versicolor	Aliphatic 1,ω-diols	Ethyl acetate, toluene or methyl *tert*-butyl ether.	0.05M Na-acetate buffer at pH 4.8	≤ 80	25°C, TEMPO	85% (v/v) Methyl *tert*-butyl ether managed to facilitate the best substrate conversion rate yield without any significant reductions in the enzyme activity.	Díaz-Rodríguez *et al.* (2014)
	Sinapic acid & Ferulic acid	Ethyl acetate	0.05M Phosphate buffer at pH 5.0 0.05M Acetate buffer at pH 4.0	80	25°C	The laccase-catalysed bioconversion of sinapic acid under these conditions in 85% yield of bis-lactone lignan **1** within 20 minutes. However, when the reaction was prolonged for 2 hours a yield reduction of 61% was observed, thus echoing the findings of Lacki and Duvnjak (1998). On the other hand, the laccase-catalysed bioconversion of ferulic acid proceeded faster in the presence of the acetate buffer, and also improved selectivity, significantly improving the bis-lactone lignan **2**	Tranchimand *et al.* (2006)

Table 3.3 gives a brief review of published authors whose work indicates the benefits of using biphasic solvent systems in the laccase catalysed oxidation/ bioconversion/ biotransformation reactions. Numerous other researchers have more or less come to similar conclusions as highlighted above, and reported an increase in the formation of the β-β and β-5 dimers of caffeic acid when between 80% and 90%, and 80% and 95% (v/v) of ethyl acetate was used, respectively. Biphasic solvent systems proved beneficial for Navarra *et al.* (2010) when they isolated "Pummerer's ketones" in substantial yields by the way of the laccase-catalysed oxidation of the *para-alkyl* phenols. Ponzoni *et al.* (2007) employed an ethyl acetate/ acetate buffer biphasic system in the laccase-catalysed dimerization of a series of hydroxystilbenes and bioactive phytoalexin resveratrol analogues. The authors report having attempted several experimental conditions (i.e. monophasic organic solvents, monophasic water/water-miscible organic solvents, and biphasic systems made of water/ water-immiscible organic solvents) and finding the biphasic system made of ethyl acetate and buffer more suitable. In the work undertaken by Ma *et al.* (2009) where they were investigating the effect of, amongst other parameters, selected organic solvents on the rate of the laccase-catalysed oxidation of (+)-catechin, (-)-epicatechin and catechol; they found that there was improved oxidation of all the tested substrates when not more than 93.5% (v/v) of water/water-immiscible organic solvents such as isooctane, hexane, toluene and dichloromethane.

In the laccase-catalysed conversion of β,β-dihalogenated secondary alcohols to the corresponding halogenated ketones, Kędziora *et al.* (2014) showed that biphasic solvent [i.e. 66% (v/v) toluene or methyl *tert*-butyl ether afforded the authors an oxidative conversion rate of up to 70%] system can promote a significantly high conversion rate to exclusively corresponding compounds. These benefits are not only applicable to simple enzyme-substrate reactions, as indicated by Ferrandi *et al.* (2012), the partitioning of reaction components into different phases may markedly improve substrate conversion even in complex reactions involving partner enzymes, multiple co-enzymes and mediators.

2.5.3. Laccase catalysed organic synthesis of some bioactive compounds

Laccases have proven more than adept as the go-to biocatalysts for the biotransformation of a range of organic compounds and bond formation reactions (i.e. carbon-carbon, carbon-oxygen, carbon-nitrogen and carbon-sulphur) under environmentally friendly conditions (Luna-Acosta *et al.* 2010; Wellington 2014). Stable bioactive compounds such as transformed antibiotics and steroid hormone dimers (e.g. 17β-estradiol) have been produced with the assistance of laccases' oxidative capabilities (Wellington 2014; Yavuz, Kaya and Aytekin 2014).

2.5.3.1. Oxidative decomposition reactions

Nishida and Fukuzumi (1978) presented one of the early examples of an oxidative decomposition reaction when they reported a white-rot fungus (i.e. *Trametes* sp.) facilitated conversion of ferulic acid to coniferyl alcohol in the presence of atmospheric oxygen, glucose and ethanol. Besides coniferyl alcohol, 2-methoxyquinone, dihydroconiferyl alcohol, 2-methoxyhydroquinone, vanillyl alcohol, coniferyl aldehyde and vanillic acid were amongst the other products produces under these oxidative composition conditions (Nishida and Fukuzumi 1978; Wellington 2014). The work published by Resch *et al.* (2011) decades' later revealed laccases to be responsible for the oxidative decomposition reaction previously reported by Nishida and Fukuzumi (1978) when they showed the conversion of ferulic acid to vanillin by laccase from *P. cinnabarinus* (Figure 2.27).

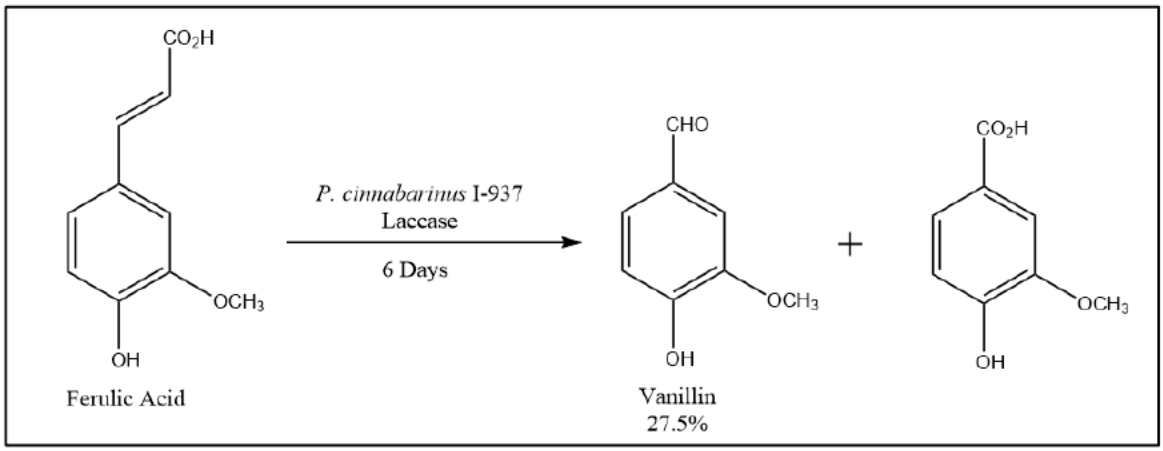
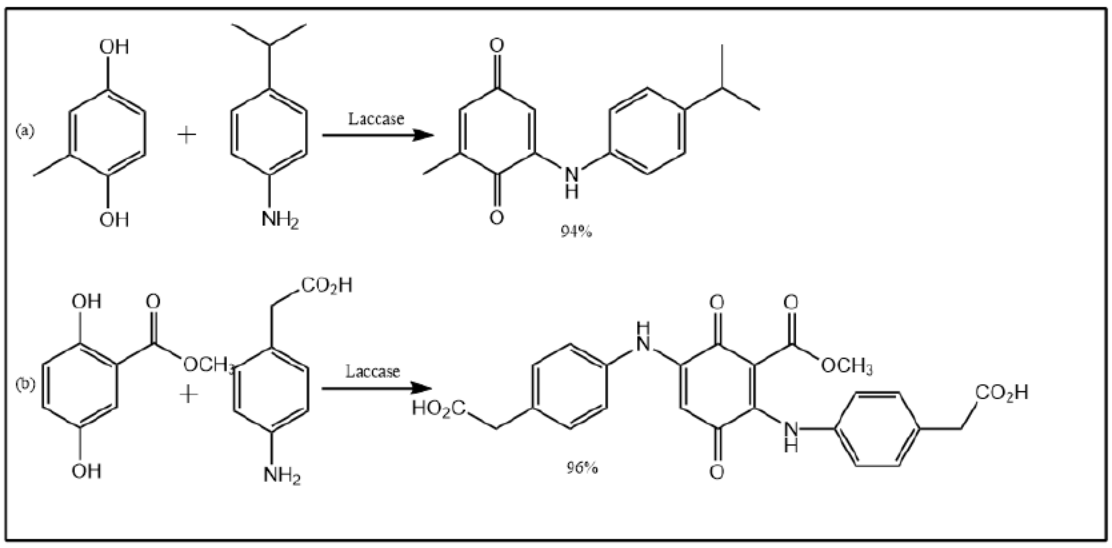


Figure 2.27: The synthesis of vanillin from the oxidative decomposition of ferulic acid catalysed by laccases from *P. cinnabarinus* I-937 (Wellington 2014).

2.5.3.2. Nuclear amination reactions (C-N bond formation)

Amination reactions are fast relying on laccases as one of the essential catalysts in the synthesis of intricate organic molecules. These enzymes present an environmentally benign way of synthesising biologically significant (C-N) molecules (Mikolasch *et al.* 2008a). Novel antibiotics and aminoquinone molecules are amongst the numerous illustrations of laccase-catalysed amination reactions' products (Mikolasch *et al.* 2008a; Piscitelli, Amore and Faraco 2012; Wellington 2014). Laccases from different sources have been shown to give good yields of aminoquinone when alkylated quinone and primary aromatic amines, albeit at significantly varying reaction paths (Niedermeyer, Mikolasch and Lalk 2005). In a study conducted by Niedermeyer, Mikolasch and Lalk (2005) to synthesise mono- and di-aminated benzoquinones with fungal laccases from different sources, they concluded that the laccase from one source favoured the formation of mono-aminated products while the other laccase favoured the di-aminated product in a range of different conditions (Figure 2.28).



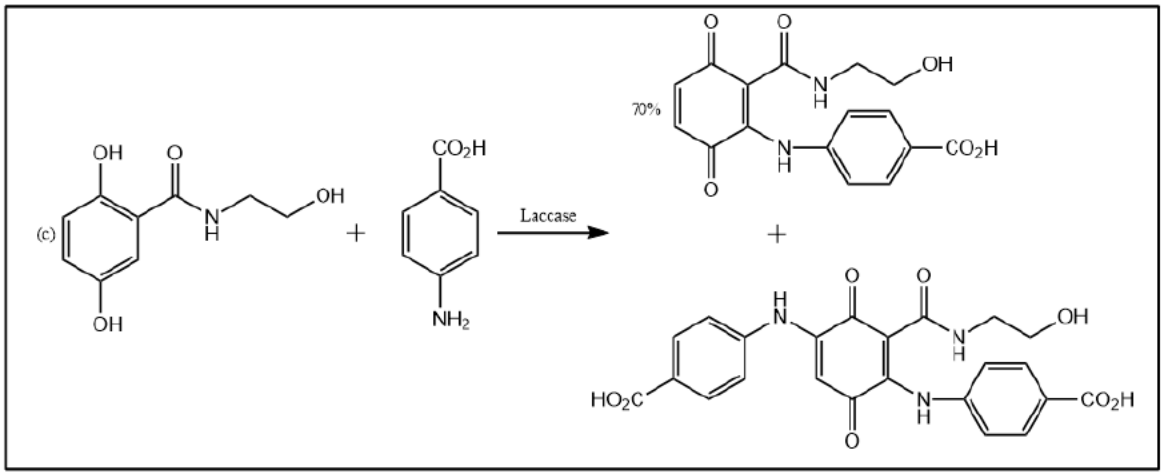


Figure 2.28: The laccase-catalysed (a) mono-amination, (b) di-amination, and mono- and di-amination of p-hydroquinone (Wellington 2014).

Mikolasch *et al.* (2008b) used laccase from *M. thermophila* and *Trametes* sp. to demonstrate laccases' capability to initiate *para*-hydroquinone's nuclear amination with primary amines, resulting in the formation of mono- and di-aminated quinones (Figure 2.29 a). In another study by Wellington, Steenkamp and Brady (2010), a commercial laccase, Denilite® II Base, from Novozyme® was used to demonstrate the nuclear deamination of p-hydroquinone with aliphatic and aromatic amines. Di-aminated products were favoured over mono-aminated products, albeit at a significantly low yield (Figure 2.29 b).

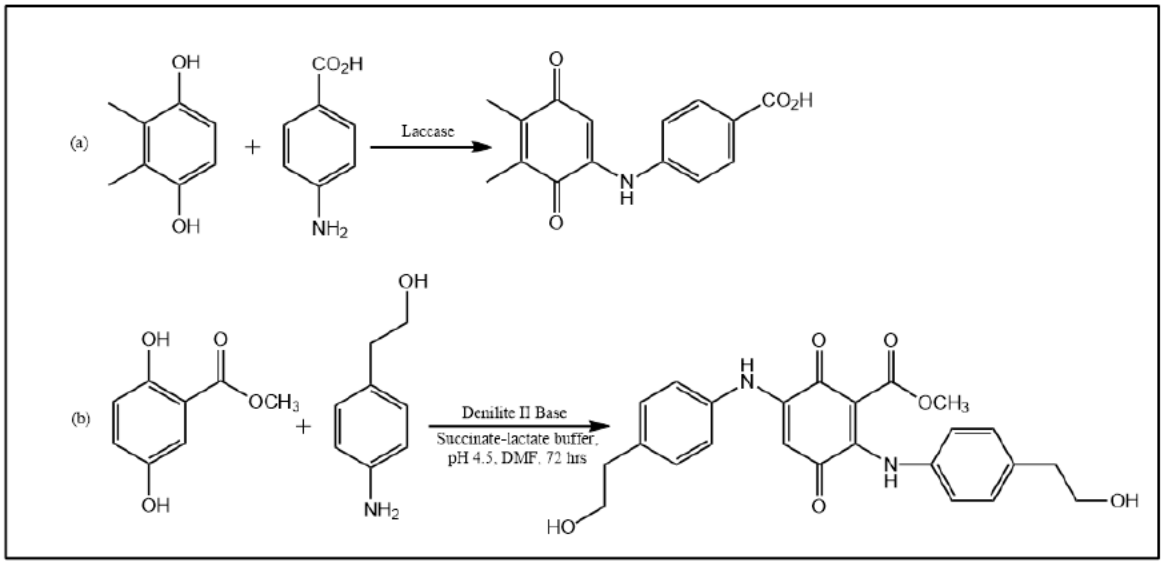


Figure 2.29: The laccase-catalysed (a) mono-amination and (b) di-amination of *p*-hydroquinone (Wellington, Steenkamp and Brady 2010).

2.5.3.3. Thiol-bond (C-S) formation reactions

Even though laccase-catalysed thiol-bond formation reactions possess numerous biochemical and biotechnological benefits, the number of reported literature in this type of laccase-catalysed reactions is very scarce (Wellington 2014). However, in a study conducted by Wellington *et al.* (2012) on the one-pot laccase-catalysed synthesis of 1,4-naphthoquinone-2,3-bis-sulphides from alkyl and aryl thiols, reaction yields depended on the thiol/ 1,4-naphthoquinone-2,3-bis-sulphides ratio, nucleophilicity, pH and substrate solubility. The authors also reported the inhibitory effect of thiols on laccase, thus suggesting a need for thiol resistant laccases to improve the conversion rates of thiol-bond formation reactions. The work published by Schlippert *et al.* (2016) demonstrated a laccase-catalysed 1,4-Michael addition reaction between *p*-hydroquinones and aromatic thiols, in which they showed laccases to be formidable catalysts for an environmentally benign synthesis of heteromolecular thiolated products (Figure 2.30), thus further illustrating the pivotal role of laccases in these types of reactions.

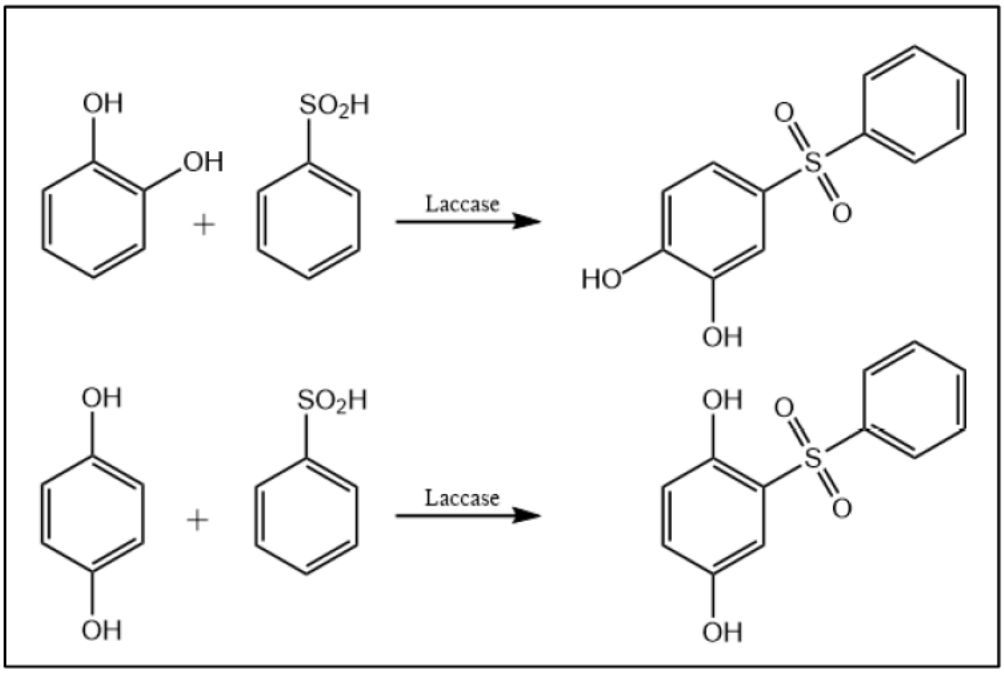


Figure 2.30: Laccase-catalysed thiol-bond formation reactions (Schlippert *et al.* 2016).

2.5.3.4. Oxidative coupling (C-O bond formation) reactions

The ability of fungal laccases to catalyse the oxidative coupling of aliphatic alcohols with dihydroxylated aromatic compounds was well illustrated by Manda *et al.* (2007) when they used a laccase from *T. villosa* to form homo- and hetero-molecular products from the oxidative coupling of selected alcohols with dihydroxylated aromatic substrates (Figure 2.31).

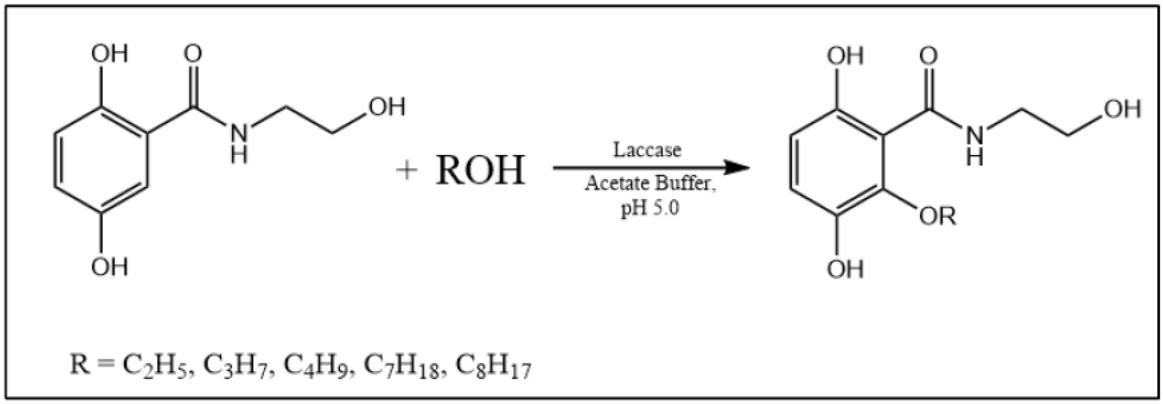


Figure 2.31: An illustration of a laccase-catalysed oxidative coupling reaction between an alcohol and a dihydroxylated aromatic compound (Manda *et al.* 2007).

However, these reactions proceed (which often occur in the presence of organic solvents, alcohols or aqueous solutions) simultaneously with some undesired side reactions due to the aromatic and aliphatic alcohols being potential substrates of laccase catalysis, thus leading to the formation of homo-molecular products, and this significantly reduces the yield of the targeted hetero-molecular product (Manda *et al.* 2007; Mikolasch and Schauer 2009a). Therefore, there is still an urgent need for reaction engineering that will suppress the formation of homo-molecular products while improving the hetero-molecular product yields.

2.5.3.5. C-C bond formation (dimerization and trimerisation) reactions

Naturally, all organic compounds are formed by a carbon-carbon bond frame, therefore the synthesis of novel organic compounds strongly hinges on understanding how these bonds can be formed in vitro. A few reports of plausible biocatalysis reactions for the synthesis of C-C bonds are available in the literature (Resch *et al.* 2011).

An example of these C-C bond formation reactions was illustrated by Witayakran and Ragauskas (2007) when they used laccase from *Trametes villosa* to facilitate C-C bond formation between quinones and dienes via the Diel-Alder reactions (Figure 2.32). The work by Ciecholewski *et al*. (2005) where they carried out the laccase-catalysed dimerization of salicylic esters was one of the earlier attempts at this type of reactions (Figure 2.33).

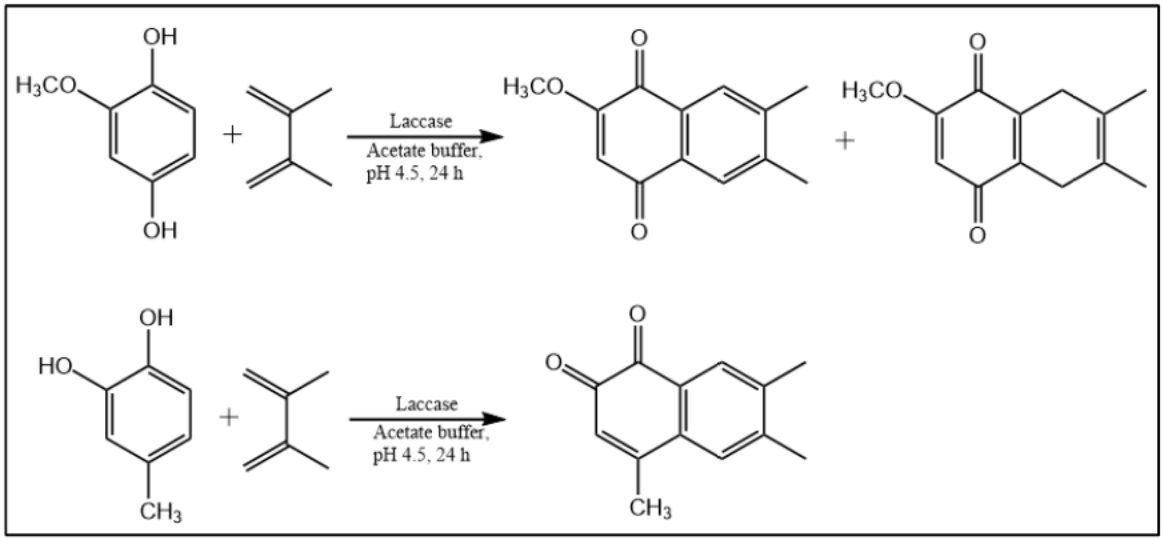


Figure 2.32: The laccase-catalysed oxidative coupling of quinone with dienes via Diel-Alder reactions to synthesise naphthoquinones (Witayakran and Ragauskas 2007).

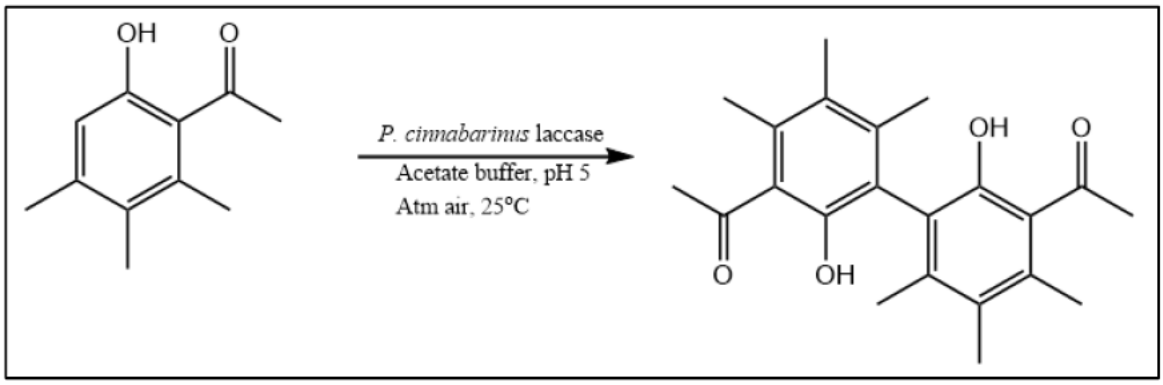


Figure 2.33: The laccase-catalysed oxidative homo-coupling of salicylic acid (Ciecholewski *et al.* 2005).

In another significant study, the domino reactions approach was used to synthesise the derivatives of dispiropyrimidinones through the oxidative coupling capabilities of a laccase from *A. bisporus* at near-standard conditions. The product yields were considerably high at 77% and 90%, respectively (Hajdok *et al.* 2009). Laccase-catalysed homo-molecular coupling reactions have also been used to synthesise numerous functionalised biaryls such as in the biotransformation of 5,6,7,8-tetrahydronaphthalen-2-ol (Intra *et al.* 2005). In another study, a laccase from *T. villosa* was used to facilitate a self-coupling reaction of bisphenol, in a phosphate buffer at room temperature (Figure 2.34) (Uchida *et al.* 2001).

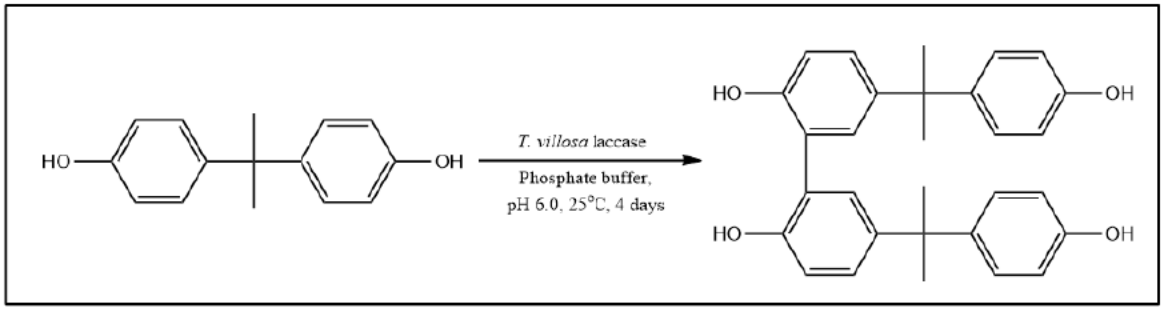


Figure 2.34: The laccase-catalysed self-coupling reaction of bisphenol (Mogharabi and Faramarzi 2014).

The dimerization of 3,5,4'-trihydroxystilbene illustrated the influence exerted by the source of laccase on the product yield when laccases from *M. thermophyla* and *T. pubescens* were used. Laccases from *T. pubescens* facilitated a significantly lower manifestation of the product (18% yield) than those from *M. thermophyla* (31% yield) (Figure 2.35) (Nicotra *et al.* 2004a).

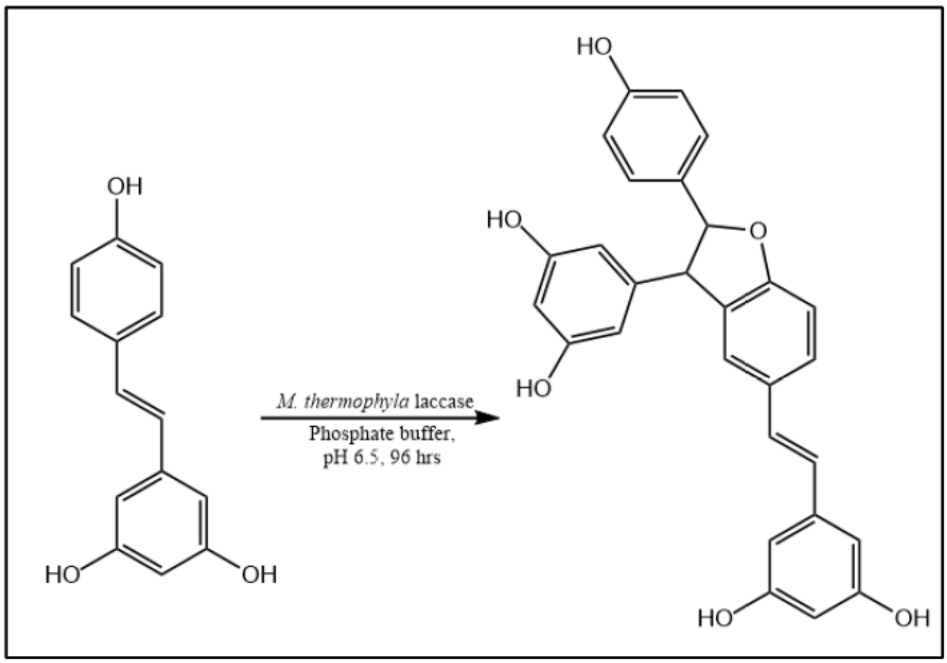


Figure 2.35: The dimerization of 3,5,4'-trihydroxystilbene by laccases from *M. thermophyla* (Nicotra *et al.* 2004a).

2.5.4. Mediators of laccase catalysed organic synthesis reactions

Mediators are generally small molecules that play an 'electron shuttling' role in laccase-catalysed oxidative reactions. The mediator enters the catalytic pocket of the enzyme, and once oxidised it diffuses out and subsequently oxidises any substrate that, due to steric hindrance or size, was unable to directly interact with the catalytic site (Banci, Ciofi-Baffoni and Tien 1999). The oxidised mediator intermediates can utilise an oxidation mechanism that is not feasible to the enzyme, thus expanding its accessible substrate range (Hildén *et al.* 2000). At first, mediators received widespread popularity when used in the laccase-catalysed bleaching of kraft pulp, thus presenting them as an important tool for future biotechnological applications. Consequently, laccase mediator systems (LMS) were utilised in the oxidative synthesis of various compounds (Johannes and Majcherczyk 2000). The mediators' ability to extend the laccases' substrate range was first demonstrated by Bourbonnais and Paice (1990) when they produced veratraldehyde and benzaldehyde from the cleavage of the 1-(3,4-dimethoxy phenyl)-2-phenoxy-ethane-1,2-diol dimer by laccase from *Coriolus versicolor* or *Trametes versicolor* in the presence of ABTS as a mediator. This publication also revealed the propensity of laccases to oxidise non-phenolic compounds

in the presence of a suitable mediator at a time when these enzymes were believed to only oxidise phenolic lignin model compounds (Bourbonnais and Paice 1990).

Several investigations on the laccase-catalysed delignification processes have demonstrated that mediators are pivotal in the oxidation of non-phenolic constituents, especially benzyl alcohol groups in lignin-model compounds [e.g., the synthesis of the ketone alderone from the oxidation of alderol] (Barreca *et al.* 2003). Subsequent works applied mediators in the biotransformation of aliphatic, benzylic, allylic, and propargyl alcohols to matching ketones and aldehydes (Potthast *et al.* 1996; Fritz-Langhals and Kunath 1998; Fabbrini *et al.* 2001; Barilli *et al.* 2004). In a study investigating the laccase-catalysed oxidation of several compounds resembling residual kraft lignin in the presence of ABTS and HBT as mediators, the authors suggested that since these mediators are small enough to access the inner structure of lignin with greater efficiency than the rather large enzyme, they can be seen as diffusible lignin oxidising agents (Crestini and Argyropoulos 1998). The ability of mediators to extend substrate range was further demonstrated and utilised by Bourbonnais and Paice (1992), where its addition drastically improved the demethylation rate of pulp. Several molecules have been reported to demonstrate this phenomenon, such as veratryl 3-hydroxyanthranilic acid with laccases from *P. cinnabarinus* (Eggert *et al.* 1996). Ganachaud *et al.* (2008) demonstrated the positive effect of TEMPO as a mediator in the laccase-catalysed synthesis of an indole trimer (i.e. 2,2-bis(3'-indolyl) indoxyl). The presence of TEMPO reduced reaction time from 48 to 24 hours, and the yield was improved by 28% (Figure 2.36). When evaluating the efficiency of 12 different mediators in the oxidative conversion of 4-methoxybenzyl alcohol, a model substrate, Fabbrini, Galli and Gentili (2002) showed that TEMPO was the most efficient mediator for these types of reactions. The TEMPO-laccase collaboration was also implicated in the selective oxidation of hydroxyl groups of cellulose and other polysaccharides (Viikari, Kruus and Buchert 1999; Jetten *et al.* 2004). Similar procedures have been employed in the oxidative conversion of some natural glycosides and other compounds such as glycosylated saponin and asiaticoside (Monti *et al.* 2005; Baratto *et al.* 2006). Furthermore, meagre investigations have demonstrated the oxidative conversion of aromatic methyls, alkenes and dibenzyl ethers to respective aldehydes, albeit with generally low yields for alkenes and dibenzyl ethers (Potthast *et al.* 1995b; Fritz-Langhals and Kunath 1998; Niku-Paavola and Viikari 2000; Fabbrini *et al.* 2001).

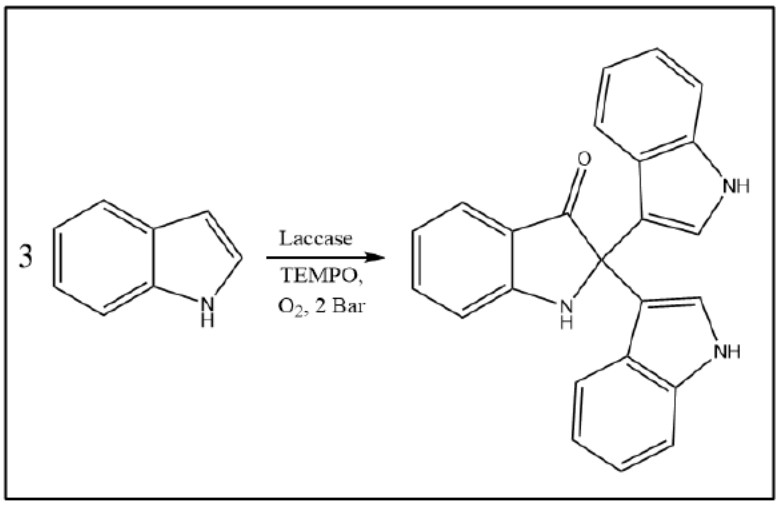


Figure 2.36: The laccase-catalysed trimerisation of indole in the presence of TEMPO as a mediator (Ganachaud *et al.* 2008).

With the vast number of mediators, it is important to note that not all mediators are a perfect fit for all reactions. Specifications such as the redox potential of both the enzyme and the mediator (which in turn is dependent on the molecular structure and stability), and the type of substrate (influences the reaction mechanism, product type, and yield) (Bourbonnais *et al.* 1997). It is generally accepted that depending on their chemical structure, mediators follow three possible oxidation mechanisms: (i) electron-transfer mechanism for compounds such as ABTS; (ii) N-OH compounds such as HBT, HPI or VLA prefer the radical hydrogen transfer mechanism; and (iii) the ionic oxidation mechanism is employed by mediators such as TEMPO (d'Acunzo *et al.* 2002; Baiocco *et al.* 2003; Galli and Gentili 2004).

2.5.4.1. Natural mediators against synthetic mediators

ABTS and HBT are examples of some synthetic mediators with proven efficiency in enhancing the oxidative capabilities of laccases, thus allowing the oxidation of aromatic compounds with high redox potentials. However, their high cost and potential to yield toxic intermediates make them undesirable in industrial applications (Morozova *et al.* 2007b; Camarero *et al.* 2008). The convenience of environmentally benign and low-cost natural mediators such as catechol, syringaldehyde (SA), violuric acid (VA), acetosyringone (AS) and 4-hydroxybenzoic acid (4-HBA) can facilitate the use of laccase-mediator systems in industrial biotechnology processes (Torres-Duarte *et al.* 2009; Song *et al.* 2020). In a study by Moldes *et al.* (2008) where they compared the efficiency of selected natural [i.e.

syringaldehyde and vanillin] and synthetic mediators [i.e. 1-hydroxy benzotriazole, violuric acid, and promazine] in laccase-catalysed bleaching of kraft pulp. They concluded that out of the tested natural mediators, syringaldehyde was the only one that improved delignification and brightness albeit with lower efficiency compared to the tested synthetic mediators. However, syringaldehyde had an advantage over the synthetic mediators in that it presented a lower extent of laccase inactivation (Moldes *et al.* 2008).

In a similar study, the efficiency of natural and synthetic mediators in the laccase-catalysed oxidation of selected polycyclic aromatic compounds was investigated. The prominent natural mediators [i.e. phenol, aniline, 4-hydroxybenzoic acid and 4-hydroxy benzyl alcohol] were found to have similar efficiency as the tested synthetic mediators [i.e. ABTS and 1-hydroxy benzotriazole (HBT)], and some of the tested natural compounds [i.e. methionine, cysteine, and reduced glutathione] exhibited some mediator activity (Johannes and Majcherczyk 2000). In a recent study by Zeng, Qin and Xia (2017) where they looked at the laccase-catalysed degradation of the herbicide isoproturon in the presence of nominated mediators, the synthetic mediators were found to be effective when compared to natural mediators. However, the degradation rate rapidly declined in the presence of synthetic mediators, thus revealing their inhibitory effect on laccases. In a contrary study investigating the laccase-catalysed transformation of halogenated organic pesticides, the author found that the natural compounds acetosyringone and syringaldehyde were the best mediators. Syringaldehyde was found to be the most efficient and behaved like a "true" mediator [i.e. highest rate of pesticide transformation was achieved with a significantly low concentration] (Torres-Duarte *et al.* 2009).

2.5.5. The effect of buffer pH and temperature in laccase-catalysed organic synthesis of bioactive compounds

Temperature and pH are amongst the reaction conditions that play an important role in the laccase-catalysed and oxidation dependant organic synthesis of bioactive compounds. For instance, in a study investigating the effect of pH on laccases' (from *Rhizoctonia praticola* and *T. versicolor*) ability to convert Syringic and vanillic acid into corresponding products, the authors found that the reaction pathways for the conversion of both substrates were largely dependent on the incubation pH of the reaction mixture. At pH values ranging between 6.0 and 8.0 syringic acid yielded two products, while four products were formed at pH values above 8.0. A maximal yield of 2-methoxy-1,4-benzoquinone was obtained from the conversion of vanillic acid at pH 4.0 (Leonowicz, Edgehill and Bollag 1984). When Kim and Nicell (2006) were investigating the dependency of bisphenol A conversion on pH, they obtained results supporting their original speculations that the formation of different products at different pHs may be due to the varying degrees of interaction between the substrate and enzyme. Also, different pHs cause different degrees of enzyme instability which in turn impact the structure and overall charge of the active site, thus causing it to favour oxidation of substrates at certain regions depending on the extent of active site distortion (Kim and Nicell 2006). For instance, fungal laccases are typically more efficient in the acidic range (i.e. pH 3.5 to 5.0) because at these levels the substrates are generally hydrogen atom donors. Furthermore, it is important to note that for phenolic substrates the conversion rates are optimal in the basic range, thus bacterial over fungal laccases (i.e. fungal laccases lose their stability in higher pH values). This behaviour can also be seen as stemming from the balance of redox potential differences between the substrate and inhibition of the T2/T3 copper site after the binding of the OH ion (Witayakran and Ragauskas 2009). Another pH related factor that might have an impact on the enzyme stability and reaction efficiency is the concentration of the buffer species (Kim and Nicell 2006).

Much like in many other enzyme-catalysed reactions, there is generally direct proportionality between the temperature and laccase activity, albeit causing a gradual loss in enzyme activity by denaturation. As the temperature is increased, the added thermal energy causes conformational changes to the active site (i.e. resulting from the distortion of

covalent bonds as the hydrogen bonds and/ or hydrophobic interactions are weakened) (Daniel *et al.* 2008; Daniel *et al.* 2009).

2.6. Separation techniques used in organic synthesis

Numerous synthetic reactions yield mixtures compounds, and reasonable knowledge of the separation procedures is required to obtain the desired compounds in their pure form. The differences in physical properties of the mixture constituents are paramount for choosing the correct separation protocol (Roberts *et al*. 1977; Skoog *et al.* 2013). Chromatography, a biophysical technique, enables separation, purification and identification of mixture components for further quantitative and qualitative analysis. For example, proteins are purified based on their size and shape, total charge, hydrophobic functional groups, and binding affinity with the stationary phase. Ion exchange, surface adsorption, partitioning, and size exclusion are some of the separation techniques based on interaction type and molecular characteristics. Some chromatography techniques are based on the stationary phase (i.e. column, thin layer, and paper chromatography), with column chromatography being the most common method of purification (Coskun 2016). The effective separation factors include molecular characteristics concerning differences in molecular weights, adsorption (liquid-solid), affinity, and partitioning (liquid-solid) (Cuatrecasas, Wilchek and Anfinsen 1968; Porath 1997). Due to these differences, some components of the mixture interact more with the stationary phase, thus progressing slowly with the mobile phase through the column while others progress rapidly through the column with the mobile phase (Gerberding and Byers 1998). Below, the principles of the relevant forms of chromatography are going to be discussed.

2.6.1. Thin-layer chromatography (TLC)

This is a solid-liquid adsorption method where the stationary phase/ solid adsorbent substance (either silica gel, cellulose or alumina) is coated on either glass, plastic or aluminium plates. The mobile phase ascends through the thin stationary phase submerged in the solvent (i.e. mobile phase) by capillary action. The mixture, which is spotted on the lower part of the plate, ascends with the solvent and its constituents move at different rates depending on their polarity and those of the stationary phase and mobile phase (Roberts *et al.* 1977; Middleditch *et al.* 2005). Fluorescence, radioactivity or a specific chemical

substance (i.e. a visible reactive product is produced, thus making it easy to identify the migration positions of the different components in the mixture) can be used to visualise colourless samples under UV or room light. Each molecule in the mixture can be qualitatively described using the relative mobility, expressed as R_f, which is measured by calculating the ratio between the distance travelled by the molecule and that travelled by the solvent (Donald *et al.* 1976).

2.6.2. Column chromatography

This is essentially a solid-liquid adsorption method where the stationary phase is packed into a column, onto either a selective filtration material (i.e. glass wool, cotton wool or filter). The analyte, in solid or liquid form, is applied to the column bed and washed through the column with a suitable mobile phase (solvent or buffer). Depending on the type of the stationary phase matrix, the analyte constituents are separated according to size, shape, net charge or molecular properties like polarity. The sample fractions are collected at the bottom of the column in a time- and volume-dependent manner (Das and Dasgupta 1998). Due to the analyte movement only occurring in the mobile phase, the rate at which the analyte migrates depends on the portion of time it spends in that phase. This portion of time is small for components of the analyte that are strongly retained by the stationary phase and large where they are more likely to be retained in the mobile phase. The time it takes for each component of the analyte to reach the detector is called the retention time (t_R). The void time (measure by determining the migration time of an unretained analyte through the column) is an important parameter because it provides the means to measure the average migration time of the mobile phase, which in turn is used to determine the retention time, which is expressed as follows (Skoog *et al.* 2013):

$$t_R = t_S + t_M,$$

where t_S is the time spent by the analyte in the stationary phase, and t_M is the time spent in the mobile phase. The notable negative aspect of this method is that it generally requires large volumes of the mobile phase to elute a small amount of the analyte, and is generally not reusable (Chalmers 1971). The efficiency at which the chromatographic column separates the components of the analyte partially depends on the comparative rates of elution of the different components. Sequentially, these comparative rates are determined by the concentrations of the analyte in the two phases. The extent to which the analyte components

are distributed between the two phases is described by the distribution constant (K_C) (Skoog *et al*. 2013):

$$K_c = \frac{C_S}{C_M},$$

where C_S is the molar analytical concentration of the analyte in the stationary phase, C_M is the molar analytical concentration of the analyte in the mobile phase.

2.6.3. High-performance liquid chromatography (HPLC)

This method is an extremely important improvement of column chromatography, where the mobile phase passes through the column under high pressure (i.e. 10 – 400 Atm) and with a high flow rate which enables the completion of the analysis within a short time (Regnier 1983). The HPLC is often classified by the separation mechanism which include: 1) chiral chromatography; 2) partitioning/ liquid-liquid chromatography; 3) ion-exchange chromatography; 4) affinity chromatography; 5) adsorption/ liquid-solid chromatography; and 6) size-exclusion chromatography (Skoog *et al.* 2013). The method permits the use of much smaller particles [i.e. more surface area] in the stationary phase than would be possible with traditional column chromatography (Roberts *et al*. 1977).

2.6.3.1. HPLC columns

Originally, HPLC columns were packed with pellicular particles which were spherical, non-porous, glass or polymer beads with diameters ranging from 30 to 40 mm. The pellicular particles would be lined with a thin porous layer of ion exchange, silica, polystyrene-divinyl benzene synthetic resin, or an alumina resin. Over the years these large pellicular particles have been replaced by porous microparticles with diameters ranging from 3 to 10 mm. The particles are composed of ion exchange, silica, polystyrene-divinyl benzene synthetic resin, or an alumina resin, with silica being the most common form of packing used. The silica particles regularly have their surface chemically or physically bound to thin organic films. This type of packing is also referred to as "bonded-phase" (Roberts *et al*. 1977; Skoog *et al.* 2013). Many of the bonded-phase packings are made through the reaction of an organo-chloro-silane with the hydroxide groups on the surface of silica particles via hydrochloric acid facilitated hydrolysis, thus producing an organo-siloxane (Figure 2.37).

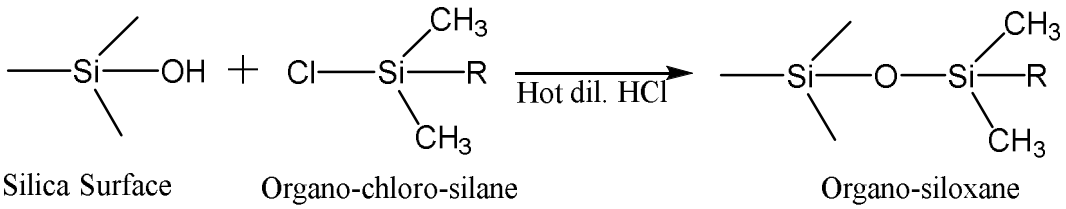


Figure 2.37: The formation of an organo-siloxane bonded-phase on the surface of a silica particle (Skoog *et al.* 2013).

The "R" symbolises a straight-chain octyl- or octyl decyl-group which is often found attached to this position. Aliphatic amines, nitriles, aromatic hydrocarbons and ethers are some of the other organic functional groups that have been attached to silica surfaces, thus enabling the availability of a broad spectrum of polarities (Skoog *etoal.* 2013). Due to the spectrum of polarities that could be achieved from varying the type of organic functional groups attached to silica, a possibility for the existence of two types of partition chromatographs with the HPLC method are presented. The early form was based on highly polar stationary phases such as triethylene glycol and is termed normal-phase chromatography. In this form of partition chromatography, the molecules with the smallest polarity are eluted first; therefore, the elution time can be decreased by increasing the polarity of the mobile phase. Normal-phase chromatography requires relatively nonpolar solvents as the mobile phase (e.g., hexane or i-propyl ether). The opposite of this is reverse-phase chromatography where the stationary phase is often a nonpolar hydrocarbon. In reverse-phase chromatography, the molecules with the most polarity are eluted first, and their retention time is increased by an increase in the polarity of the mobile phase Reverse-phase chromatography requires relatively polar solvents such as tetrahydrofuran, methanol, water or acetonitrile (Snyder, Kirkland and Dolan 2010).

2.6.3.2. HPLC Detectors

After the appropriate column and solvent has separated the components of the analyte and they are eluted from the column, they go past a suitable detector. Numerous detectors have been investigated and used with HPLC separations, and the desirable HPLC detectors are expected to have the following characteristics: 1) Adequate sensitivity, 2) Good stability and reproducibility, 3) Linear response to analytes spanning over several orders of magnitudes, 4) A short response time independent of the flow rate, 5) Highly stable and easy to use, 6) A similarity of response towards all analytes, and 7) It must be non-destructive to the sample (Skoog *et al.* 2013). Due to the varying sample types, there is no highly sensitive universal detector available for HPLC. Therefore, the detector employed is dependent on the nature of the analyte. The popular detectors used in HPLC are based on the absorption of the UV-vis spectrum. Due to the similarities in the UV-vis absorption profiles of countless organic compounds, photometers often make use of the 254 and 280 nm wavelengths (produced by a mercury source). Deuterium- or tungsten-filament sources also serve as a simple resource for the detection of absorbing molecules. Owing to their superior versatility, spectrophotometric detectors are widely preferred. Photodiode-array (PDA) detectors are used in modern instruments as they enable the visualisation of the complete absorption spectrum of the analyte (Skoog *et al.* 2013; Vickrey 2020).

2.7. Analytical techniques for identification and characterising of organic compounds

Chemists must be able to determine the structure of the compounds which they have synthesised and/ or purified. Nowadays numerous different instrumental techniques are employed for the identification and characterization of organic compounds. Through these instrumental techniques, a small amount of the analyte can provide a lot of information. One such technique is the UV-vis spectroscopy which affords the researchers/ technician information about conjugate double bond harbouring organic compounds. Furthermore, mass spectrometry (MS) and infrared spectroscopy (IR) can be employed. The MS enables the determination of molecular mass, molecular formula, and some structural features of the organic compounds; while infrared spectroscopy enables the determination of the type of functional groups that an organic compound has. Nuclear magnetic resonance (NMR) spectroscopy, a more versatile instrumental technique, affords the researchers/ technicians

with information about the hydrocarbon framework of the organic compounds (Bruice 2016). The principles of liquid chromatography-mass spectrometry (with a focus on mass spectrometry), one of the instrumental techniques used for the identification and characterization of organic compounds, will be discussed below.

2.7.1. Liquid chromatography-mass spectrometry (LC-MS)

Liquid chromatography-mass spectrometry, as the name suggests, is an instrumental technique that brings together the separation power of the HPLC with the characterization capabilities of the MS. Essentially, the MS is another detector that can be added onto the HPLC, albeit with an ability to rapidly determine the molecular weight and molecular formula from a very small amount of the analyte (Allwood and Goodacre 2010; Skoog *et al.* 2013). Here, a very small volume of the analyte from the HPLC is introduced to the MS, where it is evaporated and ionized through the removal of an electron from each molecule. There are numerous ways for ionization to be undertaken. The most common ionization sources are matrix-assisted laser desorption/ionization (MALDI), electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI).

2.7.1.1. Ionization sources

MALDI is most preferable for the analysis of large molecules such as peptides, polymers, and proteins. The analyte is embedded onto a matrix that absorbs energy at the wavelength of the nitrogen ultraviolet laser, therefore, when the laser is applied to the matrix in a vacuum it generates ions of the analyte (Kumar and Vijayan 2014). The ESI is considered to be the most useful ionization source, it involves passing the liquid sample through a stainless steel capillary tube that is kept at a high negative or positive potential difference of about 3-5kV. Charged droplets emerge from the tip of the capillary tube and are vaporized, thus increasing the surface charge of the analyte molecules which are then passed into the low-pressure region of the ion source in their gaseous state (Pratima and Gadikar 2018). However, ESI has some undesirable characteristics such as the formation of adducts, low tolerance towards salts, quenching, and a tendency to suppress the ionization of some species due to the presence of a different species in high concentrations (Allwood and Goodacre 2010).

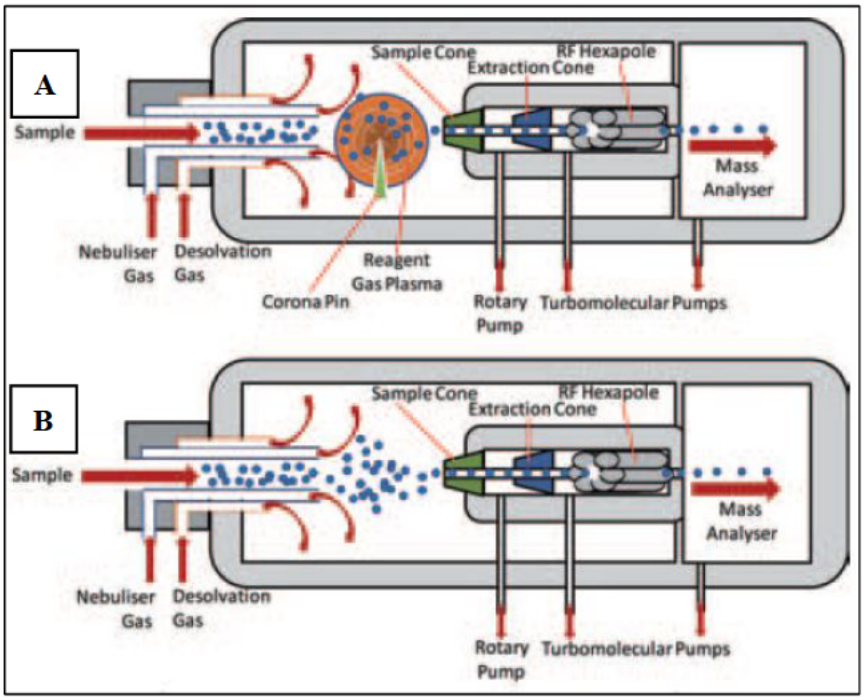


Figure 2.38: An illustration demonstrating the ionization of an analyte ionisation by way of (A) atmospheric pressure chemical ionisation (APCI) and (B) electrospray ionisation (ESI) (Allwood and Goodacre 2010).

The APCI involves evaporation/ desolvation of the sample and a vapour-phase charge transfer reaction to generate ions. This technique is widely used for less polar and non-polar samples with moderate molecular weights. Here, a sample harbouring solvent is nebulised through a constricted capillary tube into a large heating chamber, where it is vaporised under atmospheric pressure at temperatures ranging between 250°C and 400°C. The droplets produced from the vaporisation process are ionised through chemical reactions (Kumar and Vijayan 2014; Pratima and Gadikar 2018).

2.7.1.2. Mass analysers

Due to the weakened molecular bonds resulting from the removal of an electron, in many cases, these radical cations are further broken down into cations, radicals, and neutral molecules. The ionized analytes are transferred to a mass analyser where they are separated based on their respective mass to charge (*m/z*) ratios and presented to the detector (Kumar and Vijayan 2014; Pratima and Gadikar 2018). The quadrupole (Q) or triple quadrupole (QQQ), time of flight (TOF), ion trap (IT) and Fourier transfer ion cyclotron resonance (FTICR) are the commonly used mass analysers.

The single quadrupole is comprised of four parallel metal rods, with each opposing pair of rods electrically connected, and an RF voltage is applied between them. Due to the positive and negative rods being positioned 180° out of phase, the superimposition of the direct current (DC) voltage with the RF voltage creates a quadrupolar field. This quadrupolar field is an ion sorter as it only allows ions within a certain mass range to pass through. The range of masses through which the quadrupole analyser scans can be controlled by varying the DC voltage. The passage rate of specific mass ions through the quadrupole depends on the RF to DC voltage ratio (Allwood and Goodacre 2010; Kumar and Vijayan 2014; Pratima and Gadikar 2018).

The triple quadrupole (QQQ) mass analyser, on the other hand, consists of three linearly arranged quadrupole cells. The first quadrupole that is encountered by the ionised analyte functions like the classical single quadrupole (i.e. it sorts the ions according to the specified ion mass range), while the second non-RF quadrupole functions as a collision cell, where a stream of collision gas (e.g. N, Ar, He) is passed through the trapped ions at a collision energy of ~ 30 electron volts and pressure of ~ 0.4 – 1.3 kPa. In a process called collision-induced decomposition (CID), the collision of the ions with the gas causes the formation of product ions (or fragments). The final quadrupole functions as a filter that allows for the detection of the fragments produced from the second quadrupole. Thus, the QQQ mass analyser may be regarded as an in-space tandem-MS analyser that is suited for directed MS analysis of intact ions and MS-MS structural analysis of organic compounds (Allwood and Goodacre 2010).

The time of flight (TOF) mass analyser is based on the idea that the ions' velocity through the flight/ drift tube is inversely proportional to their mass (i.e. the ions with a smaller *m/z* reach the detector first) (Allwood and Goodacre 2010; Kumar and Vijayan 2014; Pratima and Gadikar 2018). In early instruments, the TOF mass analysers were positioned linearly to the ion source, such that the ions passed linearly from the ionizer to the detector on the opposite end of the flight/ drift tube. However, these days TOF mass analysers are generally positioned orthogonally to the ion source, thus significantly increasing the distance travelled by the ion in the flight/ drift tube. Due to the greater time and space separation of ions of similar *m/z*, the resolution is improved resolution and the precision of the linear TOF/MS is enhanced by more thani10 folds. Passing the accelerated ions through the reflectron further improves the resolution and precision. Reflectrons, also known as ion mirrors, are composed of a series of rings whose purpose is to create an electrostatic retardation field. The depth at which the ions penetrate the retardation field is directly proportional to their flight velocity. Therefore, the higher velocity ions are retained for a longer time, thus enabling the separation of ions with similar *m/z* but different flight velocities (Allwood and Goodacre 2010).

Ion trap (IT) mass analysers employ an amalgamation of magnetic or electric fields to arrest or "trap" ions. Numerous ion trap configurations exist, such as electrostatic traps (also known as Ortbitraps), 3D ion traps (also known as the Paul ion trap), magnetic field-based traps (ion cyclotron resonance), and a linear ion trap (2D trap) (Allwood and Goodacre 2010; Kumar and Vijayan 2014; Pratima and Gadikar 2018). The electrostatic trap arrests ions by causing them to orbit around the inner spindle (i.e. a stable flight path) by balancing their electrostatic attraction by their inertia emanating from an RF only trap. The 3D ion trap essentially employs the same principles as the quadrupole mass analyser because it makes use of both the DC and RF oscillating electric fields. However, in place of parallel rods, there are two hyperbolic metal electrodes and a ring electrode positioned halfway between them. The ions are trapped in a circular path depending on the electric field applied. Through the use of a strong magnetic field, the ion cyclotron resonance (ICR) trap causes radial orbiting of the ions such that their orbiting frequency in the magnetic field is a function of their *m/z*. The linear ion trap facilitates the trapping by using a set of quadrupole rods that are coupled with electrodes on opposite ends, thus giving it a two-fold functionality as either a quadrupole mass filter or ion trap (Clarke 2017).

2.7.1.3. Mass spectrometry detectors

Mass spectrometers employ electron multipliers, dynolyte photomultipliers and microchannel plate detectors. Secondary electron emission is primarily responsible for the operation of electron multipliers. This process takes place when ions or electrons strike a surface and cause a secondary release of electrons from the surface atoms. The type of incident particles, their energy and characteristics of the surface are the determinants of the number of secondary electrons released. The stream of secondary electrons is magnetically focused by copper-beryllium dynodes, and converted into an electric current that can be detected electronically with appropriate software which presents it as a mass spectrum. This type of detector is very common with quadrupole and ion trap mass spectrometers. In the Dynolyte photomultiplier detectors, the dynode assists with the conversion of ions into electrons. The flood of electrons is then made to stick onto a phosphor, thus causing it to emit photons. The photon signal is magnified by causing the photons to strike a photomultiplier for recording (Medhe 2018). Microchannel plate (MCP) detectors essentially consist of an array of 104 -107 minute electron multipliers that are arranged parallel to one another. The multiplier matrix is made from lead glass that has been designed such that the secondary emission characteristics of each channel is optimised. This also renders the channel walls semi conductive, which is necessary for the charge replenishment via an external voltage source. Therefore, the channels may be respectively considered as continuous dynode structures. Ions hit the channels at an angle with sufficient energy so as cause a secondary release of electrons. The channels simultaneously amplify the electron number by several orders of magnitude owing to the applied electric field strength. The electrons are collected on an anode at the opposite end of the channels, thus producing an image of the electrons (Chassela *et al.* 2019).

2.8. The current study

The current study investigated the production, purification, thermodynamic and biochemical characterisation of the oxidoreductase enzyme, laccase, expressed by the white-rot fungus, *Trametes pubescens* CBS 696.94. Small scale preliminary laccase-catalysed hetero-coupling reactions between several natural antioxidants (i.e. quercetin, catechol, nordihydroguaiaretic acid, gallic acid, guaiacol, vanillic acid, pyrogallol, naringenin, caffeic acid, ferulic acid, cinnamic acid, coumaric acid and eugenol) were undertaken to produce compounds with improved antioxidant and antimicrobial activity. The reactions that showed potential were optimised and scaled up in either monophasic or biphasic cosolvent systems. Reactions were monitored using thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) to check for the presence of the desired products. Products were purified by preparative thin-layer chromatography (PTLC) and characterised by LC-MS. 2,2'-diphenyl-1-picrylhydrazyl (DPPH), ferric antioxidant power (FRAP) and 2,2'-azino-bis-3-ethybenzthiazoline-6-sulphonic acid (ABTS) radical-scavenging activity assays were used to determine the antioxidant activity of the products. The antimicrobial activity of the products against gram-negative (i.e. *Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus aureus*) and gram-positive (i.e. *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterobacter cloacae*) bacteria was tested using the Bauer-Kirby-Sherris-Turck single disk diffusion method.

3. DETERMINATION OF THERMODYNAMIC PARAMETERS AND MODES OF INHIBITION BY SELECTED PUTATIVE INHIBITORS OF *Trametes pubescens* CBS 696.94 LACCASE

3.1. ABSTRACT

In this chapter, the biochemical characterisation and the determination of the thermodynamic parameters of *Trametes pubescens* CBS 696.94 laccase, and its utilisation in the synthesis of novel hybrid compounds are presented. *T. pubescens* CBS 696.94 produced high titres of extracellular laccase (2330 ± 50 U/l). The enzyme (~58 kDa) had an optimum activity at 60°C while optimum pH varied with the substrate used. The activity was shown to drop drastically at temperatures above 60°C, while the enzyme was most stable between pH 4.5 and 5.0. Enzyme activity was enhanced when the enzyme was pre-incubated in 20 mM of $CuSO_4$. The Michaelis-Menten constants (K_m) values for ABTS, SGZ, 2,6-DMP and guaiacol were 198 µM, 211 µM, 168 µM, and 102 µM, respectively. The k_{cat} values were 103 s^{-1}, 32 s^{-1}, 12 s^{-1}, and 13 s^{-1} with corresponding catalytic efficiency values (k_{cat}/ K_m) of 5.2×10^5 s^{-1} M^{-1}, 5.8×10^4 s^{-1} M^{-1}, 1.9×10^5 s^{-1} M^{-1} and 1.2×10^5 s^{-1} M^{-1}, respectively. The $t_{1/2}$ values of the *T. pubescens* CBS 696.94 laccase at 50°C, 60°C, and 70°C were 7.8 h, 3.8 h, and 0.72 h, respectively. The enzyme deactivation energy (E_d) was 109.362 kJ/mol while ΔG, ΔH, and ΔS for thermal inactivation of the *T. pubescens* CBS 696.94 laccase were all positive. When incubated with selected inhibitors, the enzyme either exhibited non-competitive inhibition (in the presence of sodium azide and sodium dodecyl sulphate and SDS) or uncompetitive modes of inhibition (in the presence of L-cysteine, hydrogen peroxide, and dithiothreitol). The enzyme was successfully employed in the synthesis of two novel compounds, code-named $P_{QC}1$ and $P_{QC}2$, from quercetin and catechol. $P_{QC}2$ exhibited better antimicrobial activity against *Listeria monocytogenes* and *Staphylococcus aureus* in comparison with quercetin and catechol.

Keywords:

Trametes pubescens, laccase, kinetics, thermodynamics, inhibition mode, cross-coupling, quercetin, catechol, antioxidant capacity, antimicrobial activity.

3.2. INTRODUCTION

Laccases are dimeric or tetrameric glycoproteins belonging to the blue copper family of enzymes. They are capable of catalysing the oxidation of various phenolic and non-phenolic compounds to produce radicals with concomitant four-electron reduction of molecular oxygen to water (Dwivedi *et al.* 2011; Santhanam *et al.* 2011; Riva 2013). Laccases have been found in plants, insects, bacteria, but are predominant in fungi where their presence has been documented in virtually every fungus that has been tested for them. Therefore, fungal laccases are the most characterised and have been used in many biotechnological applications (Mayer and Staples 2002; Polaina and MacCabe 2007; Bertrand *et al.* 2015; Yadav *et al.* 2018). It is a common occurrence for a single fungus to yield numerous isoforms of laccase whose production is influenced by adding an inducer (Bertrand *et al.* 2015; Yuan *et al.* 2016). This indicates that inducers are able to differentially affect the expression of laccase genes at a transcriptional level. The laccase isoforms generally possess distinguishable catalytic properties, which makes their expression profile and concentrations very important. Growth parameters such as carbon or nitrogen source may significantly influence the profile of the expressed laccase isoforms (Bertrand *et al.* 2015).

In the past two decades, the ability of laccases to facilitate specific, efficient, and environmentally sustainable oxidation of phenolic/lignin model compounds, non-phenolic compounds, and some extremely obstinate pollutants (e.g., pesticides, chlorophenols, and polycyclic aromatic hydrocarbons) has invited marked attention from researchers (Kudanga *et al.* 2011b; Yuan *et al.* 2016; Kudanga *et al*. 2017). Laccases have shown potential for use in various industries such as the pulp and paper, food, pharmaceutical, and fine chemical industries, and extensive studies on their application in bioremediation are ongoing (Upadhyay *et al*. 2016; Yuan *et al.* 2016). Laccase-catalysed coupling reactions of either identical (i.e. homo-coupling) or different molecules (i.e. hetero-coupling) can be applied in organic synthesis (Kudanga *et al.* 2011b; Kudanga *et al*. 2017; Rodríguez-Couto 2019). However, their ability to facilitate coupling reactions is heavily influenced by factors such as pH, temperature, the enzymes' redox potential (i.e. substrate range determinant), sensitivity to inhibitors, organic solvents, and heavy metals encountered in the reaction environment (Moreira Neto *et al*. 2009; Afreen *et al.* 2017). In this study, laccase produced by *T. pubescens* was purified and characterised. The thermodynamic parameters were

determined with ABTS as substrate. Finally, the purified enzyme was used in the hetero-coupling of selected phenolic substrates.

3.3. MATERIALS AND METHODS

3.3.1. Chemicals and fungal strain

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

3.3.2. Production of *T. pubescens* laccase (Tp-laccase)

3.3.2.1. Maintenance of *T. pubescens* CBS 696.94

Cultures of *T. pubescens* CBS 696.94 were maintained on 3.9% (w/v) potato dextrose agar plates at 4.0°C and were sub-cultured every 30 days to maintain viability.

3.3.2.2. Growth of *T. pubescens* CBS 696.94 in shake flasks for production of Tp-laccase

A fresh culture of *T. pubescens* was grown on 3% (w/v) potato dextrose agar plates at 28°C for 5 days. Three portions of autoclaved 300 ml Trametes Defined Media (TDM; Appendix 1) were aseptically inoculated with six discs (with a size of 1.9 cm^2) of the *T. pubescens* grown on solid media. These were incubated at 28°C with agitation at 175 rpm (Adelakun 2012). On the third day, the cultures were supplemented with 3.0 ml of 1 mM xylidine (to a final concentration of 10 µM) and 0.6 ml of 1 M $CuSO_4$ (to a final concentration of 2 mM). After 5 days, the crude enzyme was harvested by filtration through a Whatman No. 1 filter paper under vacuum. The resulting filtrate was centrifuged at 13751×g (Eppendorf centrifuge 5810R) for 20 minutes and the supernatant was collected as the crude enzyme.

3.3.3. Purification of Tp-laccase

3.3.3.1. Partial purification of Tp-laccase by ammonium sulphate precipitation

Successive amounts of ammonium sulphate (50% - 80% saturation) were added to the Tp-laccase containing medium. The homogeneous distribution of the ammonium sulphate salt was facilitated by stirring the mixture for 1 hour at 4℃, followed by centrifugation at 13751×g for 15 minutes. The pellets were re-suspended in 0.1× the initial volume in 0.1 M sodium acetate buffer at pH 5.0. Enzyme activities for both pellets and supernatants were determined using the ABTS/laccase assay. Samples were dialysed against 5 l of 0.1 M sodium acetate buffer at 4°C (pH 5.0) overnight. The Lowry assay was used to determine protein concentration (Lowry *et al.* 1951). SDS-PAGE analysis of partially purified samples was carried out as per the Laemmli method (Laemmli 1970).

3.3.3.2. Determination of laccase activity

The oxidation of 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, ε_{420} = 36, 000 M^{-1} cm^{-1}) as a substrate at 420 nm was monitored and used to determine laccase activity. The reaction mixture contained 0.110 ml of ABTS (5 mM, in distilled water), 0.833 ml of 0.1 M sodium acetate buffer (pH 5.0), and 0.057 ml of the Tp-laccase (Adelakun 2012). Oxidation of ABTS was monitored over 80 seconds, and the amount of enzyme required to oxidise 1 µmol of ABTS per minute was defined as one unit of laccase (Dias *et al.* 2017). The following formula was used to calculate enzyme activity:

$$\text{Enzyme Activity} = \frac{\Delta A_{420\text{nm}} \times SV}{\varepsilon_{420\text{nm}} \times E \times d} \times DF$$

Where,

ΔA_{420nm}= Change in absorbance per minute at 420 nm

SV = Total volume (ml)

ε_{420nm} = Extinction coefficient of ABTS at 420 nm

E = Enzyme sample volume (ml)

d = Light path of the cuvette (cm)

DF = Dilution factor

3.3.3.4. Protein quantification of the Tp-laccase samples

Protein quantification of the Tp-laccase samples was carried out using the Lowry assay (Lowry *et al.* 1951). A 2 mg/ml bovine serum albumin (BSA) stock solution was prepared by dissolving 10 mg of BSA in 5 ml of distilled water. Various concentrations of BSA standards were prepared from the stock solution. In Eppendorf tubes, 20 µl of the sample was added, followed by 180 µl of distilled water. The Lowry's solution (1.0 ml) was added to the mixture and incubated at room temperature for 15 minutes. A 100 µl of 1.0 N Folin's phenol reagent was added to the mixture, followed by incubation in the dark for 30 minutes, after which the absorbance was measured at 660 nm (refer to appendix 3 for the standard curve).

3.3.3.5. Ultrafiltration

The laccase sample was centrifuged in an Amicon filtration device from Merck (30 kDa molecular weight cut-off) at 3438×g for 60 minutes at 4°C. The enzyme activity of the concentrated sample was determined, as described above. Protein concentration was determined by the Lowry assay.

3.3.3.6. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE was carried out as per the Laemmli method (Laemmli 1970) to determine the molecular weight of the enzyme and visualise and compare the purification efficiency of each step. Before SDS-PAGE analysis, the samples (40 µg in 15 µl) were prepared by mixing them with 5 µl of sample buffer (25 mM Tris-HCl buffer, pH 8.0, 2% SDS, 2% dithiothreitol, 20% glycerol and 0.02% bromophenol blue). The samples were then heated at 100°C for 5 minutes and cooled at room temperature. Samples were loaded on the gel (5% stacking and 10% resolving) and electrophoresed at 20 mA per gel at room temperature until the dye front had reached the bottom. Coomassie brilliant blue R250 (0.25% dye, 10% acetic acid, 45% ethanol and distilled water) was used to stain the gels. The gels were destained (7.5% acetic acid, 5.0% ethanol and distilled water) and photographed with a Gel Doc XR system (Bio-Rad).

3.3.3.7. Purification with anion exchange chromatography (IEC)

A 5 ml HiTrap diethyl amino ethyl (DEAE) cellulose column from Cytiva Health Life Sciences (previously GE Health Life Sciences) was used for IEC. Five column volumes of sodium acetate buffer (100 mM Na-Ac, 1.5 M NaCl, pH 5.0), 5× column volumes of 0.5 M NaOH, 3× column volumes of sodium acetate buffer (100 mM Na-Ac, 1.5 M NaCl, pH 5.0), 6× column volume of Milli-Q water were sequentially used to wash the column. Finally, the column was equilibrated with 6× column volumes of sodium acetate buffer (50 mM Na-Ac, pH 5.0). The sample port was washed with 50.0 ml Milli-Q water, and approximately 2 ml of the sample was injected. Protein elution was carried out with a salt gradient of 0 - 1.0 M NaCl at a flow rate of 1.0 ml/min. The fractions that showed appreciable laccase activity were analysed for protein concentrations and were further analysed by SDS-PAGE.

3.3.3.8. Purification with size exclusion chromatography (SEC)

Sephadex G-75 matrix (3.0 g) was suspended in 71.0 ml of 0.1 M sodium acetate buffer pH 5.0 and heated at 90°C for 2 hours and allowed to cool at room temperature. The matrix was poured into a burette plugged with cotton wool. The matrix was washed with 5× column volumes of 0.1 M sodium acetate buffer (pH 5.0) and left overnight for the resin to settle (to a final bed length of 60 cm). The enzyme sample (0.68 ml) was run through the column and eluted with the same buffer at a flow rate of 1.0 ml/min. Fractions were collected and checked for enzyme activity. The fractions with high levels of enzyme activity were then analysed for purity by SDS-PAGE. The fractions showing appreciable purity were pooled together and concentrated by ultrafiltration through a 30 kDa pore size Amicon filtration unit at 5000×g for 1 hour.

3.3.4. Biochemical characterisation of Tp-laccase

The optimum pH for oxidation of selected substrates (ABTS, syringaldazine, guaiacol, caffeic acid, *p*-coumaric acid, ferulic acid, vanillic acid and 2,6-DMP) was determined by performing laccase activity assays in different buffers [50 mM sodium citrate buffer (pH 3.0 - 5.5), 50 mM sodium phosphate buffer (pH 6.0 - 8.0) and 25 mM sodium tetraborate-HCl buffer (pH 8.5 - 9.0)] at 60°C (Kudanga *et al.* 2009). The same buffers were used to determine the pH stability of Tp-laccase by incubating the enzyme in each respective buffer for 3 hours and performing laccase activity assays at 30 minutes intervals using ABTS as

the substrate. The optimum temperature for the activity of Tp-laccase was determined by conducting laccase activity assays at different temperatures (15°C - 100°C) at the optimum pH for the substrates ABTS, SGZ and 2,6-DMP. Product formation was monitored in the wavelength range 200 – 800 nm (refer to Appendix 4 for λ_{max} values of each tested substrate).

3.3.5. Enzyme kinetics and thermal deactivation of Tp-laccase

Kinetic parameters of the purified Tp-laccase were determined at 60°C using ABTS, SGZ, guaiacol and 2,6-DMP as substrates in the concentration range 25 - 300 µM. Assays were performed at the appropriate pH for each substrate (pH 7.0 for SGZ, pH 3.5 for both ABTS and 2,6-DMP) and the K_m and K_{cat} values were determined using SigmaPlot (Systat Software Incorporated). The activation energy (E_a) was calculated according to Yadav *et al.* (2018). The deactivation energy (E_d) of the enzyme was calculated from the residual activity at different temperatures by incubating the enzyme solution in 50 mM sodium acetate buffer (pH 3.5) for ABTS at various temperatures (25 - 80°C), in the absence of substrate. Samples were taken at the desired intervals, and laccase assays were performed for the calculation of residual activities. The inactivation constants (K_d) and deactivation energy (E_d) were calculated as explained below:

The thermal denaturation is a reaction in which the rate of the enzyme deactivation (dC/dt) follows first-order kinetics in relation to the concentration of the active enzyme (C):

$$dC/dt = -k_dC \quad (1)$$

Which can also be expressed as:

$$\ln[C_t/C_0] = - k_dt \quad (2)$$

Where C_0 is the initial concentration of the enzyme and C_t is the concentration of the enzyme at time t. Since the residual enzyme activity (E) is directly proportional to the concentration of the active enzyme (C), thus,

$$E_t/E_0 = C_t/C_0$$

Now Eq. (2) can be written as:

$\ln[E_t/E_0] = - k_d t$

or

$2.303 \log [E_t/E_0] = - k_d t$

(3)

K_d is the deactivation rate constant, which is calculated from the slope of the plots of $\log[E_t/E_0]$ versus t. The half-life ($t_{1/2}$) of the enzyme is defined as the time required for the enzyme to lose half of its initial activity. It can be expressed as follows:

$t_{1/2} = 2.303 \log 2/ K_d$

(4)

The energy required for deactivation has been calculated by the Arrhenius plot using the Arrhenius equation:

$K_d = Ae^{\left(-E_d/RT\right)}$

(5)

So that,

$\ln[K_d] = - E_d/RT + \ln A$

(6)

Where E_d represents deactivation energy, R is the universal gas constant (8.314 J/K/mol), and T is the absolute temperature. Deactivation energy (E_d) involved in the deactivation of the process is calculated from the slope of a linear plot of $\ln[K_d]$ versus T^{-1}.

3.3.6. Thermodynamic parameters of the Tp-laccase

The thermodynamic parameters of irreversible inactivation of Tp-laccase were determined by rearranging the Eyring's absolute rate equation derived from the transition state theory:

$$K_d = (K_bT/h)e^{(-\Delta H/RT)}e^{(-\Delta S/RT)}$$

ΔH (change in enthalpy of deactivation), ΔG (change in free energy of inactivation), and ΔS (change in entropy of inactivation) for irreversible inactivation were calculated as follows:

$$\Delta H = E_d - RT \quad (7)$$

$$\Delta G = - RT\ln(K_dh/K_bT) \quad (8)$$

$$\Delta S = (\Delta H - \Delta G)/T \quad (9)$$

Where K_b is the Boltzmann's constant (R/N) = 1.38×10^{-23} J/K, T is the absolute temperature (K), h is the Planck's constant = 6.626×10^{-34} Js, N is the Avogadro's number = 6.02×10^{23} mol^{-1}, and R is the gas constant = 8.314 J/K/mol.

3.3.7. Effect of inhibitors on Tp-laccase activity

The effect of five different known fungal laccase inhibitors namely L-cysteine, sodium azide (NaN_3), dithiothreitol (DTT), sodium dodecyl sulphate (SDS) and hydrogen peroxide (H_2O_2) on the activity of Tp-laccase on ABTS was investigated. Inhibitors at various concentrations (0.05 - 5 mM) were pre-incubated with laccase in 50 mM sodium acetate buffer at pH 5.0 and 30°C for 60 minutes and residual activity was measured using the ABTS assay (Kudanga *et al.* 2009; Si *et al*. 2013; Shi *et al*. 2014).

3.3.8. Effect of metal ions on Tp-laccase activity

The effect of metal ions (i.e. Cu^{2+}, Na^{+}, K^{+}, Ba^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+} and Al^{3+}) on Tp-laccase was investigated. The metal ions of various concentrations (5.0 - 25 mM) were pre-incubated with the laccase in 50 mM sodium acetate buffer pH 5.0 and 30°C for 60 minutes and residual activity was measured using the ABTS assay described earlier (Section 3.3.3.2).

3.3.9. Effect of organic solvents on Tp-laccase activity

The enzyme was pre-incubated in a co-solvent system consisting of organic solvents [i.e. DMSO, acetone, ethyl acetate, methanol, dioxane or ethanol at a concentration of 50% (v/v)] and a 50 mM sodium acetate buffer (pH 5.0). The residual activity was monitored hourly for 6 hours with the ABTS assay as previously described (section 3.3.3.2).

3.3.10. Small scale preliminary oxidation of selected phenolic compounds

Selected phenolic compounds were oxidised by Tp-laccase (0.66U) in a co-solvent system consisting of a 50 mM sodium acetate buffer pH 5.0 and selected water-miscible solvents (i.e. acetone, methanol, dioxane or ethanol) at a concentration of 70% (v/v). The reaction mixtures were supplemented hourly with 0.66 U of Tp-laccase to overcome the enzyme inactivation caused by the organic solvents. The reactions took place in a shaking incubator at 37°C and 200 rpm for 6 - 12 hours (i.e. depending on the conversion rate of the substrate). The formation of dimers was monitored using thin-layer chromatography (refer to section 3.3.11.1).

3.3.10.1 Coupling of catechol onto quercetin

Quercetin was dissolved in a co-solvent system consisting of a 50 mM sodium acetate buffer pH 5.0 and dioxane at a concentration of 50% (v/v) to a final concentration of 5 mM followed by the dissolution of catechol in the same mixture to a final concentration of 5 mM. The mixture was vortexed to ensure homogeneity. This was followed by the addition of 0.66 U laccase. The reaction mixture was kept in a shaking incubator at 37.0°C and 200 rpm for 6 hours. The formation of the coupling products was monitored hourly using thin-layer chromatography (TLC).

3.3.11. Chromatographic separation of oxidation products

3.3.11.1. Thin layer chromatography (TLC)

Aluminium-backed silica gel 60 F254 plates (Merck) were used for TLC analysis. Toluene:Dioxane:Acetic acid [11:2.5:0.4 (v/v/v)] was used as the mobile phase. The plates were exposed to UV light at 254 nm for the visualisation of the compounds.

3.3.11.2. High-performance liquid chromatography (HPLC)

An equal volume of ice-cold methanol was added to the reaction mixtures to stop the coupling reactions and the mixtures were left on ice for 20 minutes. Thereafter, the reactions were centrifuged at 14 000×g, 4°C for 15 minutes. The supernatants were filtered through 0.2 µm nylon filters into clean 2 ml vials and analysed by HPLC. A Prominence HPLC system (Shimadzu) was used. A Sunfire C18 5.0 µm, 4.6×150 mm reversed-phase column (Waters) was used for the separation of the reaction products. Gradient elution with 0.1% formic acid (solvent A) and acetonitrile (solvent B) was employed. The gradient was set-up as follows: 98% A to 0% A (20 min); 0% A to 98% A (20 - 21 min); 98% A (21 - 30 min) at a flow rate of 1.0 ml/min. The peaks were analysed using the LabSolutions data management software (Shimadzu).

3.3.12. Purification of oxidation products

Preparative TLC was used to purify the oxidation products of interest. Aluminium-backed silica gel 60 F254 plates (Merck) were used for TLC analysis. Toluene:Dioxane:Acetic acid [11:2.5:0.4 (v/v/v)] was used as the mobile phase. The plates were exposed to UV light at 254 nm for the visualisation of the compounds. The oxidation products of interest were scraped from the TLC plates, dissolved in methanol and filtered through a 0.2 µm nylon filter to remove the silica.

3.3.13. Characterisation of oxidation products

Liquid chromatography-mass spectrometry (LC-MS) was used to characterise the purified (or partially purified) oxidation products.

The Prominence Shimadzu HPLC system was coupled with a Shimadzu LCMS 2020 mass spectrometer. The oxidation products were separated as described in section 3.3.11.2. An injection volume of 20 µl and an oven temperature of 35°C was used. The MS spectra were obtained in negative mode using the ESI ionizer with an electrospray voltage set at +3500 V. The dry gas flow was set to 9 l/min at a temperature of 350°C and nebulizer gas pressure of 35 psi.

3.3.14. Determination of antioxidant activity

The ferric reducing antioxidant power (FRAP) assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ABTS scavenging assays were used to determine antioxidant activity.

3.3.14.1. DPPH scavenging effect

The reaction mixture contained 3.9 ml of a 63.4 µM DPPH solution (dissolved in methanol) and 0.1 ml of various concentrations of the purified oxidation products dissolved in methanol. The mixture was incubated at room temperature and in the dark for 60 minutes. The decrease in absorbance was monitored spectrophotometrically at 517 nm and the results were expressed as C_{50} values. The C_{50} value is defined as the concentration of the antioxidant required to achieve a 50% decrease in the DPPH radical concentration (Adelakun *et al.* 2012). Methanol was used as the negative control.

3.3.14.2. ABTS radical scavenging effect

The ABTS radicals were produced by mixing a 2.0 mM ABTS solution with 7.0 mM $K_2S_2O_8$ and incubating in the dark for 24 hours at room temperature. The ABTS radicals' absorbance (at 734 nm) was adjusted to 0.7 by diluting with 95% methanol before use. Test solutions were mixed with the diluted ABTS radical solution to effective concentrations of 5, 10, 20, and 30 µM, and their absorbance was recorded after 6 minutes of incubation. Methanol was used as the negative control.

3.3.14.3. FRAP assay

The ferric reducing antioxidant power of the phenolic substrates and the corresponding purified oxidative products was measured according to the method by Ahmed *et al*. (2015), with minor modifications. The working solution was prepared by mixing 25 ml of a 300 mM sodium acetate buffer pH 3.6 with 2.5 ml of a 10 mM TPTZ solution and 2.5 ml of a 20 mM ferric chloride solution. Before use, the mixture was incubated at 37°C for 15 minutes. Iron sulphate (5 µM to 100 µM) was used as a standard. The mixture of the test compound and the working solution was incubated in the dark for 30 minutes, followed by the determination of the absorbance at 593 nm. The results were reported as FRAP values (i.e. µM of Fe^{3+} converted to Fe^{2+}). Methanol was used as the negative controls.

3.3.15. Determination of antimicrobial activity

The agar well diffusion method was used to determine the antibacterial activity of the coupling products. A 100 µl of each standardised bacterium (0.5 McFarland turbidity standard) was aseptically spread on Petri dishes containing solidified Mueller Hinton agar. Sterilised absorbent paper discs with a diameter of ~ 6 mm (Whatman No. 2 filter paper) were impregnated with 100 µl of a 5 mM starting substrates or respective product and air-dried (Kim *et al*. 1995). The dried discs were placed in plates that were labelled accordingly for each American Type Culture Collection (ATCC) bacterium [Gram-positive *Bacillus cereus* (ATCC 10876), *Listeria monocytogenes* (ATCC 19111), and *Staphylococcus aureus* (ATCC 29213); and Gram-negative *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), and *Enterobacter cloacae* (ATCC BAA1143)]. The contents of the discs were allowed to diffuse for 1 hour at 4°C and incubated at 37°C for 24 hours. Respective coupling product resuspension solvents were used as negative controls. Upon completion of the incubation period, the diameters of the zones of inhibition around each disc were measured to the nearest millimetre along two axes, 90° to each other and the mean of the two readings was recorded (Alabi *et al.* 2012). All experimental determinations were carried out in triplicate.

3.3.16. Determination of the minimum inhibitory concentration (MIC)

The minimum inhibitory concentration was carried out using the micro broth technique in microtiter plate wells (Corning) and 0.5 McFarland bacterial suspension standard (CLSI, 2000). Suspension broth and solvent and bacterial broth only were used as negative and positive controls. All experimental determinations were done in triplicate. The lowest compound concentration that visibly inhibited growth was recorded as the MIC.

3.3.17. Statistical analysis for the antioxidant and antimicrobial activities

Antimicrobial and antioxidant activity data were subjected to one-way analysis of variance (ANOVA) and sample differences were determined by Duncan's Multiple Range testing using SPSS version 16. *P*-values < 0.05 were regarded as significant.

3.4 RESULTS AND DISCUSSION

3.4.1 Growth of *T. pubescens* in flasks for the production of Tp-laccase

Owing to the innumerable biotechnological applications of laccases there has been an increase in research aimed at optimizing the production of this enzyme in different microorganisms (Niladevi and Prema 2008). In the current study, the production of laccase for use in the modification of bioactive compounds was performed in 5-litre shake flasks. Laccase is an extracellular enzyme, therefore, *T. pubescens* secreted it into the medium. An initial laccase activity of 0.11 ± 0.021 U/m1 was observed on day 1 of the fermentation. This gradually increased to 1.05 ± 0.031 U/ml by day 3. A repertoire of phenolic and aromatic compounds can be used as inducers to increase the production of laccase by fungi, particularly by white-rot fungi (Terrón *et al.* 2004). Also, an addition of $CuSO_4$ as a co-inducer (i.e. supplies Cu^{2+} ions in the medium) has been shown to enhance laccase activity (Haibo *et al.* 2009; Zhu *et al.* 2011; Si *et al.* 2013). In the current study, a combination of xylidine and $CuSO_4$ was used to induce and enhance enzyme production. On the third day of fermentation, the cultures were supplemented with xylidine and $CuSO_4$. This resulted in an increased production of laccase on days 4 and 5. The activity dropped on days 6 and 7, possibly due to protease activity or insolubility of the laccase. The highest laccase activity (2.3 U/ml) was observed and harvested on the 5^{th} day (Figure 3.19). The harvested crude enzyme was purified before further use.

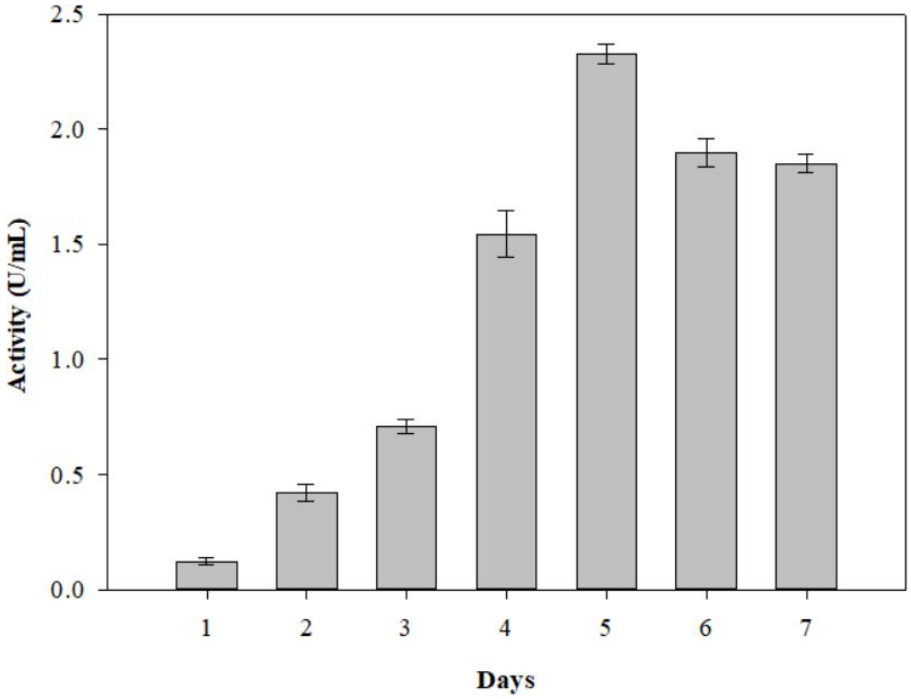


Figure 3.1: Effect of incubation time on the production of laccase by *Trametes pubescens* in Trametes defined medium at 28°C.

3.4.2 Purification of Tp-laccase

Partial purification using ammonium sulphate precipitation is a classical method in protein biochemistry. Ammonium sulphate is the most preferred salt for this method due to having a combination of a polyvalent anion (SO_4^{2-}) and a univalent cation (NH_4^+), and protein structure stabilisation (while concomitantly reducing its solubility) effect due to its kosmotrope status (Dennison 2013; Park *et al*. 2015). The water molecules are preferentially accreted to the sulphate ion, thus excluding the proteins from a proportion of water that is proportional to the salt concentration. This ultimately brings the proteins to their solubility limit (Dennison 2013). Before purification of Tp-laccase, medium precipitate and fungal cell mass were removed by centrifugation of the harvested medium. Subsequently, the crude laccase was partially purified by ammonium sulphate precipitation. The summary of Tp-laccase purification is shown in Table 3.1.

Table 3.1: Purification table for the purification of Tp-laccase CBS 696.5

Stage	Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Recovery (%)	Purification (fold)
Crude	3000	6990	4230	1.65	100	1
$SO_4(NH_4)_2$	450	1382	351	3.94	19.8	2.4
Ultrafiltration	24	1236	126	9.80	17.7	5.9
IEC (DEAE)	50	149	6	24.8	2.1	15.0
SEC (Seph. G-75)	6	7	0.18	41.3	0.11	25.0

After the sequential increase of ammonium sulphate saturation from 50% (w/v) to 80% (w/v), the enzyme activity of the pellet was found to be the highest at 80% saturation. Enzyme activities were similar between the pellets obtained at 80% (w/v) and 90% (w/v) ammonium sulphate saturation. However, the pellet obtained at 80% (w/v) ammonium sulphate saturation had the highest specific activity, thus showing that this pellet had the most significant amount of the desired enzyme per unit mass of total protein (Appendix 5). The partially purified enzyme was further purified and concentrated by subjecting it to ultrafiltration through a 50 kDa molecular weight cut-off (MWCO) ultrafiltration unit. When the concentrated protein sample was subjected to ion-exchange chromatography, two isoforms of the enzyme were eluted (Figure 3.2). This was not surprising because it is a common occurrence for fungal species such as *T. pubescens* to produce more than one isoform of laccase with distinguishable catalytic properties (Adelakun 2012; Bertrand *et al.* 2015; Yuan *et al.* 2016). The first isoform (TpL1) did not bind to the column matrix (DEAE) i.e., it eluted from the column before the ionic strength of the mobile phase was increased. This alludes to the mobile phase pH being below the isoelectric point of TpL1 (i.e. the isoform had a net positive charge). The second isoform (TpL2) eluted at NaCl concentrations ranging between ~ 0.2 M and 0.4 M. It is therefore apparent that at this mobile phase pH, TpL2 had an overall negative charge (Dennison 2013).

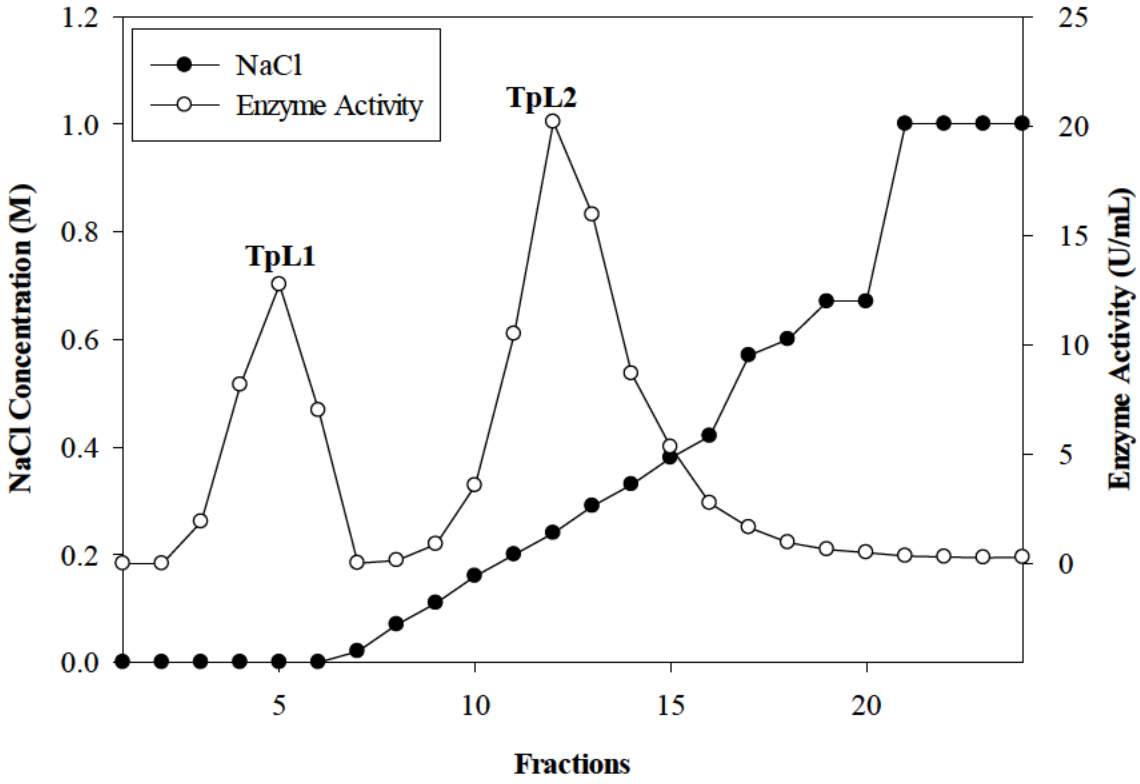


Figure 3.2: Ion exchange (DEAE) chromatogram showing the elution profile of TpL1 and TpL2 isoforms during a salt gradient of 0 - 1 M NaCl.

All the fractions obtained were analysed for protein content and laccase activity, and the specific activity was calculated. Both the Lowry and Bradford assays were used for the determination of the protein content and had standard curves with good correlation (R^2 = 0.9919 and R^2 = 0.9978, respectively, Appendix 3). However, the Lowry assay was preferred for further experimentation as it presented less variation than the Bradford assay. The fractions from ion-exchange chromatography showing activity were analysed by SDS-PAGE, and those with high laccase activity and similar band profiles were pooled together. These were further purified by size exclusion chromatography. The purified enzyme gave a single dominant protein band when analysed by SDS-PAGE with an apparent molecular weight of ~58 kDa (Figure 3.3 A). This is closer to the lower limit of the molecular weight range of 60 - 100 kDa that is reported for fungal laccases (Baldrian 2006; Hildén, Hakala and Lundell 2009; Rodríguez-Couto 2019). Zymograms of TpL1 and TpL2 revealed that *T. pubescens* produces a mixture of a monomer and a dimer (Figure 3.3 B), which was in line with what was reported by Adelakun (2012). The monomer band possessed much more activity than the dimer band.

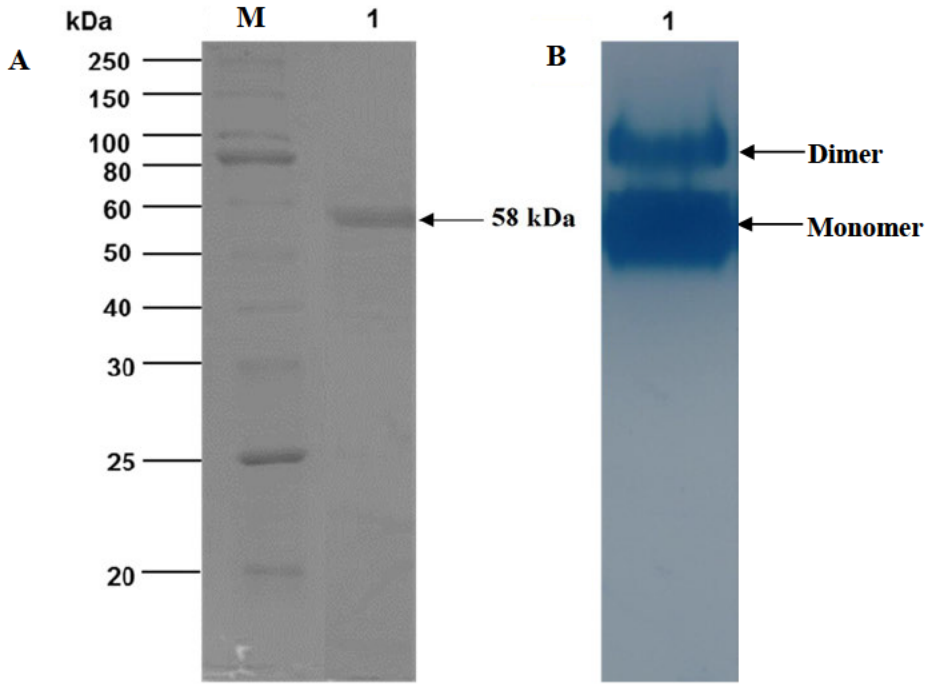


Figure 3.3: (A) SDS-PAGE gel of purified laccase stained with coomassie brilliant blue R250. Lane M: protein molecular weight marker, lane 1: purified laccase fraction after gel filtration chromatography. (B) Native-PAGE analysis of laccase, bands were developed with 5 mM ABTS.

3.4.3 Biochemical characterization of Tp-laccase

The activity of laccase at different temperatures when oxidising either ABTS, syringaldazine or 2,6-DMP was evaluated. The enzyme showed the highest activities of 340.0 ± 3.63 U/mL, 16.16 ± 1.80 U/mL and 300.0 ± 8.39 U/mL, respectively at 60.0°C (Figure 3.4). Even though Si *et al*. (2013) reported an optimum of 50°C for a laccase from a similar *T. pubescens* strain, others have reported an optimum activity between 50°C - 60°C (Galhaup *et al.* 2002; Si *et al*. 2013). However, Gaitan *et al.* (2011) and Hilden *et al*. (2009) reported an optimum activity at exactly 60°C with a sudden drop at temperatures higher than 60°C (Hildén *et al*. 2009; Gaitan *et al.* 2011). This was also observed in this study (Figure 3.4).

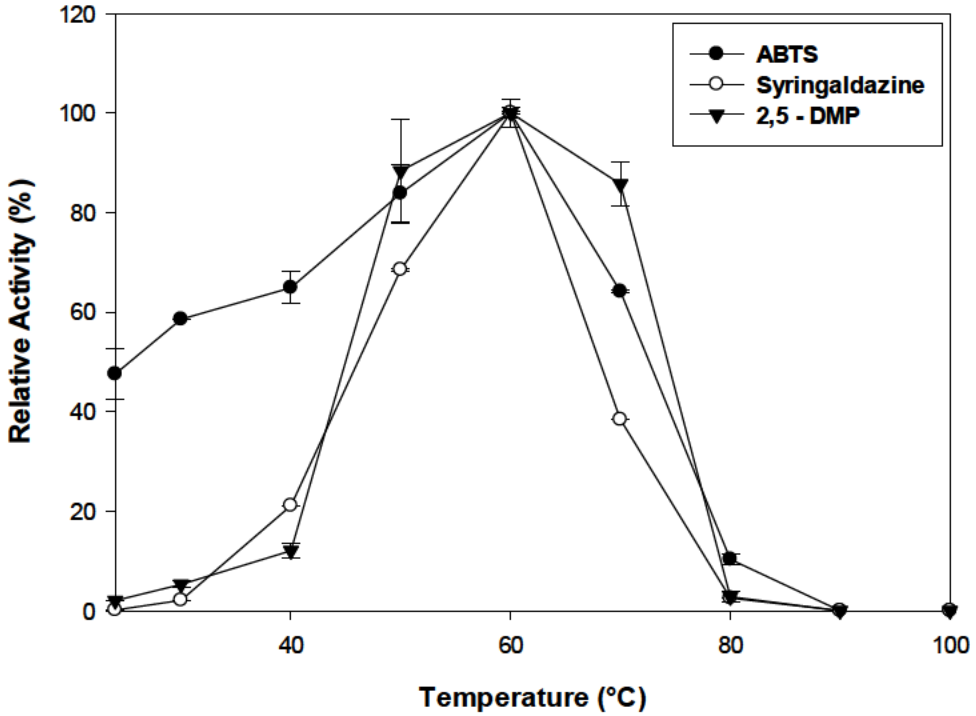


Figure 3.4: Effect of temperature on the oxidation of common laccase substrates by Tp-laccase. All values are means of three replicates ± standard deviation obtained by monitoring product formation by UV-Vis spectrometry (ABTS at 420 nm; syringaldazine at 530 nm and 2,6-DMP at 470 nm).

The thermostability of laccase, when incubated in temperatures ranging from 25°C - 70°C for 3 hours, was investigated. The enzyme showed appreciable stability between 25°C and 60°C (Figure 3.5). This is consistent with previous findings by authors who reported thermostability between 4°C and 70°C (Galhaup *et al.* 2002; Gaitan *et al.* 2011; Si *et al.* 2013). Similarly, other researchers observed a sudden drop in stability at temperatures above 60°C (Baldrian 2006; Yan *et al.* 2015).

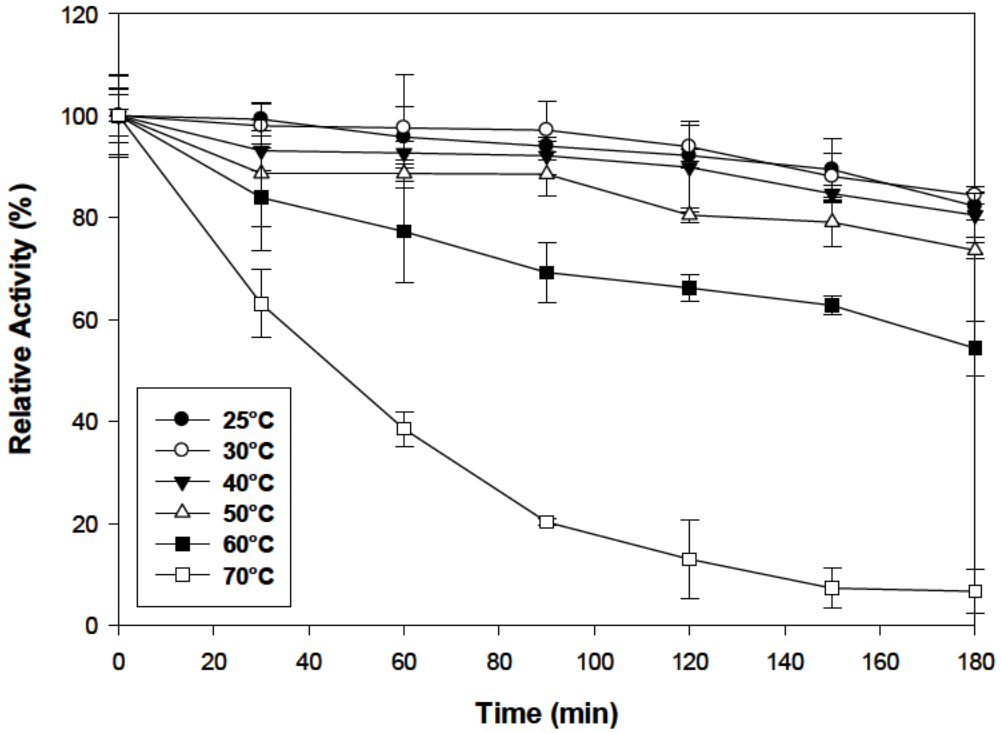


Figure 3.5: Temperature stability of Tp-laccase during the oxidation of ABTS. All values are means of three replicates ± standard deviation obtained by monitoring product formation at 420 nm. The activity at 100% was 6.62 ± 0.36 U/ml.

Laccase was active over a broad pH range of 3.0 - 8.0 for ABTS and 2,6-DMP, and 3.0 - 8.5 for syringaldazine with an optimum at pH 3.5 for ABTS and 2,6-DMP and pH 7.0 for syringaldazine (Figure 3.6). This is consistent with previous reports which showed that the pH optimum of laccase is substrate-dependent (Galhaup *et al.* 2002; Kudanga *et al.* 2009). At the optimum pH values, the laccase activities were 143.44 ± 1.41 U/ml, 26.54 ± 2.67 U/ml and 80.61 ± 3.35 U/ml for ABTS, 2,6-DMP and syringaldazine, respectively. The apparent differences in pH optima for different substrates appear to be dependent upon the chemical structures of the substrate, e.g., when a substrate is a phenol (i.e. an organic hydrogen donor), the optimal pH ranges from 3.5 - 6.0, but if the substrate is an electron donor like ABTS, the laccase activity gradually decreases from pH 2.5 - 7.0 (Morozova *et al.* 2007a; Sampaio *et al.* 2016).

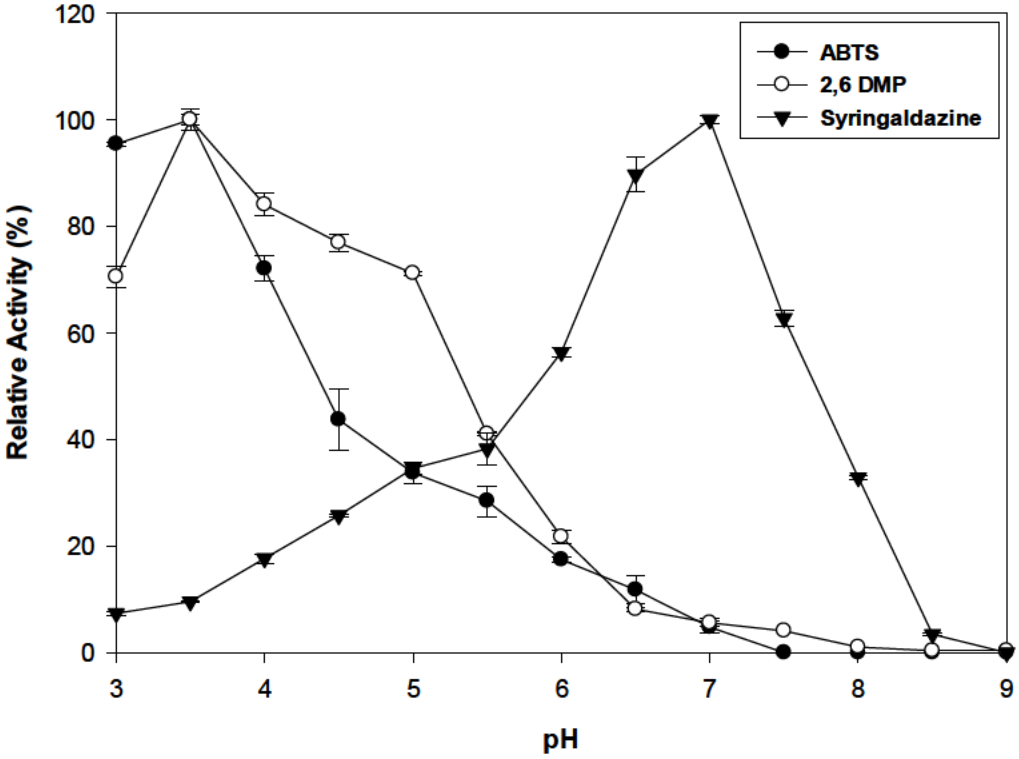


Figure 3.6: Effect of pH on the oxidation of common laccase substrates by Tp-laccase. All values are means of three replicates ± standard deviation and were obtained by monitoring product formation (ABTS at 420 nm; 2,6-DMP at 470 nm and syringaldazine at 530 nm).

The effect of pH on the oxidation of selected laccase substrates (i.e. guaiacol, caffeic acid, *p*-coumaric acid, ferulic acid and vanillic acid) revealed that the selected substrates could be easily oxidised within the pH range 4.0 - 6.0 (Figure 3.7). The optimum pH values were 4.0, 4.5, 5.0, 6.0 and 6.0 for caffeic acid, guaiacol, *p*-coumaric acid, ferulic acid and vanillic acid, respectively.

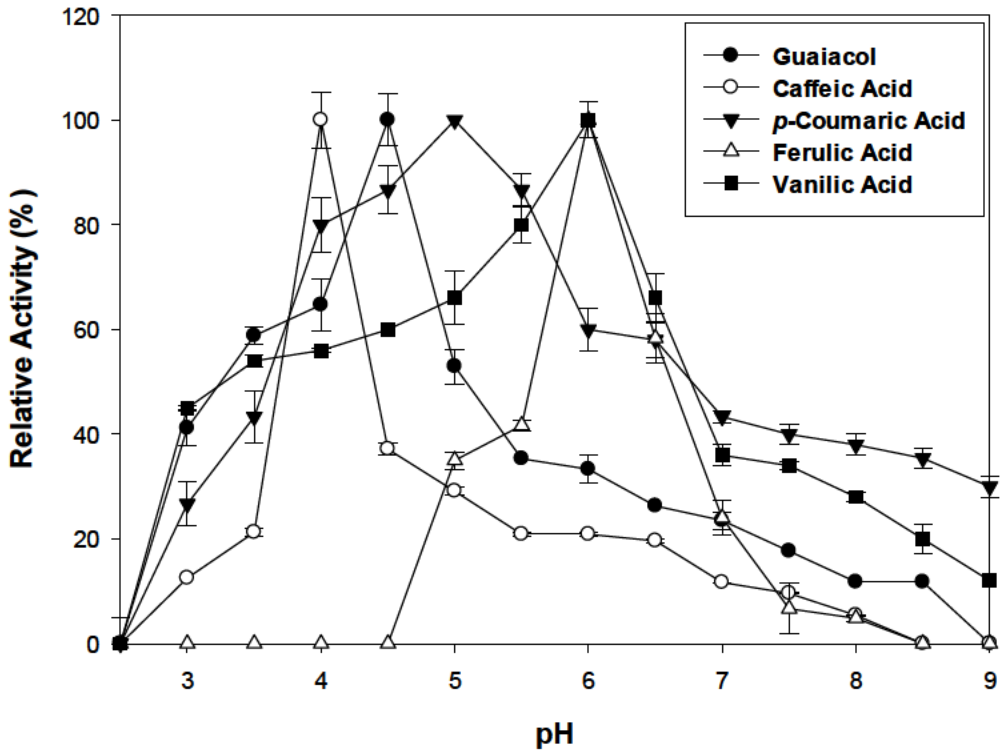


Figure 3.7: Effect of pH on the oxidation of selected phenolic substrates by Tp-laccase. All values are means of three replicates ± standard deviation obtained by monitoring product formation (guaiacol at 470 nm; caffeic acid at 370 nm; *p*-coumaric acid at 340 nm; ferulic acid at 362 nm and vanillic acid at 310 nm).

The stability of laccase at different pH values ranging from pH 3.0 - 9.0 is shown in Figure 3.8. The enzyme showed appreciable stability (at least 65% residual activity) within the investigated pH range and was most stable at pH 4.0 - 5.0, retaining over 90% activity after three hours. The enzyme has been reported to be highly stable over a broad pH range (4.5 - 10.0) while maintaining 75% of its original activity (Si *et al*. 2013). Galhaup *et al.* (2002) reported maximal stability at pH 5.0. Fungal laccases typically show their highest pH stability at acidic pH values; however, the enzyme still shows significantly high activity even at basic pH (Figure 3.8).

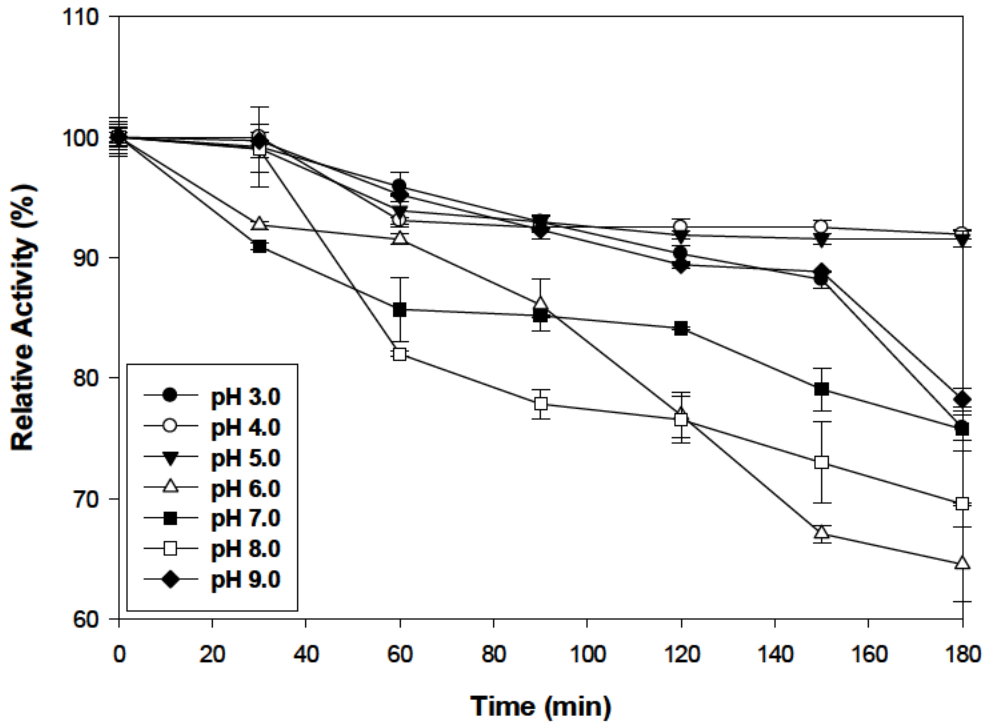


Figure 3.8: pH stability of Tp-laccase during the oxidation of ABTS. All values are means of three replicates ± standard deviation obtained by monitoring product formation at 420 nm. The activity at 100% was 5.89 ± 0.91 U/ml.

Apart from Cu^{2+} at 20 mM, all the heavy metal cations investigated reduced/inhibited enzyme activity with Al^{3+} showing a clear inverse relationship between cation concentration and enzyme activity (Figure 3.9). Previous studies have also reported the enhancement of laccase activity by Cu^{2+} at selected concentrations (Haibo *et al.* 2009; Zhu *et al.* 2011; Si *et al.* 2013). Mg^{2+} gave rise to a pattern that seems to show an increase in laccase activity with increasing cation concentration from 5 mM – 20 mM, however, the overall effect is inhibition of laccase and this is contrary to some previous reports (Guo *et al.* 2011; Zhu *et al.* 2011; Si *et al.* 2013). In the presence of Al^{3+}, laccase activity is inversely proportional to the cation concentration. This comes as no surprise since Al^{3+} has been reported to have an inhibitory effect on other fungal laccases (Si *et al.* 2013).

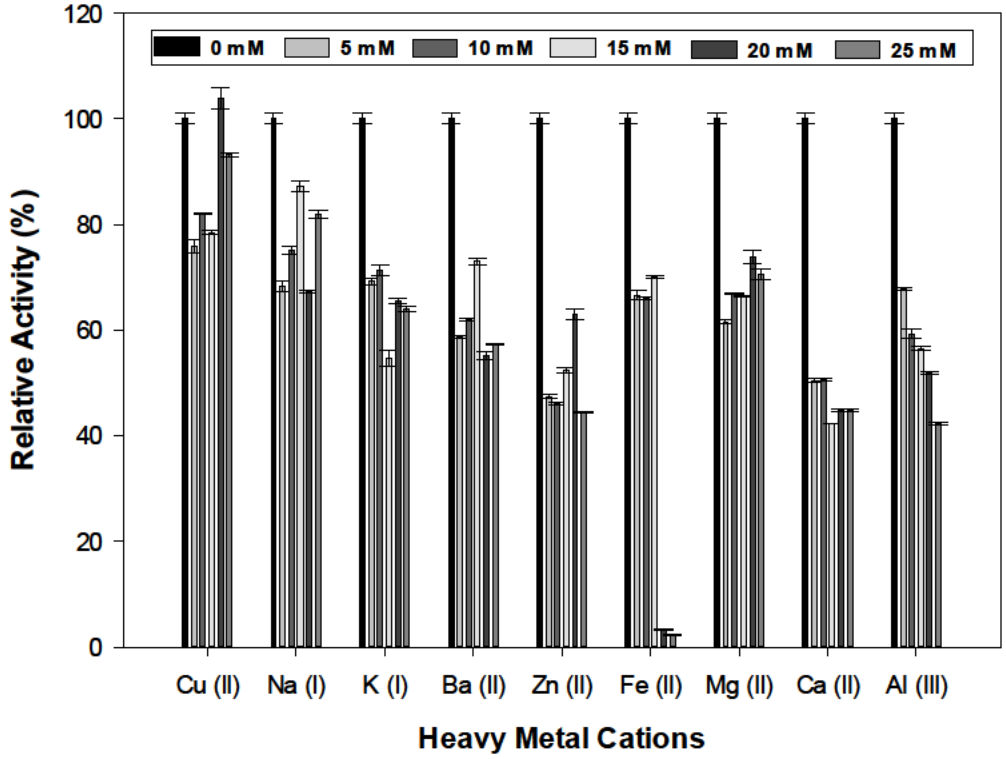


Figure 3.9: Effect of heavy metal cations on the oxidation of ABTS by Tp-laccase. All values are means of three replicates ± standard deviation. 100% activity = 161.6 ± 17.07 U/ml.

A summary of the changes in the catalytic parameters of Tp-laccase (i.e. ΔK_m and ΔV_{max}) imposed by five putative inhibitors, the apparent types of inhibition and the inhibition modes is depicted in Table 3.2. Upon incubating the enzyme with either sodium azide (NaN_3) or sodium dodecyl sulphate (SDS), the Michaeli-Menten constant (K_m), an indicator of substrate binding efficiency or binding affinity, remained unchanged while a significant decrease in the maximal rate of the reaction (V_{max}) was observed (Appendices 6 a and b). It is therefore apparent that the effect of NaN_3 and SDS on the enzyme is that of non-competitive inhibitors. This indicates that SDS and NaN_3 bind to an allosteric site of Tp-laccase which does not affect the substrate binding affinity (Palmer and Bonner 2011; Komoda and Matsunaga 2015; Delaune and Alsayouri 2019). Incubation of the enzyme with either L-cysteine, hydrogen peroxide or dithiothreitol resulted in the reduction of both the substrate-binding affinity and maximal rate (Appendices 6 c to e). This is characteristic of uncompetitive inhibition, wherein the inhibitor only binds to the enzyme-substrate complex (Delaune and Alsayouri 2019). The binding of the substrate to the active site could reveal an

inhibitor binding site by causing a conformational change to occur in the enzyme or the inhibitor could have a second interaction with a substrate-bound enzyme (Palmer and Bonner 2011). In an attempt to remove the inhibitor, the enzyme-inhibitor mixtures were dialysed and concentrated. The activity was recovered to ~89% for the enzyme previously incubated with NaN_3, thus indicating that it is a reversible inhibitor. This was expected because the azide ions are known to reversibly chelate Cu^{2+} ions of laccases (Couto and Herrera 2006; Gaur *et al.* 2018). On the contrary, laccases previously incubated with SDS, L-cysteine, H_2O_2 and DTT did not recover their activity. Although this observation might somewhat have been expected for SDS, L-cysteine and DTT, it was unexpected for H_2O_2 because it is known to reversibly form complexes with Type-2 Cu^{2+} ions (Skounas *et al.* 2010). This type of interaction is normally easily reversed by dialysis. Furthermore, it would not have been out-of-place to expect hydrogen peroxide to impose kinetic parameter changes similar to those of a competitive inhibitor due to it binding directly to the Type-2 Cu^{2+} ions of the active site (Brändén *et al.* 1971). The inhibitor strength followed the order NaN_3 > SDS > L-cysteine > H_2O_2 > DTT.

Table 3.2: The effect of selected inhibitors on laccase activity and their most plausible mode of inhibition

Inhibitor	[a]IC_{50} (mM)	Inhibition Type	ΔK_m	ΔV_{max}	Inhibition Mode
NaN_3	0.08	Reversible	0	[b]Neg	Non-competitive
SDS	1.10	Irreversible	0	[b]Neg	Non-competitive
L-cysteine	1.30	Irreversible	[b]Neg	[b]Neg	Uncompetitive
H_2O_2	2.00	Irreversible	[b]Neg	[b]Neg	Uncompetitive
DTT	2.70	Irreversible	[b]Neg	[b]Neg	Uncompetitive

[a]IC_{50} is defined as the concentration of the inhibitor that causes a 50% reduction in enzyme activity.

[b]Neg = a reduction in the magnitude of the kinetic parameter.

Due to the perception that water played a pivotal part in the activity, stability and conformational flexibility of the enzyme, enzyme catalysed reactions have been undertaken in aqueous media (Klibanov 1989). Owing to water activity in the reaction medium, the amount of enzyme-bound water molecules were observed to regulate enzyme activity (Halling 1994). The hydrophilicity of many organic substrates has prompted the employment

of non-conventional media such as organic solvents (Leon *et al.* 1998). This type of media, especially biphasic, can improve enzyme efficiency by maintaining activity in the aqueous phase while product recovery is seamlessly facilitated in the organic phase (Sellek and Chaudhuri 1999). The effect of selected water-miscible and water-immiscible organic solvents on the activity of Tp-laccase is shown in Figure 3.10, thus revealing the suitable solvent to apply in coupling reactions. The enzyme retained ~80% residual activity when incubated with 50% (v/v) of DMSO, ethyl acetate and dioxane for 6 hours (Figure 3.10). Approximately 58% of residual activity was retained after 6 hours with acetone as co-solvent, while ~80% activity was lost within 3 hours of incubation with ethanol and methanol, and ~10% residual activity remained after 6 hours. Generally, organic solvents are known to reduce enzyme activity, more so in monophasic systems fostered by solvents such as ethanol, dioxane, DMSO, acetone and methanol.

The rapid enzyme activity reduction imposed by acetone, ethanol and methanol is due to their ability to directly interact with the enzyme. This leads to an exchange of the solvent molecules with those of the water in the active centre, resulting in the distortion and irreversible inactivation of the enzyme (Dordick 1989; Zheng and Ornstein 1996; Bell *et al*. 1997). On the other hand, the enzyme was highly tolerant of dioxane and DMSO which also foster monophasic systems. Doukyu and Ogino (2010) reported rapid deactivation of laccase by DMSO, which is contrary to what was observed here. This could be due, in part, to the enzyme source and purification processes (Rogalski *et al.* 1995; Rogalski *et al.* 1999; Rodakiewicz-Nowak *et al.* 2000). The presence of ethyl acetate in the solution is an example of a biphasic solvent system. This arrangement, albeit with its challenges, is preferred over monophasic solvent systems because it facilitates efficient product separation and enzyme regeneration due to the infinitesimal interaction with the organic system, thus offering significantly lower rates of enzyme inhibition (Doukyu and Ogino 2010; Adelakun 2012).

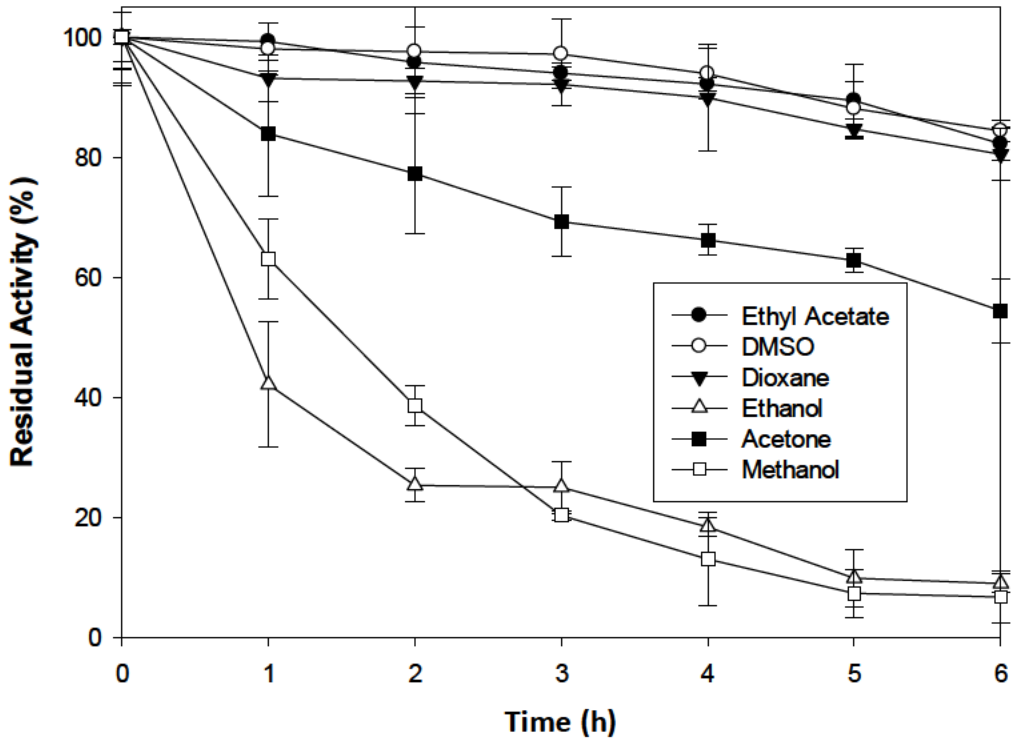


Figure 3.10: The effect of selected organic solvents on the activity of Tp-laccase. The mixtures contained 50% (v/v) of each organic solvent. All values are means of three replicates ± standard deviation. The activity at 100% was 6.62 ± 0.83 U/ml.

3.4.4 Enzyme kinetics and thermodynamic characterization of Tp-laccase

The K_m and k_{cat} values of the Tp-laccase were compared to other reported fungal laccases (Table 3.3). The K_m values for ABTS were higher than most of the fungal laccases being compared (Table 3.3). This indicates that Tp-laccase has a slightly lower binding affinity towards ABTS when compared with the other fungal laccases (Bisswanger 2014). The enzyme had a higher binding affinity (i.e. lower K_m values) for the other tested substrates (guaiacol, syringaldazine and 2,6-DMP) in comparison to most of the other fungal laccases (Shleev *et al.* 2007; Shi *et al.* 2014; Sung-Jong and Su-Jin 2017). The catalytic efficiency of Tp-laccase, an indicator of potency in applications, was found to be better for ABTS, 2,6-DMP and guaiacol but lower for syringaldazine when compared to some fungal laccases (Shleev *et al.* 2007; Shrestha *et al.* 2016; Sung-Jong and Su-Jin 2017)

Table 3.3: Kinetic parameters for Tp-laccase-catalysed oxidation of ABTS, 2,6-DMP, SGZ and guaiacol

Source of laccase	ABTS			2,6-DMP			SGZ			Guaiacol			References
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} M^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} M^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} M^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} M^{-1}$)	
T. pubescens	198	103	5.2×10^5	168	32	1.9×10^5	211	12	5.8×10^4	102	13	1.2×10^5	This study
T. pubescens	50	150	3.0×10^6	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	220	136	6.2×10^5	Shleev *et al.* (2007)
Trematosphaeria mangrovei	1400	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	Atalla *et al.* (2013)
Echinodontiaceae taxodii	41	*ND*	*ND*	133	*ND*	*ND*	*ND*	*ND*	*ND*	779	*ND*	*ND*	Shi *et al.* (2014)
Ganoderma lucidum	110	4.1	3.7×10^4	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	Shrestha *et al.* (2016)
Mycetinis scorodonius	27	72	2.7×10^6	602	68	1.1×10^5	*ND*	*ND*	*ND*	2772	223	8.2×10^4	Sung-Jong and Su-Jin (2017)

ND = Not determined.

The ability of Tp-laccase to withstand thermal inactivation is the key determinant of its thermostability (Figure 3.11). Enzymes with higher deactivation energy (E_d) values are more resistant to thermal inactivation. The thermal inactivation of the enzyme begins with its unfolding and subsequently becomes inactivated (Yadav *et al.* 2018). In many cases, upon reduction of input thermal energy to below inactivation energy (cooling), the enzyme reverts to its native conformation. However, keeping the enzyme at high temperatures for a prolonged time can permanently inactivate it (Das *et al.* 2012; Kumar and Satyanarayana 2013). In the current study, Tp-laccase was found to have a significantly high E_d value of 109.36 kJ mol^{-1}, albeit lower than those of typical bacterial laccases (Yadav *et al.* 2018). Higher E_d values mean delayed conformational changes when exposed to high temperatures. Furthermore, thermodynamic parameters such as ΔG, ΔH, and ΔS were also calculated for Tp-laccase (Table 3.4). The reaction feasibility is mainly determined by measuring the magnitude of change in Gibbs free energy (ΔG), particularly the conversion of the enzyme-substrate complex to the product. A lower ΔG is preferred as it favours the viability of a reaction. The enzyme was found to exhibit a lower ΔG when compared to the fungal laccase from *Daedalea flavida* (Singha and Panda 2015). The determination of ΔG for Tp-laccase was done at a higher temperature than that of *D. flavida* laccase. This confirms the robustness of Tp-laccase over the *D. flavida* laccase (Singha and Panda 2015). The positive ΔG values for the Tp-laccase indicates the non-spontaneity of its thermal denaturation. The enthalpy (ΔH), an indicator of the energy required for thermal denaturation of the enzyme, was found to be higher than those of some documented fungal (Singha and Panda 2015; Nelson and Anne 2021) and bacterial (Chauhan, Goradia and Jha 2018) laccases. This indicates that higher thermal energy is required to break down the enzyme's stabilising bonds. The destabilisation of enzyme bonds causes an increase in the disorder of the enzyme structure, which is measured by the change in entropy (ΔS). Enzyme stability generally has an inverse relationship with the change in entropy (Singha and Panda 2015; Yadav *et al.* 2018). To the best of our knowledge, this is the first report on the evaluation of thermodynamic parameters of a laccase from *T. pubescens*.

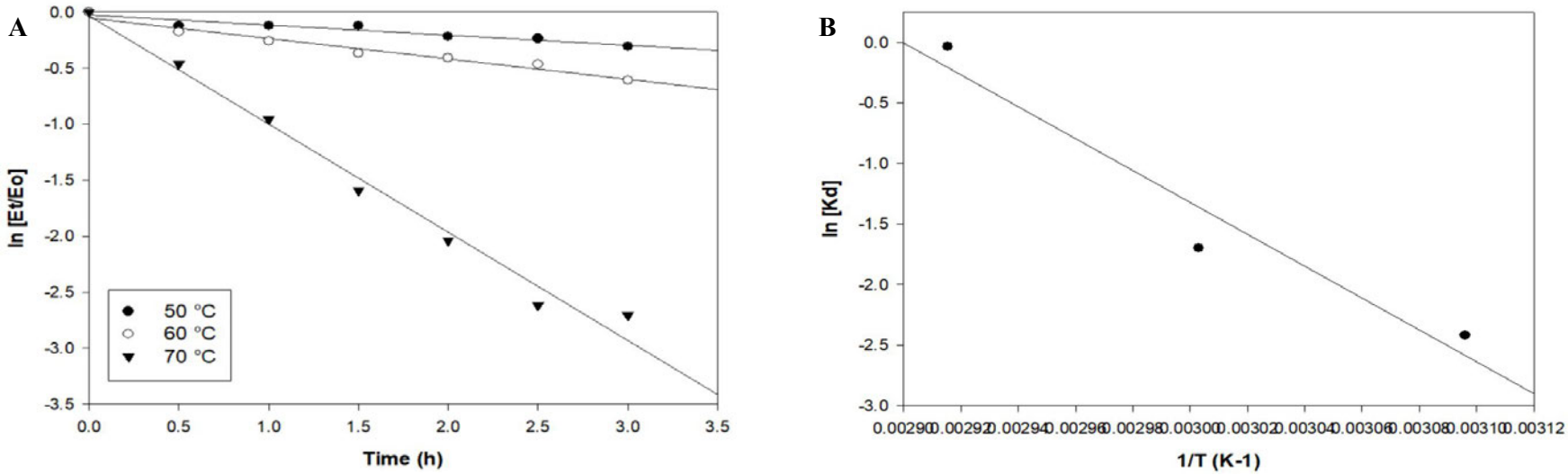


Figure 3.11: (A) Plot of ln[E_t/E_0] versus time (h) for the calculation of the deactivation constant (K_d) and $T_{1/2}$ of Tp-laccase at different temperatures [50°C, 60°C, and 70°C]. (B) Arrhenius plot of Tp-laccase for the calculation of deactivation energy (E_d).

Table 3.4: Thermodynamic parameters of Tp-laccase measured during thermal deactivation at various temperatures

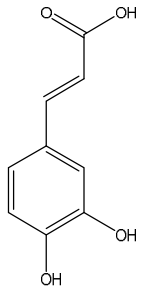
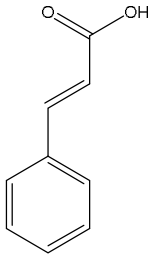
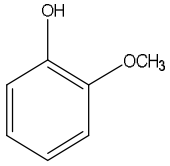
Temperature (K)	K_d (h^{-1})	$T_{1/2}$ (h)	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J $mol^{-1}K^{-1}$)
323.15	0.0889	7.8	106.68	85.82	64.57
333.15	0.183	3.8	106.59	86.56	60.15
343.15	0.965	0.72	106.51	84.51	64.15

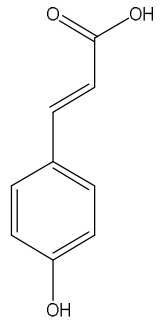
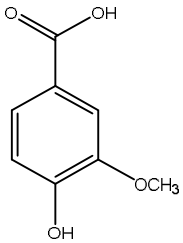
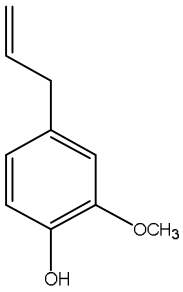
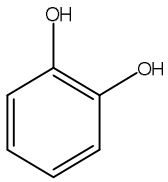
3.4.5. Preliminary coupling reactions

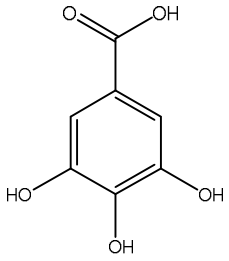
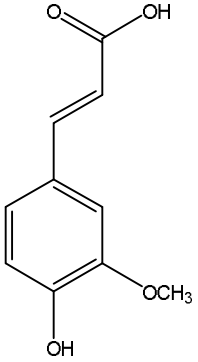
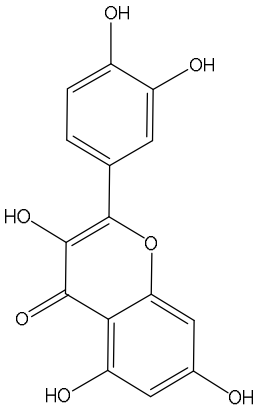
3.4.5.1. Oxidation reactions for substrate screening

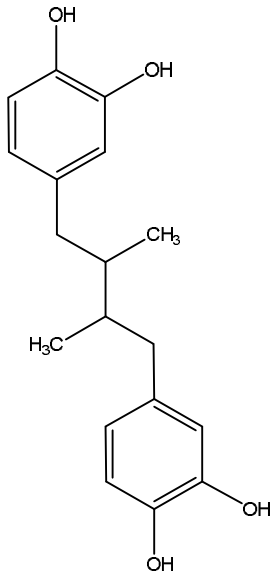
The upsurge in industrial interest in bioactive compounds has prompted the need to develop economically efficient and environmentally benign enzymatic processes to produce bioactive compounds. Due to their wide substrate range, laccases are good candidates for organic synthesis. In this regard, the ability of Tp-laccase to oxidise selected phenolic compounds was investigated (Table 3.5).

Table 3.5: Phenolic compounds screened for oxidation by *Tp*-laccase

Substrate	Reaction Outcome	Product's LC-MS signal (*m/z*)	Remarks
 Caffeic acid MW: 180.16 g/mol	(+)	357 dimer	Caffeic acid was oxidised owing to the hydroxide groups on the meta- and para- positions of its benzene ring.
 Cinnamic acid MW: 148.16 g/mol	(-)	293 dimer	Cinnamic acid probably was not oxidised owing to the absence of phenolic groups.
 Guaiacol MW: 124.14 g/mol	(+)	245 dimer 367 trimer	Guaiacol is easily oxidised by laccase due to its ortho- methoxy group (Kudanga *et al*. 2017).

 *p*-Coumaric acid MW: 164.05 g/mol	(+)	325 dimer	The unsaturated carbon-carbon bond between the phenyl ring and the carboxylic group is largely responsible for the laccase-catalysed oxidation of *p*-coumaric acid.
 Vanillic acid MW: 168.14 g/mol	(+)	333 dimer	The electron-donating methoxy group counteracts the effect of the electron-withdrawing carboxylic group, thus making it easy for laccase to oxidise vanillic acid.
 Eugenol MW: 164.20 g/mol	(+)	325 dimer	The electron-donating methoxy group makes it a good laccase substrate.
 Catechol MW: 110.10 g/mol	(+)	218 dimer	The hydroxyl groups in the C-1 and C-2 positions of catechol are good electron donor. They, therefore, enable the oxidation of the molecule by laccase

 Gallic acid MW: 170.12 g/mol	(+)	338 dimer	The para- hydroxyl group of gallic acid is highly susceptible electron donor which makes it easy to oxidise.
 Ferulic acid MW: 194.18 g/mol	(+)	385 dimer	The electron-donating methoxy group makes it easy to oxide the hydroxyl group.
 Quercetin MW: 302.24 g/mol	(+)	601 dimer	There are several electron-donating groups on the quercetin molecule making it easy to oxidise the molecule.

 Nordihydroguaiaretic acid MW: 302.37 g/mol	(+)	601 dimer	There are several electron-donating groups on the nordihydroguaiaretic acid molecule hence the molecule can be easily oxidised.

(+) = Substrate oxidised by Tp-laccase; (-) = Substrate not oxidised by Tp-laccase.

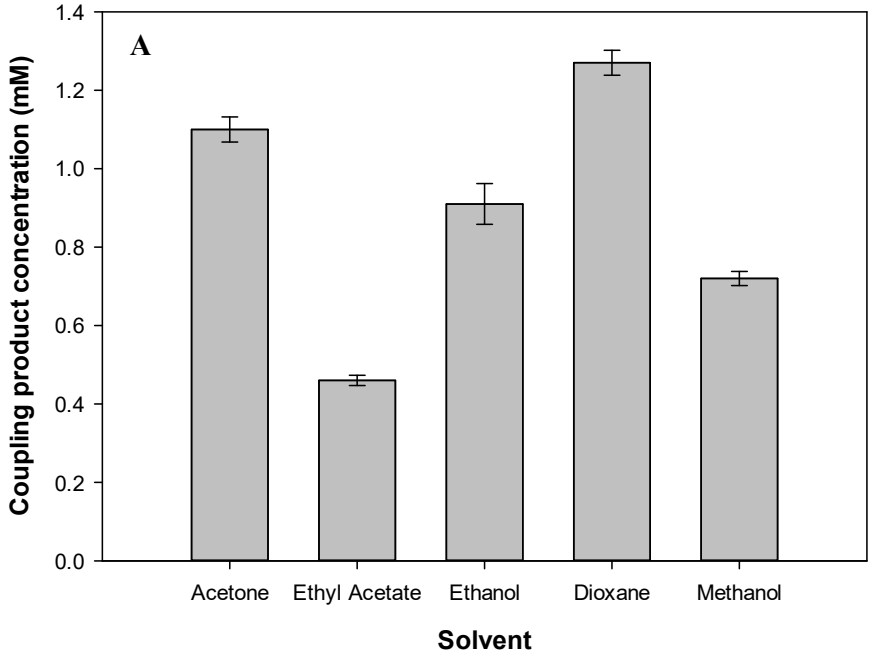
Substrates that showed distinct production formation (as evidenced by TLC analysis) were used as candidates for hetero-coupling reactions, specifically quercetin, nordihydroguaiaretic acid, catechol and gallic acid.

3.4.6. Coupling catechol onto quercetin

Reactions in which two organic molecules are linked together with the aid of a catalyst are termed coupling reactions. These reactions are termed homo-coupling when two identical molecules are linked together, or hetero-coupling, when two different molecules are joined together. Due to an increasing interest in environmentally benign methods of organic synthesis, enzymes such as laccases have been employed as catalysts in coupling reactions. An example was the laccase-catalysed enrichment of naringenin with hydroxylated and/or methoxylated phenolic molecules (Prasetyo *et al*. 2011). In the current study, the coupling of catechol with quercetin is reported. Quercetin has been reported to health benefits including antioxidant, anti-inflammatory, anti-cancer and antiviral properties (Li *et al*. 2016). Unlike catechol and its water-soluble derivatives quercetin is insoluble in water but quite soluble in some organic solvents (Li *et al.* 2016; Zheng *et al.* 2017; Zhang *et al.* 2018). The application of organic solvents is not out of place in synthetic chemistry. They promote synthetic reactions by providing suitable kinetic and thermodynamic conditions (Koskinen and Klibanov 1996). For instance, some hydrogen

bond-forming solvents (with optimal concentration of water) have been shown to activate the enzymes such as oxidoreductases by improving their conformational flexibility, thus allowing for better interaction of the substrate with the active site and positively impacting on the reaction kinetics (Klibanov 1989). Furthermore, the reduction of the thermodynamic activity of water has been shown to shift the thermodynamic equilibria towards the production of compounds that would be virtually unattainable under normal (aqueous) reaction conditions (Vic *et al*. 1997; Deschrevel *et al.* 2003). Therefore, the coupling reactions were undertaken in organic solvents to solubilise both reactants.

Monophasic and biphasic systems were investigated using acetone, ethyl acetate, ethanol, dioxane or methanol as cosolvent. The highest product ($P_{QC}1$) concentration was observed in the presence of dioxane as co-solvent (Figure 3.12 A) at 50% v/v solvent concentration (Figure 3.12 B) and reaction time of 6 hours (Figure 3.12 C). The reactions that were carried out in water-miscible solvents (acetone, ethanol, dioxane, and methanol) produced higher concentrations of the desired product when compared to those carried out in water-immiscible solvents (ethyl acetate). Monophasic solvent systems such as the ones used in this study have been reported to exhibit substrate conversion rates comparable to those in buffers, albeit for a short time and low substrate concentrations (Milstein *et al.* 1989; Lu *et al.* 2012; Liu *et al.* 2019). These types of solvent systems offer high regulation capabilities by minimising the accumulation of excess substrate and product around the enzyme (Adelakun 2012).



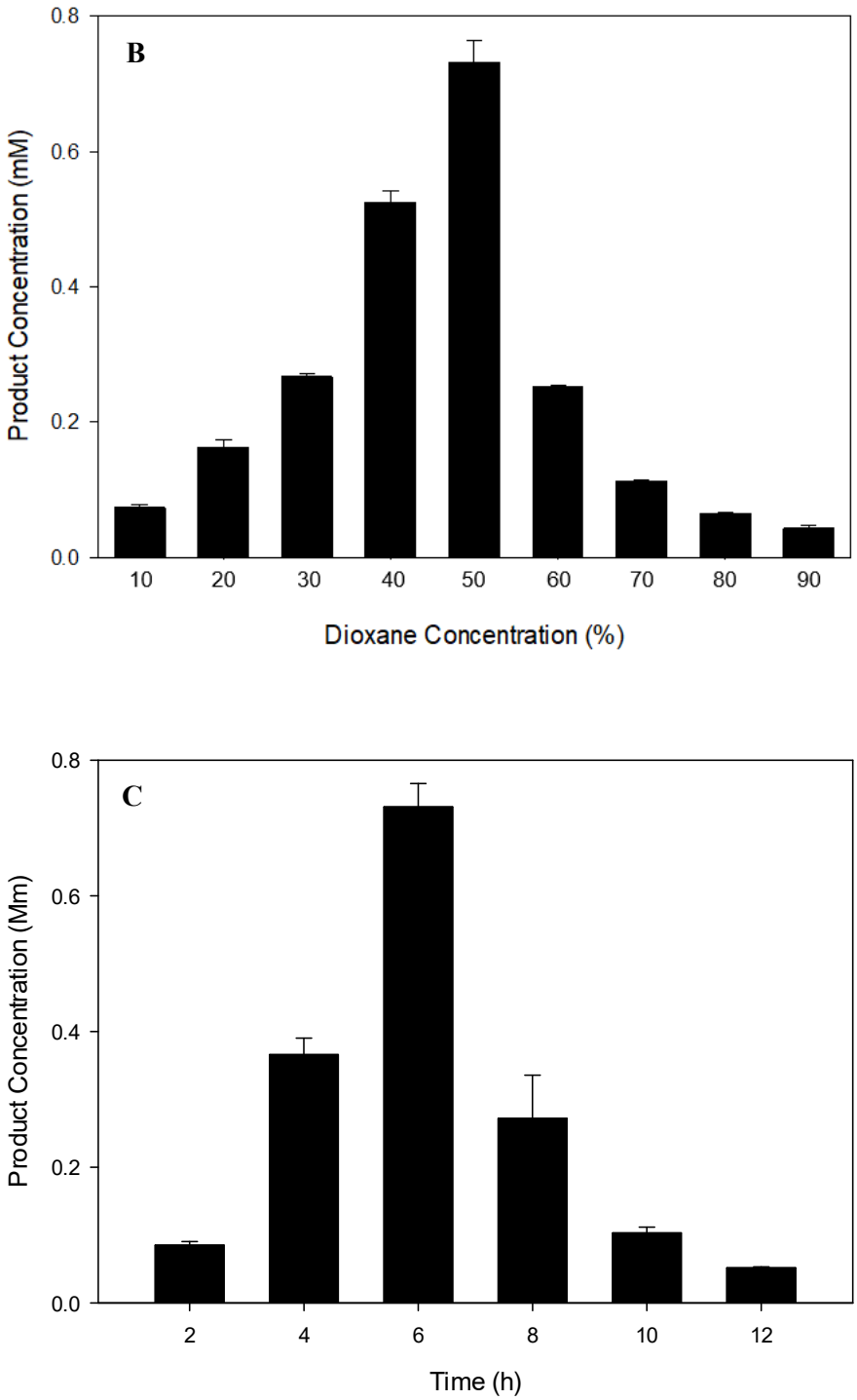


Figure 3.12: The effect of (A) organic co-solvents type, (B) concentration, and (C) reaction duration on the formation of the quercetin-catechol ($P_{QC}1$) coupling product. All values are means of three replicates ± standard deviation.

The Tp-laccase-catalysed coupling reaction resulted in the formation of two products with R_f – values of 0.17 ($P_{QC}1$) and 0.35 ($P_{QC}2$) (Figure 3.13). The lower R_f value of $P_{QC}1$ shows that it is more polar compared to $P_{QC}2$. HPLC chromatograms support this observation (Figure 3.15 A and C). Due to the nature of the reverse phased column, molecules exhibiting higher polarity are eluted before those with lower polarity (Snyder *et al*. 2010).

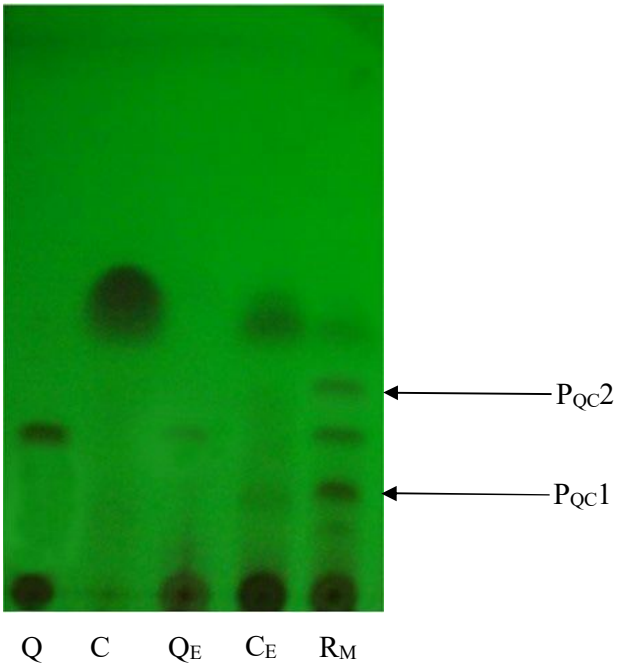


Figure 3.13: Thin layer chromatogram of products of Tp-laccase catalysed coupling of catechol onto quercetin. Lane Q: quercetin only; Lane C: catechol only; Lane Q_E: quercetin + laccase; Lane C_E: catechol + laccase; and Lane R_M: quercetin + catechol + laccase.

3.4.6.1 Purification and characterisation of quercetin-catechol coupling products

The product bands were recovered from the TLC plate and dissolved in methanol. When secondary TLC analysis was conducted, single product bands were observed (Figure 3.14, L_1 and L_2).

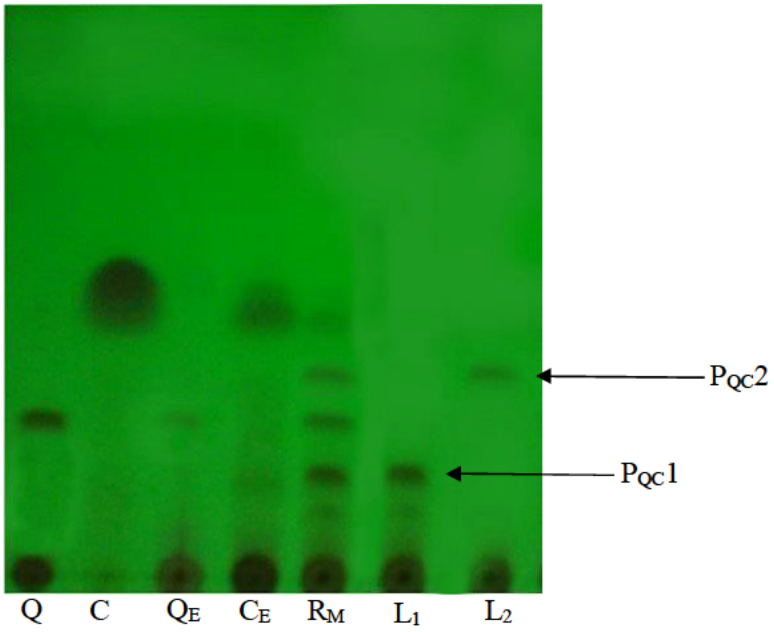
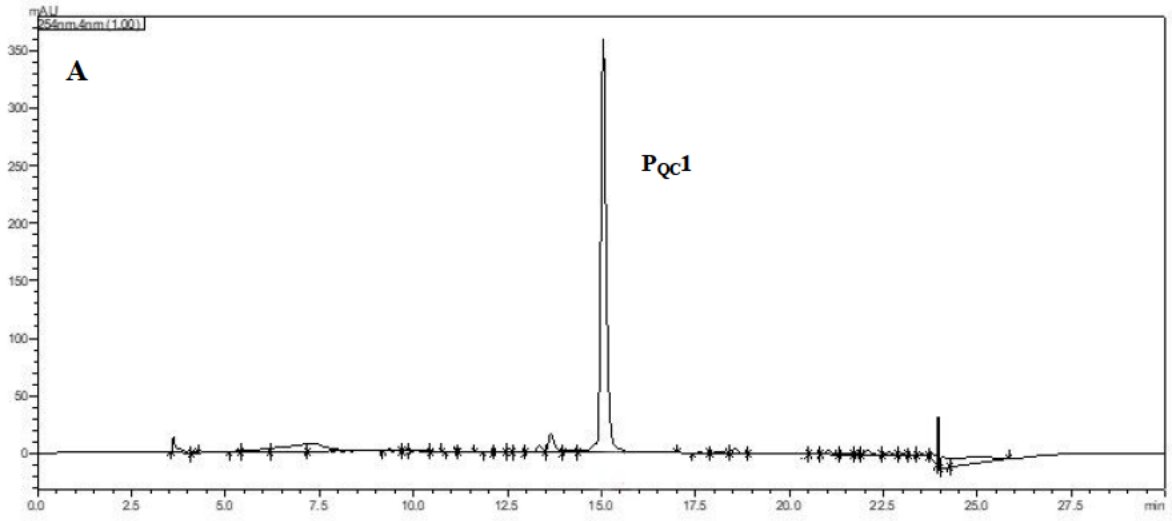
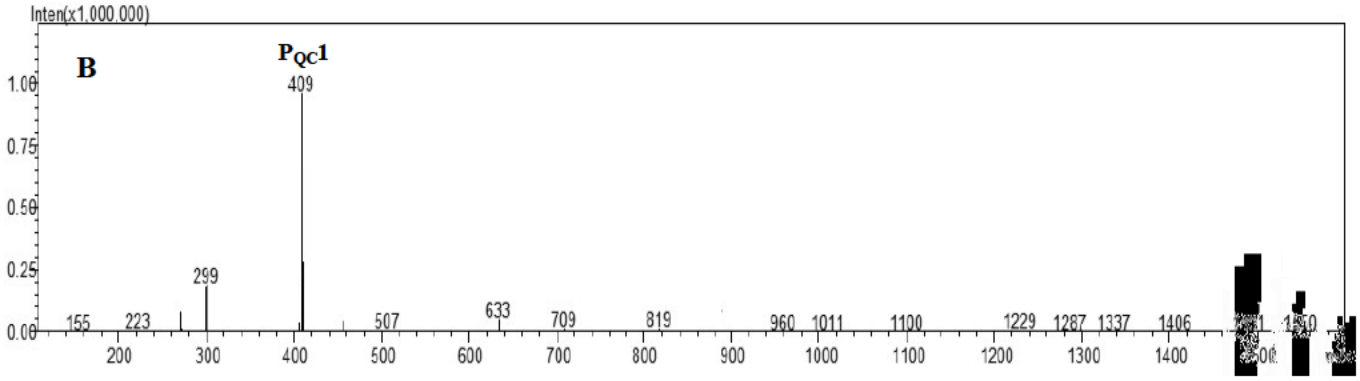
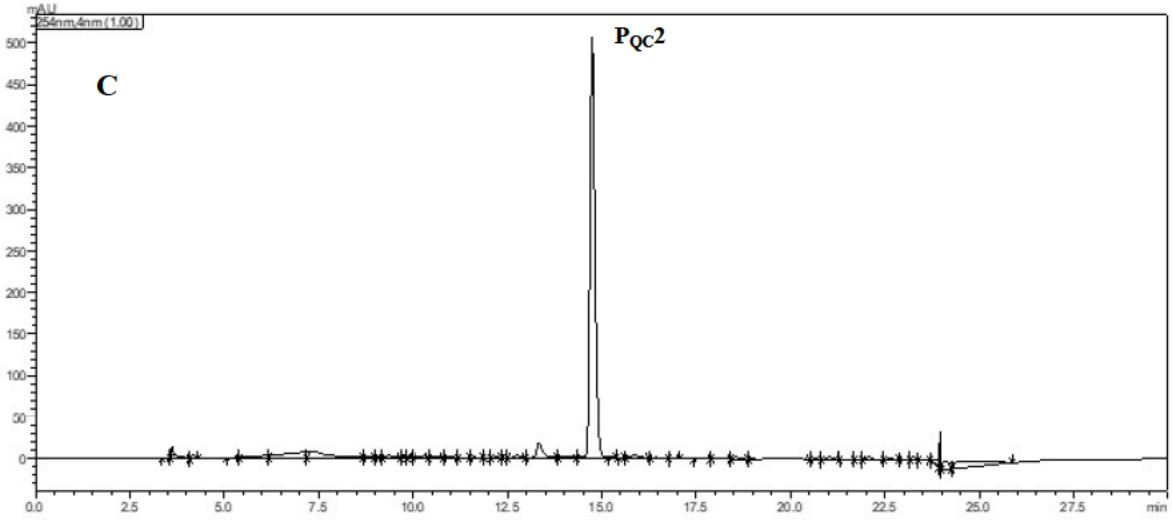


Figure 3.14: Thin layer chromatogram of laccase-catalysed hetero coupling of quercetin and catechol. The image compares the migration of substrates (Q and C), the contents of oxidation reactions (Q_E and C_E) and the coupling reaction (R_M), and the purified coupling products (L_1 and L_2).

P_{QC} 1 and 2 were recovered with yields of 12.9% and 7.84%. The purified products were further analysed and characterised by LC-MS. LC-MS analysis of the purified products revealed the retention time and mass spectrum signal of $P_{QC}1$ at 15.133 minutes and *m/z* 409 (Figure 3.15 A and B), and those of $P_{QC}2$ were 14.746 and *m/z* 709 (Figure 3.15 C and D). This suggests the coupling of one molecule of quercetin to one molecule of catechol (*m/z* 409) and two molecules of quercetin to one molecule of catechol (*m/z* 709), respectively. Structural changes of this nature usually lead to an improved antioxidant activity emanating from an increase in the number of hydroxyl and/or methoxy groups (Adelakun 2012).


mAU
254nm,4nm (1.00)
A
PQC1
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
27.5
min


Inten(x1,000,000)
B
PQC1
409
299
155
223
507
633
709
819
960
1011
1100
1229
1287
1337
1406


mAU
254nm,4nm (1.00)
C
PQC2
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
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22.5
25.0
27.5
min

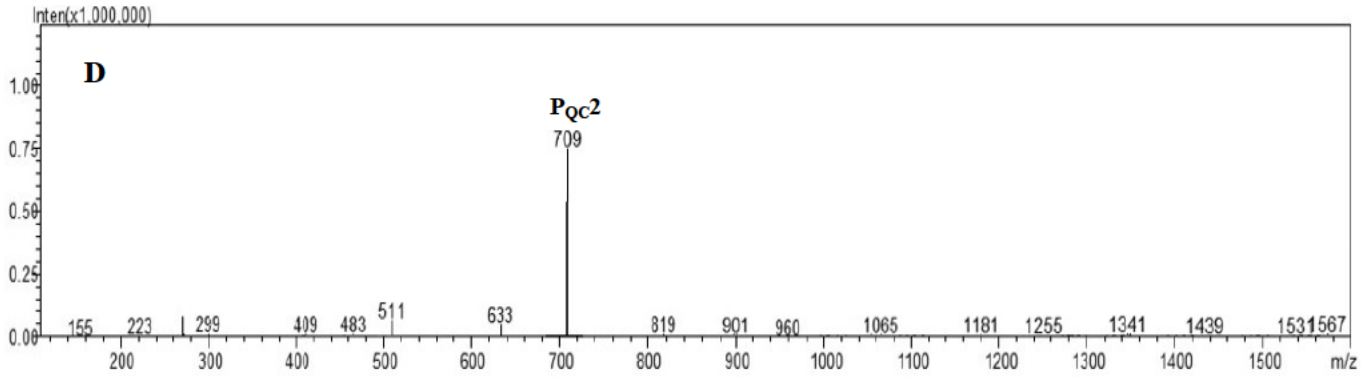


Figure 3.15: (A) HPLC chromatogram of product 1 ($P_{QC}1$), (B) MS spectrum of product 1 ($P_{QC}1$), (C) HPLC chromatogram of product 2 ($P_{QC}2$), and (D) MS spectrum of product 2 ($P_{QC}2$).

Based on the LC-MS results, the possible reaction pathway for the formation of the two products is proposed (Figure 3.17). The predicted product structures were suggested based on the ortho-directing hydroxyl groups on positions 3' and 4' of the quercetin molecule, the steric hindrance presented by position 6', and the existence of a free carbon on position 5' (Figure 3.16). Also, the density functional theory calculations by Marković *et al.* (2013) revealed that the 4' hydroxyl group is deprotonated first due to exhibiting the lowest bond dissociation energy and proton affinity. The reaction is initiated by laccase through the abstraction of an electron from quercetin, resulting in a radical which subsequently forms a resonance structure through the rearrangement of the π-electrons. The catechol is simultaneously oxidised by the enzyme, and the resulting radical nucleophilically attacks the quercetin radical on position 5' thereby forming an ether linkage of product 1 (Adelakun 2012a). The catechol moiety on product 1 is further oxidised by laccase and is nucleophilically attacked by the quercetin radical, resulting in the formation of the secondary ether linkage of product 2.

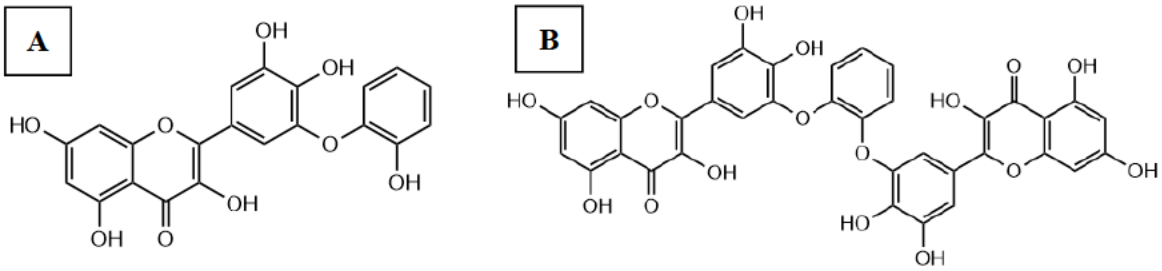


Figure 3.16: The proposed chemical structures of (A) $P_{QC}1$ and (B) $P_{QC}2$ formed during the Tp-laccase-catalysed coupling of catechol onto quercetin.

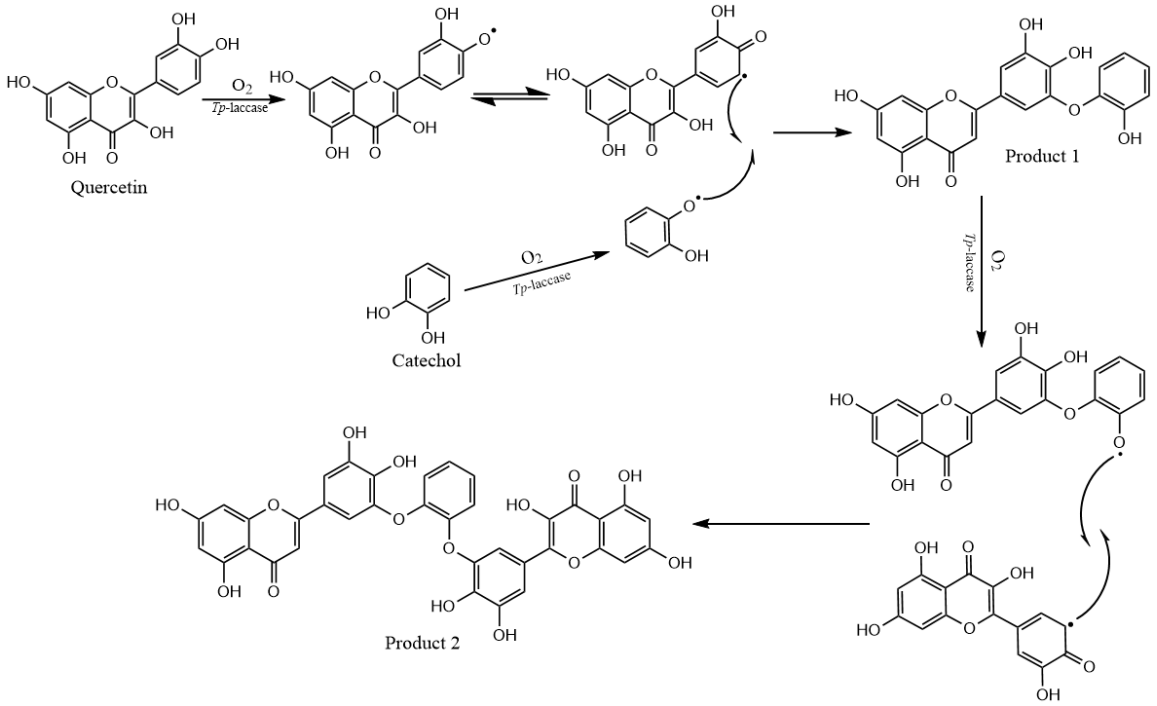


Figure 3.17: The proposed reaction pathway for the formation of the quercetin-catechol coupling product.

3.4.6.2. Antioxidant properties of quercetin-catechol coupling products

The antioxidant activities of $P_{QC}1$ and $P_{QC}2$ were compared to those of catechol and quercetin using ABTS, DPPH, and FRAP assays. The ABTS and DPPH assays evaluate the radical scavenging potential of the products to reduce the $ABTS^{\bullet+}$ and DPPH• to their stable forms (Nemadziva *et al.* 2018; Wang *et al.* 2018). The determination of the concentration of the test compound required to reduce the initial concentrations of $ABTS^{\bullet+}$ and DPPH• by 50% is an extensively employed approach to measuring antioxidant activity. The IC_{50} value is inversely proportional to the antioxidant activity of the test compound (Olszowy and Dawidowicz 2018). On the other hand, the FRAP assay tests the compounds ability to reduce the $[Fe(III)(TPTZ)_2]^{3+}$ complex to the $[Fe(II)(TPTZ)_2]^{2+}$ complex (Powell 2016). One FRAP unit is defined as the reduction of 1 mole of the $[Fe(III)(TPTZ)_2]^{3+}$ complex to the $[Fe(II)(TPTZ)_2]^{2+}$ complex (Huang *et al*. 2005).

The antioxidant activities of catechol, quercetin, and coupling products are shown in Table 3.6. Based on the principle that coupling the substrates increases the number of hydroxyl groups (Kudanga *et al*. 2017), it is a plausible expectation for the products to exhibit enhanced antioxidant activity. However, both products had significantly lower antioxidant activities when compared to quercetin. Quercetin has been proven to exercise its antioxidant activity through the B-ring and, to a lesser extent, the C-ring which is often hindered by the association of the would-be electron donor to a π-bonding carbon that is close to the electron-withdrawing carbonyl group (Bruice 2016; Zheng *et al*. 2017; Kumar *et al*. 2018). In a study by Zheng *et al.* (2017) the glycosylation of quercetin via the B-ring (particularly through an ether linkage, as observed here) presented quercetin glycosides with significantly lower antioxidant activities. It is for this reason that an ether linkage is proposed in Figure 3.17 rather than a C-C linkage which would leave more free OH groups and increase antioxidant activity. Nonetheless, both products exhibited better solubility in ethanol when compared with quercetin.

Table 3.6: The antioxidant activities of catechol, quercetin, $P_{QC}1$, and $P_{QC}2$ as determined by the DPPH, ABTS, and FRAP assays

Compound	Molecular Weight (g/mol)	ABTS C_{50} (μM)[a]	DPPH C_{50} (μM)[a]	FRAP (μM)[b]
Catechol	110.10	13.83 ± 0.345^{c}	20.32 ± 1.063^{c}	18.21 ± 0.328^{c}
Quercetin	302.24	3.79 ± 0.132^{d}	4.97 ± 0.348^{d}	68.31 ± 1.63^{d}
Product 1 ($P_{QC}1$)	410.33	6.82 ± 0.016^{e}	10.74 ± 0.186^{e}	28.67 ± 1.085^{e}
Product 2 ($P_{QC}2$)	710.58	32.16 ± 0.952^{f}	57.76 ± 0.218^{f}	6.89 ± 0.509^{f}

All values are means of three replicates ± standard deviation.

Values with different superscripts [c, d, e, f] in the same column are significantly different ($p < 0.05$) as shown by Duncan's multiple range test.

[a]Concentration (μM) of substrates or products required to reduce [$ABTS^{\bullet+}$] or [$DPPH^{\bullet}$] by 50%.

[b]The FRAP value of substrates and products (μM) is equivalent to the amount of $[Fe(III)(TPTZ)2]^{3+}$ converted to $[Fe(II)(TPTZ)2]^{2+}$ by 1 mM of the sample.

3.4.6.3. Antimicrobial properties of the quercetin-catechol coupling products

The antimicrobial activities of $P_{QC}1$ and $P_{QC}2$ on selected ATCC bacteria were compared to those of catechol and quercetin using a method outlined by Alabi *et al.* (2012). $P_{QC}2$ exhibited a significantly higher antimicrobial activity against the gram-positive *L. monocytogenes* and *S. aureus* when compared with both catechol and quercetin. High antimicrobial activity was further exhibited by $P_{QC}2$ against the gram-negative *E. cloacae*, albeit lower than that displayed by quercetin (Table 3.7). Phenolic compounds such as quercetin and catechol are well known for their antimicrobial activities, which are largely attributed to their intrinsic ability to cause structural and/or functional damage to bacterial cell membranes (Silva *et al.* 2018). Antimicrobial activity has been reported to be influenced by the number and position of the hydroxyl groups (Borges *et al.* 2013). The results, with regards to the antimicrobial activity of $P_{QC}2$, add to the notion that increasing the degree of hydroxylation results in enhanced antimicrobial activity (Borges *et al*. 2013) (Table 3.7). Through their hydroxyl groups and/ phenolic rings, they are capable of forming destructive complexes with bacterial proteins and membranes (Nitiema *et al.* 2012).

Numerous studies have confirmed that the hydroxylation of flavonols such as quercetin in positions C-5 and C-7 imbues them with antimicrobial activity against Gram-positive strains such as *S. aureus* and *L. monocytogenes* (Woźnicka *et al.* 2013). This explains the superior antimicrobial activity of $P_{QC}2$ over quercetin against *S. aureus* and *L. monocytogenes* as it possesses twice the number of C-5 and C-7 hydroxyl groups. Hydroxylation of the B and C rings may also have a positive impact on antimicrobial activity. However, some flavonols have been shown to exhibit lower activity when hydroxylated on C-4', such as in the case of kaempferol and galangin (Farhadi *et al.* 2019). The hydroxylation of kaempferol on C-4' causes it to exhibit less activity than galangin (has no hydroxylation on C-4') against *S. aureus* (Echeverría *et al.* 2017). The low activity of $P_{QC}1$, when compared to quercetin, may allude to the unavailability of C-4' due to the steric hindrance presented by the binding of catechol on C-5' (Adelakun 2012).

Similar behaviour has been reported for catechol (1,2-benzenediol) when compared to some of its isomers (i.e. 1,3-benzenediol and 1,4-benzenediol). While catechol exhibited activity against both Gram-positive (e.g. *S. aureus*) and Gram-negative (e.g. *Salmonella enterica*), 1,3-benzenediol (C-1,3 hydroxylated) exhibited no antibacterial activity against the tested bacteria,

and 1,4-benzenediol exhibited no activity against *S. aureus* (Kim and Lee 2014). However, highly hydroxylated phenolic compounds (e.g. $P_{QC}2$) have been reported to be more effective against gram-positive bacteria due to their easy interaction with the cell wall peptidoglycan (Nitiema *et al.* 2012), subsequently modifying the permeability and/or rigidity (Bouarab-Chibane *et al.* 2019). This could explain why *L. monocytogenes* and *S. aureus* are more sensitive to the highly hydroxylated $P_{QC}2$.

Table 3.7: The antimicrobial activity of the coupling products $P_{QC}1$ and $P_{QC}2$ against selected gram-positive and gram-negative bacteria in comparison to quercetin and catechol

Microorganism Type	Catechol	Quercetin	$P_{QC}1$	$P_{QC}2$
	ZI (mm)	ZI (mm)	ZI (mm)	ZI (mm)
B. cereus	5.45 ± 0.21^{a}	3.21 ± 0.07^{b}	NA	NA
L. monocytogenes	NA	11.7 ± 0.53^{b}	NA	17.8 ± 0.96^{d}
S. aureus	5.40 ± 0.23^{a}	7.83 ± 0.24^{b}	3.59 ± 0.55^{c}	10.6 ± 0.73^{d}
P. aeruginosa	7.43 ± 0.81^{a}	6.72 ± 0.36^{b}	NA	NA
E. coli	6.62 ± 0.57^{a}	NA	NA	NA
E. cloacae	NA	16.4 ± 1.28^{b}	6.58 ± 0.34^{c}	13.4 ± 0.17^{d}

All values are means of three replicates ± standard deviation.

Values with different superscripts $^{[a, b, c, d]}$ in the same row are significantly different ($p < 0.05$) as shown by Duncan's multiple range. NA = No Activity. ZI = Zone of inhibition diameter to the nearest millimetre (mm). The concentrations of Catechol, Quercetin, $P_{QC}1$ and $P_{QC}2$ were 5mM.

The impact of catechol, quercetin, $P_{QC}1$, and $P_{QC}2$ on selected Gram-positive and -negative bacteria species is presented in Table 3.8. Only the bacterial species that showed sensitivity to the compounds (Table 3.7) were used for the determination of the MIC. The compounds were used at final concentrations in the range of 25 – 500 µg/ml. When comparing the results of all the experiments, it is clear that *L. monocytogenes* and *S. aureus* were the most sensitive to $P_{QC}2$ at a concentration of 200 µg/ml and 150 µg/ml, respectively. *E. cloacae* was most sensitive to quercetin at 150 µg/ml. The sensitivity of *S. aureus* to quercetin has been previously reported (Jaisinghani 2017). This has been attributed to the formation of strong complexes between quercetin and the heavy metals of some bacteria resulting in the inhibition of metalloenzymes (Cushnie and Lamb 2005), which may subsequently cause numerous metabolic disturbances (due to impaired ion channel functions) (Duda-Chodak 2012). The activity of catechol against different bacterial species, albeit low, has been demonstrated (Kim and Lee 2014; Kord Forooshani *et al.* 2019; Meng *et al.* 2019), and it is consistent with the current results.

Table 3.8: The minimum inhibitory concentrations (MICs) of the coupling products on sensitive bacteria in comparison with catechol and quercetin

Microorganism Type	Catechol	Quercetin	$P_{QC}1$	$P_{QC}2$
	MIC (µg/ml)	MIC (µg/ml)	MIC (µg/ml)	MIC (µg/ml)
L. monocytogenes	NA	300	NA	200
S. aureus	350	250	400	150
E. cloacae	NA	150	350	250

NA = No Activity.

3.5 CONCLUSION

A 58 kDa laccase was successfully produced from *T. pubescens*, purified and biochemically characterised. The enzyme optimum pH was substrate dependant. The optimum temperature of the enzyme was 60°C for all tested substrates and the enzyme was fairly stable up to a temperature of 60°C. Apart from sodium azide, all tested inhibitors irreversibly inhibited the Tp-laccase and the modes of inhibition were either non-competitive or uncompetitive. Tp-laccase's Michealis-Menten constants (K_m) for the tested substrates were in the order: SGZ > ABTS > 2,6-DMP > guaiacol, suggesting that guaiacol was the preferred substrate. The catalytic efficiencies for tested substrates were comparable to those of similar laccases. The deactivation energy and enthalpy of the enzyme were found to be higher than those reported for other fungal laccases, while the change in Gibb's free energy was lower. The coupling of quercetin and catechol resulted in the production of the heterodimer (yield 15.6 ± 1.26%) and heterotrimer (yield 9.8 ± 1.12%). The heterodimer exhibited inferior antioxidant activity, while the heterotrimer displayed enhanced antimicrobial activity against *L. monocytogenes* and *S. aureus* with minimal inhibitory concentrations of 200 µg/ml and 150 µg/ml, respectively. This study demonstrates that laccases from *T. pubescens* can be used to form hybrid phenolic compounds from catechol and quercetin. The increased number of hydroxyl groups could potentially enhance both the antioxidant and/or antimicrobial activities of the products. The products may also exhibit improvements in other physicochemical properties, as evidenced by the increase in solubility.

4. LACCASE-CATALYSED HETERO-COUPLING OF SELECTED PHENOLIC COMPOUNDS

4.1 ABSTRACT

Plant-based phenolic compounds display a vast array of pharmacological properties. However, these benefits generally come with inherent challenges such as low solubility, poor bioavailability and low antioxidant capacity. Enzyme facilitated modification of these compounds has the potential to improve these properties. In this study, we investigated the laccase-catalysed hetero-coupling of phenolic compounds as a way of improving antioxidant and antibacterial activities. In a biocatalysis system consisting of 50% or 60% dioxane, a 5'-5' quercetin/NDGA heterodimer (*m/z* 601; [M] = 602) and a 5-5' gallic acid/NDGA heterodimer (*m/z* 469; [M] = 470) were produced. The antioxidant activity of the heterodimers was approximately 2-fold higher than that of the parent compounds and 5'-5' quercetin/NDGA heterodimer also showed enhanced antimicrobial activity against gram-positive and gram-negative bacteria.

Keywords:
Trametes pubescens, laccase, quercetin, nordihydroguaiaretic acid, gallic acid, hetero coupling, antioxidant activity, antimicrobial activity.

4.2 INTRODUCTION

The various pharmacological properties of plant-based phenolic compounds have attracted a great deal of attention from researchers over the years (Jimenez-Garcia *et al.* 2018). Quercetin, gallic acid and nordihydroguaiaretic acid (NDGA) are examples of such plant-based compounds whose properties have been keenly investigated. They have been reported to exhibit ant carcinogenic, anti-inflammatory, antioxidant and antimicrobial activities owing mainly to the number and position of hydroxyl substituents on their benzene rings (David *et al*. 2016; Gonçalves and Romano 2017; Kahkeshani *et al.* 2019; Mala John *et al.* 2020). Studies on structure-activity relationships have provided the basis for structural manipulations to improve properties of phenolic compounds (Vermerris and Nicholson 2007; Aouf *et al.* 2013; Wang *et al*. 2010; Coimbra *et al*. 2011; Torres *et al*. 2012; Esfanjani and Jafari 2017).

Traditionally, chemical processes have been used to modify phenolic compounds (Ran *et al*. 2008; Torres *et al*. 2008). However, these processes are generally detrimental to the environment because they are usually energy-intensive, use toxic chemicals, and generate chemical wastes (Sheldon 2016). Consequently, green catalysis has been suggested as a sustainable alternative (Saleh and Koller 2018). Enzyme-catalysed modifications consume little energy, have high stereo- and regio-selectivity, and have few reaction steps (Truppo 2017; Chapman *et al*. 2018). Literature is awash with enzyme-facilitated processes in which biocatalysts such as peroxidases have been used to modify phenolic compounds (Kamel *et al*. 1977; Savic *et al*. 2016; Yadav *et al*. 2017; Manda *et al.* 2020). The requirement of hydrogen peroxide as a co-factor renders peroxide-facilitated processes undesirable due to additional cost and potential health and occupational hazards (Pandey *et al.* 2017). Consequently, laccases are now the preferred biocatalysts for the modification and synthesis of bioactive compounds (Kudanga *et al*. 2017).

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multi-copper oxidase enzymes belonging to the blue copper family of enzymes. They catalyse the oxidation of various non-phenolic and phenolic compounds to produce radicals with concomitant four-electron reduction of molecular oxygen to water (Cohen *et al*. 2002; Dwivedi *et al*. 2011; Santhanam *et al*. 2011; Riva 2013). Unlike the co-factor requiring enzymes such as peroxidases, laccases do not need additional co-factors to catalyse reactions. They employ molecular oxygen as the final electron acceptor. The radicals generated in the laccase-catalysed reactions can cross-couple to form oligomers of the substrates (Kudanga *et al*. 2017). The additional electron-donating groups of the oligomers have been reported to confer enhanced pharmacological and physicochemical properties (Lu *et al.* 2006a; Wolfe and Liu 2008). Therefore, this study employed *Trametes pubescens* laccase for the coupling of quercetin with NDGA, and gallic acid with NDGA as a way of synthesising new phenolic compounds with enhanced antioxidant and/or antimicrobial activities.

4.3 MATERIALS AND METHODS

4.3.1. Chemicals and enzymes

Quercetin, nordihydroguaiaretic, gallic acid, and other chemicals used were of analytical grade and were purchased from Merck and Sigma Aldrich. As previously mentioned (section 3.3.1), the *T. pubescens* CBS 696.94 strain used for the production of laccase was obtained from BOKU, University of Natural Resources and Life Sciences, Vienna, Austria.

4.3.2. Determination of enzyme activity

Laccase activity was determined as previously described in section 3.3.3.2.

4.3.3. Laccase-catalysed coupling reactions

The coupling reactions were undertaken in a monophasic system consisting of a 50 mM sodium acetate buffer pH 5.0 and an appropriate water-miscible co-solvent (i.e., dioxane, ethanol, methanol or acetone). The co-solvents were used at a concentration of 70% (v/v), and each of the substrates had an effective concentration of 5 mM. The 100 ml mixture was thoroughly mixed to ensure homogeneity before adding the enzyme. Laccase was added to a final activity of 0.66 U/ml. The reaction mixtures were incubated at 37°C, with shaking at 200 rpm for 6 hours. Product formation was monitored hourly using thin-layer chromatography (TLC).

4.3.4. The effect of co-solvent concentration and reaction time

The effect of co-solvent concentration on product formation was determined. The reactions were undertaken in a monophasic system consisting of a 50 mM sodium acetate buffer pH 5.0 and a range of concentrations of the co-solvent (10 – 90%, v/v). The concentration of each substrate was kept at 5 mM. The reactions were initiated with 0.66 U/ml of laccase and incubated at 37°C, with shaking at 200 rpm for 12 hours. Product formation was monitored hourly by TLC and HPLC analysis.

4.3.5. Chromatographic separation of coupling products

Aluminium-backed silica gel 60 F254 plates (Merck) were used for TLC analysis. Toluene:dioxane:acetic acid [11:2.5:0.4 (v/v/v)] or toluene:ethyl acetate:formic acid [7:5:1

(v/v/v)] was used as the mobile phase. The plates were exposed to UV light at 254 nm for the visualisation of the compounds.

4.3.6. High-performance liquid chromatography (HPLC)

HPLC analysis was carried out as previously described in section 3.3.11.2.

4.3.7. Purification of the coupling products

Preparative TLC was used to purify the products of interest using the mobile phases described in section 4.3.5. The plates were exposed to UV light at 254 nm for the visualisation of the compounds. The products of interest were scraped from the TLC plates, dissolved in methanol and filtered through a 0.2 µm nylon filter (Merck) to remove the silica gel particles.

4.3.8. Characterisation of the products

Liquid chromatography-mass spectrometry (LC-MS) was used to characterise the purified (or partially purified) products. LC-MS analysis was carried out as previously described in section 3.3.13.

4.3.9. Determination of antioxidant activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic) acid (ABTS) scavenging assay and the ferric reducing antioxidant power (FRAP) assay were used to determine antioxidant activity as previously described in section 3.3.14.1.

4.3.10. Determination of antimicrobial activity

Antibacterial activity was assessed by the agar well diffusion method and the minimum inhibitory concentrations were determined as previously described in sections 3.3.15 and 3.3.16, respectively.

4.3.11. Statistical analysis for the antioxidant and antimicrobial activities

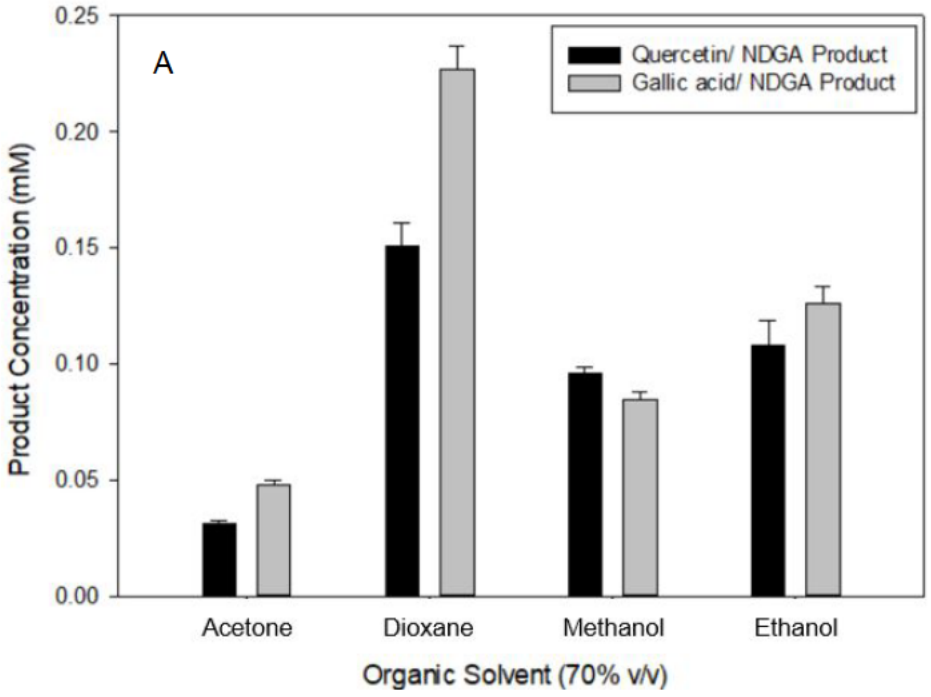
Antimicrobial and antioxidant activity data were subjected to statistical analysis as described in section 3.3.17.

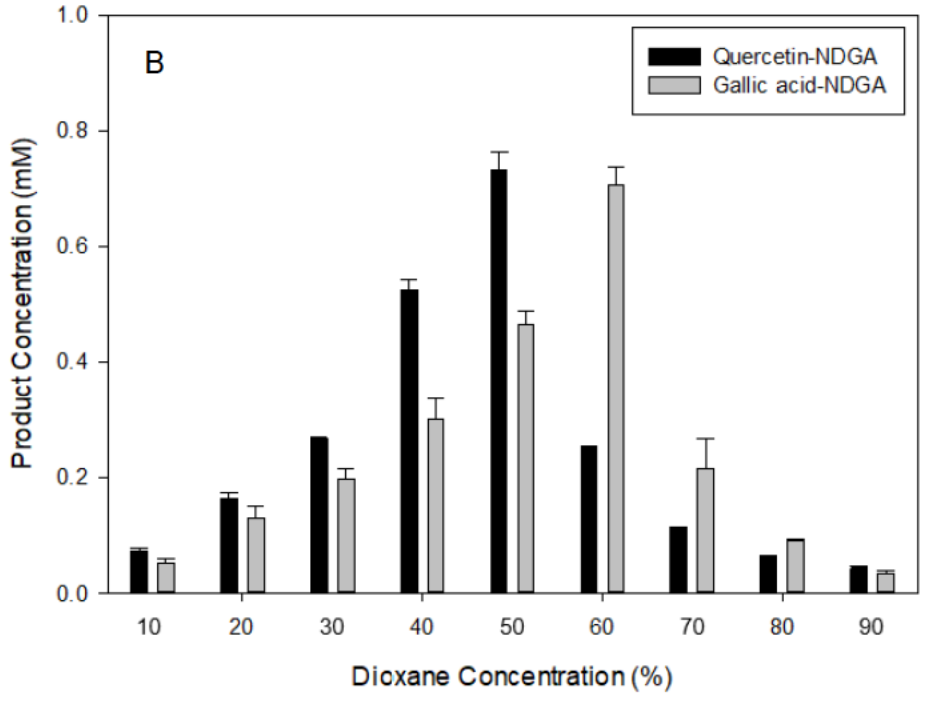
4.4 RESULTS AND DISCUSSION

4.4.1. Co-solvent and reaction duration effect on coupling product formation

Most laccase substrates are hydrophobic and not soluble in aqueous buffer systems. Therefore, all coupling reactions were carried out in organic co-solvent systems. The use of organic solvents often results in reduced reaction rates stemming from the interaction of the solvents with the enzyme and elimination of water from the active centre (Dordick 1989; Bell *et al*. 1997). This may, subsequently, cause structural distortion of the enzyme which may result in its irreversible inactivation (Zheng and Ornstein 1996). Nonetheless, the successful employment of organic co-solvents in laccase-catalysed reactions has been demonstrated and attributed to the organic solvents' intrinsic biocatalysis advantages (Burton 2003a; Riva 2006a; Mohtashami *et al*. 2019). These advantages include: optimisation of substrate specificity and enantio-selectivity; solubilisation of non-polar substrates; a reversal of the thermodynamic equilibrium of hydrolysis reactions; removal of water-dependent side reactions; and elimination of microbial contaminations (Kudanga *et al*. 2017). The extent of substrate solvation varies per solvent and may dictate thermodynamic activity, and the partitioning coefficient of both the substrates and the product. Consequently, solvent selection heavily hinges on the substrate, enzyme source and chemical properties of the solvent (Rogalski *et al*. 1995; Rogalski *et al*. 1999; Rodakiewicz-Nowak *et al*. 2000; Gogoi *et al*. 2009). Therefore, several solvents namely methanol, ethanol, dioxane and acetone were investigated for their effect on the formation of the respective coupling products.

Dioxane was identified as the best co-solvent for the laccase-catalysed production of quercetin-NDGA (P_{QN}) and gallic acid-NDGA (P_{GN}) hybrid molecules. The tested co-solvents best facilitated product formation in the order dioxane > ethanol > methanol > acetone (Figure 4.1A). The most effective dioxane concentrations were found to be 50% and 60% (v/v), which yielded 14.71 ± 0.59 and 14.12 ± 0.53 for P_{QN} and P_{GN}, respectively (Figure 4.1B). Both products were found to be at their highest yields at 6 hours, after which the products began to polymerise as observed by the reduction in their yields (Figure 4.1C) and the appearance of new heavier side products (precipitate). Water-miscible solvents have been reported to influence the selectivity of laccase-catalysed reactions (Intra *et al*. 2005). The better product selectivity exhibited by dioxane can be attributed to its lower dielectric constant which may reduce the collision rates between radicals. Although this slows down product formation, it reduces polymerisation, effectively increasing the yield of the desired product.


A
Quercetin/ NDGA Product
Gallic acid/ NDGA Product
Product Concentration (mM)
0.25
0.20
0.15
0.10
0.05
0.00
Acetone
Dioxane
Methanol
Ethanol
Organic Solvent (70% v/v)


B
Quercetin-NDGA
Gallic acid-NDGA
Product Concentration (mM)
1.0
0.8
0.6
0.4
0.2
0.0
10
20
30
40
50
60
70
80
90
Dioxane Concentration (%)

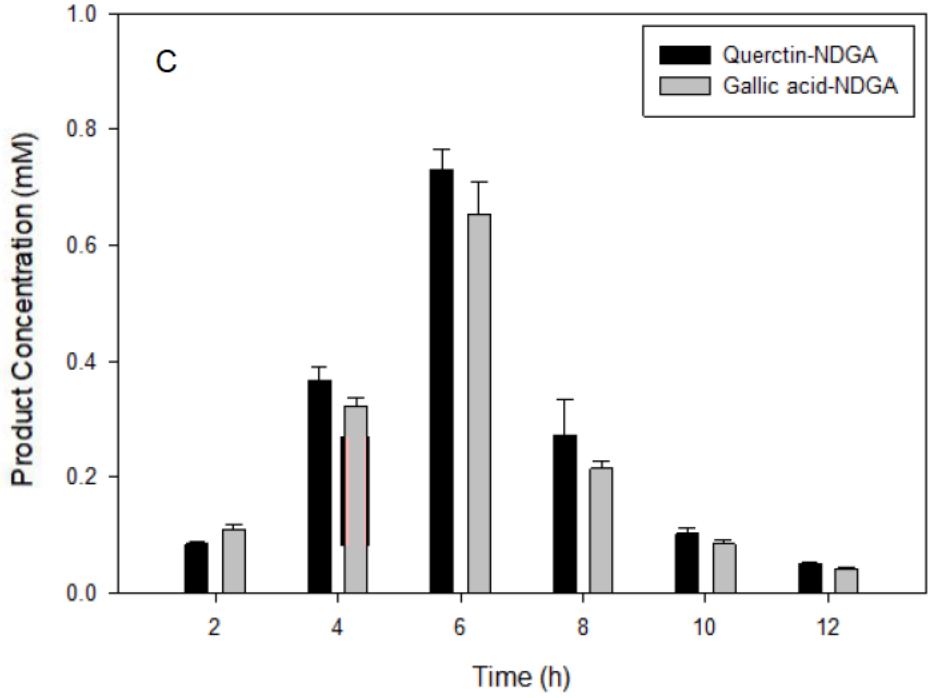
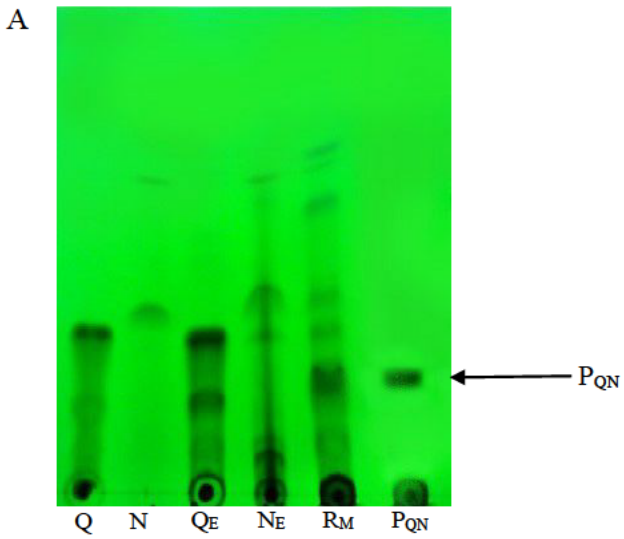


Figure 4.1: The effect of the organic co-solvent type (A), concentration (B), and reaction time (C) on the formation of the quercetin- nordihydroguaiaretic acid (NDGA) and gallic acid-NDGA coupling products. All values are means of three replicates ± standard deviation.

4.4.2. Laccase-catalysed coupling of quercetin and nordihydroguaiaretic acid

Laccase-catalysed coupling of quercetin with NDGA resulted in the formation of one product code-named P_{QN} (Figure 4.2A). The purity of the product was confirmed by HPLC (retention time 16.102 minutes) (Figure 4.2B). The product was recovered (yield of 15%) and further analysed by LC-MS (t_R 16.605 minutes; *m/z* 601 ($[M-H]^-$) (Figure 4.3). This indicates the formation of a hetero-dimer of quercetin and nordihydroguaiaretic acid.



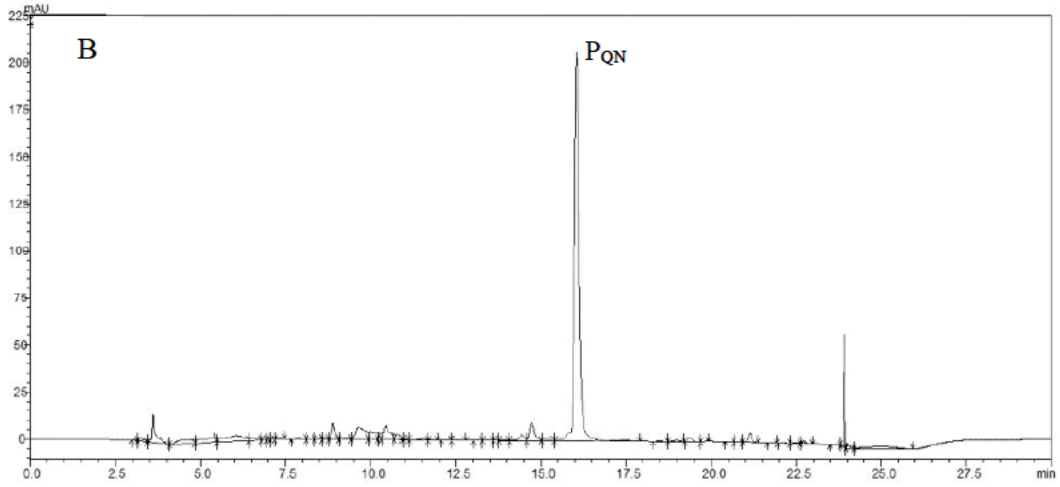


Figure 4.2: TLC (A) and HPLC (B) analysis of the quercetin-nordihydroguaiaretic acid product synthesised via a laccase-catalysed reaction. The migration of substrates (Q and N), homo-coupling reaction contents (Q_E and N_E), hetero-coupling reaction contents (R_M), and the purified coupling product (P_{QN}) are clearly differentiated. The HPLC chromatogram shows the extent of product purification.

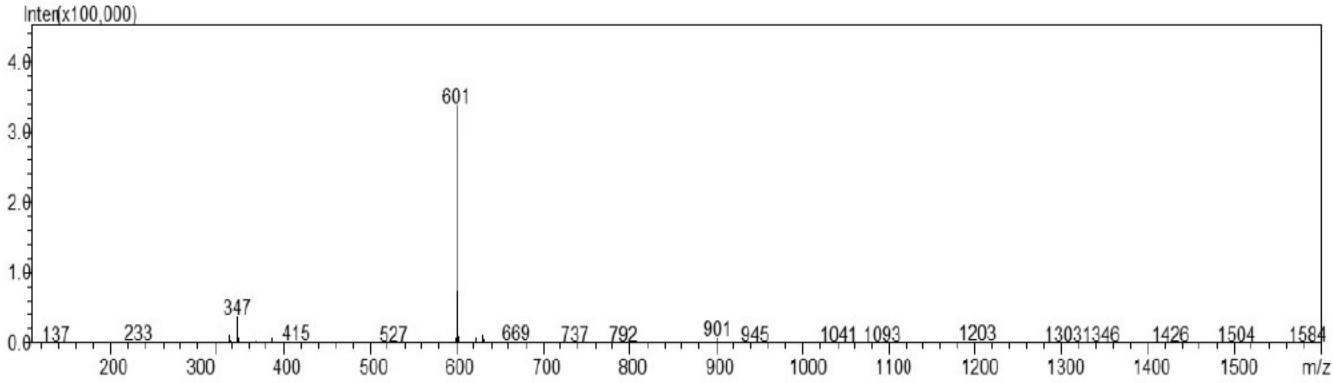


Figure 4.3: The mass spectrum of the product formed during a laccase-catalysed coupling reaction of quercetin and nordihydroguaiaretic acid.

Based on LC-MS results and literature on the oxidation patterns of both quercetin and nordihydroguaiaretic acid, a reaction mechanism was proposed (Figure 4.4). Since both substrates were present in solution during the initiation of the reaction, both were simultaneously oxidised by Tp-laccase. However, due to the low bond dissociation energy of the 4'-OH group (~312 kJ/mol) on quercetin when compared to that of the 4,4'-OH group (351 kJ/mol) on nordihydroguaiaretic acid (Marković *et al*. 2010; Evgeny and Taisa 2011), it can be postulated that the quercetin radicals accumulate more rapidly than those of nordihydroguaiaretic acid. The unpaired electrons spontaneously move around, occupying different positions (due to π-electron

conjugation) to achieve resonance stabilisation of the radicalised molecule (Fourré *et al.* 2016). The collision of the 5'-radical of quercetin with the 5'-radical of nordihydroguaiaretic acid forms a stabilised hetero-dimer through the 5'-5' carbon-carbon bond. Carbon-carbon bond linkages are generally preferred over the corresponding ether linkages because they have a low heat of formation (ΔH_f) (del Río and Gutiérrez 2008).

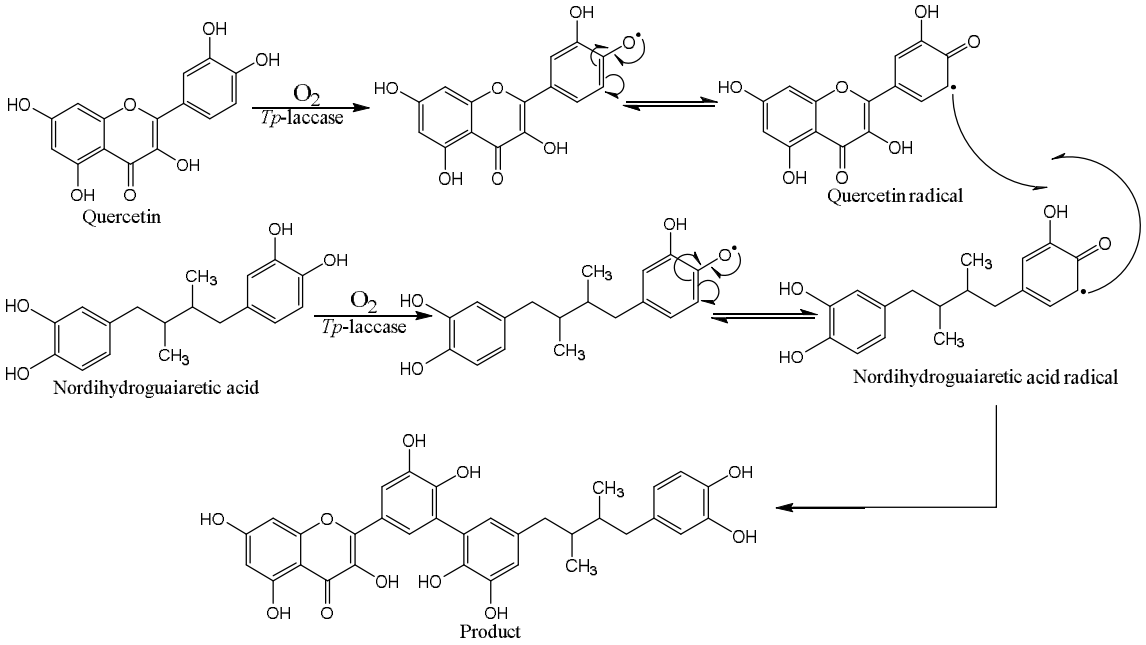


Figure 4.4: The proposed reaction mechanism for the formation of the quercetin-nordihydroguaiaretic acid hetero-dimer via a Tp-laccase catalysed synthesis reaction.

4.4.2.1. Antioxidant properties of the quercetin and NDGA coupling product

The antioxidant activity of the quercetin/NDGA hetero-dimer was compared to those of the parent compounds (i.e. quercetin and NDGA) using the ABTS, DPPH, and FRAP assays. Plant-derived phenolic compounds are well known as reducing agents and scavengers of reactive oxygen and nitrogen species. Both quercetin and NDGA have had their numerous biological activities documented, and their ability to scavenge radicals has been demonstrated (David *et al.* 2016; Gonçalves and Romano 2017; Kahkeshani *et al*. 2019; John *et al*. 2020). The radical scavenging ability of the quercetin/NDGA heterodimer was compared to those of the parent compounds. The heterodimer performed better than the parent compounds (Table 4.1). The antioxidant activities of the heterodimer were ~2 folds higher when compared to NDGA, and

were enhanced by ~25% when compared to quercetin. Phenolic compounds owe their radical scavenging abilities to structural characteristics, such as hydroxyl groups that enable the donation of the hydrogen atom, and the propensity to form stable phenoxyl radicals (Ahmed *et al*. 2015).

Table 4.1: The antioxidant activities of quercetin, nordihydroguaiaretic acid and their hetero-dimer. All values are means of three replicates ± standard deviation

Compound	Molecular Weight (g/mol)	ABTS C_{50} (μM)[a]	DPPH C_{50} (μM)[a]	FRAP (μM)[b]
NDGA	302.37	5.62 ± 0.381[c]	7.86 ± 0.406[c]	49.18 ± 2.03[c]
Quercetin	302.24	3.79 ± 0.132[d]	4.97 ± 0.348[d]	68.31 ± 1.63[d]
Hetero-dimer	602.61	2.98 ± 0.092[e]	3.84 ± 0.304[e]	78.02 ± 1.96[e]

All values are means of three replicates ± standard deviation.

Values with different superscripts [c, d, e] in the same column are significantly different ($p < 0.05$) as shown by Duncan's multiple range test.

[a]Concentration (μM) of substrates or products required to reduce [$ABTS^{\bullet+}$] or [$DPPH^{\bullet}$] by 50%.

[b]The FRAP value of substrates and products (μM) is equivalent to the amount of $[Fe(III)(TPTZ)2]^{3+}$ converted to $[Fe(II)(TPTZ)2]^{2+}$ by 1 mM of the sample.

The hydroxyl groups on the ortho-catechol ring (B) of quercetin provides the group with a higher acidity capacity, thus making the ortho 3', 4' dihydroxy substitution in this ring critical for the high antioxidant activity of quercetin (Ozgen, Kilinc and Selamoğlu 2016). Furthermore, the analysis of the electron spin density of the 4'-quercetin radical by Vasilescu and Girma (2002) revealed a partial involvement of ring C in the scavenging activities of quercetin. Nordihydroguaiaretic acid, on the other hand, consists of two catechol rings that confer radical scavenging activity (Floriano-Sánchez *et al.* 2006). Due to the symmetrical nature of nordihydroguaiaretic acid, any of its catechol rings can donate electrons (Manda *et al*. 2020). The hetero coupling of quercetin and NDGA combines the antioxidant effects of the parent compounds resulting in improved antioxidant activity. Increasing the number of hydroxyl groups generally improves antioxidant activity (Kudanga *et al*. 2017). The enhancement of the antioxidant capacity means the antioxidant does not necessarily have to be used at high concentrations. This in turn may assist in the reduction of the prooxidant and cytotoxic effects that are observed at higher concentrations of NDGA (Lambert *et al*. 2004; Floriano-Sánchez *et al*. 2006).

4.4.2.2. Antimicrobial properties of the coupling product

The quercetin/NDGA heterodimer exhibited higher antimicrobial activity against *L. monocytogenes*, *S. aureus*, *E. coli* and *E. cloacae* (Table 4.2 and 4.3). The product inhibited the growth of both gram-positive and gram-negative bacteria. This can be attributed to the abundance of hydroxyl groups that enable the heterodimers to interact with the peptidoglycan cell wall of gram-positive bacteria while the methyl groups enable easy interaction and formation of complexes with the cell membrane constituents of the gram-negative bacteria (Sarbu *et al.* 2019). Through hydroxyl groups and/or phenolic rings phenolic compounds form disparaging complexes with bacterial proteins and membranes (Nitiema *et al*. 2012; Borges *et al*. 2013).

Table 4.2: The antimicrobial activity of the heterodimer against selected gram-positive and gram-negative bacteria in comparison to parent compounds

Microorganism Type	NDGA	Quercetin	P_{QN}
	ZI (mm)	ZI (mm)	ZI (mm)
B. cereus	9.35 ± 1.04^{a}	3.21 ± 0.07^{a}	7.09 ± 0.56^{a}
L. monocytogenes	NA	11.7 ± 0.53	18.2 ± 1.92
S. aureus	9.82 ± 0.84	7.83 ± 0.24	12.4 ± 0.27
P. aeruginosa	16.1 ± 0.34^{a}	6.72 ± 0.36^{b}	NA
E. coli	12.4 ± 0.96^{a}	NA	16.1 ± 1.06^{c}
E. cloacae	13.9 ± 0.86	16.4 ± 1.28	17.5 ± 1.74

All values are means of three replicates ± standard deviation.

Values with different superscripts [a, b, c] in the same row are significantly different ($p < 0.05$) as shown by Duncan's multiple range test.

NA = No Activity. ZI = Zone of inhibition diameter to the nearest millimetre (mm). The concentrations of NDGA, quercetin and P_{QN} were 5 mM.

Table 4.3: The minimum inhibitory concentrations of the heterodimer on sensitive bacteria in comparison to parent compounds

#Microorganism Type	NDGA	Quercetin	P_{QN}
	MIC (µg/mL)	MIC (µg/mL)	MIC (µg/mL)
L. monocytogenes	NA	300	200
S. aureus	150	250	100
E. coli	100	NA	50
E. cloacae	125	150	50

NA = No Activity

4.4.3. Laccase-catalysed coupling of nordihydroguaiaretic acid and gallic acid

Coupling of NDGA and gallic acid resulted in the formation of a single cross-coupling product (P_{GN}) with an R_f value of 0.59 (Figure 4.5A) and retention time of 12.192 minutes (Figure 4.5B). The product was recovered (yield of 15.2%) and further analysed by LC-MS (Figure 4.6).

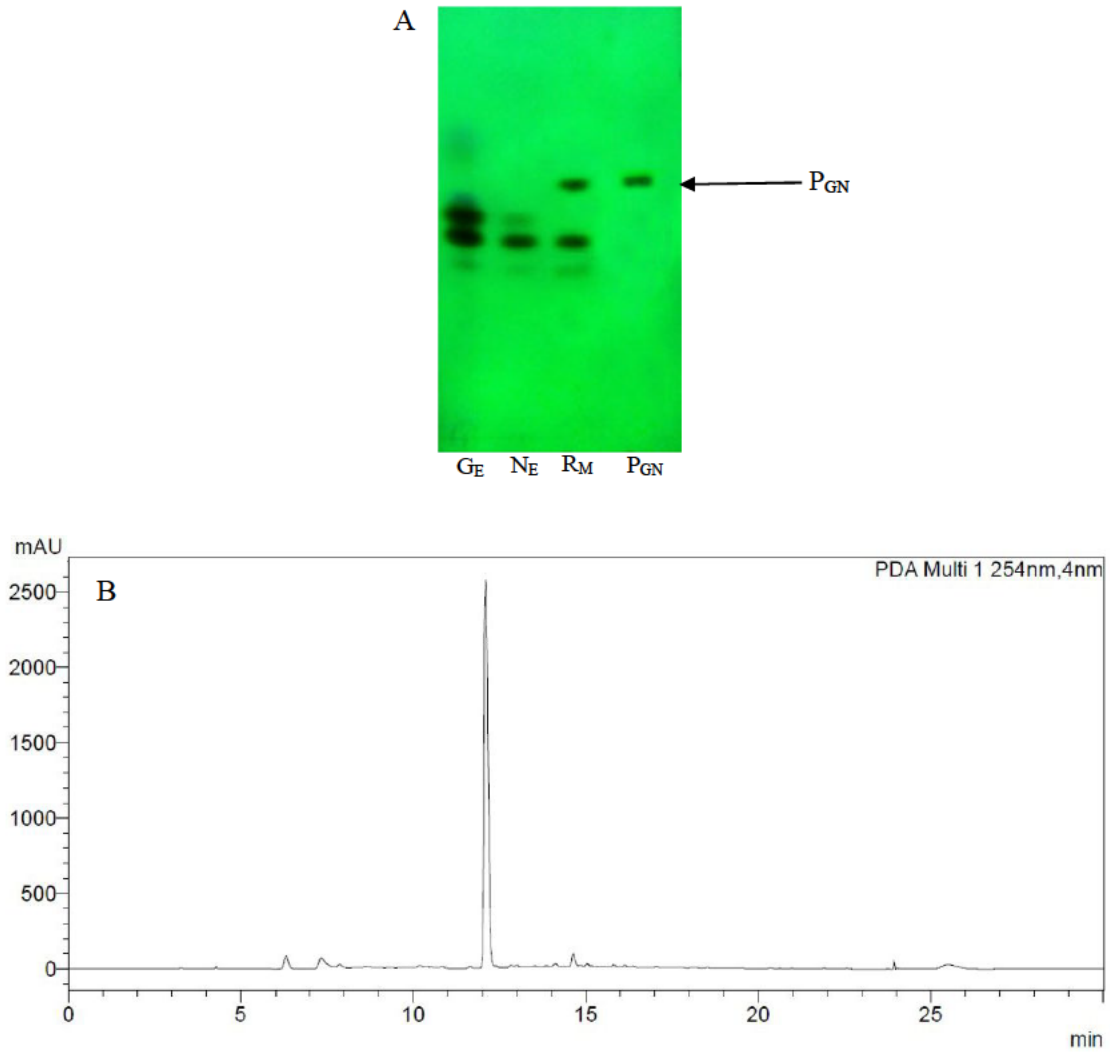


Figure 4.5: Thin layer chromatogram (A) and high-performance liquid chromatogram (B) of laccase-catalysed gallic acid-nordihydroguaiaretic acid coupling. (G_E – gallic acid + laccase, N_E - nordihydroguaiaretic acid + laccase, R_M - hetero-coupling reaction, and P_{GN} - the purified coupling product.

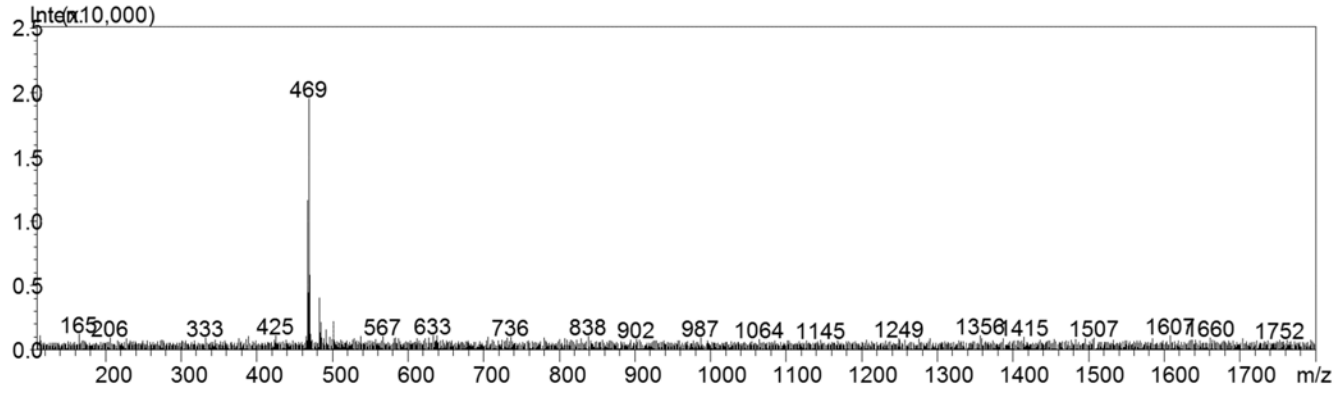


Figure 4.6: Mass spectrum of the product formed during a laccase-catalysed coupling reaction of gallic acid and nordihydroguaiaretic acid.

The mass to charge ratio of the product (*m/z* 469 ($[M-H]^-$) indicate the formation of a heterodimer of gallic acid and nordihydroguaiaretic acid. Based on these results and literature on the oxidation patterns of both gallic acid and nordihydroguaiaretic acid, a reaction mechanism was proposed (Figure 4.7). Since both substrates were present in solution during the initiation of the reaction, both were simultaneously oxidised by Tp-laccase. All three aromatic hydroxyl groups of gallic acid are susceptible to oxidation, albeit at varying rates due to their different pKa values and bond dissociation energies (Eslami *et al.* 2010). The slight difference in the bond dissociation energies of the 4-OH group (347 kJ/mol) on gallic acid and the 4,4'-OH group (351 kJ/mol) on nordihydroguaiaretic acid makes it reasonable to postulate that the radicals of the two compounds will accumulate at approximately the same rate in the reaction vessel (Marković *et al*. 2010; Evgeny and Taisa 2011).

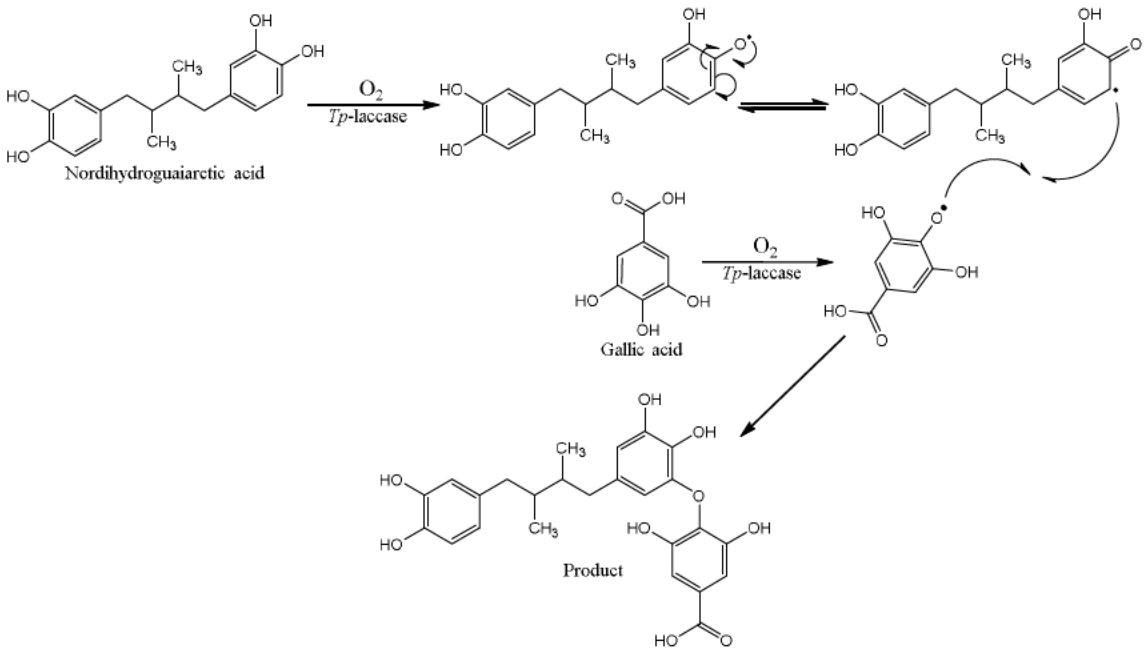


Figure 4.7: The proposed reaction mechanism for the formation of the gallic acid-nordihydroguaiaretic acid heterodimer via a Tp-laccase catalysed synthesis reaction.

4.4.3.1. Antioxidant properties of the coupling products

The antioxidant activity of the gallic acid/NDGA heterodimer was compared to those of the parent compounds (i.e. gallic acid and NDGA) using the ABTS, DPPH, and FRAP assays (Table 15).

Table 4.4: The antioxidant activities of gallic acid, nordihydroguaiaretic acid and their heterodimer

Compound	Molecular Weight (g/mol)	ABTS C_{50} (µM)[a]	DPPH C_{50} (µM)[a]	FRAP (µM)[b]
NDGA	302.37	5.62 ± 0.381[c]	7.86 ± 0.406[c]	49.18 ± 2.03[c]
Gallic acid	170.12	7.23 ± 0.516[d]	11.6 ± 0.592[d]	38.92 ± 1.09[d]
Hetero-dimer	470.49	3.28 ± 0.092[e]	3.84 ± 0.304[e]	68.15 ± 2.06[e]

All values are means of three replicates ± standard deviation.

Values with different superscripts [c, d, e] in the same column are significantly different ($p < 0.05$) as shown by Duncan's multiple range test.

[a]Concentration (µM) of substrates or products required to reduce [$ABTS^{\bullet+}$] or [$DPPH^{\bullet}$] by 50%.

[b]The FRAP value of substrates and products (μM) is equivalent to the amount of $[Fe(III)(TPTZ)2]^{3+}$ converted to $[Fe(II)(TPTZ)2]^{2+}$ by 1 mM of the sample.

The bioactive capabilities of environmentally derive phenolic compounds have attracted a lot of attention from researchers over the years. Both gallic acid and nordihydroguaiaretic acid have had their proficiencies reported on, and their radical scavenging abilities have been demonstrated (Floriano-Sánchez *et al.* 2006; Galano *et al.* 2010; Marino *et al*. 2014; Badhani *et al*. 2015). The radical scavenging ability of the gallic acid/NDGA heterodimer was compared to those of the parent compounds. The heterodimer performed better than the parent compounds. A summary of the molecular weights and antioxidant activities of quercetin, nordihydroguaiaretic acid and their heterodimer (P_{GN}) are provided in Table 4.4. The DPPH and ABTS radical scavenging abilities of the heterodimer were better by almost 2-fold when compared to both parent compounds. The ferric reducing power of the heterodimer was higher by almost 2-fold when compared to both parent compounds. Phenolic compounds owe their radical scavenging abilities to structural characteristics, such as hydroxyl groups that enable the donation of the hydrogen atom, and the propensity to form stable phenoxyl radicals (Ahmed *et al*. 2015). The aromatic hydroxyl groups of gallic acid are all capable of exercising their reducing ability (Badhani *et al*. 2015). This ability is partly can exacerbated by changes in pH of the surrounding environment, particularly towards alkalinity, due to the different pKa (4 – 11.4) values assigned to these aromatic hydroxyl groups (Eslami *et al*. 2010). Structure-function studies of gallic acid have revealed that its radical scavenging efficiency is not only dependent on hydroxyl groups but also steric freedom particularly that of the *para*-substituted hydroxyl group (4-OH) (Lu *et al.* 2006b). The *ortho*-substituted hydroxyl groups tend to stabilise the radicalised form of gallic acid, which culminates into a reduced hydrogen bond dissociation enthalpy and hence higher antioxidant activity. The ortho-substituted hydroxyl groups achieve this by forming intramolecular hydrogen bonds with the para-substituted hydroxyl group (Badhani *et al*. 2015).

Nordihydroguaiaretic acid, on the other hand, consists of two catechol rings that confer cytoprotective effects as a strong scavenging agent of multiple types of reactive oxygen and nitrogen species (Floriano-Sánchez *et al*. 2006). Due to the symmetrical nature of nordihydroguaiaretic acid, any of its catechol rings can donate electrons (Manda *et al*. 2020). Therefore, the laccase catalysed coupling of gallic acid and nordihydroguaiaretic resulted in a 5-5' heterodimer with better antioxidant activity and increased stability of the resulting radical due to a higher number of electron centres. This also supports the principle that increasing the number

of hydroxyl groups can improve antioxidant activity (Kudanga *et al*. 2017). The synergistic enhancement of the stability of the resultant radicals and antioxidant capacity may assist in the reduction of the prooxidant and cytotoxic properties of that are observed at higher concentrations of nordihydroguaiaretic acid (Lambert *et al*. 2004; Floriano-Sánchez *et al*. 2006). Several researchers have demonstrated laccase-catalysed polymerisation of plant-derived phenolic compounds results in compounds with antioxidant capacities that are better than those of their monomeric parent compounds (Chung *et al*. 2003; Kurisawa *et al*. 2004).

4.5 CONCLUSION

This study demonstrates that laccases from *T. pubescens* can be used to form hybrid phenolic compounds from nordihydroguaiaretic acid and quercetin, and nordihydroguaiaretic acid and gallic acid. The increased number of hydroxyl groups contributed to enhancing the antioxidant and/or antimicrobial activities of the products. The products may also exhibit improvements in other physicochemical properties.

5. GENERAL DISCUSSION

The past century has seen an overwhelming upsurge of research on natural antioxidants, primarily due to rising awareness and knowledge about the carcinogenicity of synthetic antioxidants (Buisman *et al*. 1998; McCarthy *et al*. 2001). Natural phenolic compounds have a wide range of possible applications including use in pharmaceuticals, food products, dietary supplements and cosmetics (Augustyniak *et al*. 2010). However, undesirable properties exhibited by some of these compounds, coupled with concerns over the environmental impacts of chemical synthesis have prompted intensified research in biocatalysis (Stamatis *et al*. 2001; Torres *et al*. 2008; Wang *et al*. 2010; Carocho and Ferreira 2013). The ability of laccases to oxidatively transform an extensive range of compounds is unsurpassed and makes them ideal catalysts for industrial applications. Their physiological roles such as lignin and secondary metabolite biosynthesis and oxidation of flavonoids in plants inform their biochemical and catalytic properties (Caparrós-Ruiz *et al*. 2006; Pourcel *et al*. 2007; Turlapati *et al*. 2011; He *et al*. 2019; Li *et al*. 2019).

In the current study, high titres of extracellular laccase (2330 ± 50 U/l) were produced from *T. pubescens*, which were significantly higher than those reported in literature (Gonzalez *et al.* 2013). This could be attributed to the differences in culture conditions, i.e. xylidine and 2 mM of $CuSO_4$ were used as co-inducers in the current study while Gonzalez *et al*. (2013) only used $CuSO_4$. The purification of the enzyme was achieved via a 4-step protocol; $(NH_4)_2SO_4$ precipitation, ultrafiltration, anion exchange and size exclusion chromatography. $(NH_4)_2SO_4$ is the most preferred salting-out agent due to possession of polyvalent anion (SO_4^{2-}) and a univalent cation (NH_4^+), and its ability to stabilise protein structure while concomitantly reducing its solubility (Dennison 2013; Park *et al*. 2015).

Subjecting the ultrafiltrated crude enzyme to anion-exchange chromatography resulted in the separation of two eluent isoforms of the enzyme. This comes as no surprise because it is a common occurrence for fungal species such as *T. pubescens* to produce more than one isoform of laccase with distinguishable chemical and catalytic properties (Adelakun 2012b; Bertrand *et al.* 2015; Yuan *et al.* 2016). The first isoform (TpL1) showed no affinity for the diethylaminoethyl (DEAE) cellulose matrix, thus implying that the mobile phase pH was below its isoelectric point. The second isoform (TpL2) was eluted at NaCl concentrations ranging between ~ 0.2 M and 0.4 M. It can therefore be deduced that TpL1 comprises a proportionally

higher number of amino acids bearing basic side-chains, and this alludes to the presence of conformational differences between TpL1 and TpL2 which may in turn impact on the enzyme activity (Dennison 2013). The observation that the catalytic activity of TpL2 was 2-fold higher than that of TpL1 is consistent with this deduction. A zymogram confirmed the production of two laccase isoforms by *T. pubescens* as observed in anion exchange chromatography. TpL2 was further purified with size exclusion chromatography (SEC), and the SDS-PAGE analysis of the purified enzyme gave a single dominant protein band with an apparent molecular weight of ~58 kDa. This is similar to the approximate molecular weight range of 60 – 100 kDa that is recorded for fungal laccases (Baldrian 2006; Hildén *et al.* 2009; Rodríguez-Couto 2019).

The purified enzyme was biochemically characterised by determining the pH and temperature properties, the effect of organic solvents and heavy metals and susceptibility to putative inhibitors. The optimum pH of the enzyme (60°C) was consistent with that reported in literature for similar enzymes (Galhaup *et al*. 2002; Kunamneni *et al*. 2007a; Hilden *et al*. 2009; Gaitan *et al*. 2011). Furthermore, the enzyme displayed thermal stability between 25°C and 60°C, with a drastic drop in activity at temperatures above 60°C. This was consistent with previously recorded thermostability values of *T. pubescens* laccases (Gaitan *et al*. 2011; Si *et al*. 2013; Yan *et al*. 2015).

The optimum pH of the enzyme was substrate-dependent, and the highest activities were observed at pH 3.5 for ABTS and 2,6-DMP, and pH 7.0 for syringaldazine. The dependency of pH optimum on the substrate was also observed with typical lignin model substrates, i.e. guaiacol, caffeic acid, *p*-coumaric acid, ferulic acid and vanillic acid (Kudanga *et al*. 2009). This is mostly attributed to the OH^- inhibition at the T1/T2 trinuclear cluster and the differences in redox potential between substrates and T1 copper. Furthermore, during an investigation of the kinetic parameters' dependence on pH, Garzillo *et al.* (2001) concluded that non-phenolic substrates bind to the enzyme via a histidine residue, whereas phenolic substrates may require a histidine and an acid residue (i.e. aspartic acid). Nonetheless, the pH dependency of *T. pubescens* laccase is synonymous to that of the other described fungal laccases, i.e. monotonic curves for nonphenolic substrates and bell-shaped pH profile for the phenolic substrates (Stepanova *et al*. 2003; Morozova *et al*. 2007a; Gaitan *et al*. 2011; Shi *et al*. 2014; Sampaio *et al*. 2016). The enzyme was most stable between pH 4.0 and 5.0 and was fairly stable at all tested pH values, retaining at least 65% activity after 3 hours. Fungal laccases have been reported to be stable at pH values ranging from 4.5 – 10.0, maintaining 75% of their original activity after 72 hours (Si

et al. 2013). Similarly, Galhaup *et al*. (2002) reported a 92.5% residual activity between 4.0 and 5.0.

Since the substrates were insoluble in water, the effect of various organic co-solvents on enzyme activity was determined. The enzyme was most stable in DMSO, ethyl acetate and dioxane, while acetone caused a moderate reduction in enzyme activity. On the contrary, Doukyu and Ogino (2010) reported rapid enzyme deactivation by DMSO. These contrasting findings could be due to different enzyme sources and purification processes (Rodakiewicz *et al*. 2000). The enzyme's stability in ethyl acetate is not surprising. Ethyl acetate imposes a biphasic system in which the enzyme is confined to the aqueous phase and has infinitesimal contact with the organic solvent (Adelakun 2012). This arrangement, albeit with its hurdles, is preferred due to its facilitation of efficient product separation and enzyme regeneration (Doukyu and Ogino 2010; Adelakun 2012). The enzyme was least active in ethanol and methanol as co-solvents. This may be due to the solvent-facilitated conformational distorting and irreversible inactivation of the enzyme (Dordick 1989; Zheng and Ornstein 1996; Bell, Janssen and Halling 1997).

Understanding the behaviour of Tp-laccase in the presence of heavy metal ions and inhibitors is important since some of these chemicals are encountered in potential application environments, and may give insight on key residues in the active site (El-Shora *et al*. 2008; Si *et al*. 2013). Apart from Cu^{2+} ions at 20 mM concentration, the enzyme was inhibited by all tested heavy metals. Enhancement of laccase activity by selected concentrations of Cu^{2+} has been reported (Haibo *et al*. 2009; Zhu *et al*. 2011; Si *et al*. 2013), and there are previous reports on the inhibitory effect of Al^{3+} (Si *et al*. 2013) and the other investigated ions (Guo *et al*. 2011; Shi *et al*. 2014). The non-competitive inhibition of NaN_3 and SDS can be ascribed to the presence of an allosteric site on the enzyme (Palmer and Bonner 2011; Komoda and Matsunaga 2015; Delaune and Alsayouri 2019). L-cysteine, hydrogen peroxide and dithiothreitol uncompetitively inhibited laccase as evident from the reduction of both the substrate-binding affinity and maximal rate. Therefore, L-cysteine, hydrogen peroxide and dithiothreitol bind to the enzyme-substrate complex (Delaune and Alsayouri 2019). The inhibition of Tp-laccase provides insight into the mechanism of action of the enzyme. Specific inhibitors are often employed to elucidate critical residues for catalytic activity (Pratt and Cornely 2021). For instance, the inhibition of Tp-laccase by group-specific compounds such as L-cysteine, dithiothreitol, and NaN_3 eludes to the presence and significance of thiol groups on its catalytic activity (Si *et al*. 2013).

Understanding substrate conversion rates of an enzyme offer an avenue through which it can be accurately studied under various conditions (Engelking 2015). This provides a basis for the optimal application of the enzyme in different scenarios. The determination of the kinetic parameters was undertaken with ABTS, guaiacol, syringaldazine and 2,6-DMP, and they were compared with previously published literature on fungal laccases. A slightly lower ABTS-binding affinity was demonstrated by the enzyme in this study, while the guaiacol-, syringaldazine- and 2,6-DMP-binding affinities were higher or comparable to those in the literature (Shleev *et al*. 2007; Kalyani *et al*. 2012; Bisswanger 2014; Shi *et al*. 2014; Ling *et al*. 2015; Sung-Jong and Su-Jin 2017). The enzyme demonstrated a catalytic efficiency that was comparable with some fungal laccases (Shleev *et al*. 2007; Shrestha *et al*. 2016; Sung-Jong and Su-Jin 2017). Significant differences in reaction conditions and enzyme sources might be the reason for the differences observed.

The ability of the enzyme to withstand thermal inactivation is the key determinant of its thermostability. Enzymes with higher deactivation energy (E_d) values are more resistant to thermal inactivation and can be easily applied at higher temperatures. In the current study, the enzyme was found to have a significantly high Ed value of 109.36 kJ/mol, albeit lower than those of typical bacterial laccases (Yadav *et al*. 2018). This implies a delay in conformational changes when the enzyme is exposed to high temperatures. Therefore, there is a prospect of applying Tp-laccase in various environmentally benign processes that require thermostable enzymes in the textile, and pulp and paper industry (Hilden *et al*. 2009). The reaction feasibility is mainly determined by measuring the magnitude of change in Gibbs free energy (ΔG), particularly the conversion of the enzyme-substrate complex to the product. A lower ΔG indicates a readily feasible reaction. The thermal deactivation of the enzyme was found to be non-spontaneous as evident from the positive ΔG. The enthalpy (ΔH), an indicator of the energy required for thermal denaturation of the enzyme, was found to be higher than that of the previously documented fungal laccase (Singha and Panda 2015). This indicates that higher thermal energy is required to break down the stabilising bonds of the enzyme. To the best of our knowledge, this is the first report on the evaluation of thermodynamic parameters for a laccase from *T. pubescens*.

The need for economically efficient and environmentally benign approaches for producing bioactive compounds has led to the intensification of interest in laccases as catalysts in organic synthesis. In the current study, *T. pubescens* laccase-catalysed hetero-coupling of selected plant-derived phenolic compounds was investigated. The formation of the coupling products was

solvent type-, concentration and time-dependent. Quercetin/catechol coupling products were best synthesised in a monophasic system comprising 50% dioxane (reaction time 6 hours). LC-MS analysis of both the crude and purified products revealed a heterodimer ($P_{QC}1$) and a heterotrimer ($P_{QC}2$). Both products displayed low antioxidant activities when compared to quercetin, while $P_{QC}1$ had enhanced activity when compared to catechol. This can be ascribed to the increased number of hydroxyl following the coupling of the two molecules (Kudanga *et al.* 2017).

Comparison of the antimicrobial activities of $P_{QC}1$ and $P_{QC}2$ with those of catechol and quercetin showed that $P_{QC}2$ was a better antimicrobial compound against *L. monocytogenes* and *S. aureus*. Furthermore, $P_{QC}2$ displayed high antimicrobial activity against *E. cloacae*, albeit inferior to that of quercetin. These findings are consistent with the notion that increasing the degree of hydroxylation results in enhanced antimicrobial activity (Borges *et al*. 2013). However, highly hydroxylated phenolic compounds have been reported to be more effective against gram-positive bacteria due to their interaction with the peptidoglycan in the cell wall (Nitiema *et al*. 2012). The compounds modify the permeability and/ or rigidity of the cell wall, and inhibit cellular functions through hydrogen bonding with cellular enzymes (Bouarab-Chibane *et al*. 2019). This could explain the sensitivity of *L. monocytogenes* and *S. aureus* to the highly hydroxylated $P_{QC}2$.

A reaction mixture comprising of 50% dioxane and a reaction time of 6 hours were optimal for the formation of the quercetin/NDGA coupling product (a heterodimer, P_{QN}). The reaction mechanism and structure were proposed based on LC-MS data and published oxidation patterns of both quercetin and NDGA. The 4'-OH on quercetin and NDGA, respectively, have been reported to exhibit the lowest bond dissociation energies of the two substrates (Marković *et al*. 2010; Evgeny and Taisa 2011), thus making the ideal positions for the simultaneous oxidation of quercetin and NDGA. The π-electron conjugation enables the resonance stabilisation of the 5'-quercetin and 5'-NDGA radicals (Fourré *et al*. 2016). The subsequent collision of the two radicals results in a stable 5'-5' heterodimer. The low heat of formation (ΔH_f) of C-C bonds makes them favourable over ether linkages (del Río and Gutiérrez 2008).

The 5'-5' heterodimer displayed superior antioxidant activity compared to the substrates. This can be ascribed to the combined effects of the B- and C-ring hydroxyl groups of quercetin (Valescu and Girma 2002; Ozgen *et al*. 2016), and the additional hydroxyl groups contributed by the two catechol rings contributed by nordihydroguaiaretic acid (Floriano-Sánchez *et al*.

2006; Manda et al. 2020). Comparison of the antimicrobial activity of P_{QN} with those of NDGA and quercetin showed that P_{QN} was a better antimicrobial agent against *L. monocytogenes*, *S. aureus*, *E. coli* and *E. cloacae*. This can be attributed to the abundance of hydroxyl groups that enhance the interaction of the heterodimers with the peptidoglycan cell wall of gram-positive bacteria while the methyl groups facilitate the interaction and formation of complexes with the cell membrane constituents of the gram-negative bacteria (Sarbu *et al*. 2019).

The NDGA/gallic acid coupling product (P_{NG}) was best synthesised in a cosolvent system comprising 60% dioxane after 6 hours. The antioxidant activity of P_{NG} was determined by ABTS, DPPH and FRAP assays. The 4-O-5' heterodimer displayed superior antioxidant activity, exhibiting 1.7- and 2.2-fold increases in the ABTS radical scavenging capacity, 2.1- and 3.0-fold increases in DPPH radical scavenging activity, and 1.4- and 1.8-fold increases in FRAP units in comparison to NDGA and gallic acid, respectively. The improved antioxidant activity can be ascribed to the combined effects of the hydroxyl groups on the catechol rings of NDGA (Floriano-Sánchez *et al*. 2006; Manda *et al*. 2020), and the additional hydroxyl groups contributed by C-3 and C-5 of the gallic acid moiety (Eslami *et al*. 2010; Badhani *et al*. 2015).

The aromatic hydroxyl groups of gallic acid are all capable of exercising their reducing ability (Badhani *et al*. 2015). This ability can be exacerbated by changes in pH, particularly towards alkalinity, due to the different pKa values of the aromatic hydroxyl groups (Eslami *et al*. 2010). Structure-function studies of gallic acid revealed that its radical scavenging efficiency is not only dependent on hydroxyl groups but also steric freedom particularly that of the para-substituted hydroxyl group (Lu *et al*. 2006b). The ortho-substituted hydroxyl groups tend to stabilise the radicalised form of gallic acid through intramolecular hydrogen bonding. This reduces the hydrogen bond dissociation enthalpy and increases antioxidant activity (Badhani *et al*. 2015). NDGA, on the other hand, consists of two catechol rings that confer cytoprotective effects as a strong scavenging agent of multiple types of reactive oxygen and nitrogen species (Floriano-Sánchez *et al*. 2006). Due to the symmetrical nature of NDGA, it is capable of donating electrons through any of its catechol rings (Manda *et al*. 2020).

Overall, the results indicate the dependence of the activity of the enzyme on the type of substrate, which was evident from the evaluation of both substrate affinity and catalytic efficiency against selected common substrates. The enzyme maintained activity in the presence of heavy metal ions, organic solvents, high temperatures, and a wide pH range, which indicates the robust nature

of the enzyme for potential application in diverse environments. The enzyme also proved to be a potentially valuable tool for the green synthesis of novel bioactive compounds.

6. CONCLUSION AND RECOMMENDATIONS

6.1. CONCLUSION

Laccase facilitated biocatalysis remains an ideal approach for the synthesis of phenolic compounds, particularly, antioxidants. Owing to a clean reaction mechanism that uses molecular oxygen and produces water as the only by-product, laccases are considered green catalysts and offer an exciting opportunity for industrial applications. This study aimed to utilise laccases from *T. pubescens* CBS 696.94 in the synthesis of hybrid antioxidants from plant-derived natural phenolic antioxidants.

These major findings of this study can be summarised as follows:

- Laccase was successfully produced from *T. pubescens* CBS 696.94. The enzyme was purified using $(NH_4)_2SO_4$ precipitation, ultrafiltration, anion exchange chromatography and size exclusion chromatography. The molecular weight of the purified enzyme was ~ 58 kDa
- From the screening process, caffeic acid, guaiacol, *p*-coumaric acid, vanillic acid, eugenol, catechol, gallic acid, ferulic acid, quercetin, and nordihydroguaiaretic acid were identified as laccase substrates.
- Coupling reactions resulted in the formation of catechol and quercetin heterodimer (*m/z* 409) and heterotrimer (*m/z* 709), quercetin and nordihydroguaiaretic acid heterodimer (*m/z* 601), gallic acid and nordihydroguaiaretic acid heterodimer (*m/z* 469).
- Product yield was optimised in a monophasic system containing sodium acetate buffer pH 5.0 and 50% or 60% v/v dioxane as cosolvent at an agitation speed of 200 rpm, 37°C and a reaction time of 6 hours.
- The catechol and quercetin heterodimer showed inferior antioxidant activity, while the heterotrimer displayed enhanced antimicrobial activity against *L. monocytogenes* and *S. aureus*.
- The quercetin/nordihydroguaiaretic acid and gallic acid/ nordihydroguaiaretic heterodimers showed superior antioxidant activities compared to their respective substrates. The quercetin/nordihydroguaiaretic acid also showed enhanced antimicrobial activity against *L. monocytogenes*, *S. aureus*, *E. coli* and *E. cloacae*.

Overall, the current study demonstrated the potential of *T. pubescens* laccase as a viable biocatalyst for the synthesis of hybrid antioxidants. Four antioxidant compounds, a catechol/quercetin heterodimer and heterotrimer, a quercetin/NDGA heterodimer, and a gallic acid/ NDGA heterodimer were successfully produced. The products have potential for application as alternative antioxidants or antibacterial agents.

6.2. RECOMMENDATIONS

The application of laccases as biocatalysts offers an environmentally friendly approach for the biotransformation and/ or synthesis of bioactive compounds. Nevertheless, several considerations and modifications are required to allow the transition of this green technology from laboratories into realistic industrial processes. There is still a need to develop robust laccase variants with properties that are ideal for application in specific industrial processes. These properties include thermostability, and tolerance to inhibitors, organic solvents and extremes of pH (Mate and Alcalde 2015; Kudanga and Le Roes-Hill 2014; Kunamneni *et al*. 2008). Protein engineering offers a compelling pathway towards designing industrially relevant laccases through for example directed evolution (Rodgers *et al.* 2010; Santhanam *et al.* 2011). This method relies on an iterative two-step protocol that initially generates molecular diversity by random mutagenesis and in-vitro recombination, followed by the screening of library members with improved properties (Lutz 2010). These iterative cycles of mutagenesis and screening have been reported to enhance thermostability, tolerance to organic solvents, enantio-selectivity, and substrate specificity (Jäckel and Hilvert 2010).

One of the most noticeable phenomena of the current study was the strikingly low yields of the intended products coupled with a high rate of polymerisation as a side reaction. This limitation is consistent with numerous reports on the application of laccases for biotransformation (Lee and Liu 2003; Gavezzotti *et al.* 2014). Another key issue is producing chirally pure compounds. The stereospecificity of *in vivo* laccase-catalysed biosynthesis reactions opens a promising avenue through which stereospecificity can be achieved for *in vitro* laccase reactions (Orlandi *et al.* 2001; Zoia *et al.* 2008). Some researchers have identified key proteins for the regulation of laccase-catalysed reactions such that only a single isomer is produced (Davin and Lewis 2005). Bioprospecting for similar analogous proteins in nature, as well as enlisting molecular biology to design proteins with a similar regulatory effect can lead to the production of desired bioactive compounds. Other researchers have opted for the protection of functional groups that present a statistical and thermodynamic opportunity for the formation of undesired products through the attachment of either a chiral auxiliary compound (Orlandi *et al*. 2001) or a benzyl group (Gavezzotti *et al*. 2014). Combining this with reaction engineering may be the key to improve yields of the desired product and therefore moving laccase-catalysed synthesis of bioactive compounds from benchtop to realistic industrial applications. Other envisaged future studies could include the bioavailability, solubility and other pharmacological properties of the synthesised compounds.

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APPENDIX 1. *Trametes* defined media, and trace elements

Table: 1 L of Trametes defined media (TDM)

Glucose	10.0 g
Peptone	5.23 g
KH_2PO_4	2.00 g
$MgSO_4$	0.50 g
$CaCl_2$	0.10 g
NaCl	0.29 g
Thymine	0.05 g
Wheat bran	10.0 g

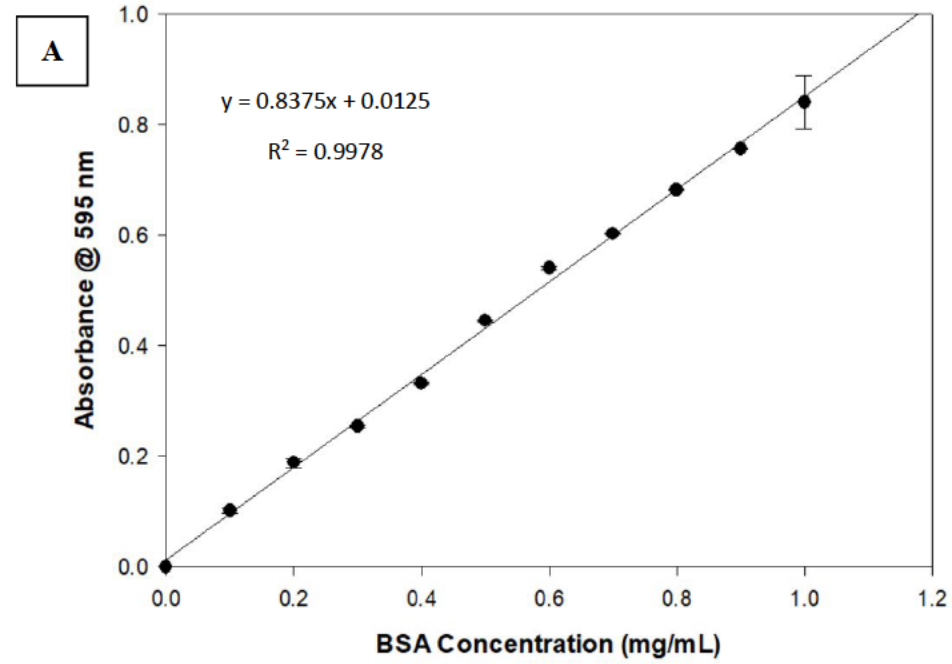
Table: 500 ml trace elements

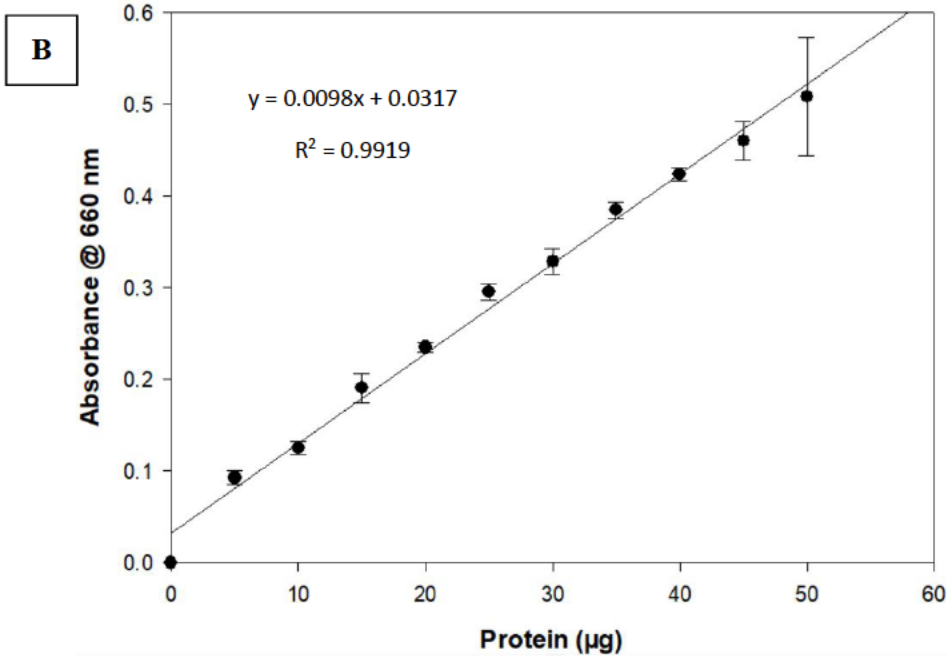
Iron Sulphate	0.28 g
Copper Sulphate	0.25 g
Zinc Chloride	0.034 g
Manganese Sulphate	0.169 g
Cobalt Chloride	0.095 g
Ammonium Molybdate	0.309 g

APPENDIX 2. Supplementary data to the fermentation process of *Trametes pubescens*

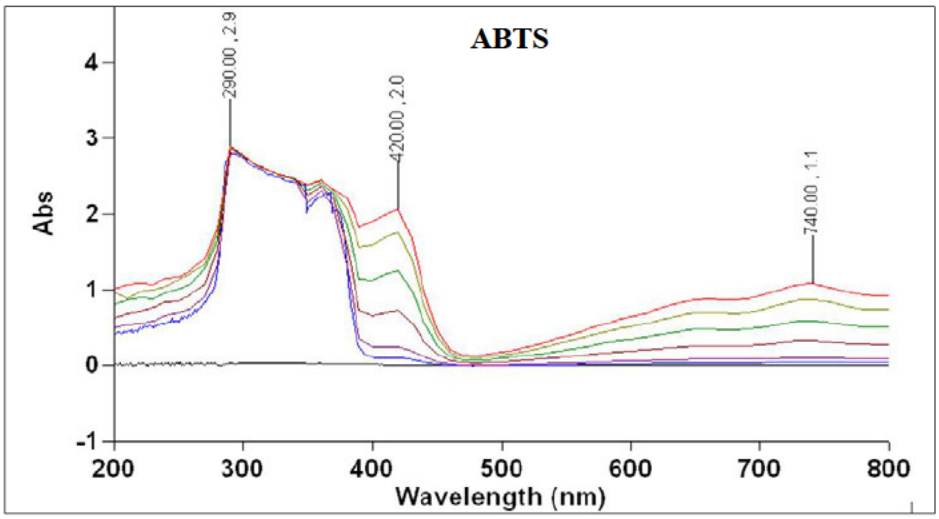
Days	Activity 1 (U/mL)	Activity 2 (U/mL)	Activity 3 (U/mL)	Average Activity (U/mL)	StdDev
Starter Culture	0.10	0.09	0.13	0.11	0.02
1	0.12	0.14	0.11	0.12	0.02
2	0.46	0.41	0.39	0.42	0.04
3	0.74	0.68	0.70	0.71	0.03
4	1.58	1.43	1.62	1.54	0.10
5	2.34	2.28	2.36	2.33	0.04
6	1.96	1.89	1.84	1.89	0.06
7	1.89	1.85	1.81	1.85	0.04

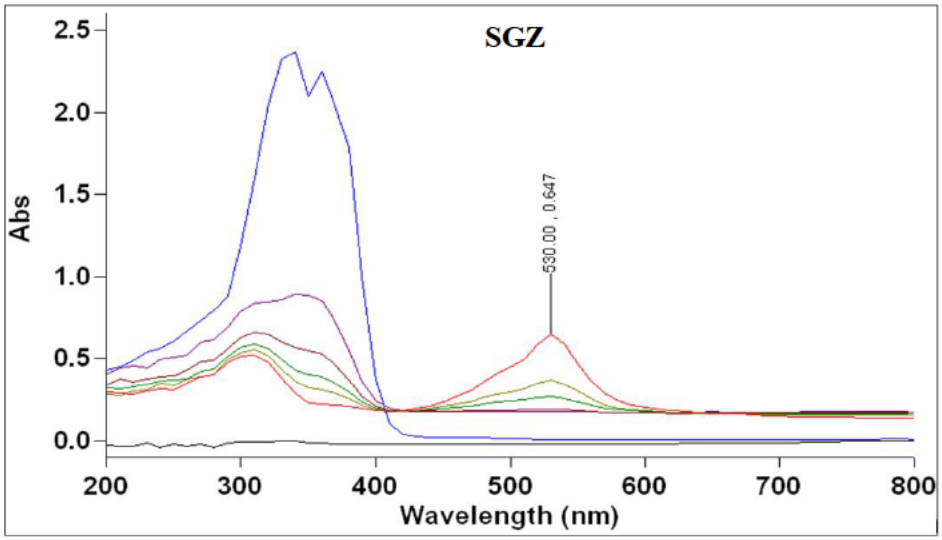
APPENDIX 3. Standard curves for the Bradford (B) and Lowry (B) protein assays

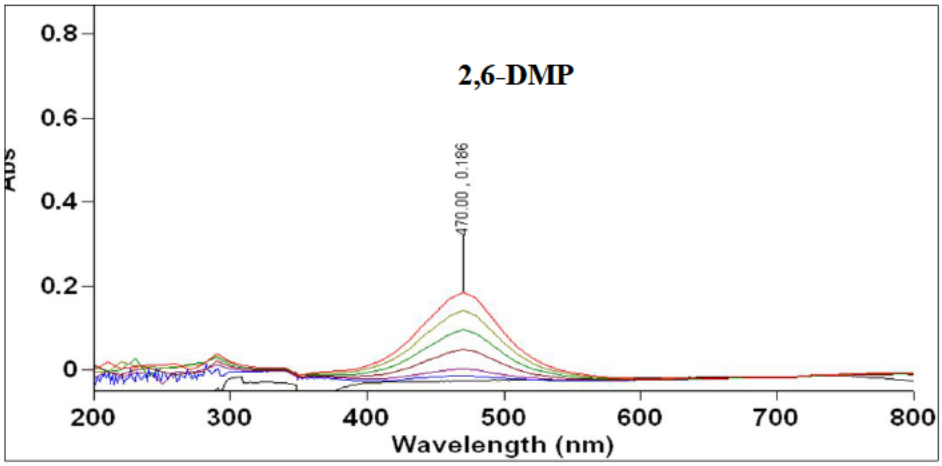




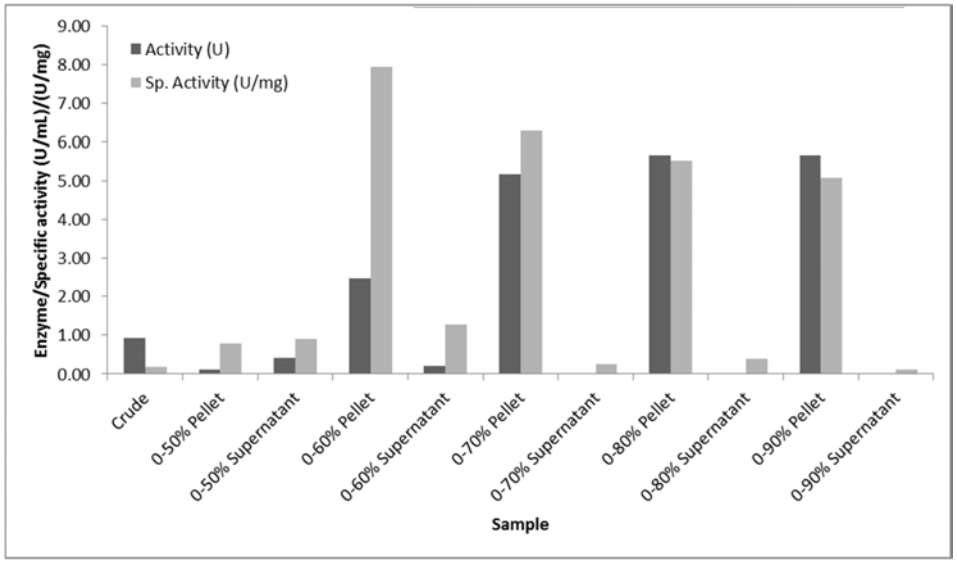
APPENDIX 4. Wavelength scans obtained from monitoring product formation in the laccase-catalysed oxidation reactions of ABTS, SGZ and 2,6-DMP



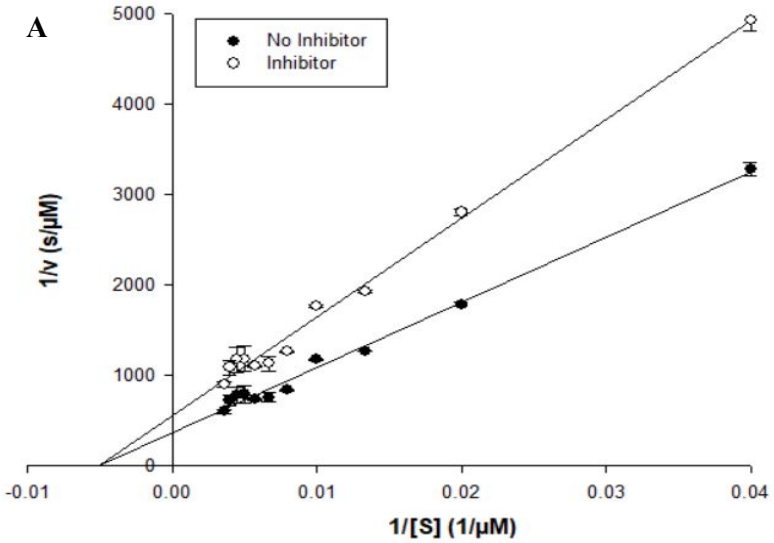




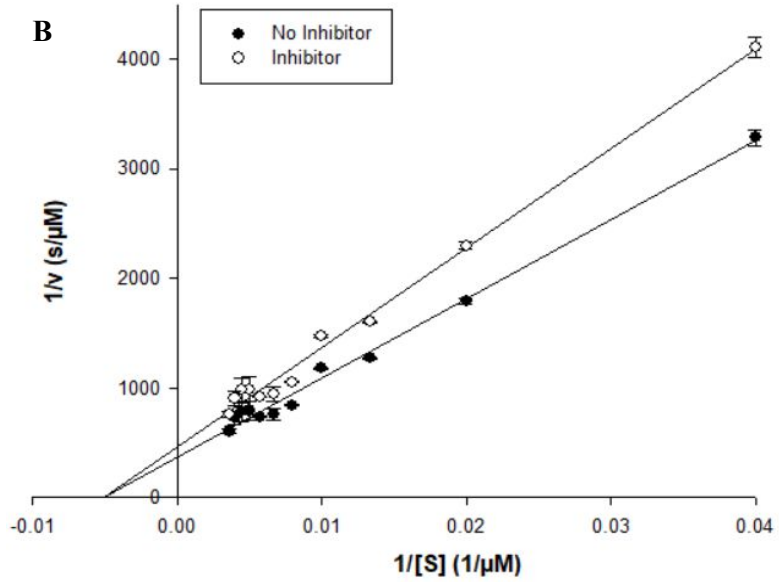
APPENDIX 5. Partial purification of Tp-laccase by ammonium sulphate precipitation



APPENDIX 6. The Lineweaver-Burk plot showing an inverse relationship between the rate of substrate (ABTS) conversion by Tp-laccase and the concentration of the substrate, both in the presence and absence of (A) NaN_3, (B) SDS, (C) DTT, (D) H_2O_2, and (E) L-cysteine. All values are means of three replicates ± standard deviation



B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Inhibitor
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1/[S] (1/μM)
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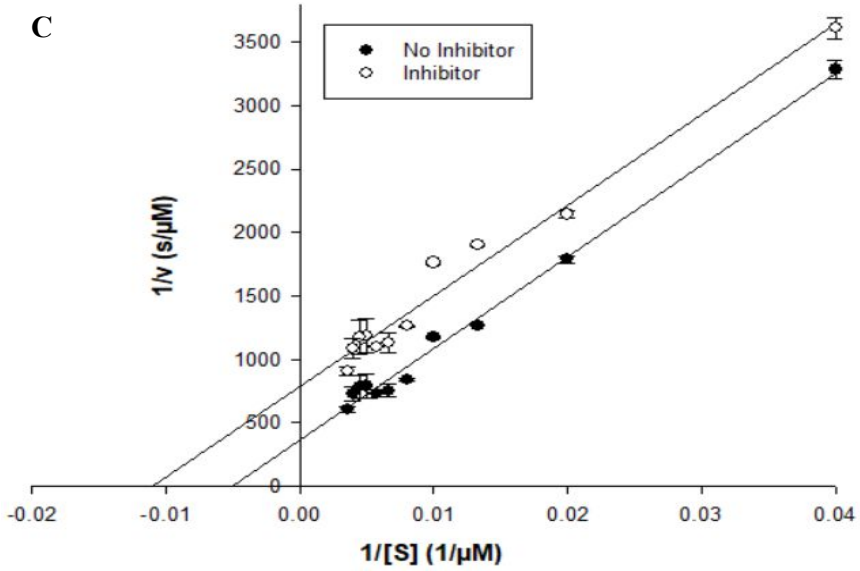
3000
2000
1000
0
-0.01
0.00
0.01
0.02
0.03
0.04

C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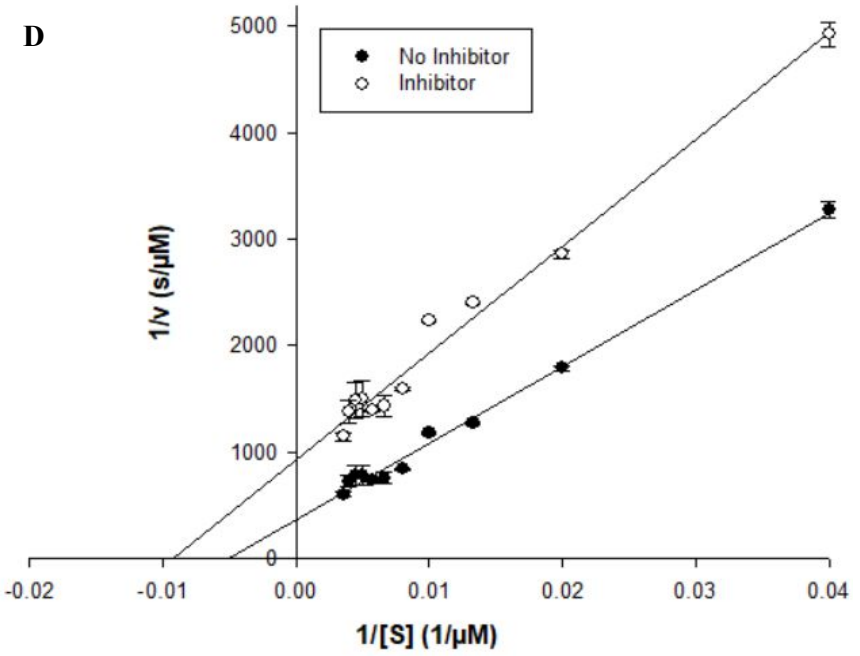
0.00
0.01
0.02
0.03
0.04

D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1000
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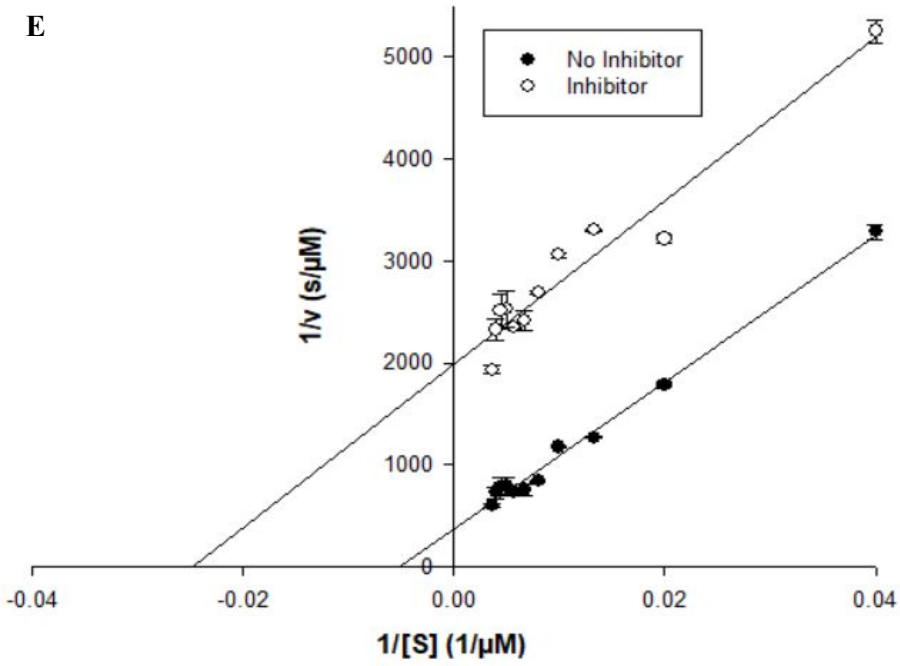
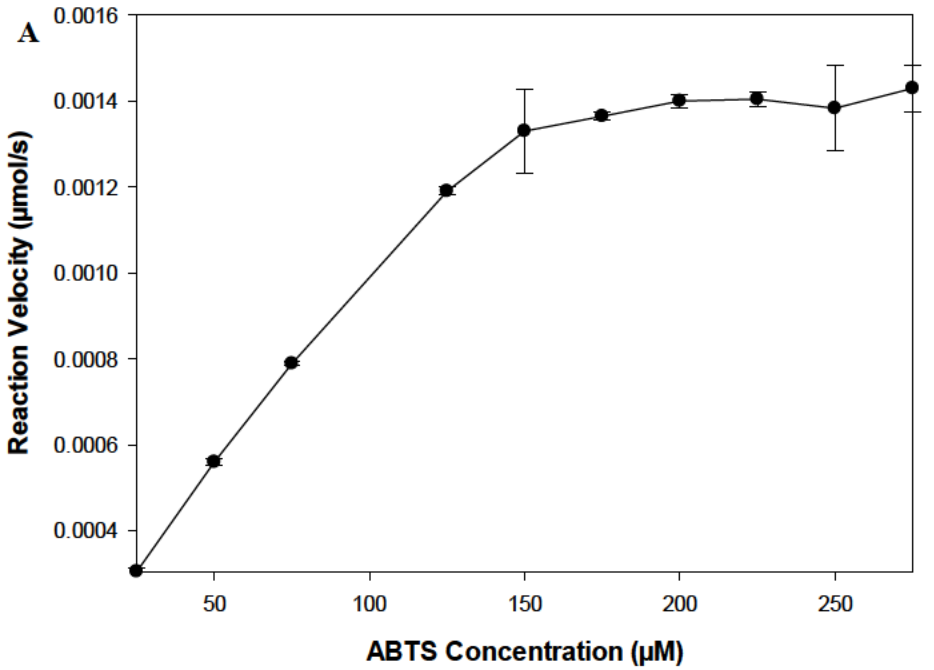
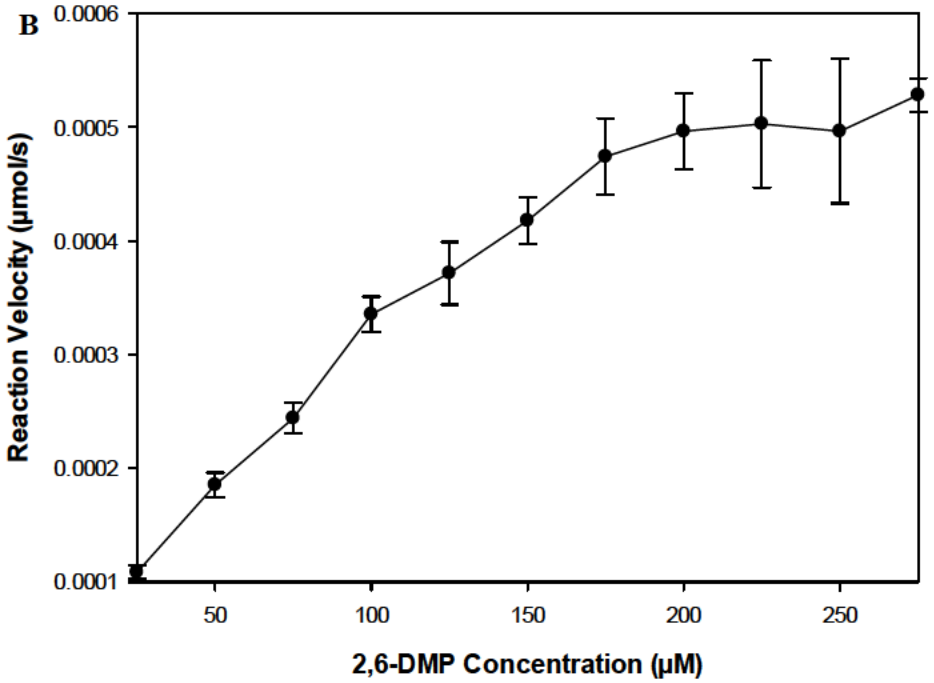
-0.01
0.00
0.01
0.02
0.03
0.04

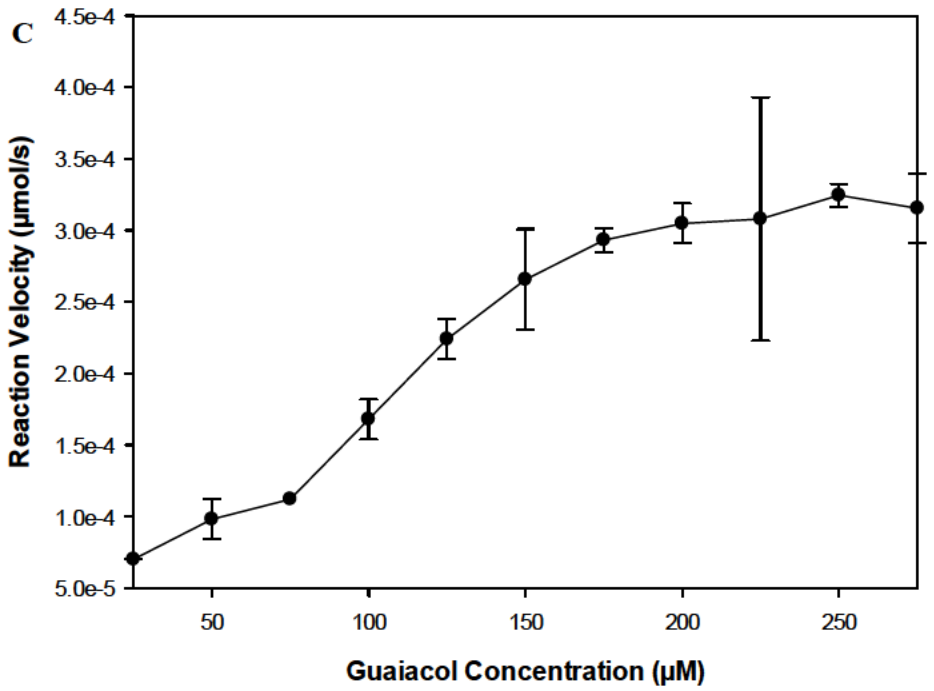
E

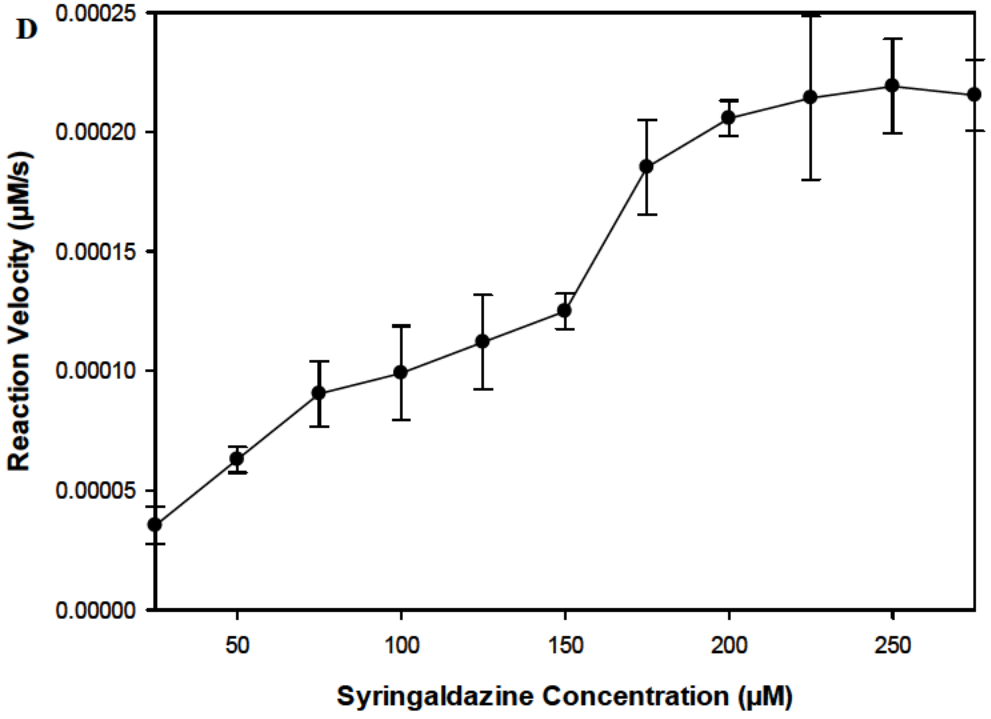

No Inhibitor
Inhibitor
1/v (s/μM)
1/[S] (1/μM)
5000
4000
3000
2000
1000
0
-0.04
-0.02
0.00
0.02
0.04

APPENDIX 7. Steady-state Michaelis-Menten plots showing the relationship between the substrate conversion rate of Tp-laccase and the concentration of selected substrates; (A) ABTS, (B) 2,6-DMP, (C) Guaiacol, and (D) SGZ. All values are means of three replicates ± standard deviation obtained by spectrophotometrically monitoring the rate at which the oxidation product is formed (ABTS at 420 nm; Syringaldazine at 530 nm and 2,6-DMP at 470 nm)

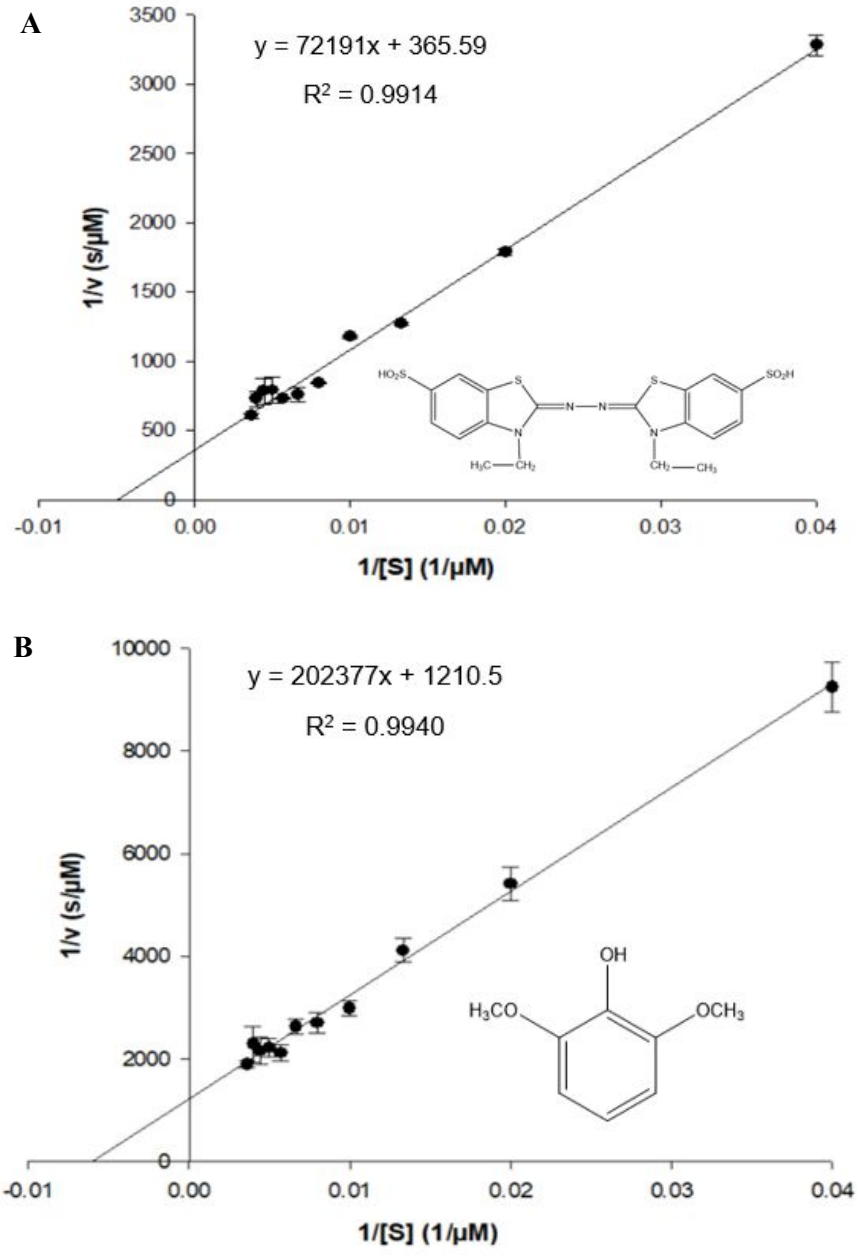




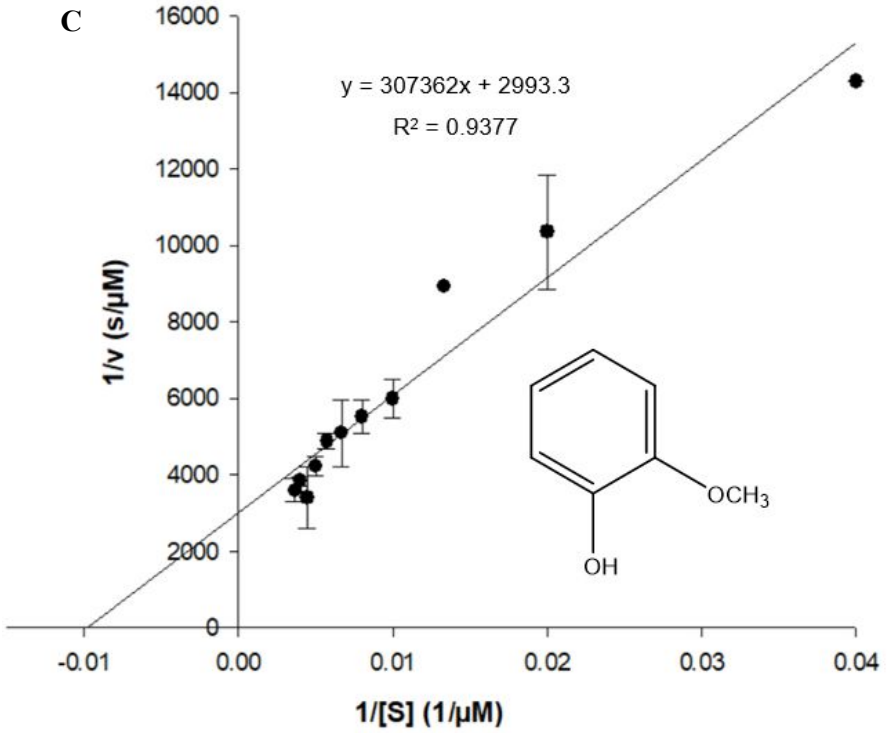

C
4.5e-4
4.0e-4
3.5e-4
3.0e-4
2.5e-4
2.0e-4
1.5e-4
1.0e-4
5.0e-5
Reaction Velocity (µmol/s)
50
100
150
200
250
Guaiacol Concentration (µM)


D
0.00025
0.00020
0.00015
0.00010
0.00005
0.00000
Reaction Velocity (µM/s)
50
100
150
200
250
Syringaldazine Concentration (µM)

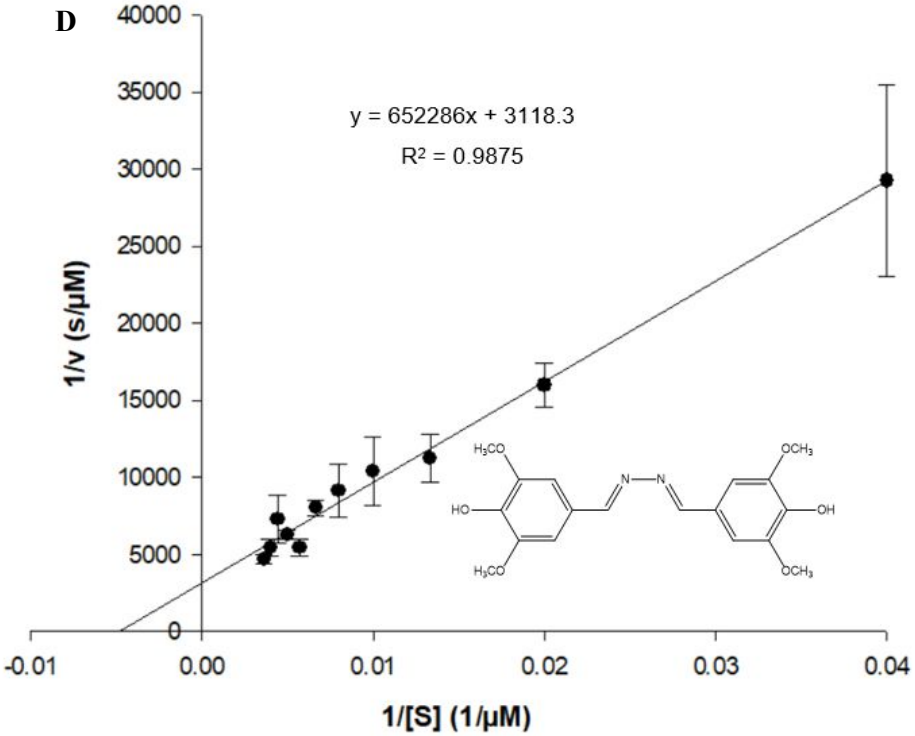
APPENDIX 8. The Lineweaver-Burk plots showing an inverse relationship between the rate of substrate conversion by Tp-laccase and the concentration of selected substrates; (A) ABTS, (B) 2,6-DMP, (C) Guaiacol, and (D) SGZ. All values are means of three replicates ± standard deviation obtained by calculations from the values obtained in the accompanying Michaelis-Menten plot (Appendix 7)



C


y = 307362x + 2993.3
R² = 0.9377
1/v (s/µM)
1/[S] (1/µM)
16000
14000
12000
10000
8000
6000
4000
2000
0
-0.01
0.00
0.01
0.02
0.03
0.04
OCH3
OH

D


y = 652286x + 3118.3
R² = 0.9875
1/v (s/µM)
1/[S] (1/µM)
40000
35000
30000
25000
20000
15000
10000
5000
0
-0.01
0.00
0.01
0.02
0.03
0.04
H3CO
HO
OCH3
OH
N
N

APPENDIX 9. The LC-MS analysis of a reaction mixture harbouring the laccase-catalysed coupling of gallic acid and nordihydroguaiaretic acid.