SCREENING, ISOLATION AND CHARACTERIZATION OF LACCASE ENZYMES FROM NAMIBIAN *TERMITOMYCES SCHIMPERI* AND *KALAHARITUBER PFEILII*

A THESIS SUBMITTED IN PARTIAL FULFILMENT

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BY

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ABSTRACT

Few reports could be found on screening, isolation and characterisation of enzymes in local Fauna and Flora and little is known about laccase enzymes from Namibia origin hence there is a niche for enzyme studies in this area. This research has qualitatively screened *Termitomyces schimperi* and *Kalaharituber pfeilii* fruiting bodies for laccase enzymatic activity using α-naphtnol and 2, 2-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid (ABTS). A clone of *T. schimperi* was also grown in the laboratory under controlled conditions. A purification protocol of laccase from *K. pfeilii* consisted of filtering the blended samples from the truffle's outer layer, supernatant precipitation with ammonium sulphate at 80% saturation, ultrafiltration, size exclusion gel chromatography, and then anion exchange chromatography with DEAE Bio-Gel. For *K. pfeilii*, the final purification step resulted in a total activity (U) of 0.172, specific activity 15.317 U/mg, yield 23.6% and a purification fold of 880 was obtained. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) showed that *K. pfeilii* was homogenous according to the size with a band appearing at 60kDA. Following the same protocol, purification of laccases from *T. schimperi* fungal combs gave a total activity (U) of 0.0094 and specific activity of 3.901 (U/mg) the yield was 0.095 % while a 4 fold purification was achieved. The laccase from the solid state media of clones of *T. schimperi* in the laboratory was also purified with the same protocol. The final step which was an elution through DEAE ion exchange chromatography resulted in total activity (U) of 1.226 and specific activity of 47.406 (U/mg). The yield was 17% and a 25 folds purification of was achieved. The research further characterised the purified enzymes based on the optimum pH and temperature. A laccase from *K. pfeilii* has optimum temperature around 60°C and showed significant activity even at

temperatures up to 80°C. The laccase from laboratory isolates of *T. schimperi* had optimum activity at 70°C and remained active even at 90°C.

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Ɛ	Extinction Coefficient
A280	Absorbance at 280 nm
ABTS	2, 2'azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
BSA	Bovine Serum Albumin
Cu	Copper
DEAE	Diethylaminoethyl
DMP	2.6-dimethoxyphenol
EC	Enzyme Commission Numbers
ET	Electron Transfer
g	Centrifugal Force
HBT	1-hydroxybenzotriazole
kDa	kilodalton
MAWF	Ministry of Agriculture, Water and Forestry
MCO	Multicopper Oxidases
MEA	Malt Extract Agar
M_r	Molecular Mass
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
T1	Type 1 (copper site)

T2/T3	Type 2/ Type 3 (copper site)
PDA	Potato Dextrous Agar
UNAM	University of Namibia
SANBio	Southern African Network for Biosciences
ZERI	Zero Emissions Research and Initiatives

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LIST OF PUBLICATIONS

V. L. Haileka, M. Kandawa-Schulz and A. Cheikhyoussef. Screening, Isolation and Partial Characterization of Laccase from Namibian *Termitomyces schimperi* and *Kalaharituber pfeilii*. Paper presented at the 2nd Annual Science Research Conference, 30-31 October 2014, University of Namibia, Windhoek, Namibia. pp.31-32

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DECLARATION

I, Vanessa L. Haileka, declare that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

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..... Date.....

DEDICATION

I dedicate this work to my family, My Mother, Ndamononghenda Haileka, to my grandmother Ester Mwenyombali Haileka, when the giants walk the earth, you were among them, and you were a phenomenal woman. Rest in Peace.

CHAPTER 1: INTRODUCTION

1.1 General Introduction-Enzymes the Major Biocatalysts

Properties of enzymes allows them to be utilized in enzyme technology as biocatalysts, either as isolated enzymes or as enzyme systems in living cells to catalyse chemical reactions on an industrial scale in a sustainable manner (Kirk, Borchert, & Fuglsang, 2002).

Enzymatic application covers the production of desired products for all human material needs for example, food, pharmaceuticals, detergents, hygiene, and environmental technology; and a wide range of analytical purposes, especially in diagnostics. In addition, their modification and optimization allow for new synthetic schemes and the solution of analytical problems (Kirk et al., 2002). The present global market for industrial enzymes is estimated to be around 4.4 billion USD, with the highest sales of technical enzymes occurring in the leather market, followed by the bioethanol market, while the food and beverage enzymes segment is third with an estimated value of 1.3 billion USD (Binod, Palkhiwala, Gaikaiwari, & Nampoothiri, 2013).

Africa has a high diversity of wild mushroom biota that is poorly researched and documented in term of their importance as a rich source of enzymes and bioactive components (Nakalembe & Kabasa, 2013).

1.2 Laccases Enzymes Roles in Biological Systems

Laccases are common enzymes in nature, especially in plants and fungi. Most studied laccases are of fungal origin especially those from classes of white-rot fungi such as *Trametes versicolor*, *Pleurotus sajor-caju* and *Ganoderma lucidum* (Giardina & Faraco, 2010). Most of the fungal laccases reported thus far are extra-cellular enzymes, whereas only few enzymes from fruiting bodies have been described so far. In fungi, laccases carry out a variety of physiological roles

during their life cycle including; morphogenesis, fungal plant pathogen/ host interaction, stress defence, and lignin degradation (Giardina & Faraco, 2010). In plants, laccases have been found in the wood and cellular walls of herbaceous species, where they participate in lignin biosynthesis (Giardina & Faraco, 2010). Bacterial laccases appear to have a role in morphogenesis, in the biosynthesis of the brown spore pigment and in the protection afforded by the spore-coat against UV light and hydrogen peroxide, and in copper homeostasis (Giardina & Faraco, 2010).The main function of the laccase-type proteins in insects is believed to be sclerotization of the cuticle in the epidermis (Giardina & Faraco, 2010). Fungal laccases have higher redox potential than bacterial or plant laccases, and their action seems to be relevant in nature (namely in the degradation of lignin) finding also some important applications in biotechnology (Silvério, Rodríguez, Tavares, Teixeira, & Macedo, 2013).

1.3 Statement of the problem

The enzyme industry is ever looking for products with characteristics of highly commercial value. Enzymatic oxidation has advantages over chemical oxidation, because reactions are carried out in mild conditions and enzymes are biodegradable and highly specific catalysts (Silvério et al., 2013). However screening for enzymes in local fauna and flora is non-existent at the moment and hence, little is known about laccases from endemic Namibian fungi species.

Contrary to other edible wild mushrooms, less attention has been paid to the indigenous information about truffles in Africa. The available information comes from some amateurs and foreign tourists' documentation, as well as from knowledge accumulation passed orally from generation to the next (Enshasy, Elsayed, Aziz, & Wadaan, 2013). This research documents the screening, isolation and purification of laccase(s) from Namibian the local fungal fauna.

1.4 Objectives of the Research

The main objective of this research was to expose value addition opportunities of local Namibian wild mushrooms, by investigating the distribution of industrially important enzymes found in Namibian endemic fungal species of the termite mushroom, *Termitomyces schimperi* and the Kalahari truffles, *Kalaharituber pfeilii*.

The specific objectives of this research include:

1. Screening, isolation and purification of laccase(s) from termite mushroom, *Termitomyces schimperi* and the Kalahari truffles *Kalaharituber Pfeilii*.
2. Measuring the enzymatic activities of the isolated enzymes.
3. Characterising the isolated enzymes based on the molecular weight, pH, and temperature.

1.5 Hypotheses of the Study

The study had two research hypotheses:

1. Laccases are thought to be nearly ubiquitous among fungi, and their presence has been documented in virtually every mushroom species examined thus far (Lettera et al., 2010). Recently, a study from Italy documented the presence of laccase in the black truffle of Périgord (Zarivi et al., 2013). Hence Namibian termite mushroom, *Termitomyces schimperi* and the Kalahari truffles *Kalaharituber Pfeilii* might possess laccase enzyme activity.

2. Due to the unique conditions these organisms grow under, it is expected that the isolated laccase enzymes have unique properties some of which could be useful for various applications such as heat and pH stability.

1.6 Significance of the Study

This study investigates the presence of laccases in the fruiting bodies of endemic Namibian fungi: *Termitomyces schimperi* and *Kalaharituber pfeilii,* by doing so it documents for the first time the presence of the laccases in these local edible fungal species. It shows that both edible fungal species although a source of food they can be a source of useful bioactive proteins of commercial value. Laccase production has been documented from different species of microorganisms, unfortunately, most of them have low yield of enzyme activities and poor thermal stability. Thermostability of laccases in this study revealed that they have a potential for industrial applications. Purification protocol of laccase enzymes in this study shows the easy and practicality of establishing purification strategies for laccase isolation in the future. It has characterized them partially in terms of heat and pH stability these factor that that provides a better understanding on their possible potential industrial applications, as well as defining their role(s) in development and nutrition related issues.

CHAPTER 2: LITERATURE REVIEW

2.1 Laccase Enzymes

2.1.1 Structure of Laccases

Laccases are either monomeric or multimeric copper containing glycoproteins (Figure 1), with molecular masses between 50 kDa and 130 kDa which may exhibit additional heterogeneity because of variable carbohydrate content or differences in copper content or because they are expressed as the products of multiple genes (Patel, Gupte, & Gahlout, 2014).

2.1.2 Biochemical Properties

Laccases are a family of enzymes within the protein superfamily of multicopper oxidases (MCOs) (Figure 1) (Demet, Wagner, & Wang, 2011). MCOs consist of four enzyme families: laccases (EC 1.10.3.2), ascorbate oxidases (EC 1.10.3.3), ferroxidases (EC 1.16.3.1) and ceruloplasmin (EC 1.16.3.1) (Figure 1) (Demet et al., 2011).

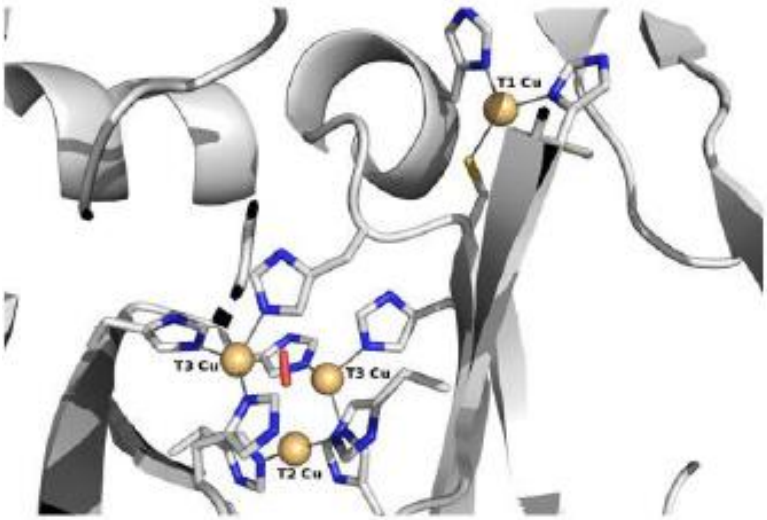


Figure 1 The structure of copper sites found in four multicopper enzymes families, copper ions are illustrated in brown spheres (Bento et al., 2010).

Laccases, which are the simplest members of the MCOs family, show a characteristic fold that comprises three cupredoxin domains, with a mononuclear copper centre localised in the third domain, and a trinuclear copper centre located in between the first and the third domains (Figure 2) (Patel et al., 2014).

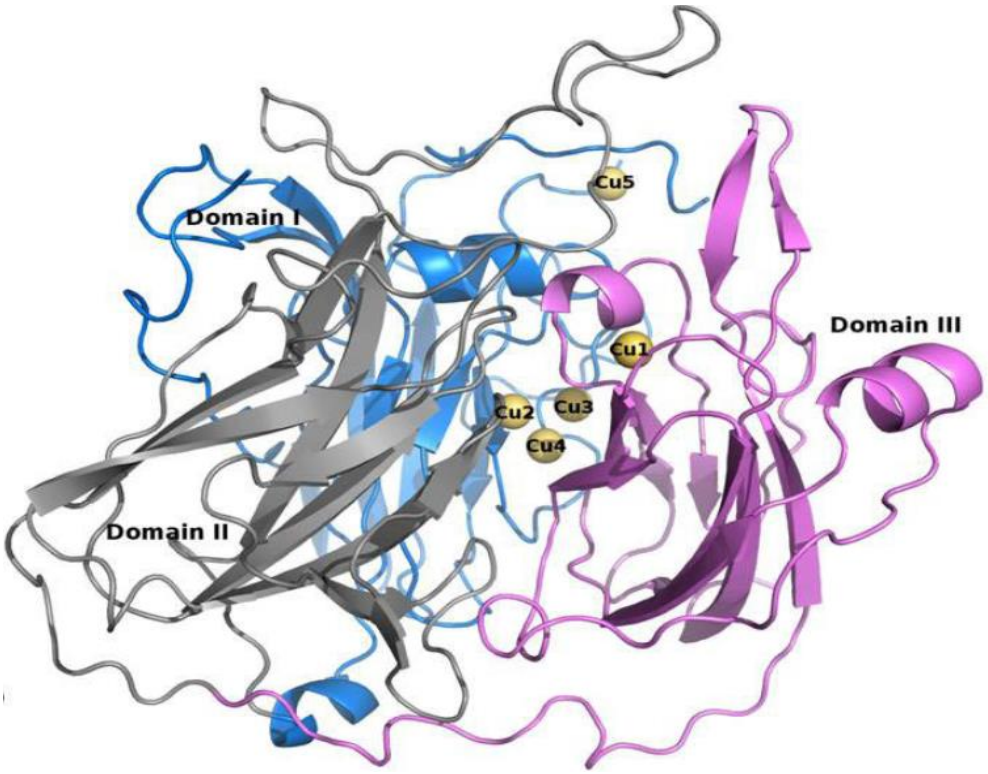


Figure 2 Three – dimensional structure of CotA laccase with each cupredoxin domain coloured in a different colours, domain I blue, domain II grey, and domain III, violet copper atoms are represented by spheres coloured in yellow (Bento et al., 2010).

The loops surrounding the one blue Type I (T1) copper forms represents the phenolic substrate-binding site of the enzyme where the substrate is oxidized, and a tri-nuclear copper cluster consisting of three Type II (T2)/Type III (T3) copper forms is where oxygen is bound, activated and reduced (Figure 3) (Viswanath, Rajesh, Janardhan, Kumar, & Narasimha, 2014).

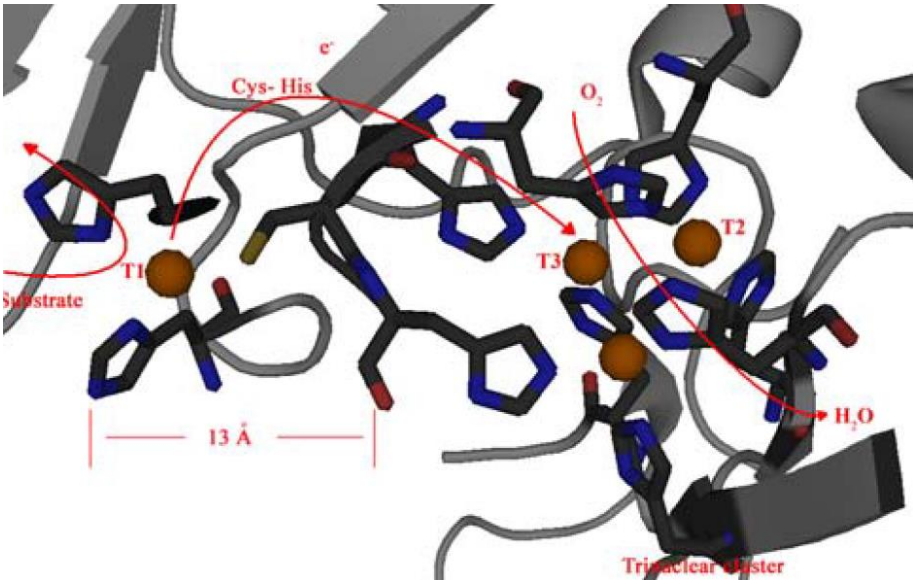


Figure 3 The structure of the laccase active site with arrows marking the flow of substrates, electrons (e^-) and O^2 (Giardina & Faraco, 2010).

2.1.3 Laccase Catalysed Reactions

Laccases are one of the oldest enzymes reported and currently arousing great interest in the scientific community because of its basic requirements (it just needs air to work and its only released by-product is water) (Figures 3&4) and huge catalytic capabilities, making it one of the ‘‘greenest’’ enzymes of the 21st century (Mate´, Garcı´a-Burgos, Garcı´a-Ruiz, Ballesteros, Camarero, & Alcalde, 2010). The substrates of laccases may vary from diphenols and polyphenols to diamines, aromatic amines, benzenethiols, and substituted phenols (Viswanat et al., 2014).

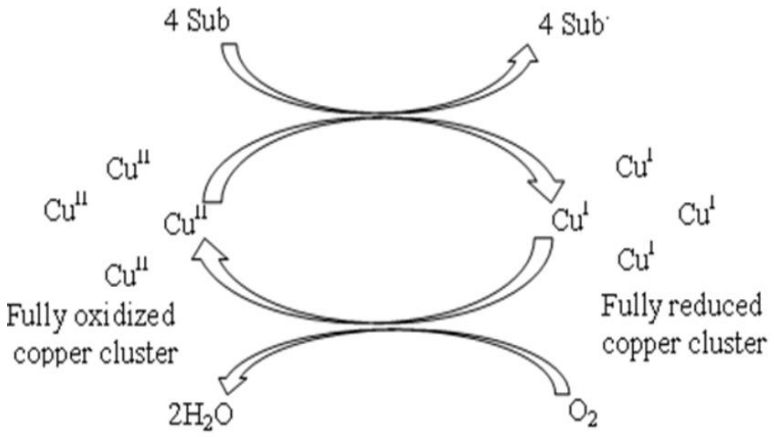


Figure 4 Diagrammatical representation of reduction of molecular oxygen to water (Chaurasia, Bharati, & Singh, 2013).

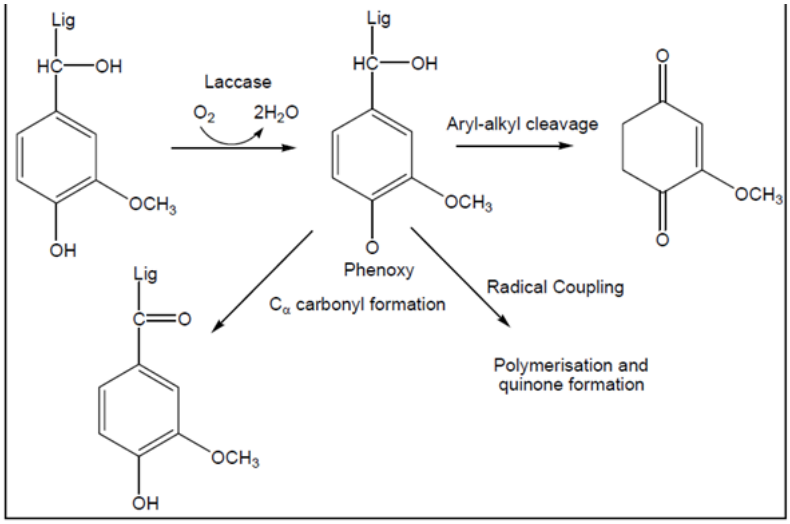


Figure 5 Oxidation of phenolic subunits of lignin by laccase (Kunamneni, Ballesteros, Plou, & Alcalde, 2007).

2,2-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), guaiacol and 2,6-dimethoxyphenol (2,6-DMP) are substrates generally used to assay a laccase activities. Syringaldazine, toluidine, and *p*-phenylene diamine are considered specific substrates for laccases (Rouvinen & Hakulinen, 2011). Mediators, acting as intermediate substrates for laccases, enable laccase to indirectly oxidize large molecules and even nonphenolic substrates, such as

nonphenolic β-1 lignin model dimer (Figure 5). The first mediator used in the laccase-mediator system (LMS) for pulp delignification was ABTS.

Laccase/mediator-catalysed oxidations of nonphenolic substrates (Figure 6) can proceed via two different mechanisms. ABTS-mediated reactions follow an ET route (Figure 7), whereas the[N–OH-type mediators, such as HBT, follow a hydrogen atom transfer (HAT) route by abstraction of a hydrogen atom from the[N–OH-type mediators, producing a [N–O] (Giardina & Faraco, 2010).

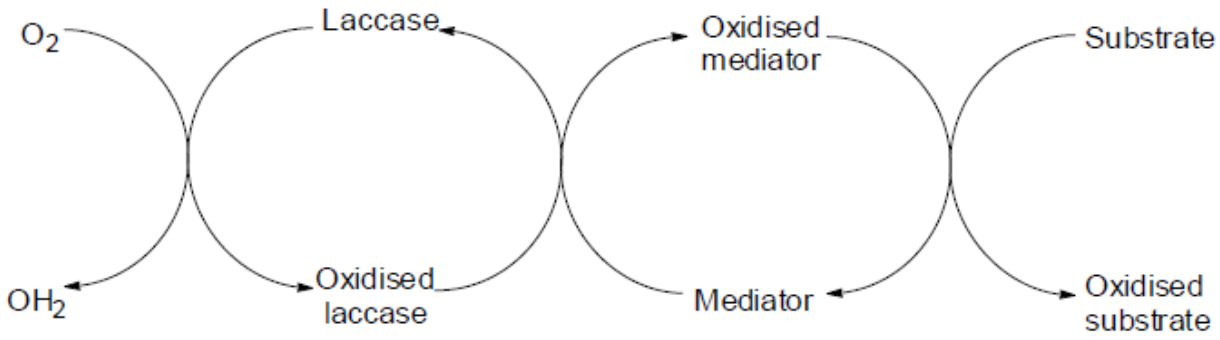


Figure 6 Catalytic cycle of a laccase-mediator oxidation system (Kunamneni, Ballesteros, Plou, & Alcalde, 2007**).**

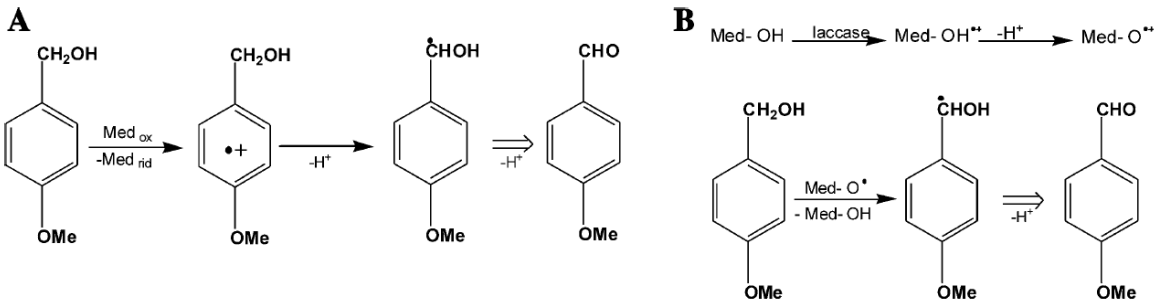


Figure 7 Laccase/mediator catalysed oxidations of nonphenolic substrates via ET route (a) and radical HAT route (b).

2.2. Industrial Applications of Laccase Enzymes

Laccase enzymatic features are suitable for several different practical applications in industrial biotechnology. Seven commercial industries where laccase enzymes are being utilized are the paper and pulp industries, textile manufacturing, washing powder, food manufacturing, pharmaceuticals production, diagnostics and environmental applications. In the paper production industries, laccases are used for pulp delignification. Laccases are known to possess the capabilities for textile dye bleaching and have the ability to oxidise a wide variety of phenolic compounds; examples include its use as a washing powder component or for exclusion of phenolic compounds from cork stoppers. These blue copper enzymes are also used as catalysts for the transformation of antibiotics and steroids such as in manufacturing of two products with anti-cancer properties, actinocin or the phytoalexin resveratrol and even as ingredients in cosmetics (Giardina & Faraco, 2010). Some investigation into Laccase phytomedicine disclosed that the fruiting bodies of the mushroom. *Agrocybe cylindracea* produce a laccase with HIV-1 reverse transcriptase inhibitory activity with an IC_{50} around 12.7 μM and anti-proliferative activity against hematoma Hep G2 cells and breast cancer MCF-7 cells, with an IC50 less than 10μM for both cell lines. The mechanism is probably protein–protein interaction (Hu, Zhang, Zhang, Wang, & Ng, 2011). Other promising applications include food improvement, development of biosensors biofuel cells and nanobiotechnology for the development of biosensors to detect various phenolic compounds, oxygen or azides (Chhaya & Modi, 2013).

For environmental applications; they are used in bioremediation of soils, water, as well as detoxification of industrial effluents mostly from the paper and pulp; textile and petrochemical industries (Kunamneni et al., 2007). Real effluents treated with laccases achieved the removal of the mutagenic character of the effluent (Giardina & Faraco, 2010).

Phenol-polluted waters are widely produced as wastes of several industrial and agricultural activities. Phenolic compounds and their derivatives are considered priority pollutants because they are harmful toward living organisms, even at low concentrations (Kunamneni et al., 2007). Oxidoreductases are able to catalyse the transformation of several phenolic compounds through an oxidative coupling reaction. This results in the formation of less soluble, high molecular weight compounds that may be easily removed from water by sedimentation or filtration (Chakroun, Mechichi, Jesus, Dhouib, & Sayadi, 2010). Laccases might also be useful in synthetic chemistry. For instance, laccase-catalysed reactions can be used for the polymerization of catechol monomers for polycatechol synthesis or for the production of inert phenolic polymers, such as poly (1-napthol). These polymers have application in wood composites, fibre bonding, laminations, coatings, and adhesives. A novel system of enzymatic polymerization for the preparation of ‘‘artificial urushi’’ polymeric films (Japanese traditional coating) has been demonstrated, using laccases.

The use of laccases is broadened by the use of synthetic and metal-complex mediators for industrial purposes, they are used in large quantities and this is very costly and not very safe for the environment. To diminish these difficulties, various methods have been tested among them production of laccase recombinants (Malarczyk, Kochmanska-Rdest, & Jarosz-Wilkolazka, 2009). Therefore, the search for economical and safe laccases production methods has been one of the main enzyme research topics in the past few decades.

2.3 Fungi as producer of extracellular Laccase enzymes

Although there are many taxonomic or physiological groups of fungi that do not produce significant amounts of laccases, these oxidative enzymes are particularly abundant in white-rot basidiomycete fungi such as *Trametes versicolor, Trametes hirsuta, Trametes ochracea,*

Trametes villosa, Trametes gallica, Cerrena maxima, Coriolopsis polyzona, Lentinus tigrinus and Pleurotus eryngii where they are capable of degrading lignin *in vivo* (Patel et al., 2014). In the case of *Pyconoporus cinnabarinus* laccase was described as the only ligninolytic enzyme produced by this species with the ability to degrade lignin (Baldrian, 2005). There are many records of laccase production by ascomycete too. Information available is helpful in finding ways to employ these fungi or their enzymes for useful purposes such as production of biofuel from plant biomass and detoxification of environmental pollutants. Concomitantly, overexpression of laccase in suitable host organisms would provide means to achieve high titres and the use of inducers could also enhance production capabilities.

2.4. Current Research on Mushrooms in Namibia

Current mushroom research in Namibia is promising and being focused on promoting mushroom production for food consumption, income generation and employment creation. Mushroom research in Namibia stems from the initiatives of the Zero Emissions Research and Initiatives (ZERI) projects, a partnership between UNAM, the Southern African Network for Biosciences (SANBio) and the Finnish Southern Africa Partnership Programme to Strengthen NEPAD/SANBio Network (BioFISA).

The ZERI concept is to turn waste into a resource, thus generating "value added" products and this is where mushrooms come in; as a spent material is used as a prime substrate for the mushroom's growth (Spinosa, 2008). Namibian scientists focused on among others on anhancing higher mushroom yields grown on local substrate materials. Molloy and colleagues (Molloy, Critchley, Kandjengo, & Mshigeni, 2003) chose the oyster mushroom, *Pleurotus sojior-caju*, and did a preliminary study on its utilization for seaweed and brewing industry waste. This species was used because it is tolerant of high temperatures, grows on a wide variety of substrates and

produces a high yield of valuable mushrooms (Molloy, Critchley, Kandjengo, & Mshigeni, 2003).

Mushrooms research is one of the active areas of research in the Ministry of Agriculture, Water and Forestry (MAWF), with the focus being on producing the exotic oyster and white button mushrooms on indigenous crop waste materials as substrates. It is demonstrated that mushrooms could be successfully grown on crop residues, straws and other agricultural wastes that are readily available, especially in the northern communal area where crop residues are in abundance. Basidiomycetes are the only organisms known to degrade lignocellulose using two alternative degradation strategies: white rot basidiomycetes break down the lignin moiety extensively before attacking the cellulose, whereas brown rot basidiomycetes cause limited lignin alteration while primarily degrading cellulose (Alfaro, Oguiza, Ramírez, & Pisabarro, 2014). These strategies depend on complex portfolios of extracellular enzymes whose production and export are environment and substrate dependent (Alfaro et al., 2014) .

However, in order to make real "value added" products, mushrooms and fungus can be grown to produce secondary products such as enzymes albeit still maintain their function in society as food. Hence, this research will be screening Namibian fungi for laccase enzymes in their fruiting bodies. This is to establish that the enzyme production does not become a competitor with the fungi's function to be a source of nutrition to human but instead become both a source of food as well as a source of industrially important enzymes such as laccases.

2.4.1 Termite Mushroom

Termite mushrooms, *Termitomyce*s sp. (locally known as *Omajova* in Otjiherero) are giant mushrooms that emerge from the base of the termite mounds during the rainy season (Hildén, Mäkelä, Lankinen, & Lundell, 2013).

Figure 8 *Termitomyces schimperi*

Although they are widely spread in Africa and Asia, they are not well characterised but currently there many studies from the continent focused on documenting their existence and exploring their macro and micro morphological characteristics. Fungus-growing termites cultivate their fungal symbionts in monocultures (Katoh et al., 2002; Moriya et al., 2005; Shinzato et al., 2005; Aanen et al., 2009) on sponge like fungus combs within the nest (e.g., Sands, 1969). The termites provide substrate, an optimal microclimate, and a competitor-free space for fungal growth (Tibuhwa, 2012).

Termitomyces schimperi, a common fungus in many African countries, belongs to the phylum Basidiomycota; Class Agaricomycetes; Order Agaricales; Family Lyophyllaceae; genus Termitomyces.

Termitomyces species are characterized morphologically based on the dome-looking shape of their perforatorium on the pileus, which is well developed, and the subterranean pseudorhiza, which are attached to the fungal comb of the termite's nest (Tang et al. 2005). In contrast to other higher basidiomycetes, T*ermitomyces* species do not form clamp connections between adjacent cells, thus making a heterokaryon difficult to differentiate from a homokaryon (Siddiquee & Yee, 2012).The volatile organic compounds VOCs of fresh fruiting bodies include more than twenty-four compounds (Enshasy et al., 2013).

2.4.2 *Kalaharituber pfeilii*

Kalaharituber pfeilii is a hypogeous ascomycete fungus it belongs to the order Pezizales, which has six families: Glaziellaceae, Discinaceae, Morchellaceae, Helvellaceae, Tuberaceae, Pezizaceae, and Pyronemataceae (Enshasy et al., 2013).

Figure 9 *Kalaharituber pfeilii* fruiting bodies

It belongs to the family Tuberaceae, in this family, most of known desert truffles species belong to the genera such as Terfezia, Delastreopsis, Balstonia, Delastria, Leucangium, Mattirolomyces, Phaeangium Picoa, Tirmania, and Tuber, of which *Kalaharituber pfeilii* is a member (Kagan-Zur & Roth-Bejerano, 2008). *Kalaharituber pfeilii* is found in the Namib Desert and Northern part of Namibia. Truffles have no stem, no gills, and its mycelium grows underground (Wang & Marcone, 2011). Wherever they are found, fruit bodies of desert truffles are collected and appreciated as a food. They are eaten fresh or dried for preservation. Their edible ascocarps are rich in fiber, proteins, vitamins, and minerals with high commercial value. In Italy and France, each kilogramme of truffles sells on the open market from €600–€6 000 (equivalent to N$7100-71000) depending on species (Wang & Marcone, 2011).

Studies on the composition and nutritional value of desert truffles have been carried out in those countries where they are known and appreciated and biochemical studies of enzymatic activities in desert truffle is immerging (Kagan-Zur & Roth-Bejerano, 2008). In various studies, it has been shown that truffles possess bioactivities including antioxidant, antiviral, antimicrobial, hepatoprotective, anti-mutagenic and anti-inflammatory activities. They are a rich sources of nutrients (protein, carbohydrate, starch, reducing sugars and low fats), micronutrients (vitamins and carotenoids) and minerals (P, K, Mn, Co, Ni, Cd, Fe) with promising bioactive properties (antioxidant and antibacterial potentials (Kumar, Jayanta, & Patra, 2014). There is a high interest in both biological scientific and culinary studies with the latter paying special attention to the aroma and nutritional characteristics of truffles but also to their biological potential.

To understand the differences between truffle species and their qualities, culinary scientists have established aroma profiles for identification purposes and methods to control postharvest quality deterioration, which help truffles to maintain their nutritional value, sensory qualities and to

extend their shelf life (Wang & Marcone, 2011). Biochemical scientist have focused on investigate the value-added benefits to truffles by demonstrating their potential biological functions in human and animals (Wang & Marcone, 2011).

2.5 Current Research on Laccase Enzymes of Truffels or Termitomyces

Almost all species of white-rot fungi were reported to produce laccase in varying degrees and the enzyme has been purified from many species (Viswanath et al., 2014). Screening and isolation of novel laccases with different physicochemical and catalytic properties is occurring frequently because of their practical applications in industrial biotechnology (Puthirasigamany, Wirges, & Zeiner, 2013). Several production strategies have been adopted along with process optimization to achieve better process economics (Lettera, Vecchio, Piscitelli, & Sannia, 2011). Laccases are promising enzymes to replace the conventional chemical processes of several industries such as: pulp and paper, textile, pharmaceutical, and nano-biotechnology (Prinz, Hönig, Schüttmann, Zorn, & Zeiner, 2014).

Researches in Taiwan isolated laccases from the habitats of the fungus growing termites mainly, the fungus combs and associated symbiotic fungus (Taprab, Johjima, Maeda, Moriya, & Trakulnaleamsai, 2005). Consequently, phenol degradation in the fungus comb is considered to be important for improving palatability of termite food, especially that containing high phenol content such as fallen leaves and bark (Taprab et al., 2005). In Italy the laccase enzyme has been documented in *Tuber melanosporum* (Zarivi et al., 2013).

One of the problems to commercialise the use of laccase is the lack of sufficient enzyme stocks. Thus, efforts have to be made in order to achieve cheap overproduction of laccase in heterologous hosts, and also their modification by chemical means or protein engineering, to obtain more robust, active and less expensive enzymes. To diminish these difficulties, various

methods have been tested and among them are the electrochemical methods and production of laccase recombinants (Malarczyk et al., 2009). Based on the literature, laccases from these funguses (truffles and Termitomyces) have special characteristics and the molecular weight, absorption, optimum temperature and pH for laccase production, and temperature and pH stability will be highlighted in this study.

2.6 Laccase Purification

Laccase purification protocol usually follows the following procedure supernatant filtration, ammonium sulphate precipitation at 80% saturation. The sample is then desalted by loading into a Sephadex-100 gel desalting column. Fractions showing activity are then loaded onto a DEAE-Cellulose ion-exchange column for final purification. The purification steps could be optimised with gradient elution buffers (Chefetz, Chen, & Hadar, 1998).

2.7. Temperature and pH stability

Neutral laccases show a remarkably high stability with respect to both pH and temperature. The enzyme contains only one copper atom/molecule instead of the usual four, along with two zinc atoms and one iron atom in each protein molecule. An important factor playing a significant role in the enzymatic catalysis is the pH of the reaction medium, which affects not only the catalytic activity of laccase, but also the redox potential of substrates (Polak & Jarosz-wilkolazka, 2012).

CHAPTER 3: METHODOLOGY

3.1. Research Design

This study employed both qualitative and quantitative approaches on screening, isolating and purifying laccases from local fungi: *Termitomyces schimperi* and *Kalaharituber pfeilii*. Qualitative design yielded information such as the indication of laccase production on Potato Dextrose Agar (PDA) using chromogenic indicators of laccase activity α -naphthol ABTS. Laccase enzyme oxidizes α-naphtnol to a deep purple complex in the area surrounding the mycelia or tissue, giving a visual confirmation for the presence of the enzyme. Quantitative purity of the enzymes was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis by determining the molecular weight of the isolated laccases. Quantitative approaches resulted in data such as quantification of protein (mg) concentration with Bradford's reagents. Enzyme activity (IU), specific activity (U/mg), recovery (%) and purification (fold) were also quantified using ABTS (2, 2'–azino-bis 3-ethylbenzthiazolin-6-sulphonic acid) as a substrate. The study is designed in such a way that species of fungi that showed laccase activity on α -naphthol were further isolated and laccase enzymes in these species were purified and characterised.

3.2 Chemicals

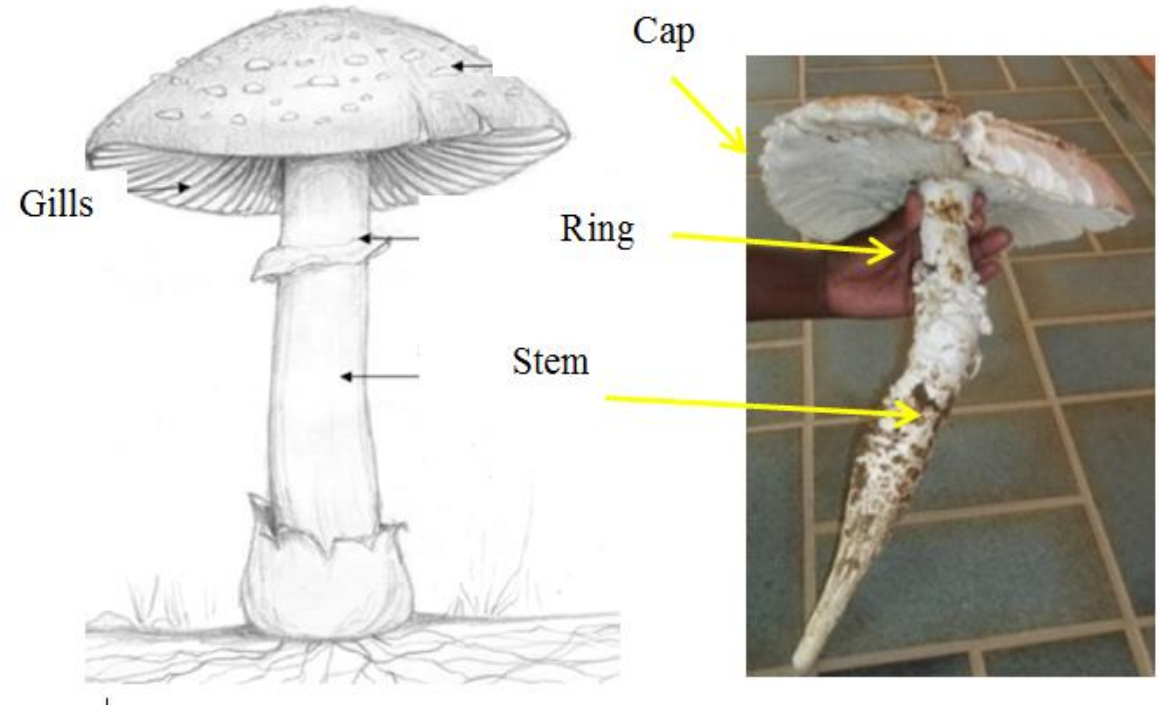
PDB (potato dextrose broth), bacteriological agar, yeast extract, malt extract, peptone and dextrose, were from Merck (Merck, Germany). The chemicals applied include sodium bicarbonate, sodium carbonate decahydrate, citric acid monohydrate, tri-sodium citrate dehydrate, citric acid monohydrate, sodium phosphate dibasic dehydrate, sodium phosphate monobasic monohydrate, acrylamide, bis-acrylamide, TEMED (N,N,N',N'-tetramethylethylenediamine), 2-mercaptoethanol, SDS (sodium dodecyl sulfate), trizma-base,

glycine, Coomassie blue, and low range markers kit, were from Bio Rad (Bio-Rad, USA). Enzyme substrates ABTS (2, 2'–azino-bis 3-ethylbenzthiazolin-6-sulphonic acid); DEAE-Sepharose and Q-Sepharose and Biogel P-100 from Bio-Rad (Bio-Rad, USA). All other reagents, chemicals, including solvents, inorganic salts, acids and bases solutions and culture media were prepared with bi-distilled water from Merck Millipore (Merck-Millipore, USA).

3.3 Sample Collection

3.3.1 Fungi Fruiting Body Samples

The wild termite mushrooms, *Termitomyces schimperi* and truffles *Kalaharituber pfeilii* were collected from Hochveld, North-East of Okahandja, Otjozondjupa Region central Namibia and Omuthiya, Oshikoto region northern Namibia, respectively. Samples were collected during the rainy season from February to May 2014. After collection, the collected fruit bodies were cleaned by gently wiping the fruiting bodies with cloth to remove soil debris. *Termitomyces schimperi* was cut into different sections based on the sections of a mushroom (Fig 10), and were crushed and preserved in citrate buffer (pH 4) and stored at -4°C. *Kalaharituber pfeilii* was also blended, preserved and stored under similar conditions



a

b

Figure 10 Parts of the mushroom highlighted on *T. schimperi* a. *Macrotermes natalensis* on a *T. schimperi* stalk (b) downloaded from: http://pixshark.com/parts-of-a-mushroom.htm

3.3.2 Fungi Cultivation under Laboratory Conditions

3.3.2.1 Solid State Growth Conditions

In order to test for laccase production under laboratory conditions *T. schimperi* fungus was cultivated for the production of extracellular enzymes and grown on Potato Dextrose Agar (PDA) in the following way: small pieces of the *T. schimperi* was put on PDA media and incubated at 31°C for 7 days. Fungal colonies that grew on this media where further propagated to new plates to isolate pure colonies. For further optimization, cultures were inoculated onto different agar media with a pH adjusted to 6.0 in Ø 90 mm petri dishes: Malt Extract Agar, Potato-Dextrose Agar and Sabouraud agar. The fungal isolates where able to grow on both media types. Media type that produced the highest laccase activity was selected for growth for the isolation and purification of the laccase enzyme.

3.3.2.2 Broth State Growth Conditions

T. schimperi pre-cultures were initiated by placing small mycelium pieces from 3-day-old (31°C) PDA plates, in 500 mL Erlenmeyer flasks containing 100 mL of nutrient broth. The submerged culture was grown at 31ºC for 10 days without shaking. Since the media on the plates showed activity of laccase once they were supplemented with $CuSO_4$ the nutrient broth media was also supplemented with the following salts (Table 1).

Table 1 Composition of nutrient broth mineral medium for the cultivation of ***T. schimperi*** under laboratory conditions.

Components of medium	**Concentration**
$MgSO_4$. 7 H_2O	0.5 g/L
NaCl	0.1 g/L
$CaCl_2$. 2 H_2O	0.1 g/L
$CuSO_4$. 5 H_2O	0.1 mg/L
$FeSO_4$. 7 H_2O	0.2 mg/L
$MnSO_4$. 4 H_2O	0.02 mg/L
$ZnCl_2$	0.15 mg/L

3.4 Screening Samples for Laccase Production

In order to see if the fungi can produce laccase enzymes, 10 days old plates of fungal colonies were sprinkled with 3 drops of α-naphthol, if the fungal colony is able to produce laccase a purple halo appears where the α-naphthol was dropped. After 10 days sample was also taken from the liquid state culture and the ability of the organism to produce the laccase enzyme was tested by adding 100µL of culture to a mixture of l mL 50Mm acetate buffer and 1mL 2mM ABTS solution.

3.4. Quantitative Determination of Extracellular Protein

Protein concentration was determined by following the Bradford method (Bradford, 1976) using bovine serum albumin (Bio-Rad, USA) as a standard. The Bradford total protein assay is the spectroscopic analytical method which is used to determine the total protein concentration in a sample. In this method, Coomassie brilliant blue G-250 dye binds to proteins and changes their

colour from brown to blue. That colour change is monitored at 595nm in UV-visible spectrophotometer (Bradford, 1979). Protein standards in different concentrations were set up, from which a standard curve was prepared. This curve was used to determine the amount of protein, mg/mL in the samples by measuring the absorbance at 595 nm using a UV spectrophotometer (UV-1601 Recording Spectrophotometer, Shimadzu, Japan).

The protein contents of eluted column effluents were estimated by spectrophotometric measurement at 280 nm. Most proteins have absorption at 280 nm, due to the presence of aromatic groups in tyrosine and tryptophan residues (Boyer, 1993).

3.5. Enzyme Assay of Laccase Activity

Oxidation of ABTS (2, 2-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid) is catalysed by laccase, resulting in the green $ABTS^{+}$ radical (Figure 11) that exhibits a maximum of absorbance at 420 nm (More et al., 2011).

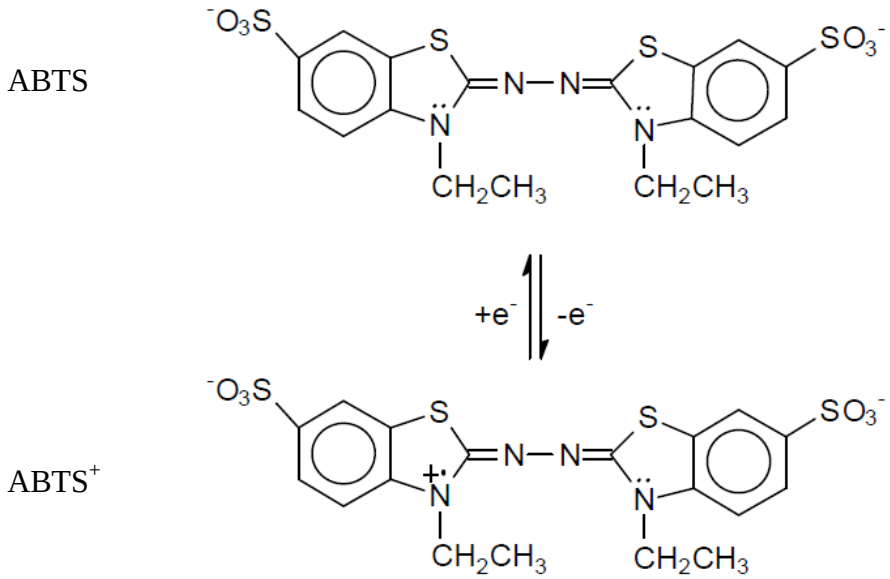


Figure 11 Oxidation mechanism of ABTS by laccase (Macherczyk et al., 1998).

For determining the enzymatic activity the broth from each flask was filtered through Whatman#1 and finally through the 0.45-mm-pore-size membrane filter, (Millipore® Millex® HN). The filtrate was then tested for the laccase activity by adding 100µl of the enzyme solution to a mixture of 1ml 50 mM acetate buffers and 1ml 2mM ABTS solution. Fungal strains oxidized ABTS to the dark green ABTS cation radicals (ABTS·+) indicating the production of extracellular laccase. Laccase activity was determined by measuring the oxidation of ABTS. The oxidation was detected by measuring the absorbance increase at 420 nm (Ɛ 36000 M cm^{-1}). The absorbance of each sample was taken after a 10 minutes interval. Control samples contained 0.1 mL of distilled water instead of enzyme solution. One unit laccase activity was defined as change in absorbance of the assay mixture at (420 nm) in 10 minutes. The change in absorbance in 10 min. was equivalent to µM of ABTS oxidized in 10 minutes. That result was then converted to µM of ABTS oxidized per min to calculate the IU. The laccase activity in U/mL (µmol cation radical released.min^{-1}.ml^{-1} enzyme) was calculated as follows:

$$\text{Laccase activity (U/mL)} = \frac{\Delta\, A470/\text{min} \times 4 \times Vt \times \text{dilution factor}}{Ɛ \times Vs}$$

Where:

V_t = final volume of reaction mixture

V_s = sample volume

Ɛ = extinction coefficient of $M^{-1}cm^{-1}$

4 = derived from unit definition and principle

An aliquot of the enzyme tests solution was incubated in 1 mL containing 50 mM sodium acetate buffer (pH 4.5) and 2 mM ABTS at 24°C. One unit of enzyme activity was defined as the

amount enzyme required producing an absorbance increase at 420 nm of 1 per minute per millilitre of reaction mixture under the aforementioned assay conditions (More et al., 2011).

Enzyme activities were measured at room temperature (20 ± 2ºC). The enzymatic activity was expressed as units (U) and defined as the amount of enzyme required to produce 1 μmol product per minute. The expressed extracellular enzymatic laccase activity was further defined in as IU.

An experiment was conducted to determine the degree of ammonium sulphate saturation required to precipitate laccase from the supernatant. Ammonium sulphate was dissolved in 5 mL of supernatant and centrifuged (7300×g; 10 min). The amount of laccase activity remaining in the supernatant was determined visually with the ABTS assay. The supernatant was then centrifuged in order to remove unwanted proteins until the supernatant showed no laccase activity.

3.6. Laccase Enzymes Purification

The pellets or supernatants were ultra-filtered through a Centrisart 1 membrane with 20-kDa molecular weight cut off (Sartorius, Germany). The membrane with sample inside was centrifuged at 4,000×g for 10 minutes and the desalted filtrate was loaded onto a hand packed Sephadex G-100 gel filtration column (Econo Column 1.5 mm × 30 mm, Bio-Rad, U.S). The samples where eluted using 50mM Sodium Acetate buffer (pH 4.5) at flow rate of 1ml/min. Fractions of 2mL where collected, for each fraction the approximate amount of protein concentration in the eluted column effluents was estimated by spectrophotometric measurement at 280 nm. Laccase activity was also determined and fractions containing laccase activity were pooled. The pooled samples were then loaded onto a hand packed DEAE-Cellulose anion exchange column (Econo Column 1.5 mm × 30 mm, Bio-Rad, U.S). The enzyme was eluted from the column with a gradient buffer. Before elution the column which was equilibrated with

50 mM phosphate buffer (pH 6.0). Ten column volumes of the same buffer were passed through the column before elution with a linearly increasing NaCl concentration gradient (0 to 0.5 M). Fractions of 2mL where collected, for each fraction the approximate amount of protein concentration in the eluted column effluents was estimated by spectrophotometric measurement at 280 nm. Laccase activity was also determined and fractions containing laccase activity were pooled, these fractions where used to characterise the purified enzymes. Protein concentration (mg/ml), total activity (U) specific activity (U/mg), yield (%) and purification (fold) were calculated at each step of purification according to More et al. (2011).

3.7 Laccase Characterisation

Various temperature and pH conditions using various buffers have been used to characterise the enzyme heat stability (More et al., 2011). To estimate the optimum temperature, the purified laccase was tested at a temperature range of 20–90 °C. To determine the optimum pH of the purified laccase, it was tested at its optimal temperature at a pH range from 3.0 to 10.0. The pH level was adjusted using the following buffers: 0.1M citrate buffer (pH 3–5), 0.1M phosphate buffer (pH 6–8), and 0.1M carbonate buffer (pH 9-10). The stability of the purified laccase at various temperatures was investigated by pre-incubating the purified laccase at different temperatures between 4 and 70 °C for 1h, followed by determination of the residual activity. The effect of pH on the laccase stability was determined by incubating the purified enzyme at 4°C in solutions of different pH levels for 24 h and determining the residual activity.

3.8 Purity Analysis on Gel Electrophoresis

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) separates proteins by their molecular weight (Laemmli, 1970) and it was used in this study to evaluate the purity of the fractions and to determine the molecular weight of the purified laccase(s).

3.9. Data analysis

Experiments were done in triplicates; means and standard deviation (SD) were calculated. One way analysis of variance (ANOVA) was conducted using Excel to compare the yield values and enzymatic activities from different mushroom species.

3.10. Research Ethics

A research permit was obtained from the postgraduate school of the University of Namibia to carry on the research activities reported in this thesis. *Termitomyces schimperi* (*Omajova*) samples were harvested in a sustainable manner whereby the bottom part of the stem was left for the termites to feed on it so the termite has longer life span. Mushrooms were collected in an ethical manner as prescribed by the Ministry of Agriculture, Water and Forestry (MAFW) guidelines.

CHAPTER 4: RESULTS

4.1. Sample Collection

In this study two different fungi from Namibia were collected (Figure 12). A basidiomycete mushroom was collected at the termite mounds and identified by its physical and morphological traits and these showed that it is *Termitomyces schimperi*. It was a giant termitophilic fungi with a subterranean elongation of the stipe also known as pseudorhiza which connected the basidiomes to the comb in the termite nest (Frøslev, Aanen, Læssøe, Rosendahl, & Asia, 2003). Ascomycete's fungi, also known as truffles were bought at the local market in Omuthiya, Oshikoto region, northern Namibia.

Figure 12 Physical features of the collected fungi in the study: *Termitomyces schimperi* (left) *Kalaharituber pfeilii* (right).

4.2. Screening Samples for Laccases Production

4.2.1 Laccase Screening in Fungal Fruiting Bodies

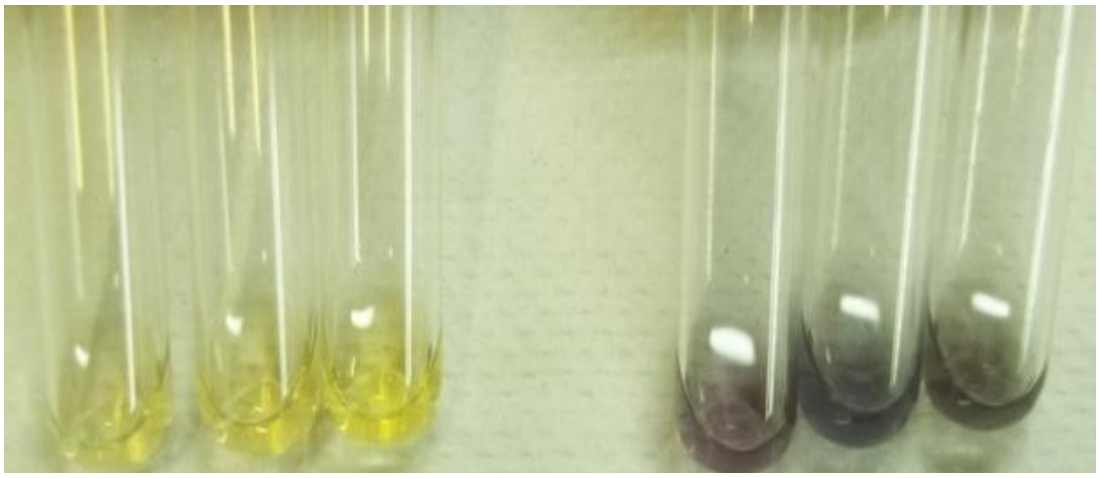
Crude enzyme extracts of fungal fruiting bodies were filtered and tested for laccase activity. Laccase positive produced an intense green colour with the ABTS as substrate (Figure 13).

Laccase activity in *T. schimperi* is detectable through its fruiting body, however there is very little activity in the stem in comparison to the cap and soil close to the mycelium, hence the study only continued with isolating laccase from these parts of the mushroom.

Figure 13 Laccase positive activities in fungal fruiting bodies; *Termitomyces schimperi* (lab isolate) **A,** soil extract **B,** cap **C,** Stem/Stipe **D**. *K. pfeilii* **E**

4.2.2 Laccase Screening in Cultivated *Termitomyces schimperi*

For preliminary screening of laccase in growth media, PDA media produced the highest activity for broth state media (Figure 14); 1mL of the growth media was taken and tested for laccase activity with α-naphthol. Activity on the α-naphthol was present in three (3) of the ten isolates, this means they could produce the laccase.



A **B**

Figure 14 Differences between laccase negative (A, yellow colour) and laccase positive (B, purple colour).

For solid state media laccase producing colonies have purple halos on PDA media containing 1% α –naphthol (Figure 15). Of such organisms, colonies representing close resemblance to *T. schimperi* were considered for further qualitative and quantitative studies. More et al. (2011) did a similar study in screening 28 samples for laccase activities using this method.

Figure 15 Laccase positive cultures of *T. schimperi* on PDA.

4.3. Enzyme Assay of Laccase Activity

Laccase activity in *T. schimperi* was distributed with the activity increasing as you go down the mushroom, with highest activity 69.662 U/mL being measured at the roots and surrounding soil of the mycelium (Table 2,3). The *K. pfeilii* produces laccase with 72.2 U/mL in the crude extract (Table 4).

4.4 Laccase Enzymes Purification

The laccase from *T. schimperi* was purified to 72.2-fold with a yield of 22.4% (Table 2), using a series of purification steps that included ammonium sulphate precipitation, gel permeation using, Sephadex G-100 column chromatography and DEAE Cellulose column chromatography. The results of the laccase purification procedure are summarized in Table 2 and 3.

Table 2 Purification summary of laccase from *T. schimperi* isolate and cultivate under laboratory conditions 31°C

Purification Step Lab isolate	**Protein (Mg)**	**Total Activity (U) Mg**	**Specific Activity (U/Mg)**	**Yield (%)**	**Purification fold**
Crude Extraction	36,667	6,859	1,898	100	1
Ammonium Sulphate Precipitation	4,354	1,112	3,904	16,214	2
Sephadex G-100 Chromatography	0,403	1,913	9,499	27,888	5
DEAE ion exchange Chromatography	0,0258	1,226	47,406	17,875	25

Table 3 Purification summary for laccase from soil near the mycelium of the mushroom *T. schimperi*

Purification Step Soil isolate.	**Protein (Mg)**	**Total Activity (U) Mg**	**Specific Activity (U/Mg)**	**Yield (%)**	**Purification fold**
Crude Extraction	7,694	6,421	0,922	100	1
Ammonium Sulphate Precipitation	0,372	0,209	0,564	2,130	0.6
Sephadex G-100 Chromatography	0,0459	0,177	0,833	1,799	0.9
DEAE ion exchange Chromatography	0,0256	0,0094	3,901	0,095	4

Table 4 Purification summary of laccase from *Kalaharituber pfeilii*

Purification Step *K. pfeilii*	**Protein (mg)**	**Total Activity (U)**	**Specific Activity (U/Mg)**	**Yield (%)**	**Purification fold**
Crude Extraction	41,481	0,722	0,017	100	1
Ammonium Sulphate Precipitation	0,544	0,284	0,525	39,4	31
Sephadex G-100 Chromatography	0,0454	0,265	5,843	36,8	336
DEAE ion exchange Chromatography	0,011	0,172	15,317	23.6	880

Ammonium Sulphate precipitation resulted in a fold purification of laccase protein from *T. schimperi* (Tables 2&3, Figures 17, 18). It appears that the ammonium sulphate precipitation was not as successful in purifying proteins from the soil near *T. schimperi* roots, the purification

was only 0.6, under the condition of operation, and a lot of protein was lost in this process (Table 3, Figures 16-18). Hence the purification processes for soil enzymes need a different approach. The chromatographic process however, increased the purification fold in *K. pfeilii* by 31% (Table 4, Figure 19). Sephadex G-100 Chromatography purified the enzyme by 5 folds in the laboratory grown isolate (Figure 18), but the purification fold on the soil samples was low again (Table 3). The gel however increases the purity of the enzyme from *K. pfeilii* by more than 300 folds (Table 4, Figure 19).

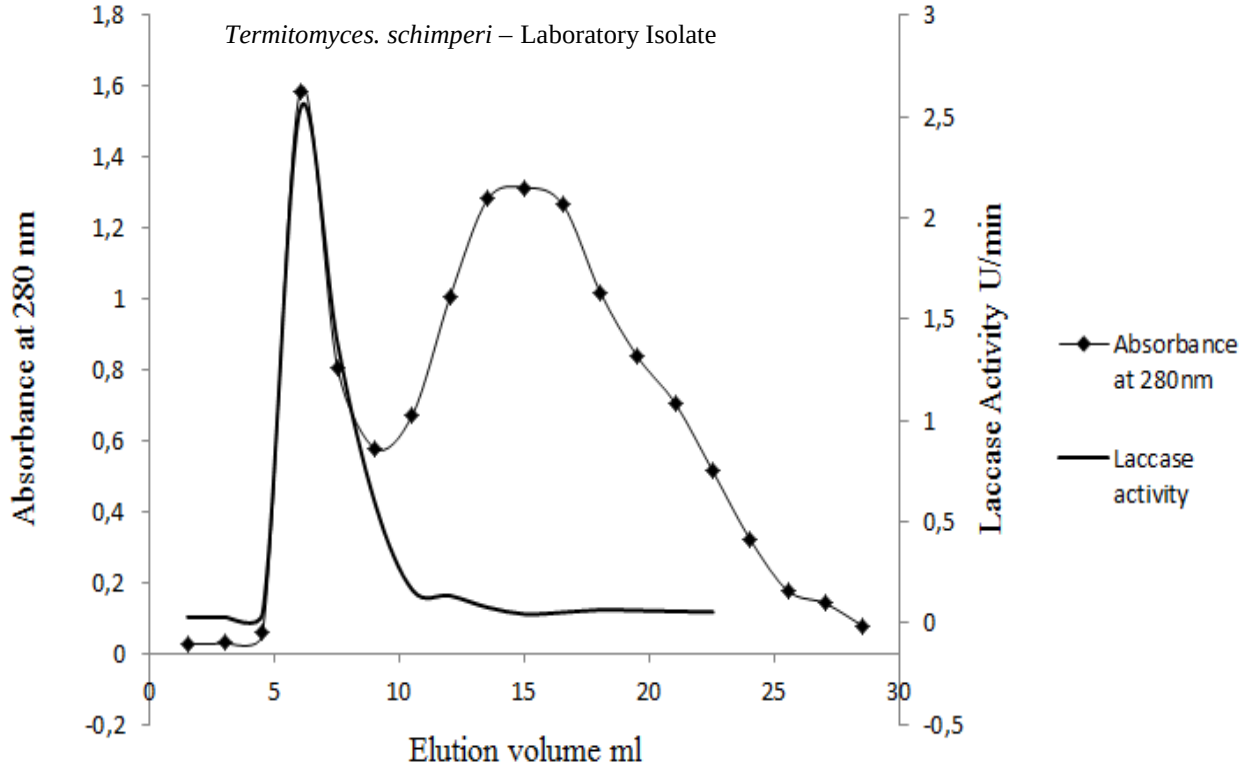


Figure 16 Sephadex G-100 gel column chromatography chromatogram of *Termitomyces schimperi* cultivated under laboratory conditions laccase in 50 Mm sodium acetate buffer, pH 4.5. (♦) Absorbance; (■) Laccase activity

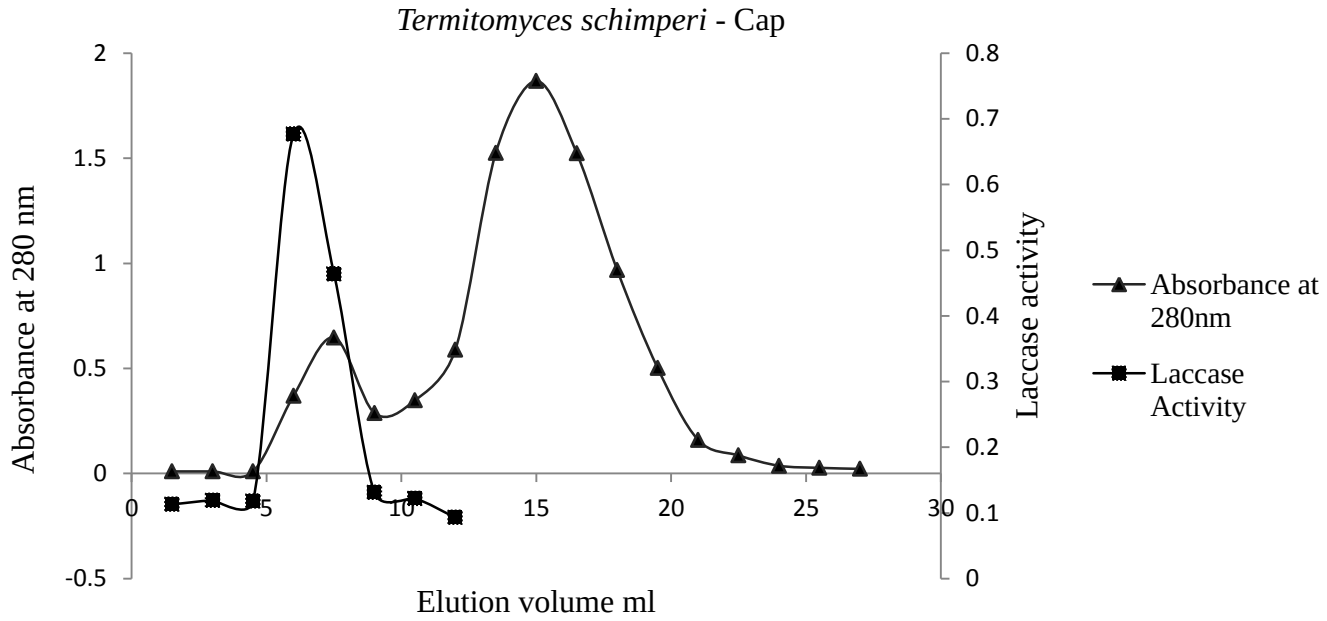


Figure 17 Sephadex G-100 gel column chromatography chromatogram of *T.schimperi* cap sample in 50 Mm sodium acetate buffer, pH 4.5. (▲) Absorbance; (■) Laccase activity

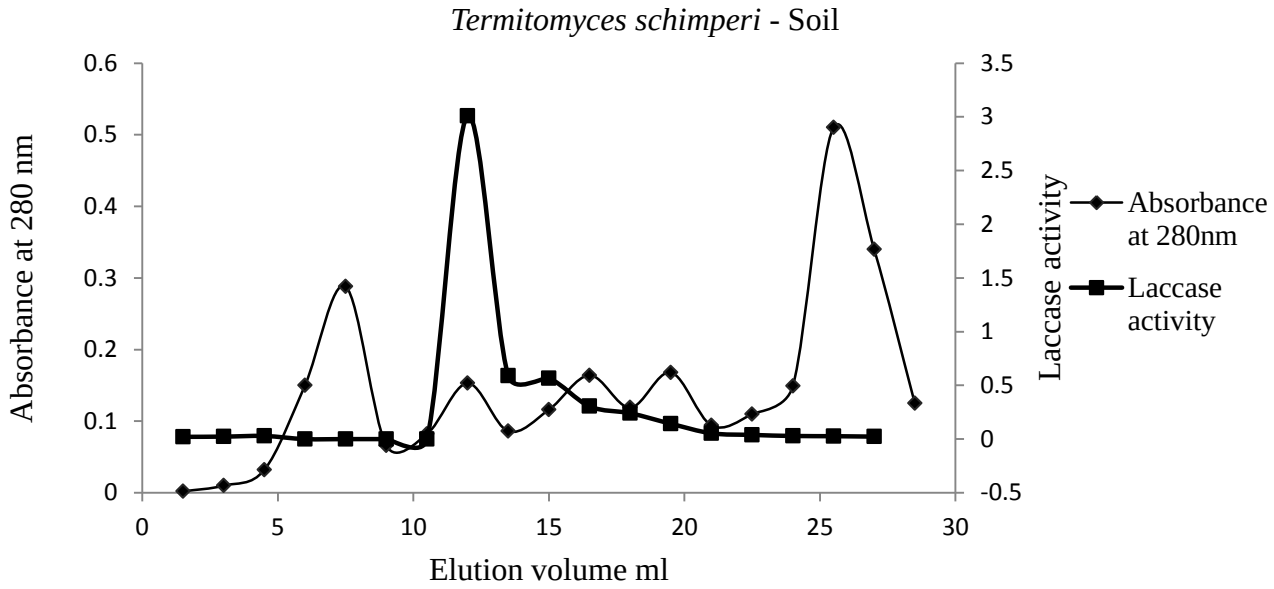


Figure 18 Sephadex G-100 gel column chromatography chromatogram of *T.schimperi* laccase soil sample in 50 Mm sodium acetate buffer, pH 4.5. (♦) Absorbance; (■) Laccase activity

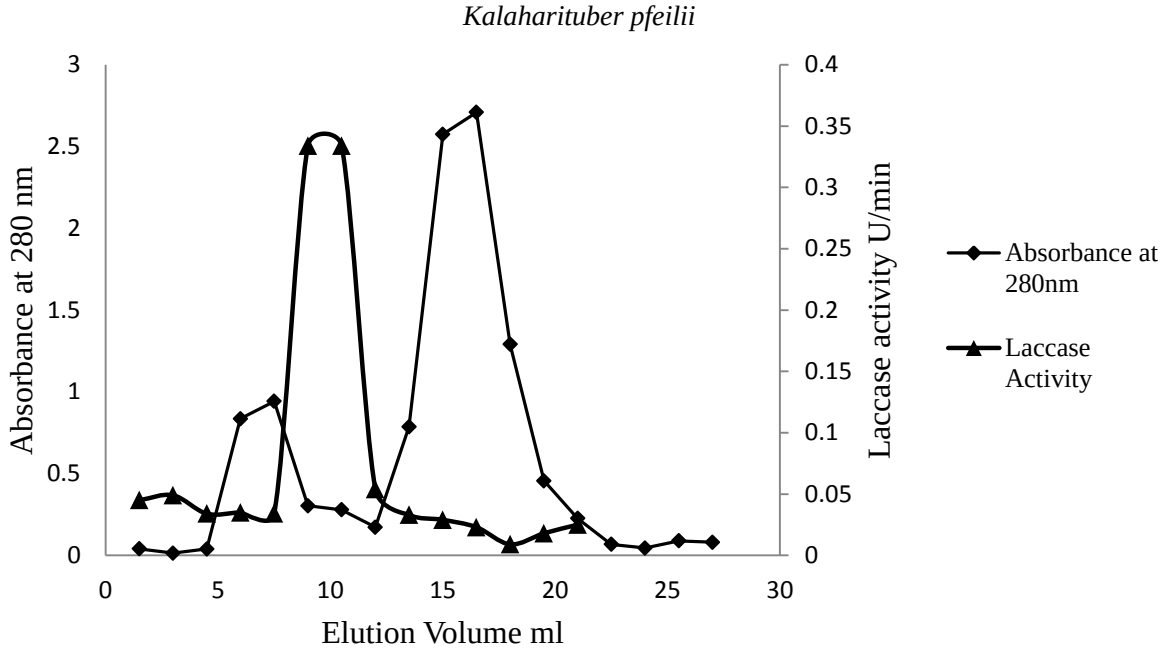


Figure 19 Sephadex G-100 gel column chromatography chromatogram of *Kalaharituber pfeilii* laccase in 50 Mm sodium acetate buffer, pH 4.5. (♦) Absorbance; (▲) Laccase activity

DEAE ion exchange chromatography purified the laccases from the soil better, eventually giving 4-fold purification. It was also 5 times better than Sephadex G-100 of the laboratory grown isolate, giving 5 times more purity (Figures 20, 21, Tables 2, 3). In the *K. pfeilii*, the final purification fold was more than 800 (Figure 22, Table 4).

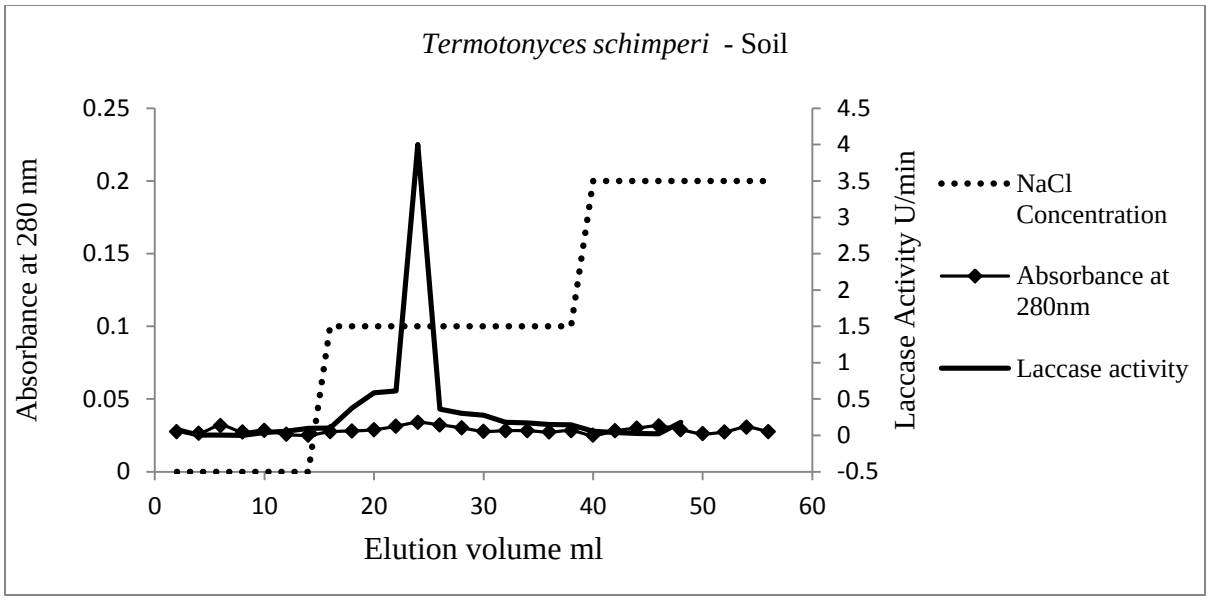


Figure 20 DEAE-Sepharose Ion exchange chromatogram of the laccase *Ternitomyces schimperi* soil sample eluted with a linearly increasing NaCl concentration gradient (0 to 0.5 M).

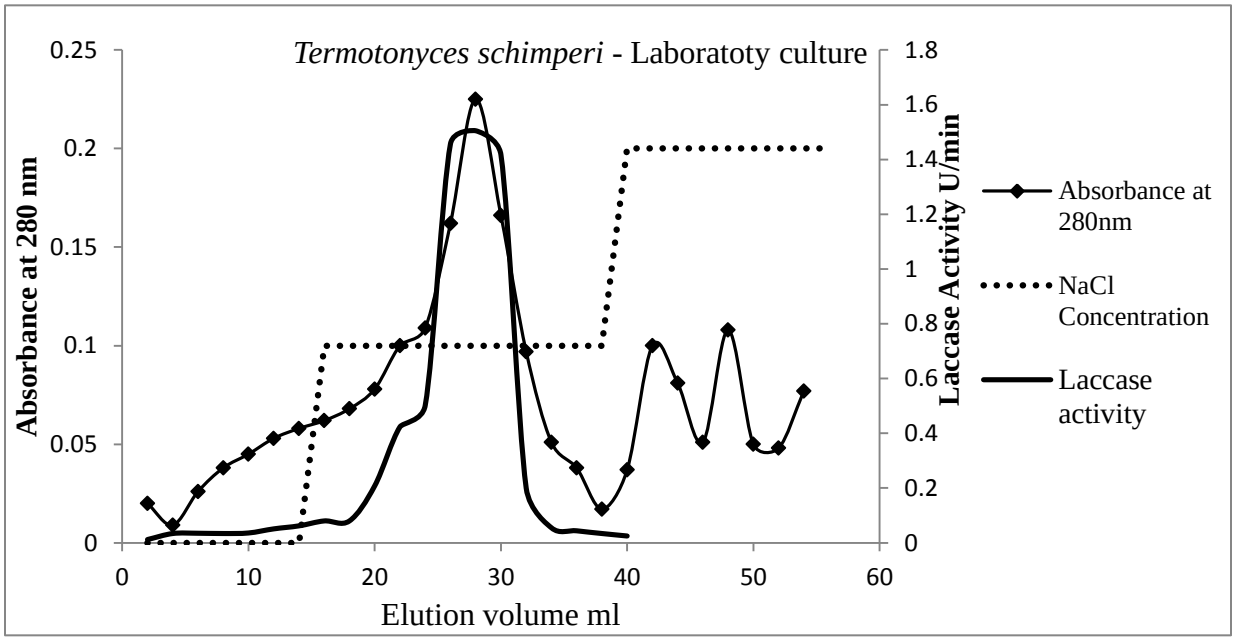


Figure 21 DEAE-Sepharose Ion exchange chromatogram of the laccase *Ternitomyces schimperi* cultivated under laboratory conditions eluted with a linearly increasing NaCl concentration gradient (0 to 0.5 M).

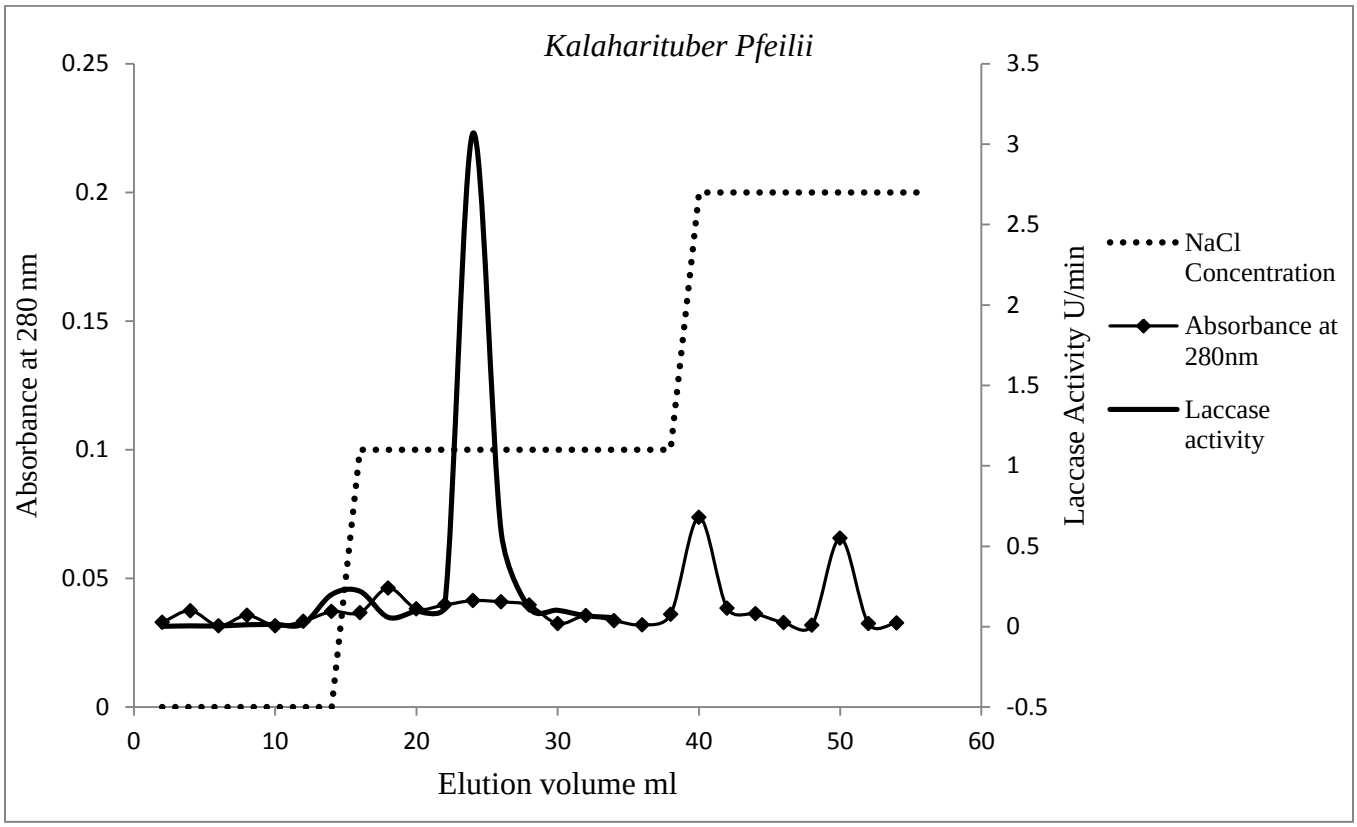


Figure 22 DEAE-Sepharose Ion exchange chromatogram of the laccase from *Kalaharituber Pfeilii* eluted with a linearly increasing NaCl concentration gradient (0 to 0.5 M).

4.5. Laccases Partial Characterisation

4.5.1. Optimum Temperature and Temperature Stability of Laccase

Laccases from *T. schimperi* fruiting body has optimum activity at 30°C for the cap and stem and 40°C for the laccase found in the roots (Figure 23).

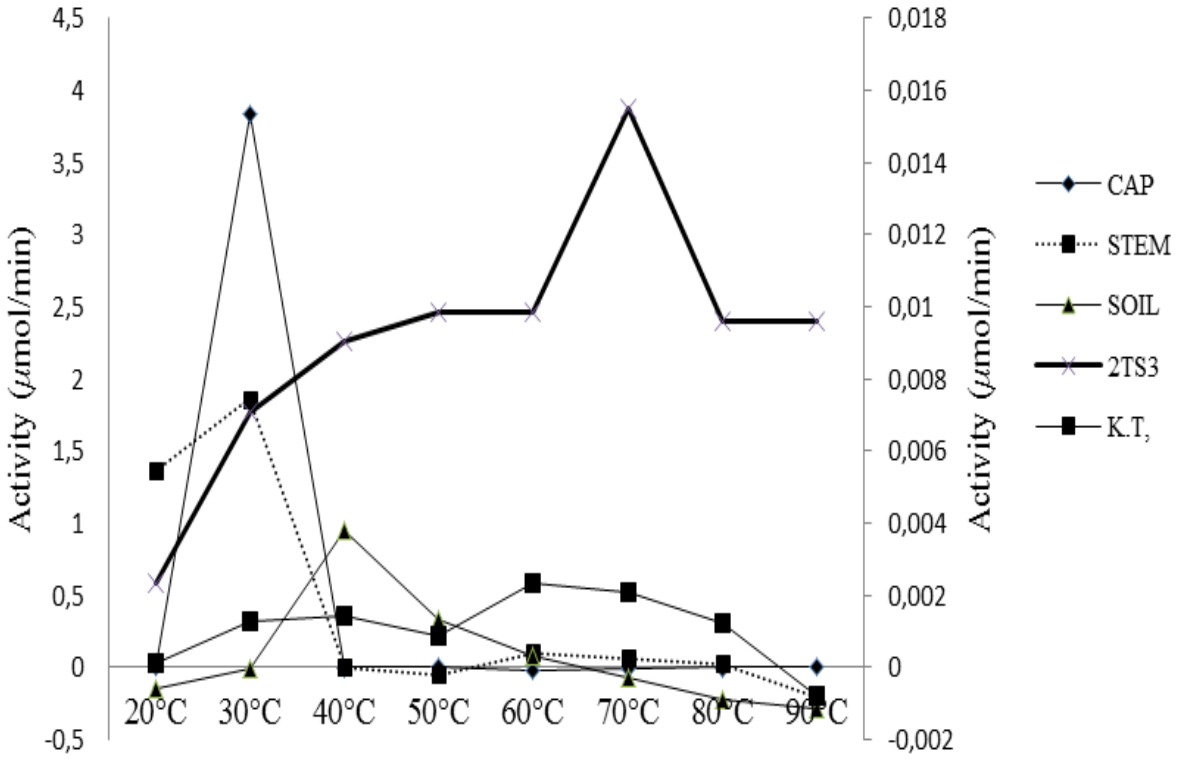


Figure 23 Optimum temperatures for purified laccases from *Termitomyces schimperi* - Cap, *Termitomyces schimperi* - Stem**,** *Termitomyces schimperi* - Fungal combs, *Termitomyces schimperi* - Laboratory Isolate and *Kalaharituber pfeilii***.**

The activity significantly drops below 20°C and above 40°C for the fruiting body enzymes. It appears that in both organs, the activity is restricted to a short temperature range with in which the enzyme is active (Figure 23). For the laccase isolated from the cap, it range detected as below as 25°C but it loses activity rapidly around 40°C. The optimal activity for the laccase in the laboratory grown organism was at 70°C, while that of *K. pfeilii* was at 60°C (Table 5). Laccases with optimum activity around 30 °C were stable at that temperature for over 6 hours, however they lost the activity in 30 minutes at temperature around 40°C(Figure 23). The laccase from *K. pfeilii* was stable at 60°C for 8hrs (Figure 23, Table 5).

Table 5 Temperature range of laccase enzymes in *K. pfeilii* and *T. schimperi* fruiting bodies

Source	**Optimum Temperature**	**Activity Temperature range**
T. schimperi - cap	30°C	20°C-40°C
T. schimperi - stem	30°C	20°C-40°C
T. schimperi - fungal combs	40°C	30°C-60°C
T. schimperi – Lab Isolate	70°C	30°C-90°C
K. pfeilii	60°C	30°C-80°C

4.5.2 Optimum pH and pH Stability of Laccase

Optimum pH for laccases is shown in Figure 24. The laccases isolated from *T. schimperi* soil have an optimum pH 7. While the laccases isolated from *T. schimperi* cultivated under laboratory condition has the highest activity at pH 5. Kalahari truffles (*K. pfeilii*) laccase activity was high at pH 3. For all the purified enzymes, the activity decreased as the pH increased.

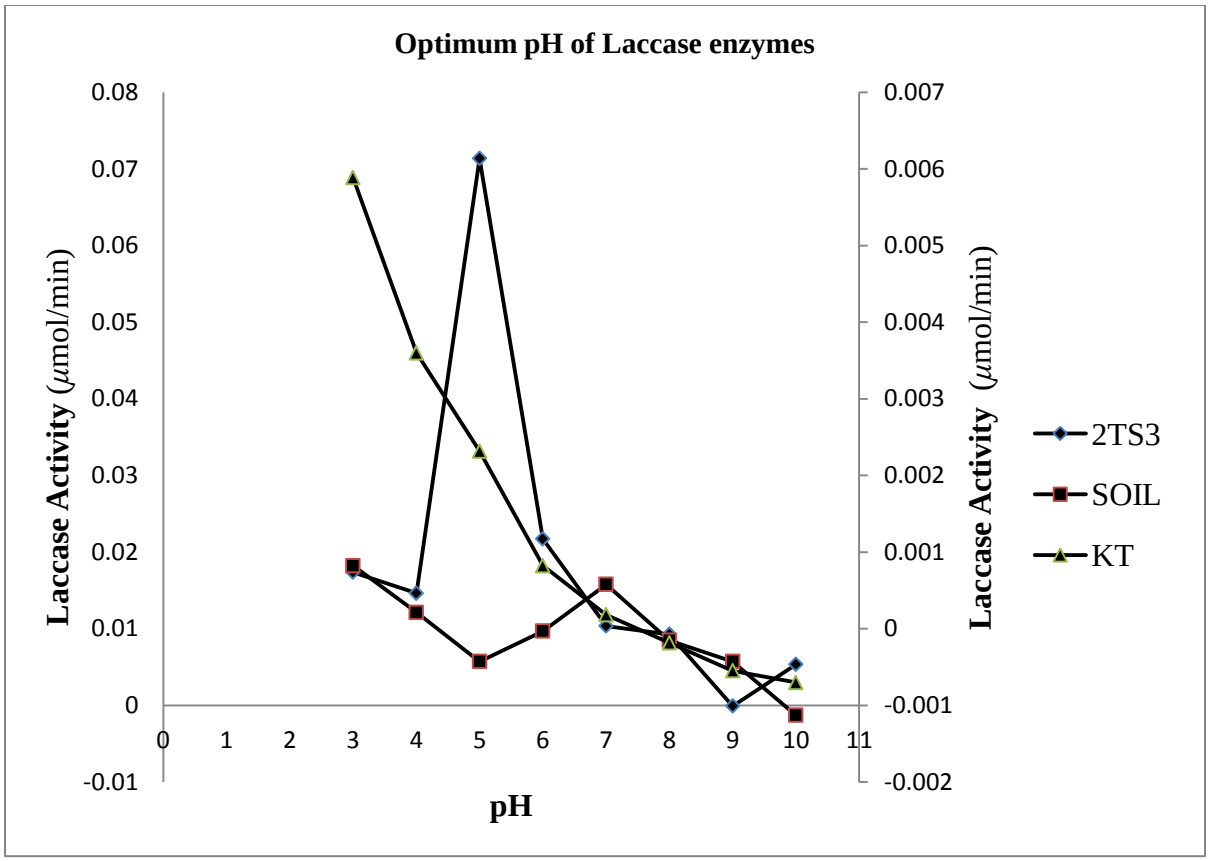


Figure 24 Optimum pH for laccases from: *Termitomyces schimperi* - Laboratory Isolate **A**, *Termitomyces schimperi* – Soil **B**, *Kalaharituber pfeilii*. the activity was measured after incubation of the enzymes with different buffers for ten minutes, the buffers where 0.1M citrate buffer (pH 3–5), 0.1M phosphate buffer (pH 6–8), and 0.1M carbonate buffer (pH 9-10).

4.6 Purity Analysis and Molecular Weight with SDS-PAGE

The SDS-PAGE for the laccases from *T. schimperi* soil (Fungal combs, laboratory cultivated) and *K. pfeilii* are shown in figures 25 and 26 respectively. In the crude extract (A) the laccase from *T. schimperi* soil (Fungal combs) (Figure 25), the protein concentration was low but after one purification step using Sephadex G-100 Gel Chromatography(B-), bands of different protein were more clear. In the last step of the purification process using DEAE- Ion exchange

Chromatograph (C), some of the bands disappeared or it could be related to lose of activity in these proteins.

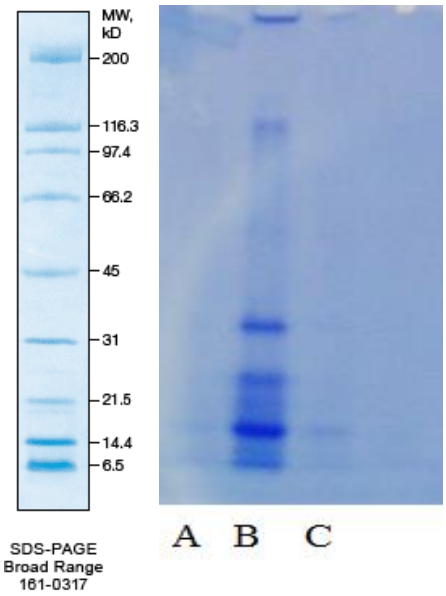


Figure 25 SDS-PAGE analyses on laccase from *T. schimperi* soil (Fungal combs) (A-C), A- crude extract; B- Sephadex G-100 Gel Chromatography; C- DEAE- Ion exchange Chromatograph. On the left side, broad molecular weight ranges marker (6.5 – 200 KD) (Bio-Rad, USA), was downloaded from http://www.bio-rad.com/en-za/product/unstained-sds-page-standards?pcp_loc=catprod .

Figure 26 shows the resulted bands from laccase extracts of *T. schimperi* (laboratory cultivated) and *K. pfeilii*. Ladders O- crude extract; P- ammonium sulphate and Q - Sephadex -100 gel chromatography for laccase from *T. schimperi* laboratory cultivated sample.

Ladders F; crude extract, K, J: ammonium sulphate; L; Sephadex-100 Gel Chromatography, M, N: DEAE- Ion exchange Chromatograph for *K. pfeilii* laccase during different purification steps.

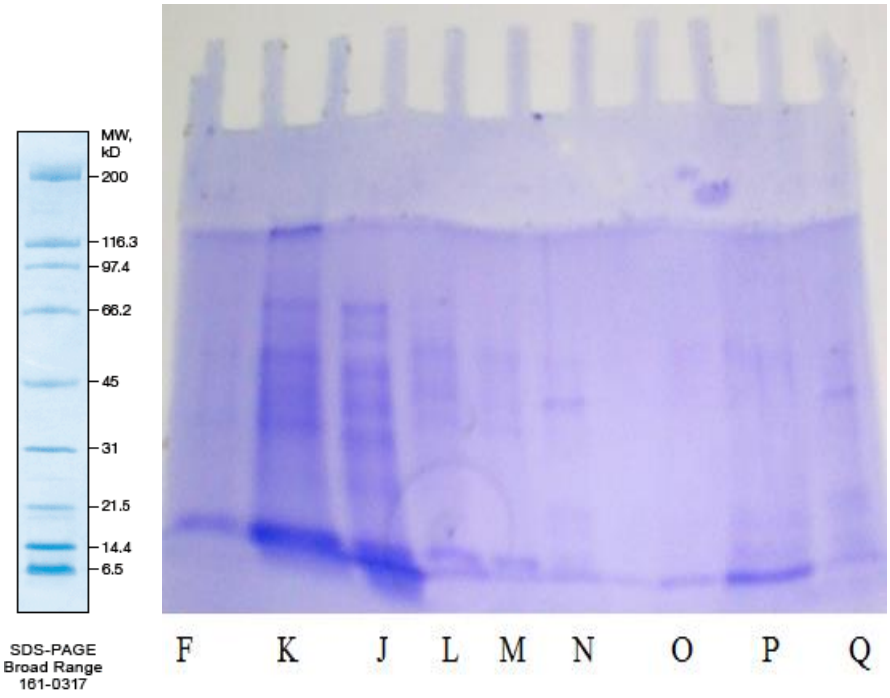


Figure 26 SDS-PAGE analyses on laccase from *T. schimperi* (Laboratory cultivated) O- crude extract; P-Ammonium Sulphate Q - Sephadex -100 Gel Chromatography; R- DEAE- Ion exchange Chromatograph. *K. pfeilii* laccase (F to N) F; crude extract, K, J: Ammonium sulphate; L; Sephadex -100 Gel Chromatography, M,N: DEAE- Ion exchange Chromatograph. On the left side, broad molecular weight ranges marker (6.5 – 200 KD) (Bio-Rad, USA), was downloaded from http://www.bio-rad.com/en-za/product/unstained-sds-page-standards?pcp_loc=catprod

CHAPTER 5 DISCUSSIONS

5.1. Screening for Laccases Activity

T. schimperi and *K. pfeilii* are endemic edible Namibia mushrooms that are collected by Namibians as food sources. Both fungi are a source of income for local vendors who sell them. Current projects in Namibia focus their attention on enhancing mushroom production for consumption and income generation purposes. However, it is well known that fungi such as these reported in this study, although edible, are also a source of biotechnological important components such as enzymes. Laccase enzymes are extracellular glycoprotein with many industrial applications, with their role being expanded by the fact that its substrate range increases with the use of mediators which help to oxidize compounds it cannot oxidize directly. This research has focused on screening the two fungi for these industrially important laccase enzymes.

The two fungi in this study are locally used as food sources in both traditional and local houses. An attempt was made to cultivate these organisms in the laboratory so that the enzyme productions will not compete with the production of food. Due to the lack of appropriate media *K. pfeilii* was not cultured. Three laboratory agar mediums where tested (Malt Extract Agar; Sabouraud Agar and Potato Dextrous Agar) for optimal growth and laccase production by *T. schimperi.* Only PDA allowed for growth of the *T. schimperi* growing and producing detectable laccase activity. It is known that these organisms could grow fungal mycelium under laboratory conditions however the challenge remains that *T. schimperi* to achieve fruiting bodies under laboratory conditions (Nobre, Koopmanschap, Baars, Sonnenberg, & Aanen, 2014).

Laccase activity was detected in all the fungus combs examined as well as in the culture supernatants of isolated symbiotic fungi. Conversely, no peroxidase activity was detected in any of the fungus combs or the symbiotic fungal cultures It has been shown that the extracellular phenol-oxidizing enzymes lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13), and laccase (EC 1.10.3.2) are responsible for the depolymerisation of lignin (13, 23). Lignin peroxidase catalyses the oxidation of various aromatic compounds to form aryl cation radicals while manganese peroxidase oxidizes Mn(II) to Mn(III), which diffuses from the enzyme and oxidizes various phenolic compounds. These enzymes require hydrogen peroxide for their activities (Taprab et al., 2005). Phenol-oxidizing enzymes in symbiotic fungi and fungus combs of termites belonging to the genera Macrotermes, Odontotermes, and Microtermes have been studied in Thailand (Taprab et al., 2005).

5.2. Laccase Enzymes Purification

Laccase activity was detected through *T. schimperi* fruiting body however, laccase is usually an extracellular enzyme (Kunamneni et al., 2007), and the detectable activity from the cap and stem was not present in sufficient enough concentration to be purified and there was not enough sample to be highly concentrated and be purified to homogeneity. The highest activity of laccase in this experiment was 47.409 U/mg and this was from the purified enzyme of the laboratory grown isolate cloned from the *T. schimperi*. The third highest activity of 3.901 (U/mg) was from fungal comb of the *T. schimperi* mushroom. In this research has also isolated the laccase enzyme from the *K. pfeilii* with a purification fold of up to 800 with DEAE (Table 5). The purified enzyme was only from the sample of the outer part of the Kalahari tuber as the white flesh part of the truffle did not have any laccase activity. Zarivi and colleagues analysed for laccase the histochemical localization of laccase *Tuber melanosporum* (Zarivi et al., 2013). And they also found that the highest laccase

expression occurs in the ectomycorrhizae, when the host plant roots are invaded by the fungal mycelium.

5.3. Effects of PH and Temperature on Laccase Stability and Activity.

Temperature and pH are two of the most important factors in production of enzymes (Dhakar & Pandey, 2013). The optimum pH of laccases is highly dependable on the substrate. When using ABTS as substrate the optimum pH are more acidic and are found in the range of pH 3.0-5.0 In general, laccase activity has a bell shaped profile with an optimal pH that varies considerably (Rouvinen & Hakulinen, 2011).

The optimum pH of the laccases in this study was only done for the DEAE ion exchange purified laccases, mainly *K. pfeilii* and the two *T. schimperi* (soil isolate) and the *T. schimperi* laboratory isolate. Similar results were obtained except for *K. pfeilii* which the isolated laccase activity goes lower than pH 3. The variation of the pH optimum may be due to changes in the reaction caused by the substrate, oxygen or the enzyme itself that isolated in this study shows that they have optimal activity in the acidic pH conditions. Both the activities decrease rapidly after pH 6. The difference in redox potential between the phenolic substrate and the T1 copper could increase oxidation of the substrate at high pH values, but the hydroxide anion (OH-) binding to the T2/T3 coppers results in an inhibition of the laccase activity due to a disruption of the internal electron transfer between the T1 and T2/T3 centers. These two opposing effects can play an important role in determining the optimal pH of the bi-phasic laccase enzymes (Kunamneni et al., 2007).

Both *T. schimperi* samples including laccases from the cap and fungal combs, where were also tested for their optimum temperature, although the temperature optimum for the cap and fungal combs was only done one the semi-purified samples eluted from the Sephadex G-100 gel filtration column. Laccases from *T. schimperi* fruiting body has optimum activity at 30◦C for the

cap and stem and 40◦C for the laccase found in the fungal comb. The activity significantly drops below 20°C and above 40°C for the fruiting body laccases. It appears that in both organs of *T. schimperi*, the activity is restricted to a short temperature range in which the enzyme is active (Table 3). The optimal activity for the laccase in the laboratory grown isolate of *T. schimperi* was at 70 °C and at 60°C for the *K. pfeilii* laccase. Laccases with optimum activity around 30°C where stable at that temperature for over 6 hours, however they lost the activity in 30 minutes at temperature around 40°C (Figure 23). Laccase from *K. pfeilii* was stable at 60°C for 8hrs.

The laboratory grown isolate of *T. schimperi* has a laccase with the highest optimum temperature of 70°C. This enzyme was also stable for about an hour at this temperature. Operatively laccases are moderately thermo-tolerant, showing optima activity at 50-55 °C, and under acidic conditions (pH 3-5); although their maximum stability occurs in the alkaline zone (pH 8-9) (Martínez et al., 2013). Comparable results of thermostability were also reported on laccase from *Agaricus blazei* (Ulrich et al., 2005) and *Cerrena unicolor* LacC2 (Lisova et al., 2010). In the present study, the laccase was active in the temperature range of 40–70 °C. A typical laccase gene codes for a protein of 500–600 amino acids and the molecular weights of laccases are usually in the range of 60 to 90 kDa as determined by SDS-PAGE (Viswanath et al., 2014). Difference between the molecular weight (MW) predicted from the peptide sequence and the experimentally obtained molecular weight is caused by glycosylation, which typically accounts for about 10–20% of the total MW (Viswanath et al., 2014).

CHAPTER 6 CONCLUSION AND RECOMMENDATIONS

K. pfeilii and *T. schimperi* are two endemic edible Namibian mushrooms that are collected by Namibians as food sources. Both fungi are a source of income for local vendors who sell them. Current projects in Namibia focus their attention on enhancing mushroom production for consumption and income generation purposes. However it is well know that fungi such as these although edible, are also a source of biotechnological important products such as enzymes. Laccase enzymes are extracellular glycoprotein with many industrial applications, with its role being expanded by the fact that its substrate range increases with the use of mediators which help to oxidise compounds it cannot oxidise directly. This research has focused on screening the two fungi for these industrially important enzymes. *T. Schimperi* produces laccases through its fruiting body. Laccases from the cap and fungal combs were isolated and purified to homogeneity with a protocol of 80% ammonium sulphate, ultrafiltration, Sephadex Bio-gel chromatography, and DEAE ion exchange chromatography. The two laccases differed slightly. It has been shown that these enzymes are produced mainly as extracellular, as the highest activity was detected in the media of the laboratory grown isolate and the in the soil close to the fungal comb of the *T. schimperi*. Another laccase enzyme was isolated from the *K. pfeilii* with a purification fold of up to 800 with DEAE ion exchange chromatography column. The *K. pfeilii* has pH activity below 3. This proves its ability to work in low acidic conditions. Laccases found in from *T. schimperi* fruiting body has optimum activity at 30°Cfor the cap and stem and 40°C for the laccase found in the fungal comb. The activity significantly drops below 20°C and above 40°C for the fruiting body enzymes. The optimal activity for the laccase in the laboratory grown organism was at was at 70°C, while that of *K. pfeilii* was at 60°C. The *K. pfeilii* the laccase was stable in at 60°C for 8hrs. High temperature stability and pH wide range stability could be used

as indicators for their interesting properties which need further studies to confirm their potentials in industrial application. Kinetics studies and expanding the characterization together with genetic determination for the genes responsible for these enzymes from local mushrooms should be perused for full understanding of these enzymes and their functions.

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