ISOLATION, PURIFICATION AND CHARACTERIZATION OF LACCASE FROM *MICROCYSTIS FLOS-AQUAE* AND ITS POTENTIAL FOR BIOREMEDIATION OF AZO DYES

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DECEMBER, 2021

DECLARATION

I hereby declared that this dissertation titled "**Isolation, Purification and Characterization of Laccase from *Microcystis flos-aquae* and Its Potential for Bioremediation of Azo Dyes**" is a record of my own research work under the supervision of Dr. Emmanuel Oluwadareus Balogun and Prof. Mohammed Nasir Shuaibu. The information derived from the literatures has been duly acknowledged in the text and list of references provided. This work has neither in whole or part been presented for the award of any certificate in any institution of learning.

Muhammad Ayyub Muhammad		
(Name)	(Signature)	(Date)

CERTIFICATION

This dissertation titled "ISOLATION, PURIFICATION AND CHARACTERIZATION OF LACCASE FROM *MICROCYSTIS FLOS-AQUAE* AND ITS POTENTIAL FOR BIOREMEDIATION OF AZO DYES" conducted by MuhammadAyyubMuhammad (P17LSBC8006) meets the regulations governing the award of the degree of Masters of Science in Biochemistry, Department of Biochemistry, Ahmadu Bello University, Zaria and is accepted for its contribution to knowledge and literary presentation.

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DEDICATION

Blessed is He in Whose Hand is the dominion; and He is able to do all things. This work is dedicated to Almighty Allah, Who through His guidance and mercy showered me with His blessings and granted me wisdom, knowledge and understanding so that I can succeed, and thus spared my life.

Also, to my beloved late maternal grandparents, my mom, my uncle and my supervisors who all have always been a source of inspiration and moral support to me.

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ABSTRACT

Discharges from manufacturing companies such as the textile industries, accounts globally for up to 280,000 tons of dye wastes annually leading to a wide range environmental pollution and health hazards. Due to the high costs of chemical treatment methods of these dyes, in Nigeria and other African countries, these dye wastes remain in the environment endangering the lives and health of aquatic animals and humans. Hence, there is need to develop a cheaper and efficient means of decontamination. A green technology that rely on enzyme-mediated bioremediation of dye pollutants is therefore considered. In this study, seven previously unexplored organisms belonging to cyanobacteria and microalgae were screened for production of laccase, an enzyme with established bioremediation activities. For the first time, it was reported herein that the cyanobacteria *Microcystis flos aquae*produce a novel extracellular laccase. The laccase was purified to homogeneity using a combination of ammonium sulphate precipitation, dialysis, anion exchange and size exclusion chromatographic techniques. The yield for the laccase was 0.55% (*w/w*) with a purification fold of 10.21, and specific activity of 0.16 mmol/min/mg at 40 °C and pH of 4. The enzyme had an estimated molecularweight of ~66 kDa on SDS-PAGE. Kinetic assessments using ABTS [2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid] as substrate indicated that the laccase has K_m and V_{max} values of 51.71 mM and 5.02 µM.min^{-1}, respectively. The activity of the enzyme is suppressed by the presence of EDTA and Fe^{2+} whereas Cu^{2+} is needed by the enzyme to be functional. The bioremediation potential of the laccase was assayed using 0.039g/L of Cibacron Brilliant Blue (CiBB) solution. The CiBB dye, which is an azo dye was rapidly decolorized within 3 hours at 40 ^{o}C and pH 4. In conclusion, the purified laccase from *Microcystis flos aquae* could be used for bioremediation of textile effluents.

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LISTS OF SYMBOLS AND ABBREVIATIONS

ABTS- 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)

ANOVA- Analysis of Variance

CiBB- Cibacron Brilliant Blue

DDT- 1,1,1-trichloro-2,2-bis (4 chlorophenyl) ethane

EC- Enzyme Commission Number

EPR- Electron Paramagnetic Resonance

K_M- Michaelis Menten's constant

LiP- lignin peroxidase

LMS- Laccase Mediator System

PAH- Polycyclic Aromatic Hydrocarbons

PCB- Polychlorinated biphenyls

PCP- Pentachlorophenols

RB5- Reactive Black 5

RRBR- Reactive Brilliant Blue R

TNT- Trinitrotoluene

Vmax- Maximum Velocity

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Exploration for efficient and green oxidation technologies has increased interest in the use of enzymes to replace conventional non-biological methods. Among these oxidant enzymes, laccases have been the subject of study because of their important role in environmental protection, where enzymatic catalysis could be more advantageous than the chemical process (Baldrian 2006; Riva 2006; Rodriguez and Toca 2006, 2007). Laccases (EC 1.10.3.2, benzenediol: oxygen oxidoreductases) are extracellular multicopper oxidases involved in the breakdown of recalcitrant aromatic compounds, which result in the reduction of oxygen to water after the oxidation of their substrates (Giardina *et al.*,2010).Molecular oxygen serves as the terminal electron acceptor and is thus reduced to two molecules of water as shown in the equation (i) below:

$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O \ldots\ldots\ldots\ldots\ldots\ldots\ldots (i)$$

ABTS (2,2'-Azino-bis-3-ethylbenzothiazo-line-6-sulfonic acid) is the commonly used substrate in laccase activity assays, followed by 2,6-dimethoxyphenol (2,6-DMP), catechol, guaiacol, and syringaldazine(Giardina *et al.,* 2010). Reports have shown that laccases have wide range of substratesusing molecular oxygen as the final electron acceptor, thus this contribute to their widespread applications in various industries, such as textile, food, biofuel, organic synthesis, bioremediation, paper and pulp, pharmaceutical, and cosmetic industries (Huang *et al.,* 2011; Pezzella *et al.,* 2013; Viswanath *et al.,* 2014; Senthivelan *et al.,* 2018; Sitarz *et al.,* 2016; Upadhyay *et al.,* 2016). Until recently, laccases and laccase-like multicopper oxidases were found in higher plants, fungi, insects, prokaryotes and lichens (Claus 2004; Lisov *et al.,* 2007).

As yet, indications for the occurrence of extracellular laccase-like phenol oxidases in algae are limited to the demonstration of the oxidation of typical laccase substrates by cell-free culture supernatants of green microalgae (La Russa *et al.,* 2008), whereas information about structural or catalytic properties of such enzymes is still missing.

While there has been more work done on the biotransformation of aromatic compounds by fungi and bacteria, the ability of algae to degrade various phenolic and other aromatic compounds, like synthetic dyes and polyaromatic hydrocarbons, is just in its early stage (Ghasemi *et al.,* 2011; Subashchandrabose *et al.,* 2013). Phenol metabolism in algae may proceed via hydroxylation and ring cleavage pathways. In view of the phylogenetic distribution of laccases, it could be expected that these enzymes are present not only in higher, but in more ancestral plants as well (McCaig *et al.,* 2005).Rönnerstrand (1943) was the first to report laccase-like enzyme activity in red algae. The presence of algal laccases has been discussed with respect to the evolution of lignin and related polymers (Viswanath *et al.,* 2014) and has also been proposed to contribute greatly in rendering diverse phenolic pollutants harmless (Subashchandrabose *et al.,* 2013; Chiaiese *et al.,* 2011; Kılıç *et al.,* 2011). In the last decade, various green algae were found to oxidize typical laccase substrates extracellularly (La Russa *et al.,* 2008; Otto *et al.,* 2014; Chiaiese *et al.,* 2011; Kılıç *et al.,* 2011).

Green algae are traditionally recognized as important primary producers, the abilities of green algae to biodegrade harmful environmental pollutants and complex natural compounds become increasingly apparent (Ghasemi, Rasoul-Amini *et al.,* 2011; Subashchandrabose *et al.,* 2013). For instance, micropollutants like the xenoestrogen 17α- ethinylestradiol (EE2) can be acted upon by green microalgae (Greca *et al.,* 2008). The algae play an important role in controlling and biomonitoring of organic pollutants in aquatic ecosystems (Kaoutar *et al.,* 2014). The use of

higher plants and bacteria for bioextraction and rendering heavy metals and organic pollutants harmless have been extensively studied (Kaoutar *et al.,* 2014). However, the use of microalgae in bioremediating polluted aquatic environment is gaining attention. Several other green algae were shown to oxidize typical laccase substrates (La Russa 2009; Chiaiese *et al.*,2011; Kılıc¸ *et al.,*2011).

Studies on bioaccumulation/biodegradation of organic xenobioticsin green algae is of great importance from environmental point of view because of widespread distribution of these compounds in agricultural applications, making them to be one of the major problems in aquatic ecosystem (Jin *et al.,* 2012). The algae haveproved to be effective in biosorption of heavy metals as well as (Suresh and Ravishankar 2004) bioremediation of colored wastewater containing triphenylmethane and azo dyes, *p*-nitrophenol and phenanthrene (Acuner and Dilek 2004; Lima *et al.,* 2003).These bioremediation capabilities of microalgae are useful for environmental sustainability (Li *et al.,*2010; Ellis *et al.,* 2012).

1.2 Statement of Research Problem

Industries such as textiles, tanneries, paper and pulp operate in Nigeria producing large volume of liquid waste (effluent) containing these dyes and are released with little or no treatment and little attention is paid by the government agencies. These effluents are discharged into nearby surface water and channel into rivers/tributaries polluting water, soil and the environment.

The contamination of soil, water, and air by toxic chemicals has become one of the major environmental problems faced by the world today. With industrialization the driving tool of the modern world, the pollution of the environment with mandate organic compounds has become a serious problem with an annual estimate of eighty billion pounds of hazardous organo-pollutants

produced in the United States and not more than 10% of these are disposed of safely (Viswanath *et al.*,2014).

The accumulation of organic pollutants in the environment can cause serious problems to the stability of many aquatic ecosystems and the resultant harmful effects on human health and the environment (Kaoutar *et al.,* 2014). Human activities involving these persistent organic pollutants have adverse effects on environment and human health (Kaoutar *et al.,* 2014).

Azo dyes represent up to 70% of dyestuff used in textile and other industries (Wang *et al.,* 2017). With about 280,000 tons of unbound xenobiotic dyes discharged annually as wastewater (Jin *et al.,* 2007). The synthetic dyes which are toxic, mutagenic and carcinogenic contains colored components (Leechart *et al.,* 2009), thus affect the balance of aquatic ecosystem and pose threat to public health due to bioaccumulation and cause soil contamination (Sriram *et al.,* 2013). In addition, the reduced intermediates of azo dyes which are the aromatic amines, possess more harmful effect than the dyes themselves (Gomi *et al.,* 2011).

1.3 Justification of the Study

Bioremediation has served as a promising technology for removing unwanted xenobiotics compared to the conventional physicochemical management practices (Sannino *et al.,* 2016).

Investigation on organic xenobiotics bioaccumulation/biodegradation in green algae is of great importance from environmental point of view.Wide spread of these organic xenobiotics has become one of the major problems in aquatic ecosystem (Jin *et al.,* 2007). In the past decade, the use of microalgae in removing toxic and harmful substancesfrom colored wastewater has gained a lot of interest in bioremediation related researches for environmental sustainability (Li *et al.,* 2010, Ellis *et al.,* 2012).

Of the over one hundred laccases that have been isolated from different microorganisms, only very few are of high enzymatic yield and activity, most of these laccases are 'common' with low yield of enzymatic activity and tolerance to extreme conditions (Kim *et al.,* 2012). Theabove-namedlimitations hinder their large-scale commercial and industrial applications. Therefore, it is necessary to search for other sources of laccases with higher yields and versatile properties.

1.4 Aim and Objectives

1.4.1 Aim of the Study

The aim of this work was to investigate different species of microalgae for laccase production, biochemically characterize the enzyme, and examine its Azo dye bioremediation potential.

1.4.2 Specific Objectives

The specific objectives are:

i. To screen different species of microalgae for laccase production.
ii. To isolate and purify the laccase from the selected microalgae.
iii. To biochemically characterize the produced laccase.
iv. To determine the potential of the enzyme in the bioremediation of Azo dye contaminated water.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Laccase (Overview)

Laccase (EC 1.10.3.2, benzenediol: oxygen oxidoreductases) is one of the few enzymes that have been studied since the nineteenth century. Yoshida was the first to discover laccase and in 1883

he identified/isolated the enzyme from the exudates of the Japanese lacquer tree, *Rhus vernicifera*. However, in 1896, for the first time, such enzyme laccase was illustrated by both Laborde and Bertrand to be a fungal enzyme (Madhavi and Lele 2009). Laccases can be defined as copper-containing enzymes that catalyze the oxidation of a wide variety of organic and inorganic substrates, including mono-, di, and polyphenols, amino phenols, methoxy phenols, aromatic amines and ascorbate which reduced concomitantly four electrons of oxygen to water (Galhaup *et al.*, 2001). Laccase belong to a group of a large blue copper proteins or blue copper oxidases; other enzymes in this group are the plant ascorbate oxidases and the mammalian plasma protein ceruloplasmin. The ability of laccase to reduce molecular oxygen to water as well as their ability to oxidize phenolic compounds has led to intensive studies of the enzymes (Madhavi and Lele 2009).

2.2 Occurrence

Laccase is widely distributed in a wide range of higher plants and fungi (Benfield *et al.*, 1964) as well as in bacteria (Diamantidis *et al.*, 2000).). Laccases in plants have been identified in trees, cabbages, turnips, beets, apples, asparagus, potatoes, pears, and various other vegetables (Madhavi and Lele 2009). Laccase is also found to be present in a dozen insects of genera that includes *Bombyx, Calliphora, Diploptera, Musca, Manduca, Lucilia, Papilio, Phormia, Rhodnius, Sarcophaga, Schistocerca, and Tenebrio* (Xu 1999). Most of the laccases described in literature were isolated from higher fungi but very few studies have been carried out on algae. Laccases have been isolated from Ascomycetes, Deuteromycetes, and Basidiomycetes fungi (Assavanig *et al.*, 1992). Laccase from *Monocillium indicum* was the first laccase to be characterized from an ascomycete showing peroxidase activity (Thakker *et al.*, 1992). Most common laccase producers are the wood rotting fungi *Trametes versicolor, Trametes hirsuta,*

Trametes ochracea, Trametes villosa, Trametes gallica, Cerena maxima etc. (Morozova *et al.*, 2007). Although laccase enzymes are widely spread in plants and fungi, laccase activity has been reported only in few bacteria and algae (Octavio *et al.*, 2008).

2.3 Molecular Properties of Laccase

Laccase is a glycosylated monomer or homodimer protein generally having fewer saccharide compounds (10-25%) in fungi, bacteria and algae than in the plant enzymes. The carbohydrate moiety is made up of monosaccharides such as hexoamines, glucose, galactose, mannose, fucose and arabinose (Rogalski and Leonowicz 2004). On SDS-PAGE, most laccases show mobilities corresponding to molecular weight of 60-100 kDa, of which 10-50% may be attributed to glycosylation. Mannose is one of the major components of the carbohydrates attached to laccase. Glycosylation in laccase is responsible for secretion, proteolytic susceptibility, activity, copper retention, and thermal stability (Xu 1999).

Most laccases studied are extracellular proteins, although intracellular laccases have been found in several fungi and insects. Laccases from fungi, bacteria and algae have isoelectric points (pI) ranging from 3-7, whereas plant laccases have pI values of up to 9. The low pH optima of the microbial enzyme may be because they are well adapted to grow under acidic conditions, while the plant laccase being intracellular have their pH optima nearer to the physiological range(Benfield *et al.*, 1964). Thus, the differences in pH optima may be because of the dissimilarity in physiological functions. In addition to their pH variations, these enzymes also differ in terms of their activity and functions. Laccases from algae, bacteria and fungi are responsible for detoxification of phenolic compounds in the medium where these microorganisms grow, while enzymes from plant sources are involved in synthetic processes such as lignin formation (Benfield *et al.*, 1964).

Purified laccases exhibit a characteristic blue appearance due to their electronic absorption around 600nm. Typical UV-Visible spectra of laccase (at resting state) shows two maxima around 280nm and 600nm and one shoulder around 330nm. The ratios of the absorbance at 280nm and 600nm are generally 14 to 30, and the ratio of the absorbance at 330nm to that at 600nm is 0.5 to 2(Leontievisky *et al.,* 1997). In the holoenzyme form, most laccases have four copper atoms per monomer. UV/visible and electron paramagnetic resonance (EPR) spectroscopy are used to classify these copper atoms into three groups. The type I copper (T1) which is responsible for the intense blue of the enzymes has a strong electronic absorption around 600nm and is detectable by EPR. The type II copper (T2) is colourless, but EPR-detectable, and the type III copper (T3) which consists of a pair of copper atoms that give a weak absorbance near the UV spectrum has no EPR signal. The binding dioxygen and four- electron reduction to water occurs at the T2 and T3 copper sites which are closely together and form a trinuclear centre (Leontievisky *et al.,* 1997).

2.4 Classification According to Substrate Specificity

Laccases are associated with three (3) types of activities:

i. Catechol oxidase or o- diphenol: oxygen oxidoreductases (EC 1.10.3.1)
ii. Laccase or p- diphenol: oxygen oxidoreductases (EC 1.10.3.2)
iii. Cresolase or monophenol monooxygenase (EC 1.18.14.1).

The above enzymes differ from one another on the basis of substrate specificity (Walker and McCallion 1980). Because laccases have an overlapping range of substrate with tyrosinase, it makes it difficult to define a particular one based on its substrate specificity. With more affinity towards the second group, laccase has been well documented to have both ortho and paradiphenol activity. While tyrosinases possess cresolase activity, laccases on the other hand

can oxidize wide range of substrates such as syringaldazine (Thurston 1994, Eggert *et al.*, 1996). However, there has been only one report of an enzyme exhibiting both tyrosinase and laccase activity (Madhavi and Lele 2009). Another obstacle in defining laccase according to substrate is that the substrate varies from one organism to another. An immobilized commercial laccase was able to degrade meta, ortho and para-substituted methoxyphenols, chlorophenols and cresols but the substituted phenols from these three types of phenols are oxidized in different orders and to different extents (Lante *et al.*,2000). Extensive research on laccase has shown that phenolic compounds that were oxidized slowly by the enzyme can be used to increase the storage stability of laccase in some microorganisms (Mai *et al.*, 2000). As there are so many potential applications of laccase, a highly stable laccase would be of tremendous benefit.

2.5 Mode of Action of Laccase

Laccase only attack the phenolic subunit of lignin, leading to alpha-carbon oxidation, alpha carbon- beta carbon cleavage, and aryl-alkyl cleavage. Laccase activity involves:

i. Reduction of the type 1 copper by a reducing substrate
ii. Internal electron transfer from type 1 to the type 2 and type 3 copper
iii. Reduction of oxygen to water at the type 2 and type 3 copper site.

The oxidation of a reducing substrate by laccase typically involves the loss of a single electron and the formation of a free (cation) radical. The radical is in general unstable and may undergo further laccase-catalyzed oxidation (e.g from quinone to phenol) or nonenzymatic reactions (e.g hydration, disproportion or polymerization) (Xu 1999). The electron transfer from substrate to type 1 copper is probably controlled by redox potential difference. A lower oxidation potential of substrate or a higher redox potential of laccase (type 1 site) often results in a higher rate of substrate oxidation (Madhavi and Lele 2009).

Different studies have shown that the binding pocket of type 1 copper site is quite shallow and has limited stearic effect on simple phenol substrate. In contrast, the oxygen binding pocket of the type 2 or type 3 copper sites appears to hinder the access of oxidizing agents other than oxygen. Activation of oxygen occurs in the trinuclear copper cluster and it likely involves chemical bond formation (Madhavi and Lele 2009). Solomon *et al.* (2008) proposed that under turnover conditions, the share of electron from substrate to the type 1 site (the initial electron acceptor from substrate) is the committed step.

2.6 Main Reaction of Laccase

During the catalytic processes of laccases, different free radical reactions are produced depending on the substrate and reaction conditions. Among the various reactions of laccases, coupling of free radicals that generate dimeric products or polymeric compounds and oxidative carboxylations are the most frequent reactions(Rogalski and Leonowicz 2004). The oxidation of substrates is coupled to reduction of molecular oxygen; generating two water molecules. For each oxygen reduced, four molecules of substrate are oxidized without hydrogen peroxide production:

$$4H^{+} + 4\,substrate + O_2 \rightarrow$$

$$2H_2O + 4\,substrate^{+} \ldots\ldots\ldots\ldots\ldots\ldots\ldots (Solomon\ et\ al., 2005).$$

Consequently, laccases are considered “ideal green” catalysts because they employ O_2 as a co-substrate and generate H_2O as a byproduct as shown in Figure 2.1.

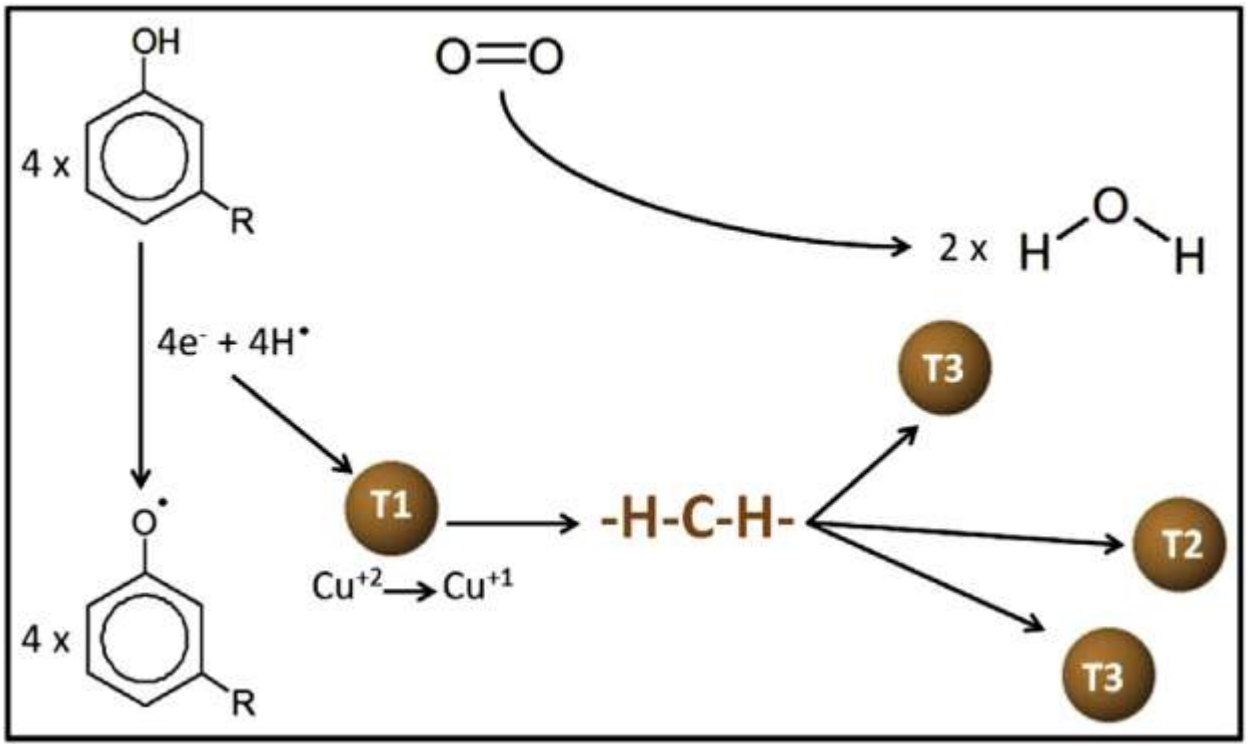


Figure 2.1: Laccase catalytic cycle(Baldrian, 2006).

Substrates are oxidized by the Cu-T1 center and electrons are transferred by a highly conserved motif: His-Cys-His (HCH) to the T2 and T3 copper centers. This is where reduction of molecular oxygen to water takes place.

2.7 Factors Involved in Laccase Production

A number of studies have shown laccases to be intracellular enzymes, but most are extracellular glycoproteins (Kunamneni *et al.*, 2007), and their production usually occurs during the secondary metabolism of different micro-organisms, and are affected by different factors such as species, culture type (stationary or agitated), aeration and culture time (Brijwani *et al.*, 2010; Elisashvili and Kachlishvili, 2009; Kunamneni *et al.*, 2007). More importantly, the major factors are carbon, nitrogen, and inducerconcentrations (Majeau *et al.*, 2010). These parameters are considered limiting for laccase large-scale production. For example, fungi grown on wood or in submerged culture results in low enzyme yield (Piscitelli *et al.*, 2010). Microbial metabolism may be "controlled" by environmental conditions and culture medium composition. Multiple

DNA regulatory sites, such as Metal Responsive Elements (MREs), Xenobiotic Responsive Elements (XREs) and Heat Shock Elements (HSEs) located 400 bp upstream of laccase gene promoter regions can be activated by certain xenobiotic compounds, heavy metals or thermal shock; influencing laccase expression (Faraco *et al.,* 2002). Increase in biomass culture concentration does not directly lead to an increase in laccase production or laccase activity. However, higher concentrations can be obtained by the addition of aromatic compounds such as 2,5-xylidine; one of the most efficient inducers (Kunamneni *et al.,* 2007; Tong *et al.,* 2007). Reports have shown that cultivation of *P. cinnabarinus* and *Trametes versicolor*for 24 h, followed by the addition of 2,5-xylidine at a concentration ranging from 0.01 mM to 1.25 mM produces a maximum laccase activity, compared to culture without an inducing agent(Kollmann *et al.,* 2005). Copper being another important inducer for laccase production has shown that its addition in low concentrations to culture media stimulates laccase production in white rot fungi (Shutova *et al.,* 2008). Palmieri *et al.*(2000) found that the addition of 150 mM of copper sulphate gave an increase in laccase activity from 6×10^2 to 30×10^3 $U \cdot L^{-1}$ compared to control culture.

Although many investigations have focused on testing different inducing agents to increase laccase production. Tong *et al.* (2007) induced laccase production in *Trametes sp*., 420, by addition of 0.5mM Cu^{+2} and 6mM o-toluidine. In this study, laccase activity values ranged approximately between 68.1×10^2 $U \cdot L^{-1}$ and 78.8×10^2 $U \cdot L^{-1}$. Tinoco *et al.,* (2001) incorporated copper and lignin simultaneously and observed an increase in growth and volumetric activity of laccase from 10^3 up to 12×10^3 $U \cdot L^{-1}$, these results open new paths towards more efficient processes in the production of laccases from fungi, bacteria, and algae.

Regarding the influence of carbon source, studies have shown that rapidly degraded substrates such as glucose, mannitol and cellobiose, usually produce high laccase activities in comparison to other substrates such as cellulose or lactose that are degraded more slowly (Mikiashvili *et al.,* 2006). In some cases, an increase in laccase activity occurs with a concomitant increase in fungal growth. Some studies have shown that specific activities of laccases can be augmentedby the appropriate carbon source. For example, Rivera-Hoyos *et al.*(2017) reported a laccase specific activity increase when glucose was used in place of fructose. More also, Rodrıguez-Couto and Toca Herrera (2006) demonstrated in *Trametes hirsuta* that sequential addition of different carbon sources, such as glucose followed by glycerol, resulted in a higher laccase production rate, compared to cultures supplemented only with glucose or cellulose. For many species, glucose is a typical repressor of laccase production. An optimal concentration of inducer agents and carbon sources depends on organism species and strain. This suggests that carbon source or inducer agent should not be assumed based on prior published experiments and the existence of different mechanisms for laccase gene regulation. With regard to the influence of nitrogen, the majority of authors report laccase production by nitrogen source exhaustion (Majeau *et al.,* 2010). Still others have found that for some strains, enzyme yield and activity does not directly depend on nitrogen (Kunamneni *et al.,* 2007). Furthermore, some authors have reported early laccase production in nitrogen rich culture media, compared to cultures with low concentration of nitrogen source (Elisashvili *et al.,* 2008a, 2008b). These data display certain ambiguity regarding the selection of nitrogen's optimum concentration for laccase production. Some studies report increases in laccase activity with low carbon-nitrogen ratio (Hou *et al.,* 2004). In contrast, other authors have reported higher laccase productions with high carbon-nitrogen ratio (Dong *et al.,* 2005).

2.8 Laccases and Chemical Mediators: Mimicking Nature

Lignin is a structural component of plant cell walls. It is a complex and amorphous polymer of aromatic nature, which comprises approximately 20-32% of the dry weight of wood (Ralph *et al.,*2007). Monomers forming lignin are p-coumaril, conyferil, and sinapyl alcohols; they differ from each other by the degree of methoxylation. These monomers produce phydroxyphenyl, guaiacyl, and syringyl phenylpropanoid units, which are capable of generating electron delocalized radicals that couple at various sites (Ralph *et al.*, 2007; Widsten and Kandelbauer, 2008). While in plant wood-tissues laccases are part of the lignin synthesizing system. Laccase is a large molecule (Rodgers *et al.,* 2010) that cannot penetrate deep into wood and because it has a low redox potential (≤0.8 V) compared to ligninolytic peroxidases (>1 V), laccase can only oxidize lignin phenolic fragments. However, the number and type of substrates oxidized by laccase can be extended by a mechanism involving the participation of redox mediators.

These mediators are compounds with low molecular weight that can easily be oxidized by laccase, producing highly reactive and unstable cationic radicals. However, at the same time these cationic radicals can oxidize complex compounds (with the exclusion of phenolic substrates) before returning to their original state (Torres *et al.,* 2003). By this mechanism, mediators act as diffusible electron transporters, allowing indirect oxidation of polymeric substrates such as lignin, penetrating even to less accessible areas of its structure. Additionally, because of mediator use, laccases are able to oxidize compounds with greater redox potential than their own; an example of this is the oxidation mediated by polycyclic aromatic hydrocarbons or PAHs (Riva, 2006). Since Bourbonnais demonstrated that mediator inclusion expanded laccase's catalytic activity toward non-phenolic substrates (Rivera-Hoyos *et al.,* 2017), more than 100 different mediators have been described with ABTS (2,2'- azino-bis (3-

ethylbenzothiazoline-6-sulfonic acid)) and the more commonly used HBT (1-hydroxybenzotriazole) (Solıs-Oba *et al.,* 2005). This laccase-mediator system is also commonly used in pulp and paper bleaching industry as well as bioremediation of xenobiotic compounds such as PAHs (Majcherczyk and Johannes, 2000; Alcalde *et al.,* 2002; Rivera-Hoyos *et al.,* 2017). Furthermore, studies have shown that the combination of two or more mediators (e.g., ABTS and HBT) can generate a synergistic effect on oxidative activity (Rivera-Hoyos *et al.,* 2017). However, the elevated chemical mediator commercial costs, their high toxicity, and lack of studies on derivative effects, in addition to inactivation caused by their cationic radical exertion on laccases, makes the laccase-mediator system implementation still limited. For these reasons, employment of natural mediators could have environmental and economic advantages. Many compounds involved in natural degradation of lignin can act as mediators in an effective manner. Such is the case for lignin degradation compounds derived from oxidized lignin units, or those secreted by white rot fungi. In a similar way the natural mediators 4-hydroxybenzoic acid, 4-hydroxybenzilic alcohol, veratrilic alcohol, syringaldehyde, acetosyringone, vanillin, and p-coumaric acid, among others, have been tested with different laccases with similar results to those obtained with artificial mediators. A clear example is the recently PAHs described degradation of benzo[a]pyrene, pyrene and anthracene using a laccase-mediator system from P. cinnabarinus and p-coumaric acid (Cañas and Camarero, 2010).

2.9 Recombinant Laccases and Heterologous Production

Obtaining laccases from native sources does not provide sufficient yield to meet industrial processes requirements. Furthermore, high cultivation and purification increase costs. For this reason, heterologous laccase expression has become a promising alternative. With readily available commercial hosts that are simple to manipulate genetically and cultivate; productivity

can become more efficient, reducing production costs (Piscitelli *et al.*, 2010). Until now results obtained from laccase's heterologous production are promising, however many difficulties encountered are yet to be solved. Among the obstacles encountered are high expression levels in combination with an optimal biological activity. The ability to produce laccases in heterologous systems in an efficient manner depends largely on changes made to the original DNA sequence(Nakamura and Goa, 2005). For example, an increase in secretion of recombinant laccases has been obtained replacing the native signaling peptide by signal sequences present in proteins with a high degree of secretion by the host ((Wang, 2004; Salony *et al.*, 2008). Inducing random mutagenesis and/or recombination to modify the protein's characteristics was another strategy employed (Rivera-Hoyos *et al.*, 2017). Results regarding the production of heterologous laccases imply that "the best host" or the "more promising" laccase still remains unknown. Currently, as a result of great advances in molecular biology and recombinant DNA technology, it is possible to explore new strategies aimed at producing laccases at industrial levels.

2.10 Potential Industrial and Biotechnological Applications of Laccase Enzyme

There have been numerous applications of laccases in the last two decades. Laccases find applications within the following fields:

2.10.1 Food industry

Laccases can be applied to certain processes that enhance or modify the colour appearance of food or beverage. In this way, an interesting application of laccases involves the elimination of undesirable phenolics, responsible for the browning, haze formation and turbidity development infruit juice, beer and wine (Minussi *et al.*, 2002). Laccases are currently of interest in baking due to their ability to cross-link biopolymers. Thus, Selinheimo *et al.*, (2006) showed that a laccase from the white-rotfungus *Trametes hirsuta* increased the maximum resistance of dough

and decreased the dough extensibility in both flour and gluten dough.In the last decade, Minussi *et al.* (2002) have described the potential applications of laccase in different aspects of the food industry such as beverage processing, ascorbic acid determination, pectin gelation, baking processes.

2.10.2 Pulp and paper industry

The industrial preparation of paper requires separation and degradation of lignin in wood pulp. Environmental concerns stimulate the replacement of conventional and polluting chlorine-based delignification/bleaching procedures. Oxygen delignification processes have been industrially introduced, but pre-treatments of wood pulp with ligninolytic enzymes might provide milder and cleaner strategies of delignification without affecting the integrity of cellulose (Susana *et al.*, 2006). Although extensive studies have been performed to develop alternative bio-bleaching systems, few enzymatic treatments exhibit the delignification/brightening capabilities of modern chemical bleaching technologies.

One of the few exceptions to this generalization is the development of LMS (Laccase Mediator System) delignification technologies for Kraft pulps. In addition, laccase is more readily available and easier to manipulate than both lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP) and LMS has already found practical applications such as the Lignozym®-process (Susana *et al.*, 2006). Several authors applied the LMS to pulp biobleaching.

However, all these biobleaching studies were focused on wood pulps and not much is known about the efficiency of the LMS on non-wood pulps including those used for manufacturing specialty papers (Giardina *et al.,* 2010). Studies have shown the potential of LMS to remove lignin-derived products responsible for colour from a high-quality flax pulp (Camarero *et al.,*2004). They showed the feasibility of LMS to substitute chlorine-containing reagents in

manufacturing of these high-price paper pulps. The capability of laccases to form reactive radicals in lignin can also be used in targeted modification of wood fibers. For example, laccases can be employed in the enzymatic binding of fibers in the production of lignocellulose based composite materials such as fiberboards(Junghanns *et al.*, 2009). Laccases have been proposed to activate the fiber bound lignin during manufacturing of the composites, thus, resulting in boards with good mechanical properties without toxic synthetic adhesives (Hüttermann *et al.,* 2001; Susana *et al.,* 2006). Another possibility of enhancing the chemical or physical properties of the fiber products is by functionalizing lignocellulosic fibers by laccases. Preliminary results have shown that laccases are able to graft various phenolic acid derivatives onto Kraft pulp fibers (Lund and Ragauskas, 2001; Chandra and Ragauskas, 2002). This ability could be useful in the future to attach chemically versatile compounds to the fiber surfaces, possibly resulting in fiber materials with completely novel properties such as hydrophobicity or charge.

2.10.3 Textile industry

The textile industry accounts for two-third of the total dyestuff market and consumes large volumes of water and chemicals for wet processing of textiles. The chemical reagents used are very diverse in chemical composition, ranging from inorganic compounds to polymers and organic products (Susana *et al.*, 2006). There are more than 100,000 commercially available dyes with over 7×10^5 tonnes of dyestuff produced annually (Zollinger, 2002). Due to their chemical structure, dyes are resistant to fading on exposure to light, water and different chemicals and most of them are difficult to decolourize due to their synthetic origin. Government legislation is becoming more stringent, especially in the developed countries, regarding the removal of dyes from industrial effluents. Concern arises, as several dyes are made from known carcinogens such as benzidine and other aromatic compounds. Most currently existing processes to treat dye

wastewater are ineffective and not economical (Susana *et al.*, 2006). Therefore, the development of processes based on laccases seems an attractive solution due to their potential in degrading dyes of diverse chemical structure (Abadulla *et al.*, 2000; Blánquez *et al.*,2004; Hou *et al.*, 2004), including synthetic dyes which are currently used in the industry (Rodríguez Couto *et al.*, 2004a, 2005).

The use of laccase in the textile industry is growing rapidly and in 1996, Novozyme (Novo Nordisk, Denmark) launched a new industrial application of laccase enzyme in denim finishing: DeniLite®, the first industrial laccase and the first bleaching enzyme with the help of a mediator molecule. Also, in 2001 the company Zytex (Zytex Pvt. Ltd., Mumbai, India) developed a formulation based on LMS capable of degrading indigo in a very specific way. The trade name of the product is Zylite(Susana *et al.*, 2006).

2.10.4 Nanobiotechnology

For the past two decades, bioelectrochemistry have been receiving increased attention. Progress on bioelectrochemistry has been integrated into analytical applications, e.g. in biosensors working as detectors in clinical and environmental analysis (Haghighi *et al.*, 2003). Since laccases are able to catalyze electron transfer reactions without additional cofactors, their use has also been studied in biosensors to detect various phenolic compounds, oxygen or azides. Moreover, biosensors for detection of morphine and codeine, catecholamines (Leite *et al.*, 2003; Ferry and Leech, 2005; Susana *et al.*,2006), plant flavonoids (Jarosz-Wilkoazka *et al.*, 2004) and also for electro immunoassay (Kuznetsov *et al.*, 2001) have been developed. Nanotechnology contributes to the development of smaller and more efficient biosensors through controlled deposition and specific adsorption of biomolecules on different types of surfaces, achieving micro and nanometer order. Regarding laccases, the immobilisation has an important influence

on the biosensor sensitivity (Freire *et al.*, 2001). Martele *et al.,* (2003) have shown that micropatterning is an efficient method for the immobilisation of laccases on a solid surface in order to develop a multi-functional biosensor.Also, Roy *et al.,* (2005) found that cross-linked enzyme crystals (CLEC) of laccase can be employed in biosensor applications, this have great advantage over the soluble enzyme. In addition, an enzyme electrode based on the co-immobilization of an osmium redox polymer and laccase on glassy carbon electrodes has been applied to ultrasensitive amperometric detection of the catecholamine neurotransmitters dopamine, epinephrine and norepinephrine, attaining nanomolar detection limits (Ferry and Leech, 2005). Laccase can also be immobilized on the negatively charged electrode (cathode) of biofuel cells that could generate power, for example, for small transmitter systems (Chen *et al.,* 2001). Biofuel cells are extremely attractive from an environmental point of view because electrical energy is generated without combusting fuel, thus, providing a cleaner source of energy.

Flatpolyelectrolyte multilayers built by alternating adsorption of oppositely charged polyelectrolytes have been used to recrystallize bacterial proteins making the building of artificial cell walls possible (Toca-Herrera *et al.,* 2005). Caruso *et al.,* (2000) showed that the encapsulated enzyme could retain 100% of its activity after incubation for 100 min with protease. The permeability properties of the wall capsule are important for the proper function of the encapsulated enzyme. Antipov *et al.,* (2002) investigated the permeability properties of hollow polyelectrolyte multilayer capsules as a function of pH and salt concentration. This mechanism together with the LbL encapsulation technique permits the development of microreactors. Also, colloidal particles covered with polyelectrolytes and phospholipids have been used to host and activate rubella virus (Fischlechner *et al.,* 2005).

2.10.5 Other applications of laccase

2.10.5.1 Role in bioremediation

Bioremediation is the use of living microorganisms to degrade environmental pollutants or to prevent pollution. In other words, it is a science for removing pollutants from the environment thus restoring the original natural surroundings by making it harmless and preventing further pollution (Sasikumar and Papinazath 2003). It can also be defined as the process that uses microorganisms or their macromolecular components to return the environment altered by contaminants to its original condition. Bioremediation may be in the bioremediation of some contaminants, such as chlorinated pesticides that are degraded by bacteria, or a more general approach may be taken, such as oil spills that are broken down using multiple techniques including the addition of biosurfactant to facilitate decomposition of crude oil by bacteria. Bioremediation may occur either in the presence of oxygen or in the absence of oxygen (Komancova *et al.,* 2003). Bioremediation can save life web and hinders the passage of dangerous and risky contaminants from one ecosystem to the other. It is often carried out on site, without causing a major disruption of normal activities. This also eliminates the need to transport quantities of waste off site and the potential threats to human health and the environment that can arise during transportation. Bioremediation has shown to be less costly than other technologies used for clean-up of hazardous waste (Vidali 2001).

Synthetic dyes are being rapidly applied in the textile, paper, leather dyeing, color photography, cosmetics, pharmaceutical, and food industries (Saratale *et al.,* 2011, Forootanfer *et al.,* 2012, Rezaie *et al.,* 2015,). These dyes are widely used in various industries across the world for different purposes particularly for textile dyeing. Every year, more than 80,000 tons of reactive dyes are produced and consumed for textile dyeing globally. Reactive dyes have been used

extensively to dye more than half of the global production of cotton, this feat is achieved as a result of their beneficial characteristic of bright colour, water-fast, and simple application techniques with low energy consumption (Hessel *et al.,* 2007). Azo dyes (contain the N = N group in their structure) identified as one of the most popular synthetic coloring agents because they could be easily and affordably synthesized and found to be stable (Maaley-Kammoun *et al.,* 2009, Shrimardi *et al.,*2012; Ashrafi *et al.,* 2013). However, in the process of textile dyeing, 50 – 60% of these water-soluble dyes do not bind to the fiber and are lost into the effluent. In addition to the high volume of water required, each year textile mills generate and discharge billions of liters of wastewater effluent full of colours, salts, and organic chemicals which have harmful effect to the environment (Hessel *et al.,* 2007, Kant, 2012). Furthermore, more studies have been carried out on the toxicity, mutagenicity, and carcinogenicity of synthetic azo dyes and/or their metabolites (Pereira *et al.,* 2009, Grassi *et al.,* 2011, Mirzadeh *et al.,* 2014). Similarly, extensive work about the harmful effects of azo dyes on the germination and growth of many environmentally important plants have been carried out (Saratale *et al.,* 2011., Mirzadeh *et al.,* 2014). So, during the last decade, development of physicochemical (Mahvi *et al.,* 2009, Maleki *et al.,* 2010) and/or biological techniques for bioremediation of wastewater rich in such complex aromatic structures have received great attention among which enzymatic removal of such pollutants is an economic and environmentally friendly procedure due to the low energy required and the minimal effect on ecosystems (Mendes *et al.,* 2011, Younes *et al.,* 2012, Asadgol *et al.,* 2014).

2.10.5.1.1 Soil bioremediation

One of the major sources of contamination in soil is Polycyclic aromatic hydrocarbons (PAHs), together with other xenobiotics therefore, their degradation is of great importance for the

environment. The catalytic properties of laccases can be used to degrade such compounds. Thus, laccases were able to mediate the coupling of reduced 2,4,6-trinitrotoluene (TNT) metabolites to an organic soil matrix, which resulted in detoxification of the munition residue (Durán and Esposito, 2000). Laccases however, were also found to degrade PAHs, which arise from natural oil deposits and utilization of fossil fuels. (Pointing, 2001).

2.10.5.1.2 Degradation of xenobiotics

Laccases exhibit a wide range substrate specificity which make them possible in oxidizing a broad range of xenobiotic compounds including chlorinated phenolics (Torres *et al.,* 2003), pesticides (Pozdnyakova *et al.,* 2004), and polycyclic aromatic hydrocarbons (Pointing, 2001). Moreover, polycyclic aromatic hydrocarbons, which arise from natural oil deposits and utilization of fossil fuels, were also found to be degraded by laccases (Pointing 2001). Laccase purified from a strain of *Coriolopsis gallica* oxidized carbozole, N-ethylcarbozole, fluorine, and dibenzothiophene in presence of 1-hydroxybenzotriazole and 2, 2'-azinobis (3-ethylbenzothiazoline)-6-sulfonic acid as free radical mediators (Bressler *et al.,* 2000). Laboratory researches have shown that phenols and aromatic amines may be removed from water by the application of laccase (Niku-Paavola and Viikari 2000). The underlying mechanism of the removal involves enzymatic oxidation of the pollutants to free radicals or quinones that undergo polymerization and partial precipitation (Buddolla *et al.,* 2004). Laccase from white-rot fungus, *Trametes hirsuta,* has been used to oxidize alkenes (Ahn *et al.,* 2002). The oxidation is the effect of a two-step process in which the enzyme first catalyzed the oxidation of primary substrate, a mediator added to the reaction, and then the oxidized mediator oxidizes the secondary substrate, the alkene, to the corresponding ketone or aldehyde(Buddolla *et al.,* 2004). In addition to substrate oxidation, laccase can also prevent soil pollutants by coupling to soil humic

substances—a process analogous to humic acid synthesis in soils (Buddolla *et al.,* 2004). The xenobiotics that can be immobilized in this way include phenolic compounds such as chlorinated phenols and anilines e.g. 3, 4-dichloroaniline or 2, 4, 6-trinitrotoluene (Buddolla *et al.,* 2004). A laccase produced in the yeast, *Pichia pastoris*, was engineered by site directed mutagenesis to improve the rate of electron transfer between the copper-containing active site of laccase and an electrode (Buddolla *et al.,* 2004). Thus, laccase may be usefully engineered to improve the efficiency of particular bioremediation processes.

2.10.5.1.3 Decolorization of dyes

Studies have shown that there were over 100,000 commercially available dyes with over 7×10^5tonnes of dyestuff produced annually(Rajaguru *et al.,* 2002). Globally, it is estimated that over 280,000 tonnes of textile dyes are discharged in textile industrial effluent every year (Jin *et al.,* 2007). Originally dye agents are of two types, natural coloring agents and organic dyestuffs. Natural colouring agents are mainly of inorganic origin (clays, earth, minerals, metal salts and even semi-precious stones, such as malachite) or organic dyestuff traditionally divided into two (2) groups, one of animal and the other of plant origin (Ackacha *et al.*,2003). Undoubtedly, plants are the most important sources of dyes, but few other organisms like lichens, insects and shellfish were also reported to be good sources of natural dyes(McMullan *et al.,* 2001). Reactive dyes cause asthma rhinitis and dermatitis, allergic contact dermatitis (Mathur *et al.,* 2005b), genotoxicity (Dogan *et al.,* 2005), carcinogenicity (De Roos *et al.*, 2005). Dyes have a very low rate of removal ratio for Biological Oxygen Demand (BOD) to Chemical Oxygen Demand (COD) (less than 0.1). Therefore, industrial effects containing dyes should be processed before their discharge into the environment (Wong *et al.,* 2003). (Enayatizamir *et al.,* 2011) observed degradation of 92% in the Azo Black Reactive 5 dye by *P. chrysosporium* after 3 days of

treatment. *P. chrysosporium*URM6181 and *Curvularia lunata*URM6179 strains decolourize effluent containing textile indigo dye by approximately 95% for 10 days of treatment (Miranda *et al.,* 2013). Laccase purified from the fungus *Trametes hirsuta* was able to degrade triarylmethane, indigoid, azo, and athraquinonic dyes used in dyeing textiles (D'Annibale *et al.*, 2000) as well as 23 industrial dyes (Ying *et al.*, 2002). Organic dyes present a wider spectrum of compound with different physical and chemical properties (Indrani *et al.,* 2016). However, synthetic dyes have quickly replaced the traditional natural dyes. They are cheap to afford and offered new colour varieties, as well as improved properties to the dyed material (Indrani *et al.*, 2016).

2.10.5.1.4 Effluent treatment

Laccases offer numerous advantages of great interest to biotechnological applications of industrial effluent treatment. Their ability to exhibit broad substrate specificity, make it possible for them to be able to bleach Kraft pulp or detoxify agricultural byproducts including olive mill wastes or coffee pulp (Heemken *et al.,* 2001). The use of laccase in the textile industry is growing very fast since, besides decolorizing textile effluents as commented above, laccase is used to bleach textiles and even to synthesize dyes (Raghukumar 2000). *Flavodon flavus* decolourized several synthetic dyes such as Azure B and Brilliant Blue R in low nitrogen medium (Soares *et al.,* 2001). Alternatively, laccase, along with stabilizers, may be suitable for treatment of wastewater (Abadulla *et al.,* 2000; Soares *et al.,* 2001). Laccase isolated from the fungus, *Flavodonflavus,* was shown to decolourize the effluent from a Kraft paper mill bleach plant (Soares *et al.,* 2001). Purified laccase from white rot basidiomycete, *Trametes villosa,* degrades bisphenol A, an endocrine-disrupting chemical (Raghukumar *et al.,* 2000). Nonylphenols have increasingly gained attention because of their potential to mimic the action of

natural hormones in vertebrates (Lyons *et al.*,2003). They result from incomplete biodegradation of nonylphenol polyethoxylates (NPEOs), which have been widely used as nonionic surfactants in industrial processes. Both nonylphenols and NPEOs are discharged into the environment, mainly due to incomplete removal of wastewater treatment facilities (Lyons *et al.*,2003). Nonylphenols are more resistant to biodegradation than their parent compound and hence are found worldwide in wastewater treatment plant effluents and rivers (Bermek *et al.,* 2002). Due to their hydrophobicity, they tend to be absorbed onto surface water particles and sediments and accumulate in aquatic organisms. Consequently, nonylphenols represent a serious environmental and human health risk. Laccases from aquatic hyphomycete, *Clavariopsis aquatica*, have proved to degrade xenoestrogen nonylphenol (Buddolla *et al.,* 2004).

2.10.5.2 Synthetic chemistry

In the near future, laccases may also be of great interest in synthetic chemistry, where they have been proposed to be useful in oxidative deprotection (Susana *et al.,* 2006) and production of complex polymers and medical agents (Mai *et al.,* 2000; Uyama and Kobayashi, 2002; Kurisawa *et al.,* 2003; Nicotra *et al.*, 2004). In the last decade, Mustafa *et al.* (2005) synthesized phenolic colorants by using an industrial laccase named Suberase® (Novo Nordisk A/S, Bagsvaerdt, Denmark).For example, laccase-based hair dyes where H_2O_2 as an oxidizing agent is replaced in the dye formulation are less irritant and easier to handle than current hair dyes (Susana *et al.,* 2006). With the aid of laboratory experiments,cosmetic and dermatological preparations containing proteins for skin lightening have been developed (Golz-Berner *et al.,* 2004).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Source of Algae

Three microalgae (*Chlorella sorokiniana, Chlorogonium sp, and Planktothrix sp*) and four cyanobacteria species (*Oscillatoria sp,Microcystis auroginosa, Microcystis flos-aquae* and *Microcystis wasenbergii*) were obtained from the freshwater microalgae culture collection of University of Texas, USA.

3.2 Collection and Identification of Algae specimen

The algae were identified on the basis of their morphological characteristics. These organisms were further cultured in their respective culture media.

3.3 Preparation of Stock Culture for Media Cultivation

Nine (9) stock solutions with constituents as shown in Table 3.1 were prepared. For each of the nine stock solutions, the salts were dissolved one after the other in the appropriate volume of distilled water until all the salts were completely dissolved. The stock solutions were then stored in the dark (Plastic bottles wrapped with aluminum foil) in the refrigerator, until required.

3.4 Preparation of Blue-Green-11 (BG-11) Media

Conical flask of 1000 mL was washed with detergent and rinsed with clean water. The flask was rinsed again with distilled water and allowed to dry. After drying, the flasks werethen covered with an aluminum foil paper and autoclaved at 121^{o}C, 15 psi for 15 minutes.

Table 3.1: BG-11 Medium stock composition for culturing of Algae

Stock No	**Stock Solution**	**Amount in 200ml distilled water**	**Volume for 1liter media**
1	$NaNO_3$	30g	10mL
2	K_2HPO_4	8.0g	1mL
3	$MnSO_4.7H_2O$	15g	1mL
4	$CaCl_2.2H_2O$	7.2g	1mL
5	Citric Acid	1.2g	1mL
6	Ammonium Ferric Citrate	1.2g	1mL
7	EDTA NA_2	0.2g	1mL
8	Na_2CO_3	4.0g	1mL
9	Trace Metal		1mL
	i. H_3BO_3	1.43g	
	ii. $NaCl_2.4H_2O$	0.923g	
	iii. $ZnSO_4.7H_2O$	0.11g	
	iv. $NaMoO_4.2H_2O$	0.193g	
	v. $CuSO_4.5H_2O$	0.04g	
	vi. $Co(NO_3)_2.6H_2O$	0.023g	

3.5 Cultivation of Algal Species

Ten milliliters of stock one was dispensed into an already autoclaved conical flask. One milliliter each of stock two to nine were added to the same flask containing the stock one. Nine hundred and eighty-two (982) milliliters of distilled water was then added to the resulting mixture of the stock solutions to make up 1 litre. The mixture was then autoclaved at 121^{o}C, 15 psi for 15minutes and allowed to cooled. The above procedure was replicated six times in six different one-litre conical flasks. Two hundred and fifty mg/mL each of the seven microorganisms were inoculated to the various conical flasks and placed under ideal conditions (air condition, specific light intensity of 40 µmolm^{-2}s^{-1}, temperature of 23$\pm$1 ^{o}C, light and dark cycle of 16 & 8 hoursfor optimum growth.

3.6Enzyme Purification

3.6.1 Ammonium sulphateprecipitation

After 15 days of growth, the culture was filtered through Whatman No.1 filter paper and centrifuged at 20,000 rpm for 10 min to remove algal cells and undissolved material. After centrifugation, the biomass was separated from the supernatant. The biomass was subjected to cell lysis by sonication on ice to break cell wall and release cellular contents. Laccase activity using ABTS as substrate was assayed for in the resulting supernatant after centrifugation. The above procedures were repeated for all the microorganisms mentioned earlier, with *Microcystis flos aquae* having the highest specific activity (15.55 µmol/mg.min) and was used for the entire enzyme purification processes. The enzyme was purified by salting-out using an aliquot of 100 mL of crude solution which was precipitated with 90% $(NH_4)_2SO_4$ (AS) at room temperature under continuous stirring. After salt dissolution, the mixture was left for 20 min at room

temperature (25°C) and was subsequently centrifuged at 20,000 rpm for 10 minutes. The precipitate was removed, and the supernatant obtained was subsequently dialyzed. The supernatant was desalted overnight at 4°C against distilled water in 3 kDa cutoff cellulose acetate dialysis tubing (Sigma-Aldrich). Laccase activity assay as well as protein concentration were estimated before and after dialysis by the Lowry assay method (1951s), using bovine serum albumin as a standard. The obtained supernatant was used for chromatographic enzyme purification.

3.6.2 DEAE-sephadex chromatography

The dialyzed sample was loaded on DEAE-Sephadex column already equilibrated with 25 mM Tris–HCl (pH 7.5). The column was washed with two column volume of the same buffer. One hundred milliliters of the adsorbed proteins were eluted from the column with a stepwise gradient of 20 mL NaCl (0.2M, 0.4M, 0.6M, 0.8M and 1M). Fractions with higher activity were pooled and re-concentrated. Laccase activity assay and total protein concentration were carried out.

3.6.3 Gel filtration chromatography

Ten milliliters of the fractions pooled from DEAE-Sephadex chromatography containing laccase activity were loaded on Sephadex G-100 column (1.5 cm × 30 cm) equilibrated with 25 mM Tris–HCl (pH 7.5). The enzyme was eluted with the same buffer and 3 ml fractions were collected at a flow rate of 1 $mL \cdot min^{-1}$. Laccase activity assay and total protein concentration were carried out on each fraction according to Niku-Paavola *et al.*(1990) and Lowry's method (1951), respectively and the concentration of laccase enzyme was adjusted to 0.1 $mg \cdot mL^{-1}$.

3.6.4 Enzyme activity

Laccase activity was measured in a 1.0 mLreaction mixturecontaining 100 µM ABTS [2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid] in 50 mM glycine-HCl buffer (pH 3.0). The reaction was started by the addition of 10 µL of enzyme solution. The substrate oxidation was monitored at 420 nm (Extinction coefficientε= 29,300/M/cm). One unit of enzyme activity was defined as the amount of enzyme required to produce 1 µM of oxidized product per min. The protein concentration of the crude sample was determined by the Lowry method using bovine serum albumin as a standard protein. The protein concentration of the collected fractions was monitored based on the absorbance at 280 nm after anion-exchange and size exclusion chromatography.

3.7 Molecular Weight Determination (SDS-PAGE) using ExpressPlusTM PAGE Gels

3.7.1 Preparation of gel buffer and gel tank

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was done following the method of Laemmli (1970), using 10% precast gels. One pack of MOPS running buffer powder (Cat No. M00138) was dissolved in 1 L of deionized water to make 1 L of 1× running buffer. ExpressPlusTM PAGE Gel was removed from the pouch and the sealing tape at the bottom of the gel cassette was peeled off. The comb from the gel cassette was gently removed and the gel was inserted into the gel running apparatus. Sufficient 1× MOPS running buffer was poured into the inner tank of the gel running apparatus to cover the sample wells by 5-7 mm. The outer tank was filled with 1× MOPS running buffer to ensure proper cooling. For best results, the buffer in the outer tank was above the top level of the sample wells. The sample wells were rinsed thoroughly with 1x running buffer to remove air bubbles and displace any storage buffer. Protein sample buffer (×4) 8µl and 32µl of the sample was mixed and heated at 95 ^{o}C for

5minutes. The protein sample was loaded directly into the wells as well as the protein standard. The tank was closed and run at 140 volts for 1hour.Once the run is finished, the gel from the gel tank was removed. The gel cassette was then opened by carefully inserting the cassette opener into the gap between the two plates. The cassette opener was wiggled up and down gently to separate the two plates. The operation was repeated along both sides of the cassette, until the two plates are completely separated. Upon opening, the plate without the gel was removed and discarded, and the gel was allowed to stay on the other plate. The gel was loosened from the plate with water and gently removed.

3.7.2 Staining and drying gels

Coomassie Staining- The staining solution was made by dissolving 0.1% Coomassie R-250 in a 40% ethanol, containing 10% acetic acid solution. Also, destaining solution was prepared using 10% ethanol, 7.5% acetic acid solution. Thereafter, the gel cassette was opened, the gel was taken out gently after electrophoresis, and placed in a staining container of 100mL staining solution. The staining container was covered and heated in a microwave oven at full power for 8 minutes. The solution was not allowed to boil, this is to prevent hazardous, flammable vapours from forming. The staining container was removed from the microwave oven and the gel was shaken gently for 5 minutes at room temperature on an orbital shaker. The staining solution was later drained and the gel was rinsed with deionized water. The stained gel was then placed in 100 mL destaining solution.

3.8 Catalytic Property of Laccase/Laccase Characterization

3.8.1 Effect of pH on activity and stability of purified laccase.

The optimum pH of the purified laccase from *Microcystis flos-aquae* was determined with ABTS substrate prepared at varying pH range from 2.0 to 11.0at room temperature. The buffer

systems used were 100 mM of Glycine-HCl (2.0– 4.0), sodium-acetate (5.0–7.0) and Tris-HCl (8.0–11.0). For pH stability, purified laccase was pre-incubated at pH 2.0–11.0 for 120 minutes and the residual activity was determined using ABTS as substrate. All experiments were run in triplicates and average values were calculated.

3.8.2 Effect of temperature on activity and stability of purified laccase

The optimum temperature of the purified laccase from *Microcystis flos-aquae* was carried out by measuring the laccase activity at optimum pH and temperatures from 20^{o}C to 80^{o}C. Thermo stability of the purified laccase was determined by incubating the enzyme at temperature from 20^{o}C to 80^{o}C with the increment of 10^{o}C for 120 minutes and residual activity was determined using ABTS as the substrate. All experiments were run in triplicates and average value was calculated.

3.8.3 Effect of inhibitor and metal ions on activity of purified laccase

The effects of a potential inhibitor (EDTA) and divalent metal ions i.e. Calcium (Ca^{2+}), Copper (Cu^{2+}), Magnesium (Mg^{2+}), Iron (Fe^{2+}) and Zinc (Zn^{2+}) at 1 mM and 5 mM concentrations on the enzyme activity were determined using the optimum pH (4.0) and temperature (40^{o}C). The purified laccase was pre-incubated with inhibitor and metal ions for 20 min respectively before assaying with ABTS and relative activity was measured. All experiments were run in triplicates and average value was calculated.

3.8.4 Kinetic studies

The kinetic constants (K_m and V_{max}) for the purified laccase were determined using ABTS as substrate at 40 °C and pH 4.0. Michaelis-Menten (K_m) parameter representing the affinity of laccase to its substrate and V_{max} value which linked to the maximum rate of the reaction was calculated by measuring the enzymatic activity at differentconcentrations of the substrate

(ABTS)(10 µM–50 µM). The assays were carried out in triplicate. K_m and V_{max} values were calculated using the Lineweaver–Burk plots of the Michaelis–Menten equation.

3.9 Dye Decolorization Studies

3.9.1 The effect of pH and temperatureon laccase-mediated decolorization

In order to investigate the influence of pH on laccase mediated decolorization, 0.039g/L CiBB (Cibacron Brilliant blue) dye solution was prepared at varying pH (2.0–11.0) at room temperature. The buffer systems used were 100 mM of Glycine-HCl (2.0– 4.0), sodium-acetate (5.0–7.0) and Tris-HCl (8.0–11.0). and the purified laccase was consequently added to the reaction mixture which was then monitored for decolorization as previously described by Ashrafi *et al.,* (2013) using ABTS as substrate. At the optimum pH of decolorization, the dye solution containing purified laccase was incubated at different temperatures (20°C–80°C) and decolorization percent was then determined after 1 hour at 580 nm. Three independent experiments were conducted and means of decolorization percent was calculated.

3.9.2 Dye decolorization

In order to assess the decolorization potential of the purified laccase from *Microcystis flos-aquae,* the previously described method of Ashrafi *et al.,* (2013) was used. The dye solution was prepared by dissolving the synthetic azo dye (Cibacron Brilliant Blue; CiBB) in sodium-acetate buffer (100 mM, pH 7.0). Thereafter, the purified enzyme (10 µl) was added into the dye solution and the reaction mixture was incubated at 40°C and 50 rpm for 3 hours. At intervals of 30 minutes, samples were taken and the absorbance was measured at 580 nm. The decolorization percent was calculated using the equationbelow:

$$\text{Decolorization (\%)} = \left(\frac{Ai - At}{Ai}\right) \times 100$$

where "*Ai*" and "*At*" are the initial absorbance and the absorbance after incubation time of the reaction mixture, respectively (Couto *et al.*, 2007; Khlifi *et al.*, 2010). Three independent experiments were conducted and the mean value for decolorization percent was calculated.

3.10 Statistical Analysis

3.10.1 Screening for laccase production by various algal species

Triplicate samples were collected and analyzed each for the various species, and mean ± standard deviation was recorded. Data was analyzed using Between-Within ANOVA (Split-Plot ANOVA) and homogeneity of Variance was tested using Tukey post-hoc test.

3.10.2 Effect of pH on activity and stability of purified laccase

Triplicate samples were collected and analyzed each for the effect of pH on enzyme activity and stability. Mean ± standard deviation was calculated. Data was analyzed using ONE WAY ANOVA (Split-Plot ANOVA) and homogeneity of Variance was tested using Tukey post-hoc test.

3.10.3 Effect of temperature on activity and stability of purified laccase

Triplicate samples were collected and analyzed each for the effect of temperature on enzyme activity and stability. Mean ± standard deviation was calculated. Data was analyzed using ONE WAY ANOVA (Split-Plot ANOVA) and homogeneity of Variance was tested using Tukey post-hoc test.

3.10.4 Effect of inhibitor and metal ions on activity of purified laccase

Triplicate samples were collected and analyzed for the effect of inhibitor and the various metal ions used in this study on the activity of the purified laccase. Mean ± standard deviation was

calculated. Data was analyzed using ONE WAY ANOVA (Split-Plot ANOVA) and homogeneity of Variance was tested using Tukey post-hoc test.

3.10.5 Effect of pH and temperature on laccase-mediated decolourization

Triplicate samples were collected and analyzed each for the effect of pH and temperature on laccase-mediated decolourization. Mean ± standard deviation was calculated. Data was analyzed using ONE WAY ANOVA (Split-Plot ANOVA) and homogeneity of Variance was tested using Tukey post-hoc test.

CHAPTER FOUR

4.0 RESULTS

4.1 Screening forLaccase Production by various Algal Species

In this study, seven (7) microorganisms belonging to cyanobacteria and green microalgae were screened for the production of laccase. Although all the organisms produced laccase extracellularly, *Microcystis flos-aquae* produced the highest in terms of yield and specific activity. The maximum production of laccase from *Microcystis flos-aquae*was obtained at day15 having specific activity of 0.33 ±0.003 µmol/mg·minwith a reduction at days 18 and 21with specific activities of 0.29 ±0.005 µmol/mg·minand0.28 ±0.002 µmol/mg·minrespectivelyFigure 4.1).

4.2 Percentage Saturation of Ammonium Sulphate Precipitation

The high laccase production observed from the cyanobacteria *Microcystis flos-aquae* led to thepurification of the enzyme using ammonium sulphate precipitation. Low activity of laccase was observed using 10% saturated ammonium sulphate which remained unchanged in 20% and 30% saturation (Figure 4.2). There was an increase in activity from 40%saturation (2.21µmol/min) until 90%saturation (11.76µmol/min) where the maximum laccase activity was obtained. At 100% saturation, there was a decrease in the activity of laccase (11.43µmol/min) (Figure 4.2).

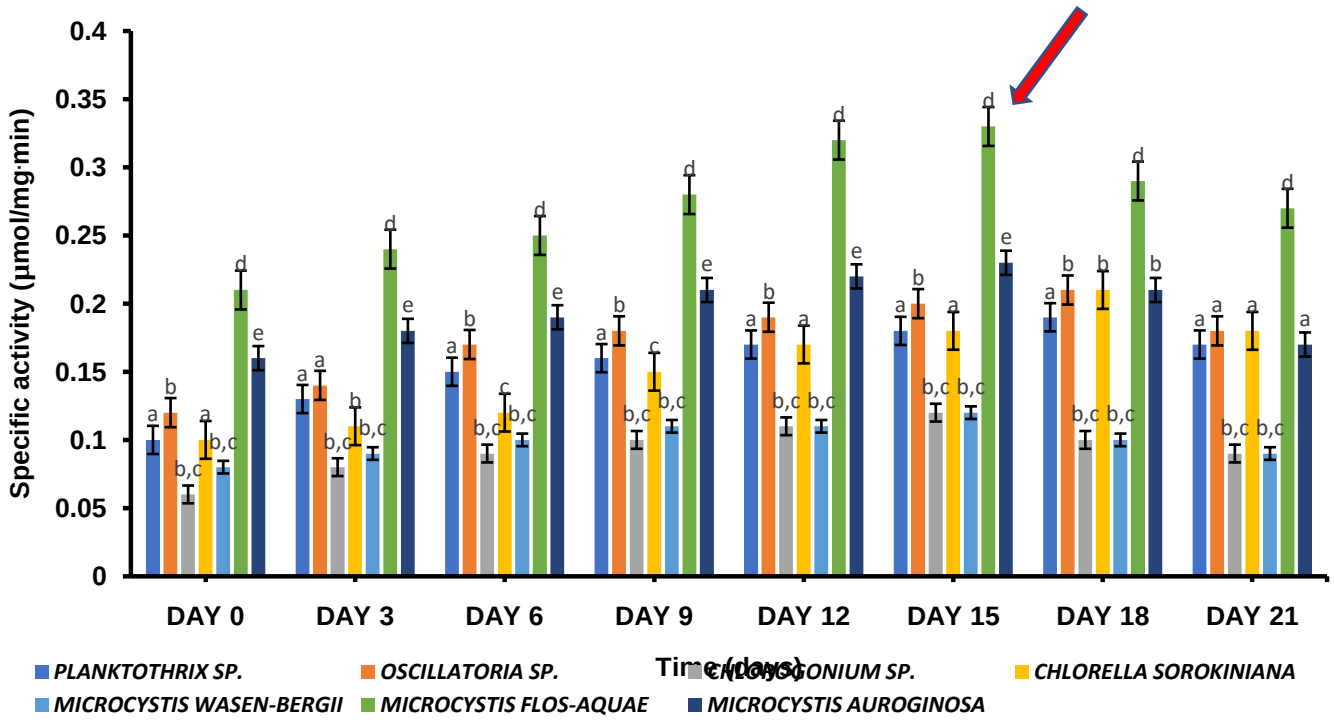


Figure 4.1: Screening for laccase production by various algal species.

Data was analyzed using SPLIT PLOT ANOVA followed by Tukey's multiple post-hoc test. Bars with different superscripts (a-e) are considered statistically significantly ($p<0.05$) different across the days and between the organisms. (n=3). The arrow in the figure indicate day with the highest yield of laccase production by *Microcystis flos-aquae.*

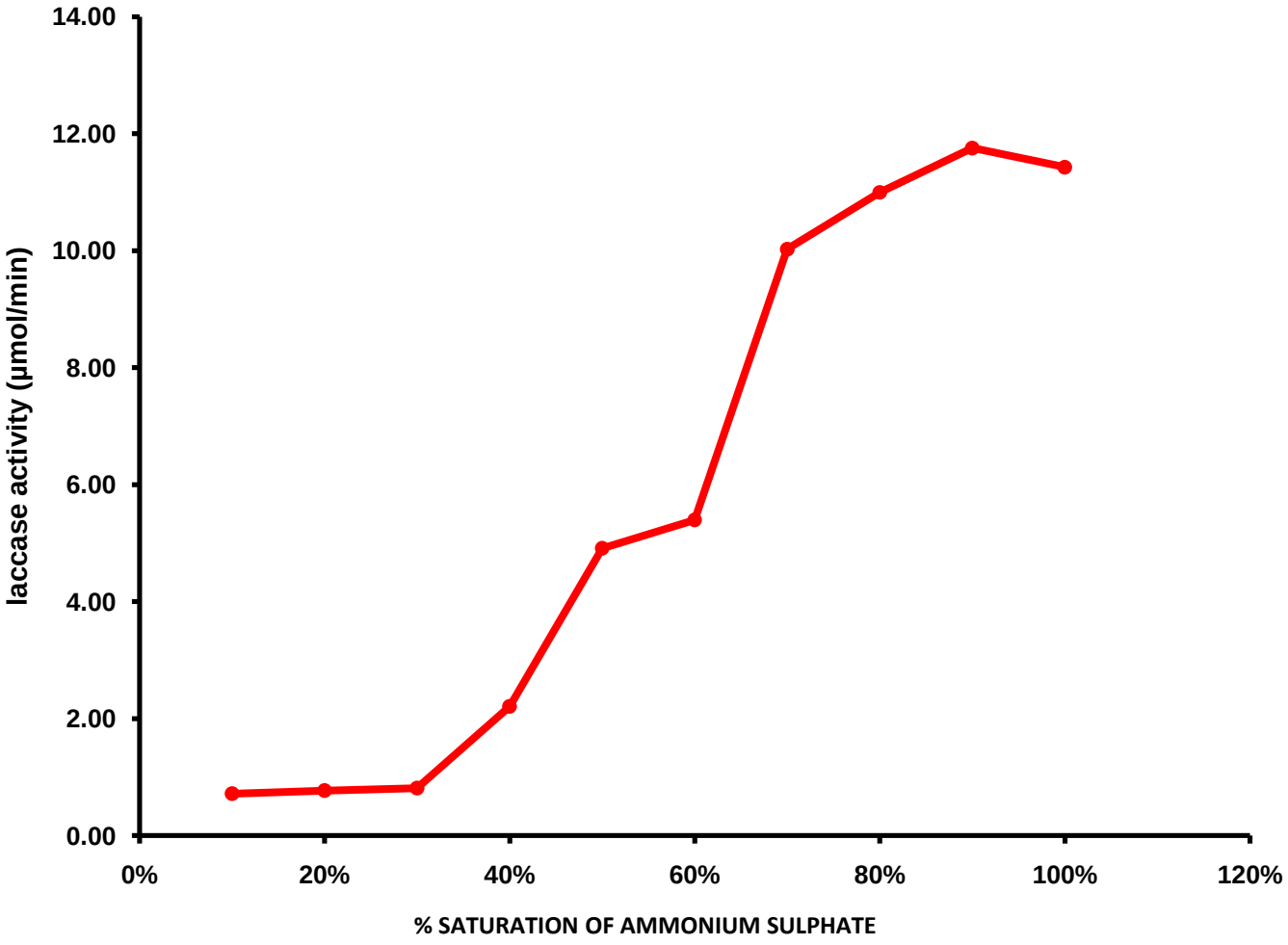


Figure 4.2: Determination of Laccase Activity based on Percentage saturation of ammoniumsulphate precipitation

4.3 Purification of *Microcystisflos-aquae*Laccase on DEAE Sephadex

Twenty milliliters of the dialyzed sample from *Microcystis flos-aquae*was subjected to anion exchange chromatography using DEAE-Sephadex exchanger, and this led to the collection of thirty (30) fractions (Figure 4.3). Exactly, three (3) milliliters of eluate was collected for each fraction at a flow rate of 10 drops per minute. Assay of laccase activity using ABTS as substratein the fractions revealed that fraction eleven (11) showed/had the highest specific activity (118.6 µmol/mg·min) with total protein concentration of 3.8mg/mL and the enzyme was purified to 7.6-fold with a yield of 1.41% (Table 4.1).

4.4: Purificationof *Microcystis flos-aquae*Laccase on Sephadex G-100

Ten milliliters of partially purified laccase from anion exchange chromatography was further subjected to size exclusion chromatography using Sephadex G-100 exchanger which led to the collection of twenty-five (25) fractions (Figure 4.3). Exactly, three (3) milliliters of eluate was collected for each fraction at a flow rate of 10 drops per minute. Investigation of laccase activity using ABTS as substrate in the fractions revealed that fraction six (6) showed/had the highest specific activity (158.73µmol/mg·min) with total protein concentration of 0.11mg/mL and the enzyme was purified to 10.21-fold with a yield of 0.55% (Table 4.1)

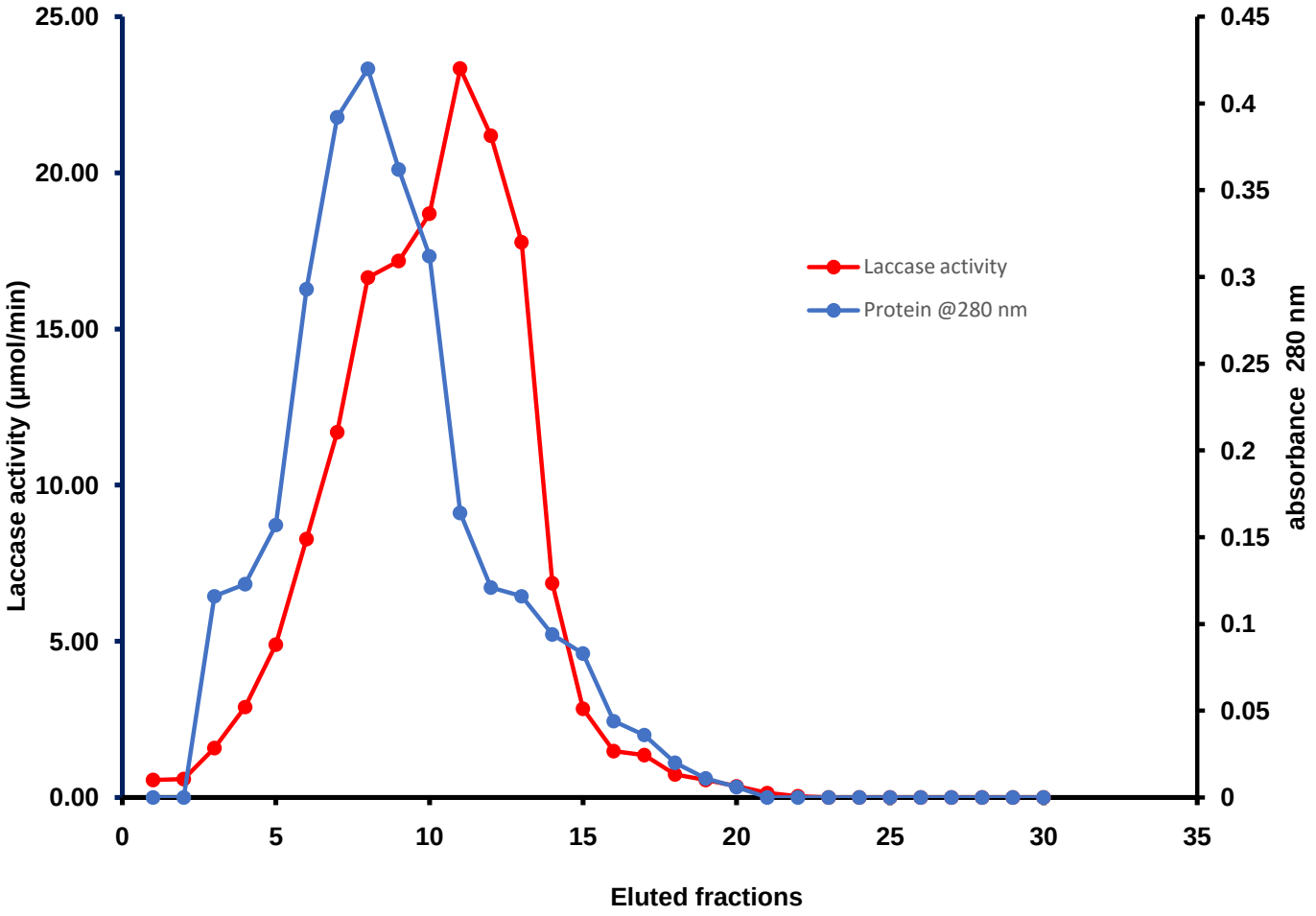


Figure 4.3: Elution profile of *Microcystisflos-aquae* laccase on DEAE Sephadex

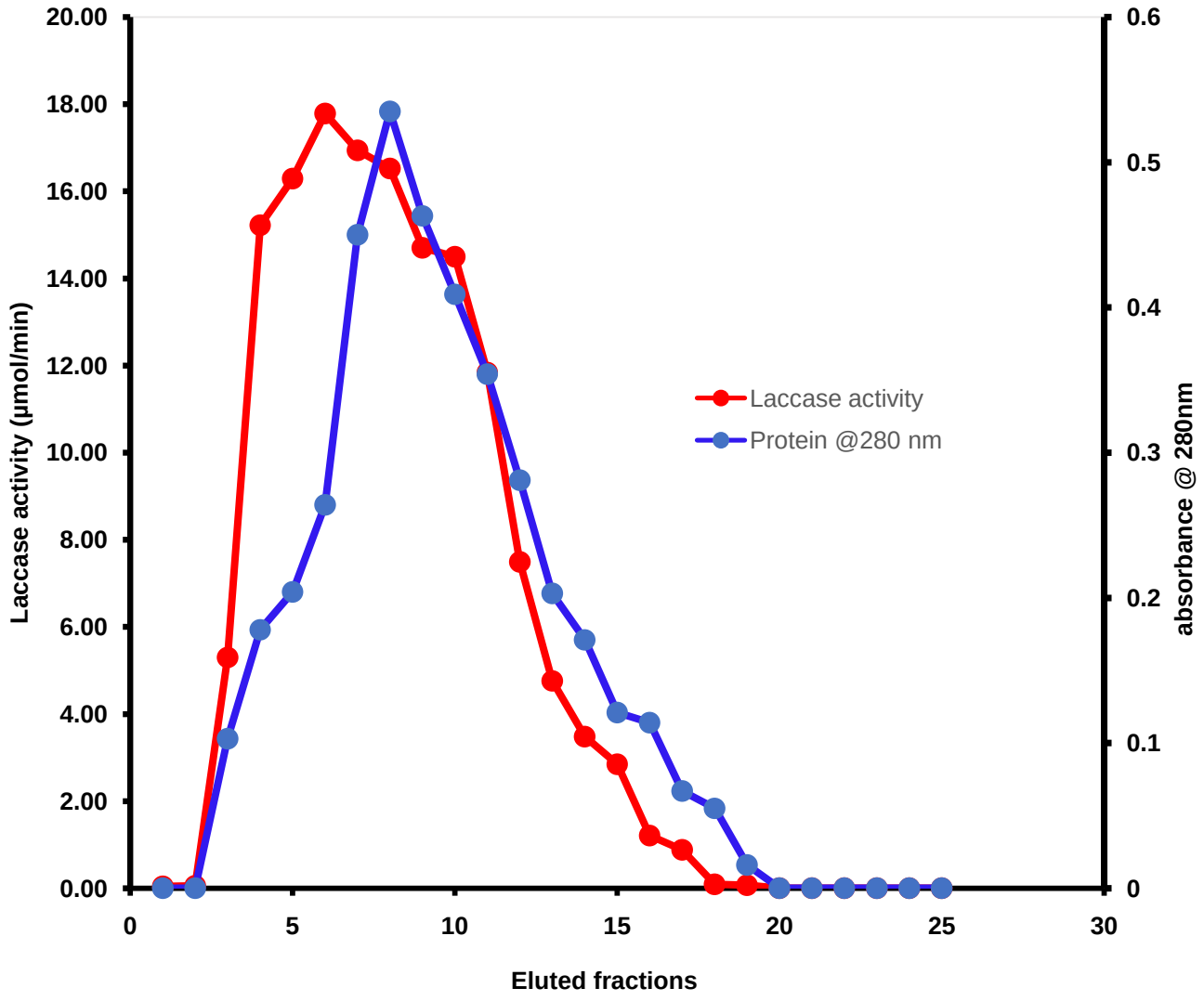


Figure 4.4: Elution profile of *Microcystis flos-aquae* laccase on Sephadex G-100

4.5 Purification Table of Laccase from *Microcystis flos-aquae*

The laccase isolated and purified from *Microcystis flos-aquae* was purified using a combination of ammonium sulphate precipitation, dialysis, anion exchange and size exclusion chromatographic methods (Table 4.1). with each purification steps, there is a decrease in total activity, this could be as a result of separation of our enzyme of interest from cluster of other proteins that might be present at each purification step. Furthermore, there is an increase in specific activity across the purification steps with approximately 0.16mmol/min/mg obtained at the end of the purification. The purity (purification fold) of the laccase increases also at each purification step from 1-fold until 10.21-fold purification was obtained, and a decreased in yield of 0.55% obtained (Table 4.1).

4.6 SDS-PAGE for Determination of Purity and Calculation of Molecular Weight of Laccase

In the present study, the laccase from *Microcystis flos aquae* was purified to near homogeneity and was estimated to be approximately 66 kDa in size on SDS-PAGE as indicated in figure 4.5. The purified laccase showed a single band on the SDS-PAGE when stained with Coomassie brilliant blue R-250, indicating the purity of the enzyme sample, thus suggesting the monomeric nature of the enzyme.

Table 4.1: Purification Table of Laccase from *Microcystis flos-aquae*

Purification steps	Volume (ml)	Protein concentration (mg/mL)	Laccase activity (µmol/min)	Total activity (µmol/min)	Total protein (g/mL)	Specific activity (µmol/mg·min)	Purification fold	Yield (%)
Crude enzyme	950	2.16	33.58	31901	2052	15.55	1	100
Ammonium sulphate precipitation	100	1.08	27.71	2771	108	25.66	1.65	8.69
Dialysis	50	0.73	24.99	1249.5	36.5	34.23	2.2	3.92
DEAE-Sephadex (IEC)	20	0.19	22.45	449	3.8	118.16	7.6	1.41
Sephadex G-100 (SEC)	10	0.11	17.46	174.6	1.1	158.73	10.21	0.55

Enzyme unit (U) is the micromole of substrate converted per minute (µmol/min) under the assay conditions.

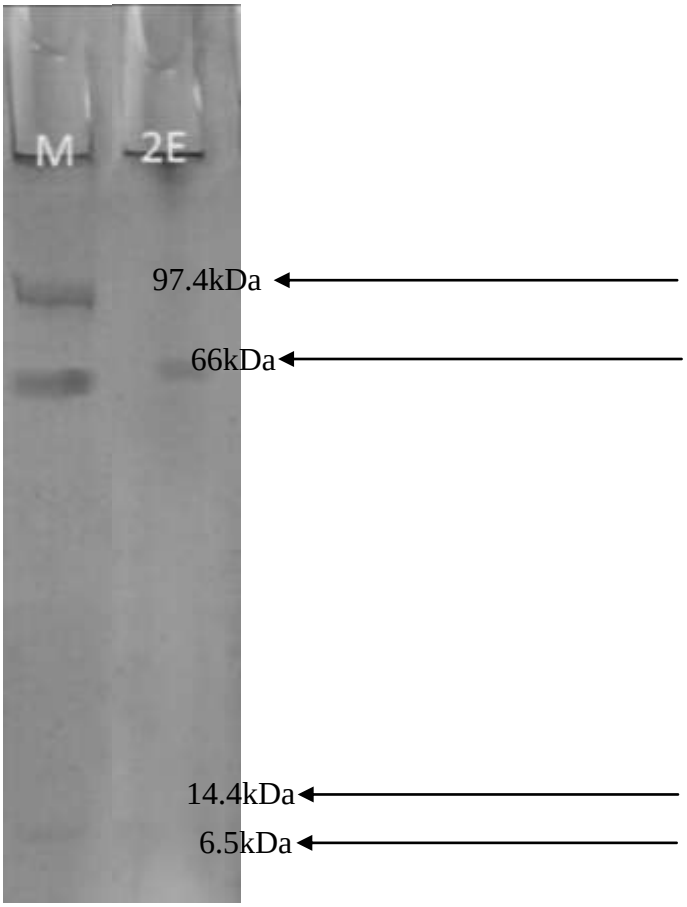


Figure 4.5: SDS-PAGE for depicting the purity and molecular weight of laccase from *Microcystis flos-aquae*

(Lane M: Protein molecular weight marker (kDa), Lane 2E: Partially purified laccase of *Microcystis flos aquae* eluted from Gel filtration chromatography)

4.6a Effect of pH on Activity and Stability of Purified Laccase

In order to characterize the purified laccase, the optimum pH of the enzyme was determined by measuring the relative activity at different pH. The relative activity of laccase at pH 2 and 3 was 67% and 71% respectively, which increases to 81% at pH 4. A reduction in the relative activity was observed from pH 5 to pH 11. (Figure 4.6a).

In the same instance, the stability of the enzyme was high at pH 2 and 3 with the enzyme being more stable at pH 4 with residual activity of 77%. There is significant decrease ($p = 0.01$) in stability from pH5 to pH 11 (Figure 4.6b).

4.6b Effect of Temperature on Activity and Stability of Purified Laccase

Similarly, the optimum temperature of the enzyme was determined by measuring the relative activity at different temperatures. The relative activity of laccase at 20^{o}C was 42%. Significant increase ($p=0.01$) in activity was obtained at temperatures of 30^{o}C and 40^{o}C with relative activity of 80% and 86% respectively(Figure 4.7a).75% of the residual activity was retained at temperature 30^{o}C to 40^{o}C respectively. In the same instance, there is drastic reduction in temperature stability of 40% to 16% from 60^{o}C to 80^{o}C (Figure 4.7b).

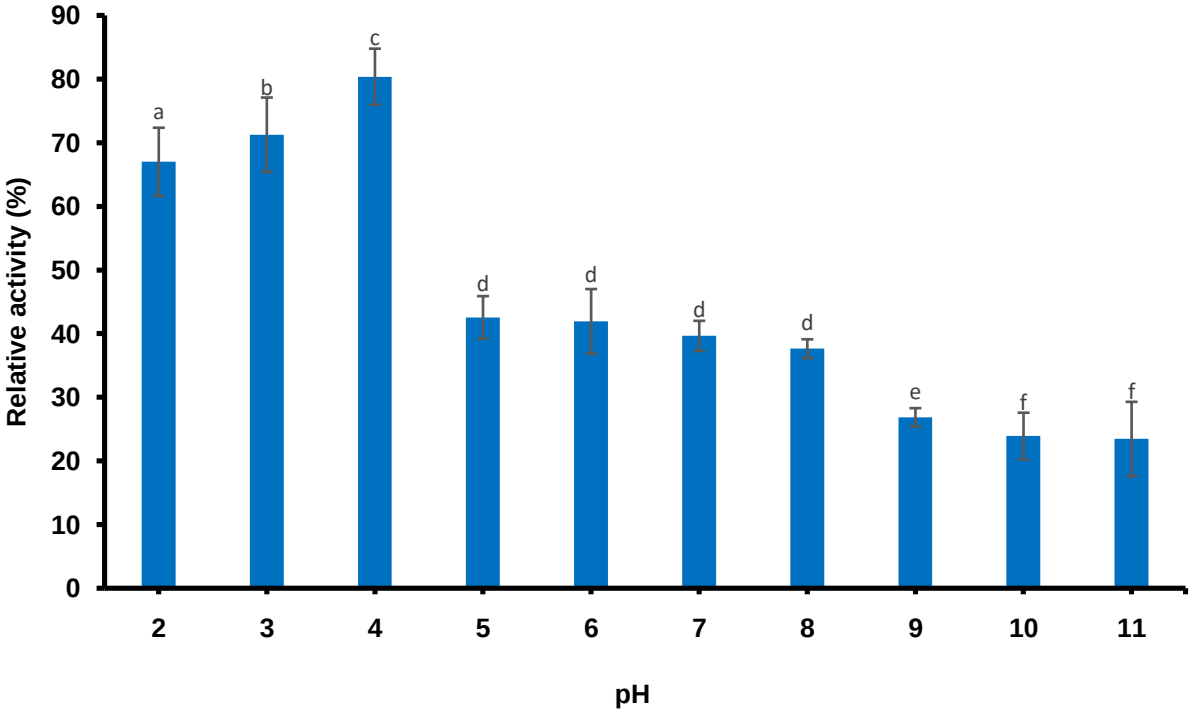


Figure 4.6a: Effect of pH on activity of purified laccase.

Data was analyzed using ONE-WAY ANOVA followed by Tukey's multiple post-hoc test. Bars with different superscripts (a-f) are considered statistically significantly (p=0.01) different across the pH. (n=3)

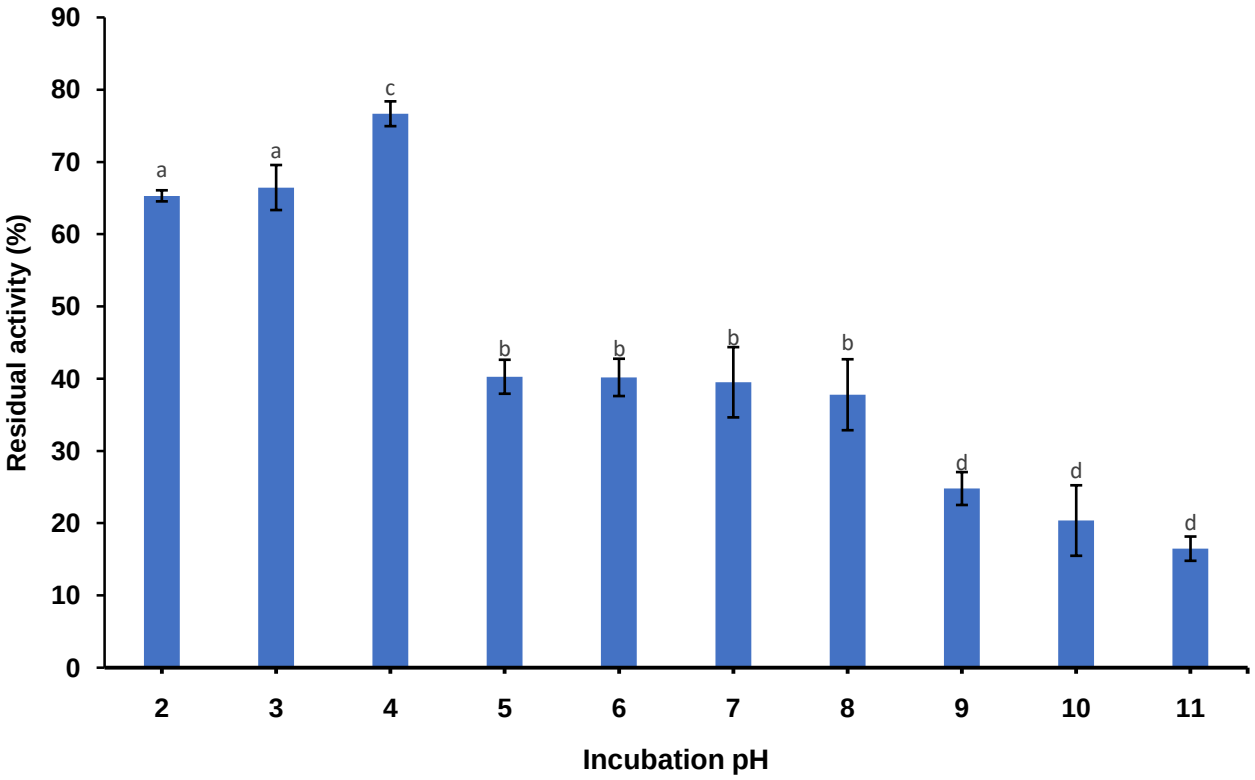


Figure 4.6b: Effect of pH on stability of purified laccase.

Data was analyzed using ONE-WAY ANOVA followed by Tukey's multiple post-hoc test. Bars with different superscripts (a-d) are considered statistically significantly (p=0.01) different across the pH. (n=3)

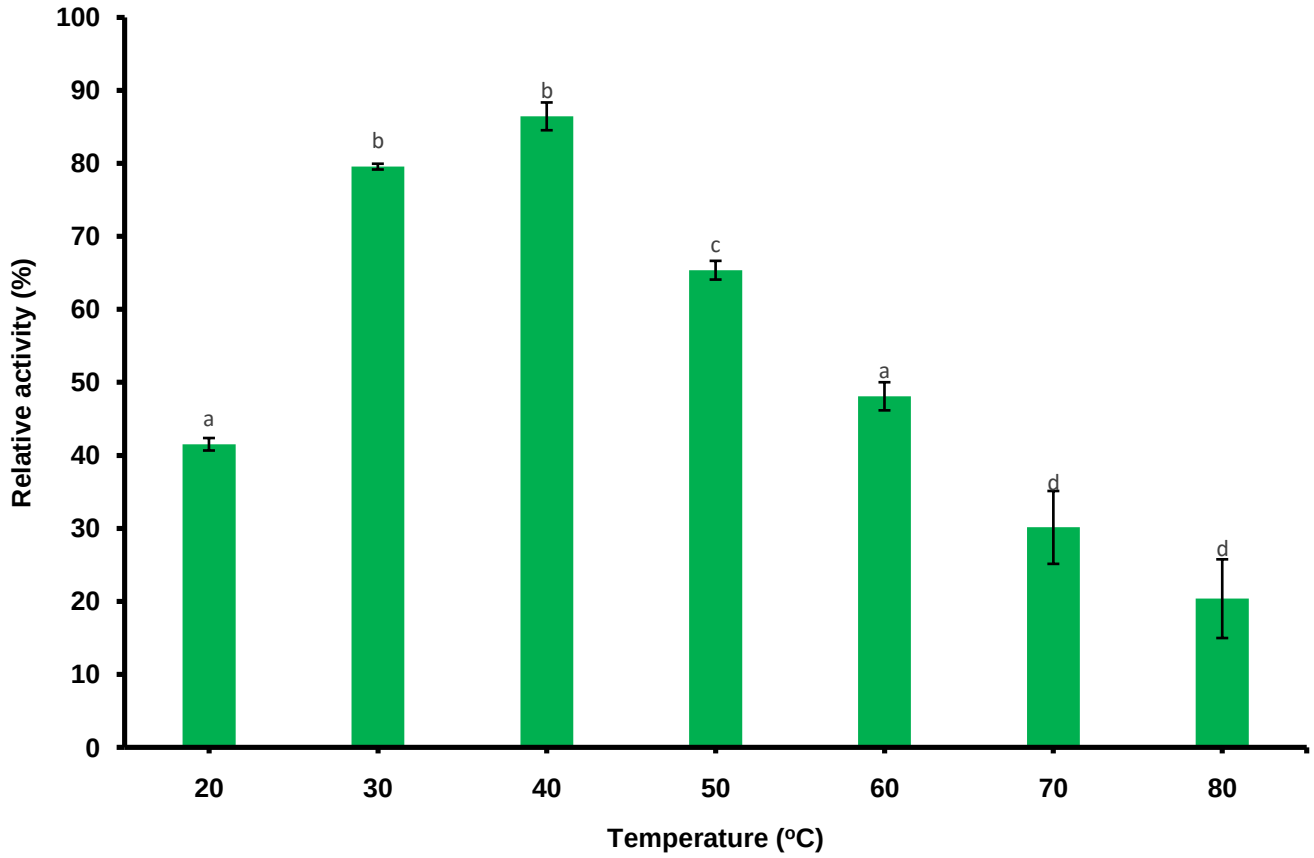


Figure 4.7a: Effect of temperature on activity of purified laccase.

Data was analyzed using ONE-WAY ANOVA followed by Tukey's multiple post-hoc test. Bars with different superscripts (a-d) are considered statistically significantly (p=0.01) different across the temperatures. (n=3)

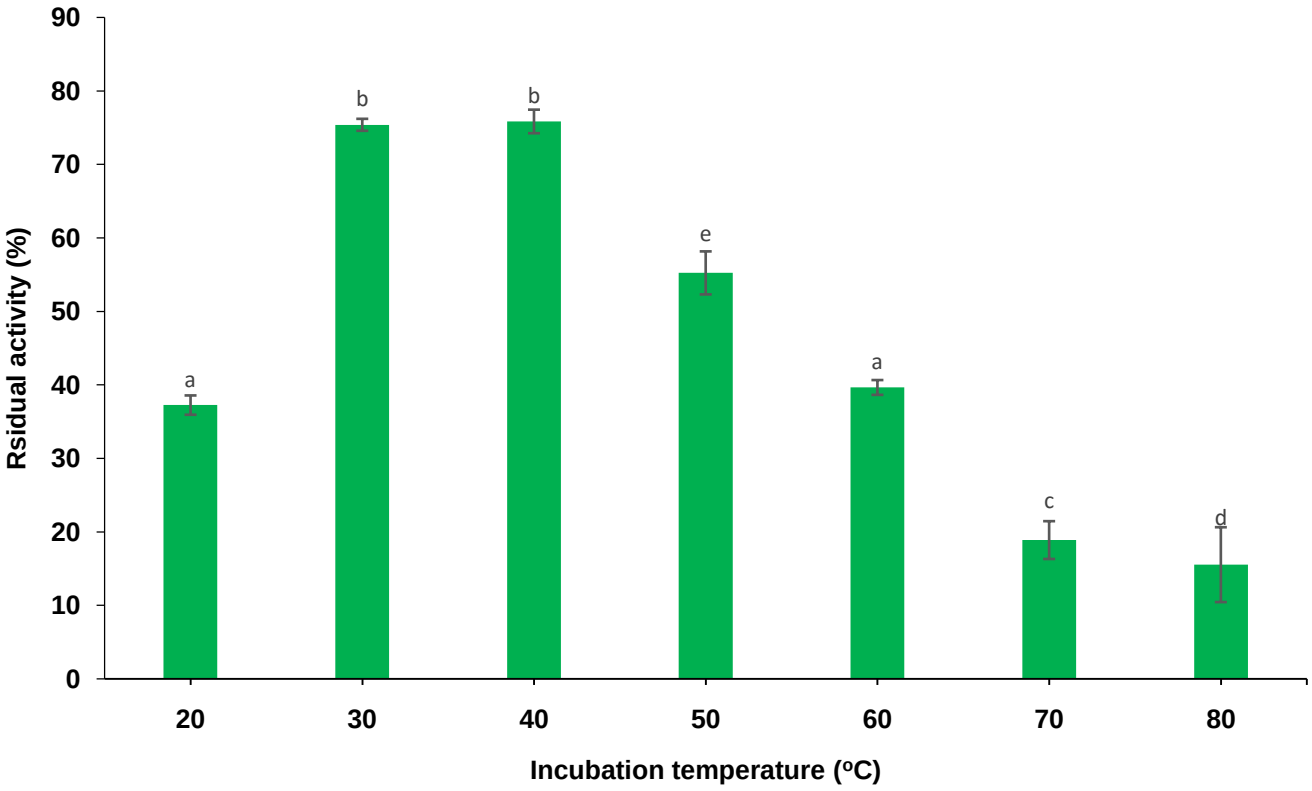


Figure 4.7b: Effect of temperature on stability of purified laccase.

Data was analyzed using ONE-WAY ANOVA followed by (Tukey's multiple post-hoc test). Bars with different superscripts (a-e) are considered statistically significantly (p=0.01) different across the temperatures. (n=3)

4.8 Effect of Metal ions and Inhibitor on Laccase Activity

The relative activity of the purified laccase was statistically increased (p=0.01-0.02) using 5mM of the metals Ca^{2+}, Mg^{2+}, and Cu^{2+} respectively. However, there is statistically decrease (p=0.01) in activity in the presence of Fe^{2+}or EDTA (Figure 4.8).

4.9Kinetics of Purified Laccase

The kinetic assessments using ABTS (2,2-azinobis-3-ethylbenzothiozoline-6-sulfonic acid) as substrate indicated that the laccase has both K_m and V_{max} values of 51.71 mM and 5.02 µmol/min respectively (Figure 4.9).

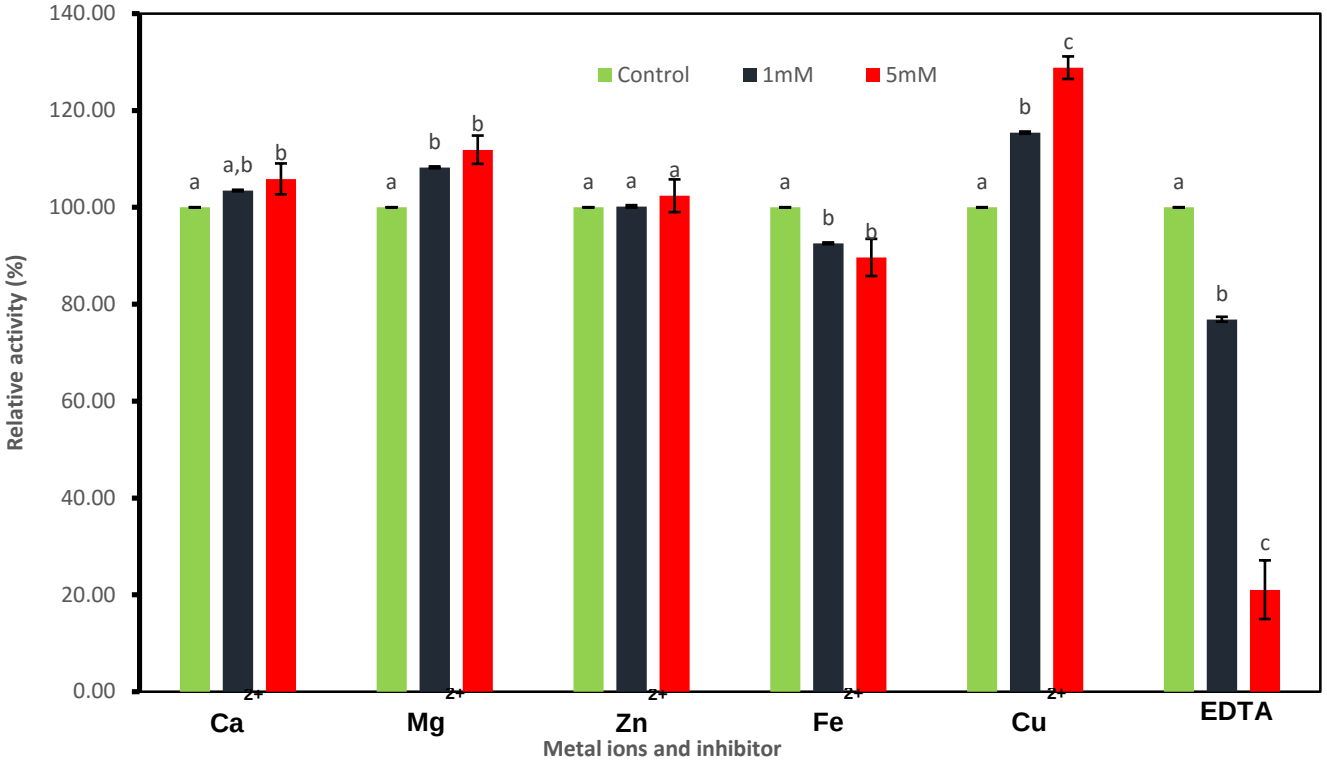


Figure 4.8: Effect of metal ions and inhibitor on laccase activity.

Data was analyzed using ONE-WAY ANOVA followed by Tukey's multiple post-hoc test. Bars with different superscripts (a-c) are considered statistically significantly (p=0.01-0.02) different across the metal ions and inhibitor. (n=3)

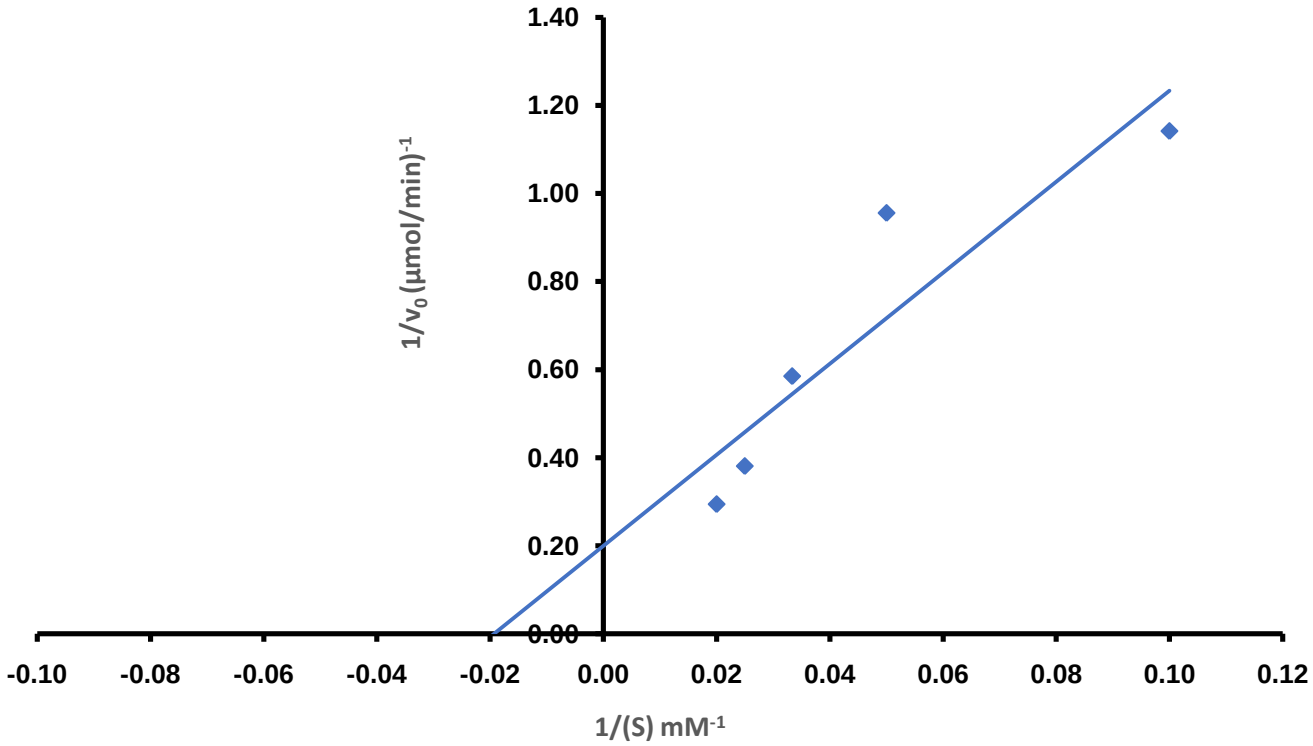


Figure 4.9: Lineweaver–Burk plot with ABTS as substrate

The kinetics of laccase enzyme (maximum velocity and Michaelis Menten's constant of 5.02 µmol·min^{-1} and 51.71 mM respectively)

4.10Effect of pH and Temperature on Laccase-catalyzed Decolorization

The effect of pH on laccase-mediated decolorization was determined by measuring the decolorization percent of Cibacron Brilliant Blue (CiBB) dye at various pHs (2-11). Sixty five percent (65%) decolorization was obtained at pH 2 with increase in decolorization at pH 3 (68%) and pH 4 (78%). Furthermore, increase in pH led to decrease in dye decolorization where 40% was recorded for both pH10 and pH11 (Figure 4.10a).

Similarly, the effect of temperature on laccase-mediated decolorization was determined by measuring the decolorization percent of CiBB at temperatures of 20^{o}C to 80^{o}C. At 20^{o}C, 41% of the dye was decolorized. There was significant ($p \leq 0.01$) increase in decolorization at 30^{o}C (69%) to 40^{o}C (73%), after which a decrease in decolorization was observed from 50^{o}C (62%) to 80^{o}C (11%) (Figure 4.10b).

4.11 Bioremediation (decolorization) Potential of Crude Laccase and Partially Purified Laccase on Decolorization of Cibacron Brilliant Blue (CiBB) Azo Dye

The bioremediation (decolorization) potential of the purified laccase was assayed at pH 4 and temperature of 40^{o}C using 0.039g/l of Cibacron Brilliant Blue (CiBB) solution. In the presence of ABTS as substrate, significant (p=0.01) increase in decolorization was evident throughout the time intervals. The purified laccase in the presence of ABTS was able to decolorize 50% of the dye compared to 13% by the crude laccase after 30 minutes. At the end of 180 minutes, it was observed that the purified laccase reduces Cibacron Brilliant Blue (CiBB) dye by 90% while only 39% decolorization was observed using the crude enzyme, this showed the high potential and efficiency of the purified laccase compare to its crude(Figure 4.11).

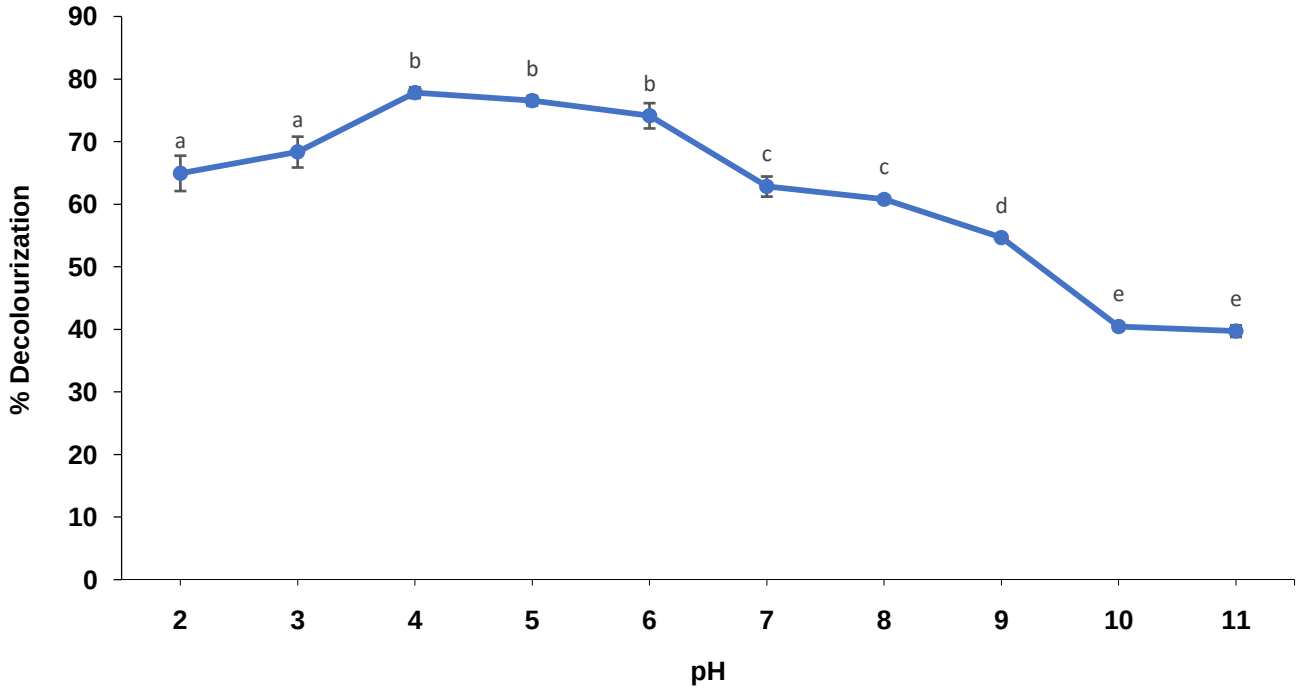


Fig. 4.10a: The effect of pH on laccase-catalyzed decolorization.

Data was analyzed using ONE-WAY ANOVA followed by Tukey's multiple post-hoc test. Bars with different superscripts (a-e) are considered statistically significant (p=0.01) across the pH. (n=3)

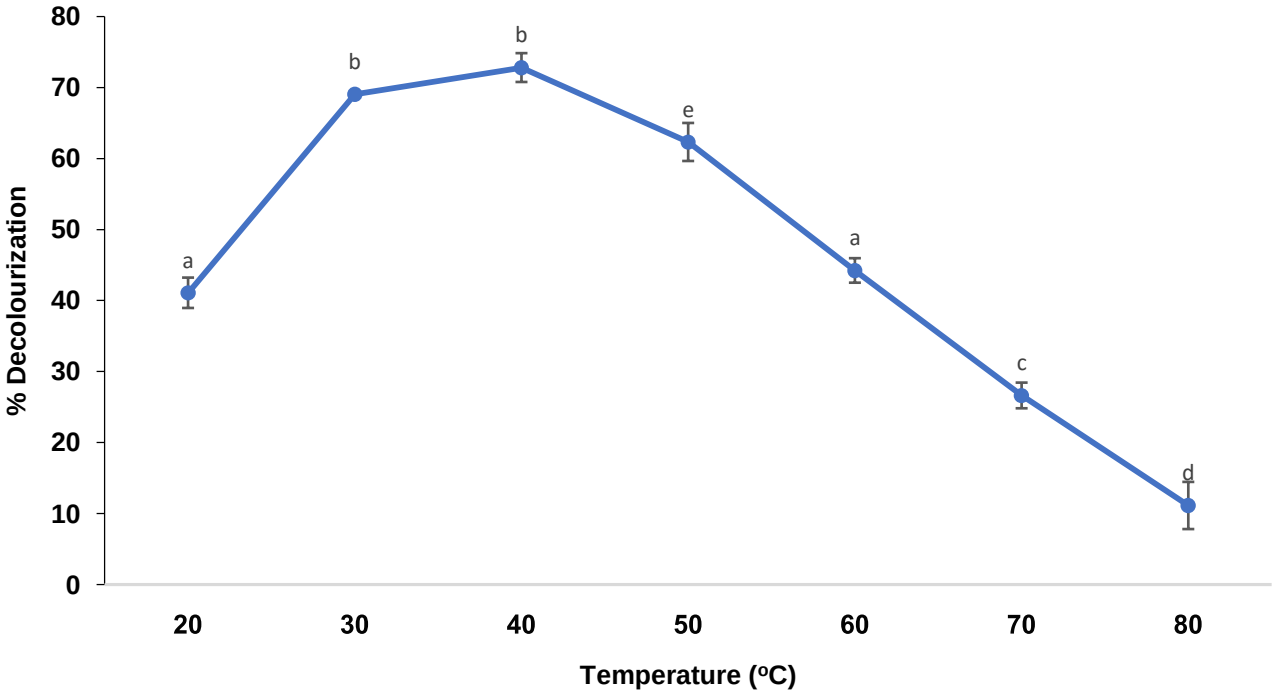


Figure 4.10b: The effect of temperature on laccase-catalyzed decolorization.

Data was analyzed using ONE-WAY ANOVA followed by Tukey's multiple post-hoc test. Bars with different superscripts (a-e) are considered significantly (p=0.01) different statistically across the temperatures. (n=3)

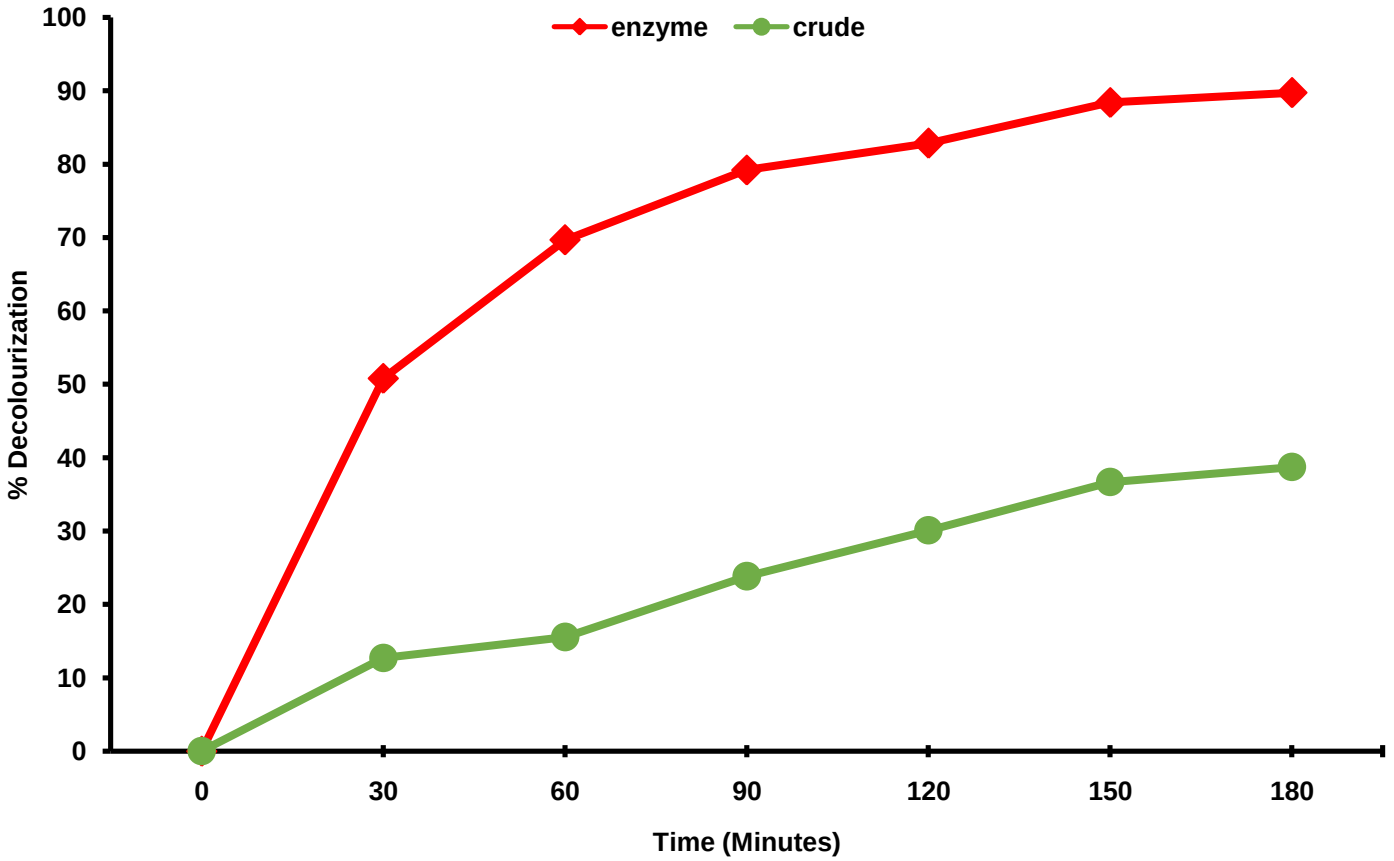


Figure 4.11: Time-dependent bioremediation (decolorization) potential of crude laccase and partially purified laccase on decolorization of Cibacron Brilliant Blue (CiBB) azo dye

CHAPTER FIVE

5.0 DISCUSSION

In this study, seven (7) microorganisms belonging to cyanobacteria (*Microcystis aurogenosa, Microcystis flos-aquae, Microcystis wasen bergii and Oscillatoria*) and green microalgae (*Chlorogonium, Chlorella sorokiniana, and Planktothrix*) were screened for production of laccase for a period of three (3) weeks. Although all the organisms produced laccase extracellularly, *Microcystis flos-aquae* producedthe highest throughout the period of experimentation. In all the organisms, there was decrease in laccase activity in the cyanobacteria after day fifteen (15) and after day eighteen (18) in the green microalgae, this could be as a result of depletion in medium contents/nutrients utilized by the organisms for their growth/and metabolism.

The extracellular laccase obtained from *Microcystis flos-aquae* was purified using ammonium sulphate precipitation, ion exchange chromatography on DEAE-Sephadex followed by gel filtration chromatography. *Microcystis flos-aquae* laccase was purified from the culture filtrate. The results showed a downhill slope of concentration and total protein with each of the passing purification step whereas an increase in specific activity and fold purification was observed, this is because our protein (enzyme) of interest is been purified from other cluster of proteins present at each purification step, thus an increase in specific activity.

The purification of laccase obtained in the present study is comparable with that of Chaurasia *et al.,* (2014) who reported a 10.42 purification fold with 12.57 % yield of laccase from *Trametes hirsutaMTCC-1171* using DEAE cellulose column chromatography. However, Yan *et al.* (2014) reported 1.37and 4.07-fold purifications with 5.78 and 11.64 % yields of laccase from *T. trogii*

S0301 using anionic exchange chromatography followed by Sephadex G-75 chromatography, respectively.

Previously, laccase purification was reported from fungi; *Pleurotus sajorcaju MTCC 141* with a 10.71-fold increment in activity and 3.46% yield; while in bacteria, 28.46-fold purification of laccase from *Bacillus tequilensis SN4* with 13.34% yield was reported (Sahay *et al.,* 2008, Sondhi *et al.,* 2014). However, in green algae *Tetracystis aeria*which was 120-fold purified had a low yield of 2.5% (Otto *et al.,* 2014). Mostly, laccase purification has been done from fungal sources till date which includes, *Trametes gibbosa, Trametes hirsuta, Trametes versicolor, Pleurotus ostreatus, and Phellinus igniarius* etc. (Baldrian P. (2006)) and only one laccase has been purified and characterized in a green alga (Otto *et al.,* 2014). Presence of laccase was reported in *Oscillatoria boryana* and *Phormidium valderianum* but there are no reports on the purification of laccase from cyanobacteria (Palanisami *et al.,* 2010). The most commonly used method for laccase purification is salt elution from an anion-exchange resin due to higher stability at neutral to alkaline pH (Patel *et al.,*2014). The extracel1ular cyanobacterial laccase from *Microcystis flos-aquae* exhibit strong binding with DEAE cellulose column and eluted fraction with peak activity at 0.2 M NaCI gradient elution. Laccases show variable binding and many studies have also reported the elution from 0.1 M to 0.3 M NaCl gradient (Binz *et al.,* 1996).

Most bacterial and fungal laccases have molecular weights of proteins ranging from 43 kDa to 110 kDa (Yaropolov *et al.,* 1994; Madhavi and Lele 2009). In this study, the approximate molecular mass of the purified laccase from *Microcystis flos-aquae* was found to be 66 kDa, which resembles the molecular mass of most fungal laccases (Yang *et al.,* 2013). The purified laccase showed single band on the SDS-PAGE, when stained with Coomassie blue R-250,

indicating the purity of the enzyme sample, thus suggesting the monomeric character of the enzyme.

The effect of pH and temperature on the activity of the purified laccase was also investigated. Laccases have different pH optima, which are dependent on the particular substrate used. (Robles *et al.,* 2000). The effect of pH on enzyme activity was analyzed by carrying out enzyme assays at different pH. The optimum pH of the purified laccase from *Microcystis flos-aquae* was 4.0 with ABTS as substrate.The enzyme was able to hold back its activity in a long range of pH, from 2.0 to 8.0 after 2 hours of incubation and found to be most stable at the optimum pH (pH 4.0). The activity of the enzyme was drastically reduced and inactivated at pH 10 and pH 11.0 which might be due to the binding of a hydroxide anion to the trinuclear coppers of laccase that interrupts the internal electron transfer from T1 to trinuclear centre and ionization of pI (Gholami-Borujeni *et al.,* 2011). It was reported that the oxidation rate of different substrate gradually decreased at higher pH which might be due to ionization of critical amino acids (Asp and Glu) (Gholami-Borujeni *et al.,* 2013). Similar results were obtained with laccase of *Cerrena unicolor* strain 137, *Peniophora sp, Carica papaya* and γ-*proteobacterium JB* (Paavola *et al.,* 2004; Michniewicz *et al.,* 2006; Sharma *et al.,* 2007; Nivedita *et al.,* 2015).

Temperature effect showed bell shaped graph. Maximum activity of purified laccase from *Microcystis flos-aquae* was recorded at 40˚C, beyond which the activity of the enzyme started decreasing. At 40˚C, 86% laccase activity was retained. Further increase in temperature resulted in drastic decrease in enzyme activity which might be as a result denaturation of the enzyme. Youn *et al.*(1995) reported that laccase from *Pleurotus ostreatus* showed an optimum temperature of 30˚C, whereas 50˚C was observed as optimal temperature in *Pleurotus florida* and *Pleurotus pulmonarius* (Youn *et al.,* 1995; De-Souza *et al.,* 2003). Thermal stability study

with the purified laccase was carried out at 20˚C to 80˚C for 180 minutes and it was found that the laccase was quite stable at 30˚C to 40˚C, retaining over 70 % of its residual activity after 180 minutes of incubation. While the enzyme was able to retained over 50% of its residual activity at 30˚C to 50˚C after 180 minutes of incubation, the enzymes lost most of its residual activity at 70˚C and 80˚C, with less than 20 % recorded at 70˚C and 80˚C. Increase in temperature resulted in decreased stability of enzyme. The thermal stability of enzymes may be influenced by the presence of hydrophobic or charged residues, which increase enzyme rigidity and restrict conformational changes during substrate binding (Somero *et al.*, 2004).

Attala *et al.,* (2015) reported the effect of metal ions on laccase activity which was found to be greatly dependent on its source as well as the type of metals used that strongly affects the catalytic activity of the enzyme. In the present study, effect of Cu^{2+}, Zn^{2+}, Ca^{2+}, Fe^{2+} and Mg^{2+} metals at 1mM and 5mM respectively was observed for the characterization of *Microcystis flos-aquae* laccase. Other scientists also reported the stimulatory effect of $Cu^{2+}$and Zn^{2+}on laccase activity in *Cladosporium cladosporioides* and *Streptomyces psammoticus* (Niladevi *et al.,* 2008). Similarly, Lorenzo *et al.* (2005) reported that addition of Cu^{2+} to the reaction mixture enhanced laccase activity at concentrations lower than 1.0 mM and that Mn^{2+} and Zn^{2+} did not affect the enzyme activity at any of the concentrations tested (0.5 mM to 80 mM). The filling of the Type I Copper binding site by Cu^{2+} could be responsible for this increased laccase activity (Shekher *et al.,* 2011; Sondhi *et al.,* 2014). EDTA was reported to be an inhibitor of metallo-enzymes due to its tendency of forming inactive complexes with inorganic prosthetic cofactors of the enzyme (Sadhasivam *et al.,* 2008). In the present study, laccase activity was significantly inhibited in the presence of 1 mM and 5mM EDTA with 23% and 79% inhibition respectively compared to controlwhich was similar to previously reported results (Sadhasivam *et al.,* 2008). Phenol

degrading laccase from the *Trichoderma atroviride* was also significantly inhibited in the presence of sodium azide, EDTA and L-cysteine (Chakroun *et al.,* 2010*).* The inhibitory effect of EDTA on laccase activity was also reported by many researchers (De-Souza *et al.,* 2003; Liu *et al.,*2010).

The kinetic parameters V_{max} and $K_m$were determined using ABTS as the substrate. Lineweaver–Burk plot confirmed that the K_m and V_{max} values of the purified *Microcystis flos-aquae* laccase were 51.71 mM and 5.02 $\mu mol \cdot min^{-1}$ respectively. Values obtained were similar to previously reported results. Vantamuri *et al.,* (2016) reported the K_m and V_{max} of purified laccase from *Marasmius sp.* BBKAV79 to be 3.03 mM and 5 $\mu mol \cdot min^{-1}$, respectively.

Maximum decolorization of the CiBB dye occurred at pH 4.0 which is the optimum pH of the enzyme. Alteration from the optimum pH of laccase negatively affected decolorization of all synthetic dyes. In the study of Ashrafi *et al.* (2013) and Mirzadeh *et al.* (2014) laccase mediated decolorization of all applied dyes was maximally achieved at acidic pH of 5. It was demonstrated that most of laccases of fungal origins maximally work at acidic pH and the enzyme activity at higher pH is decreased due to binding of hydroxide anion to the T2/T3 coppers of laccase and as a result interrupting with the internal electron transfer from T1 to T2/T3 centers (Baldrian, 2006). In the present study, the purified laccase from *Microcystis flos aquae* was able to withstand and retained most of its activity at acidic pH range, while activity with respect to decolorization was drastically reduced at a very high pH or alkaline pH. Decolorization percent of Cibacron Brilliant Blue (CiBB) dye was gradually increased by enhancing the reaction temperature from 20°C–40°C and maximum decolorization (73%) was observed at the temperature of 40 °C. The amount of decolorization of the studied dye was dropped drastically (11 % decolorization) by

elevating temperature to 80 °C which was in agreement with the findings of Ashrafi *et al.* (2013) who observed that maximum dye decolorization occurred between the temperatures of 40–60°C.

The effect of crude and purified laccase from *Microcystis flos-aquae* on decolorization of Cibacron Brilliant Blue (CiBB)was investigated in the presence of ABTS. Samples were taken at specific intervals from the reaction medium during 180 minutes of reaction and spectrophotometric measurements were performed at wavelength where the dye exhibit a maximum absorbency. The purified laccase in the presence of ABTS was able to decolorize 50% of the dye compared to 13% by the crude laccase after 30 minutes. At the end of 180 minutes, it was observed that the purified laccase reduces Cibacron Brilliant Blue (CiBB) dye by 90% while only 39% decolorization was observed using the crude enzyme. Like many fungal laccases (Soares *et al.,* 2001; Zille *et al.,* 2004; Camarero *et al.,* 2005; Pereira *et al.,* 2009) the *Trametes aeria* laccase is unable to effectively decolorize the highly recalcitrant dyes RBBR (Reactive Brilliant Blue R) and RB5 (Reactive Black 5) in the absence of redox mediators, but substantial decolorization of these dyes is achieved in the presence of ABTS. The present study suggest that the pure laccase mostly decolorizes the Cibacron Brilliant Blue (CiBB) dye compared to the crude enzyme and that the dye decolorization potential of the purified laccase was much higher in terms of extent as well as time. Many scientists have reported that the methyl or methoxy groups bound on the phenolic groups in the azo dye structure donate electrons and hence increase the activity of laccase enzyme, whereas the nitro groups in the structure attract electrons and hence inhibit the oxidation reaction catalyzed by the laccase enzyme (Chivukula *et al.,* 1995; Xu F. 1996).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The study demonstrated that a 66 kDa extracellular-copper-dependent laccase from *Microcystis flos-aquae*was purified and characterized. Seven (7) organisms belonging to the micro-algae and cyanobacteria were screened for production of laccase, with *Microcystis flos-aquae* been the best producer with optimum activity on day 15. The partially purified laccase from *Microcystis flos-aquae* has an enzyme yield of 0.55% at fold-purification of 10.21 and specific activity of approximately o.16 mmol/min/mg at which the enzyme was purified using a combination of ammonium sulphate precipitation, dialysis, anion exchange and size exclusion chromatographic methods. At optimum pH and temperature of 4.0 and 40^{o}C, respectively, the enzyme has a Km of 51.71 mM and Vmax of 5.02 µMmin^{-1} with high potential for azo dye oxidation. Furthermore, the Cibacron Brilliant Blue which is an azo dye was rapidly decolorized up to 90% within 3 hours, implying the bioremediation (decolorization) potential of the partially purified laccase on synthetic (azo) dyes.

6.2 Recommendation

i. Cloning and overexpression of the *Microcystis flos-aquae*in *E. coli* may result in higher yield and cost-effective production.

ii. Toxicity studies should also be performed to determine the safety level of these decolorized compounds.

REFERENCES

Abadulla, E., Tzanov, T., Costa, S., Robra, K. H., Cavaco-Paulo, A., and Gübitz, G. M. (2000). Decolorization and detoxification of textile dyes with a laccase from Trametes hirsuta. *Applied and environmental microbiology*, *66*(8), 3357-3362.

Ackacha, M. A., Połeć- Pawlak, K., and Jarosz, M. (2003). Identification of anthraquinone coloring matters in natural red dyestuffs by high performance liquid chromatography with ultraviolet and electrospray mass spectrometric detection. *Journal of separation science*, *26*(11), 1028-1034.

Acuner, E., and Dilek, F. B. (2004). Treatment of tectilon yellow 2G by Chlorella vulgaris. *Process Biochemistry*, *39*(5), 623-631.

Ahn, M. Y., Dec, J., Kim, J. E., and Bollag, J. M. (2002). Treatment of 2, 4- dichlorophenol polluted soil with free and immobilized laccase. *Journal of Environmental Quality*, *31*(5), 1509-1515.

Alcalde, M., Bulter, T., and Arnold, F. H. (2002). Colorimetric assays for biodegradation of polycyclic aromatic hydrocarbons by fungal laccases. *Journal of biomolecular screening*, *7*(6), 547-553.

Antipov, A. A., Sukhorukov, G. B., Leporatti, S., Radtchenko, I. L., Donath, E., and Möhwald, H. (2002). Polyelectrolyte multilayer capsule permeability control. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *198*, 535-541.

Asadgol, Z., Forootanfar, H., Rezaei, S., Mahvi, A. H., and Faramarzi, M. A. (2014). Removal of phenol and bisphenol-A catalyzed by laccase in aqueous solution. *Journal of Environmental Health science and engineering*, *12*(1), 1-5.

Ashrafi, S. D., Rezaei, S., Forootanfar, H., Mahvi, A. H., and Faramarzi, M. A. (2013). The enzymatic decolorization and detoxification of synthetic dyes by the laccase from a soil-isolated ascomycete, Paraconiothyrium variabile. *International Biodeterioration & Biodegradation*, *85*, 173-181.

Assavanig, A., Amornkitticharoen, B., Ekpaisal, N., Meevootisom, V., and Flegel, T.W. (1992) "Isolation, characterization and function of laccase from *Trichoderma*." *Applied Microbiology Biotechnology 38*, 198-200.

Attala, M.M., Kheiralla, Z.H., Hamed, E.R., Youssry. A.A., and Aty, A.A. (2015) Characterization and purification of the crude *Trematosphaeria mangrovei laccase enzyme. Egypt Pharmaceutical Journal.*, 11, 93–98.

Baldrian, P. (2006). Fungal laccases–occurrence and properties. *FEMS microbiology reviews*, *30*(2), 215-242.

Benfield, G., Bocks, S.M., Bromley, K., and Brown, B.R. (1964). Studies in fungal and plant laccases, *Phytochemistry3*, 79-88.

Bermek, H., Li, K., and Eriksson, K. E. L. (2002). Studies on mediators of manganese peroxidase for bleaching of wood pulps. *Bioresource technology*, *85*(3), 249-252.

Binz, T., and Canevascini, G. (1996). Differential production of extracellular laccase in the Dutch elm disease pathogens Ophiostoma ulmi and O. novo-ulmi. *Mycological Research*, *100*(9), 1060-1064.

Blánquez, P., Casas, N., Font, X., Gabarrell, X., Sarrà, M., Caminal, G., and Vicent, T. (2004). Mechanism of textile metal dye biotransformation by Trametes versicolor. *Water research*, *38*(8), 2166-2172.

Bressler, D. C., Fedorak, P. M., and Pickard, M. A. (2000). Oxidation of carbazole, N-ethylcarbazole, fluorene, and dibenzothiophene by the laccase of Coriolopsis gallica. *Biotechnology letters*, *22*(14), 1119-1125.

Brijwani, K., Rigdon, A., and Vadlani, P. V. (2010). Fungal laccases: production, function, and applications in food processing. *Enzyme Research*, *2010*.

Budolla, S., Noreddine, K. C., and Jacqueline, D. (2004). Production of laccase without inducer by Chaetomium species isolated from Chettaba forest situated in the East of Algeria. *African Journal of Biotechnology*, *15*(7), 207-213.

Camarero, S., Garcıa, O., Vidal, T., Colom, J., del Rıo, J. C., Gutiérrez, A., and Martınez, Á. T. (2004). Efficient bleaching of non-wood high-quality paper pulp using laccase-mediator system. *Enzyme and Microbial Technology*, *35*(2-3), 113-120.

Cañas, A. I., and Camarero, S. (2010). Laccases and their natural mediators: biotechnological tools for sustainable eco-friendly processes. *Biotechnology advances*, *28*(6), 694-705.

Caruso, F., Trau, D., Möhwald, H., and Renneberg, R. (2000). Enzyme encapsulation in layer-by-layer engineered polymer multilayer capsules. *Langmuir*, *16*(4), 1485-1488.

Chakroun, H., Mechichi, T., Martinez, M. J., Dhouib, A., and Sayadi, S. (2010). Purification and characterization of a novel laccase from the ascomycete Trichoderma atroviride: application on bioremediation of phenolic compounds. *Process Biochemistry*, *45*(4), 507-513.

Chandra, R. P., and Ragauskas, A. J. (2002). Evaluating laccase-facilitated coupling of phenolic acids to high-yield kraft pulps. *Enzyme and microbial technology*, *30*(7), 855-861.

Chaurasia, P. K., Yadav, R. S., and Yadava, S. (2014). Purification and characterization of yellow laccase from Trametes hirsuta MTCC-1171 and its application in synthesis of aromatic aldehydes. *Process Biochemistry*, *49*(10), 1647-1655.

Chen, T., Barton, S. C., Binyamin, G., Gao, Z., Zhang, Y., Kim, H. H., and Heller, A. (2001). A miniature biofuel cell. *Journal of the American Chemical Society*, *123*(35), 8630-8631.

Chiaiese, P., Palomba, F., Tatino, F., Lanzillo, C., Pinto, G., Pollio, A., & Filippone, E. (2011). Engineered tobacco and microalgae secreting the fungal laccase POXA1b reduce phenol content in olive oil mill wastewater. *Enzyme and Microbial Technology*, *49*(6-7), 540-546.

Chivukula, M., and Renganathan, V. (1995). Phenolic azo dye oxidation by laccase from Pyricularia oryzae. *Applied and Environmental Microbiology*, *61*(12), 4374-4377.

Claus, H. (2004). Laccases: structure, reactions, distribution. *Micron*, *35*(1-2), 93-96.

Couto, S. R. (2007). Decolouration of industrial azo dyes by crude laccase from Trametes hirsuta. *Journal of hazardous materials*, *148*(3), 768-770.

D'Annibale, A., Stazi, S. R., Vinciguerra, V., and Sermanni, G. G. (2000). Oxirane-immobilized Lentinula edodes laccase: stability and phenolics removal efficiency in olive mill wastewater. *Journal of biotechnology*, *77*(2-3), 265-273.

De Roos, A. J., Ray, R. M., Gao, D. L., Wernli, K. J., Fitzgibbons, E. D., Ziding, F., and Checkoway, H. (2005). Colorectal cancer incidence among female textile workers in Shanghai, China: a case-cohort analysis of occupational exposures. *Cancer Causes & Control*, *16*(10), 1177-1188.

De Souza C.G.M., Peralta R.M. (2003) J. Basic Microbiol., 43, 278-286.

Decher, G. (1997). Fuzzy nanoassemblies: toward layered polymeric multicomposites. *science*, *277*(5330), 1232-1237.

Della Greca, M., Pinto, G., Pistillo, P., Pollio, A., Previtera, L., and Temussi, F. (2008). Biotransformation of ethinylestradiol by microalgae. *Chemosphere*, *70*(11), 2047-2053.

Diamantidis, D., Effosse, A., Potier, P., and Bally, R. (2000). "Purification and characterization of the first bacterial laccase in rhizospheric bacteria," *Azospirillum lipoferum, Soil Biology and Biochemistry32*, 919-927.

Dogan, E. E., Yesilada, E., Ozata, L., and Yologlu, S. (2005). Genotoxicity testing of four textile dyes in two crosses of Drosophila using wing somatic mutation and recombination test. *Drug and chemical toxicology*, *28*(3), 289-301.

Dong, J. L., Zhang, Y. W., Zhang, R. H., Huang, W. Z., and Zhang, Y. Z. (2005). Influence of culture conditions on laccase production and isozyme patterns in the white- rot fungus Trametes gallica. *Journal of Basic Microbiology: An International Journal on Biochemistry, Physiology, Genetics, Morphology, and Ecology of Microorganisms*, *45*(3), 190-198.

Durán, N., and Esposito, E. (2000). Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. *Applied catalysis B: environmental*, *28*(2), 83-99.

Eggert, C., Temp, U., and Eriksson, K. E. (1996). The ligninolytic system of the white rot fungus Pycnoporus cinnabarinus: purification and characterization of the laccase. *Applied and environmental microbiology*, *62*(4), 1151-1158.

Elisashvili, V., and Kachlishvili, E. (2009). Physiological regulation of laccase and manganese peroxidase production by white-rot Basidiomycetes. *Journal of Biotechnology*, *144*(1), 37-42.

Elisashvili, V., Kachlishvili, E., and Penninckx, M. (2008). Effect of growth substrate, method of fermentation, and nitrogen source on lignocellulose-degrading enzymes production by white-rot basidiomycetes. *Journal of industrial Microbiology and Biotechnology*, *35*(11), 1531-1538.

Elisashvili, V., Penninckx, M., Kachlishvili, E., Tsiklauri, N., Metreveli, E., Kharziani, T., and Kvesitadze, G. (2008). Lentinus edodes and Pleurotus species lignocellulolytic enzymes activity in submerged and solid-state fermentation of lignocellulosic wastes of different composition. *Bioresource technology*, *99*(3), 457-462.

Ellis, J. T., Hengge, N. N., Sims, R. C., and Miller, C. D. (2012). Acetone, butanol, and ethanol production from wastewater algae. *Bioresource technology*, *111*, 491-495.

Enayatizamir, N., Tabandeh, F., Rodríguez-Couto, S., Yakhchali, B., Alikhani, H. A., & Mohammadi, L. (2011). Biodegradation pathway and detoxification of the diazo dye Reactive Black 5 by Phanerochaete chrysosporium. *Bioresource Technology*, *102*(22), 10359-10362.

Faraco, V., Giardina, P., Palmieri, G., and Sannia, G. (2002). Metal-activate Laccase Promoters. *Elsevier.*

Ferry, Y., and Leech, D. (2005). Amperometric detection of catecholamine neurotransmitters using electrocatalytic substrate recycling at a laccase electrode. *Electroanalysis: An International Journal Devoted to Fundamental and Practical Aspects of Electroanalysis*, *17*(2), 113-119.

Fischlechner, M., Zschörnig, O., Hofmann, J., and Donath, E. (2005). Engineering virus functionalities on colloidal polyelectrolyte lipid composites. *Angewandte Chemie International Edition*, *44*(19), 2892-2895.

Flathman, P. E., and Lanza, G. R. (1998). Phytoremediation: current views on an emerging green technology. *Journal of soil contamination*, *7*(4), 415-432.

Forootanfar, H., Moezzi, A., Aghaie-Khozani, M., Mahmoudjanlou, Y., Ameri, A., Niknejad, F., and Faramarzi, M. A. (2012). Synthetic dye decolorization by three sources of fungal laccase. *Iranian journal of environmental health science & engineering*, *9*(1), 1-10.

Freire, R. S., Durán, N., and Kubota, L. T. (2001). Effects of fungal laccase immobilization procedures for the development of a biosensor for phenol compounds. *Talanta*, *54*(4), 681-686.

G. McKay, "Waste color removal from textile effluents," *American Dyestuff Reporter*, vol. 68, no. 4, pp. 29–34, 1979.

Galhaup, C., and Haltrich, D. (2001). Enhanced formation of laccase activity by the white-rot fungus Trametes pubescens in the presence of copper. *Applied Microbiology and Biotechnology*, *56*(1), 225-232.

Ghasemi, Y., Rasoul- Amini, S., and Fotooh- Abadi, E. (2011). The biotransformation, biodegradation, and bioremediation of organic compounds by microalgae 1. *Journal of phycology*, *47*(5), 969-980.

Gholami-Borujeni, F., Faramarzi, M. A., Nejatzadeh-Barandozi, F., and Mahvi, A. H. (2013). Oxidative degradation and detoxification of textile azo dye by horseradish peroxidase enzyme. *Fresen Environ Bull*, *22*, 739-744.

Gholami-Borujeni, F., Mahvi, A. H., Nasseri, S., Faramarzi, M. A., Nabizadeh, R., and Alimohammadi, M. (2011). Enzymatic treatment and detoxification of acid orange 7 from textile wastewater. *Applied biochemistry and biotechnology*, *165*(5), 1274-1284.

Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S., and Sannia, G. (2010). Laccases: a never-ending story. *Cellular and Molecular Life Sciences*, *67*(3), 369-385.

Golz-Berner, K., Walzel, B., Zastrow, L., and Doucet, O. (2004). Cosmetic and dermatological preparation containing copper binding proteins for skin lightening. Int Pat Appl WO2004017931.

Gomi, N., Yoshida, S., Matsumoto, K., Okudomi, M., Konno, H., Hisabori, T., and Sugano, Y. (2011). Degradation of the synthetic dye amaranth by the fungus Bjerkandera adusta Dec 1: inference of the degradation pathway from an analysis of decolorized products. *Biodegradation*, *22*(6), 1239-1245.

Grassi, E., Scodeller, P., Filiel, N., Carballo, R., and Levin, L. (2011). Potential of Trametes trogii culture fluids and its purified laccase for the decolorization of different types of recalcitrant dyes without the addition of redox mediators. *International Biodeterioration & Biodegradation*, *65*(4), 635-643.

Greca, V.G., Almendáriz, J., and. Solís-Oba, M., (2008). Biotechnological treatment for colorless denim and textile wastewater treatment with laccase and ABTS. *Revista internacional de contaminación ambiental*, *24*(1), 5-11.

Haghighi, B., Gorton, L., Ruzgas, T., and Jönsson, L. J. (2003). Characterization of graphite electrodes modified with laccase from Trametes versicolor and their use for bioelectrochemical monitoring of phenolic compounds in flow injection analysis. *Analytica Chimica Acta*, *487*(1), 3-14.

Heemken, O. P., Reincke, H., Stachel, B., and Theobald, N. (2001) "The occurrence of xenoestrogens in the Elbe river and the North Sea," *Chemosphere*, *45(3)*, 245–259.

Hessel. N.T. J., Mikolasch, A., Lalk, M., Witt, S., Seefeldt, S., Hammer, E., and Lindequist, U. (2007). Novel cephalosporins synthesized by amination of 2, 5-dihydroxybenzoic acid derivatives using fungal laccases II. *Chemical and pharmaceutical bulletin*, *55*(3), 412-416.

Hou, H., Zhou, J., Wang, J., Du, C., & Yan, B. (2004). Enhancement of laccase production by Pleurotus ostreatus and its use for the decolorization of anthraquinone dye. *Process Biochemistry*, *39*(11), 1415-1419.

Huang, Y.C., Chen, Y.F., Chen, C.Y., Chen, W.L., Ciou, Y.P., and Liu, W.H. (2011). Production of ferulic acid from lignocellulolytic agricultural biomass by Thermobifida fusca thermostable esterase produced in *Yarrowia lipolytica* transformant. *Bioresour Technol., 3,* 8117–8122.

Hüttermann, A., Mai, C., and Kharazipour, A. (2001). Modification of lignin for the production of new compounded materials. *Applied microbiology and biotechnology*, *55*(4), 387-394.

Indrani jadhvav, Roshan Vaniwal, Divya Shrivastaava and Kapilesh Jadhav,. Review article: microorganism-Based Treatment of Azo Dyes, *Journal of Environmental Science and Technology*; 9(2):188-197, 2016.

Indrani. J., Vasniwal, R., Shrivastava, D., and Jadhav, K. (2016). Microorganism-based treatment of azo dyes. *Journal of Environmental Science and Technology*, *9*(2), 188.

Jarosz-Wilkołazka, A., Ruzgas, T., and Gorton, L. (2004). Use of laccase-modified electrode for amperometric detection of plant flavonoids. *Enzyme and Microbial Technology*, *35*(2-3), 238-241.

Jeon, J. R., Baldrian, P., Murugesan, K., and Chang, Y. S. (2012). Laccase- catalysed oxidations of naturally occurring phenols: from in vivo biosynthetic pathways to green synthetic applications. *Microbial biotechnology*, *5*(3), 318-332.

Jin, X. C., Liu, G. Q., Xu, Z. H., and Tao, W. Y. (2007). Decolorization of a dye industry effluent by Aspergillus fumigatus XC6. *Applied microbiology and biotechnology*, *74*(1), 239-243.

Junghanns, C., Pecyna, M. J., Böhm, D., Jehmlich, N., Martin, C., Von Bergen, M., and Schlosser, D. (2009). Biochemical and molecular genetic characterisation of a novel laccase produced by the aquatic ascomycete Phoma sp. UHH 5-1-03. *Applied microbiology and biotechnology*, *84*(6), 1095-1105.

Kant, K.S. (2012). Laccase from an alkalitolerant basidiomycetes Crinipellis sp. RCK- 1: Production optimization by response surface methodology. *Journal of basic microbiology*, *52*(4), 397-407.

Kaoutar. C. B., Sánchez, E., and Baghour, M. (2014). The role of algae in bioremediation of organic pollutants. *J. Iss. ISSN*, *2360*, 8803.

Khlifi, R., Belbahri, L., Woodward, S., Ellouz, M., Dhouib, A., Sayadi, S., and Mechichi, T. (2010). Decolourization and detoxification of textile industry wastewater by the laccase-mediator system. *Journal of Hazardous Materials*, *175*(1-3), 802-808.

Kılıç, N. K., Karatay, S. E., Duygu, E., & Dönmez, G. (2011). Potential of Gonium spp. in synthetic reactive dye removal, possible role of laccases and stimulation by triacontanol hormone. *Water, Air, & Soil Pollution*, *222*(1), 297-303.

Kim H, Lee S, Ryu S, and Choi H.T. (2012). Decolorization of remazol brilliant blue R by a purified laccase of polyporus brumalis. Appl. Biochem. Biotechnol. 166(1): 159-164.

Kollmann, A., Boyer, F. D., Ducrot, P. H., Kerhoas, L., Jolivalt, C., Touton, I., and Mougin, C. (2005). Oligomeric compounds formed from 2, 5-xylidine (2, 5-dimethylaniline) are potent enhancers of laccase production in Trametes versicolor ATCC 32745. *Applied microbiology and biotechnology*, *68*(2), 251-258.

Komancová, M., Jurčová, I., Kochánková, L., and Burkhard, J. (2003). Metabolic pathways of polychlorinated biphenyls degradation by Pseudomonas sp. 2. *Chemosphere*, *50*(4), 537-543.

Kruus, K., Niku-Paavola, M. L., and Viikari, L. (2001). Laccase—a useful enzyme for modification of biopolymers. In *Biorelated polymers* (pp. 255-261). Springer, Boston, MA.

Kunamneni, A., Ballesteros, A., Plou, F. J., and Alcalde, M. (2007). Fungal laccase—a versatile enzyme for biotechnological applications. *Communicating current research and educational topics and trends in applied microbiology*, *1*, 233-245.

Kurisawa, M., Chung, J. E., Uyama, H., and Kobayashi, S. (2003). Enzymatic synthesis and antioxidant properties of poly (rutin). *Biomacromolecules*, *4*(5), 1394-1399.

Kuznetsov, B. A., Shumakovich, G. P., Koroleva, O. V., & Yaropolov, A. I. (2001). On applicability of laccase as label in the mediated and mediatorless electroimmunoassay: effect of distance on the direct electron transfer between laccase and electrode. *Biosensors and Bioelectronics*, *16*(1-2), 73-84.

La Russa M. (2009). Green algae selection useful for phyco-remediation of olive-mill wastewaters and increase of their lipid content by genetic engineering. *Ph.D. Thesis*, University of Naples Federico II, Naples.

La Russa, M., De Biasi, M. G., Chiaiese, P., Palomba, F., Pollio, A., Pinto, G., and Filippone, E. (2008, September). Screening of green microalgae species for extracellular phenoloxidase activity useful for wastewater phycoremediation. In *Proceedings of the 4th European Bioremediation Conference, Chania, Greece (September 03–06, 2008)*.

Lante, A., Crapisi, A., Krastanov, A., and Spettoli, P. (2000). Biodegradation of phenols by laccase immobilised in a membrane reactor. *Process Biochemistry*, *36*(1-2), 51-58.

Leechart, P., Nakbanpote, W., and Thiravetyan, P. (2009). Application of 'waste'wood-shaving bottom ash for adsorption of azo reactive dye. *Journal of environmental management*, *90*(2), 912-920.

Leite, O. D., Lupetti, K. O., Fatibello-Filho, O., Vieira, I. C., and de M Barbosa, A. (2003). Synergic effect studies of the bi-enzymatic system laccase–peroxidase in a voltammetric biosensor for catecholamines. *Talanta*, *59*(5), 889-896.

Leontievsky, A., Myasoedova, N., Pozdnyakova, N., and Golovleva, L. (1997). Yellow'laccase of Panus tigrinus oxidizes non-phenolic substrates without electron-transfer mediators. *FEBS letters*, *413*(3), 446-448.

Li M, Zhang G, Wang H, Ng T (2010). Purification and characterization of a laccase from the edible wild mushroom *Tricholoma mongolicum. Journal of Environmental Science and Technology*. *20(7),* 1069-1076.

Lima, N., Rodríguez Couto, S., Rodríguez, A., Paterson, R. R. M., and Teixeira, J. A. (2003). Laccase activity from the fungus Trametes hirsuta using an air- lift bioreactor. *Letters in applied microbiology*, *42*(6), 612-616.

Lisov, A. V., Zavarzina, A. G., Zavarzin, A. A., and Leontievsky, A. A. (2007). Laccases produced by lichens of the order Peltigerales. *FEMS microbiology letters*, *275*(1), 46-52.

Liu, Z., Zhang, D., Hua, Z., Li, J., Du, G., and Chen, J. (2010). Improvement of laccase production and its properties by low-energy ion implantation. *Bioprocess and biosystems engineering*, *33*(5), 639-646.

Lorenzo, M., Moldes, D., Couto, S. R., and Sanromán, M. A. A. (2005). Inhibition of laccase activity from Trametes versicolor by heavy metals and organic compounds. *Chemosphere*, *60*(8), 1124-1128.

Lowry O. H., Rosebrough A. L., and Randall R. J. (1951) Protein Measurement with the Folin Phenol Reagent. *Journal of biological chemistry*. 1951; 193: 265-275.

Lund, M., and Ragauskas, A. (2001). Enzymatic modification of kraft lignin through oxidative coupling with water-soluble phenols. *Applied Microbiology and Biotechnology*, *55*(6), 699-703.

Lyons, J. I., Newell, S. Y., Buchan, A., and Moran, M. A. (2003). Diversity of ascomycete laccase gene sequences in a southeastern US salt marsh. *Microbial Ecology*, *45*(3), 270-281.

Maalej-Kammoun, M., Zouari-Mechichi, H., Belbahri, L., Woodward, S., and Mechichi, T. (2009). Malachite green decolourization and detoxification by the laccase from a newly isolated strain of Trametes sp. *International Biodeterioration & Biodegradation*, *63*(5), 600-606.

Mabrouk, A. M., Kheiralla, Z. H., Hamed, E. R., Youssry, A. A., and Abd El Aty, A. A. (2012). Characterization and purification of the crude Trematosphaeria mangrovei laccase enzyme. *Egyptian Pharmaceutical Journal*, *11*(2), 93.

Madhavi, V., and Lele, S. S. (2009). Laccase: properties and applications. *BioResources*, *4*(4), 1694-1717.

Mahvi, A. H., Ghanbarian, M., Nasseri, S., and Khairi, A. (2009). Mineralization and discoloration of textile wastewater by TiO2 nanoparticles. *Desalination*, *239*(1-3), 309-316.

Mai, C., Majcherczyk, A., and Hüttermann, A. (2000). Chemo-enzymatic synthesis and characterization of graft copolymers from lignin and acrylic compounds. *Enzyme and microbial technology*, *27*(1-2), 167-175.

Majcherczyk, A., and Johannes, C. (2000). Radical mediated indirect oxidation of a PEG-coupled polycyclic aromatic hydrocarbon (PAH) model compound by fungal laccase. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1474*(2), 157-162.

Majeau, J. A., Brar, S. K., and Tyagi, R. D. (2010). Laccases for removal of recalcitrant and emerging pollutants. *Bioresource technology*, *101*(7), 2331-2350.

Maleki A., Mahvi A.H., Ebrahimi R., and Zandsalimi Y. (2010) Study of photochemical and sonochemical processes efficiency for degradation of dyes in aqueous solution. *Korean Journal of Chemical Engineering;* 27:1805–10.

Marques de Souza, C. G., and Peralta, R. M. (2003). Purification and characterization of the main laccase produced by the white- rot fungus Pleurotus pulmonarius on wheat bran solid state medium. *Journal of Basic Microbiology: An International Journal on Biochemistry, Physiology, Genetics, Morphology, and Ecology of Microorganisms*, *43*(4), 278-286.

Martelé, Y., Callewaert, K., Naessens, K., Van Daele, P., Baets, R., and Schacht, E. (2003). Controlled patterning of biomolecules on solid surfaces. *Materials Science and Engineering: C*, *23*(3), 341-345.

McCaig, B.C., Meagher, R.B., Dean, J.F. (2005) Gene structure and molecular analysis of the laccase-like multicopper oxidase (LMCO) gene family in Arabidopsis thaliana. *Planta, 221*, 619–636

McMullan, G., Meehan, C., Conneely, A., Kirby, N., Robinson, T., Nigam, P., and Smyth, W.F. (2001). Microbial decolourisation and degradation of textile dyes. *Applied microbiology and biotechnology*, *56*(1), 81-87.

Mendes, S., Farinha, A., Ramos, C. G., Leitão, J. H., Viegas, C. A., and Martins, L. O. (2011). Synergistic action of azoreductase and laccase leads to maximal decolourization and detoxification of model dye-containing wastewaters. *Bioresource technology*, *102*(21), 9852-9859.

Michniewicz, A., Ullrich, R., Ledakowicz, S., and Hofrichter, M. (2006). The white-rot fungus Cerrena unicolor strain 137 produces two laccase isoforms with different physico-chemical and catalytic properties. *Applied Microbiology and Biotechnology*, *69*(6), 682-688.

Mikiashvili, N., Wasser, S. P., Nevo, E., and Elisashvili, V. (2006). Effects of carbon and nitrogen sources on Pleurotus ostreatus ligninolytic enzyme activity. *World Journal of Microbiology and Biotechnology*, *22*(9), 999-1002.

Minussi, R. C., Pastore, G. M., and Durán, N. (2002). Potential applications of laccase in the food industry. *Trends in Food Science & Technology*, *13*(6-7), 205-216.

Mirzadeh, S. S., Khezri, S. M., Rezaei, S., Forootanfar, H., Mahvi, A. H., and Faramarzi, M. A. (2014). Decolorization of two synthetic dyes using the purified laccase of Paraconiothyrium variabile immobilized on porous silica beads. *Journal of Environmental Health Science and Engineering*, *12*(1), 1-9.

Morozova, O., Perez-Perez, M.C., Barbosa-Rodrıguez, I., Ricardo, J., and Villase~nor Ortega, F. (2007). Laccases. In: Guevara-Gonzalez, R.G., Torres-Pacheco, I. (Eds), Advances in Agricultural and Food Biotechnology. Research Signpost, Kerala, India, pp. 323e340.

Mustafa, R., Muniglia, L., Rovel, B., and Girardin, M. (2005). Phenolic colorants obtained by enzymatic synthesis using a fungal laccase in a hydro-organic biphasic system. *Food research international*, *38*(8-9), 995-1000.

Nakamura, K., and Go, N. (2005). Function and molecular evolution of multicopper blue proteins. *Cell. Mol. Life Sci. 62*, 2050-2066.

Neifar, M., Jaouani, A., Ellouze-Ghorbel, R., Ellouze-Chaabouni, S., and Penninckx, M.J. (2009) Effect of culturing processes and copper addition on laccase production by the white-rot fungu *Fomes fomentarius* MUCL 35117. Lett. *Appl. Microbiol. 49*, 73-78.

Nicotra. S., Cramarossa, M.R., Mucci, A., Pagnoni, U.M., Riva, S., and Forti, L. (2004) Biotransformation of resveratrol: synthesis of trans-dehydrodimers catalyzed by laccases from *Myceliophtora thermophyla* and from *Trametes pubescens*. *Tetrahedron 60*, 595–600.

Niku-Paavola, M. L., and Viikari, L. (2000). Enzymatic oxidation of alkenes. *Journal of Molecular Catalysis B: Enzymatic*, *10*(4), 435-444.

Niku-Paavola, M.L., Karhunen, E., Kentelinen, A., Viikari, L., Lundell, T., and Hatakka, A. (1990). The effect of culture conditions on the production of lignin modifying enzymes by the white rot fungus *Phlebia radiata. Journal of Biotechnology, 13*, 211–221.

Niladevi. K.N., Jacob. N., and Prema. P. (2008) Evidence for a halo-tolerantalkaline laccase in *Streptomyces psammoticus*: Purification and characterization. *Process Biochem*. *43*, 654–660.

Nivedita. J., Veda. P., Pandey, U., and Dwivedi, N. (2014). Purification of a thermostable alkaline laccase from papaya (*Carica papaya*) using affinity chromatography. *Int J Biol Macromol.72*, 326–332. https://doi.org/ 10.1016/j.ijbiomac.2014.08.032 PMID: 25192855.

Octavio. L., Tlecuitl-Beristain, S., Sánchez, C., Robson, G. D., and Díaz-Godínez, G. (2008). Laccases of Pleurotus ostreatus observed at different phases of its growth in submerged fermentation: production of a novel laccase isoform. *Mycological Research*, *112*(9), 1080-1084.

Otto. B., Schlosser. D. (2014). First laccase in green algae: purification and characterization of an extracellular phenol oxidase from *Tetracystis aeria. Planta*, 240:1225–1236. https://doi.org/10.1007/s00425- 014-2144-9 PMID: 25115562

Paavola. M.L., Fagerstrom. R., Kruus, K., and Viikari. L. (2004). Thermostable laccase from the white- rot fungus *Peniophora sp.Enzyme and Microbial Technol., 35*, 100–102.

Palanisami, S., and Uma, L. (2010). Laccase and polyphenol oxidase activities of marine cyanobacteria: a study with Poly R 478 decolourization. *World J. Microbiol. Biotechnol., 26*, 63–69.

Palmieri, G., Giardina, P., Bianco, C., Fontanella, B., and Sannia, G. (2000). Copper induction of laccase isoenzymes in the ligninolytic fungus Pleurotus ostreatus. *Applied and environmental microbiology*, *66*(3), 920-924.

Patel H, Gupte M. Gahlout A. A purification and characterization of an extracellular laccase from solidstate culture of Pleurotus ostreatus HP-1, 3 Biotech. 2014; 4: 77–84. https://doi.org/10.1007/s13205- 013-0129-1 PMID: 28324461

Pereira, L., Coelho, A. V., Viegas, C. A., Dos Santos, M. M. C., Robalo, M. P., and Martins, L. O. (2009). Enzymatic biotransformation of the azo dye Sudan Orange G with bacterial CotA-laccase. *Journal of biotechnology*, *139*(1), 68-77.

Pezzella, C., Lettera, V., Piscitelli, A., Giardina, P., and Sannia, G. (2013). Transcriptional analysis of *Pleurotus ostreatus laccase genes*. *Appl Microbiol Biotechnol97*, 705–717.

Piscitelli, A., Pezzella, C., Giardina, P., Faraco, and V., Giovanni, S., (2010). Heterologous laccase production and its role in industrial applications. *Bioeng. Bugs 1*, 25-262.

Pointing, S. B. (2001). "Feasibility of bioremediation by white-rot fungi," *Applied Microbiology and Biotechnology*, *57, (1-2),* 20–33.

Pozdnyakova, N. N., Rodakiewicz-Nowak, J., and Turkovskaya, O. V. (2004). Catalytic properties of yellow laccase from Pleurotus ostreatus D1. *Journal of Molecular Catalysis B: Enzymatic*, *30*(1), 19-24.

Raghukumar, C. (2000). Fungi from marine habitats: an application in bioremediation *Mycological research*, *104*(10), 1222-1226.

Rajagurup, P., Vidya. L., Baskorasethupathi, V., Kumar, P.A., Palanivel, M., and Kalaiselvi, K. (2002). Genotoxicity evaluation of polluted ground water in human peripheral blood lymphocytes using the comet assay. *Mutat. Res./Gentet.Toxicol. Environ. Mutagen.,56*, 81-87.

Ralph, J., Brunow, G., Boerjan, and W., (2007). Lignins. In: Ltd, J.W.S. (Ed), eLS: *Encyclopedia of Life Sciences*. http://www.els.net. Chichester.

Rezaei, S., Tahmasbi, H., Mogharabi, M., Ameri, A., Forootanfar, H., Khoshayand, M. R., and Faramarzi, M. A. (2015). Laccase-catalyzed decolorization and detoxification of Acid Blue 92: statistical optimization, microtoxicity, kinetics, and energetics. *Journal of Environmental Health Science and Engineering*, *13*(1), 1-9.

Riva, S. (2006) Laccases: blue enzymes for green chemistry. *TRENDS Biotechnol24*, 219–226.

Rivera-Hoyos, C. M., Morales-Álvarez, E. D., Chaparro-Núñez, L. E., Daza, C. E., Poutou-Piñales, R. A., and Pedroza-Rodríguez, A. M. (2017). Decolorization and detoxification of Malachite Green by Ganoderma lucidum: key operating parameters and adsorption studies. *Journal of Environmental Engineering*, *143*(4), 04016093.

Robles, A., Lucas, R., Cienfuegos, & G., Gálvez, A. (2000). Phenol-oxidase (laccase) activity in strains of the hyphomycete Chalara paradoxa isolated from olive mill wastewater disposal ponds. *Enzyme Microb. Technol. 26*, 484–490.

Rodgers, C.J., Blanford, C.F., Giddens, S.R., Skamnioti, P., Armstrong, F.A., and Gurr, S.J. (2010). Designer laccases: a vogue for high-potential fungal enzymes? *Trends Biotechnol. 28*, 63-72.

Rodrıguez-Couto, S., and Toca-Herrera, J.L. (2006). Industrial and biotechnological applications of laccases: a review. *Biotechnol. Adv. 24*, 500-513.

Rogalski, J., and Leonowicz, A. (2004). Microbial enzymes: production and applications. Laccase. *Concise encyclopedia of bioresource technology*, 533-542.

Rönnerstrand S. (1943) Untersuchungen über Oxydase, Peroxydase und Ascorbinsäure in einigen Meeresalgen. Dissertation, University of Lund; C. W. Lindström, Lund

Roy, J.J., Abraham, T.E., Abhijith, K.S., Sujith-kumar, P.V., and Thakur, M.S. (2005). Biosensor for the determination of phenols based on Cross-Linked Enzyme Crystals (CLEC) of laccase. *Biosens Bioelectron, 21*, 206–11.

Sadhasivam. S., Savitha. S., Swaminathan. K., and Lin. F.H. (2008;) Production purification and characterization of mid redox potential laccase from a newly isolated Trichoderma harzianum WL. *Process Biochem., 43*, 736–74.

Sahay, R., Yadav, R.S.S., and Yadav, K.D.S. (2008). Purification and characterization of extracellular laccase secreted by *Pleurotus sajor-caju MTCC 141*. *Chin J Biotech., 24*, 2068–2073.

Salony, G. N., Baranwal, R., Chhabra, M., Mishra, S., Chaudhuri, T. K., and Bisaria, V. S. (2008). Laccase of Cyathus bulleri: structural, catalytic characterization and expression in Escherichia coli. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, *1784*(2), 259-268.

Sannino, F., Addorisio, V., Mateo, C., and Guisan, J. M. (2016). Oxidation of phenyl compounds using strongly stable immobilized-stabilized laccase from Trametes versicolor. *Process Biochemistry*, *48*(8), 1174-1180.

Saratale, R. G., Saratale, G. D., Chang, J. S., and Govindwar, S. P. (2011). Bacterial decolorization and degradation of azo dyes: a review. *Journal of the Taiwan institute of Chemical Engineers*, *42*(1), 138-157.

Saratale, R. G., Saratale, G. D., Kalyani, D. C., Chang, J. S. and Govindwar, S. P. (2009) "Enhanced decolorization and biodegradation of textile azo dye Scarlet R by using developed microbial consortium-GR," *Bioresource Technology, 100(9),* 2493–2500.

Sasikumar, C. S., and Papinazath, T. (2003, December). Environmental management: bioremediation of polluted environment. In *Proceedings of the Third International Conference on Environment and Health* (pp. 465-469). Department of Geography, University of Madras, Chennai and Faculty of Environmental Studies, York University, New York.

Selinheimo, E., Kruus, K., Buchert, J., Hopia, A., and Autio, K. (2006). Effects of laccase, xylanase and their combination on the rheological properties of wheat doughs. *Journal of Cereal Science*, *43*(2), 152-159.

Senthilvelan, T., Kanagaraj, J., and Panda, R. C. (2018). Effective bioremoval of syntan using fungal laccase to reduce pollution from effluent. *International Journal of Environmental Science and Technology*, *15*(7), 1429-1440.

Sharma, P., Goel, R., and Capalash, N. (2007). Bacterial laccases. *World journal of microbiology and biotechnology*, *23*(6), 823-832.

Shekher, R., Sehgal, S., Kamthania, M., and Kumar, A. (2011). "Laccase: Microbial sources, production, purification, and potential biotechnological applications," *Enzyme research (1),* 1-11. DOI: 10.4061/2011/217861

Shirmardi, M., Mesdaghinia, A., Mahvi, A. H., Nasseri, S., and Nabizadeh, R. (2012). Kinetics and equilibrium studies on adsorption of acid red 18 (Azo-Dye) using multiwall carbon nanotubes (MWCNTs) from aqueous solution. *E-Journal of Chemistry*, *9*(4), 2371-2383.

Shutova, V. V., Revin, V. V., and Myakushina, Y. A. (2008). The effect of copper ions on the production of laccase by the fungus Lentinus (Panus) tigrinus. *Applied biochemistry and microbiology*, *44*(6), 619-623.

Sitarz, A. K., Mikkelsen, J. D., and Meyer, A. S. (2016). Structure, functionality and tuning up of laccases for lignocellulose and other industrial applications. *Critical reviews in biotechnology*, *36*(1), 70-86.

Soares, G. M., Costa-Ferreira, M., and de Amorim, M. P. (2001). Decolorization of an anthraquinone-type dye using a laccase formulation. *Bioresource Technology*, *79*(2), 171-177.

Solís-Oba, M., Ugalde-Saldívar, V. M., González, I., and Viniegra-González, G. (2005). An electrochemical–spectrophotometrical study of the oxidized forms of the mediator 2, 2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) produced by immobilized laccase. *Journal of Electroanalytical Chemistry*, *579*(1), 59-66.

Solomon, E. I., Yoon, J., Aznar, C. P., Palmer, A. E., Andersson, K. K., Britt, R. D., and Quintanar, L (2005). Spectroscopic and electronic structure studies of the trinuclear Cu cluster active site of the multicopper oxidase laccase: nature of its coordination unsaturation. *Journal of the American Chemical Society*, *127*(40), 13832-13845.

Somero, G. N. (2004). Adaptation of enzymes to temperature: searching for basic "strategies". *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *139*(3), 321-333.

Sondhi, S., Sharma, P., Saini, S., Puri, N., and Gupta, N. (2014). Purification and characterization of an extracellular, thermo-alkali-stable, metal tolerant laccase from Bacillus tequilensis SN4. *PloS one*, *9*(5), 96951.

Sriram, W. M., Eyiolawi, S. A., Salawu, K. O., and Onilude, A. A. (2013). Decolourization of synthetic dyes by laccase enzyme produced by Kluyveromyces dobzhanskii DW1 and Pichia manshurica DW2. *African Journal of Biotechnology*, *18*(1), 1-11.

Subashchandrabose, S. R., Ramakrishnan, B., Megharaj, M., Venkateswarlu, K., and Naidu, R. (2013). Mixotrophic cyanobacteria and microalgae as distinctive biological agents for organic pollutant degradation. *Environment international*, *51*, 59-72.

Suresh, B., & Ravishankar, G. A. (2004). Phytoremediation—a novel and promising approach for environmental clean-up. *Critical reviews in biotechnology*, *24*(2-3), 97-124.

Susana. C., Ibarra, D., Martínez, M. J., and Martínez, Á. T. (2006). Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. *Applied and environmental microbiology*, *71*(4), 1775-1784.

Thakker, G. D., Evans, C. S., and Rao, K. K. (1992). Purification and characterization of laccase from Monocillium indicum Saxena. *Applied Microbiology and Biotechnology*, *37*(3), 321-323.

Thurston C. F. (1994). The structure and function of fungal laccases. Microbiology, 140: 19-26.

Tinoco, R., Pickard, M. A., and Vazquez-Duhalt, R. (2001). Kinetic differences of purified laccases from six Pleurotus ostreatus strains. *Letters in Applied Microbiology*, *32*(5), 331-335.

Toca- Herrera, J. L., Krastev, R., Bosio, V., Küpcü, S., Pum, D., Fery, A., and Sleytr, U. B. (2005). Recrystallization of bacterial S- layers on flat polyelectrolyte surfaces and hollow polyelectrolyte capsules. *Small*, *1*(3), 339-348.

Tong, P., Hong, Y., Xiao, Y., Zhang, M., Tu, X., and Cui, T. (2007). High production of laccase by a new basidiomycete, Trametes sp. *Biotechnology letters*, *29*(2), 295-301.

Torres, E., Bustos-Jaimes, I., and Le Borgne, S. (2003). Potential use of oxidative enzymes for the detoxification of organic pollutants. *Applied Catalysis B: Environmental*, *46*(1), 1-15.

Upadhyay, P., Shrivastava, R., and Agrawal, P. K. (2016). Bioprospecting and biotechnological applications of fungal laccase. *3 Biotechnology*, *6*(1), 15.

Uyama, H., and Kobayashi, S. (2002). Enzyme-catalyzed polymerization to functional polymers. *Journal of Molecular Catalysis B: Enzymatic*, *19*, 117-127.

Vantamuri, A. B., and Kaliwal, B. B. (2016). Purification and characterization of laccase from Marasmius species BBKAV79 and effective decolorization of selected textile dyes. *3 Biotechnology*, *6*(2), 1-10.

Vidali, M. (2001). Bioremediation. an overview. *Pure and applied chemistry*, *73*(7), 1163-1172.

Viswanath, B., Rajesh, B., Janardhan, A., Kumar, A. P., and Narasimha, G. (2014). Fungal laccases and their applications in bioremediation. *Enzyme research*, *2014*.

Walker, J.W.R., and McCallion, R.F. (1980), "The selective inhibition of ortho and paradiphenol oxidases. *Phytochemistry19*, 373-373.

Wang, C. Du., H. Hou, J. Zhou, J., and Yan, B.(2013) "Enhancement of laccase production by Pleurotus ostreatus and its use for the decolorization of anthraquinone dye," Process Biochemistry, vol. 39, no. 11, pp. 1415–1419.

Wang, H. X., and Ng, T. B. (2004). Purification of a novel low-molecular-mass laccase with HIV-1 reverse transcriptase inhibitory activity from the mushroom Tricholoma giganteum. *Biochemical and biophysical research communications*, *315*(2), 450-454.

Wang, S. S., Ning, Y. J., Wang, S. N., Zhang, J., Zhang, G. Q., and Chen, Q. J. (2017). Purification, characterization, and cloning of an extracellular laccase with potent dye decolorizing ability from white rot fungus Cerrena unicolor GSM-01. *International journal of biological macromolecules*, *95*, 920-927.

Wesenberg, D., Kyriakides, I., and Agathos, S. N. (2003). White-rot fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnology advances*, *22*(1-2), 161-187.

Widsten, P., & Kandelbauer, A. (2008). Laccase applications in the forest products industry: a review. *Enzyme and microbial technology*, *42*(4), 293-307.

Wong, P. W., Teng, T. T., and Mohammed Z. Z. (2003). Removal of Disperse Dye and Reactive Dye by Coagulation-Flocculation Method. *Proc. Envirn*., 2:264-269.

Xu, F. (1996). Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. *Biochemistry*, *35*(23), 7608-7614.

Xu, F. (1999). "Laccase in Flickinger M.C. and Drew S.W. (eds), *Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, Bioseperation,"* John Wiley & Sons Inc., New York, pp. 1545-1554.

Yan, J., Chen, D., Yang, E., Niu, J., Chen, Y., and Chagan, I. (2014). Purification and characterization of a thermotolerant laccase isoform in Trametes trogii strain and its potential in dye decolorization. *International Biodeterioration & Biodegradation*, *93*, 186-194.

Yang, Y., Ding, Y., Liao, X., and Cai, Y. (2013). Purification and characterization of a new laccase from Shiraia sp. SUPER-H168. *Process Biochemistry*, *48*(2), 351-357.

Yaropolov, A. I., Skorobogat'Ko, O. V., Vartanov, S. S., and Varfolomeyev, S. D. (1994). Laccase. *Applied Biochemistry and Biotechnology*, *49*(3), 257-280.

Ying, G. G., Williams, B., and Kookana, R. (2002). Environmental fate of alkylphenols and alkylphenol ethoxylates—a review. *Environment international*, *28*(3), 215-226.

Youn, H. D., Hah, Y. C., & Kang, S. O. (1995). Role of laccase in lignin degradation by white-rot fungi. *FEMS Microbiology Letters*, *132*(3), 183-188.

Younes S. B., Bouallagui Z., and Sayadi, S. (2012) Catalytic behavior and detoxifying ability of an atypical homotrimeric laccase from the thermophilic strain Scytalidium thermophilum on selected azo and triarylmethane dyes. J Mol Catal B Enzym; 79:41–8.

Zollinger, H. (2002). Synthesis, properties and applications of organic dyes and pigments. Colour chemistry. New York: John Wiley-VCH Publishers; 92100.

APPENDICES

Appendix I

Formulas used during preparation of Reagents

For solid compounds, the grams needed for a particular compound was calculated using;

$$Amount\ (g) = \frac{Molar\ concentration \times Molar\ mass \times Required\ volume\ (m/s)}{1000}$$

For liquid samples prepared from the stock, the concentration of the stock was calculated using;

Concentration (M1) = % Purity X Specific gravity X 10
molecular weight

Thereafter, the volume (V) required from the amber to prepare the solution was calculated by the formula;

Volume of stock (V) = *Needed concentration (M2)* X *Volume to be prepared (V2)*
Stock concentration (M1)

Appendix II

Formula for Calculating Purification of an Enzyme

During the purification profile, Total Protein (mg/ml) was determined using;

Total protein (mg/ml)= *Total activity*
Specific activity

However, purification fold was calculated as;

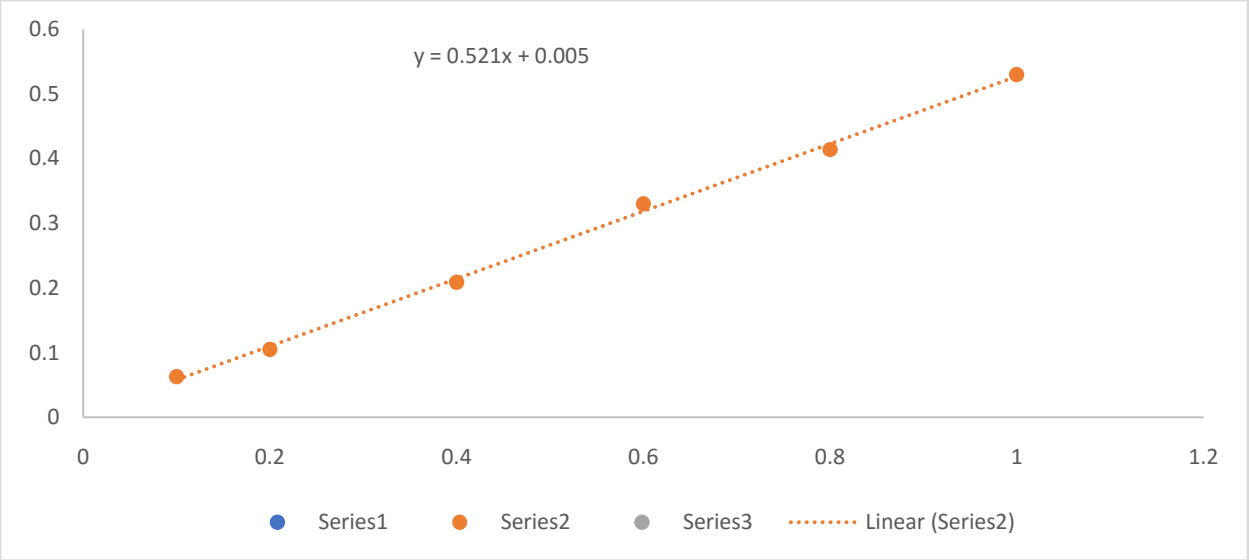
$$\text{Purification fold} = \frac{\text{Specific activity of protein at a purification step}}{\text{Crude specific activity}}$$

% yield and Enzyme activity were calculated using the following formulas respectively;

$$\% \text{ Yield} = \frac{\textit{Total activity of a protein at a step X 100}}{\textit{Total activity of crude}}$$

$$\text{Enzyme activity (mol/mins)} = \frac{\text{Absorbance}}{\text{Incubation time}}$$

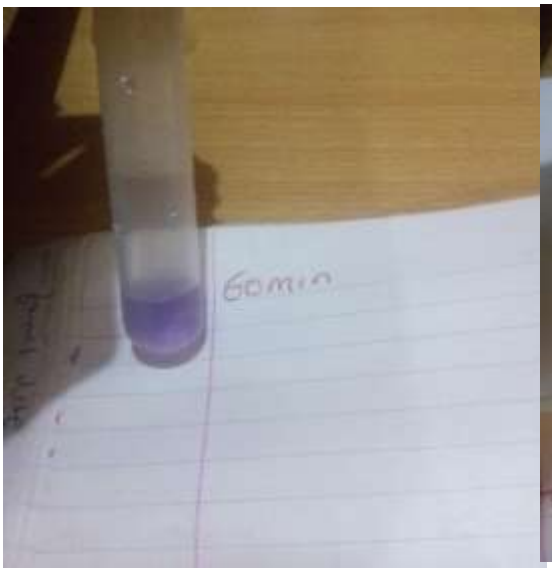
Appendix III



Standard curve for Total Protein standard

Appendix IV

(A) initial dye concentration **(B) Decolorization at 30 minutes**



(C) Decolorization at60 minutes(D) Decolorization at90 minutes

Pictorial representation of Bioremediation (decolorization) potential of crude laccase and partially purified laccase on decolorization of Cibacron Brilliant Blue (CiBB) azo dye

(E) Decolorization at120 minutes(F) Decolorization at180 minutes

Pictorial representation of Bioremediation (decolorization) potential of crude laccase and partially purified laccase on decolorization of Cibacron Brilliant Blue (CiBB) azo dye

Appendix V

NIGERIAN SOCIETY OF BIOCHEMISTRY AND MOLECULAR BIOLOGY (NSBMB)

38th SCIENTIFIC CONFERENCE AND ANNUAL GENERAL MEETING

SOKOTO 2020

Usmanu Danfodiyo University, Sokoto

Department of Biochemistry

29 May, 2021

Dear Author(s): Muhammad Ayyub Muhammad; Emmanuel Oluwadareus Balogun; Abdullahi Balarabe Sallau; Mathias Ahii Chia; Mohammed Nasir Shuaibu

ACCEPTANCE LETTER

We are pleased to inform you that after review by the Technical Sub-committee, your abstract titled **"Isolation, Purification and Characterization of Laccase from *Microcystis flos aquae* and its effects on decolorization of Cibacron Brilliant Blue (azo) dye."** has been accepted for Oral presentation at the forthcoming 38th Annual Conference of the Nigerian Society of Biochemistry and Molecular Biology (NSBMB) to be hosted by Usmanu Danfodiyo University, Sokoto, Nigeria between the 6th and 10th Jun, 2021.

Accept our warmest regards.

Sincerely,

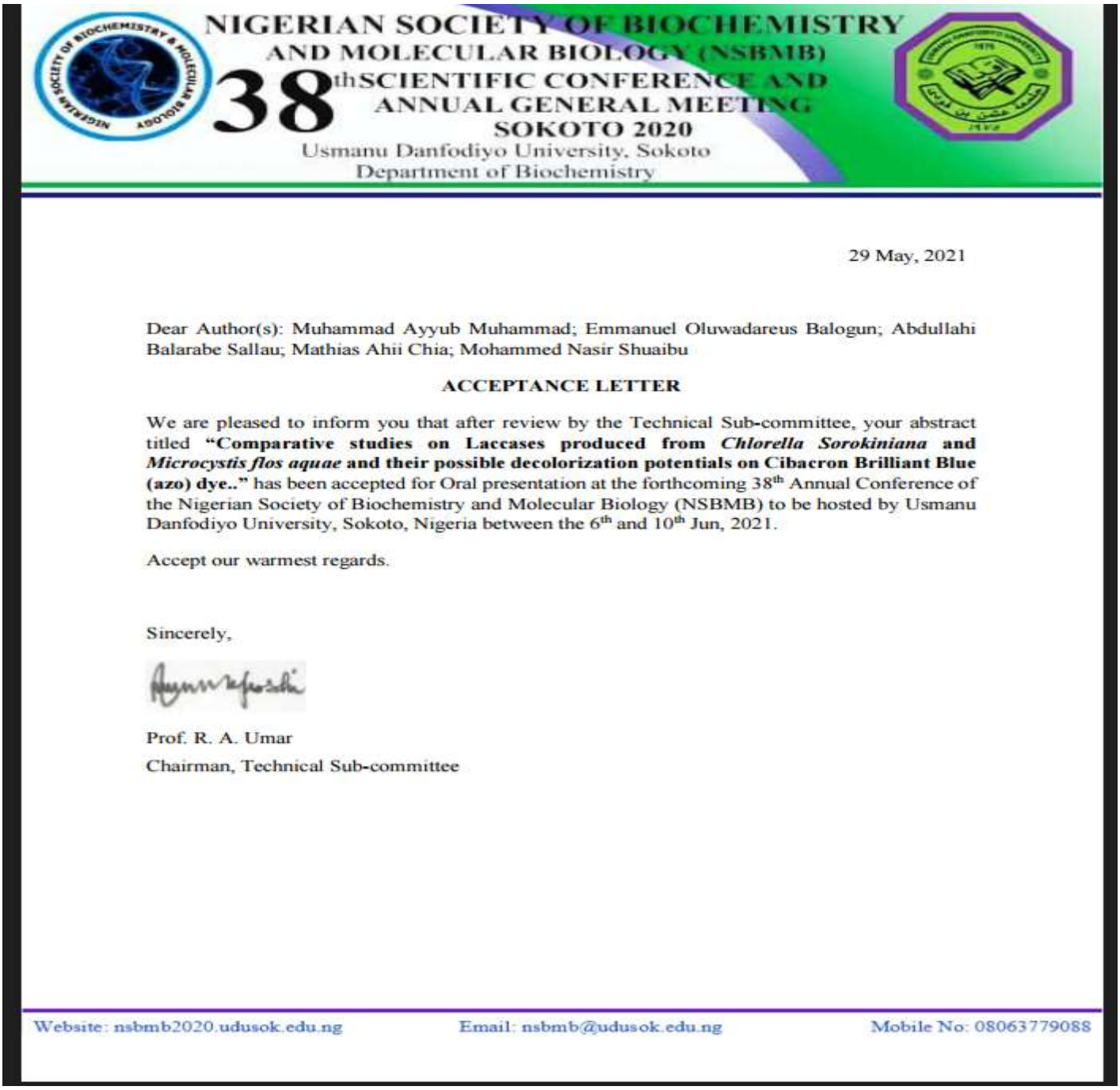
Prof. R. A. Umar

Chairman, Technical Sub-committee

Website: nsbmb2020.udusok.edu.ng Email: nsbmb2@udusok.edu.ng Mobile No: 08063779088

Paper presented on "Isolation, purification and characterization of laccase from *Microcystis flos aquae* and its effects on decolorization of Cibacron Brilliant Blue (azo) dye"

Appendix VI



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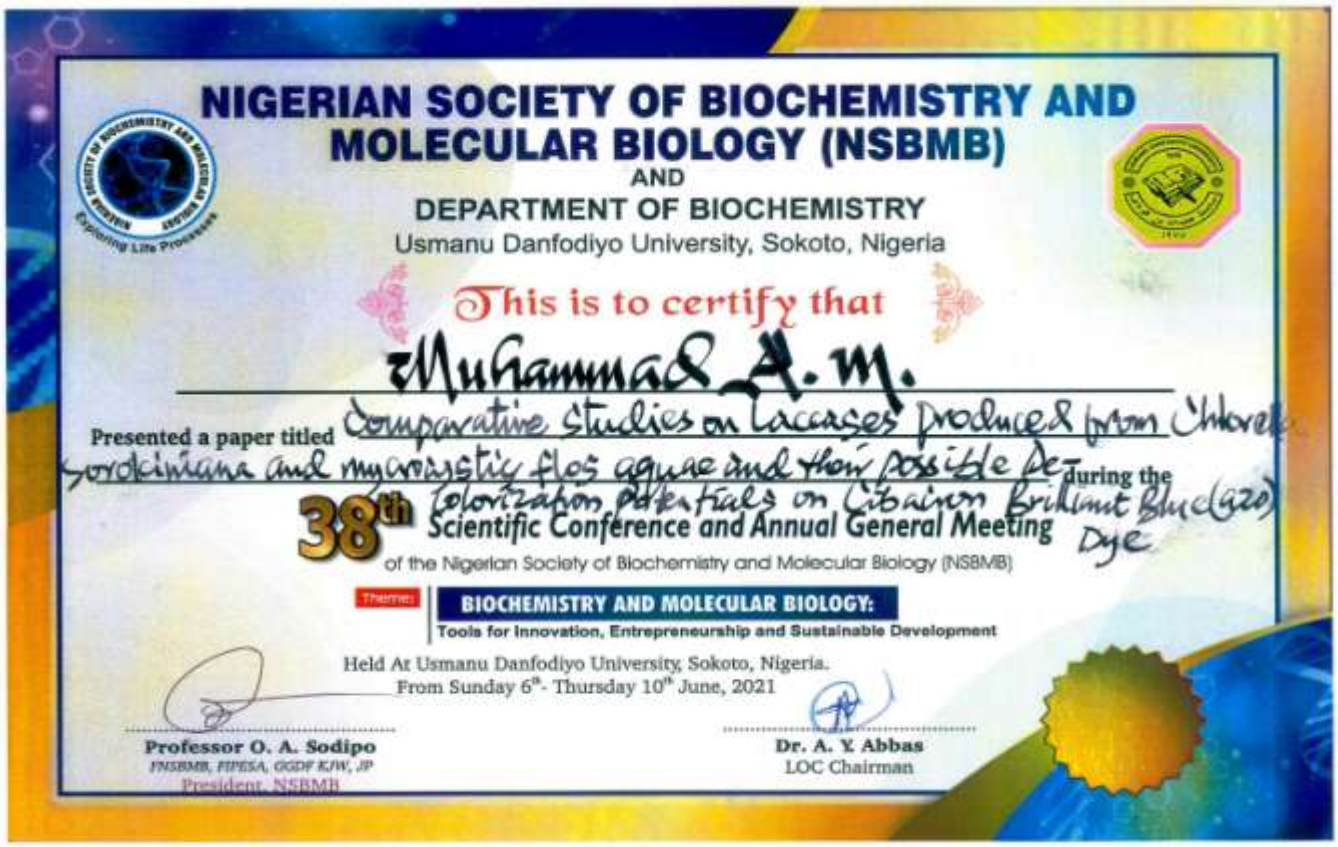
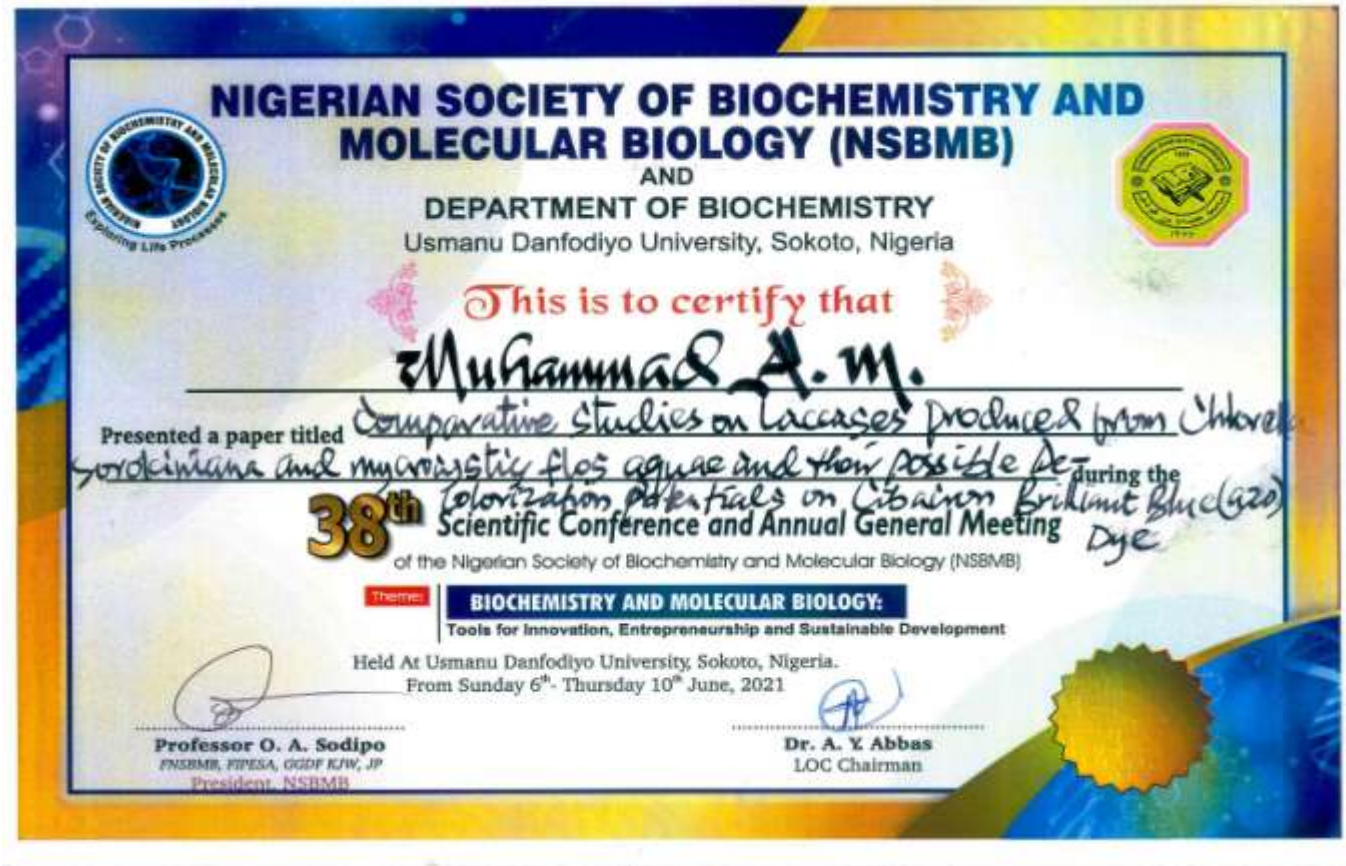
Appendix VII



(A) Certificate on the papers presented at the 38th Scientific Conference and Annual General Meeting of the Nigerian Society of Biochemistry and Molecular Biology (NSBMB).

Appendix VIII



NIGERIAN SOCIETY OF BIOCHEMISTRY AND MOLECULAR BIOLOGY (NSBMB)

AND

DEPARTMENT OF BIOCHEMISTRY

Usmanu Danfodiyo University, Sokoto, Nigeria

This is to certify that

Muhammad A. M.

Presented a paper titled Comparative Studies on Laccases produced from Chlorella sorokiniana and mycrasstic flos aquae and their possible De- during the

38th Scientific Conference and Annual General Meeting

of the Nigerian Society of Biochemistry and Molecular Biology (NSBMB)

Theme: BIOCHEMISTRY AND MOLECULAR BIOLOGY:

Tools for Innovation, Entrepreneurship and Sustainable Development

Held At Usmanu Danfodiyo University, Sokoto, Nigeria.

From Sunday 6th- Thursday 10th June, 2021

Professor O. A. Sodipo

FNSBMB, FIPESA, OGDF KJW, JP

President, NSBMB

Dr. A. Y. Abbas

LOC Chairman

(B) Certificate on the papers presented at the 38th Scientific Conference and Annual General Meeting of the Nigerian Society of Biochemistry and Molecular Biology (NSBMB).