SUBSTRATE SPECIFICITY OF LACCASE FROM *Pleurotus pulmonarius* TOWARDS AROMATIC COMPOUNDS

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TITLE PAGE

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A DISSERTATION SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF NIGERIA, NSUKKA IN PARTIAL FUFILMENT OF THE REQUIREMENT FOR THE AWARD OF MASTER OF SCIENCE (M. Sc.) IN BIOCHEMISTRY (ENZYMOLOGY)

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FEBRUARY, 2020

CERTIFICATION

Isiwu, Chukwurobe Vitus, a postgraduate student of the Department of Biochemistry, with Registration Number, PG/M.Sc/17/03280 in the Department of Biochemistry, Faculty of Biological Sciences, University of Nigeria, Nsukka has satisfactorily completed the requirements for the course work and research for the award of the degree of Master of Science (M. Sc.) degree in Biochemistry (Enzymology and Protein Chemistry). The work embodied in this dissertation is original and has not been submitted in part or in full for any other diploma or degree of this or any other University.

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External Examiner

DEDICATION

This work is dedicated to Almighty God, the great grand architect of the Universe

ACKNOWLEDGEMENTS

My profound gratitude goes to the Almighty God for his protection and guidance throughout the course of the work. My appreciation also goes to my supervisors, Prof. F. C. Chilaka and Dr. A. L. Ezugwu for their painstaking supervision during the course of this work. I also wish to thank Dr. T. C. Ezike for his tremendous effort towards the success of this work. I humbly wish to thank Dr. P. E. Joshua, the postgraduate coordinator for his tremendous effort and fatherly love shown to me during the course of this work. I also want to use this opportunity to thank my brother Dr. E. C. Isiwu for his moral and financial support.

ABSTRACT

This study was carried out with aim of ascertaining the substrate specificity of laccase from *Pleurotus pulmonarius* towards aromatic compounds. A pure culture of *Pleurotus pulmonarius* was obtained after it has been sub-cultured from its stock culture and grown on potatoes dextrose agar (PDA). The pure culture of *Pleurotus pulmonarius* showed a positive result when screened for laccases expression using 2, 2- azinobis 3- ethyl-benzothazoline 6-sulfonate (ABTS) as substrate. A pilot study for laccase production was carried out for 14 days of which day nine (9) showed the day of maximum laccase activity of 590 U/ml. The total volume of 1000 ml of the enzyme was produced, in solid state fermentation system (SSFs) using wheat bran as the lignocellulosic based substrate, which served as the crude. The 80 % ammonium sulphate saturation was found suitable to precipitate protein with highest laccase specific activity of 3252 U/mg. After dialysis, the specific activity was observed to be 7310 U/mg. The purification folds after ammonium sulphate saturation, dialysis and gel filtration were 1.27, 2.85 and 3.71, respectively. The percentage yields after ammonium sulphate saturation, dialysis and gel filtration were 3.78, 2.68 and 1.90, respectively. Laccases from *Pleurotus pulmonarius* showed activity within the broad range of pH of (3.0 to 6.5) and temperature range of 30 $^{\circ}$C - 70 $^{\circ}$C. The optimum pH was 4.0 with an activity of 566.124 U/ml, while optimum temperature was observed at 50 ^{0}C with an activity of 557.234 U/ml. In the effect of substrate concentration, 90 µM of ABTS gave highest activity of 61.24 µmol/min, with Vmax of 90.91 µmol/min and K_M of 31.27 µM. The laccase specificity for ABTS, α-Naphthol, 2, 6-DMP and O-dianisidine were found to be 2.91, 3.50, 20.83 and 27.03, respectively. The high specificity of laccase for various aromatic compounds makes laccase a promising enzyme for biodegradation of dyes which is eco-friendly.

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LIST OF ABBREVIATIONS

MCOs	Multi Copper Oxidases
ABTS	2, 2- azinobis 3- ethyl-benzothazoline 6-sulfonic acid
2, 6-DMP	2, 6-Dimethoxyphenol
PDA	Potatoes Dextrose Agar
DABSA	2,5-diaminobenzenesulfonic acid ()
SSFs	Solid State Fermentation system
pH	Pondius Hydronium
Mg^{2+}	Magnesium ion
Fe^{2+}	Iron II ion
Ca^{2+}	Calcium ion
Zn^{2+}	Zinc ion
MREs	Metal Response Elements
XREs	Xenobiotics Response Elements
HSREs	Heat Shock Response Elements
PCP	Pentachlorophenol
2, 4-DCP	2, 4-dichlorophenol
BSA	Bovine Serum Albumin

CHAPTER ONE

INTRODUCTION

Laccases (EC 1.10.3.2) belong to the family of blue multi-copper oxidases (MCOs), which catalyze one-electron oxidation of donor substrates, resulting in reduction of molecular oxygen to water (Rajesh *et al*., 2016). Laccase is an enzyme that oxidizes phenolic substrates such as α-naphthol, 2, 6-Dimethoxyphenol, guaiacol and ABTS (2, 2- azinobis 3- ethyl-benzothazoline 6-sulfonate) (Francesca *et al*., 2006). Laccase was first described by Yoshida in 1883 when he extracted it from the exudates of the Japanese lacquer tree *Rhus vernicifera*, from which the name laccase was derived (Yoshida, 1883**).** Laccases are produced by va3rious sources like fungi, bacteria, plants and insects (Imran *et al*., 2012). The redox potentials of laccases vary and are generally classified as low, medium and high- redox potential function (Mate and Alcalde, 2015). Laccases from ascomycetes and basidiomycetes form medium and high redox potential having an oxidation potential ranging from +710 to +790 mV, respectively (Rodgers *et al*., 2010). Multi-copper oxidases contain a minimum of four coppers divided into type 1(T1), type 2 (T2) and binuclear type 3 (T3) which consists of Cu^{2+} and Cu^{3+} (Stephen and Edward, 2015). Oxidation of substrates takes place near T1, and electrons are transferred through the protein via the Cys-His pathway to the T2/T3 trinuclear copper cluster (Rukmakesh *et al*., 2018). The ability of laccases to act on a range of substrates and also to detoxify a lot of pollutants have made them to be usable for several purposes in many industries including paper, pulp, textile and petrochemical industries (Couto and Toca, 2006). Though, some substrates cannot be directly oxidized by laccases, either because of their large size which restricts them from gaining access into the enzyme active site or because of their high redox potential. This hindrance can be overcome by using suitable chemical mediators which are oxidized by the laccase and their oxidized forms are then able to interact with high redox potential substrate targets (Arora and Sharma, 2010). Enzyme processes are potentially energy saving and also save investing in special equipment resistant to heat, pressure or corrosion. Due to their efficiencies and their specific actions, the mild conditions in which they work, and their high biodegradability, enzymes are well suited for a wide range of industrial applications. Enzymes work only on renewable raw materials. Fruit, cereals, milk, fats, cotton, leather and wood are some typical candidates for enzymatic conversion in industry. Enzymes are used in the textile industry

because they accelerate reactions, act only on specific substrates, operate under mild conditions, are safe and easy to control, can replace harsh chemicals and enzymes are biologically degradable.

1.1 Optimum Conditions for Laccase Production

To achieve maximum laccase production, nutritional conditions such as carbon, nitrogen and inducer sources must be optimized as well as physical conditions such as pH, agitation and inoculum size (Jie *et al.,* 2017). The addition of copper before inoculation effectively increases the laccase production compared to the addition of copper some hours after inoculation (Murugesan and Vembu, 2016).

1.2 Effect of Carbon and Nitrogen Source on Laccase Production

The organism grown in the defined medium contains 0.1 %w/v yeast extract and 1 % (w/v) different carbon and nitrogen sources. Glucose, mannose, maltose, fructose, and lactose are the most commonly used carbon sources. High glucose and sucrose concentration in the production media reduce the production of laccase by obstructing the initiation process. Many previous studies have proved that both the nature and concentration of nitrogen sources are powerful nutritional factors regulating ligninolytic enzyme production by wood-rotting basidiomycetes (Mikiashvili *et al*., 2005). This problem of obstruction in enzyme production can be improved by using polymeric substrates like cellulose. Yeast extract, peptone, urea, $(NH_4)_2SO_4$, and $NaNO_3$ are the commonly used nitrogen sources in laccase production and is commonly triggered by nitrogen depletion (Nona *et al*., 2006) but some nitrogen strains do not affect the enzyme activity.

1.3 Effect of pH on Laccase Production

The pH of the culture medium influenced the growth of the organism. In general, fungi grow over a wide range of pH, with the optimum pH being situated mostly in the acid range (Nurdan *et al*., 2005). The pH does not only affect the growth but also the production and activity of metabolites due to the effect of pH on cell permeability and the availability of certain divalent metal ions such as $Mg^{2+,}$ Fe^{2+}, Ca^{2+} and $Zn^{2+.}$ The influence of pH on the growth, production and activity of ligninolytic enzymes and the degradation of aromatic compounds such as lignin and environmental pollutants by basidiomycetes have been studied severally by different scholars.

1.4 Effect of Temperature on Laccase Production

The optimal temperature of laccase is dependent on the strain type. It has been found that 25 ºC is the optimal temperature for laccase production in presence of light, but, in case of dark, the optimal temperature is 30 ºC (Afreen *et al*., 2018). The optimum temperature range for laccase production is between 30ºC and 35 ºC (Rajesh *et al*., 2016). The laccase from *Ganoderma lucidum* is almost fully active in the temperature range of 40 ºC– 70 ºC, with maximum activity at 50 ºC (Peng *et al*., 2019). Laccase produced by *T. modesta* was fully active at 50 ºC and was very stable at 40 ºC but half-life decreased to 120min at higher temperature (60 ºC) (Nyanhongo *et al*., 2002).

1.5 Mechanism of Catalysis of Laccases

Laccase attacks the phenolic compounds leading to Cα oxidation, Cα-Cβ cleavage and aryl-alkyl cleavage (Pankaj *et al*., 2013). Similar enzymes lacking the Cu atom responsible for the blue colour are called yellow or white laccases. For laccases to perform their catalytic functions, they depend on Cu atoms that are distributed at the three different copper centers (Ram and Pankaj, 2014). Type-1 or blue copper centre, Type-2 or normal copper centre and Type-3 or coupled binuclear copper centres, differ in their characteristics electronic paramagnetic resonance (EPR) signals (Claudia *et al*., 2013). The organic substrate is oxidized by one electron at the active site of the laccase generating a reaction radical which further reacts non-enzymatically. The electron is received at type1 Cu and is shuttled to the trinuclear cluster where oxygen is reduced to water. It has been proposed that the mononuclear copper type1 function as the primary electron acceptor extracting electron from the reducing substrate and delivering it to the trinuclear site where oxygen is reduced to water and the oxidized form of the enzyme is regenerated.

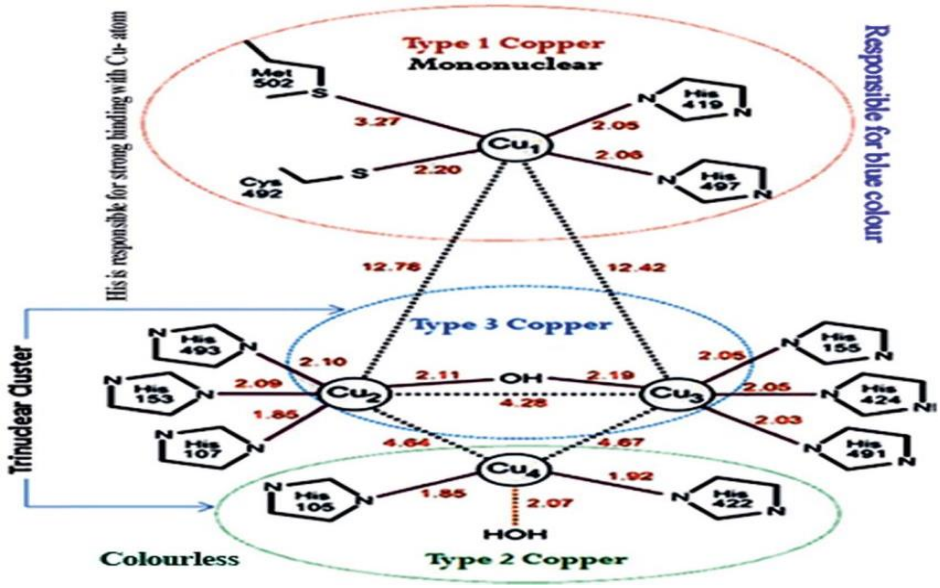

Figure 1: Active site of laccases, showing (T1 site) and a three nuclear copper cluster (T2/T3 site) consisting of one T2 copper ion and two T3 copper ions (Ram and Pankaj, 2014)

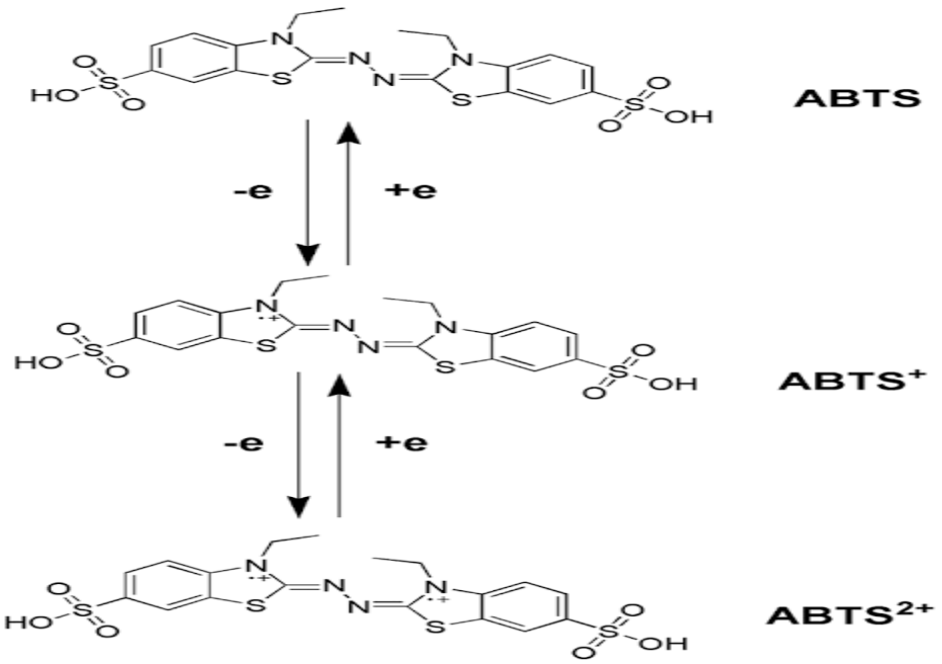

Figure 2: Oxidation/reduction of ABTS by Laccase (Gerhard, 2017)

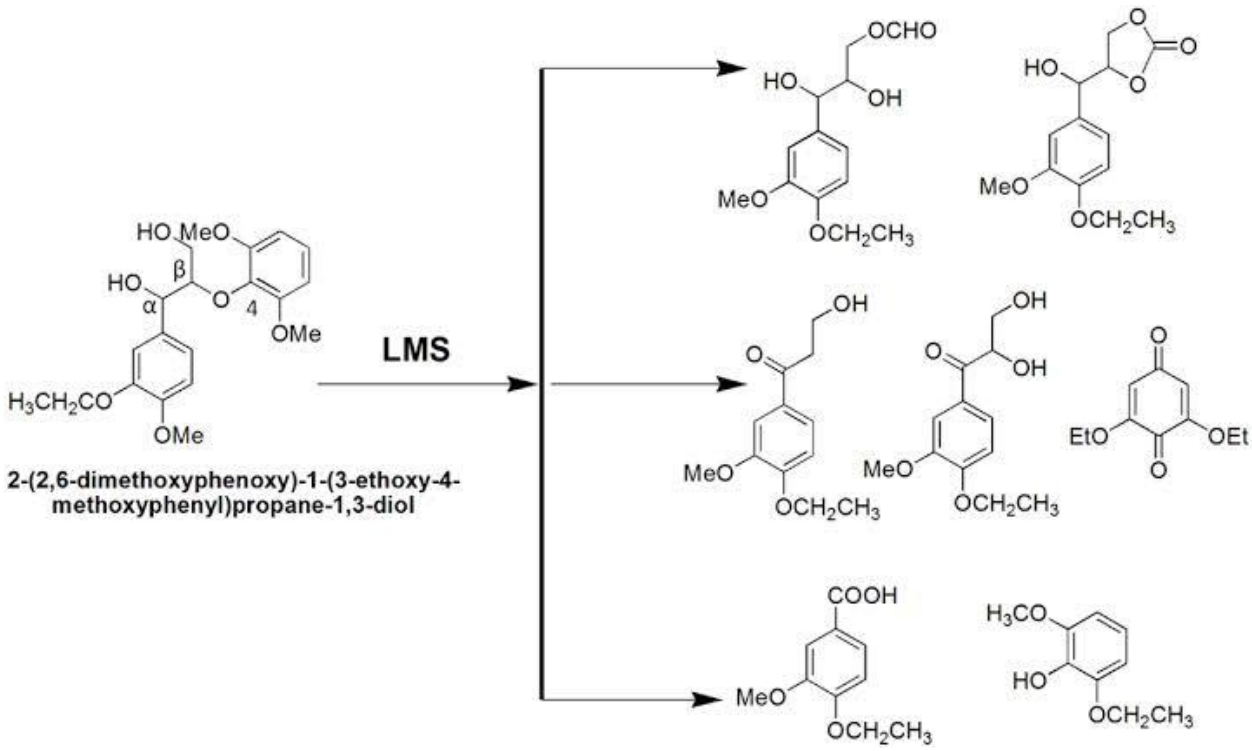

Figure 3: Lignin biodegradation with laccase-mediator systems (Lew *et al*., 2014)

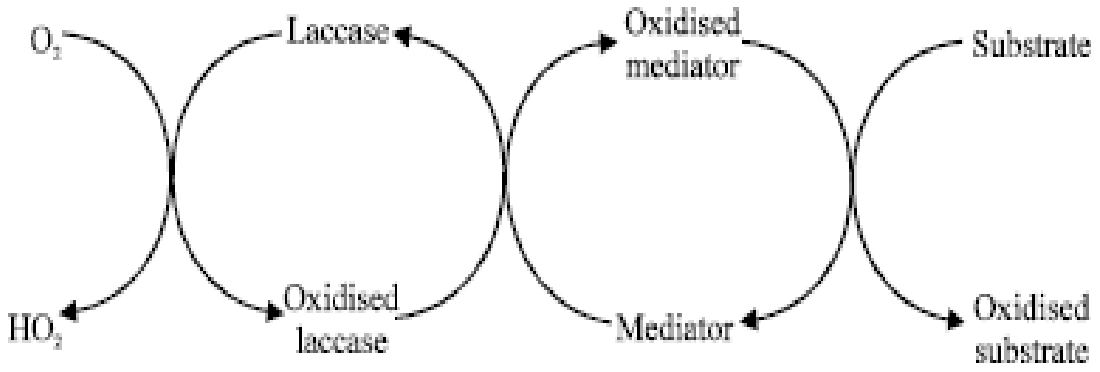

Figure 4: Catalytic cycle of a laccase-mediator oxidation system (Johann *et al.*, 2010).

1.6 Functions of Laccases

a. Laccases play a role in the formation of lignin by promoting the oxidative coupling of monolignols , a family of naturally occurring phenols

b. Laccases produced by fungus *Pleurotus ostreatus*, play a role in the degredation of lignin, and can therefore be classified as lignin-modifying enzyme

c. Laccases produced by fungi can facilitate the biosynthesis of melanin pigments (Lee *et al*., 2019)

d. Laccass catalyse ring cleavages of aromatic compounds

1.7 Natural Laccase Producers

1.7.1 Fungi

Laccases of fungal origins have been the most intensively studied. Fungal laccases are implicated in both intra- and extra-cellular physiological processes including delignification, morphogenesis, pigmentation, and pathogenesis (Forootanfar and Faramarzi, 2015). Among fungi, ascomycetes, basidiomycetes, and deuteromycetes can produce laccases, and white-rot basidiomycetes are the most efficient lignin degraders and laccase producers (Arora and Sharma, 2010). *Pleurotus ostreatus* and *Trametes versicolor* can be regarded as the model organisms in basic and applied laccase research. Other well-known laccase-producing basidiomycetes include *Agaricus bisporus*, *Cerrena unicolor*, *Coprinopsis cinerea*, *Coriolopsis gallica*, *Cryptococcus neoformans*, *Cyathus bulleri*, *Fomes fomentarius*, *Ganoderma lucidum*, *Panus rudis*, *Phlebia radiata*, *Polyporus brumalis*, *Pycnoporus cinnabarinus*, *Pycnoporus sanguineus*, *Rigidoporus microporus*, *Schizophyllum commune*, as well as various Pleurotus (e.g*., P. eryngii, P. florida, P. pulmonarius, and P. sajor-caju)* and Trametes (e.g., *T. hirsuta*, *T. pubescens, T. trogii, and T. villosa)* species (Forootanfar and Faramarzi, 2015). Laccase yields are variable depending on the species and strain, but most naturally occurring species appear to be poor laccase producers. However, screening and selection of promising laccase producers from nature, followed by optimization of culture conditions, still constitute a viable and effective approach to obtain organisms with tremendous laccase synthesis ability (Elisashvili and Kachlishvili, 2009). The genus Cerrena with high laccase yields and application potentials deserves attention, and the properties of its laccase can be even more desirable compared to the commercial ones. However, *Cerrena species* are relatively less studied especially compared with *Trametes species*. *Cerrena unicolor,* is a medicinal mushroom with antitumor and other activities (Matuszewska *et al.,* 2016).

1.7.2 Insects

Insects producing laccases are found among insects of genera *Drosophilia, Oryctes, Manduca, Diploptera, Musca, Papilio, Bombyx, Calliphora, Lucilia, Phormia, Rhodnius, Sarcophaga, Schistocerca*. The insect laccase is long and the terminal sequence is characterized by by unique domain consisting of several conserved cystine, aromatic and charged residues (Mishra *et al*., 2015). In a research conducted by Sharma and Kuhad (2008), two isoforms of laccase 2 gene have been found to catalyze larval, pupal and adult cuticle tanning in *Tribolium castaneum*.

1.7.3 Bacteria

Till date, laccases have mostly been isolated from fungi and plants, whereas laccase from bacteria has not been well studied. Bacterial laccases have several unique properties that are not characteristics of fungal laccases such as stability at high temperature and high pH. Bacteria produce these enzymes either extracellularly or intracellularly and their activity is in a wide range of temperature and pH (Prakram *et al*., 2017). The laccase-producing bacteria are mainly Gram positive. e.g., *Bacillus, Geobacillus, Streptomyces, Rhodococcus, Staphylococcus, Azospirillum, Lysinibacillus and Aquisalibacillus (* Dhiman and Shirkot 2015).

1.7.4 Plants

Plant sources of laccase include Trees (such as Mango Pine), cabbage, turnip, beets, apples, potatoes, pears and various vegetables (Mishra *et al*., 2015). However, in recent time, laccase have been found to be expressed in embyo of maize.

1.8 Regulations of Laccase Expression

Laccase production is improved by fermentation technology development following the optimization of the medium composition and cultivation parameters (Forootanfar and Faramarzi, 2015). Enhancing laccase yields is essential to promote industrial applications and lower the production costs of the enzyme, which relies on understanding of laccase expression regulation (Janusz *et al*., 2013). Numerous publications and reviews have been devoted to expression regulation of laccases. Expression of laccase isozyme genes is regulated differentially throughout fermentation. In response to medium composition, such as metal ions, xenobiotics as well as nutrient types and levels, laccase expression analysis has been performed on mRNA and protein levels, and the distinct responses of species, strains as well as genes no doubt paint a complex

picture of laccase expression regulation. In accordance, various cis-acting responsive elements have been identified in laccase promoter regions, such as metal response element (MREs), copper-responsive transcription factor binding sites (ACE1), xenobiotic response elements (XREs), antioxidant response elements (AREs), heat shock response elements (HSEs), CreA binding sites (CreA), and NIT2 binding sites (NIT2) (Janusz *et al.*, 2013). Copper ions are probably the most used inducer in laccase production, and the ACE1 binding site represents the most well- understood regulatory element in the laccase promoter region. Copper ions interact with the transcription factor ACE1 in *Polyporus brumalis* or CUF1 in *C. neoformans* (Jiang *et al.*, 2009) to increase laccase expression.

1.9 Cultivation and Production of Laccases

Laccases can be produced by two major fermentation methods; the submerged fermentation and solid state fermentation.

1.9.1 Submerged Fermentation

This involves the growing of microorganisms in high oxygen concentrated liquid nutrient medium. This method of enzyme production produces enzymes by microorganisms in a liquid nutrient media. The major problem associated with the fungal submerged fermentations is the viscosity of the broth. When fungal cell grows, mycelium is formed which hinders impeller action, due to this limitation occurring in oxygen and mass transfer. To deal with this problem, different strategies have been employed. Bioreactor operates in continuous manner for obtaining high efficiency. In this, *Trametes versicolor* is employed which decolorizes the synthetic dye, and for this purpose pulsed system has been developed (Lema *et al*., 2001). Broth viscosity, oxygen, and mass transfer problems are also solved by cell immobilization. Luke and Burton (2001) reported that continuous laccase production takes place without enzyme deactivation for a period of 4 months due to the immobilization of the *Neurospora crassa* on membrane. For bioremediation of pentachlorophenol (PCP) and 2, 4-dichlorophenol (2, 4 DCP), nylon mesh is used for comparing the free cell culture of *T*rametes *versicolour* with immobilized cultures. An investigation by Couto *et al*. (2004) showed that, in fixed bed bioreactors, stainless steel showed the highest laccase activity among different synthetic materials which were used as carriers for the immobilization of *Trametes hirsute.*

1.9.2 Solid State Fermentation

This method is suitable for the production of enzymes by using natural substrates such as agricultural residues because they mimic the conditions under which the fungi grow naturally (Brijwani *et al*., 2005). The lignin, cellulose and hemicelluloses are rich in sugar and promote fungal growth in ferment or and make the process more economical (Couto & Toca-Herrera, 2006). The major drawback is the bioreactor design in which heat and mass transfer is limited. Different bioreactor configurations have been studied for laccase production such as immersion configuration, expanded bed, tray, inert (nylon) and non-inert support (barley bran) in which tray configuration gave the best response (Couto *et al*., 2003). Laccase production by both solid-state and submerged fermentation is higher in case of rice bran than other substrates. The rice bran inductive capability is based on the phenolic compounds such as ferulic acid, and vanillic acid which induce the laccase production.

1.10 Industrial Applications of Laccases

1.10.1 Wine and Beer Stabilization

Wine stabilization is one of the main processes demanding laccases in food industry. Polyphenols in wine play a major role in determining aroma, color and taste of wine, which are strictly related to the particular phenolic composition. However, the same compound is also the main actor involved in madeirization process causing turbidity, color intensification and aroma and flavor alteration. Laccase treatment aimed at reducing polyphenol content has been reported as an effective method to preserve wine quality, since the use of enzymes would selectively remove target specific polyphenols without undesirably altering wine's organoleptic characteristics. They can be used in wine and beer stabilization, fruit juice processing, baking, improvement of food sensory parameters and sugar beet pectin gelation. Laccases can be used to reduce or eliminate the high concentration of phenolic compounds in wines, which alter their taste, colour, sensations of tasting and organoleptic properties (Osma *et al*., 2010). Flavonoids are benzo-c-pyrone derivatives consisting of phenolic and pyrane rings (Uyama, 2007) endowed with biological and pharmacological effects, including antioxidant, anti-mutagenic, anti-carcinogenic, anti-viral and anti-inflammatory properties (Es-Safi, 2007). High molecular weight polyphenols have been reported to exhibit enhanced biological properties with longer circulation

time *in vivo* (Hagerman *et al*., 1998). As a fact, in the last decade it has been demonstrated that oxidative coupling of flavonoids generates polymers with increased antioxidant activity. Kurisawa *et al*. (2003) have employed a Myceliophthora laccase to catalyze the oxidative polymerization of catechin—a flavonoid found in green tea and wine in a mixture of a polar organic solvent and buffer. When compared with monomeric catechin, the synthesized polymer exhibited significantly improved superoxide scavenging activity and xanthine oxidase (XO) inhibitory activity. Jadahav and Singhal (2014) used a laccase conjugated to Arabic gum to perform catechin polymerization. Free laccase produced water-insoluble cross-linked oligomer, whereas conjugated laccase produced water-soluble linear oligomer endowed with high antioxidant activity and reducing power. An improved antioxidant activity if compared with the corresponding monomer has also been observed in the polymerization of quercetin and kaempferol with a laccase from *Ustilago maydis* (Desentis *et al*., 2006). More recently, the oxidative grafting of flavonoids to modify polymer properties is gaining increased attention.

1.10.2 Textiles Industry

Laccases have been very useful in textile industries owing to their ability to bleach denin fabrics (Iracheta-Cardenas *et al*., 2016) and also enhance the whiteness in the conventional peroxide bleaching of cotton (Tzanov *et al.,* 2003). Laccases also have the capacity to oxidize various aromatic compounds such as phenols and anilines to concomitantly promote non-enzymatic homo- and/or hetero-coupling reactions yielding a colour palette of different valuable dyes for textiles (Sousa *et al*., 2014). Laccases have been used to dye cotton as well as wool fabrics with hetero-polymeric dyes generated *in situ* by the oxidative hetero-coupling of colourless precursors and modifiers initiated by laccase (Hadzhiyska *et al*., 2006). In the first attempt of cellulose textiles dyeing by Hadzhiyska *et al*. (2006), cotton cellulose was dyed *in situ* by a polymeric dye generated by oxidative coupling of colorless 2, 5-diaminobenzenesulfonic acid (DABSA) and 1-hydroxyphenol (catechol) with laccase-treated fibers, obtaining up to 70 % of dye fixation. However, it has been reported that all fabrics showed high wash fastness but low light and friction resistance. Schroder *et al*. (2007) used a response surface methodology (RSM) approach to determine the best conditions for laccase-induced coating of flax fibers and fabrics by a *Trametes hirsuta* laccase. Different phenols have been analyzed for their potential as monomers for enzyme-catalyzed polymerization: all the methoxyphenols showed different coloration with weak fastness properties. The utilization of natural flavonoids present in the cotton as anchors to

attach other phenolic compounds to the fiber surface have been reported (Kim *et al*., 2007; 2008). *Trametes hirsuta* laccase was used to catalyze the oxidation of flavonoids in solution producing quinines, further polymerized and grafted onto surface of the cotton, providing a yellow to brown coloration, depending on the external flavonoids used and on the reaction conditions. The natural flavonoids present in the cotton were found to play an important role on the grafting reaction, improving dyeing and color fastness. The washing and friction fastness test of flavonoids colorized cotton showed good results, confirming the feasibility of this new and promising coloration technique. An important improvement in the wet rubbing fastness of the dyed sample has been observed by Blanco and coworkers (Blanco *et al*., 2009). In their work, there is a covalent fixation of the *in situ* generated polymeric pigment. Aromatic amine moieties of DABSA introduced onto tosylated cotton were coupled and copolymerized with a catechol into colored product covalently fixed on the fabric upon oxidation with laccase. The controlled amination of cellulose in a first step, and the subsequent coloration allowed for up to 95 % pigment fixation on the fabric. Tzanov *et al*. (2003) have also proved the ability of laccases for wool dyeing. They used a dye bath prepared with a dye precursor (DABSA), dye modifiers (catechol and resorcinol) and laccase, in the absence of any dyeing auxiliaries, carrying out the reaction at pH and temperature values safe to the wool material. Furthermore, they have shown that by prolonging the contact time between wool, enzyme, precursor and modifier, deeper colors were obtained in contrast to the conventional process, where deeper colors are attained by increasing the amount of dye. In situ laccase-assisted overdyeing using catechol and catechin as dye precursors has been reported by Guimares *et al*. (2011). Laccase-generated polymers gave rise to new coloration states from dark brown to green-yellow and replaced dyes in the overdyeing process. Through this process, overdyeing of denim was successfully achieved with acceptable levels in terms of durability. Also leather was colored by laccase action using dihydroxynaphthalenes as substrates (Suparno *et al*., 2007). The colored products are lightfast and bound to the natural collagen by a covalent tanning manner.

1.10.3 Pulp and Paper Industry

The traditional method of separating and degrading lignin in wood during paper production involves the use of chlorine-containing reagents (Virk *et al*., 2012). Thus, for decades, there have been attempts to replace these conventional chlorine-based delignification processes with cleaner and milder strategies, paying special attention to the pre-treatment of wood pulp with ligninolytic

oxidoreductases. Laccases are preferred to peroxidases in delignification because the reaction is fuelled by O_2 rather than H_2O_2 and unlike peroxidases, and their activity is not inhibited by the co-substrate (Diana *et al*., 2016).

1.10.4 Bioremediation and Biodegradation.

Rapid industrialization and extensive use of pesticides for better agricultural productivity have lead to the contamination of soil, water, and air which constitutes serious environmental problem today. Polychlorinated biphenyls (PCB), benzene, toluene, ethyl benzene, xylene (BTEX), polycyclic aromatic hydro- carbons (PAH), pentachlorophenol (PCP), 1,1,1-trichloro- 2,2-bis (4-chlorophenyl) ethane (DDT), and trinitrotoluene (TNT) are the substances which are known for their carcinogenic as well as mutagenic effects and are persistent in the environment. Wide varieties of hazardous chemicals that are not echo friendly are being controlled by the use of fungi (Riva, 2006).

1.10.5 Food Industry

Increase in the supply of quality foods can be addressed by application of enzymes, particularly laccases in food industries. Use of enzymes in food industries have improves basic processes towards achieving better products with desired qualities (Rajendra, *et al*., 2016). Fat modification and sweetener technology are other vital areas of research where enzymes are greatly applied (Li *et al*. 2012). The application of enzymes in food industry is categorized into different sectors, such as baking, dairy, juice production and brewing. Microbial enzymes are efficiently utilized worldwide in bakery to improve dough stability, crumb softness and texture, and shelf life of products. The use of enzymes in dairy industry is owing to the increased uses of microbial enzymes in cheese processing followed by the beverages industry. One of the most common food processing techniques worldwide is bread making. The use of enzymes in the manufacturing of bread shows their high value in efficiency of production and quality control. Amylase in combination with other enzymes are added to the bread flour to enhance efficiency of retaining the moisture and to increase softness, freshness and shelf life of products and whiteness. Transglutaminase (EC 2.3.2.13) is used in baking industry to enhance the quality of flour, the amount and texture of bread and the texture of cooked pasta (Kieliszek and Misiewicz 2014). Lipases are also used to improve the flavor content of bakery products by liberating short-

chain fatty acids through esterification and to prolong the shelf life of the bakery products (Adrio and Demain 2014).

1.10.6 Pharmaceutical and Analytical Industry

Enzymes play very important roles in the pharmaceutical industries and diagnostic laboratories. Industrial enzymes are extensively used as therapeutic drugs in health issues related with enzymatic deficiency and digestive disorders. Enzymes are as well used by employing diagnostic procedures such as ELISA and diabetes testing kits (Mane and Tale 2015). Enzymes such as Chitosanase (EC 3.2.1.132) catalyze hydrolysis of chitosan to biologically active chitosan oligosaccharides, which is potent antimicrobial and antioxidant activities, lowering of blood cholesterol and high blood pressure, controlling arthritis, protective effects against infections and improving antitumor properties (Thadathil and Velappan 2014).

1.10.7 Laccase-Mediated Polymer Synthesis

In the frame of industrial application, laccase ability to synthesize polymers is of outstanding importance. Indeed, polymeric materials are essential in everyday life, being present in many and different fields, such as food, textile, electronics, communications, transportations, pharmacy, and medicine (Kobayashi and Makino, 2009). Even if current polymer production schemes are highly optimized, the huge quantity of annually produced polymers demands a greener technology, particularly in the perspective of toxic formaldehyde reduction. Hence, it is not surprising that enzymes are being engaged for polymer synthesis (Kobayashi and Makino, 2009); with laccases and peroxidases dominating this field (Hollmann and Arends, 2012). In a polymerization reaction, laccase achieves its natural work with the initial formation of radicals from a typical substrate (aromatic with hydroxyl group or groups). Then, radicals can undergo polymerization reactions towards homopolymeric materials (Mikolasch and Schauer, 2009). Radical polymerization lacks any control on the structure and morphology of the final product, thus if a defined molecule is pursued, it is necessary to find opportune reaction conditions, as reviewed by Hollmann and Arends (2012). Despite the proved capability, short-term implementation of enzyme-mediated polymer synthesis on industrial scale is still hampered by economic issues. However, the high efforts put in this field may lead to a fast revolution in polymer chemistry in the next decade. Polymer synthesis by laccases has already been reviewed

by different authors (Mikolasch and Schauer, 2009; Kobayashi and Makino, 2009; Hollmann and Arends, 2012).

1.11 *Pleurotus pulmonarius*

Pleurotus species, belonging to Pleurotaceae family, are edible macrofungi cultivated worldwide particularly in South East Asia, Europe, and Africa. *Pleurotus pulmonarius* is similar to *P. ostreatus* but the cap of *Pleurotus pulmonarius* are much paler and smaller than P. *ostreatus* and develops more of a stem. *Pleurotus pulmonarius* also prefers warmer weather than *P. ostreatus* and has low cost production, palatability, and high biological efficiency (Stamets, 2000). *Pleurotus pulmonarius* is widespread in temperate and subtropical forests throughout the world. In the eastern United States, this species is generally found on hardwoods while in the west it is commonly found on conifers (Stamets, 2000).

Figure 5: *Pleurotus pulmonarius* in its natural habitat (Gunde and Cimerman, 1995)

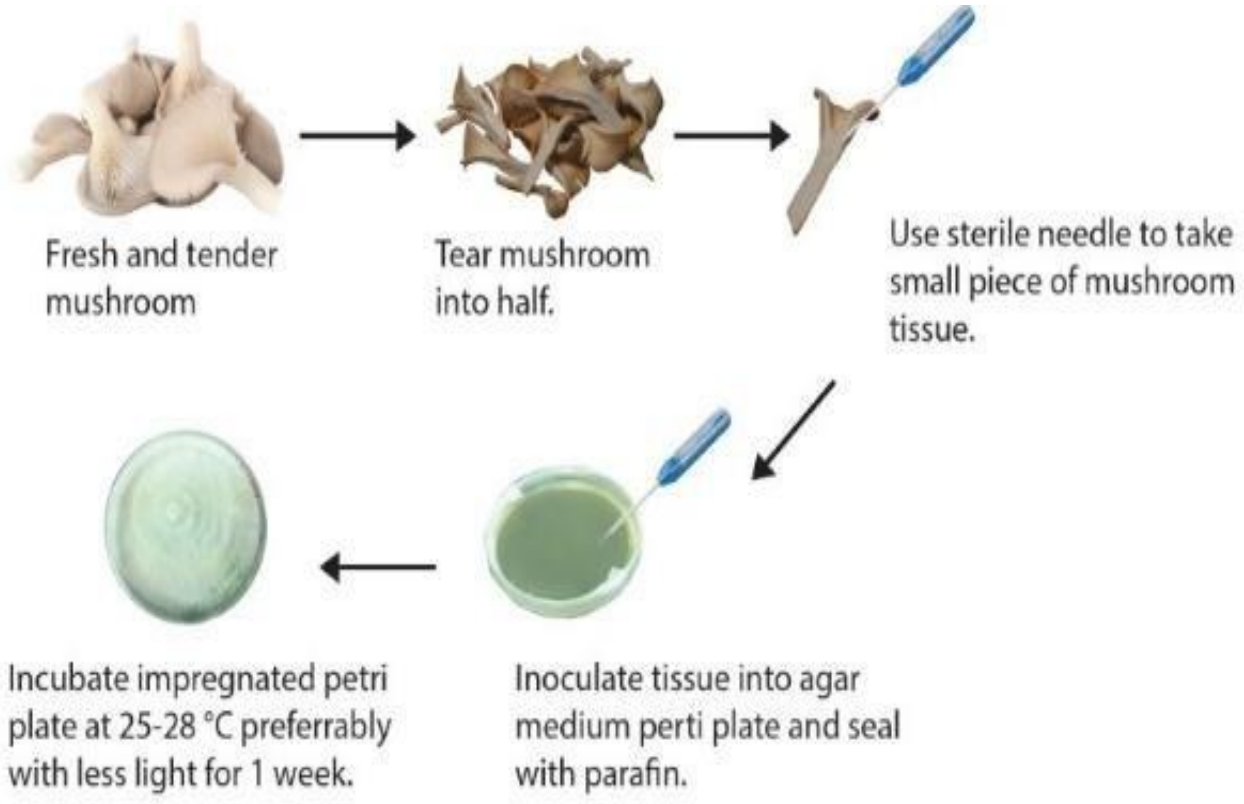

Figure 6: Summary diagram of mushroom tissue culture technique (Farhat, 2018)

1.12 Scientific Classification

Kingdom	Fungi
Division	*Basidiomycota*
Class	*Agaricomycetes*
Order	*Agaricales*
Family	*Pleurotaceae*
Genus	*Pleurotus*
Species	*pulmonarius*

1.13 Clinical Importance of *Pleurotus pulmonarius*

1.13.1 Analgestics

A polysaccharide known as β-D-Glucan extracted from *P. pulmonarius* reduces sensitivity to pain in mice (Baggio *et al*., 2010) and could serve as an attractive basis for analgestic medications.

1.13.2 Anti-inflammation

Methanol extract of P. pulmonarius shows potent anti-inflammatory properties when compared to the standard reference drugs diclofenac and cisplatin, respectively (Jose *et al*., 2002).

1.13.3 Anti-carcinogenic

Lavi *et al*. (2010) in their study concluded that extracts of *P. pulmonarius* may slow the proliferation of cancer cells with high galectin-3 levels, while at the same time downregulate tumour cell adherence - which is directly related to the progression and spread of cancer and this suggests that the extracts may serve as a useful adjuvant to cancer therapies.

1.13.4 Anti-diabetics: Hot water extract of P. pulmonarius upon oral administration showed a significant antihyperglycemic effect, reduced mortality of alloxan induced diabetic mice by 50% approximately as well as showing synergistic effect with the antidiabetic drug glibenclamide, supporting the possibility of effective combination therapy of glibenclamide and *P. pulmonarius* for diabetes (Badole *et al.,* 2008).

1.14 Justification for the Study

This research work was geared towards achieving the optimum conditions necessary for laccase production from *Pleurotus pulmonarius* as well as ascertaining the specificity of laccase towards various aromatic compounds.

1.15 Aim and Specific Objectives of the Study

1.15.1 Aim of the Study

This study was aimed at the substrate specificity determination of laccase from *Pleurotus pulmonarius* towards aromatic compounds.

1.15.2 Specific Objectives of the Study

This research was designed to:

1. Investigate the cultivation conditions/methods that will promote the expression of laccase using the above fungi.
2. Subculture *Pleurotus pulmonarius* from microbial culture collection, Enzymology and Protein Chemistry unit, University of Nigeria, Nsukka.
3. Produce crude laccase under solid state fermentation method using wheat bran as carbon source.
4. Purify crude laccase extract using ammonium sulphate precipitation, dialysis and gel filtration (Sephadex G-200).
5. Characterize the purified laccase by determining the optimum pH, temperature, effect of substrate concentration on enzyme activity, kinetic properties of the enzyme (V_{max} and K_M) and substrate specificity for various aromatic compounds.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and Reagents

All chemicals used in the research were of analytical grade and were obtained directly from the manufacturer or purchased from a commercial dealer in Nsukka, Enugu State, Nigeria. They include: ABTS [(2, 2'Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)], Ammonium sulphate, Sephadex G-100, Bovine Serum Albumin (BSA), Tris-hydrochloric acid (Tris-HCl), Acetic acid (CH_3COOH), Potato Dextrose Agar (PDA), Sodium acetate (CH_3COONa) Disodium Hydrogen phosphate (Na_2HPO_4), Sodium dihydrogen phosphate (NaHPO4), Sodium molybdate ($NaMo_4$), Copper sulphate ($CuSO_4$), Magnesium sulphate ($MgSO_4$) Iron sulphate ($FeSO_4$), Calcium chloride ($CaCl_2$), Zinc sulphate ($ZnSO_4$), Potassium Dihydrogen Phosphate, Thiamine (Vitamin B1), Glucose ($C_6H_{12}O_6$), Mercuric chloride ($HgCl_2$), Sodium cyanide (NaCN), Ethylene diamine tetraacetic acid (EDTA), Urea, Sodium dedocyl sulfate (SDS), Sodium Azide (NaN_3) are all manufactured by Sigma Aldrich, Germany.

2.1.2 Equipment and Apparatus

Equipment and Apparatus used for the present study were obtained from the laboratory unit of Department of Biochemistry. They include: pH meter (Searchtech Model PHS-3C, U.K), Spectrophotometer (Jenway 6405, England), Weighing balance (Huaxiang Electronic HX502T, China), Orbital shaker (MKV-300, LH Fermentation Ltd., U.K.), Petri dishes (Narang Medical Limited, India), Magnetic stirrer (B.Bran Scientific and Instrument Company, England), Stainless Steel Thermostatic Water Bath (SSY-H, China), Refrigerator (PZ Cussons & Haier, Qingdao, China), Centrifuge (Model 800, China), Beakers (Pyrex, England), Conical flasks (Pyrex, England), Pipette (Pyrex, England), Measuring cylinder (Pyrex England).

2.2 Methods

2.2.1 Fungi collection

The fungi were sub cultured from the stock culture in microbial culture collection, Enzymology and Protein Chemistry Unit, University of Nigeria, Nsukka.

2.2.2 Fungi screening

The sub-cultured fungi were screened for laccase enzyme using 3mM ABTS (2-2'-Azino-bis-[3-ethyl benzthiazoline-6-sulfonic acid]) by a method used by Senthivelan *et al*. (2019). This was done by adding a drop of ABTS on the fungi grown on petri-dish followed by incubation for seven days. The blue-green decolonization around the area showed the expression of the laccase.

2.2.3 Time Course Study for Maximum Laccase Production

This was carried out under stationary condition with 20 ml Basal medium proposed by Tien and Kirk (1988) containing Ammonium tartarate (0.22g), KH_2O_4 (0.2g), $MgSO_4.7H_2O$ (0.05g), Thiamine (0.001g), $CuSO_4$ (0.08g), $CaCl_2$ (0.01g), $FeSO_4$ (0.03g), $NaMo_4$ (0.05g), $ZnSO_4$ (0.05g), (pH 4.5) supplemented with 5g of wheat bran in 250ml Erlenmeyler flask and incubated for 0–14 days. Each flask was inoculated with three (3) plugs of one (1) cm diameter cut from the growing potatoes dextrose agar (PDA) cultures and incubated at 30 ^{0}C. The activity was checked every day within the 14 -days to determine the day of highest laccase production.

2.2.4 Mass Production of Laccase

Laccase was mass produced under the solid state fermentation using Tien and Kirk 1998 basal medium supplemented with 5g of wheat bran. A quantity of 1000ml of basal medium was prepared fromAmmonium tartarate (0.22g), KH_2PO_4 (0.2g). $MgSO_4.7H_20$ (0.05g), thiamine (0.001g), $CuSO_4$(0.08g), $CaCl_2$ (0.01g), $NaMO_4$ (0.05g), $MnSO_4$ (0.07g), $FeSO_4$ (0.03g), glucose (10g), $ZnSO_4$(0.05g). A 5g of wheat bran was measured and put into thirty 250ml of conical flasks each and sterilized. A volume of basal medium was added to each flask. Two agar plugs of 0.8cm was cut off from the growing edge of the pure fungi and inoculated into each conical flask and incubated for nine (9) days under stationary condition.

2.2.5 Enzyme Harvesting

After the ninth day, 30 ml of Na_2HPO_4/NaH_2PO_4 buffer pH 6.0 was added to each conical flask and put into an orbital shaker for 15 minutes to initiate agitation and ensure mixing of the enzyme and buffer. The resulting mixture was filtered using a muslim cloth. The filtrate was centrifuged at 4,000 rpm for 20 minutes to remove mycelin and the supernatant was assayed for activity.

2.2.6 Determination of Laccase Activity

Laccase activity was assayed by the modified method of Shin and Lee (2000). The assay was carried out by monitoring the rate of oxidation of 2, 2-azino bis(3-ethylenethiazoine)-6 sulphonic acid (ABTS) to a cation radical (ABTS-azine) by the crude enzyme at 25 $^{\circ}$C in 100 mM sodium acetate buffer pH 4.5. The concentration of the cation radical responsible for the intense bluegreen colour was correlated to enzyme activity. The oxidation was monitored at 420 nm ($\varepsilon 420=36\ mM^{-1}cm^{-1}$). Enzyme activity was expressed in international unit (IU) where IU is defined as the amount of enzyme forming 1 joule of product per minute. The reaction mixture contained 2.8ml 0.1 M sodium acetate buffer pH 4.5, 0.1 ml of 1 M ABTS and 0.1 ml of enzyme. The laccase activity in U (μmole cation radical released min^{-1},) was calculated as follows

$$U/mol = \frac{\Delta ABS.\ Min^{-1} \times V}{v \times \varepsilon \times d}$$

Where:

V = Total reaction volume (ml)

v = Enzyme volume (ml)

ε =Extinction coefficient of ABTS at 420nm ($\varepsilon 420=36\ mM^{-1}cm^{-1}$)

d= light path of cuvette (cm)

2.2.7 Determination of Protein Concentration

The protein concentration was measured using the Lowry *et al*. (1951) using bovine serum albumin as a protein standard.

2.2.8 Ammonium Sulphate Precipitation Profile

Ammonium sulphate precipitation profile was carried out as described by Wenk and Fernandis (2008). This was done at different ammonium sulphate saturations ranging from 20-100% ammonium sulphate at an intervals of 10%. Therefore, nine collection tubes containing 10ml of enzyme each were labelled 20% through 100%. Different grams of ammonium sulphate corresponding to the percentage saturation were measured and poured into tubes containing 10ml of crude enzyme each and were allowed to stand for 24 hrs. Each precipitate was centrifuged at 4000 rpm for 15 min and assayed for activity.

2.2.9 Ammonium Sulphate Precipitation

A known quantity of ammonium sulphate was carefully dissolved in 500ml of crude enzyme and stirred gently until the salt had completely dissolved. It was allowed to stand for 24 hours. The precipitate was centrifuged at 4,000 rpm for 15 min to remove the mycelin. Both the supernatant and pellet were assayed for activity.

2.2.10 Dialysis

Dialysis was carried out using 15ml of precipitated enzyme in 800 ml of 0.1 M Na_2HPO_4/NaH_2PO_4 buffer (pH7.0) for 12 hrs with the buffer being changed after six hrs.

2.2.11 Gel filtration

Gel filtration was carried out using sephadex G-200 packed into a column (1.5cm by 6.7cm) equilibrated with Na_2HPO_4/NaH_2PO_4 buffer (pH 6.0). A volume of 10ml of dialysed enzyme was introduced into a gel chromatographic coloumn and purified via gel filtration. The fractions were collected at thr flow rate of 5ml per 17 munites. The protein concentration of each fraction was monitored spectrophotometrically at 280 nm. Laccase activity was monitored spectrophotometrically as described above. The active fractions were pooled, stored at -10 ^oC for further studies.

2.2.12 Characterization of Laccase

2.2.12.1 Optimum pH

The optimum pH of laccase was carried out by a method described by Atalla *et al*. (2013). Purified Laccase were assayed at different pH ranging from 3.0 - 7.0 using 0.1 M sodium acetate buffer (pH 3.5 - 5.5), phosphate buffer (pH 6.0 - 7.0) at interval of 0.5 to determine the optimum pH

2.2.12.2 Optimum Temperature

Laccase activity was assayed at different temperature ranging from 30-90 ^{o}C at optimum pH as described by Anne *et al*. (2008) at 5 ^{o}C intervals. The substrate (ABTS) and buffer (Acetate buffer) were incubated for 10 minutes at respective temperature for equilibration in water bath prior to the assay.

2.2.12.3 Effect of Substrate Concentration on Laccase Activity

The Michaelis-Menten kinetic parameters were determined by a method used by Singhal *et al*. (2012). The Michaelis-Menten kinetic constant KM and V_{max} were determined by using different concentration of ABTS ranging from 10 – 120μM. Laccase activity was determined at different concentrations of the substrate keeping the enzyme concentration constant.

2.2.12.4 Substrate Specificity of Laccase for Aromatic Compounds

The Michaelis-Menten kinetic parameters were determined by a method used by Singhal *et al*. (2012), using different aromatic compounds (abts, α-naphthol, 2, 6-DMP and O-dianisidine) as substrates. Laccase activity was determined at different concentrations of the different substrates keeping the enzyme concentration constant. The Michaelis-Menten kinetic constant K_M and Vmax were determined by different concentrations of different aromatic compounds.

Hence, specificity was calculated for each of the aromatic substrate using the formular; $Vmax/K_{M.}$

CHAPTER THREE

RESULTS

3.1: Pure culture of *Pleurotus pulmonarius*

Plate 1 shows a mono culture of *Pleurotus pulmonarius* that was obtained after several culturing and sub culturing.

3.2 Fungal Screening

Plate 2 shows a positive result of laccase expression upon oxidation of ABTS to a blue-green colouration.

3.3: Incubation Period for Laccase Production

Figure 6 shows that the highest activity of laccase was observed on the day nine (9) after a fourteen (14) day pilot study was conducted to ascertain the day of maximum laccase expression.

Plate 1: Pure culture of *Pleurotus pulmonarius*

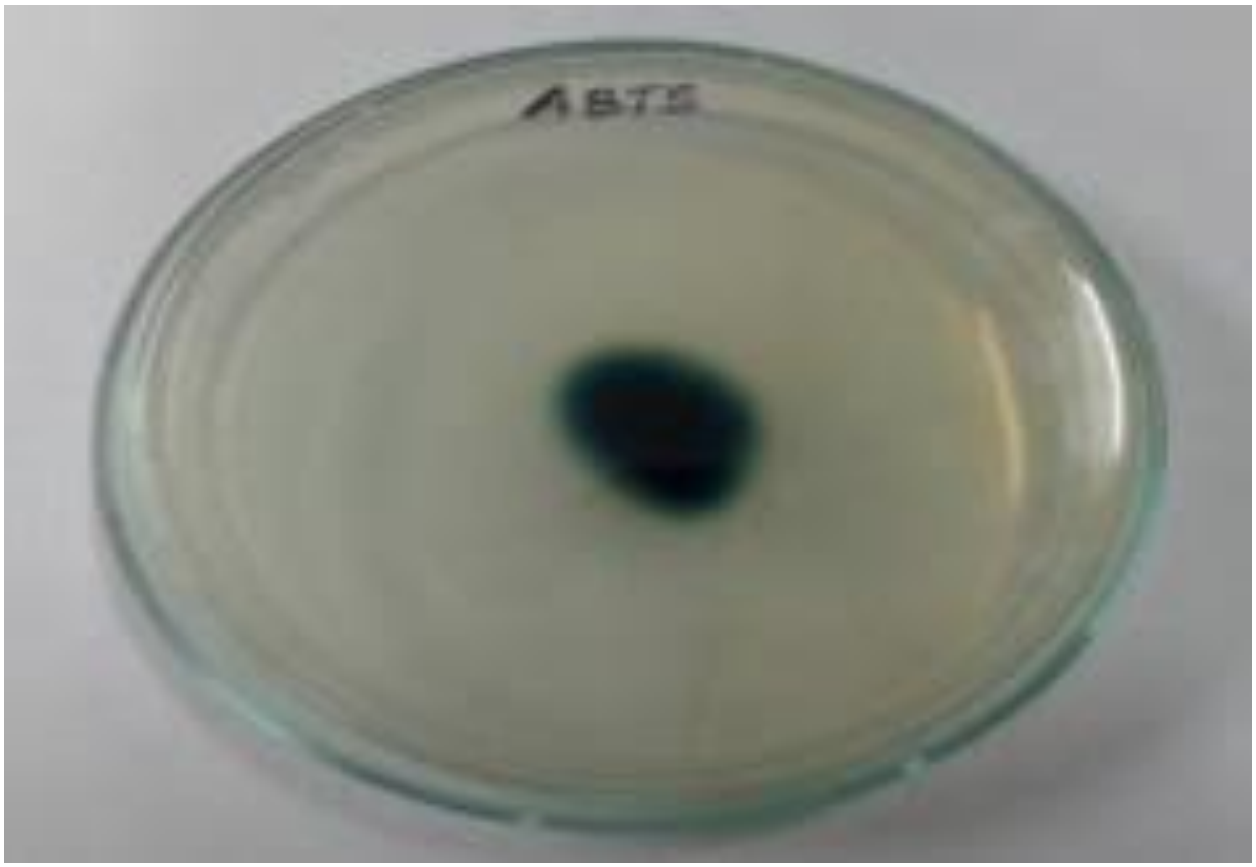

Plate 2: Screening of *Pleurotus pulmonarius* for laccase expression using ABTS

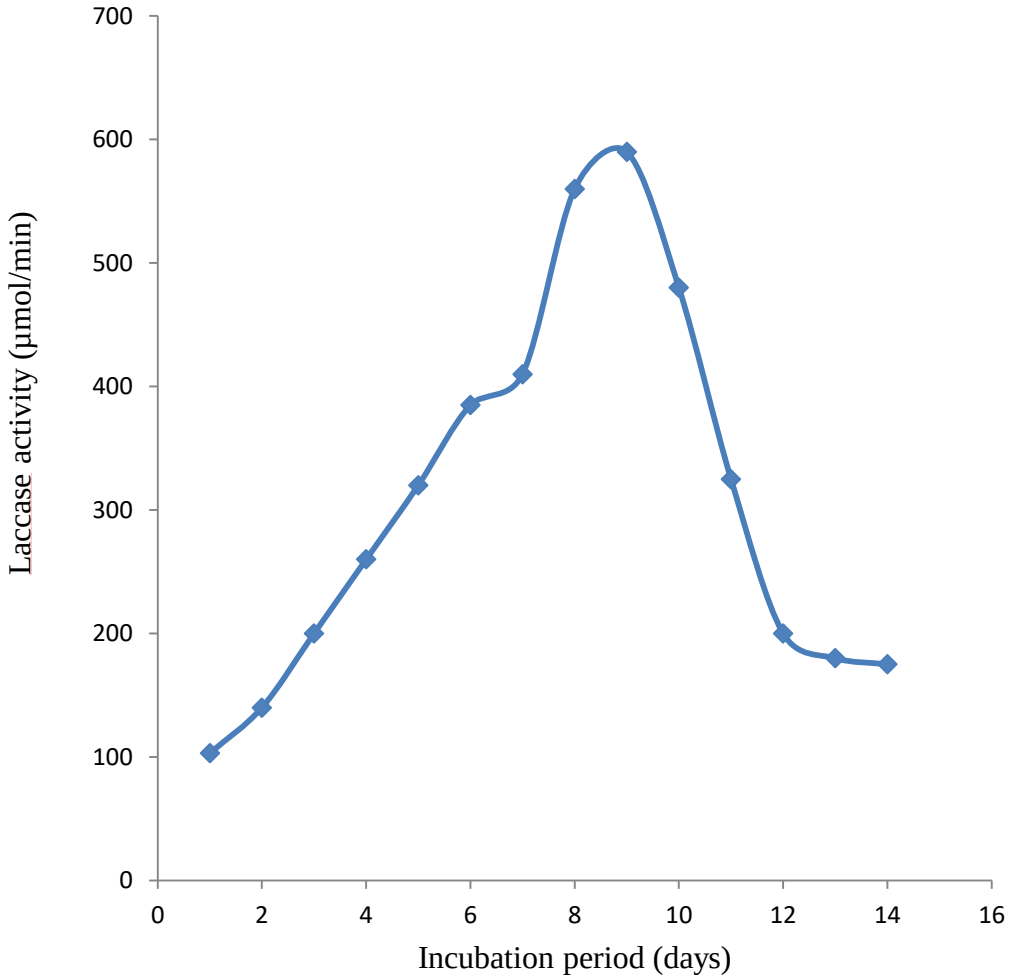

Figure 7: Incubation period for maximum laccase production

3.4: Ammonium Sulphate Precipitation

Figure 7 shows ammonium sulphate saturation profile. 80 % ammonium sulphate saturation was found suitable to precipitate protein with highest laccase activity.

3.5: Elution profile of laccase from *P.pulmonarius*

Figure 8 shows elution profile of laccase from *P. pulmonarius.* A total of 50 fractions were collected at the end of the gel filtration using Sephadex G-200. Highest laccase activity was observed in fraction 35.

3.6: Purification Table of Laccase from *P. pulmonarius*

Table 1 shows the summary of the purification of laccase from ammonium sulphate precipitation to gel filtration. The specific activity of laccase increased as the purification steps increased from ammonium sulphate precipitation to gel filtration. Crude with the specific activity of 2566 U/mg increased to 9515 U/mg after gel filtration with a purification fold and yield of 3.7 and 1.90 respectively.

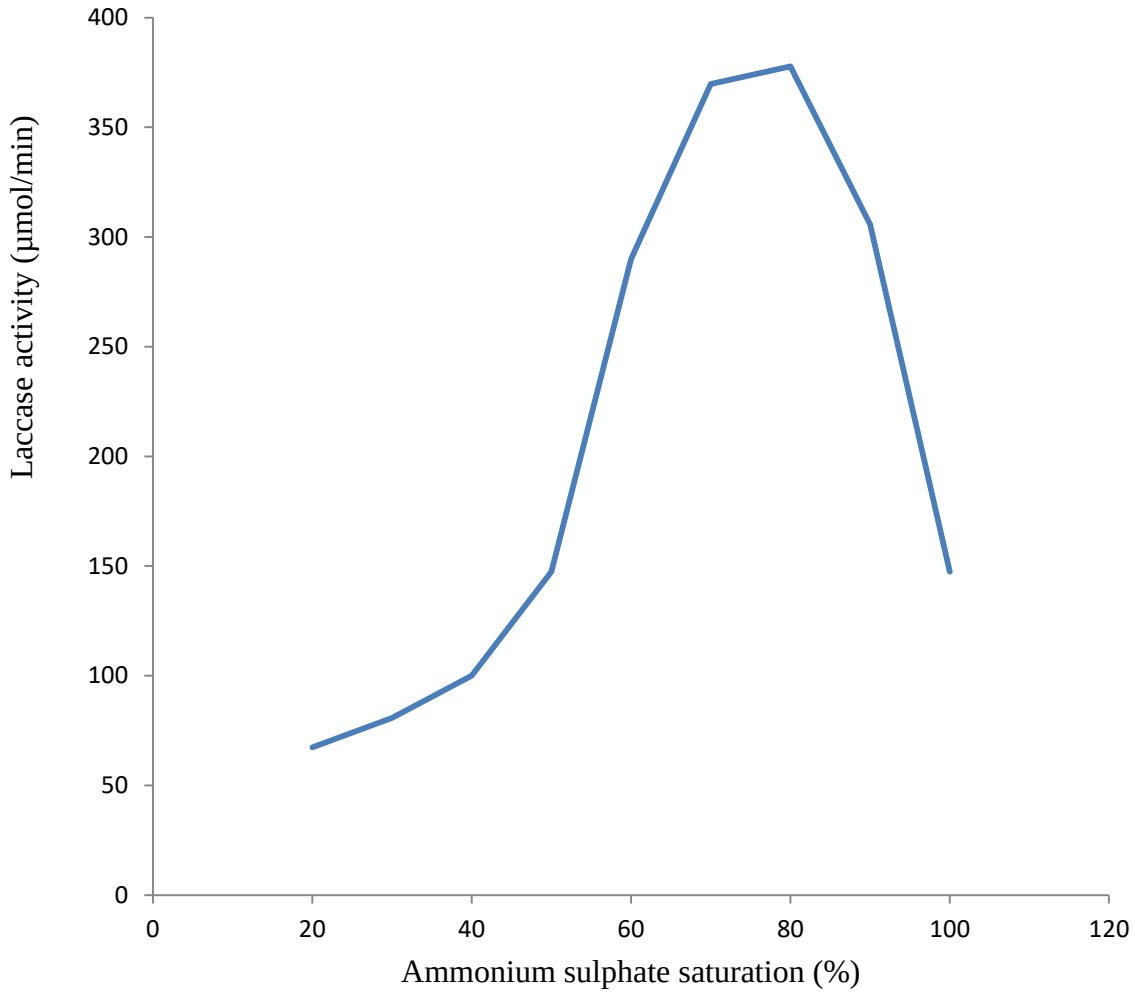

Figure 8: Ammonium sulphate saturation profile of laccase from *P. pulmonarius*

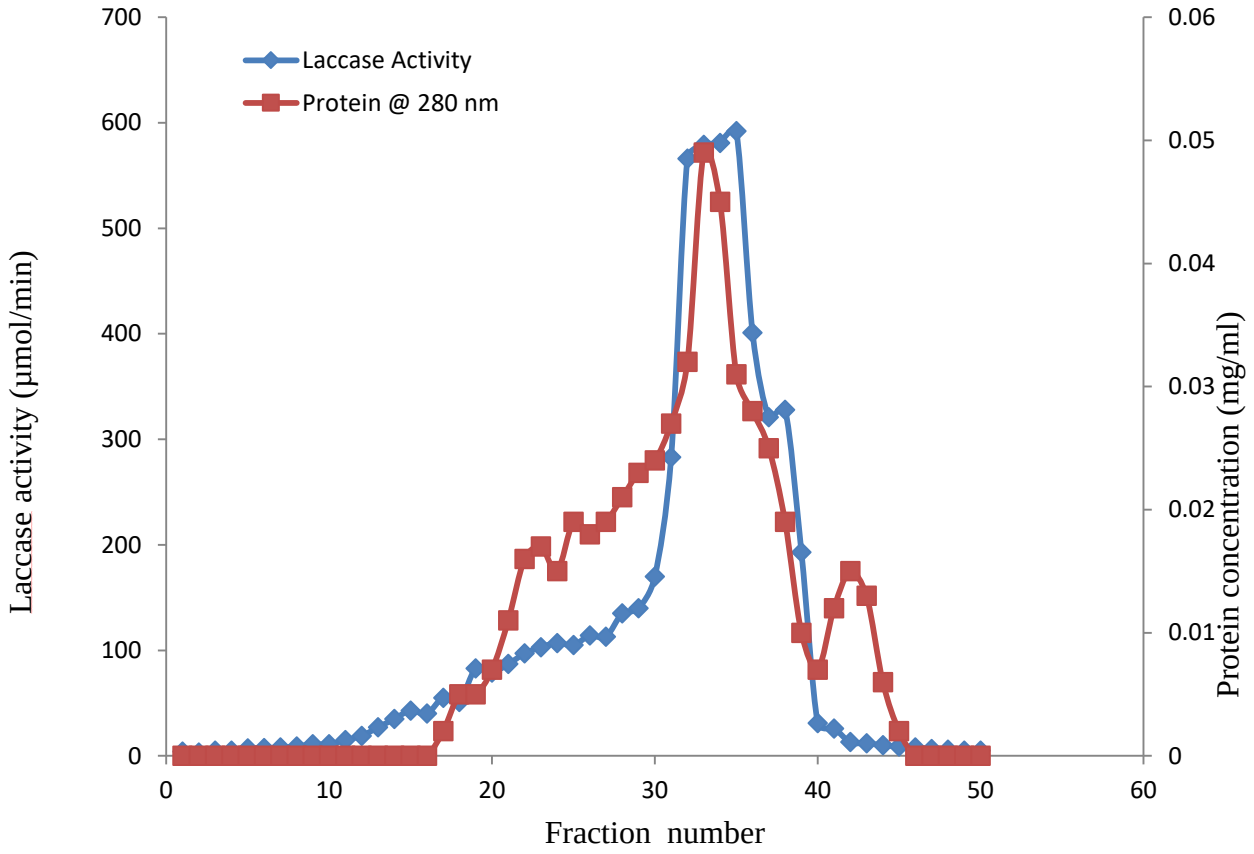

Figure 9: Elution profile of laccase from *P.pulmonarius*

Table 1: Purification table of laccase from *P. pulmonarius*

	Volume of enzyme (ml)	**Total protein (mg/ml)**	**Total activity (U)**	**Specific activity (U/mg)**	**Purification fold**	**Yield (%)**
Crude enzyme	500	152	390000	2566	1	100
Ammonium sulphate saturation (80%)	50	4.54	14750	3252	1.27	3.78
Dialysis	25	1.43	10454	7310	2.85	2.68
Gel filtration (Sephadex G-200)	20	0.78	7422	9515	3.71	1.90

3.7 Characterization of Laccase from *Pleurotus pulmonarius*

The partially purified laccase was characterized based on:
Optimum pH
Optimum temperature
Effect of substrate concentration
Kinetic properties of the enzyme (V_{max} and K_M)
Substrate specificity

3.7.1 Optimum pH

Figure 9 shows the pH effect on laccase activity. The activity of laccase increased from pH 3.0 to the optimum pH of 4.0 with an activity of 566.124 U/ml. Further increase in pH perturbed the conformation of the enzyme and resulted in decrease in the activity of the laccase.

3.7.2 Optimum Temperature

Figure 10 shows the effect of temperature on laccase activity. The activity of laccase increased with an increase in temperature as a result of effective collision between the substrate and the enzyme molecules. The optimum temperature was observed at 50 ºC with an activity of 557.234 U/ml after which the activity started decreasing. Further increase in temperature distorts the conformation of the enzyme, followed by denaturation.

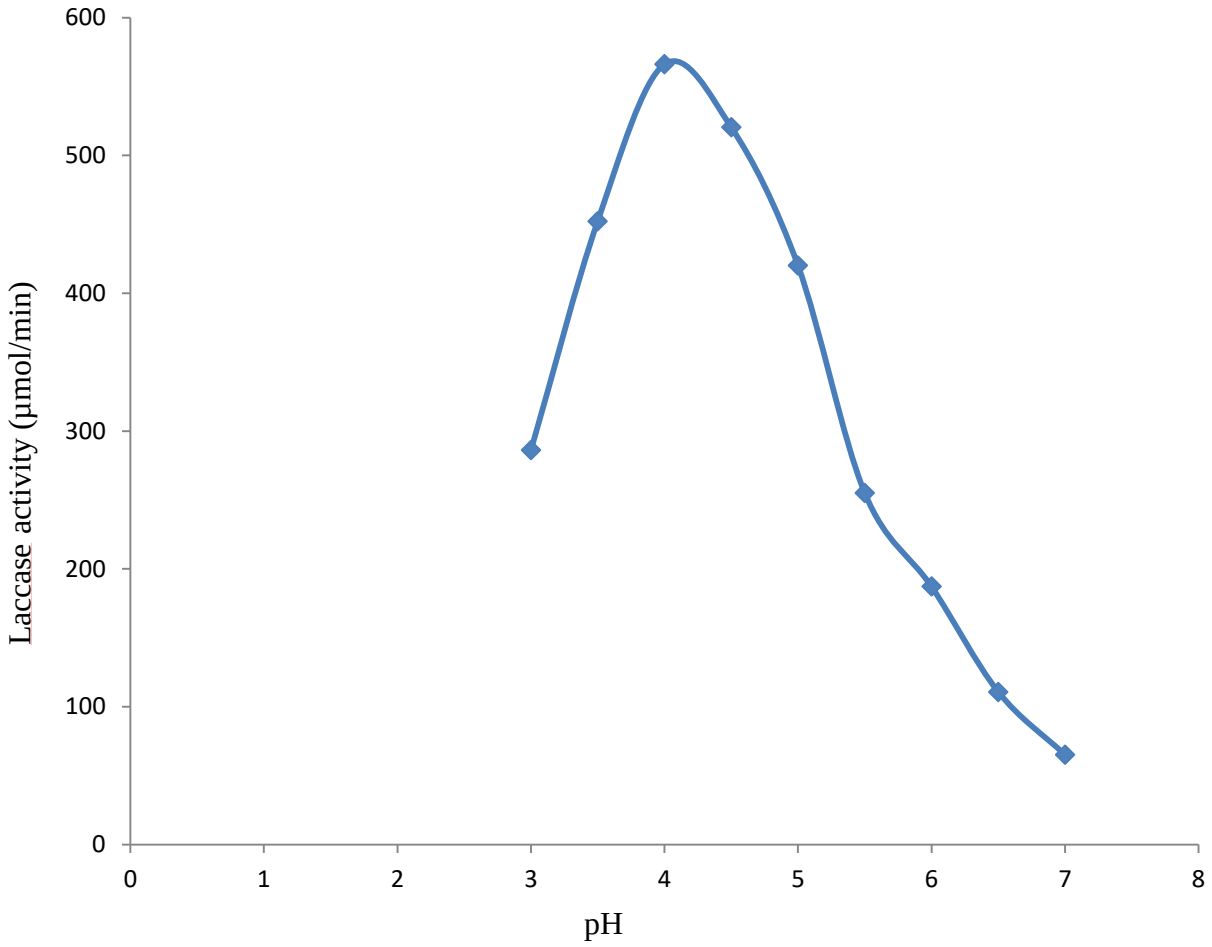

Figure 10: Optimum pH of laccase from *P. pulmonarius* was recorded at 4.0

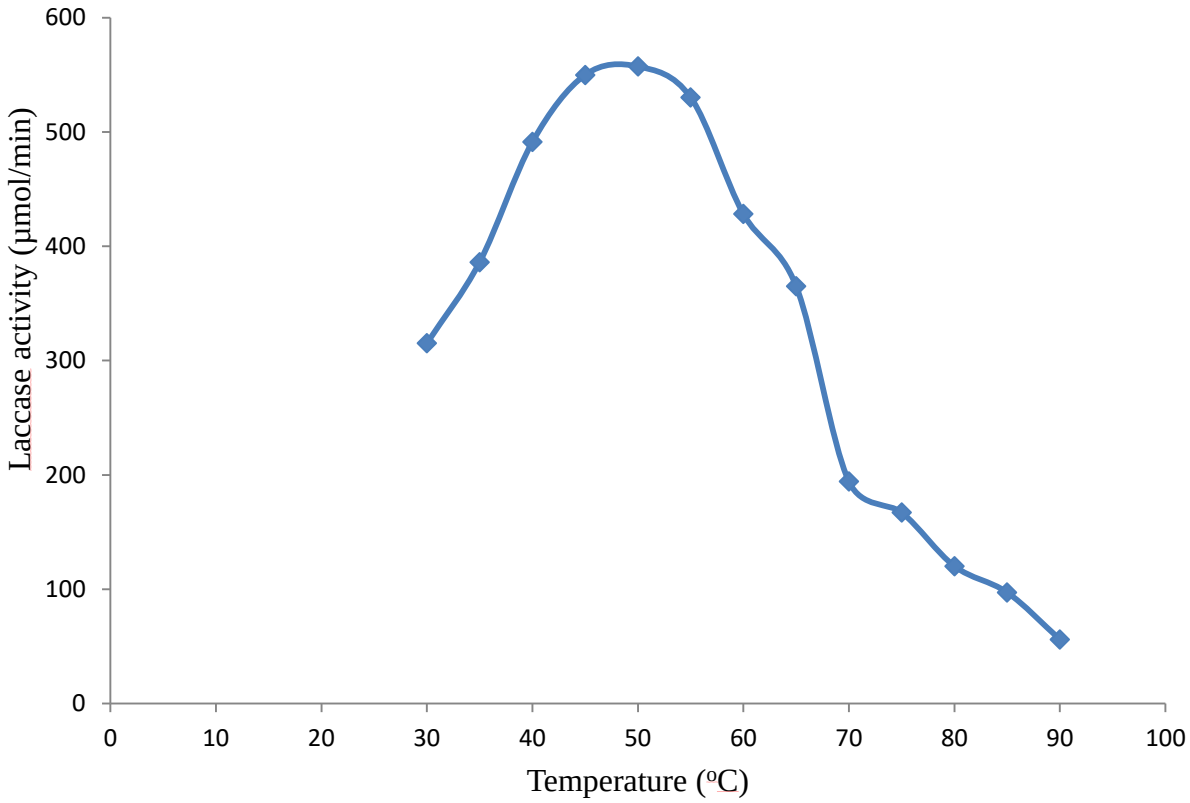

Figure 11: Optimum temperature of Laccase from *P. pulmonarius* was recorded at 50 °C

3.7.3 Effect of Substrate [ABTS] Concentration on Laccase Activity

Figure 11 shows the effect of substrate [ABTS] concentration on laccase activity. Increase in substrate concentration increased the activity of the laccase until a concentration of 90 μM with an activity of 61.24 μmol/min. Further increase in substrate concentration had no effect on the activity of the laccase. Hence, zero order reaction.

3.7.4: Line-Weaver Burk Plot Showing the Vmax and K_M for ABTS

Figure 12 shows the double reciprocal plot of initial velocity of laccase. The Michaelis constant (K_M) and maximum velocity (Vmax) obtained from Line-weaver Burk plot of initial velocity at various substrate concentrations were 31.27 μM and 90.91 μmol/min respectively.

3.7.5: Effect of Substrate [α-naphthol] Concentration on Laccase Activity

Figure 13 shows the effect of substrate [α-naphthol] concentration on laccase activity. The activity of laccase increased with an increase in the concentration of α-naphthol until the laccase active sites were saturated. There was no observable increase in the activity of laccase on further increase in substrate concentration.

3.7.6: Line-Weaver Burk Plot Showing the Vmax and K_M for α-naphthol

Figure 14 shows the double reciprocal plot of initial velocity of laccase. The Michaelis constant (K_M) and maximum velocity (Vmax) obtained from Line-weaver Burk plot of initial velocity at various substrate concentrations were 31.78 μM and 111.11 μmol/min respectively.

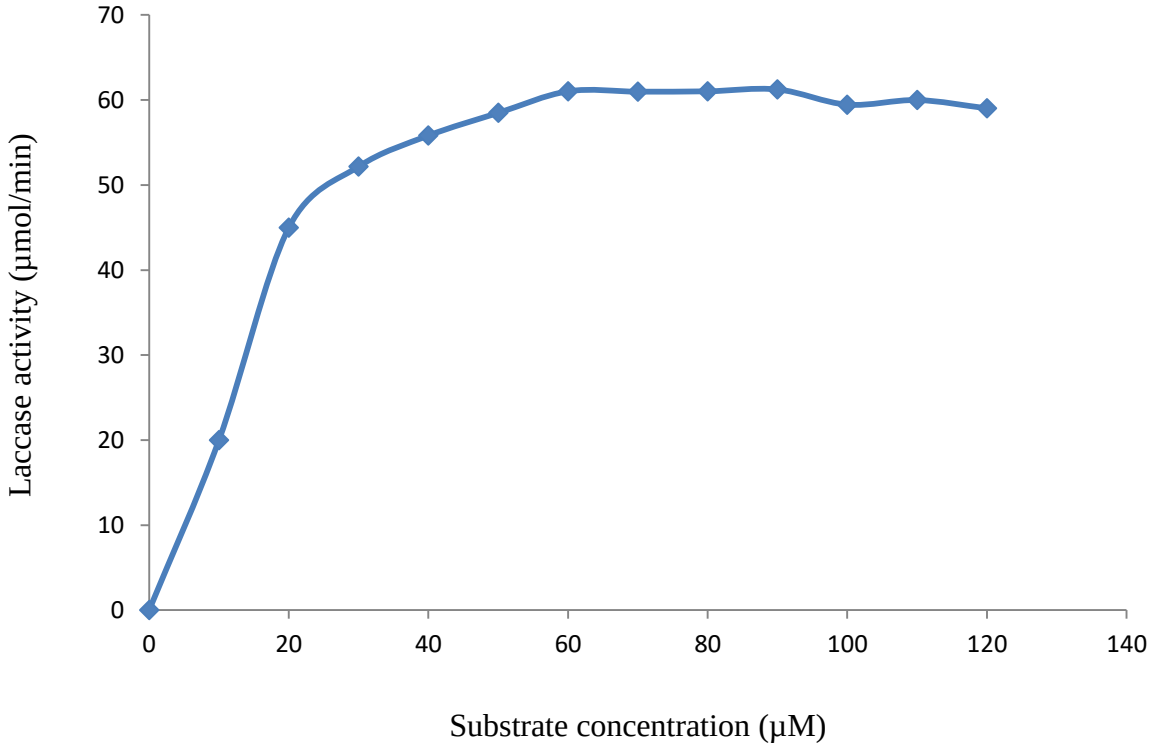

Figure 12: Michaelis-Menten plot showing the effect of substrate [ABTS] concentration on laccase activity

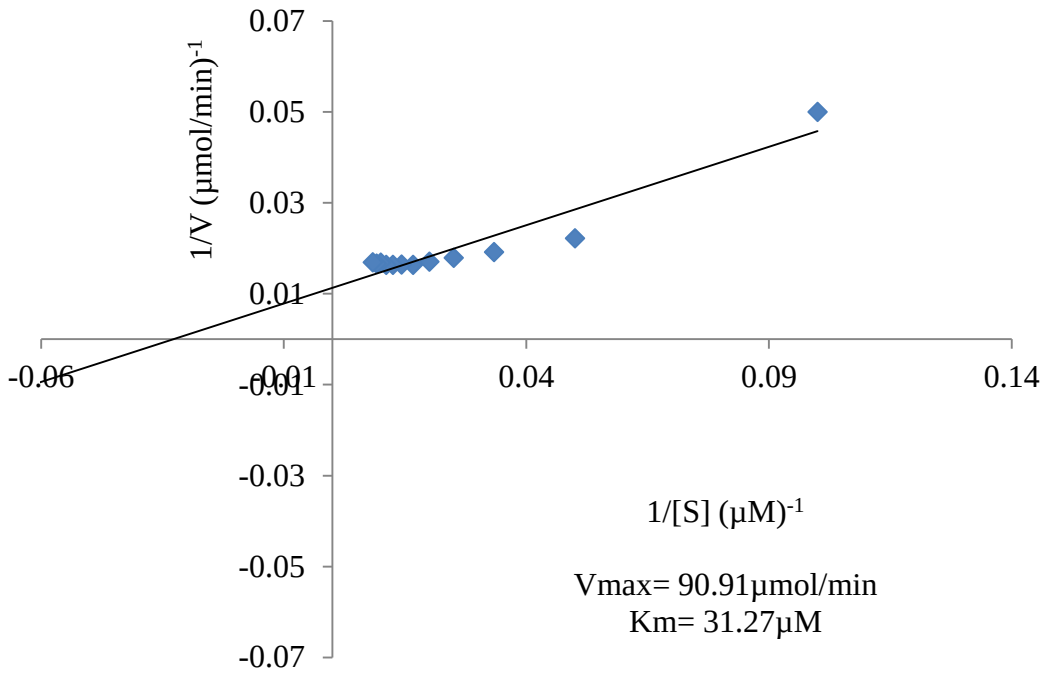

Figure 13: Line-Weaver Burk plot showing the Vmax and K_M for ABTS

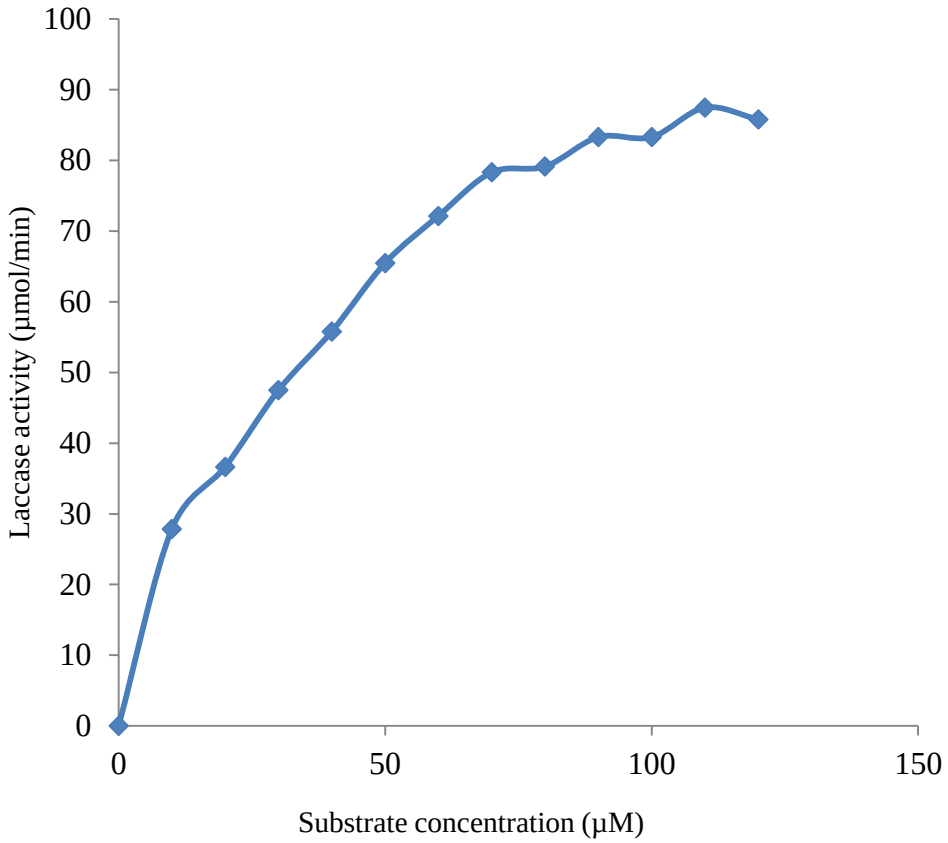

Figure 14: Michaelis-Menten plots showing the effect of substrate [α-naphthol] concentration on laccase activity

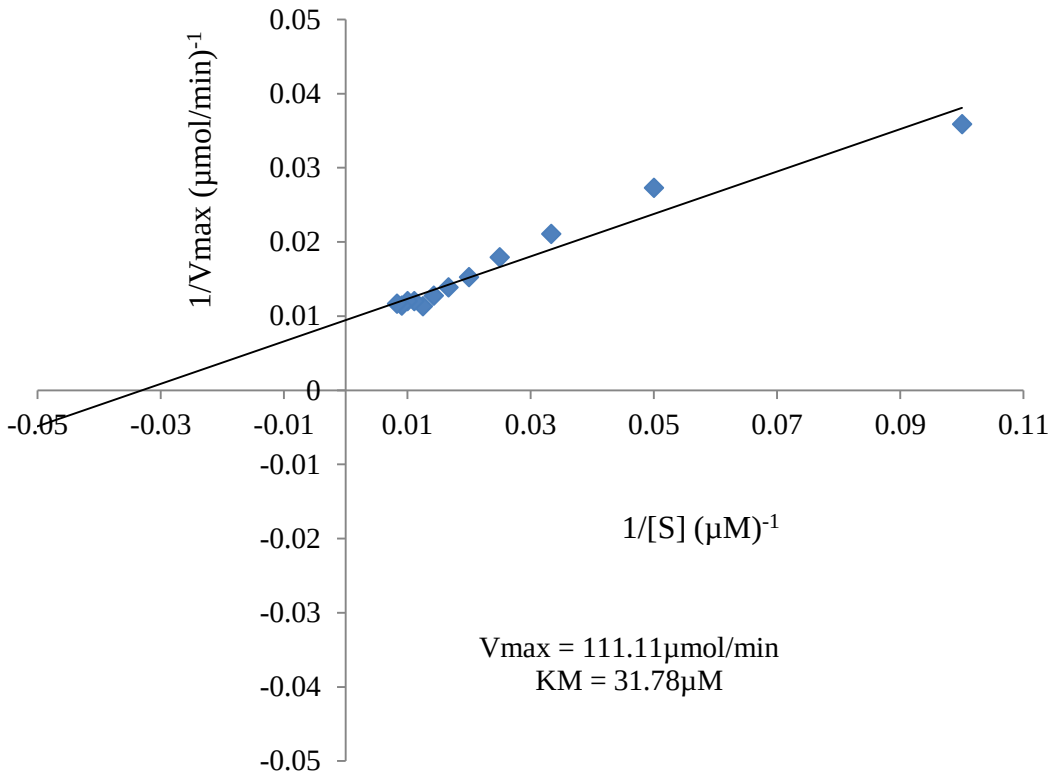

Figure 15: Line-Weaver Burk plot showing the Vmax and K_M for α-naphthol

3.7.7: Effect of Substrate [2,6-DMP] Concentration on Laccase Activity

The activity of laccase increased with an increase in the concentration of 2, 6-DMP until the laccase active sites were saturated. There was no observable increase in the activity of laccase on further increase in 2, 6-DMP concentration.

3.7.8: Line-Weaver Burk Plot Showing the Vmax and K_M for 2,6-DMP

The Michaelis constant (K_M) and maximum velocity (Vmax) obtained from Line-weaver Burk plot of initial velocity at various substrate concentrations were 48 μM and 1000 μmol/min, respectively.

3.7.9: Effect of Substrate [O-dianisidine] Concentration on Laccase Activity

The activity of laccase increased with an increase in the concentration of O-dianisidine until the laccase active sites were saturated. There was no observable increase in the activity of laccase on further increase in O-dianisidine concentration.

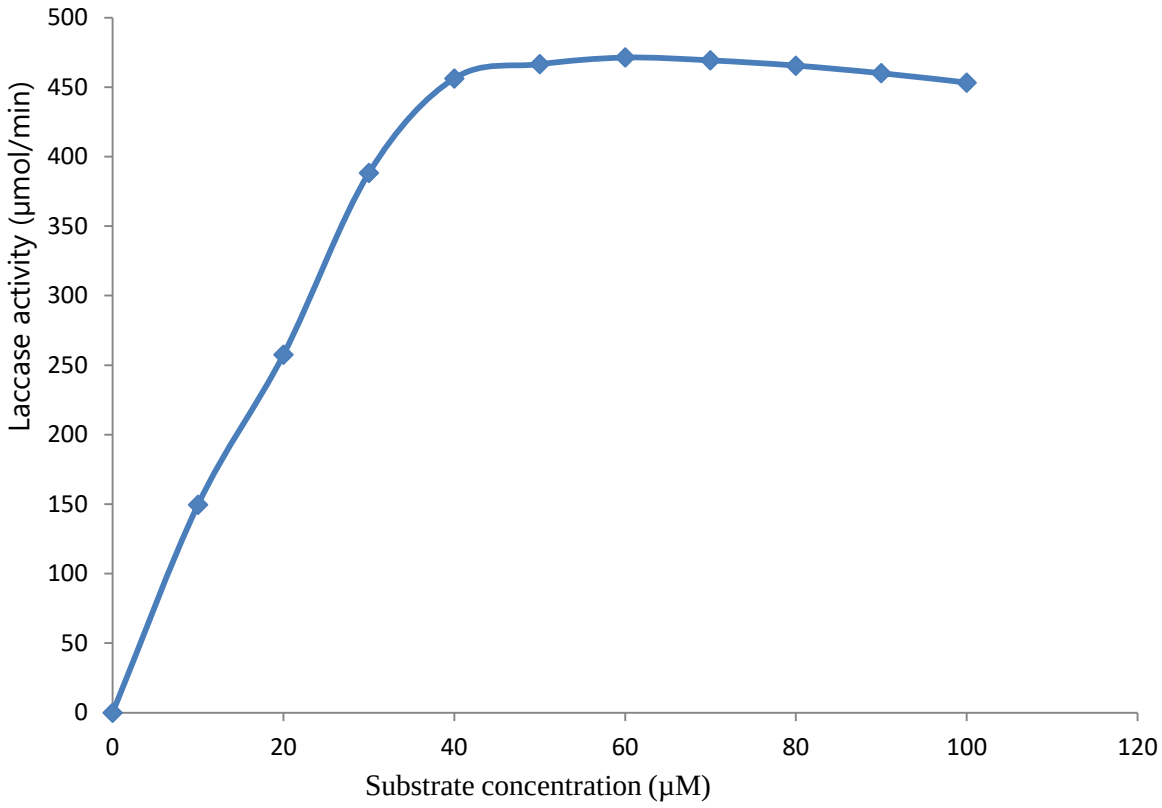

Figure 16: Michaelis-Menten plot showing the effect of substrate [2,6-DMP] concentration on laccase activity

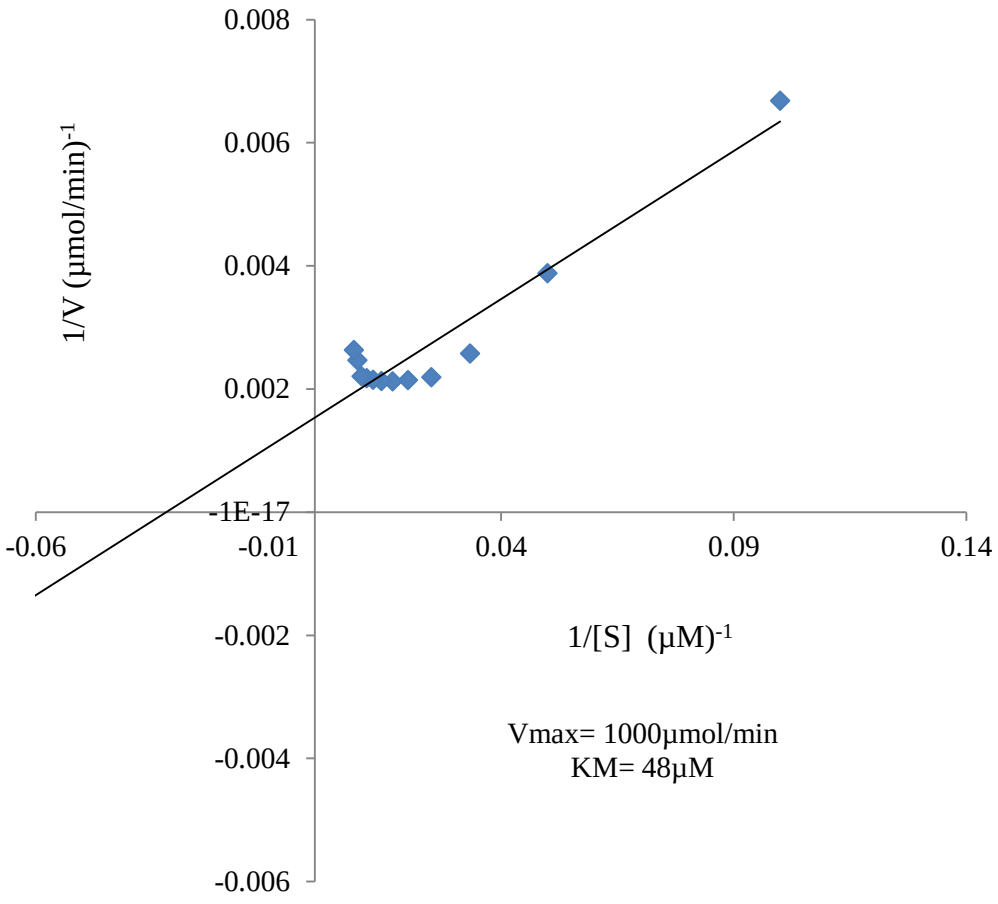

Figure 17: Line-Weaver Burk plot showing the Vmax and Km for 2,6-DMP

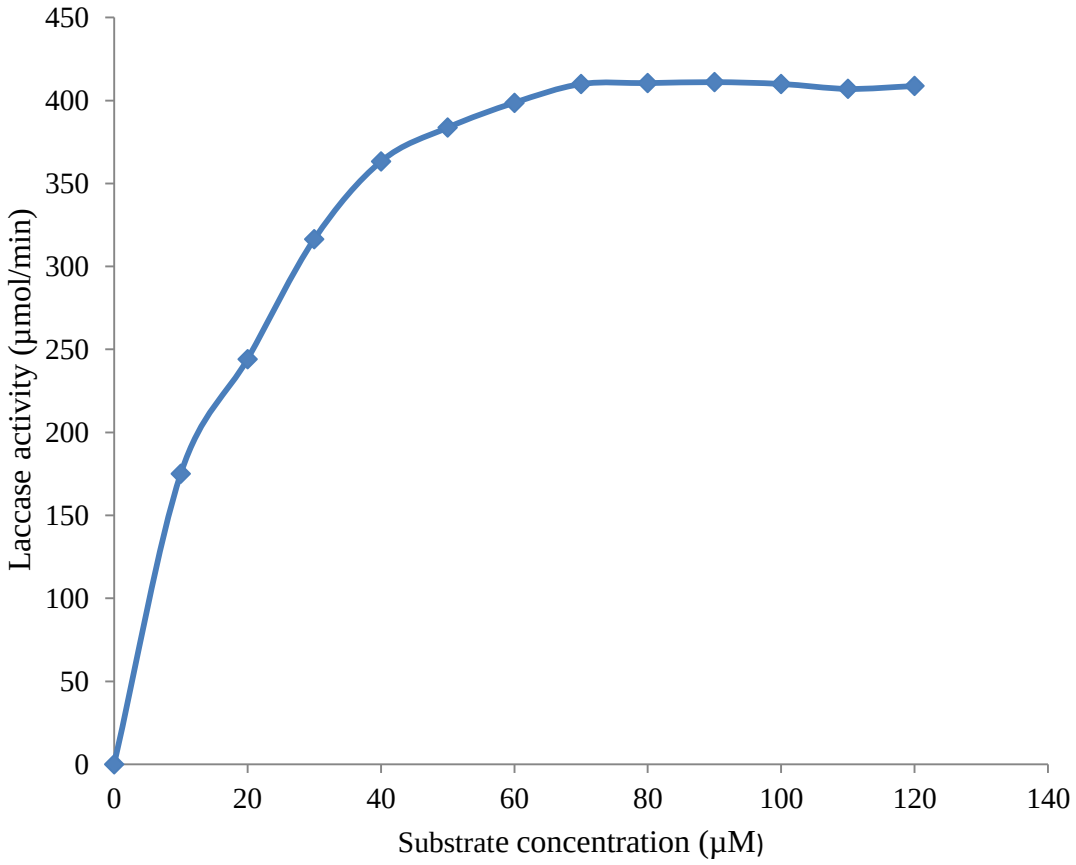

Figure 18: Michaelis-Menten curve showing the effect of substrate [O-dianisidine] concentration on laccase activity

3.7.10: Line-Weaver Burk Plot Showing the Vmax and K_M for O-dianisidine

The Michaelis constant (K_M) and maximum velocity (Vmax) obtained from Line-weaver Burk plot of initial velocity at various O-dianisidine concentrations were 18.5 μM and 500 μmol/min espectively.

3.8: Substrate Specificity Table of Laccase from *P. pulmonarius* Towards Aromatic Compounds

Table 2 shows the summary of the kinetic parameters of some selected aromatic compounds and the specificity of laccase towards those selected compounds. The result shows that laccase has higher specificity to phenolic compounds than non-phenolic compounds. Hence, the specificity of laccase for ABTS, α-naphthol, 2, 6-DMP and O-dianisidine are 2.91, 3.50, 20.83 and 27.03 respectively.

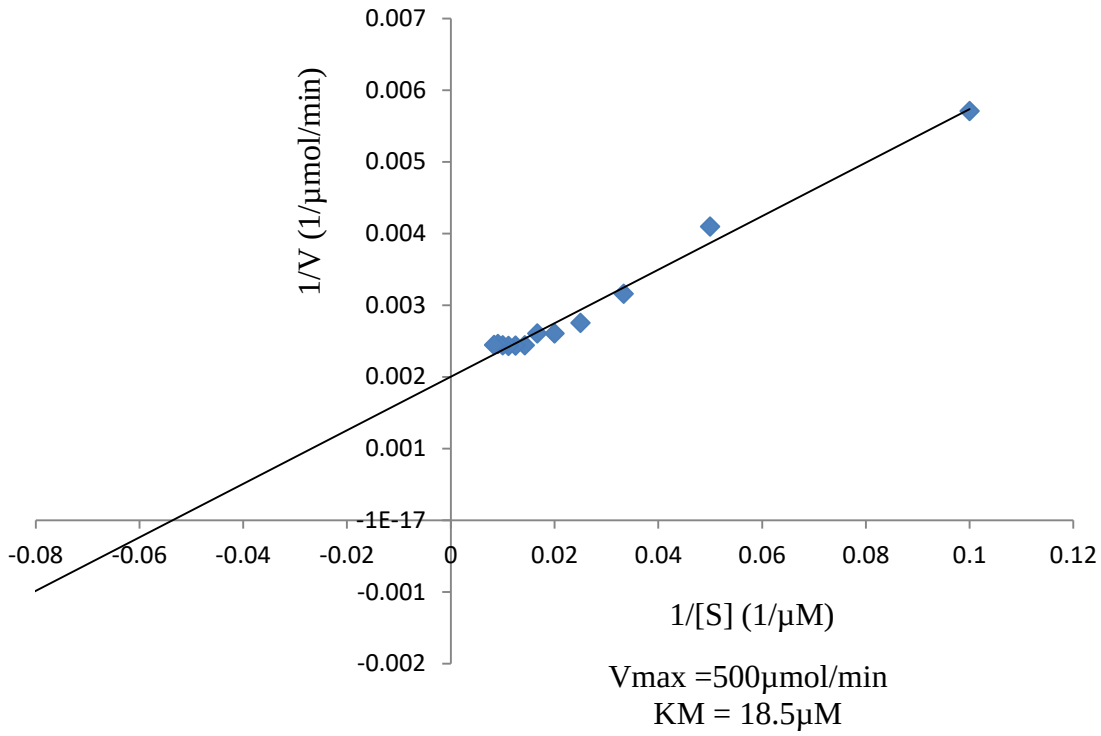

Figure 19: Line-Weaver Burk plot showing the Vmax and K_M for O-dianisidine

Table 2: Substrate specificity of laccase from *P. pulmonarius* towards some aromatic compounds

Substrate	**Vmax (µmol/min)**	**Km (µM)**	**Specificity**
ABTS	90.91	31.27	2.91
α-naphthol	111.11	31.78	3.50
2, 6-DMP	1000	48	20.83
O-dianisidine	500	18.5	27.03

CHAPTER FOUR

DISCUSSION

The production of laccase from white rot fungi depends on the medium composition and culture conditions. The presence of lignocellulosic monomers or aromaric monomers induces the expression of laccase. Wheat bran was used as the lignocellulosic monomer which served as the carbon source for laccase production from fungi. According to Papinutti *et al*. (2006), wheat bran was considered the best substrate for laccase production during solid-state fermentation because wheat bran contains a high amount of carbohydrates that can be utilized as the carbon source. Laccase producing fungi are generally selected based on the primary screening in agar plate containing a suitable substrate. Laccase producing organism (*Pleurotus pulmonarius*) showed purple coloured zone around the colony on PDA plate amended with ABTS (plate 2). Sathiyavathi *et al*. (2011) also used ABTS and guaiacol as an indicator for the confirmation of the laccase positive isolates.

After a fourteen day pilot study, day nine (9) was found suitable for mass production of laccase from *Pleurotus pulmonarius* with an activity of 590 U/ml (figure 7). This result was in tune with that of Sivakumar *et al*. (2010) who reported that the optimum day for laccase production was from 8 to 10 days. The result of Shafiq *et al*. (2016) showed that the optimum day for the production of laccase was on the 9th day. According to Hendro *et al*. (2012), *Marasmius sp.* on different substrates displayed higher laccase activity of 1116.11 U/L on the 10th day. The variation in the duration for the maximum expression of enzyme depends on the species of fungi used, pH, temperature and the culture conditions (Patel *et al*., 2009). Incubation temperature is a vital factor affecting the fermentation process in SSFs because both growth of fungi and production of enzyme are sensitive to temperature. However, high temperatures lead to adverse effect on the metabolic activity of the microorganism, thereby leading to the denaturation of the key enzymes. Xin and Geng (2011) have also reported that lower temperature retards the metabolic rate of *Trametes versicolor.* Fungi grow over a range of pH but mostly grow better in the acidic range (Nurdan *et al*., 2005). The effect of pH on the growth, production and activity of metabolites is due to its effect on cell permeability. The pH of the culture media strongly affects many enzymatic reactions and transport of compounds a cross the cell membrane as they are

sensitive to the concentration of hydrogen ions present in the medium (Adiveppa and Basappa, 2015). This may be attributed to the poor mycelia growth at an elevated pH which may restrict the laccase production.

The highest activity was obtained at 80 % ammonium sulphate saturation was found suitable to precipitate laccase of highest activity (figure 8). The ionic strength of the ammonium sulphate at 80 % is high and the interaction of the salt with the water molecules is greater than the interaction of protein with the water molecules. At 80 % ammonium sulphate saturation, the unfolded proteins retained their native conformation, leading to high activity observed at 80 % saturation. This hydrophilic interaction of ammonium sulphate salt with the water molecules made way for protein-protein interaction that causes the precipitation of proteins out of the solution. This result conforms to that of Yan *et al*. (2014) who reported 80 % ammonium sulphate saturation for laccase activity using *Trametes trogii.* This result shows that laccase is hydrophilic in nature as high concentration of salt was required to precipitate the protein with highest laccase activity. The precipitated laccase was then desalted by dialysis to remove low molecular weight molecules that might perturb the activity.

Upon dialysis, the volume of the enzyme increased as a result of simple diffusion created by concentration gradient. The volume of the final dialyzed enzyme increased as a result of diffusion of the buffer solution into the enzyme; that is from the higher concentration of water molecules (buffer) to lower concentration (the enzyme). Gel filtration was carried out in a column using sephadex G-200. Ptotein with highest laccase activity was eluted in fractio 35 (figure 9). Gel filtration involves separation of proteins based on size. Proteins of larger molecules elute first and those of smaller size elute later. The specific activity of laccase after ammonium sulphate precipitation, dialysis and gel filtration were found to be 3252 U/mg, 7310 U/mg, and 9515 U/mg with purification folds of 1.27, 2.85 and 3.71, respectively (table 1). This suggests that the enzyme became purer as the purification steps increased. The percentage yield is the amount of activity recovered or retained after every purification step. Yield tends to decrease following the increase in the purification fold because proteins are being lost at each level of purification.

Enzyme activities are significantly affected by pH and temperature. The optimum pH was observed to be 4.0 (figure 10). At pH 4.0, the catalytic amino acid side chains achieved their best conformational state thereby increasing the rate of catalysis. However, further increase in pH deprotonates the amino acid side chains which results in the reduction of the kinetic barriers that led to loss of proper folding and activity.

The optimum temperature was recorded at 50 ^{0}C (figure 11). This is in accordance with reports by Atalla *et al*. (2013). Moon *et al*. (2005) reported 50 ºC as the optimum temperature of laccase from *Trametes versicolor*. Slight variation in temperature could be as a result of substrate used and the fungi species. The increase in temperature from 30 $^{\circ}$C to 50 $^{\circ}$C increases the activity of laccase due to increase in the rate of effective collision between the substrates and the enzyme molecules.

Temperatures above 50 $^{\circ}$C lead to decrease in the activity of laccase because of possible denaturation of the three dimensional (3-D) structure of the protein. At higher temperature of 80 $^{\circ}$C and above, the enzyme denatured and the activity was lost.

The increase in substrate concentration increased the activity of the enzyme until a particular concentration of substrate where further increase in substrate concentration no longer affects the rate of reaction (figure 12, 14, 16 and 18). The kinetic plots from these results obeyed Michaelis-Menten curve. The Vmax and K_M for various substrates (ABTS, α-naphthol, 2, 6-DMP and O-dianisidine) are summarized in table 2. Michaelis constant, K_M is a kinetic term that denotes the substrate concentration at half the maximum velocity. The low K_M values show that low concentration of substrates can saturate the active sites of the enzyme. Hence, laccase has affinity for aromatic compounds. The maximum velocity V_{max} gives information on the turn over number of an enzyme which is defined as the number of mole of substrate converted into product per active site of the enzyme per second, when the enzyme is fully saturated with substrate. Laccase showed highest specificity for phenolic aromatic compounds (α-naphthol, O-dianisidine, 2, 6-DMP) more than non-phenolic aromatic compounds (ABTS). This result could be attributed to the fact that laccases catalyze phenolic substrates by abstracting one electron and one proton from the hydroxyl group and form phenoxy radicals that are stabilized by resonance into the respective quinone structures or covalently coupled to polymeric products (Mikolasch and

Schauer, 2009). Laccase attacks the phenolic compounds leading to Cα oxidation, Cα-Cβ cleavage and aryl-alkyl cleavage (Pankaj, *et al*., 2013). The enzymatic oxidation of phenolic compounds and anilines by laccases generate radicals that react with each other to form dimmers, oligomers or polymers covalently coupled by C–C, C–O and C–N bonds (Harald, 2004). It has been described that laccases can promote homo or hetero molecular coupling reactions. During reactions between phenols or quinonoid systems and primary amines, new C–O, C–N or N–N products are formed. Aromatic amines have been mainly used as nucleophiles in phenol reactions catalyzed by laccases. Hence, the specificity of laccase for ABTS, α-naphthol, 2,6-DMP and O-dianisidine were found to be 2.91, 3.50, 20.83 and 27.03 respectively (table 2).

4.2 Conclusion

Since the first discovery of laccase over a century, much work has been done by different scholars to enquire into the truth about the natural sources, biochemistry, sequences, production, and application of laccase class of enzymes. The research work tried to ascertain the kinetic parameters that could be used to determine the specificity of laccase towards different aromatic compounds. The high specificity of laccase from *P. pulmonarius* towards aromatic compounds could be useful in industries where biodegradation of aromatic effluents iss required.

4.3 Suggestions for Further Studies

1. The effect of divalent metal ions on laccase activity
2. The effect of inhibitors on laccase activities
3. The ph stability of laccase
4. The temperature stability of laccase
5 Immobilization of laccase

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