DECOLORIZATION OF SYNTHETIC DYE USING PARTIALLY PURIFIED PEROXIDASE FROM GREEN CABBAGE (*BRASSICA OLERACEA*)

BY

VICTOR, CLIFF CHINEMEREM
(PG/M.Sc/12/64202)

DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF NIGERIA
NSUKKA

OCTOBER, 2014

TITLE PAGE

DECOLORIZATION OF SYNTHETIC DYE USING PARTIALLY PURIFIED PEROXIDASE FROM GREEN CABBAGE (*BRASSICA OLERACEA*)

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENT FOR THE AWARD OF DEGREE OF

MASTER OF SCIENCE (M.Sc.) IN INDUSTRIAL BIOCHEMISTRY

AND BIOTECHNOLOGY, UNIVERSITY OF NIGERIA NSUKKA.

BY

VICTOR CLIFF CHINEMEREM

(PG/M.Sc/12/64202)

DEPARTMENT OF BIOCHEMISTRY

UNIVERSITY OF NIGERIA

NSUKKA

SUPERVISOR: PROF. I.N.E. ONWURAH

CERTIFICATION

VICTOR, Cliff Chinemerem, a postgraduate student with Registration number PG/M.Sc/12/64202 in the Department of Biochemistry has satisfactorily completed the requirement for the award of the Degree of Masters in Science (M.Sc.) in Industrial Biochemistry and Biotechnology. The work embodied in this report is original and has not been submitted in part or full for any other diploma or degree of this or any other higher institution.

---	---
Prof. I.N.E. Onwurah	Prof. O.F.C. Nwodo
(Supervisor)	(Head of Department)

External Examiner

DEDICATION

This research work is dedicated to God Almighty and to my family

ACKNOWLEDGEMENT

My profound gratitude and appreciation go to my able lecturers and supervisors Prof. F.C. Chilaka, Prof. I.N.E Onwurah and Dr. S.O.O. Eze for their guidance and supervision on this work. I am indebted to Prof. P.O. Ukoha who contributed immensely in the analysis of the spectral result and to Mr. Arinze Ezugwu and Akudo Osuiji for their candid contributions.

My appreciation also goes to my friends and classmates; Uche, Tochukwu, Onos, Dominic, Christopher, Nonso, Ig, Kelvin, Emenike, Emeka, Festus, Paul, Venture, Chisom, Maximus, Robert, Okey, Frank, Geraldine, Dikachi, Angela, Onyinye, Tochi, Mary, Judith, Oge, Zoba, Rita,Chidiogo, Charity, Nwanneka, just to mention a few. Thanks for being there and making me to laugh.

Finally, I am grateful to God for my parents, Mr. & Mrs. Leonard N. Victor, my only brother, Chimezie and my precious cousin Chioma for their love, care, financial and motivational support.

ABSTRACT

Peroxidase was extracted from cabbage and was purified in three different purification processes. It was first purified by ammonium sulphate precipitation and highest peroxidase activity was observed at 80% saturation. Hence, 80% saturation was used to mass produce the enzyme. The enzyme was again purified by dialysis which tends to remove salt as impurity from the precipitated enzyme. The enzyme was further purified by gel filtration which further removed salts and other proteins as impurities. The resulting enzyme was characterized to determine the optimum pH and temperature. The optimum pH and temperature were respectively 5.0 and 45^{o}C. The Km and Vmax obtained from Lineweaver-Burk plot of initial velocities at different concentration of H_2O_2 were found to be 3.68mM and 37.04U/ml respectively. Also, Km and Vmax of o-dianisidine were found to be 9.89mM and 28.57U/ml respectively. The enzymatic activity of this cabbage peroxidase with hydrogen peroxide on synthetic dyes was investigated and was found to be very effective in the treatment and decolorization of these dyes. This partially purified enzyme could decolorize many synthetic dyes; Azo Brilliant Black, Azo Trypan Blue, Azo Blue 5,

Azo Citrus Red 2, Azo Yellow 6, Azo Pink, Azo Purple, Vat Green 11 and Vat Orange 9. Azo Trypan Blue and Vat Orange 11 had the highest and least percentage decolorization of 88.62 and 12% respectively after contact time of 1 hour. The cabbage peroxidase was found to decolorize Azo dyes more and had little effect on Vat dyes. This peroxidase could be an important source for dye and waste water decolorization.

TABLE OF CONTENTS

CHAPTER TWO: RESULTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

INTRODUCTION

Large amounts of chemically different dyes are used for various industrial applications such as textile dyeing, paper and pulp, leather and plastics (Park *et al*., 2007). Textile dyes represent a major class of organic pollutants that are found in the waste effluent discharged by these different industries (Kalsoom *et al*., 2013). Approximately 20% of the dye load is lost in the dyeing residues during textile processing which represents one of the greatest environmental problems faced by the sector (Guarantini and Zanoni, 2000). These dyes are designed to be resistant to light, water and oxidizing agents and are therefore the most problematic groups of pollutants, considered as xenobiotics that are not easily biodegradable (Ong *et al*., 2011). The dye effluent contains chemicals that are toxic, carcinogenic, mutagenic, or teratogenic to various aquatic species and humans (Celebi *et al*., 2012). Among the textile dyes, azo dyes account for 60-70% of all textile dyestuffs used and show the largest spectrum of colours (Bae and Freeman, 2007). They are the most common group of synthetic colorants released into the environment (Saratale *et al*., 2011). The discharge of azo dyes into water bodies presents human and ecological risks, since both the original dyes and their biotransformation products can show toxic effects, mainly causing DNA damage. Therefore, the development of non-genotoxic dyes and investment in research to find effective treatments for effluents and drinking water is required, in order to avoid environmental and human exposure to these compounds and prevent the deleterious effects they can have on humans and aquatic organisms.

The treatment of dye wastewater involves chemical and physical methods such as adsorption, coagulation, oxidation, filtration and ionizing radiation. All these methods have different decolorization capabilities, operating speed and proven to be costly while producing large amounts of sludge (Leelakriangsak and Borisut, 2012). Biological processes have received increasing interest as a viable alternative owing to their cost effectiveness, ability to produce less sludge and environmental friendliness (Banat *et al*., 1996). However, synthetic dyes containing various substituents such as

nitro and sulfonic groups are not uniformly susceptible to bio-decolorization in conventional aerobic processes. Enzymatic approach has gained considerable interest in the decolorization/degradation of textile and other industrially important dyes present in wastewater. This strategy is ecofriendly and useful in comparison to conventional chemical, physical and biological treatments, which have inherent serious limitations. Stability, activity and specificity of an enzyme are the fundamental parameters that control the development of an industrial application (Torres and Ayala, 2010).

Many studies have demonstrated that fungi are able to degrade dyes and this capability to degrade dye is due to the extracellular, non-specific and non-stereoselective enzyme system (Bezalel *et al*., 1997). Peroxidases have been reported as excellent oxidant agents to degrade dyes (Kirby *et al.*, 1995). Husain (2010) reported that many aromatic dyes could be decolorized by peroxidase through precipitation or breaking of the aromatic ring structure. Several bacterial, fungal and plant peroxidases have been used for decolorization of synthetic textile dyes. Fungal extracted peroxidases have been mostly studied in dye removal processes (Novotny *et al*., 2001). Decolorization of different azo dyes by *Phanerochaete chrysosporium* RP 78 under optimized conditions was studied by reaction mechanism via azo dye (Ghasemi *et al*., 2010). Bacterial lignin peroxidases from *Pseudomonas aeruginosa* and *Serratia marcescens* have been shown to give 50% to 58% decolourization effect on textile dye-based effluent (Bholay *et al*., 2012). However, using peroxidases from microorganisms to decolorize dyes involves high cost and therefore alternative sources such as plants are now considered (Chanwun *et al*., 2013). Among the plant peroxidases, the most studied are native or recombinant horseradish peroxidases, HRP (Shrivastav, 2003 and Tiirola *et al*., 2006). HRP has been shown to have the ability to precipitate and degrade aromatic azo compounds in the presence of H_2O_2 (Bhunia *et al*, 2001). It has been utilized for the removal of halogenated phenols and pentachlorophenol (Meizler *et al*., 2011; Li *et al*., 2011). Plant peroxidases have been extracted from African oil bean seeds, sorghum, tea leaf, wheat germ, green pea and papaya fruit oil (Lee and Klein, 1990; Silva *et al*., 1990; Converso and Fernandez, 1995; Kvaratskhelia *et al*., 1997; Eze *et al*., 2000; Eze, 2012;). Other peroxidases, such as peroxidases from *Allium sativum*, *Ipomoea batatas*, *Raphanus sativus*, *Sorghum bicolor* and soybean peroxidase have also been applied to phenol removal

(Al-Ansari *et al*., 2010 and Diao *et al*., 2011). Peroxidase has been extracted from red cabbage as reported by Ghahfarrokhi *et al*. (2013) but peroxidase from green cabbage is poorly studied. This research is therefore focused on the extraction, characterization, purification of peroxidase from green cabbage and its application on decolorization of industrial synthetic dyes.

1.1 Peroxidase

The name peroxidase was first used by Linossier, who isolated it from pus in 1898. They are one of the most extensively studied groups of enzymes (Azevedo *et al*., 2003). They are widely distributed in nature and are found in plants, microorganisms and animals where they catalyze the reduction of hydrogen peroxide (H_2O_2) to water (Bania and Mahanta, 2012). They use various peroxides (ROOH) as electron acceptors to catalyze a number of oxidative reactions. In mammals, they are implicated in biological processes as various as immune system or hormone regulations. In plants, they are involved in auxin metabolism, lignin and suberin formation, cross-linking of cell wall components, defense against pathogens or cell elongation. They also show bad effect on the quality of vegetables during post-harvest senescence, oxidation of phenolic substances, starch-sugar conversion and post-harvest demethylation of pectic substances leading to softening of plant tissues during ripening (Ghahfarrokhi *et al*., 2013). Humans contain more than 30 peroxidases whereas *Arabidopsis thaliana* has about 130 peroxidases that are grouped in 13 different families and nine subfamilies (Koua *et al*., 2009). Peroxidase families from prokaryotic organisms, protists and fungi have been shown to promote virulence (Brenot *et al*., 2004; Missall *et al*., 2005 and Pineyro *et al*., 2008). Commercially, peroxidases find application in biotransformations, bioremediation, in Analytical Biochemistry and as specific reagents such as bleaching agents. Peroxidases are classified as haem peroxidases and non-haem peroxidases and distributed between thirteen superfamilies and fifty subfamilies (Passardi et *al*., 2007).

1.1.1 Enzyme Commission Classification of peroxidase

Peroxidases can be found under the same enzyme classification number EC.1.11.1.x, donor: hydrogenperoxide oxidoreductase (Fleischmann *et al*., 2004). Currently, 15 different EC numbers have been ascribed to peroxidase, from EC 1.11.1.1 to EC 1.11.1.16, excluding EC 1.11.1.14 (Passardi *et al*., 2007). Due to the presence of dual enzymatic domains, other peroxidase families were classified with

the following numbers: EC 1.13.11.44, EC 1.14.99.1, EC 1.6.3.1 and EC 4.1.1.44. To date, certain peroxidases do not possess an EC number and can only be classified in EC 1.11.1.7. Two particular cases are also observed for EC 1.11.1.2 (NADPH peroxidase) and EC 1.11.1.3 (fatty acid peroxidase). NADPH peroxidase activities have been observed in different studies (Hochman and Goldberg, 1991). However there is no known peroxidase sequence that has been assigned to this EC number, probably due to the fact that none of the peroxidases known so far have a predominant NADPH peroxidase activity. Peroxidasins, peroxinectins, other non-animal peroxidases, dyptype peroxidases, hybrid ascorbate cytochrome c peroxidase and other class II peroxidases do not possess an EC number. The two independent EC numbers (1.11.1.9 and 1.11.1.12) both correspond to glutathione peroxidase and are based on the electron acceptor (hydrogen peroxide or lipid peroxide, respectively).

Table 1: The International Union of Biochemistry classification of peroxidases

EC number	Recommended name	Abbreviation in PeroxiBase
EC 1.11.1.1	NADH peroxidase	Nadprx
EC 1.11.1.2	NADPH peroxidase	No sequence available
EC 1.11.1.3	Fatty acid peroxidase	No sequence available
EC 1.11.1.5	Cytochrome C peroxidase	CcP, DiHCcP
EC 1.11.1.6	Catalase	Kat, Cp
EC 1.11.1.7	Peroxidase	POX
EC 1.11.1.8	Iodide peroxidase	TPO
EC 1.11.1.9	Glutathione peroxidase	GPx
EC 1.11.1.10	Chloride peroxidase	Halprx, HalNprx, HalVprx
EC 1.11.1.11	1-ascorbate Superoxide	APX
EC 1.11.1.12	Phospholipidhydroperoxide glutathione peroxidase	GPX
EC 1.11.1.13	Manganese peroxidase	MnP
EC 1.11.1.14	Lignin peroxidase	Lip
EC 1.11.1.16	Versatile peroxidase	VP
EC 1.13.11.44	Linoleate diol synthase	LDS
EC 1.14.99.1	Prostaglandinendoperoxide synthase	PGHS
EC 1.6.3.1	NAD(P)H oxidase	DuOx
EC 4.1.1.44	4-carboxymuconolactone Decarboxylase	AhpD, CMD, CMDn, HCMD,HCMDn, DCMD, DCMDn, Alkyprx, Alkyprxn

(Feischman *et al*., 2004).

1.1.2 Haem-Based and non-Haem based Classification

An important number of haem and non-haem peroxidase sequences are annotated and classified in the peroxidase database, PeroxiBase. PeroxiBase contains about 5800 peroxidase sequences classified as haem peroxidases and non-haem peroxidases and distributed between thirteen superfamilies and fifty subfamilies, (Passardi *et al.*, 2007). Haem and non-haem peroxidases are found in all kingdoms.

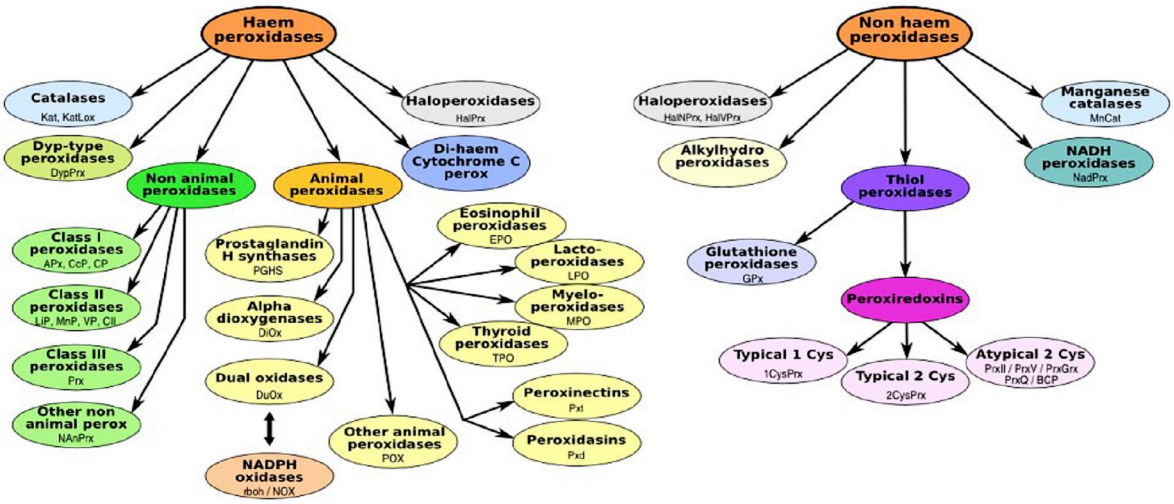


Figure 1: Schematic representation of the phylogenic relationships between the different protein classes and families found in PeroxiBase (Koua *et al*., 2009).

1.1.2.1 Haem based peroxidase

Haem peroxidase is found in plants, animals and microorganisms. They contain ferriprotoporphyrin IX (haematin or haem) as a prosthetic group (Rodrigo *et al*., 1996). Out of 6,861 known peroxidase sequences collected in PeroxiBase, more than 73% of them code for haem-containing peroxidases. In the majority of cases, haem b is the prosthetic group and its evolutionary highly conserved amino acid surroundings influence its reactivity (Torres and Ayala, 2010). Haem peroxidases tend to promote rather than inhibit oxidative damage. Genes encoding haem peroxidases can be found in almost all kingdoms of life. They are grouped in two major superfamilies: one mainly found in bacteria, fungi and plants, Passardi et *al.* (2007) and a second mainly found in animals, fungi and bacteria (Daiyasu and Toh, 2000 and Furtmuller *et al*., 2006). Members of the superfamily of plant/fungal/bacterial peroxidases (non-animal peroxidases) have been identified in the majority of the living organisms except animals. The second superfamily described as ñanimal peroxidasesò comprises a

group of homologous proteins mainly found in animals. The mammalian haem peroxidase plays a major role in both disease prevention and human pathologies (Koua *et al*., 2009). Some mammalian haem peroxidases use H_2O_2 to generate more aggressive oxidants to fight intruding microorganisms (Flohe and Ursini, 2008).

In addition to these two large superfamilies, smaller protein families are classified as capable to reduce peroxide molecules. Examples are Catalase (Kat) that can also oxidize hydrogen peroxide, dihaem cytochrome C peroxidases (DiHC*c*P), dyptype peroxidases (DypPrx), haloperoxidases with (HalPrx) or without (HalNPrx, HalVPrx) haem.

1.1.2.2. Non haem peroxidase

Non-haem peroxidases are not evolutionarily linked and form five independent superfamilies. These are alkylhydroperoxidase, NADH peroxidase (NadPrx), manganese catalases (MnCat) and thiol peroxidases. The largest one is the thiol peroxidase, which currently contains more than 1000 members grouped in two different subfamilies (Glutathione peroxidases and Peroxiredoxines).

1.1.3. Plant Peroxidases

Plant Peroxidases (PODs) are haem peroxidases. In the presence of peroxide, they oxidize a wide range of phenolic compounds, such as guaiacol, o-dianisidine, pyrogallol, chlorogenic acid, catechin, and catechol (Onsa *et al*., 2004). They are divided into three classes based on their structural and catalytic properties. The overall primary sequences and the 3-dimentional structure of these three peroxidases are quite different, implying that these subfamily genes evolve from distinct ancestral genes (Taurog, 1999). The amino acid sequences were found to be highly variable among the members of the plant peroxidase superfamily with less than 20% identity in the most divergent cases (Hiraga *et al*., 2001).

Table 2: The three classes of plant peroxidases.

CLASS	Member (EC Number)	Origin	Molecular weight (KDa)
I	Cytochrome c peroxidase (EC 1.11.1.5)	Yeast and Bacterium	32-63
	Catalase-peroxidase (EC 1.11.1.6)	Bacterium and Fungi	150-240
	Ascorbate peroxidase (EC 1.11.1.11)	Plant	30-58
II	Manganese-dependent peroxidase (EC 1.11.1.13)	Fungi	43-49
	Ligninase (EC 1.11.1.14)	Fungi	40-43
III	Peroxidase (EC 1.11.17, POX)	Plant	28-60

(Das *et al*., 2011)

1.1.3.1. Class I: Peroxidases of prokaryotic origin

Members of this class of peroxidases can be found in organelles of prokaryotic origin, namely in plastids and mitochondria and include yeast cytochrome c peroxidase (Poulos *et al*., 1980 and Finzel *et al*., 1984). These also include chloroplast and cytosolic ascorbate peroxidase and catalase-peroxidase (Regelsberger *et al*., 2001). A common feature of these peroxidases is the lack of bound carbohydrates, disulphide bonds, calcium ions and signal peptides for secretion (Azevedo *et al*., 2003). Ascorbate peroxidase has been purified from bovine eye and its N-terminal sequence was found to be homologous to that of the plant enzyme, implying that ascorbate peroxidase is not plant-specific (Wada *et al*., 1998). They have also been found in insects (Mathews *et al*., 1997). Plant ascorbate peroxidases (APXs) are found in several cellular compartments. In *Arabidopsis thaliana*, for example, the presence of eight isozymes has been confirmed: soluble cytosolic (APX1, APX2, APX6), bound to the microsome membrane (APX3, APX4, APX5), and chloroplast sAPX and tAPX (Panchuk *et al*., 2002). Ascorbate peroxidases play an important role in controlling the concentration of oxygen radicals that participate in signal transduction in many naturally occurring physiological processes in the cell.

1.1.3.2. Class II: Secreted fungal peroxidases

Class II peroxidases include lignin peroxidase (LiP) and manganese peroxidase (MnP), both from white-rot fungi such as *Phanerchaete chrysosporium*, *Phlebia radiata* and *Lentinula edodes*, Poulos *et al*. (1993) and Sundaramoorthy *et al*. (1994), the peroxidase from the hyphomycete *Arthromyces ramosus*, Kunishima *et al*. (1994) and the black inkcap mushroom peroxidase from *Coprinus cinereus* (Petersen *et al*., 1994). They have a signal peptide sequence responsible for their secretion through the endoplasmic reticulum. They possess about 5% carbohydrates, two calcium ions and four conserved disulphide bonds (Azevedo *et al*., 2003).

1.1.3.3. Class III: Classical secretory plant peroxidases (EC1.11.1.7)

Horseradish peroxidase (HRP), African oil bean seeds peroxidase, peanut peroxidase, soybean peroxidase, turnip peroxidase, tobacco peroxidase, tomato peroxidase, barley peroxidase and cabbage peroxidase are examples of class III peroxidases (Hosoya *et al*., 1960; Evans, 1968; Puppo *et al*., 1980; Mader and Fuss, 1986; Schuller *et al*., 1996; Henriksen *et al*., 1998; Belcarz *et al*., 2008 and Eze, 2012). The enzyme is reported to exist in both soluble and membrane-bound forms (Robinson, 1991). It can be found in vacuoles, tonoplast, plasmalemma, and inside and outside the cell wall and has a variety of functions (Thongsook and Barrett, 2005).

Genes encoding class III plant peroxidases are present in all land plants and form large multigenic families (Passardi *et al*., 2004). One of the roles of this peroxidase in plant defense is the reinforcement of cell wall physical barriers and lignification (Bowles, 1990**)**. Members of all classes of the plant peroxidase superfamily contain 10 common Ŭ-helices (Hiraga *et al*., 2001). Enzymes of class I and II have one specific helix while class III peroxidase has 3 specific helices (Schuller *et al*., 1996; Gajhede *et al*., 1997). Individual plant species possess a common set of peroxidases (PODs) with similar characteristics among species. Total amino acid sequence identity is sometimes less than 30% within the same plant species. However, nearly 90% of residues are identical among PODs of different plant species (Kjaersgard *et al*., 1997).

Class III plant peroxidases (PODs) exist as isoenzyme in individual plant species. Theorell isolated two forms of peroxidases from horseradish roots (Theorell, 1942). HRP I was basic and contained a low carbohydrate content, while HRP II was neutral and highly glycosylated (Azevedo *et al*., 2003). The isoenzymes have distinct physical, chemical and kinetic properties arising from small differences in their amino acid sequence (Soltis and Soltis, 1990). This suggests its involvement in various physiological processes (Hiraga *et al*., 2001). Peroxidase isoenzymes in cabbage are not numerous. The biggest amounts of isoforms are found at juvenile stage, during flowering induction (Duchovskiene and Siksnianiene, 2001).

Some authors have suggested more unusual plant peroxidases that do not fit into these classes, such as chloroperoxidases and diheme peroxidases , as group four (Smith and Veitch, 1998). Because class III peroxidases are induced by fungi, Sasaki *et al*.(2004), bacteria, Young *et al*. (1995) and Lavania *et al*. (2006), viruses, Hiraga *et al*.(2001) and viroids, Vera *et al*. (1993), they are considered as pathogenesis-related (PR) proteins, belonging to the PR-protein 9 subfamily (VanLoon *et al*., 2006).

1.1.4 Structure of Plant Peroxidase

The three-dimensional structures of plant peroxidases from *Arabidopsis*, barley, horseradish, peanut and soybean have been determined by X-ray crystallography together with the structures of several catalytic intermediates and substrate complexes that are relevant to enzyme function. On this basis, specific roles for particular amino acid residues and structural motifs or regions have been proposed or in some cases, confirmed. Some of these have been investigated experimentally using site-directed mutagenesis and other techniques (Veitch, 2004). The first full structure of plant peroxidase was solved by molecular replacement based on the pea cytosolic ascorbate peroxidase (Schuller *et al*., 1996). In 1976, Welinder determined the first complete primary structure of horseradish peroxidase (Welinder, 1976).

Plant peroxidases comprise a single polypeptide chain of approximately 300 amino acid residues, iron(III) protoporphyrin IX (haem group) at the centre and two calcium atoms located at the distal and proximal to the haem plane and are linked to the haem-binding region by a network of hydrogen bonds. The molecular weight is

approximately 40kDa (Azevedo *et al.*, 2003). The distal calcium is coordinated by 6 amino residues; Asp43, Asp50, Ser52 (side chain), Asp43, Val46, Gly48 (carbonyl) and one structural water in HRP c. The proximal calcium is coordinated by also 7 amino acid residues; Thr171, Asp222, Thr225, Asp230 (side chain), Thr171, Thr225 and Ile228 (carbonyl). These calcium sites are thought to play an important role in defining the haem pocket architecture (Howes *et al.*, 2001). On calcium loss, enzyme activity decreases by 40% (Haschke and Friedhoff, 1978). His170 forms coordinate bond to haem iron atom (proximal side) and the bond can be broken in acid solution. Asp247 carboxylate side-chain helps to control imidazolate character of His170 ring. The second coordination distal side of the haem plane is unoccupied in the resting state of the enzyme but available to hydrogen peroxide during enzyme turnover. The propionate side chains of the haem form hydrogen bonds with neighbouring residues. Also the organic porphyrin is in *Van der Waals* contact with hydrophobic amino acids (Dunford, 1999).

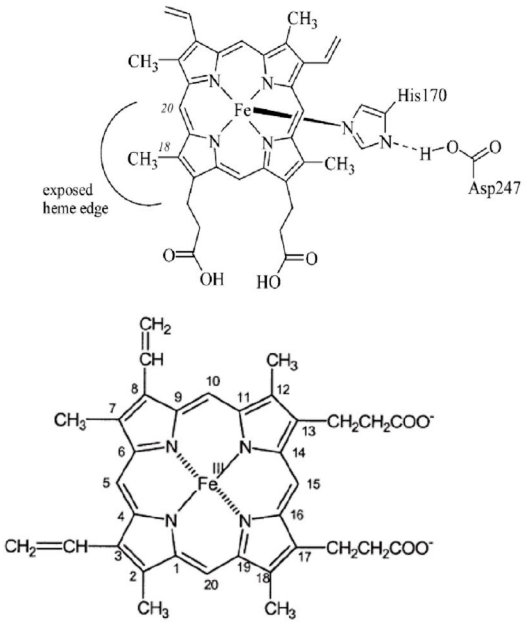


Figure 2: The interaction between haem/ His170 in HRP C and the native haem structure.

The N-terminal residue of HRP C is blocked by pyroglutamate and the C-terminus is heterogenous, with some molecules lacking the terminal residue, Ser308 (Veitch, 2004). Other structural elements of importance are four disulphide linkages based on

the invariant Cyt 11-91, 44-49, 97-301, 177-207 (HRP C numbering) and a buried salt bridge motif between Asp99 and Arg123 residues (Veitch, 2003). Nine potential N-glycosylation sites can be recognised in the primary sequence and of these, eight are occupied (Veitch, 2003). A branched heptasaccharide accounts for 75 to 80% of the glycans but the carbohydrate profile of HRP C is heterogeneous, and many minor glycans have also been characterized (Yang *et al*., 1996). N-linked glycans of all plant glycoproteins are covalently bonded through an amide bond to asparagine residues (Ueda and Agawa, 1999). HRP glycans are composed of mannose (Man), xylose (Xyl), fructose (Fuc) and N-acetylglucosamine (GlcNAc) and account for 20% of the total enzyme molecular weight (Azevedo *et al*., 2003). The main function of the glycans is to maintain the protein conformational structure and to increase the protein solubility in water (Huystee and McManus, 1998). Glycans have no effect on specific activity and reaction kinetics of the enzyme but greatly reduce the solubility in salt solution (Tams and Welinder, 1995). Glycosylation may also stabilize the polypeptide chain against uncontrolled proteolysis and free radical induced protein crosslinking (Hiner, 1995).

The structure of the enzyme is largely Ŭ-helical although there is also a small region of ß-sheet. All the three classes of plant peroxidase contain the 10 prominent helices found in HRP C, lettered helices A to J (Schuller *et al*., 1996). There are two domains. The haem occupies the crevice between both domains (the distal and proximal), sandwiched between helix B and helix F (Welinder and Gajhede, 1993). HRP contains three extra Ŭ-helices (DNjFNjand FNjj which are not found in other classes. The small helix, DNjis inserted between helices D and E and is common to class III peroxidase. The most distinctive feature of class III peroxidases is a long insertion (34 amino acid residues in HRP C) and is maintained by a disulphide bridge (between Cys177 and Cys209). This helical region varies in length and amino acid composition (Gajhede *et al*., 1997).

Table 3: The secondary structure of the recombinant HRP isoenzyme c.

Secondary structure	Common name	Amino acid residues	
		Beginning	Ending
α-Helix	A	Val14	Ser28
α-Helix	B	Ile32	Cys44
α-Helix	C	Phe77	Ala90
α-Helix	D	Cys97	Leu111
α-Helix	D′	Leu131	Asn137
α-Helix	E	Leu145	Asn154
α-Helix	F	Ser160	Leu166
β-Strand	1	Lys174	Gln176
α-Helix	F′	Met181	Leu184
α-Helix	F″	Thr199	Leu208
β-Strand	2	Leu218	Gln220
α-Helix	G	Lys232	Glu238
α-Helix	H	Gln245	Ser252
α-Helix	I	Ile260	Asn268
α-Helix	J	Thr270	Met284

(Gajhede *et al*., 1997)

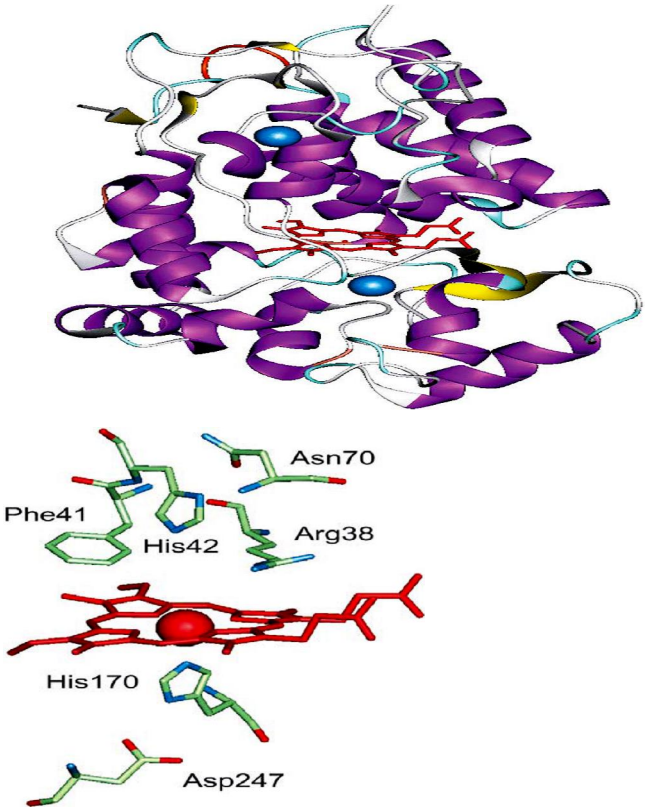


Figure 3: Three-dimensional representation of the X-ray crystal structure and key amino acid residues in the haem-binding region of horseradish peroxidase isoenzyme C (Veitch, 2004).

1.1.5 The Mechanism of Action of Peroxidase

The mechanism of catalysis of horseradish peroxidase and in particular, the HRP C isoenzyme, has been investigated extensively (Veitch and Smith, 2001). The enzyme is highly specific to its peroxide substrate, of which H_2O_2 is the most common. In the presence of peroxide, PODs from plant tissues are able to oxidize a wide range of phenolic compounds, such as guaiacol, pyrogallol, chlorogenic acid, catechin, and catechol (Onsa *et al*., 2004). Oxidation of a wide range of organic compounds has led to the speculation that the enzyme may be associated with losses in color, flavor, and nutritional value of raw and processed foods (Robinson, 1987). It plays an important role in the browning of processed yam (Eze *et al*., 2010).

Despite the differences in the proteins, active sites, and even prosthetic groups, the catalytic mechanisms of all the peroxidases are sufficiently similar and they can be viewed, despite their differences, from a common perspective. The common overall reaction of the peroxidases can be written as in the following equation;

$$H_2O_2 + 2AH = 2H_2O + 2A^{\cdot}$$

Where AH and $A^{\cdot}$ represent a reducing substrate and its oxidized radical product, respectively (Veitch, 2003). Typical reducing substrates include aromatic phenols, phenolic acids, indoles, amines and sulfonates. Ascorbate is the substrate for the reduction of hydrogen peroxide in ascorbate peroxidase and the enzyme is sensitive to ascorbate concentration (Dabrowska *et al*., 2007). If it is too low (lower than 20 µM) the enzymes lose stability and their activity declines (Shigeoka *et al*., 2002). Ascorbate peroxidase (class I), are used by plants to regulate levels of intracellular hydrogen peroxide (Mittler, 2002).

The reaction is a three-step cyclic process, in which the enzyme is first oxidized by H_2O_2 and then reduced back to the native form in two sequential steps involving the formation of two enzyme intermediates, Compounds I and II (Azevedo *et al*., 2003).

The steps involved are as follow;

(i) The first step consists of the cleavage of the H_2O_2 molecule, with the concomitant production of water. This usually involves binding of the H_2O_2 to the haem iron atom to produce a ferric hydroperoxide intermediate

[Fe(III)ï OOH]. This elusive intermediate (Compound 0) was first observed by Baek and Van Wart in the reaction of HRP with H_2O_2 (Baek and Van Wart, 1989). The conversion of Compound 0 to Compound I requires the cleavage and the protonation of the distal oxygen of the ferric hydroperoxide complex leading to the formation of the ferryl species with the elimination of the distal oxygen as a molecule of water. One of the oxygen atoms of H_2O_2 is incorporation into Compound I. It is now known that compound I contains an oxoferryl group (Fe^{IV}=O), in which the iron is in a 4^+ oxidation state and a porphyrin ´-cation radical. The crystal structure of Compound I has been determined after its generation by reaction of ferric HRP with peracetic acid (Berglund *et al.*, 2002).

(ii) Compound I oxidizes a wide range of reducing substrate molecules (AH) by a mechanism involving a single-electron transfer, leading to the formation of the second enzyme intermediate called Compound II. Compounds I and II, the critical catalytic intermediates, are readily distinguished from the resting ferric state of the protein by their UVï visible absorption spectra.

(iii)Compound II, which also contains an oxoferryl group (Fe^{IV}=O), is then reduced by a second substrate molecule (AH) to the native ferric enzyme (Fe^{III}). The oxygen accepts two protons to form a water molecule and is released from the haem. Compound III designates a complex in which a molecule of oxygen is bound end-on to the ferrous iron of the peroxidase. It is usually formed when there is a large excess of H_2O_2 (de Montellano, 2010). It is not ordinarily a catalytically active intermediate, although it may play a role in the oxidation of isoniazid by the catalaseï peroxidase KatG of *Mycobacterium tuberculosis* (Ghiladi *et al*., 2005).

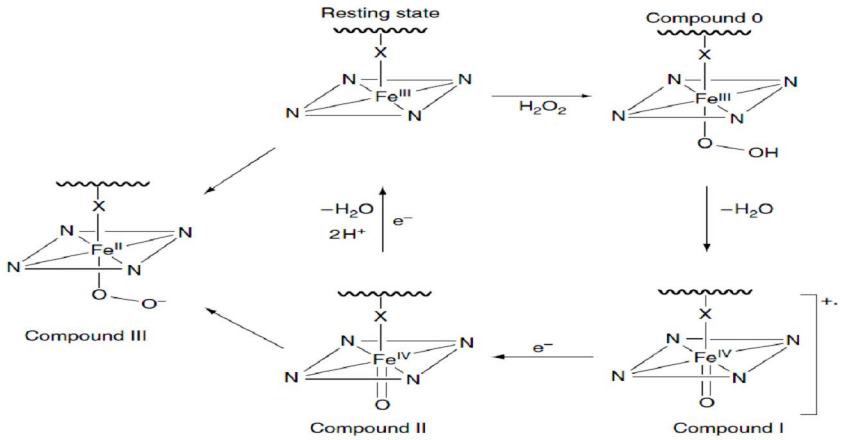


Figure 4: Generic peroxidase catalytic cycle. The square of four nitrogens around the iron atom is a representation of the prosthetic heme group of the peroxidase (de Montellano, 2010).

The prosthetic haem in the resting peroxidases is in the ferric state. In HRP and most peroxidases, the iron is five coordinate, high spin, Smulevich *et al*. (1994), with a histidine as the proximal iron ligand and a water molecule in the distal side that is not coordinated to the iron (de Montellano, 2010).

The catalytic cycle of horseradish peroxidase (HRP C) with ferulate as reducing substrate is shown in figure 5.

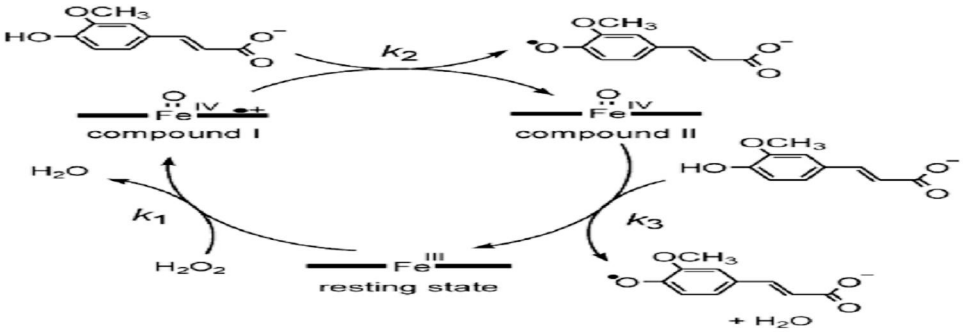


Figure 5: The catalytic cycle of horseradish peroxidase (HRP C) with ferulate (Azevedo et al., 2003).

Ferulic acid (FA) is a phenolic cinnamic acid derivative found in the plant cell wall, which acts as an *in vivo* substrate of plant peroxidases (Azevedo *et al*., 2003). The first step in the catalytic cycle is the reaction between H_2O_2 and the Fe (III) resting state of the enzyme to generate compound I, a high oxidation state intermediate comprising an Fe(IV)-oxoferryl centre and a porphyrin-based cation radical. A transient intermediate (compound 0) formed prior to compound I has been detected in reactions between HRP C and H_2O_2 at low temperatures and described as

an Fe(III)-hydroperoxy complex. Molecular dynamics simulations of these peroxide-bound complexes have been carried out (Filizola and Loew, 2000). It has been shown that the free radical produced by peroxidase only move on the surface of the enzyme and not in solution (Xialing and Lin, 2009).

Table 4: Common Substrates for Plant Peroxidase

Common name	Synonym	Detection method
ABTS	2,2′-Azino-di(3-ethylbenzothiazolin-6-sulfonate)	Spectrophotometric
Benzidine	4,4′-Diaminobiphenyl	Spectrophotometric
TMB	3,3′,5,5′-Tetramethylbenzidine	Spectrophotometric
DAB	3,3′-Diaminobenzidine	Spectrophotometric
Guaiacol	2-Methoxyphenol	Spectrophotometric
Pyrogallol	1,2,3-Trihidroxybenzene	Spectrophotometric
Phenol	Hydroxybenzene	Spectrophotometric
p-Cresol	4-Methylphenol	Spectrophotometric
o-Dianisidine	3,3′-Dimethoxybenzidine	Spectrophotometric
p-Toluidine	1-Amino-4-methylbenzene	Spectrophotometric
Tolidine	3,3′-Dimethylbenzidine	Spectrophotometric
Hydroquinone	1,4-Dihydroxybenzene	Spectrophotometric
Resorcinol	1,3-Dihydroxybenzene	Spectrophotometric
Catechol	1,2- Dihydroxybenzene	Spectrophotometric
4-Aminoantipyrine	4-Amino-2,3-dimethyl-1-phenyl-3-pyrazolinone	Spectrophotometric
p-Anisidine	1-Amino-4-methoxyphenol	Spectrophotometric
o-Phenylenediamine	1,2-Diaminophenol	Spectrophotometric
Luminol	3-Aminophthalhydrazide	Fluorimetric
Ferulic acid	4-Hydroxy-3-methoxycinnamic acid	Fluorimetric
Caffeic acid	3,4-Dihydroxycinnamic acid	Fluorimetric

(Azevedo, 2003)

1.1.6 Peroxidase Activity

Peroxidase activity involves donating electrons that bind to other substrates such as ferricynides and ascobate, in order to break them into harmless components (Bansal *et al*., 2012). Class III peroxidase activity is higher in roots than aerial parts and increased with the age of the plant (Cosio and Dunand, 2011). Colorimetrical, electrochemical and chemiluminescent methods are used for the detection of peroxidase activity (Mackova *et al*., 2001). Classical colorimetric methods are generally based on the monitoring of the formation of a coloured product from a colourless oxygen acceptor (Conyers and Kidwell, 1991). These oxygen acceptor (H

donor) compounds include benzidine, *o*-tolidine, *o*-toluidine, pyrogallol, *o*-dianisidine, *o*-phenylendiamine, guaiacol, 4-chloro-1-naphthol etc. The chemiluminescent method is based on the oxidation of cyclic diacylhydrazides accompanied by the emission of light (Arakawa *et al*., 1979). Enhanced chemiluminescent (ECL) is achieved by performing the oxidation of luminol with HRP in the presence of chemical enhancers such as certain phenols, naphthols, etc. (Thorpe and Kricka, 1986). The main advantages of ECL are that the light emission is intense, easily measured, the peroxidase activity can be assayed in seconds and is sensitive (Mackova *et al*., 2001).

1.1.7 Inhibition of Peroxidase

Many peroxidase inhibitors have been used in horseradish peroxidase mediated immunostaining and *in situ* hybridization to quench background peroxidase activity. Citrate and pyrophosphate inhibit the peroxidase-catalyzed oxidation of indoleacetic acid when cerium or manganese is used as the metallic cofactor (Mudd and Burris, 1959). Phenolic inhibitors such as 7-Hydroxy-2,3-dihydrobenzofuran derivatives, metabolites of a carbamate insecticide carbofuran, inhibits indoleacetic acid (IAA) oxidase which interferes with IAA-induced spectral change in the Soret band of horseradish peroxidase (HRP). The onset of IAA degradation required transformed HRP intermediates. The inhibitors, when added before IAA, protected HRP from reacting with IAA, thus preventing formation of highly reactive enzyme intermediates, and consequently, IAA degradation (Lee, 1977). The cytochemical reaction for peroxidase is partially inhibited by methanol. The addition of a small amount of sodium nitroferricyanide to the absolute methanol causes further inhibition (Straus, 1971). Sodium azide has been reported to inhibit peroxidase activity irreversibly (Saini *et al*., 1995). However, Saboora *et al.* (2012) demonstrated that peroxidase from turnip was inhibited by sodium cyanide rather than sodium azide. Astilbin (5,7,3',4'-tetrahydroxy-2,3-dihydroflavonol-3-ɓ-O-rhamnoside) is also an efficient inhibitor of peroxidase activity (Petacci *et al*., 2010). It has been associated with a large range of biological activities, such as lowering total cholesterol in the liver, Igarashi *et al*. (1996), protection against oxidative damage to the mitochondria and erythrocyte haemolysis in the liver (Haraguchi *et al*., 1996)

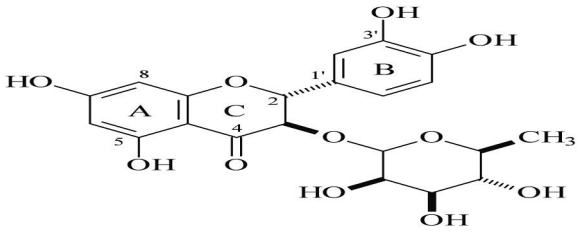


Figure 6: Structure of Astilbin (5,7,3',4'-tetrahydroxy-2,3-dihydroflavonol-3-β-O-rhamnoside)

1.1.8 Functions of Plant Peroxidase

Peroxidase is relatively stable at high temperature. It has been shown that POD can recover its activity after heat treatment (Anthon and Barrett, 2002). This property of the enzyme has been associated with deterioration of food quality during storage (Eze, 2012). It also affects the organoleptic properties such as colour, taste and aroma, causing off-flavours, off-colours, browning and nutritional damage (Mdluli, 2005). POD has been used as a model enzyme in the study of protein structure, enzyme reactions and enzyme function. Studies have shown that POD plays a role in plantôs lignifications, suberization, cross-linking of cell wall structural proteins, auxin metabolism, self-defence against pathogens, senescence, salt tolerance and oxidative stress. The primary function of ascorbate peroxidase is peroxidase detoxification (Raven, 2003).

1.1.9 Industrial Application of Plant Peroxidase

Peroxidase has a high commercial value, due to its versatile and wide applicability, from organic synthesis to biomedicine. Reduction of peroxidases at the expense of electron donating substrates makes peroxidases useful in a number of biotechnological applications. They have the potential to decrease environmental pollution by bioremediation of wastewater containing phenols, cresols and chlorinated phenols, for biopulping and decolourization of synthetic textile azo-dyes (Bansal *et al.*, 2012). They are also used in analytical applications in diagnostic kits for quantification of cholesterol, glucose, uric acid, lactose etc., in the fine chemical and pharmaceutical industries, and it is most common enzyme used for labeling an antibody in Enzyme-linked Immunosorbent Assay (ELISA).

1.1.9.1 Enzyme label and diagnostic kits

HRP is used as an enzyme label in medical diagnostics and research processes. Universal covalent conjugates of proteins, antibodies and other molecules with HRP, offer a wide range of amplifying possibilities. They are useful and versatile tools for ultra-sensitive detection in nucleic acid detection (Azevedo *et al.*, 2003). The ability of peroxidase to yield chromogenic products at low concentrations and its relatively good stability makes it useful for the preparation of enzyme conjugated antibodies and application in diagnostic kits (Bansal *et al*., 2012). HRP is one of the many biological components used in the diagnostic test kits. The most widely used test kits are the glucose, uric acid and cholesterol blood sensors. The assay for the determination of uric acid is based on the following reaction;

Uric acid $+O_2 + 2H_2O$ Uricase Ÿ Aallantoin $+ H_2O_2 + CO_2$

H_2O_2 + 4-aminophenazone + phenol peroxidase Ÿ p-(Benzoquinone) monoiminephenazone

Analysis of uric acid in human serum from ten different patients using either the kit containing turnip peroxidase or a commercially available kit, gave the same results (Hamid, 2009).

1.1.9.2 Bioremediation and wastewater treatment

Peroxidases have been applied to the bioremediation of waste waters contaminated with phenols, cresols and chlorinated phenols (Hamid, 2009). Phenol, substituted-phenols and azo dyes constitute examples of such hazardous compounds which can be found in wastewaters of a wide variety of industries (Nicell *et al*., 1993). Conventional methods of removing such pollutants such as adsorption, sedimentation, coagulation, and filtration result in a secondary waste which in itself is a problem to dispose (Ahmad and Puasa, 2007; Riera-Torres *et al*., 2010 and Amini *et al*., 2011;). HRP together with H_2O_2 have been used to remove phenolic compounds from synthetic model effluents and also from real industrial effluents (Zhang and Nicell, 2000). The addition of natural coagulants such as chitosan or mineral coagulants such as aluminium sulphate can aid in the precipitation of polymerization products and stabilization of HRP (Cooper and Nicell, 1996). The presence of chitosan has been shown to reduce the final toxicity (Wagner and Nicell, 2002).

1.1.9.3 Degradation of pesticides, polychlorinated aromatic hydrocarbons (PAHs)

Less than 5% of pesticides applied in controlling the harmful effects of insects, microorganisms and grasses on plants actually reach the target organisms. The remaining percentages leach down to subsoil and contaminate the groundwater (Kookana *et al*., 1998). The quality of soils, ground water, continental and coastal waters as well as the air, is compromised by this contamination (Surekha *et al*., 2008). Subsoil and groundwater pollution are the major consequences/outcomes environmental effects of pesticides application (Nawaz *et al*., 2011). This exposure is associated with chronic health problems or health symptoms such as respiratory problems, memory disorders, dermatologic conditions, cancer, depression, neurologic deficits, miscarriages and birth defects (McCauley *et al*., 2006). Peroxidases extracted from various species have great potential to transform several pesticides into harmless form(s). Transformation of organophosphorus pesticides by white-rot fungi has been studied (Jauregui *et al*., 2003). PAHs are composed of two or more fused aromatic rings and are components of crude oil, creosote and coal (Harayama 1997). PAHs are oxidized by peroxidases such as lignin peroxidase and manganese peroxidase (Harford-Cross *et al*., 2000; Weber *et al.,* 2008)

1.1.9.4 Organic and polymer synthesis

Plant peroxidase has been shown to exhibit a remarkable chemo- and stereospecific chemical transformations. They are able to catalyze numerous selective oxidations of reducing substrates and to resolve chiral hydroperoxides by enantioselectively reducing them to alcohols (Hoch *et al*., 1997; Adam *et al*., 1998). Examples include free-radical polymerization of vinyl monomers, such as acrylamide, acrylic acid and methacrylates, such as methyl, phenylethyl, 2-hydroxyethyl methacrylate (Karla and Gross, 2002). Using an anionic peroxidase purified from the African oil palm tree, an enzymatic synthesis of the polyelectrolyte complex of polyaniline and also sulfonated polystyrene has been developed (Sakharov *et al*., 2003).

1.1.9.5 Deodorization of manure

Plant peroxidase could be used as an enzymatic source in the deodorization of swine slurry (Govere *et al*., 2007). Odorant compounds such as phenols, indoles, volatile fatty acids, ammonia, hydrogen sulfide and mercaptans are either initially present in manure or result from anaerobic transformation of animal wastes (Zahn *et al*., 1997). A 100% reduction in the concentration of phenolic odorants without reoccurrence within 72 hours was achieved by using HRP (Govere *et al*., 2005).

1.1.9.6 Decolourization of dyes

Dye wastes represent one of the most problematic groups of pollutants, considered as xenobiotics that are not easily biodegradable (Ong *et al*., 2011). This dye effluent may contain chemicals that are toxic, carcinogenic, mutagenic, or teratogenic to various fish species (Celebi et al., 2012). Several physico-chemical methods such as adsorption, chemical treatment and ion pair extractions have been adopted and proven to be costly while producing large amounts of sludge (Leelakriangsak and Borisut, 2012). Peroxidases have been reported as excellent oxidant agents to degrade dyes (Kirby *et al*., 1995). Several bacterial, fungal and plant peroxidases have been used for decolorization of synthetic textile dyes. Decolorization of different azo dyes by *Phanerochaete chrysosporium* RP 78 under optimized conditions was studied by reaction mechanism via azo dye (Ghasemi *et al*., 2010). Peroxidase produced by *Pseudomonas sp.* was used in the biodegradation of Malachite green via a proposed mechanism.

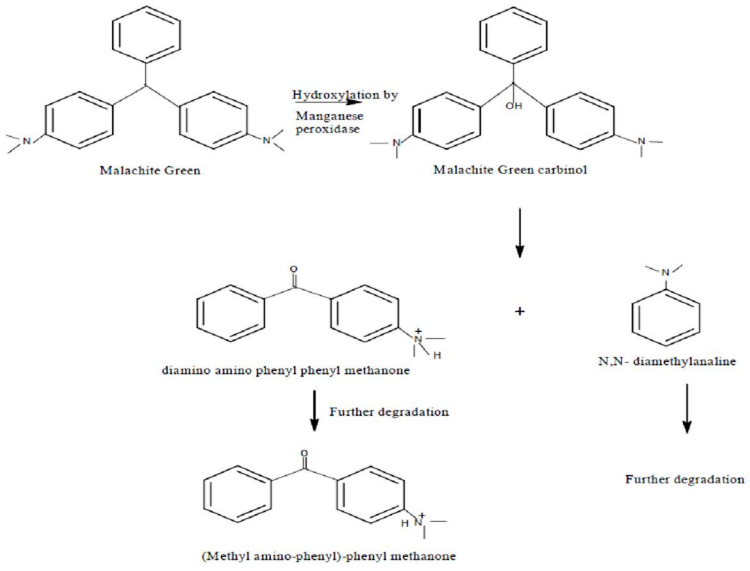


Figure 7: The proposed biodegradation pathways of Malachite green by *Psedomonas sp.* strain DY 1 (Bansal *et al*., 2012).

1.2 Dyes

Dyes are complex aromatic compounds, which are normally used for colouration of various substrates like leather, textiles, papers, fur, hair, drugs, cosmetics, waxes, greases and plastics (Maddhinni *et al*., 2006). Textile dyes are aromatic compounds representing a major class of organic pollutants that are found in the waste effluent discharged by different industries such as textile, petroleum refining, paper and pulp, leather and plastics, wood preservation, etc (Kalsoom *et al*., 2013). Dyes are normally water-soluble or water dispersible organic compounds that are capable of being absorbed into the substrate destroying the crystal structure of the substance. The dye molecules are usually chemically bonded to the surface and become a part of the material on which it is applied. The chemical constituents of the dye are mainly phenolic compounds. The colour intensity of the dye molecule depends on how strongly it absorbs radiation in the visible region, which extends from 400 to 700 nm. Today Asia (India, Japan, Korea and China) has become the largest dyestuff market, accounting for about 42% of the value of the global dyestuff market.

Table 5: Wavelengths and their Complementary colours

Colour absorbed	Wavelength absorbed (nm)	Colour observed
Red	647-700	Green
Orange	585-647	Cyan (Green-Blue)
Yellow	570-585	Blue
Green	491-570	Red
Blue	424-491	Yellow
Violet	400-424	Yellow-Green

1.2.1. Chromophores and Auxochrome

The partial structures necessary for colour (unsaturated groups that can undergo ´-´* and n-´* transitions) are called *chromophores*. The color in dyes is the consequence of the presence of a chromophore. Chromophores in dyes are generally large systems of conjugated double bonds (alternating double single bonds). It is this delocalized electron system that absorbs the energy from the light. The presence of some other groups caused an intensification of colour. These groups are called *auxochromes*. Dyes also contain auxochromes, which are a group of atoms attached to a chromophore that modify the ability of that chromophore to absorb light. They can also provide a site by which the dye can chemically bond to the fabric. They cannot undergo ´-´* transitions, but can undergo transition of n electrons.

Examples of chemical groups that are chromophores and auxochromes

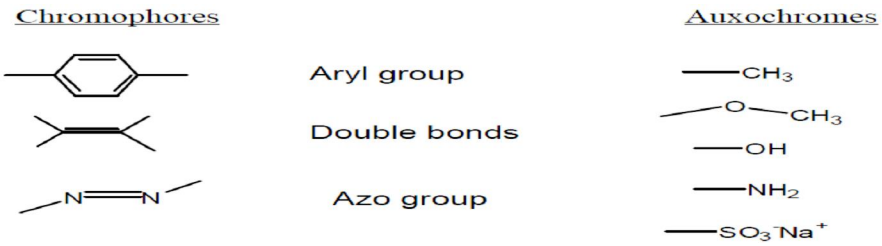


Figure 8: Some examples of chromophores and auxochrome

1.2.2 Vat dyes

The vat dyes are insoluble complex polycyclic molecules based on the quinone structure (ketoforms). They are reduced with sodium hydrosulphite in a strongly alkaline medium to give soluble leuco forms that have a great affinity for cellulose. An example of a vat dye is Vat Blue 4 (Indanthrene).

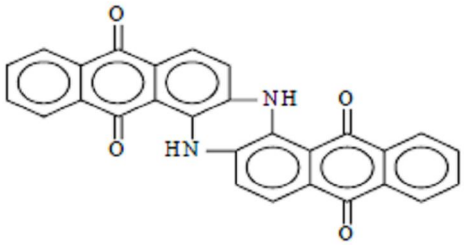


Figure 9: Structure of Vat Blue 4

1.2.3 Azo dyes

Azo dye accounts for almost 80% of annual production of commercial dyes all over the world (Fu and Viraraghavan, 2001). They are the most common group of

synthetic colorants released into the environment (Saratale *et al.*, 2011). They contain at least one azo group (-N=N-) attached to one or often two aromatic rings. Dye molecules bind to the fiber molecules in cloth via electrostatic attraction, by *van der Waals* forces, through hydrogen bonding or even by covalent bonds. Azo dyes are largely resistant to biodegradation and persist in conventional wastewater treatment processes (Stolz, 2001). Some azo dyes, their precursors and a number of their reaction products are carcinogenic (Brown and De Vito 1993). Methyl yellow, methyl orange, methyl red, congo red and alizarine yellow are some of the examples.

1.2.3.1 Synthesis of Azo Dye

Azo dyes are prepared in a two-step reaction, the first being the synthesis of an aromatic diazonium ion from an aniline derivative. The next step is coupling of the diazonium salt with an aromatic compound.

1. **Diazotization:** This involves reacting primary amine (NH_2) with sodium nitrite ($NaNO_2$). The primary amines include Aminobenzene, 2-amino-1,5-naphthalenedisulfonic acid, 3,5-dimethoxy aniline, 6-amino-2-naphthalenesulfonic acid, 6-amino-1-naphthalene, 4-aminobenzenesulphonic acid, 4-nitroaniline e.t.c.
2. **Coupling**: This involves the coupling of the diazonium salt with an aromatic compound.

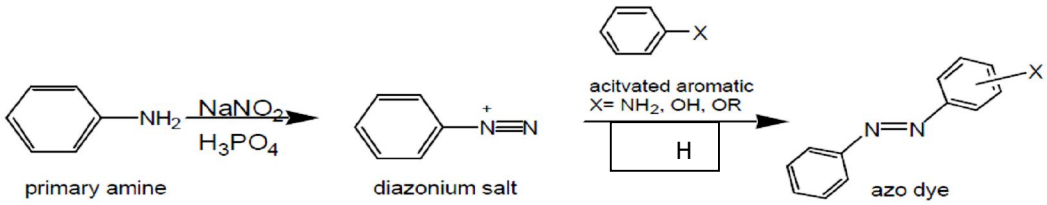
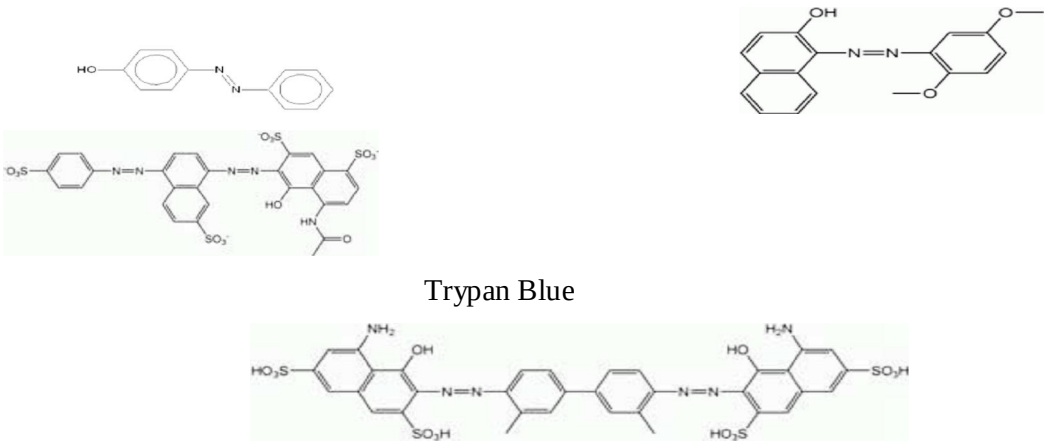


Figure 10: The general synthesis of Azo dye

The aromatic ring can be substituted with different functional groups (auxochromes) and these affect the colour of the dye.

1.2.3.2 Some Examples of Azo Dyes

Azo Yellow 6 Citrus Red 2 Brilliant Black



Trypan Blue

Figure 11: Molecular structure of some Azo dyes.

1.2.3.3 Azo Dyes and Their Mutagenic Effects

The azo dyes show good fiber-fixation (about 85%) properties as compared other synthetic dyes and this explains why so much dye is released into the environment, representing the other 10 to 15% of the amount used. Most of these dyes found are not degraded by the conventional treatments and this shows a wide range of toxic effects on aquatic life and higher organisms. Azo dyes can also be absorbed after skin exposure from the use of cosmetic products (Chequer *et al*., 2011). Tsuboy *et al*. (2007) analyzed the mutagenic, cytotoxic and genotoxic effects of the azo dye CI Disperse Blue 291, and the results clearly showed that this azo dye caused dose-dependent effects, inducing the formation of micronuclei (MNs), DNA fragmentation and increasing the apoptotic index in human hepatoma cells (HepG2). The mutagenic, carcinogenic and toxic effects of the azo dyes can be a result of direct action by the compound itself, or the formation of free radicals and aryl amine derivatives generated during the reductive biotransformation of the azo bond (Rajaguru *et al*., 1999). It may also be caused by products obtained after oxidation via cytochrome P450 (Umbuzeiro *et al*., 2005). The consequent generation of aromatic amines by dyes when in contact with sweat, saliva or gastric juices is used to classify dyes as harmful (Pielesz *et al*., 2002). Some such aromatic amines are carcinogenic and can accumulate in food chains. It has been shown that rats and mice exposed to specific azo dye arylamines or their derivatives developed cancer, mainly in the liver (Weisburger, 2002). Azo bond reduction leading to the production of aromatic amines has been demonstrated under a variety of conditions, including those encountered in the digestive tract of mammals (Pinheiro *et al*., 2004). Therefore, the majority of

possible hazards arising from the use of azo dyes are now being directed at their reduction products.

1.2.3.4 Metabolism of Azo Dyes in Humans

Following oral or skin exposure to azo dyes, humans can subsequently be exposed to bio-transformation products obtained by the action of intestinal microorganisms or that of others present on the skin or due to reactions in the liver (Esancy *et al*., 1990; Chadwick *et al*., 1992; Stahlmann *et al*., 2006). The metabolic pathways the azo dyes actually follow depend on several factors, such as,

a. the mode of administration;
b. the degree of absorption from the gastro-intestinal tract after oral ingestion;
c. the extent of biliary excretion, particularly after exposure to different routes other than the oral one;
d. genetic differences in the occurrence and activity of hepatic reducing-enzyme systems;
e. differences in the intestinal flora;
f. the relative activity and specificity of the hepatic and intestinal systems, particularly those responsible for reducing the azo link (Walker, 1970).

Biotransformation may produce less harmful compounds, but it may also form bioactive xenobiotics, ie, compounds showing greater toxicity. The main routes involved in the biotransformation of dyes are oxidation, reduction, hydrolysis and conjugation, which are catalyzed by enzymes (Zollinger, 1991; Hunger, 1994). However, in humans, biological reductions and oxidations of azo dyes are responsible for the possible presence of toxic amines in the organism (Pielesz *et al*., 2002).

1.2.3.4.1 Oxidative Metabolism

Highly lipid-soluble azo dyes, with chemical structures containing amino groups, either alkylamino or acetylamino, but without sulfonated groups, are preferentially biotransformed by oxidative reactions. Oxidation processes are mainly catalyzed by a microsomal monooxygenase system represented by cytochrome P450 (Hunger, 1994). The general mechanism of metabolic oxidation involves an electron transport chain, which first transfers an electron to the P-450-Fe^{3+} complex, which, on reduction, receives an oxygen atom and in the final steps, leads to the formation of an oxidation product in the organism (Chequer *et al*., 2011).

There are three different oxidation pathways of importance for azo dyes:

i. C-Hydroxylation-ring hydroxylation, probably via an epoxidation mechanism and subsequent rearrangement to a phenol;
ii. N-Hydroxylation at primary or secondary amino groups, or with acetyl amino groups in the liver. This reaction is followed by esterification with glucuronate or sulfate. The activated esters, which are water-soluble, can be excreted, or the ester group can split off with the formation of a nitrenium compound ($-NH^{+}$), which can covalently bind to a nucleophilic group of the DNA.
iii. Demethylation, which is the stepwise oxidation of the methyl groups of dialkylamino compounds, and the N-hydroxy derivative so formed can be further demethylated or react to form a nitrenium compound (Hunger, 1994).

Studies on the metabolism and carcinogenicity of N,N-dimethylaminoazobenzene (Butter Yellow), a classical hepatocarcinogen in rats, have shown that N-methylaminoazobenzenes are mainly metabolized by N-demethylation (Chequer *et al*., 2011).

1.2.3.4.2 Reductive Mechanism

Reductive cleavage of the azo linkages is probably the most toxicologically important metabolic reaction of azo compounds. This reaction can be catalyzed by mammalian enzymes, especially in the liver, Kennelly *et al*. (1982) or intestinal, Hartman *et al*. (1978) or skin bacteria such as *Staphylococcus aureus* (Platzek *et al*., 1999; Golka *et al*., 2004). The first catabolic step in the reduction of azo dyes is the cleavage of the azo bond, producing aromatic amines accompanied by a loss of colour of the dye (Cerniglia *et al*., 1986). If the dyes are completely reduced to aromatic amines, they can then be oxidized to N-hydroxyderivates by P450 enzymes and N-hydroxylamines can cause DNA damage. Nam and Reganathan (2000) demonstrated that both NADH and NADPH are capable of reducing azo dyes in the absence of any enzyme, under mildly acidic conditions. Azo dyes containing nitro groups can also be metabolized by the nitroreductases produced by microorganisms (Umbuzeiro *et al*., 2005).

1.3 O-dianisidine (3,3-Dimethoxybenzidine)

O-dianisidine (Molecular Formula: $C_{14}H_{16}N_2O_2$, molecular weight: 244.293 g/mol, Melting point: 135-139 ºC and density of 1.178g/cm^3) is used for reaction of peroxidase where it donates proton H^+ for the reduction of the enzyme. It is carcinogenic. It is soluble in ethanol (200 mg/mL - clear, violet-brown solution) or aqueous acids and almost insoluble in water. O-dianisidine is used for the detection of Au, Co, Cu, SCN-, V; determination of Au, NO2-, Ce(IV) (spectrophotometrically). There is evidence that dogs, rats, and humans metabolize o-dianisidine.

1.4 Hydrogen Peroxide (H_2O_2)

H_2O_2 plays an important role in clinical, chemical, biological and environmental fields. It helps to oxidize peroxidase into a catalytically active form that is capable of reacting with the phenolic contaminant. Peroxidase in-turn degrades H_2O_2 into water and oxygen. However, the mechanism of action of hydrogen peroxide on peroxidase was proposed using peroxidase-catalase superfamily. The stability of peroxidases has been found to be reduced by hydrogen peroxide (Park and Clark, 2006). The third enzyme specie (compound III) is produced when ferric peroxidases are exposed to an excess of H_2O_2. Compound III is a peroxy-Fe^{III}- porphyrin free radical, best described intermediate leading to the irreversible deactivation of the enzyme (Valderrama, 2010).

1.5 Cabbage

Cabbage *(Brassica oleracea* L. var. *capitata)* is a member of the *Brassicaceae* (Mustard) family. This family includes broccoli, brussels sprouts, cauliflower, kale, mustard (greens), and collards. Collectively, these crops are referred to as *cole* crops or *crucifers*. Cabbage is one of the most important dietary vegetables consumed in the world (Kusznierewicz *et al*., 2007). Worldwide, China is the leading producer and consumer of cabbage. It is cultivated for its head, which consists of water (92.8%), protein (1.4 mg), calcium (55.0 mg) and iron (0.8 mg). The leaves are eaten raw in salads or cooked (Adeniji *et al*., 2010). Nutritionally, cabbage is a good source of vitamins (A, C, E, and K), antioxidant enzymes (catalase and peroxidase) and other phytochemicals, such as glucosinolates or sulphur-containing compounds (Kurilich and Juvik 1999; Kopsell *et al*., 2004). The presence of phytochemicals, vitamins and provitamins, has been considered of great nutritional interest in the prevention of chronic diseases, such as cancer, arteriosclerosis, nephritis, diabetes mellitus,

rheumatism, ischemic and cardiovascular diseases and also in the aging process, in which oxidants or free radicals are involved (Chu *et al*., 2002; Pulido *et al*., 2000; Behl and Moosmann, 2002). Stoewsand (1995) attributed the cancer chemopreventive effects of *Brassica* vegetables to two types of phytochemicals: certain glucosinolates and *S*-methyl cysteine sulfoxide. However, peroxidase shows bad effect on the quality of cabbage. It causes post-harvest senescence, oxidation of phenolic substances, starch-sugar conversion and post-harvest demethylation of pectic substances leading to softening of the plant tissues during ripening (Ghahfarrokhi *et al*., 2013).

Plate 1: A typical mature cabbage (*Brassica oleracea L.*)

1.6. Aim and Objectives

This study is aimed at using peroxidase extracted from cabbage to decolorize some synthetic dyes.

It is designed to achieve the following specific objectives.

- Extraction of plant peroxidase from cabbage
- Assay for peroxidase activity using o-dianisidine
- Purification of peroxidase via ammonium sulphate precipitation, desalting (dialysis) and gel filtration.
- Treatment of the synthetic dyes with the partially purified form of the enzyme and hydrogen peroxide.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Materials

2.1.1. Equipment and Sources

The major equipment used for in this work were obtained from the departmental laboratory and are listed below;

Blender machine- Philips

Centrifuge- Finland Nigeria 80-2B

Magnet Stirrer- AM-3250B Surgi Friend Medicals, England

Water bath- Model DK

Weighing balance- Ohaus Dial-O-Gram, Ohaus Cooperation, N.J. USA

pH meter- Ecosan pH meter, Singapore

UV/ visible spectrophotometer- Jenway 6405

Refrigerator- Thermocool

2.1.2. Chemicals and Reagents

The reagents used for the study were of analytical grade and they include; the synthetic dyes (Azo Trypan Blue, Azo Blue 5, Azo Yellow 6, Citrus Red 2, Brilliant Black, Azo Pink, Azo Purple, Vat Orange 11 and Vat Green 9) which are the products of BASF Chemical Company, Germany and were gotten from the Clothing and Textile Unit, Fine and Applied Arts Department, Faculty of Arts, University of Nigeria Nsukka, Enugu State Nigeria.

The chemical used in this study were sourced as follows, Bovine serum albumin (BSA) - Bio Rad Laboratories (India); Folin Ciocalteau- Sigma-Aldrich (USA); O-

dianisidine- Sigma-Aldrich (Germany); Sephadex G25- PFC (Pharmacy Fine Chemicals); Hydrogen Peroxide- BDH Chemicals Ltd, Poole, England; di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4)- BDH Chemicals Ltd Poole England; Ammonium sulphate- Burgone Urbidges & Co (Mumbai India).

2.1.3 Plant material

Fresh cabbage head were bought from the Ogige market, Nsukka, Enugu State and were identify by the Department of Plant Science and Biotechnology, University of Nigeria Nsukka.

2.2. Methods

2.2.1. Extraction of Peroxidase

The cabbage (250g) was washed and blended with 500ml of phosphate buffer (0.05M) pH 6.0. The mixture was left for 24hours with frequent stirring with the magnetic stirrer. The homogenate was filtered with double-layered cheesecloth. The filtrate was centrifuged at 4000rmp for 30 minutes. The supernatant (the crude enzyme extract) were collected and stored at temperature below 5^oC.

2.2.2. Protein determination

Protein content of the crude enzyme extract was determined by the method of Lowry *et al*. (1951), using serum albumin (BSA) as the standard protein.

The reaction mixture contained 0.0- 1.0ml of BSA solution (at 0.1ml interval) in test tubes arranged in triplicates. The volume was made up to 1ml with distilled water. 5ml of solution E was added and allowed to stand at room temperature for about 10minutes. Then 0.5ml of solution C was added and stirred vigorously. The total volume of each test tube was 6.5ml. After standing for 30minutes, absorbance was read at 750nm using visible spectrophotometer. The mixture without the BSA solution was used as the blank. The absorbance of the crude enzyme was also determined. The mixture containing the crude enzyme extract contains all the reaction mixture without the BSA solution but 0.5ml of the crude enzyme instead.

2.2.3. Determination of enzyme activity

Peroxidase activity was determined using the method of Eze *et al*., (2000) with little modification.

The assay mixture contained 2.7ml of sodium phosphate buffer pH 6.0 (0.05M), 0.1ml of 0.8% H_2O_2 (0.025M), 0.1ml of 1% o-dianisidine and 0.1ml of the enzyme extract, giving a total volume of 3ml. The mixture was added in the order above. The mixture without the enzyme was first put in a cuvette before adding the enzyme. Immediately the enzyme was added, the change in absorbance due to oxidation of o-dianisidine in the presence of H_2O_2 was monitored using visible spectrophotometer at 460nm. The readings were taken for every 30seconds for 5minutes. The sodium phosphate buffer (0.05M) pH 6.0 was used as a blank.

One unit of the enzyme activity was defined as the amount of enzyme that gave an absorbance change of 0.1/min at 30^{o}C.

The variables below were used to calculate the purification steps of the experiments using the method of Khurshid *et al.* (2012).

Reaction rate (activity) $= \frac{(Change\ in\ Optic\ Density\ (\Delta OD) at\ 460nm)}{time\ interval}$

Since the volume of the enzyme used was 0.1ml and the time interval was 60secs (1min)

Activity$- \Delta OD\ at\ 460nm \times \frac{10}{1}$ (µmol/min)

Activity $(U/ml) = \frac{(\Delta OD)/min \times V \times Df}{\varepsilon \times v}$

Where V is total volume of reaction mixture= 3ml, *v* is enzyme volume= 0.1ml, Df is the dilution factor and ὐ is micromolar extinction coefficient of O-dianisidine= 11.3 $mM^{-1}.cm^{-1}$ at 460nm (Chanwun *et al*., 2012)

Therefore, Activity $= \Delta OD \times 2.655 \times Dilution\ factor$

Specific Activity $\left(\frac{Unit}{mg}\right) = \frac{Activity\left(\frac{U}{ml}\right)}{protein\ concentration\left(\frac{mg}{ml}\right)}$

Where total units = $\frac{units}{ml} \times total\ volume$

Percentage yield $= \frac{Total\ unit\ of\ purified\ enzyme}{Total\ unit\ of\ crude\ enzyme} \times 100$

Purification fold$= \frac{Specific\ Activity\ of\ purified\ enzyme}{Specific\ Activity\ of\ crude\ enzyme}$

2.2.4. Purification of peroxidase from *Brassica oleracea* (cabbage)

2.24.1. Ammonium Sulphate Precipitation Profile

Ammonium sulphate salt precipitation profile was carried out to determine the concentration of the salt that would give the highest precipitation of the peroxidase. This was done at different ammonium sulphate saturation ranging from 10% to 90% at interval of 10% in each test tube containing 10ml of the crude enzyme. A beaker containing the crude enzyme and a stir bar was placed on magnetic stirrer plate. While sample is stirring, ammonium sulphate of a desired saturation level was added slowly. Once total volume of ammonium sulphate was added, the mixture was poured into a test tube and allowed to stand at 4^{o}C for 30 hours. After then, the test tubes were centrifuged at 4000rpm for 30mins and the pellets were re-dissolved in 1ml of phosphate buffer pH 6.0 (0.05). Protein concentration and peroxidase activity of the precipitate were assayed to determine the percentage of ammonium sulphate that has the highest protein concentration and peroxidase activity.

2.2.4.2. Ammonium sulphate precipitation of the total crude

From the assayed precipitates, highest peroxidase activity and protein concentration was achieved at 80% ammonium sulphate saturation and was therefore used to precipitate 300ml of the crude enzyme. This was done by adding 154.8g of ammonium sulphate salt slowly in 300ml of the crude enzyme in a beaker placed on a magnet stirrer plate, until the salt was completely dissolved. The mixture was maintained at 4^{o}C for 30hours. The solution was centrifuged at 4000rpm for 30minutes after which the pellet was collected and dissolved with phosphate buffer pH 6.0. Ammonium sulphate was redissolved in the supernatant for more precipitation. It was maintained at 4^{o}C for 24hours, centrifuged and the pellet was also collected. The dissolved pellets were taken as the partially purified peroxidase. Protein concentration and peroxidase activity of the precipitate were then determined.

2.2.4.3. Desalting of protein (Dialysis)

The precipitated peroxidase was desalted by dialysis using the 10cm pretreated dialysis bag. One end of the dialysis bag was tightly tied and the precipitated enzyme was introduced inside the bag before the other end of the bag was tied. The dialysis bag was suspended in a beaker containing sodium phosphate buffer pH 6.0 (0.05M) placed on a magnet stirrer plate and allowed for 18hours with continuous stirring. The buffer was changed every 6hours with the intention of removing the ions and other low molecular weight substances that have diffused from the dialysis bag into the buffer. This is to avoid them from diffusing back to the dialysis bag once equilibrium is established. The diasylate was also assayed for peroxidase activity and protein concentration while the remaining sample was stored at -10^{o}C.

2.2.4.4. Gel Filtration Chromatography

2.2.4.4.1. Preparation of Sephadex G-25 gel

20g of sephadex G-25 gel was weighed and dissolved in distilled water and allowed to swell for 3 days with constant changing of the distilled water every 6 hours.

2.2.4.4.2. Introduction of enzyme and collection of fraction

Partially purified peroxidase (10ml) obtained from ammonium sulphate precipitation was subjected to gel filtration using sephadex G-25 gel. The gel was packed to the height of 17cm in a glass column (50cm by 2.5cm). The sodium phosphate buffer pH 6.0 (0.05M) was used to wash down the gel bed until it equilibrated (pH of eluted equals 6.0) and allowed to settle under gravity. After equilibration, the sample was introduced and was eluted using the same phosphate buffer. A total of 45 fractions of 5ml each were collected at drop rate of 5ml/9mins. The protein concentration of each fraction was assayed using UV/ Visible spectrophotometer at 280nm. The peroxidase activity of each fraction was also assayed and the fractions that showed highest peroxidase activity were pooled together after which the total volume was measured, recorded and stored at -10^{o}C.

2.2.5. Characterization of the enzyme

2.2.5.1. Effect of pH on peroxidase activity

The optimum pH value for peroxidase was determined by assaying enzyme activity at different pH. The assay was performed by using sodium acetate buffer (0.05M) pH 3.5-5.5, sodium phosphate buffer (0.05) pH 6.0-7.5 and Tris-HCl buffer (0.05M) pH 8.0-9.0 at 0.5 intervals. Peroxidase activity was assayed by using o-dianisidine substrate and the enzyme was introduced as stated in session 2.2.3.

2.2.5.2. Effect of Temperature on peroxidase activity

The optimum temperature of peroxidase was determined by incubating the peroxidase solution at 30-70^{o}C (interval of 5^{o}C) for 1 hour using pH 5.0. The activity was then assayed using the method described in session 2.2.3.

2.2.5.3. Kinetic study of the enzyme

2.2.5.3.1. Effects of different H_2O_2 concentration on peroxidase activity

Different concentrations of H_2O_2 (1-24mM) were prepared and used to assay for the peroxidase activity using acetate buffer pH of 5 at 45^{o}C. The change in activity was plotted against the change in H_2O_2, hence, the K_M and V_{max} were determined.

2.2.5.3.2. Effect of different O–dianisidine on peroxidase activity

Different concentrations of O-dianisidine (1mM-10mM) were prepared and used to assay for peroxidase activity as described in session 2.2.3. The change in activity was calculated and plotted against change in o-dianisidine concentration; hence, K_M and V_{max} were assayed.

2.2.8. Dye treatment with peroxidase

The solution of each dye was prepared by dissolving 0.5g of each dye in 500ml distilled water. After the individual preparation of the various dyes, they were scanned using UV/Visible Spectrophotometer to determine the wavelength of the dyes that have the highest peak at wavelength range of 200-850nm. Then the buffer, enzyme and H_2O_2 are added and scanned respectively to determine the effect of the cabbage peroxidase on the various dyes. This is done as follows;

Dye solution + scan

Dye + Buffer + scan

Dye + Buffer + Enzyme + scan

Dye + Buffer + Enzyme + 15minutes + scan

Dye + Buffer + Enzyme + 15minutes + H_2O_2+ 30minutes + scan

Dye + Buffer + Enzyme + 15minutes + H_2O_2 + 30minutes + 1hour + scan

Dye + Buffer + Enzyme + 15minutes + H_2O_2+ 30minutes + 1hour + 20hour + scan

Each of the reaction mixture contained 2.7ml of acetate buffer (0.05M) pH 5.0, 0.1ml of the dye solution, 0.1ml of H_2O_2 and 0.1ml of the peroxidase. The total volume of the reaction mixture is 3ml.

2.2.8.1. The percentage decolorization

The percentage decolorization of each dye was calculated by taking the absorbance of each dye mixture containing the dye, buffer, H_2O_2 and enzyme and added in the same order. The absorbance is taken immediately the enzyme is added after which is incubated for 1hour before the final absorbance was taken. The percentage decolorization is calculated thus;

Percentage Decolorization$= \frac{Ai - Af}{At} \times 100$

Where A_i ï initial absorbance before incubation

A_f ï final absorbance after incubation

CHAPTER THREE

RESULTS

3.1 Cabbage peroxidase extraction

A known volume, 1500ml of the crude enzyme was extracted from the cabbage.

3.2 Studies on the crude enzyme

Protein concentration of the crude peroxidase extracted from the cabbage was found to be 0.942mg/ml. Peroxidase activity of the crude enzyme which was monitored by the change in absorbance at 460nm due to the oxidation of the o-dianisidine in the presence of H_2O_2 and peroxidase was found to be 3.98U/ml.

3.3 Ammonium sulphate precipitation profile for cabbage peroxidase

Crude peroxidase when saturated from 20-90% with ammonium sulphate was found to give the highest precipitation of the enzyme at 80%. The peroxidase activity increased with increasing ammonium sulphate saturation until it reached 80% saturation. Hence 80% saturation was used to precipitate 300ml of the crude enzyme.

3.4 Studies on the ammonium sulphate precipitated enzyme

After ammonium sulphate precipitation, the protein concentration was found to be 0.801mg/ml while the peroxidase activity was found to be 15.93U/ml.

3.5 Studies on the dialyzed enzyme

After dialysis, the protein concentration reduced to 0.289mg/ml while the peroxidase activity increased to 20.71U/ml.

3.6 Studies on the purified enzyme after gel filtration

After gel filtration, the chromatogram showed four sharp protein concentration peaks at fractions 11, 12, 23 and 25, read at 280nm. Peroxidase activity (U/ml) peaks were observed at fractions 11, 12, 13, 14, 15.

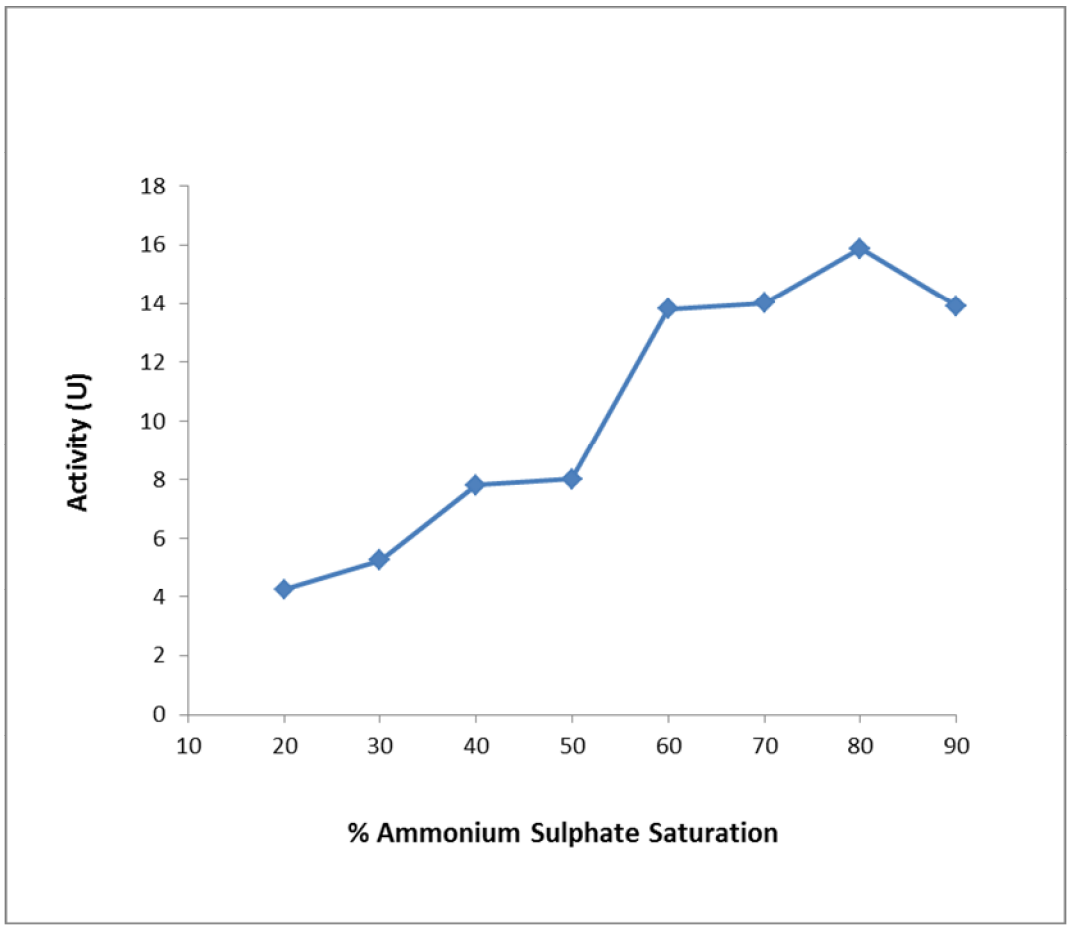


Figure 12: Ammonium sulphate precipitation profile for peroxidase extracted from cabbage

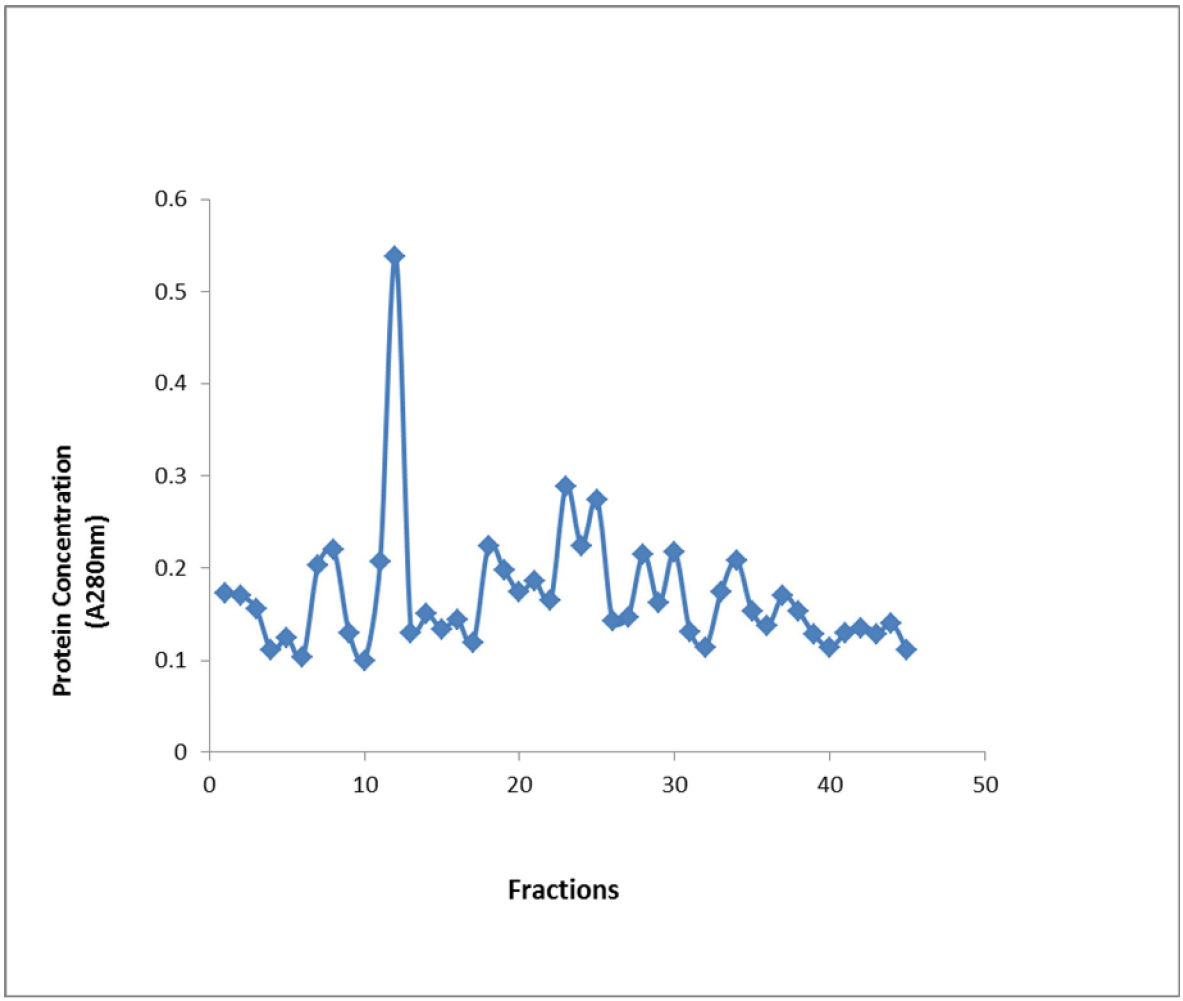


Figure 13: Gel filtration chromatography profile for protein concentration

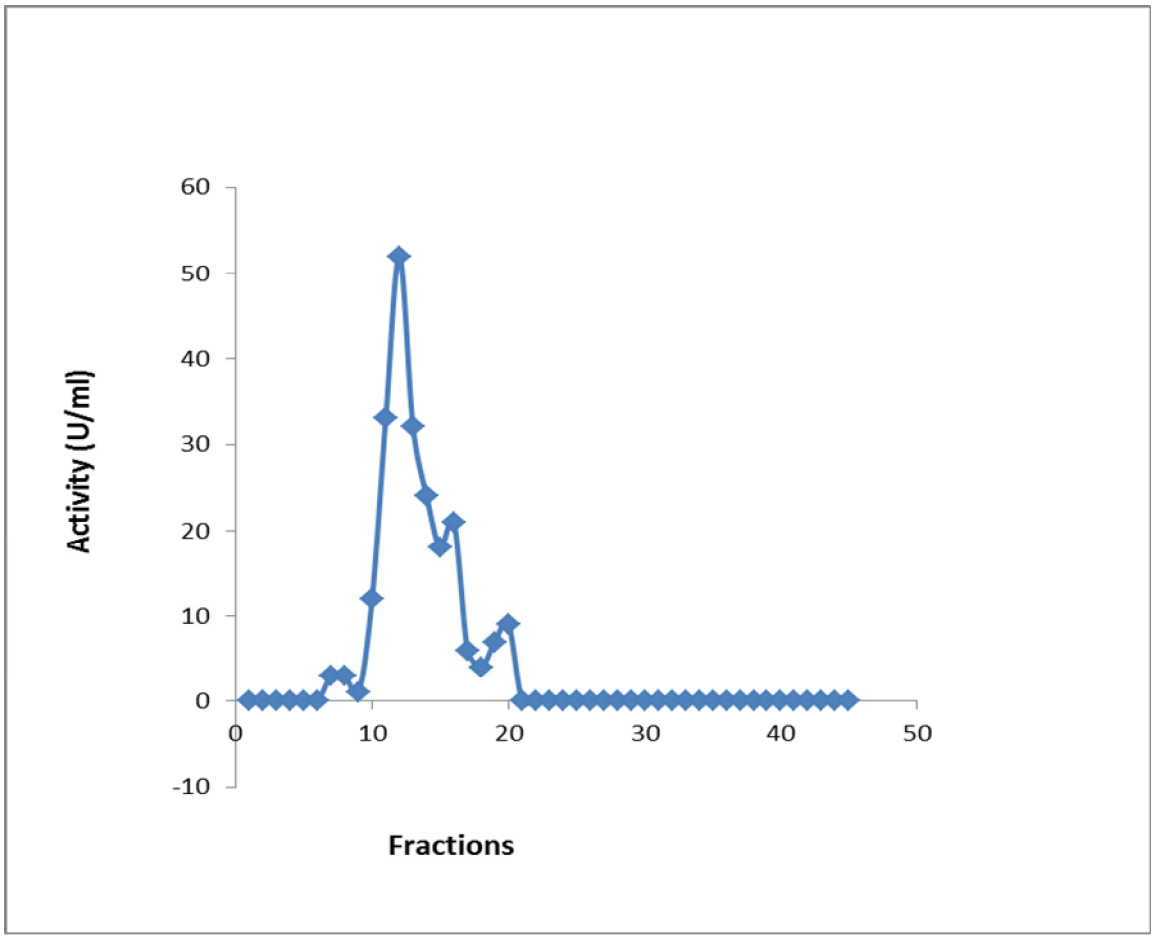


Figure 14: Gel filtration chromatography profile for peroxidase activity

3.7 Change in protein concentration of partially purified enzyme

Protein concentration of the crude peroxidase was found to be 0.94mg/ml which decreased after each purification step with the dialyzed enzyme having the least value.

3.8 Changes in Activity of the partially purified peroxidase

Peroxidase activity was found to increase after each purification step with the peroxidase after gel filtration having the highest activity.

3.9 Specific Activity of Partially purified Enzyme

The specific activity of the crude cabbage peroxidase was found to be 7.5U/mg. This value increased in each purification step with the dialyzed enzyme having the highest.

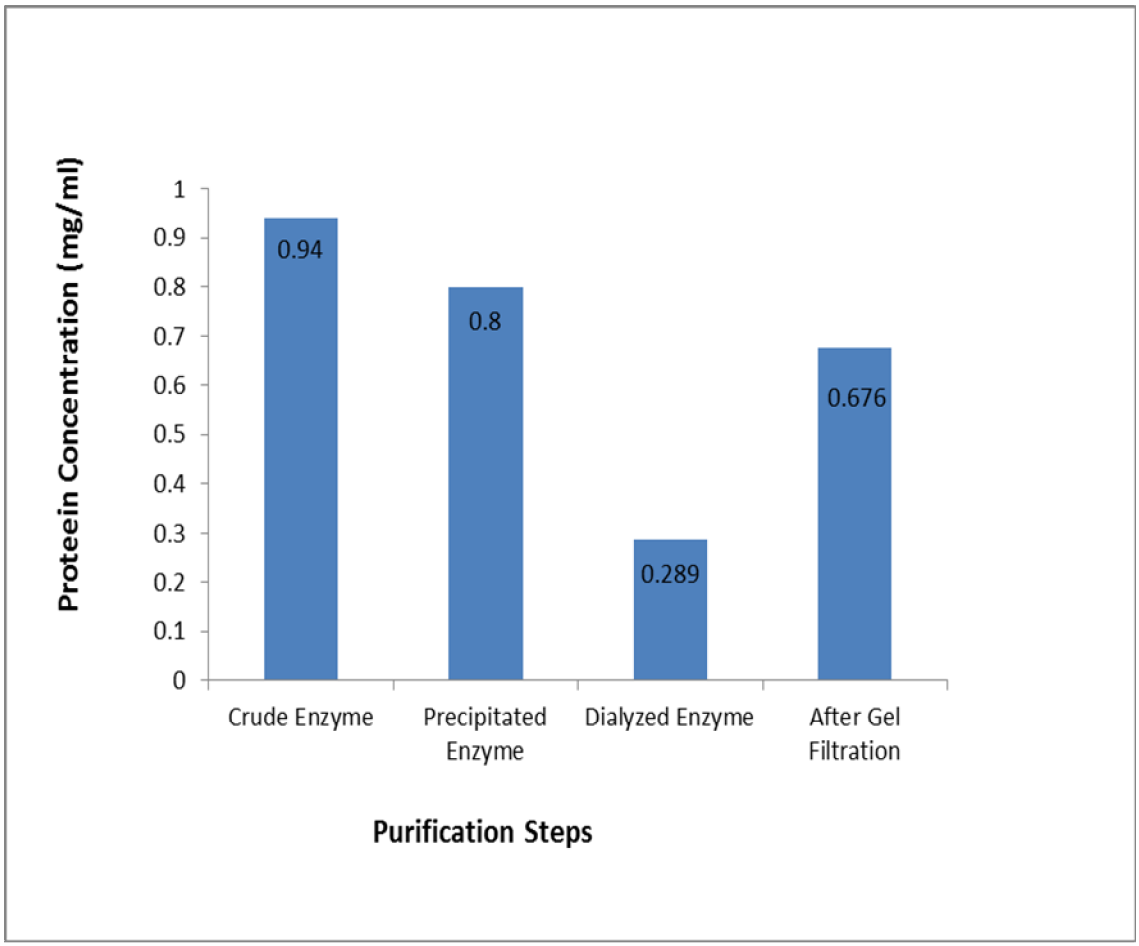


Figure 15: Protein concentration of the partially purified peroxidase

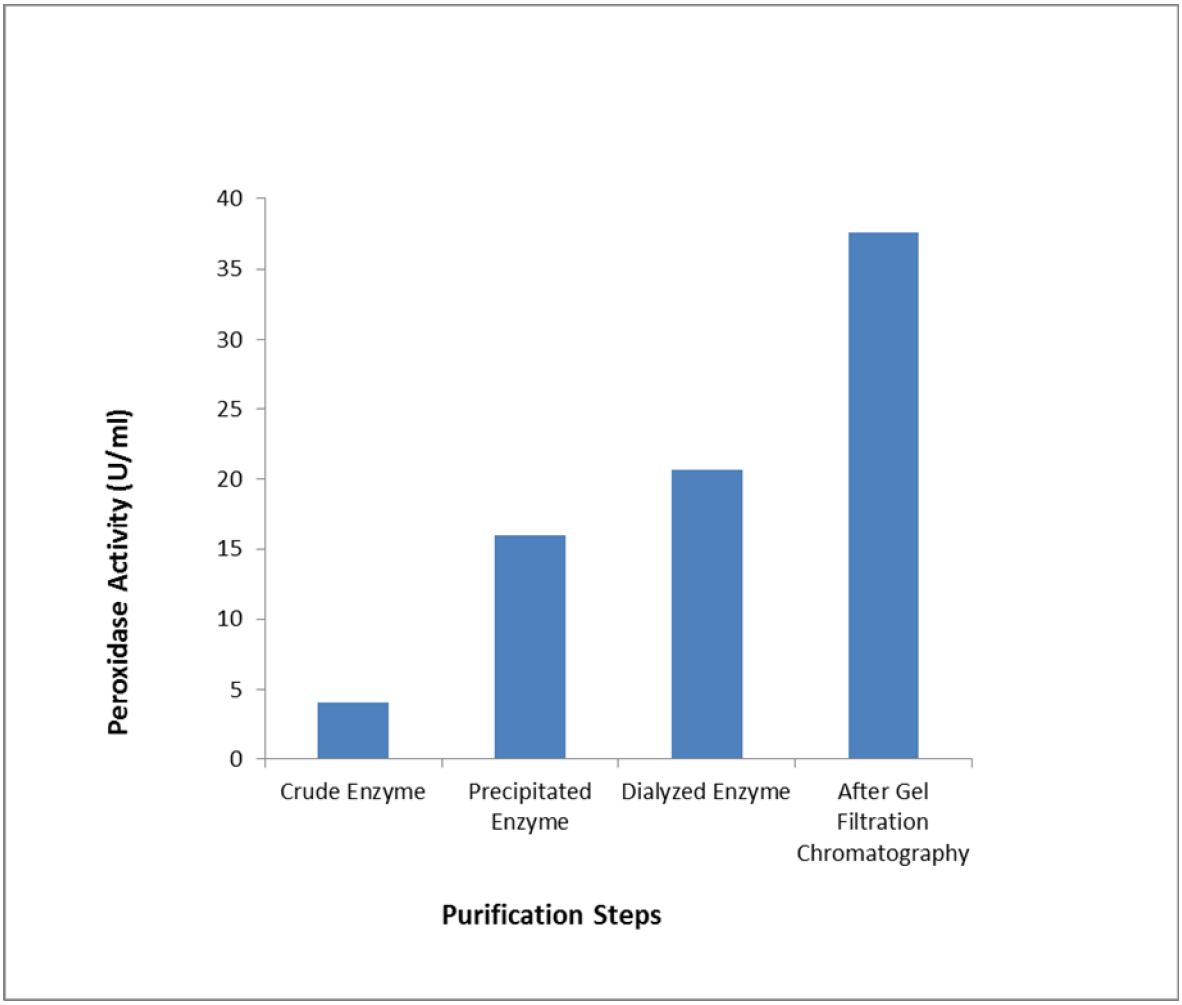


Figure 16: Peroxidase Activity of the partially purified cabbage peroxidase

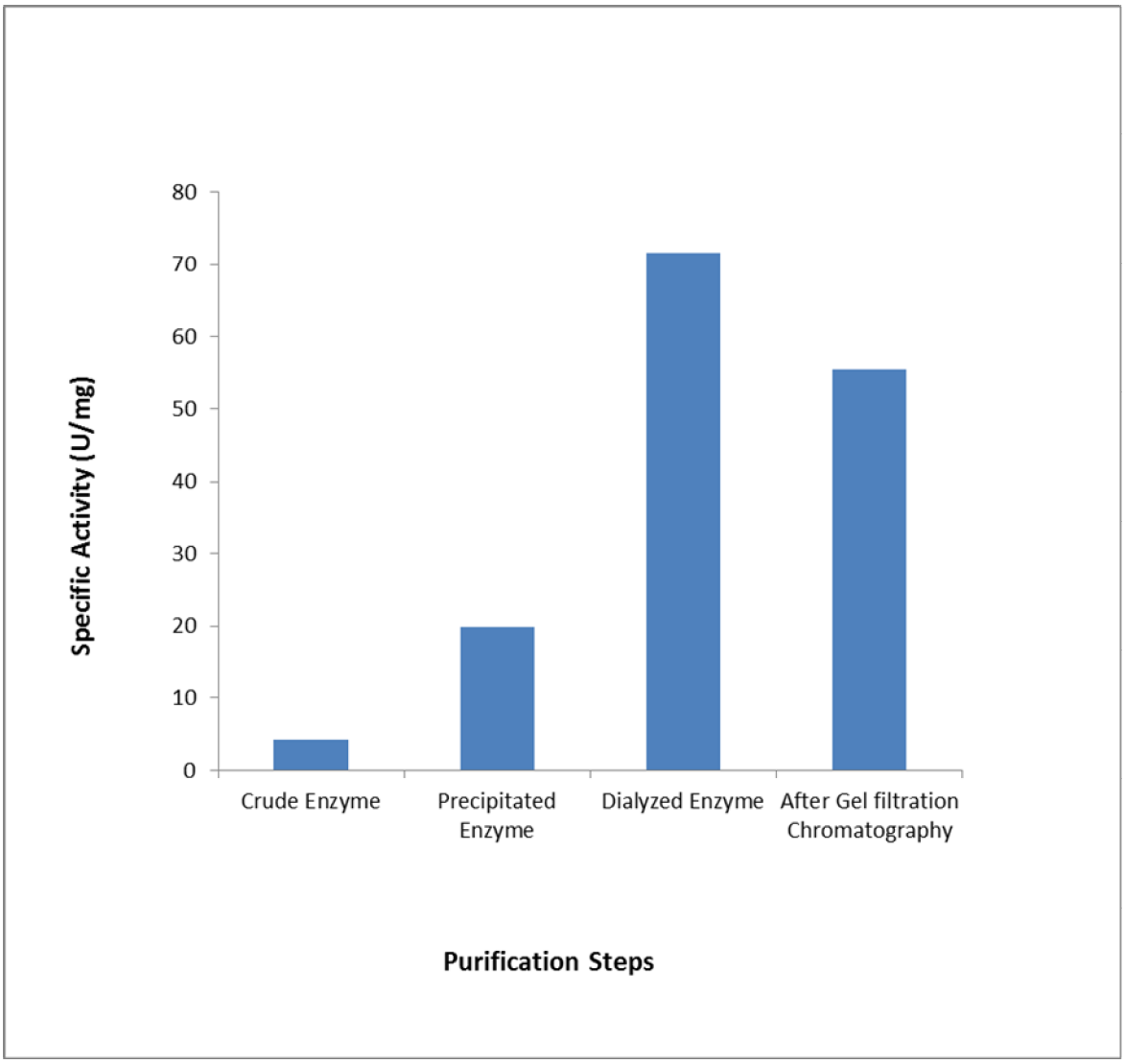


Figure 17: Specific activity of the partially purified peroxidase from cabbage.

Table 6: Purification result for *Brassica oleracea*

Purification step	Volume of Enzyme (ml)	Protein Conc. (mg/ml)	Peroxidase Activity (U/ml)	Specific Activity (U/mg)	Total Activity(U)	Purification Fold	% yield
Crude Enzyme	1500	0.94	3.98	4.23	5970	1	100
90% $(NH4)_2SO4$ precipitation	300	0.80	15.93	19.91	4779	4.71	80.05
Dialyzed Enzyme	30	0.289	20.71	71.66	621.3	16.94	10.41
Gel filtrated Enzyme	15	0.676	37.57	55.58	563.55	13.14	9.44

3.10 Characterization of cabbage peroxidase

3.10.1 Effects of pH change on peroxidase activity

The optimum pH for the peroxidase extracted from cabbage was found to be pH 5.0. An increase or decrease in pH beyond or below this optimum pH showed a decline in peroxidase activity. At pH of 7.5 no peroxidase activity was observed.

3.10.2 Effects of temperature on peroxidase activity

Figure 19 below shows the effect of temperature change on peroxidase activity. The optimum temperature of cabbage peroxidase was found to be 45^0C. An increase or decrease in temperature beyond or below this optimum value showed a decline in peroxidase activity. At 70^oC, the enzyme activity was still observed but drastically reduced.

3.10.3 Effects of substrate concentration on peroxidase activity

3.10.3.1 Effect of hydrogen peroxide concentration on peroxidase activity

Peroxidase activity was found to increase gradually with increase in H_2O_2 concentration until it reached its peak at 20 mM. Thereafter, increase in the concentration of the hydrogen peroxide caused no change on the peroxidase activity.

3.10.3.2 Determination of Kinetic parameters of cabbage peroxidase using H_2O_2

Kinetic parameters V_{max} and K_m of the peroxidase using H_2O_2 were calculated from Lineweaver-Burk plot.

3.10.3.3 Effect of different concentration of o-dianisidine on peroxidase activity

Peroxidase activity was found to increase gradually at different concentration of o-dianisidine until it reached its peak at 9mM. At its peak, increase in the concentration of the o-dianisidine caused no change on the peroxidase activity.

3.10.3.4 Determination of kinetic parameters using o-dianisidine

The V_{max} and K_m of the peroxidase using o-dianisidine were calculated from the Lineweaver-Burk plot.

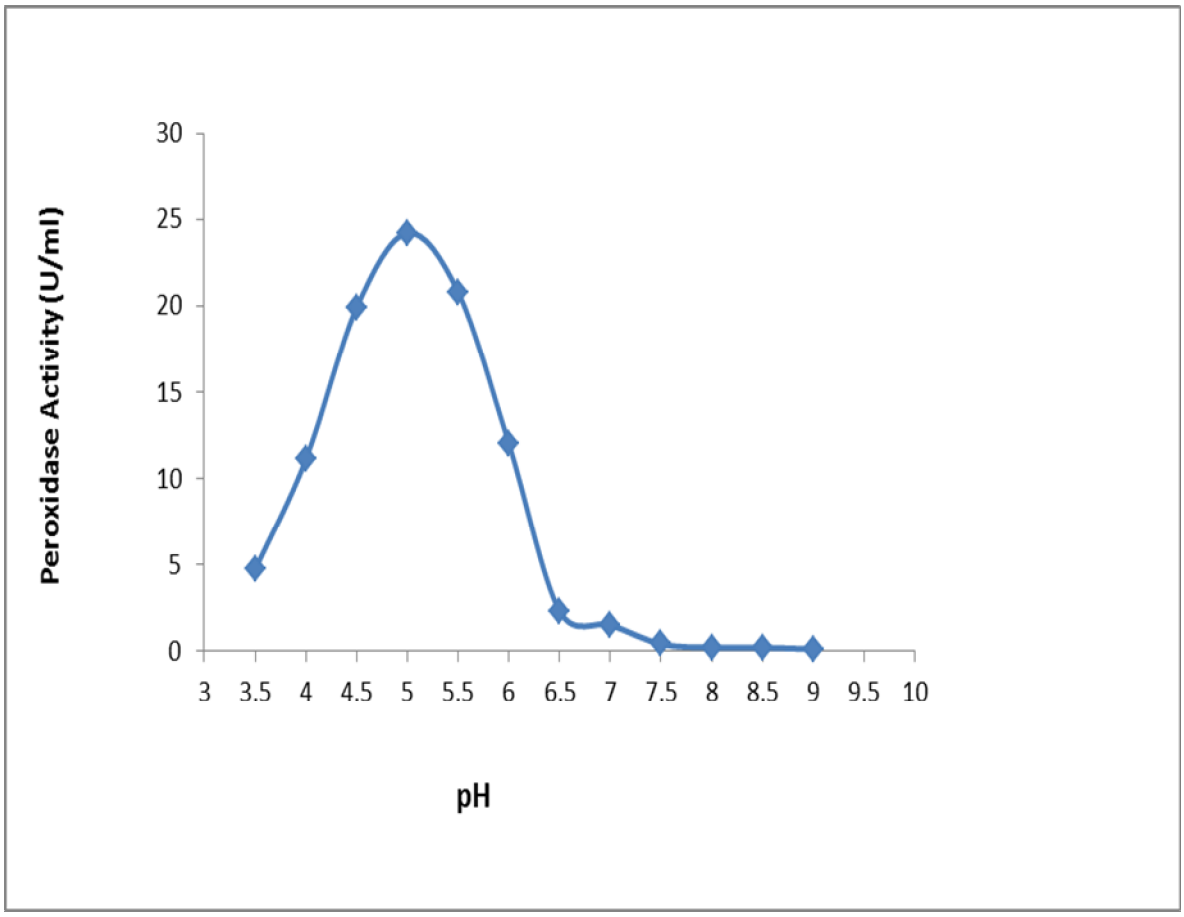


Figure 18: Effect of pH on peroxidase activity

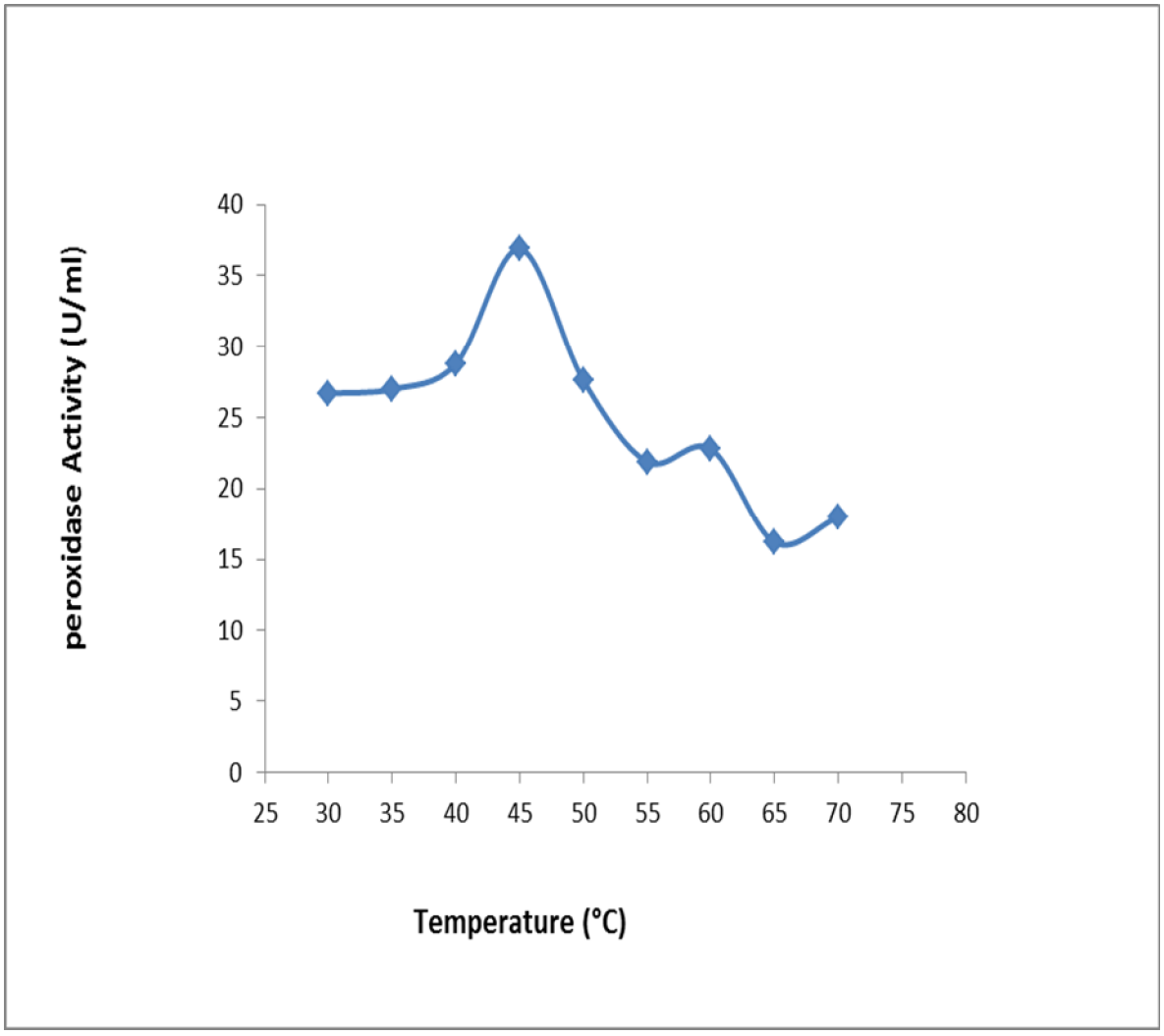


Figure 19: Effect of temperature change on peroxidase activity

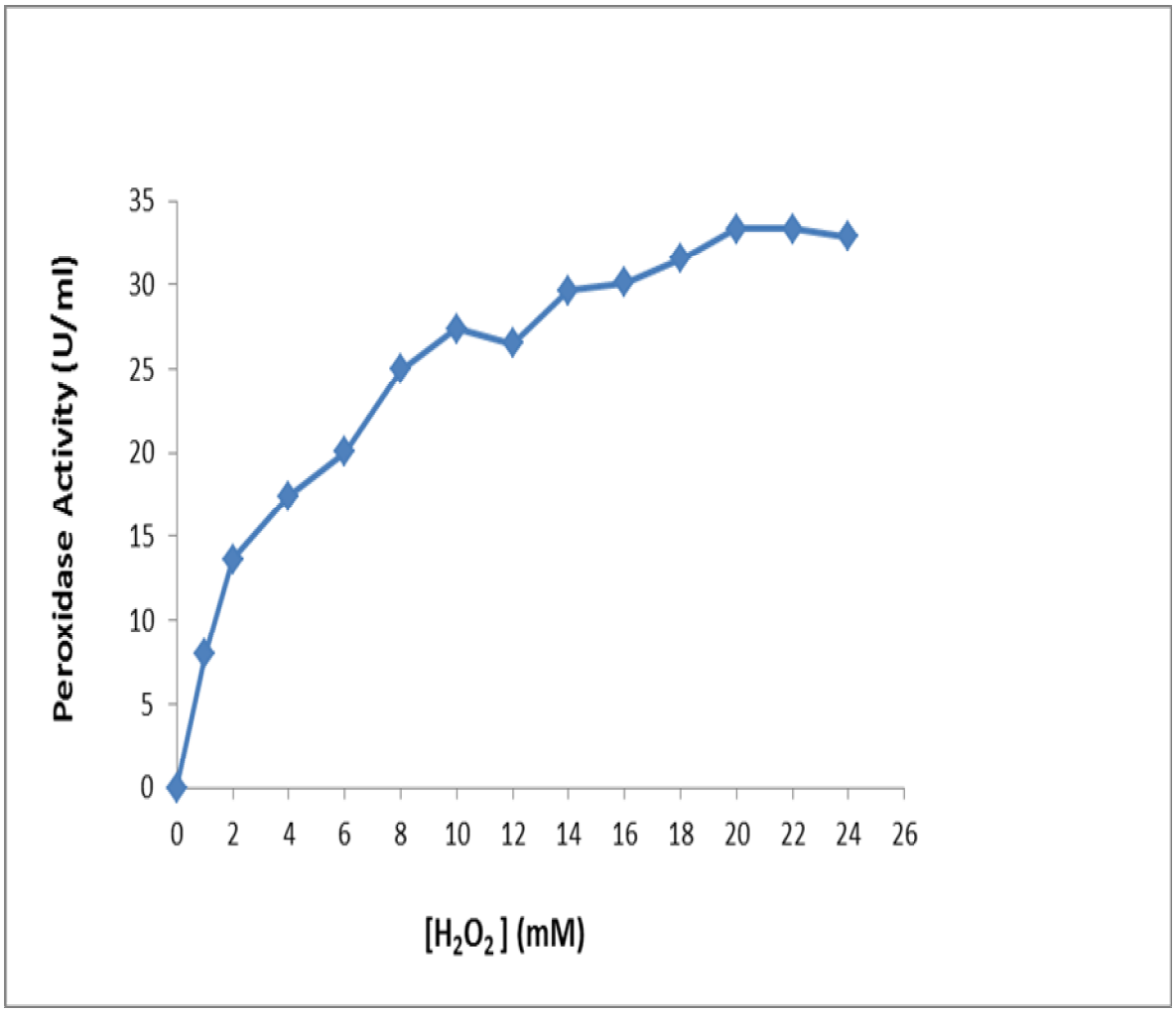


Figure 20: Michaelis-Menten's plot for hydrogen peroxide

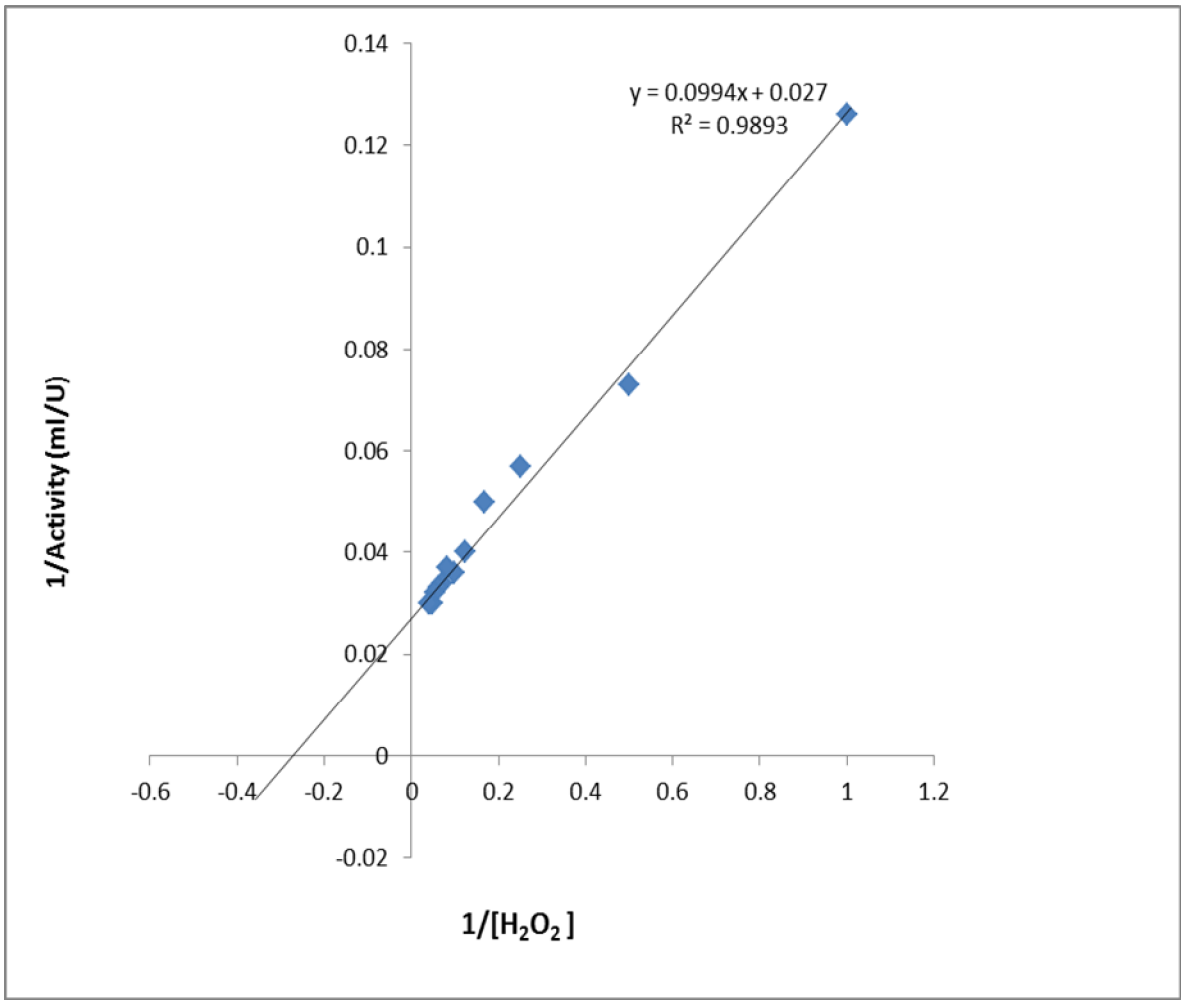


Figure 21: Lineweaver-Burk plot 1/Activit against 1/[H_2O_2]

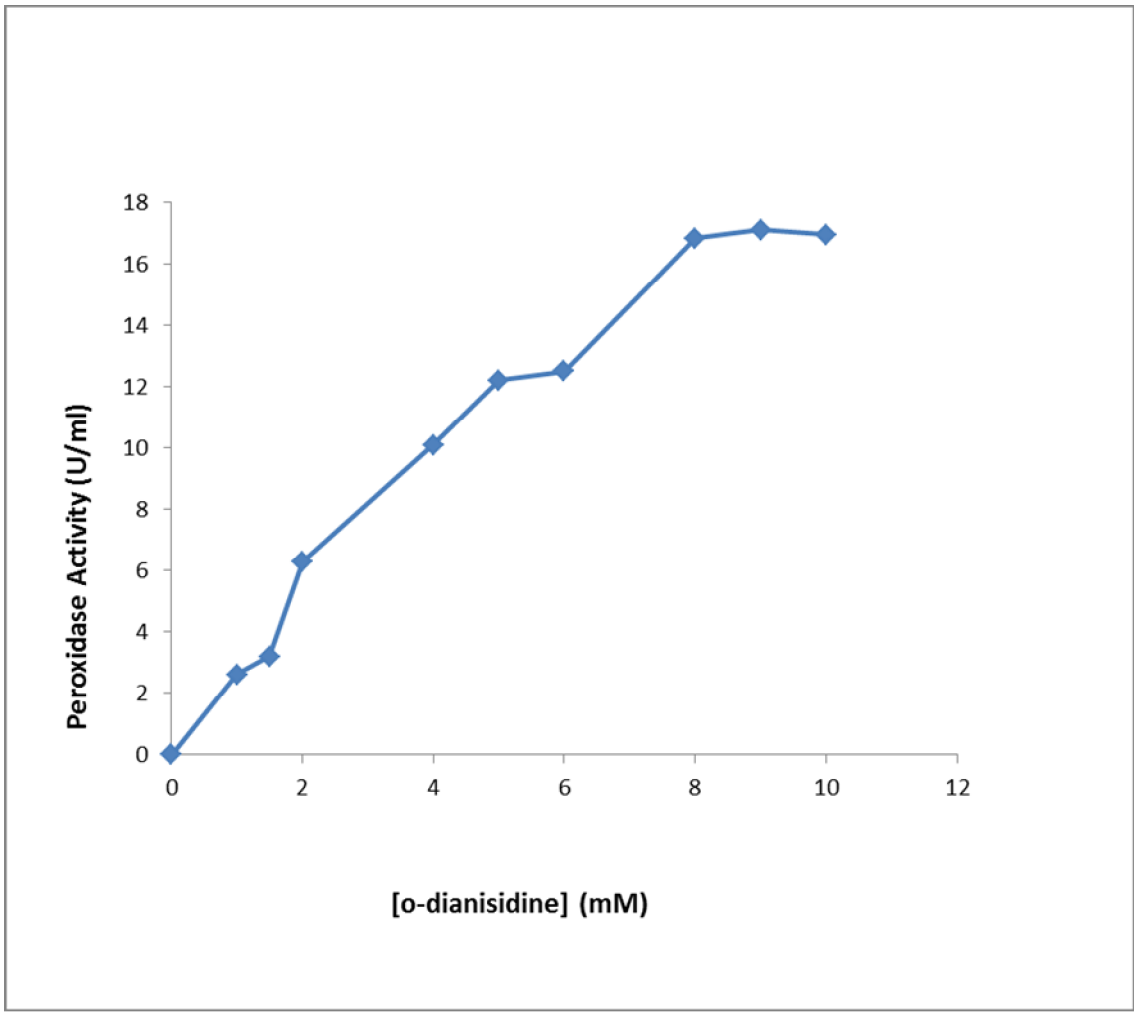


Figure 22: Michaelis-Menten's plot for o-dianisidine

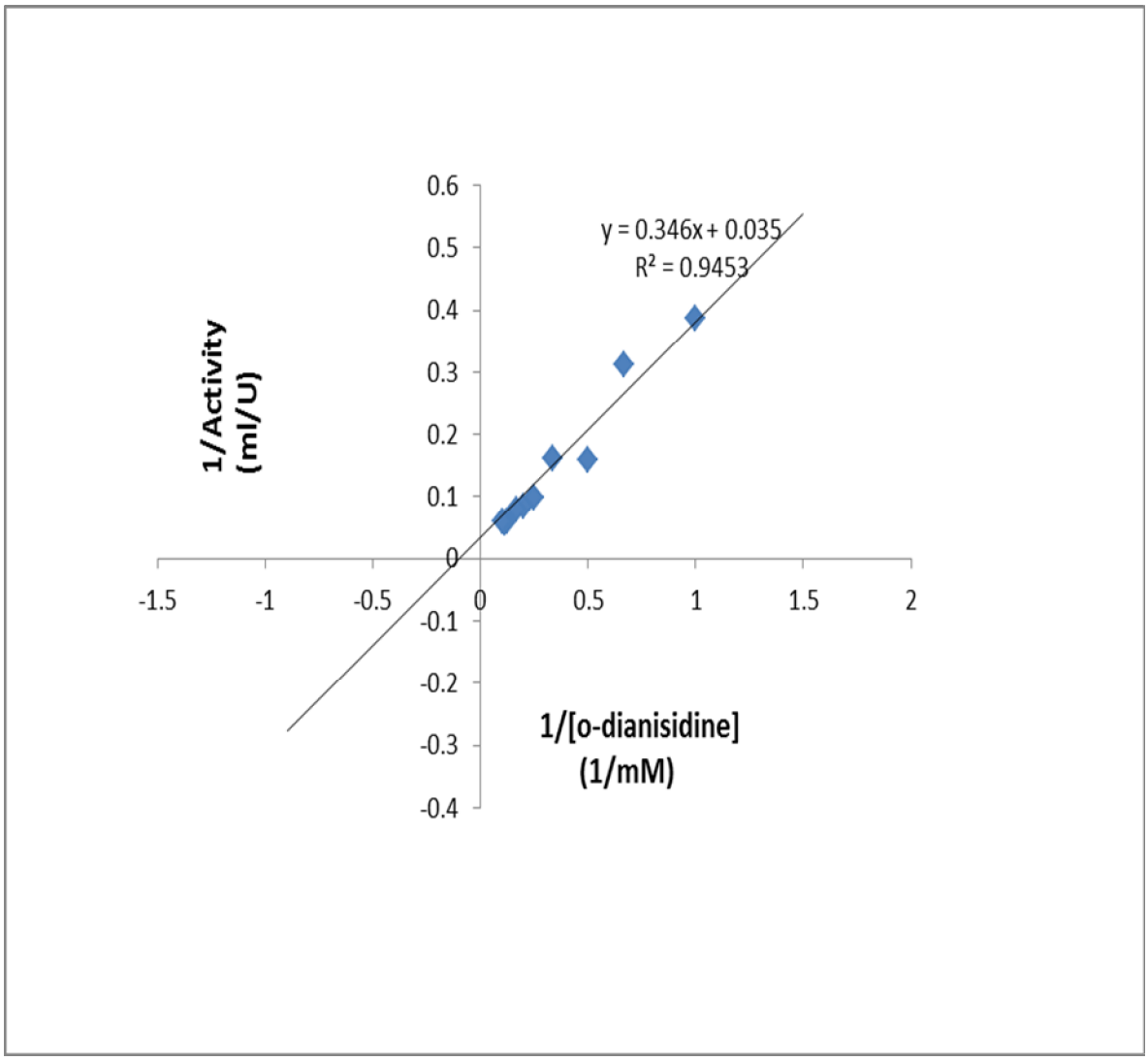


Figure 23: Lineweaver-Burk plot of o-dianisidine

Table 7: Characterization table for cabbage peroxidase

Properties	Results
pH	5.0
Optimum Temperature (^{0}C)	45
Vmax (U/ml) H_2O_2	37.04
Km (mM)	3.68
Vmax (U/ml) o-dianisidine	28.57
Km (mM)	9.89

3.11 Dye treatment with cabbage peroxidase

Azo Brilliant Black, Azo Trypan Blue, Azo Blue 5, Azo Yellow 6, Azo Citrus Red 2, Azo Pink, Azo Purple, Vat Green 9 and Vat Orange 11 were treated with cabbage peroxidase to determine the level of decolorization by the peroxidase on the different dyes.

3.11.1 Spectral result of the different dyes

3.11.1.1 Azo Brilliant Black

Figure 24 below shows the spectral results of Azo Brilliant black. The dye alone has absorbance peak of 0.463 at 219nm (UV region) and 0.148 at 614nm (visible region). After the addition of buffer and enzyme in a step by step approach, there was a change in the peaks (0.303, 0.475 for UV at 224nm and 0.089, 0.119 for Visible at 609nm respectively). There was an increase in the absorbance (0.723, 224nm at UV) in addition of H_2O_2 while the absorbance at the visible region also changed (0.106 at 609nm). There was a shift in the wavelength (from 224 to 229nm) after 1hour at the UV region with simultaneous increase in the absorbance. Further shift was observed after 20 hours and reduction in the absorbance (0.701, 269nm).

3.11.1.2 Azo Trypan Blue

Figure 25 shows the spectral reading of the Azo Trypan Blue. The dye alone had absorbance of 0.308 at 619nm (Visible region) which reduced after the addition of enzyme and H_2O_2 to 0.281 and 0.201 respectively. Further reductions were observed after 1hour and 20 hours contact times which were 0.169 and 0.114 respectively. At 20hours, there was a shift in the wavelength from 619 to 659nm. At the UV region, addition of buffer, enzyme and H_2O_2 led to the increase of absorbance; 0.371, 0.53 and 0.995 respectively at 224nm. After 1hour contact time, the absorbance increased also to 1.051. There was shift in UV region after 20hours from 224 to 269nm.

3.11.1.3 Azo Blue 5

Figure 26 below shows the spectral reading of Azo Blue 5. The absorbance was found to decrease with the addition of enzyme and H_2O_2. It also decreased after contact time of 1hour and 20hours. There was also shift after 20hours contact time from 659 to 694nm.

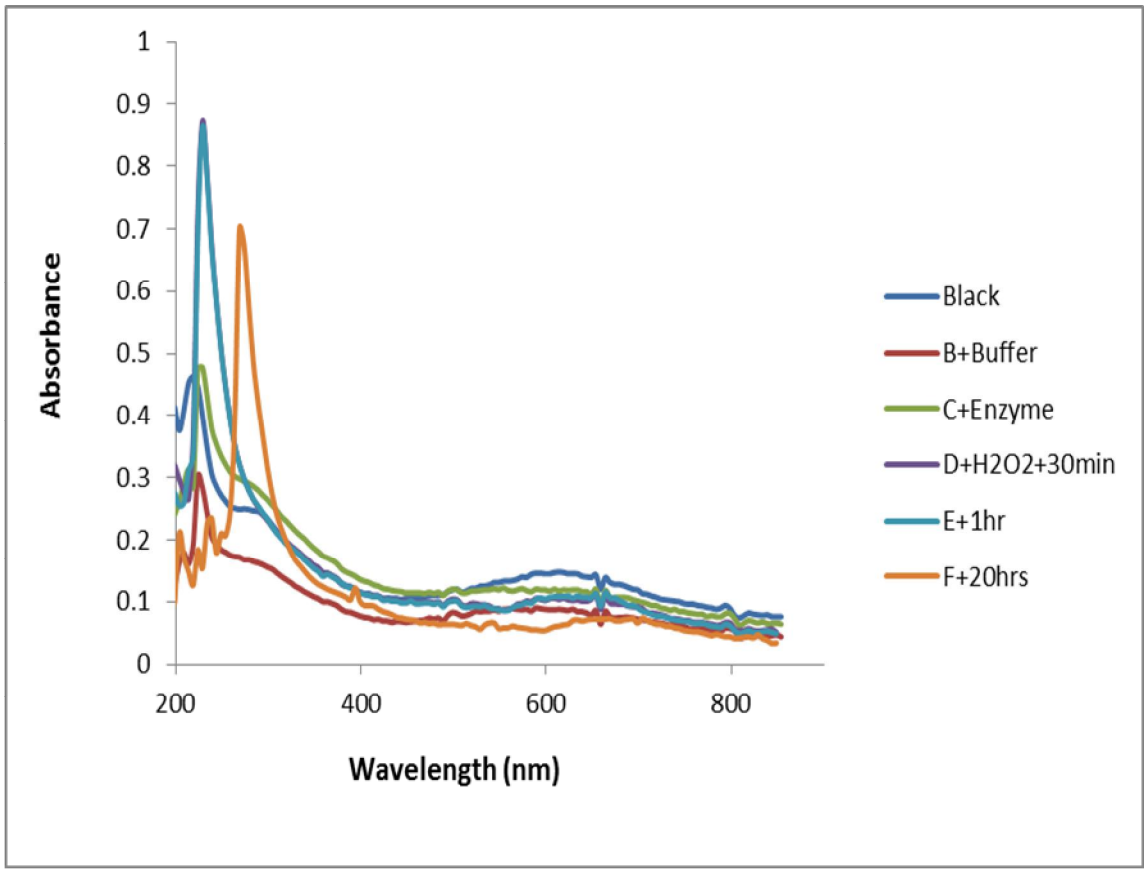


Figure 24: UV-Visible Spectral result of Azo Brilliant Black in the absence and presence of enzyme

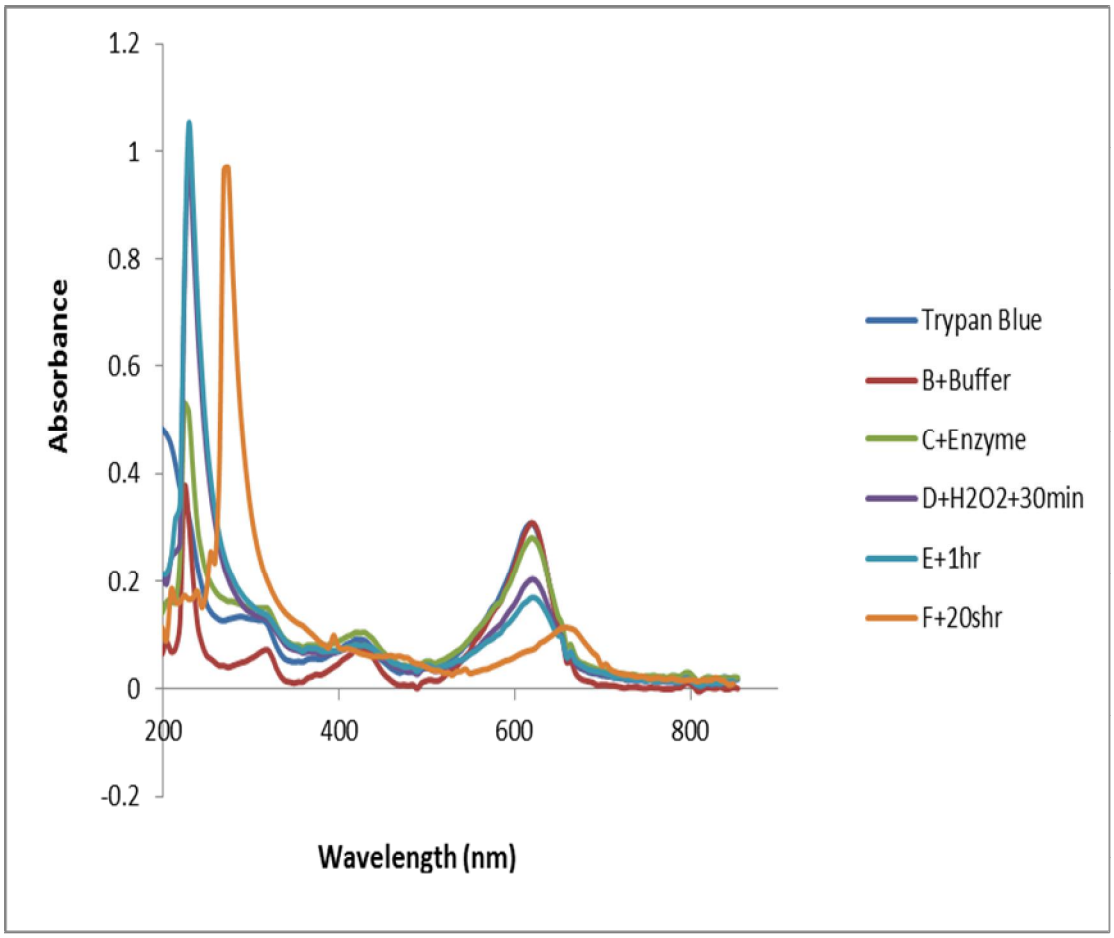


Figure 25: UV-Visible Spectral Reading of Azo Trypan Blue in the Absence and presence of Enzyme.

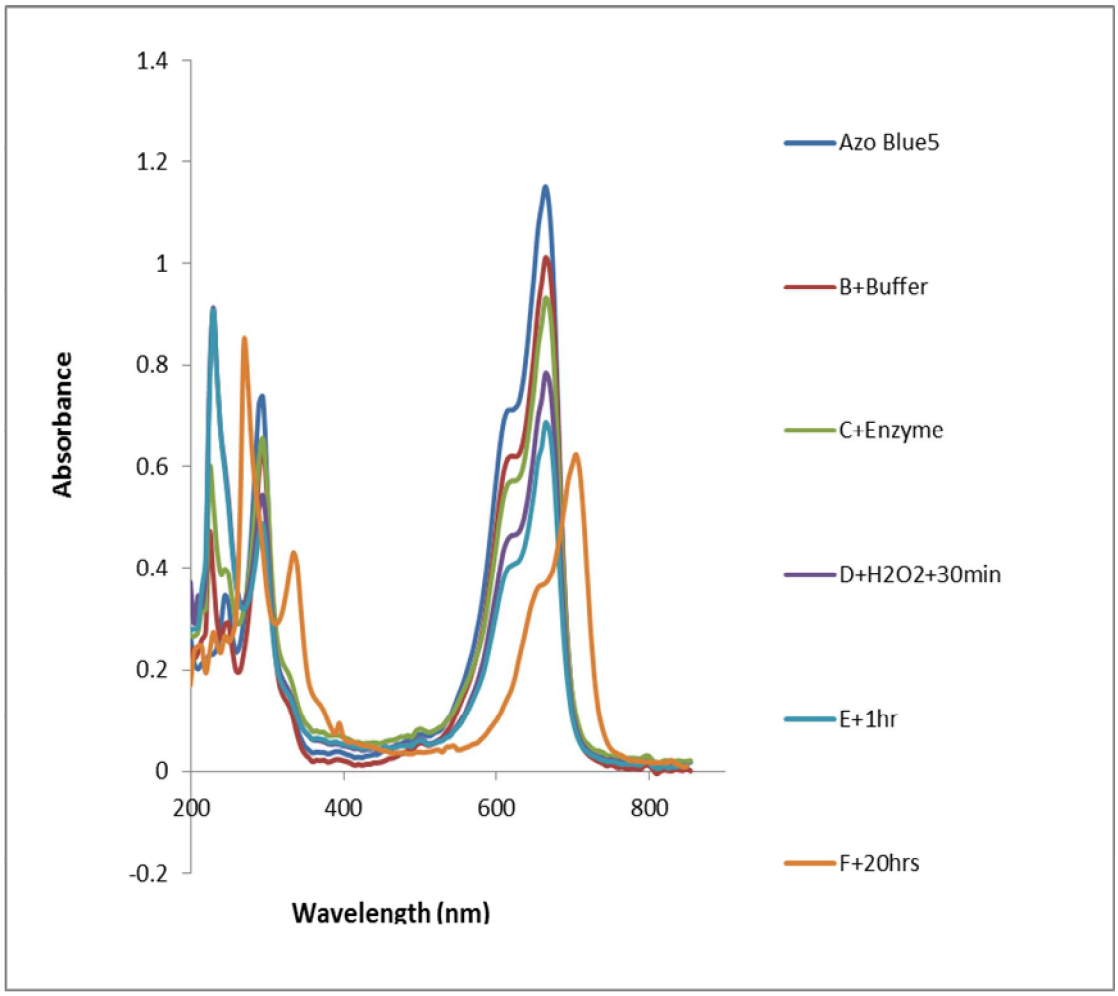


Figure 26: UV-Visible Spectral Reading of Azo Blue 5 in the presence and absence of enzyme.

3.11.1.4 Azo Yellow 6

Figure 27 below shows the spectral result of Azo Yellow 6. From the result, addition of enzyme and H_2O_2 to the dye, showed a significant difference. After 20hours contact time, it was observed that there was a shift to the right and absorbance decreased both at the UV and visible regions.

3.11.1.5 Azo Citrus Red 2

Figure 28 below shows the spectral result of Azo Citrus Red 2. The absorbance peak of the dye alone (1.688 at 544nm) was found to decrease when the enzyme and H_2O_2 were added, having values of 1.126 and 0.991 respectively. There was further decrease after contact time of 1hour and 20 hours. At 20 hours, there was a shift to the right from 544 to 584nm.

3.11.1.6 Azo Pink

Figure 29 shows the spectral result of Azo Pink. There was no significant effect of the enzymatic reaction. After 20hours contact time, a shift in wavelength from 549 to 584nm was also observed.

3.11.1.7 Azo Purple

Figure 30 shows the spectral reading of Azo Purple. There was a slight significant decrease in the absorbance when compared with the dye alone.

3.11.1.8 Vat Green 9 and Vat Orange 11

Figures 31 and 32 show the spectral result of Vat Green 9 and Vat Orange 11 respectively. There was no significant effect of the enzymatic reaction to the dyes.

3.11.2 Percentage Decolorization of the dyes

Percentage decolorization of each dye was calculated after 1hour. From the result, the dye that had the highest % decolorization was Azo Trypan blue. It had 88.62% after 1hour contact time

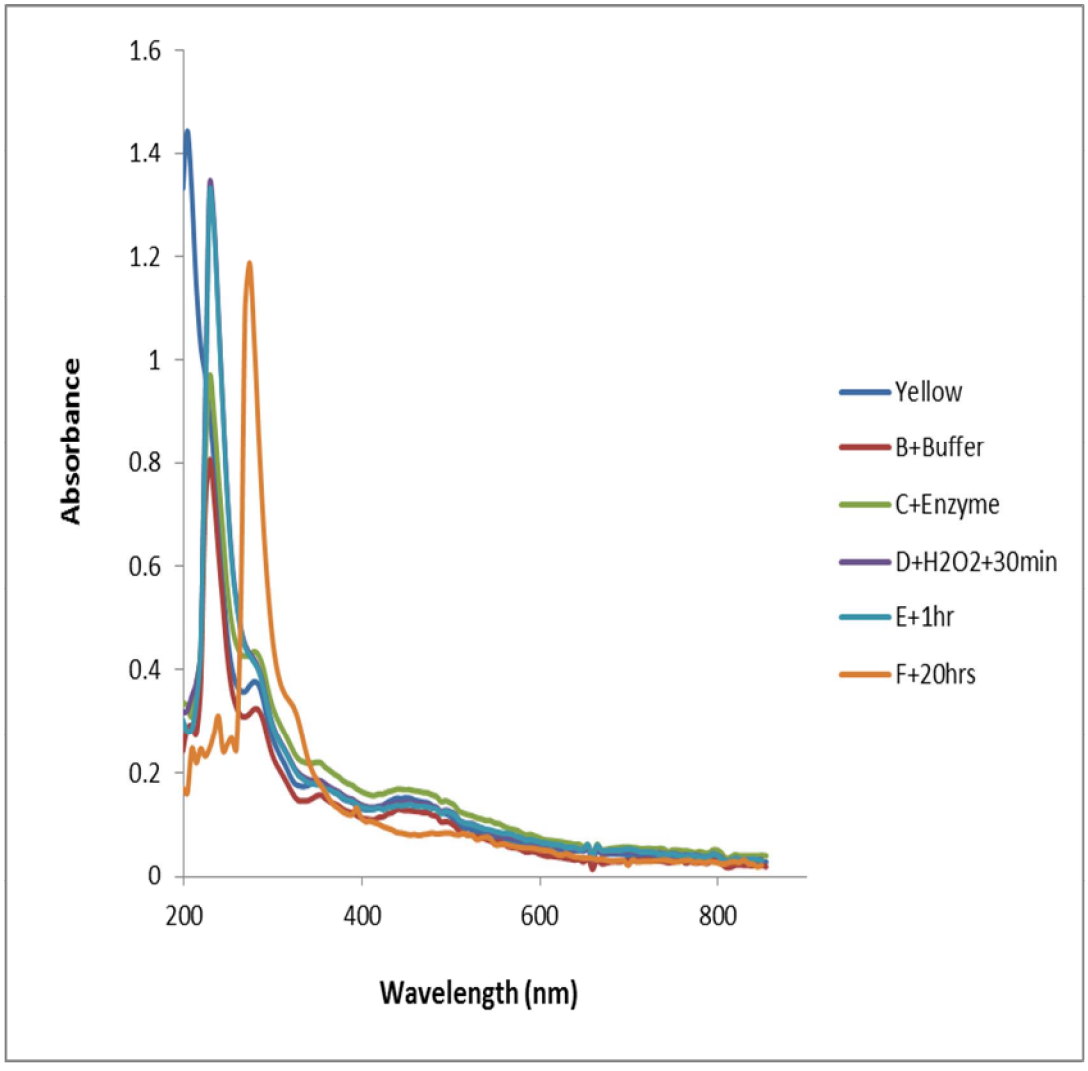


Figure 27: UV-Visible Spectral reading of Azo Yellow 6 in the presence and absence of enzyme.

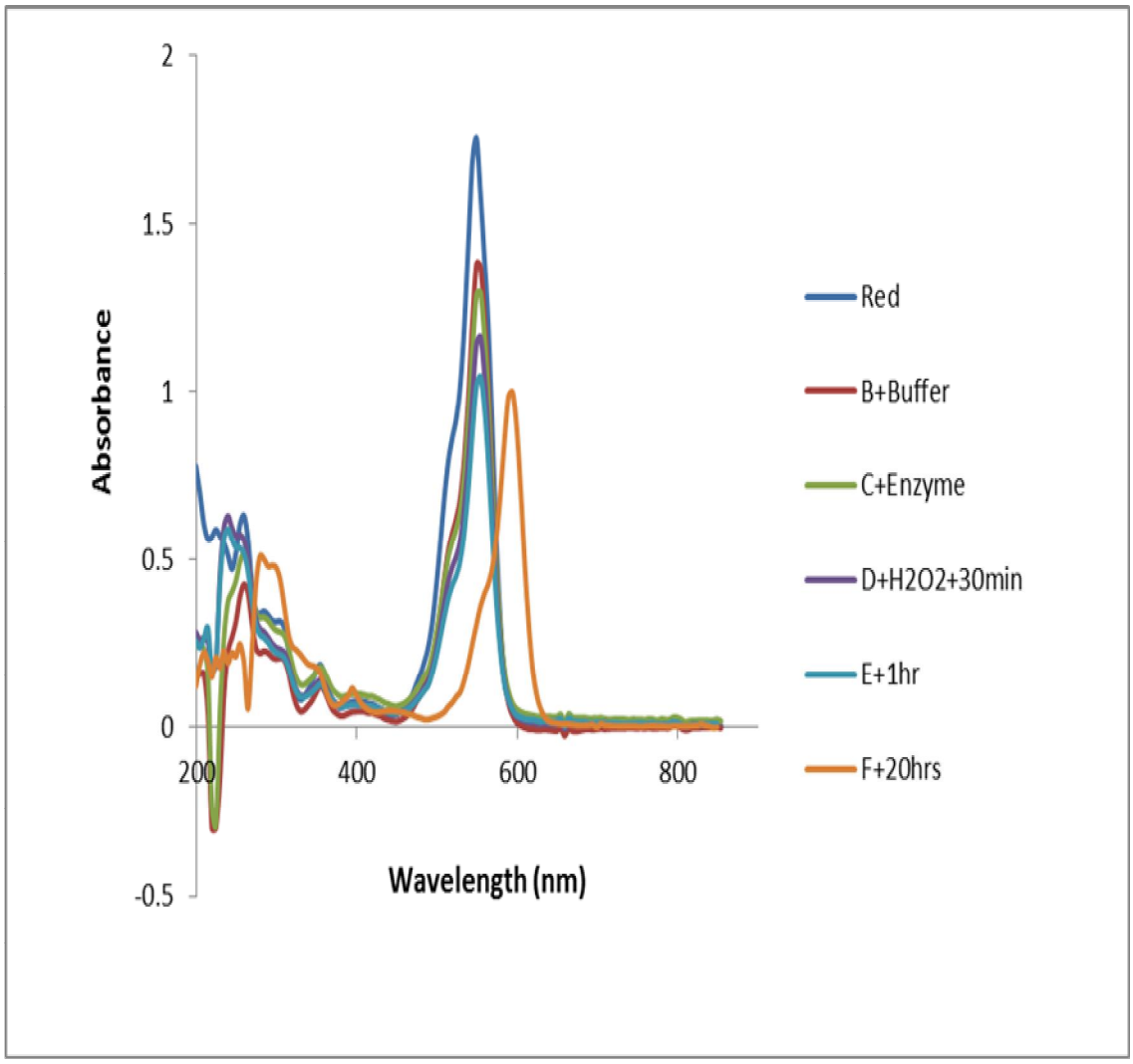


Figure 28: UV-Visible Spectral reading of Azo Citrus Red 2 in the presence and absence of enzyme.

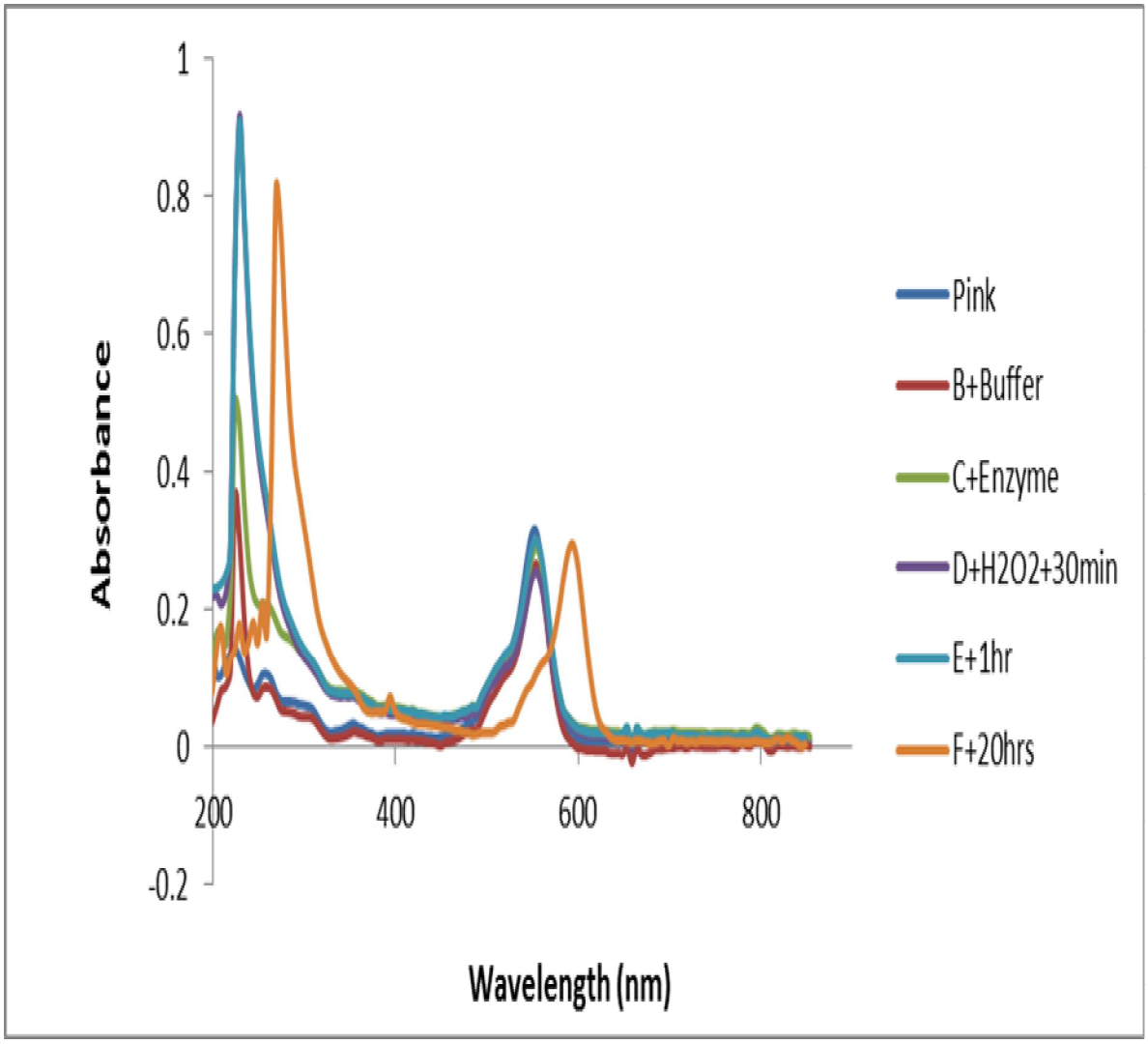


Figure 29: UV-Visible Spectral reading of Azo Pink in the presence and absence of enzyme.

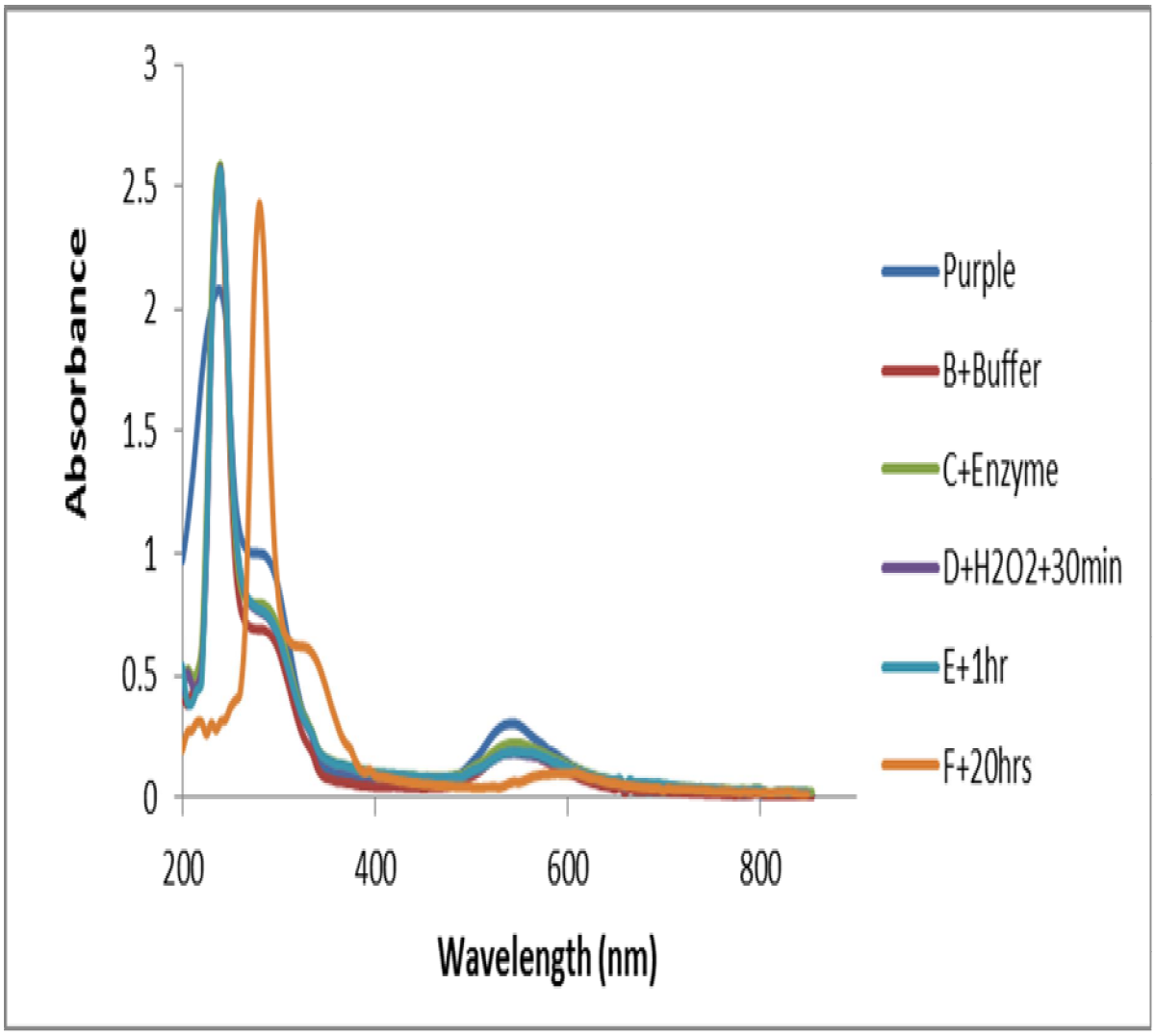


Figure 30: Spectral reading of Azo Purple in the presence and absence of enzyme

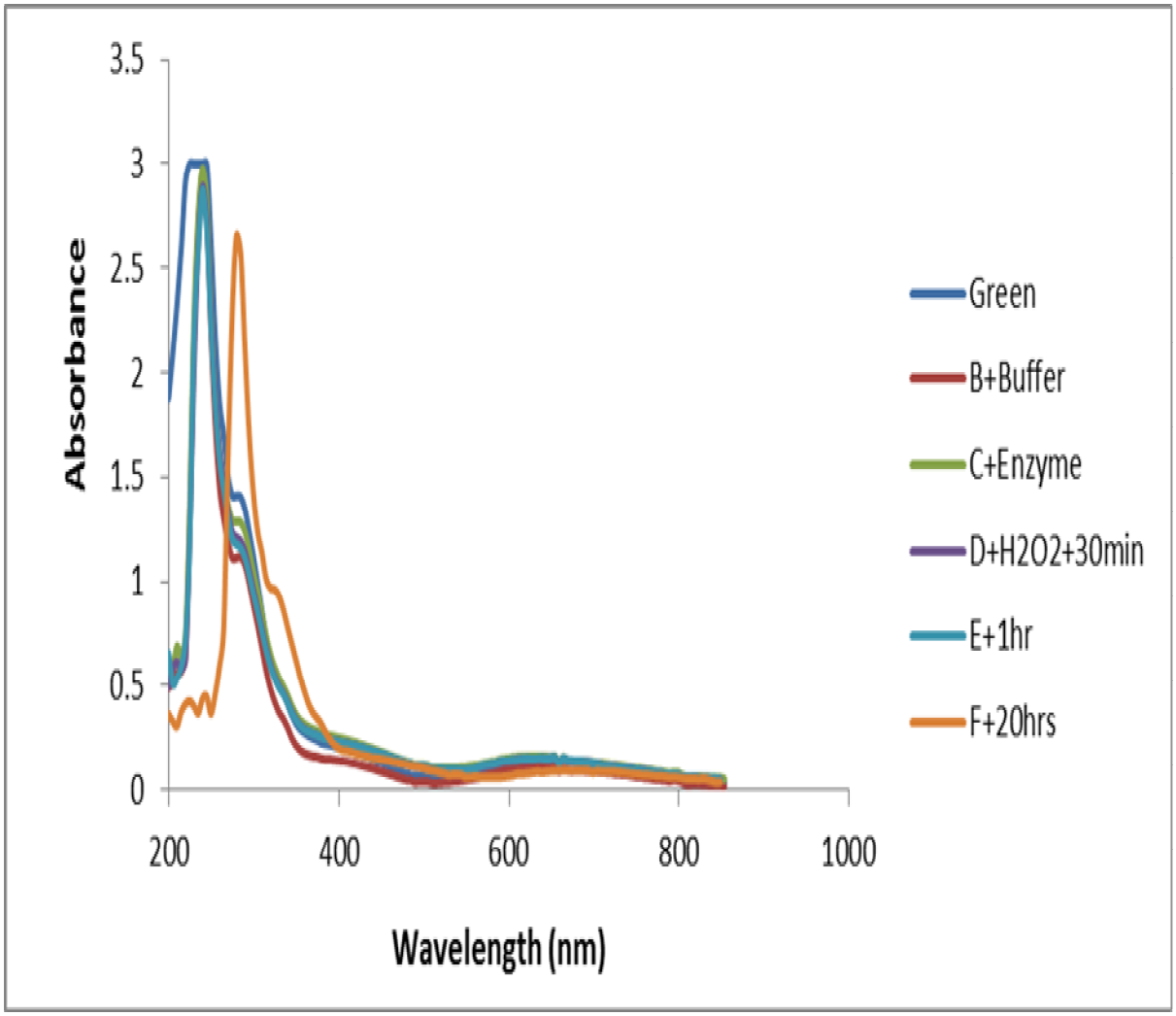


Figure 31: UV-Visible Spectral reading of Vat Green 9 in the presence and absence of enzyme.

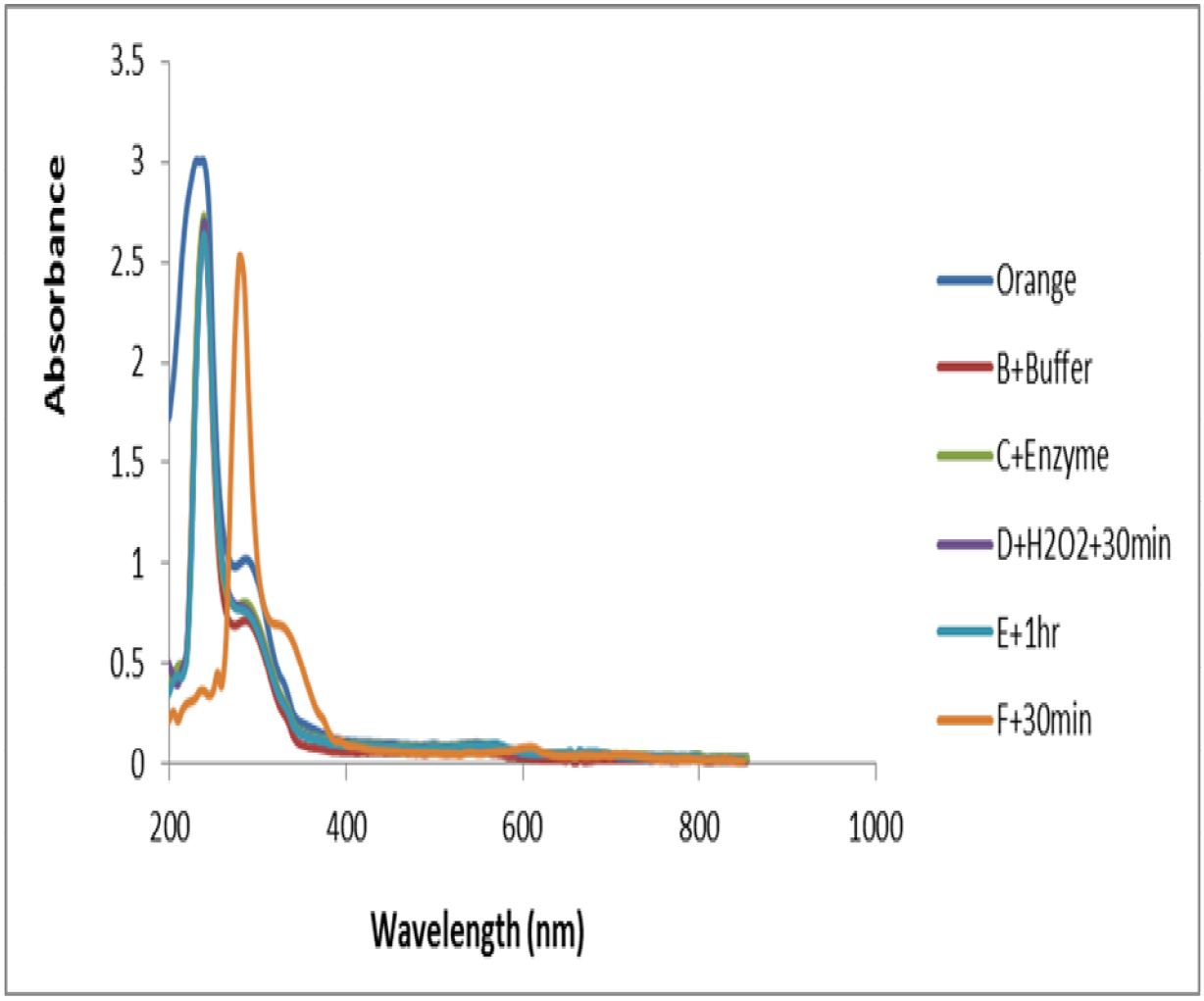


Figure 32: UV-Visible Spectral reading of Vat Orange 11 in the presence and absence of enzyme.

Figure 33: Picture of the dye solutions before decolorization.

Figure 34: Picture of the different dye solutions after decolorization.

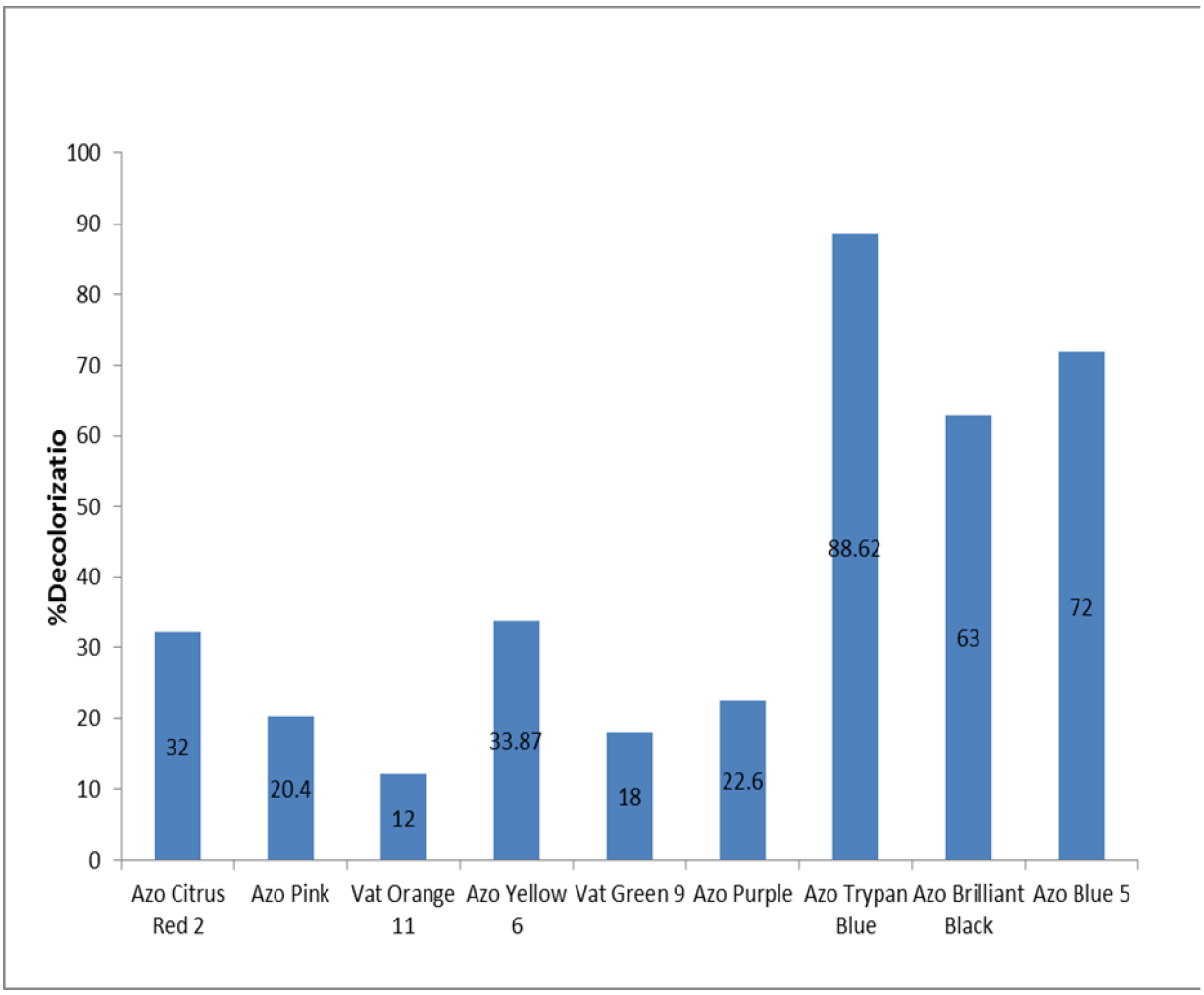


Figure 35: %Decolourization of the dyes treated with cabbage peroxidase after 1hour

CHAPTER FOUR

DISCUSSION

Peroxidase (POD) is an oxidoreductase that catalyzes a reaction in which hydrogen peroxide acts as the acceptor and another compound acts as the donor of hydrogen atoms. They are widely spread enzymes in plants, microbes and animal tissues. The broad substrate specificity, multifunctional properties and availability of peroxidases from different sources allows us to apply these enzymes in various biological and biochemical processes.

In this study, 80% $(NH_4)_2SO_4$ saturation was found suitable for precipitation of cabbage peroxidase. After ammonium precipitation profile, peroxidase activity was found to be 15.93 U/ml. Eze *et al*. (2000) reported 70% ammonium sulphate saturation for peroxidase from sorghum. Saboora *et al*. (2012) reported 80% ammonium sulphate saturation for peroxidase from turnip. Rehman *et al*. (1999) reported 85% for peroxidase from tomato, horseradish legumes and horseradish roots. Silva *et al*. (2012) reported ammonium sulphate saturation of 85% for sweet potato. Interestingly, Belcarz *et al*. (2012) reported 75% saturation for peroxidase from spring cabbage.

Ammonium sulphate is the most commonly used reagent for salting out proteins because of its high solubility which permits achievement of solution with high ionic strength (Rehman *et al*., 1999). This causes the reversible precipitation of the protein and is non-denaturing to the protein structure. The principle behind this is that proteins have polar amino acids such as glycine, serine etc. Attractive interactions between the nearby oppositely charged groups form ion pairs or salt bridges. Water as a powerful solvent, interacts with these surface amino acids and keep them in

solution. At low concentration of the salt, solubility of the proteins usually increases slightly. But at high concentrations of salt, the solubility of the proteins drops sharply and the proteins precipitate out. Completely ionized salts have more affinity for water molecules than protein hence addition of salts takes up water molecule from the protein. Therefore the ionic interactions between water molecules and protein are reduced and as a result hydrophobic interactions dominate. The hydrophobic amino acid patches present in all the proteins attract each other and forms aggregates. Ammonium sulphate precipitation is however best used to optimize yield rather than to optimize purity (Ward and Swiatek, 2009).

After ammonium sulphate precipitation, the enzyme was desalted by subjecting it to 18 hours dialysis. The peroxidase activity after dialysis was found to be 20.71 U/ml and this portrayed increase in purity of the enzyme as a result of the removal of salt by diffusion. The dialysate was applied to a sephadex G-25 gel filtration column equilibrated with phosphate buffer (0.05M), pH 6.0. The activity after gel filtration chromatography was found to be 37.57U/ml. This increased activity shows that the peroxidase was further purified by the removal of other impurities like other proteins and salts that interfered with the peroxidase activity. Therefore, peroxidase activity increased simultaneously with increase in purification steps.

The protein concentration of the crude extract was found to be 0.94mg/ml which reduced to 0.8, 0.289 and 0.676mg/ml after ammonium sulphate precipitation, dialysis and gel filtration respectively. The drastic reduction in protein concentration after dialysis may be attributed to denaturation of the enzyme arising from the environmental factors such as the handling and temperature. The reduction in protein concentration generally resulted from the removal of other proteins and salt which could serve as impurities and hence, increased peroxidase activity. The protein concentration is, however, estimated because peroxidase, as an enzyme is also a protein, and contributes to the concentration of the protein present, though there could be other proteins present.

The partially purified peroxidase was further characterized based on effects of pH, temperature and substrate (H_2O_2 and O-dianisidine) concentration. The optimum pH of the partially purified cabbage peroxidase was found to be 5.0. This is contrarily to pH 6.0 reported by Bania and Mahanta (2012) using o-dianisidine as the H donor.

Belcarz *et al*. (2007) reported optimum pH of 6.0 from spring cabbage peroxidase using guaiacol as the H donor. Peroxidases purified from various sources have been reported to have their pH optima mostly in the region of 4.5-7. Using guaiacol as the H donor, broccoli peroxidase had maximum activity at approximately pH 4-5 for the acidic peroxidase and pH 6 for both the neutral and basic peroxidases as reported by Thongsook and Barrett (2005). Duarte-Vazquez *et al*. (2000) reported optimum pH for acidic turnip Peroxidases as between 5 and 5.5 with ABTS as H donor. Saboora *et al*., (2012) reported optimum pH of 6.0-6.5 for turnip using guaiacol as the H donor. Civello *et al*. (1995) reported pH 6 for peroxidase from strawberry. Silva *et al*. (2012) reported optimum pH 7 for peroxidase from turnip using guaiacol as the H donor. Singh *et al*. (2010) reported optimum pH 4.5 for peroxidase from apple using o-dianisidine as the H donor. Chanwun *et al*. (2013) reported an optimum pH of 5 for peroxidase from rubber trees (*Hevea brasiliensis*) using o-dianisidine as the H^+ donor. Nwanguma and Eze (1995) reported optimum pH of 5.5 for sorghum using o-dianisidine as the H donor. A rapid decrease in activity was found on either the basic or acidic side of this pH optimum. This is because the release of the haem group from the active site of the enzyme is pH dependent, occurring more rapidly below pH 4 and leading to a loss of POD activity. This activity decrease might have occurred mainly by ionic alterations of the enzyme that alter the form of the enzyme and consequently the active site (Silva *et al*., 2012). At pH 7 the enzyme is completely inactivated.

The optimum temperature for the cabbage peroxidase was found to be 45ºC. At temperature of 30-40^{o}C, the peroxidase activity increased slightly and the peak was found at 45^{o}C. Above 45^{o}C, the peroxidase activity decreased steadily up to 70^{o}C at which the enzymatic activity was not lost completely. Optimum temperature for cabbage peroxidase is comparable with other reports too. Optimum temperature range of 40-50^{o}C for cabbage using o-dianisidine as the H donor was reported by Bania and Mahanta (2012). Belcarz *et al*. (2012) reported optimum temperature of 40^{o}C for peroxidase extracted from spring cabbage using guaiacol as the H donor. Hu *et al*. (2012) reported the optimum temperature of 45^{o}C for peroxidase from lettuce stem. Interestingly, Tabatabaie *et al*. (2002) reported the optimum temperature of 50^{o}C for peroxidase from cabbage and at this temperature about 90% of the enzyme activity was observed. Singh *et al*. (2010) reported optimum temperature of 40^{o}C for peroxidase from apple using o-dianisidine as the H donor. However, from all

indication, it appears that the optimum pH and temperature for the maximum peroxidase activity varies with the enzyme source, the isoenzyme composition, the H donor substrate, and the buffer used for the analysis.

Effect of substrate concentration on the partially purified enzyme was studied using hydrogen peroxide and o-dianisidine. Hydrogen peroxide concentrations were prepared from 1-24mM while o-dianisidine concentrations were prepared from 1-10mM. The peroxidase activity was found to increase with increase in hydrogen peroxide and o-dianisidine concentration until [H_2O_2] and [o-dianisidine] reached 20mM and 8mM respectively, after which further increase in [H_2O_2] and [o-dianisidine] did not cause any increase in the peroxidase activity.

The Michaelis-Menten constants (K_M) and the maximum velocity (V_{max}) for the oxidation of o-dianisidine in the presence of hydrogen peroxide and the enzyme were determined from Lineweaver-Burk plots (1/V vs 1/[S]) by following the standard assay conditions at optimum temperature and pH conditions, as shown in figures 21 and 23. The K_M and V_{max} of the hydrogen peroxide obtained were respectively 3.68mM and 37.04U/ml. The K_M and V_{max} of the o-dianisidine obtained were 9.89mM and 28.57 U/ml respectively. The K_M value of the hydrogen peroxide was roughly 2 times higher than 1.937 Mm/ml obtained by Eze (2012) from African oil bean seed. Thongsook and Barrett (2005) reported K_M value of 9.731mM of hydrogen peroxide for basic peroxidase from broccoli using guaiacol as the H donor. This indicates that peroxidase from cabbage has lower affinity for hydrogen peroxide than peroxidases from oil bean seed and has higher affinity than broccoli. Interestingly, Daurte-Vazquez *et al*. (2001) reported Km value of 3.7mM for peroxidase from turnip using guaiacol as H donor. V_{max} value for H_2O_2 can be compared with those obtained from acidic, basic and neutral peroxidase from broccoli which were 5.7mM/min, 11.7mM/min, and 438 mM/min respectively using guaiacol as the H donor (Thongsook and Barrett, 2005). Hu *et al.* (2012) reported H_2O_2 V_{max} values of 10585 and 1146 U/ml for peroxidase from lettuce stem using guaiacol and ABTS respectively as the H donor. The O-dianisidine K_M value for the cabbage peroxidase obtained is comparable with 6.7-13.8mM using guaiacol as the H donor for the oxidation of peroxidase from Korean radish root as reported by Lee and Kim (1994). Halpin *et al*. (1989) reported 10.2 mM for green peas. From the result K_M value for

H_2O_2 (3.68 mM) is lower than K_M for o-dianisidine (9.89mM) and this shows that peroxidase from cabbage has more affinity for H_2O_2 than the H donor, o-dianisidine.

The results of the effect of pH, temperature and substrate concentration (including k_m and V_{max}) gotten from this study could be of relevance to industries using peroxidase from *Brassica oleracea* in order to get the maximal activity of the enzyme to ensure the effectiveness of their industrial application of *B. oleracea*.

Application of the partially purified cabbage peroxidase was also evaluated. It has been shown that plant peroxidase is efficient for synthetic dye decolorization and degradation. It is explicitly observed by the decrease in concentration and color disappearance in the dye solutions under study. This study evaluates the ability of cabbage peroxidase to decolourize 7 azo dyes (Trypan Blue, Azo blue 5, Azo Brilliant Black, Azo Yellow 6, Azo Citrus Red 2, Azo Pink and Azo Purple) and 2 vat dyes (Vat Green 9 and Vat Orange 11). Of the nine dyes, Azo Trypan Blue had the highest % decolorization (88.62%) followed by Azo Blue 5 and Brilliant Black with % decolorization of 72 and 63% respectively after contact time of I hour. Chanwun *et al*. (2013) reported % decolorization of 83 and 88 for aniline blue and water blue respectively which are both triphenylmethane derived dyes using peroxidase from rubber trees after contact time of 1 hour. The Azo Citrus Red 2 had % decolorization of 32. Azo yellow 6 showed % decolorization of 33.87%. About 70% decolorization of Azo yellow 12 was obtained due to HRP-catalyzed reaction at pH of 4 after contact time of 1 hour as reported by Maddhinni *et al*. (2006). Azo Pink and Azo Purple had 20.4 and 22.6 respectively. The two vat dyes (Vat Green 9 and Vat Orange 11) had % decolorization of 18 and 12% respectively. Chanwun *et al*. (2013) reported % decolorization of 68 for Brilliant Green. This shows that peroxidase is more effective in decolorizing Azo dyes than Vat dyes. This could be as a result of the differences in their chemical structures. Azo dyes have free hydroxyl group (-OH) that are easily oxidized to provide H which is required to reduce the oxidized form of peroxidase for the reaction circle to continue. Unlike Azo dyes, Vat dyes have insoluble complex polycyclic molecules based on the quinone structure (ketoforms).

The spectral results of the various dyes were also evaluated. For Azo Brilliant Black, dye absorbs at 219 and 614nm. Addition of buffer shows shift in $ę_{max}$ to 224

and 589nm. This means that there was likely interaction of dye, buffer and enzyme; the mixture absorbing at 224 and 654nm shows strong interaction leading to bathochromic effect which suggests that a likely new specie was formed. Addition of H_2O_2 did not show any change after 1hour contact time. After 20hours contact time, $ę_{max}$ changed to 694nm. Oxidation had taken place leading to new species. In Azo Blue 5, there was a strong interaction after 20hours contact time. New species were likely formed due to the oxidation of the dye. Vat green 9 showed shift to 689 and 279nm after 20hours. This showed that there was a strong interaction due to the oxidation of the dye leading to the formation of new species. Vat Orange 11, Azo Pink, Azo Purple and Azo Yellow 6 showed shift in $ę_{max}$ which could be as result of the oxidation of the dye, leading to the formation of new species. H_2O_2 serves as an initiator of the peroxidase activity. It helps to oxidize peroxidase into a catalytically active form that is capable of reacting with the phenolic contaminant (Preethi *et al.*, 2013). After 1 hour incubation (at 30^oC), the concentration of the dye (spectral peak) was reduced drastically. A little reduction was noticed after 20 hours incubation of the dye-peroxidase mixture. In most cases, there was no much significant difference in absorbance after 1hour contact time. This suggests that 1hour is enough for the enzymatic reaction.

4.1 Conclusion

The cabbage peroxidase treatment of azo dyes could be applied for the detoxification and remediation of wastewater effluents especially from textile industry. Indeed, this novel process could be considered ecologically friendly, simple to handle, economical (since no chemical reagent is needed except hydrogen peroxide) and can react on extreme condition. The work shows also that the enzymatic activity could also decolorize vat (indigo and anthraquinone) dyes.

4.2 Recommendations

Our study provided a new perspective for the use of this enzyme in environmental remediation. Further studies should focus on further purification of the cabbage peroxidase to enhance its activity and characterization of different peroxidase isoezyme in cabbage. Mass production of the enzyme should be achieved by genetic engineering. Further research should be done to elucidate the reaction mechanism and

the identification of the products obtained from the decolorization process by this enzyme.

REFERENCES

Adam, W., Lazarus, M., Hoch, U., Korb, M.N., Saha-Moller, C.R. and Schreier, P. (1998). Horseradish peroxidase-catalyzed enantioselective reduction of racemic hydroperoxy homoallylic alcohols: a novel enzymatic method for the preparation of optically active, unsaturated diols and hydroperoxy alcohols. *Journal of Organic Chemistry,* **63**:6123ï 6127.

Adeniji, O.T., Swai, I., Oluoch, M.O., Tanyongana, R. and Aloyce, A. (2010). Evaluation of head yield and participatory selection of horticultural characters in cabbage (*Brassica oleraceae* var. *capitata*). *Journal of Plant Breeding and Crop Science,* **2**(8): 243-250.

Ahmad, A.L. and Puasa, S.W. (2007). Reactive dyes decolourization from an aqueous solution by combined coagulation/micellar-enhanced ultrafiltration process. *Chemical Engineering Journal*, **132**:257ï 265.

Al-Ansari, M. M., Modaressi, K., Taylor, K. E., Bewtra, J. K. and Biswas, N. (2010). Soybean peroxidase-catalyzed oxidative polymerization of phenols in coal-tar wastewater: comparison of additives. *Environmental Engineering Science,* **27**: 967ï 975.

Amini, M., Arami, N., Mahmoodi, M. and Akbari, A. (2011). Dye removal from colored textile wastewater using acrylic grafted nanomembrane. *Desalination*, **267**:107ï 113.

Anthon, G.E. and Barrett, D.M. (2002). Kinetic parameters for the thermal inactivation of quality-related enzymes in carrots and potatoes. *Agricultural and Food Chemistry,* **50**: 4119-4125.

Arakawa, H., Maeda, M. and Tsuji, A. (1979). Chemiluminescence enzyme immunoassay of cortisol using peroxidase as a label. *Analytical Biochemistry,* **97**: 248-254.

Azevedo, A.M., Martins, V.C., Prazeres, D.M., Vojinovic´, V., Cabral, J.M. and Fonseca, L.P. (2003). Horseradish peroxidase: a valuable tool in biotechnology. Biotechnology Annual Review, 9 (3):1387-2656. azo dyes by *Pleurotus ostreatus*. *Dyes and Pigments,* **73**(3): 322-325.

Bae, J.S. and Freeman, H.S. (2007). Aquatic toxicity evaluation of new direct dyes to the *Daphnia magna. Dyes and Pigments*, **73**(1): 81-85.

Baek, H.K. and Van Wart, H.E. (1989). Elementary steps in the formation of horseradish peroxidase compound I: direct observation of compound 0, a new intermediate with a hyperporphyrin spectrum. *Biochemistry,* **28**:5714ï 5719.

Bania, I. and Mahanta, R. (2012). Evaluation of peroxidase from from various plant sources. *International Journal of Scientific and Research Publication,* **2** (5): 2250-3153.

Banat, I.M., Nigam, P., Singh, D. and Marchant, R. (1996). Microbial decolorization of textile-dye-containing effluents: a review. *Bioresources and Technology*, **58**: 217ï 227.

Bansal, N., Kumari, L. and Kanwar, S.S. (2012). Peroxidase and its applications. *International Journal of Institutional Pharmacy and Life Sciences,* **2**(2):1-28.

Behl, C. and Moosmann, B. (2002). Antioxidant neuroprotection in *Alzheimer's* disease as preventive and therapeutic approach. *Free Radical Biology and Medicine,* **33**: 182-191.

Belcarz, A., Ginalska, G., Kowalewska, B. and Kulesza, P. (2008). Spring cabbage peroxidases ï Potential tool in biocatalysis and bioelectrocatalysis. *Phytochemistry*, **69**(3): 627-636.

Berglund, G.I., Carlsson, G.H. and Smith, A.T. (2002). The catalytic pathway of horseradish peroxidase at high resolution. *Nature*, **417**:463ï 468.

Bezalel, L., Hadar, Y. and Cerniglia, C.E. (1997). Enzymatic mechanisms involved in phenanthrene degradation by the white rot fungus *Pleurotus ostreatus*. *Applied Environmental Microbiol*ogy, **63**:2495ï 2501.

Bholay, A. D., Borkhataria, B.V., Jadhav, P.U., Palekar, K.S., Dhalkari, M.V., Nalawade, P.M. (2012). Bacterial lignin peroxidase: a tool for biobleaching and biodegradation of industrial effluents. *Universal Journal of Environmental Research and Technology*, **2**(1): 58-64.

Bhunia, A., Durani, S. and Wangikar, P.P. (2001). Horseradish peroxidase catalyzed degradation of industrially important dyes. *Biotechnology and Bioengineering,* **72**:562ï 567.

Bowles, D.J. (1990). Defense-related proteins in higher plants. *Annual Review of Biochemistry,* **59**: 837ï 907.

Brenot, A., King, K. Y., Janowiak, B., Griffith, O. and Caparon, M. G. (2004). Contribution of glutathione peroxidase to the virulence of *Streptococcus pyogenes*. *Infection and Immunity,* **72**: 408ï 413.

Brown, M.A. and De Vito, S.C. (1993). Predicting azo dye toxicity. *Critical Review in Environmental Science and Technology,* **23**:249ï 324.

Celebi, M., Altikatoglu, M., Akdeste, Z.M. and Yēddēām, H. (2012). Determination of decolorization properties of reactive Blue 19 dye using Horseradish Peroxidase enzyme. *Turkish Journal of Biochemistry* **37** (2): 200ï 206.

Cerniglia, C.E, Zhuo, Z., Manning, B.W., Federle, T.W. and Heflich, R.H. (1986). Mutagenic activation of the benzidine-based dye Direct Black 38 by human intestinal microflora. *Mutation Research,* **175**(1):11-16.

Chadwick, R.W., George, S.E. and Claxton, L.D. (1992). Role of the gastrointestinal mucosa andmicroflora in the bioactivation of dietary and environmental mutagens or carcinogens. *Drug Metabolism Reviews*, **24**(4): 425-492.

Chanwun, T., Muhamad, N., Chirapongsatonkul, N. and Churngchow, N. (2013). *Hevea brasiliensis* cell suspension peroxidase: purification, characterization and application for dye decolorization. *AMB Express*, 3: 1-9.

Chequer, F.M.D., Dorta, D.J. and de Oliveira, D.P. (2011). Azo Dyes and Their Metabolites: Does the Discharge of the Azo Dye into Water Bodies Represent Human and Ecological Risks? In: Treating Textile Effluent, Ed; Peter Hauser, In Tech, University Campus STeP Ri Slavka Krautzeka Rijeka, Croatia, pp 27-48.

Chu, Y.F., J. Sun, X. Wu and R.H. Liu. 2002. Antioxidant and antiproliferative activities of common vegetables. *Journal of Agricultural and Food Chemistry,* **50**: 6910-6916.

Civello, P.M., Martinez, G.A., Chares, A.R. and Anon, M.C. (1995). Peroxidase from strawberry fruit (*Fragaria anannassa* Ducd.): Partial purification and determination of some properties. *Journal of Agricultural and Food Chemistry,* **43**: 2596-2601.

Converso, D. A. and Fernandez, M. E. (1995). Peroxidase isozymes from wheat germ: purification and properties. *Phytochemistry*, **40**: 1341.

Conyers, S. M. and Kidwell, D. A. (1991). Chromogenic substrates for horseradish peroxidase. *Analytical Biochemistry*, **192**: 207-213.

Cooper, V.A. and Nicell, J.A. (1996). Removal of phenols from a foundry wastewater using horseradish peroxidase. *Water Research,* **30**: 954ï 964.

Cosio, C. and Dunand, C. (2011). Transcriptome analysis of various flower and silique development stages indicates a set of class III peroxidase genes potentially involved in pod shattering in *Arabidopsis thaliana*. *BMC Genomics*, 11:528-544.

DŃbrowska, G., Kata, A.,Goc, A., SzechyGska-Hebda, M. and Skrzypek, E. (2007). Characteristics of the plant ascorbate peroxidase family. *Acta Biologica Cracoviensia* **49 (**1): 7ï 17.

Daiyasu, H. and Toh, H. (2000). Molecular evolution of the myeloperoxidase family. *Journal of Molecular Evolution*, **51**: 433ï 445.

Das, M.K., Sharma, R.S., Mishra, V. (2011). A novel cationic peroxidase (VanPrx) from a hemi-parasitic plant (*Viscum angulatum*) of western ghats (India): Purification, characterization and kinetic properties. *Journal of Molecular Catalysis B: Enzyme*, **71**: 63-70.

de Montellano, P.R. (2010). Catalytic mechanism of heme peroxidase. In: Torres, E. and Ayala, M. (Eds), Springer Heidelberg Dordrecht, London New York, Pp 79-107.

Diao, M., Ouédraogo, N., Baba-Moussa, L., Savadogo, P. W., NôGuessan, A. G., Bassolé, I. H. N. and Dicko, M. H. (2011). Biodepollution of wastewater containing phenolic compounds from leather industry by plant peroxidases. *Biodegradation,* **22**: 389ï 396.

Duarte-Vazquez, M. A., Garcia-Almendarez, B. E., Regalado, C. and Whitaker, J. R. (2001). Purification and properties of a neutral peroxidase isozyme from turnip (*Brassica napus* L. V*ar. purple top white globe*) roots. *Journal of Agricultural and Food Chemistry*, **49**: 4450-4456.

Duchovskiene, Z. and Siksnianiene, J. (2001). Peroxidase isoenzymic spectra of cabbage, Kohlrabin and red beet during flowering induction and generative development. *Luthuanian Institute of Horticulture,* **2**: 14-16.

Dunford, H.B. (1999). Heme Peroxidases. New York: John Wiley and Sons.

Esancy, J.F., Freeman, H.S. and Claxton, L.D. (1990). The effect of alkoxy substituents on themutagenicity of some aminoazobenzene dyes and their reductive-cleavage products. *Mutation Research*, **238**(1): 1ï 22.

Evans, J.J. (1968). Peroxidases from the extreme dwarf tomato plant. Identification, isolation, and partial perification. *Plant Physiology*, **43**:1037ï 1041.

Eze, S.O.O., Chilaka, F.C. and Nwanguma, B.C. (2000). Purification and characterization of sorghum (KSV8) peroxidase. *Plant Production Resource Journal*, **5**: 51-59.

Eze, S.O.O., Chilaka, F.C. and Nwanguma, B.C. (2010). Studies on thermodynamics and kinetics of thermo-inactivation of some quality-related enzymes in white yam (*Dioscorea rotundata*). *Journal of Thermodynamics and Catalysis,* **1**(1): 1-5.

Eze, S.O.O. (2012). Kinetic analysis of the thermostability of peroxidase from African oil bean (*Pentaclethra macrophylla Benth*) seeds. *Jounal of Biochemical Technology,* **4**(1): 459-463.

Filizola, M. and Loew, G.H. (2000). Role of protein environment in horseradish peroxidase compound I formation: molecular dynamics simulations of horseradish peroxidaseï HOOH complex. *Journal of American Chemistry Society,* **122**: 18ï 25.

Finzel, B.C., Poulos, T.L. and Kraut, J. (1984). Crystal structure of yeast cytochrome c peroxidase refined at 1.7-A resolution. *Journal of Biology and Chemistry*, **259**:13027ï 13036.

Fleischmann, A., Darsow, M., Degtyarenko, K., Fleischmann, W., Boyce, S., Axelsen, K.B., Bairoch, A., Schomburg, D., Tipton, K.F. and Apweiler, R. (2004). IntEnz, the integrated relational enzyme database. *Nucleic Acids Res*., **32**: 434ï 437.

Flohe, L. and Ursini, F. (2008), Peroxidase: a term of many meanings. *Antioxidant and Redox Signaling*, **10**: 1485ï 1490.

Fu, Y. and Viraraghavan, T. (2001). Fungal decolorization of dye wastewaters: a review. *Bioresource Technol*ogy, **79**: 251ï 262.

Furtmuller, P.G., Zederbauer, M., Jantschko, W., Helm, J., Bogner, M., Jakopitsch, C. and Obinger, C. (2006). Active site structure and catalytic mechanisms of human peroxidases. *Archives of Biochemistry and Biophys*ics, **445**: 199ï 213.

Ghahfarrokhi, I.S., Garmakhany, A.D. and Mousavi, S.M. (2013). Estimation of peroxidase activity in red cabbage by Artificial Neutral Network (ANN). *International Association for Cereals Science and Technology*, 5(2): 163-167.

Gajhede, M., Schuller, D.J., Henriksen, A., Smith, A.T., and Poulos, T.L. (1997). Crystal structure of horseradish peroxidase C at 2.15 Å resolution. *Natural Structural Biology,* **4:** 1032ï 1038.

Ghasemi, F., Tabandeh, F. and Bambai, B. (2010). Decolorization of different azo dyes by *phanerochaete chrysosporium* RP78 under optimal condition. *International journal of Environmental Science and Technology***, 7:** 457-464.

Ghiladi, R.A., Medzihradszky, K.F., Rusnak, F.M. (2005). Correlation between isoniazid resistance and superoxide reactivity in *Mycobacterium tuberculosis* KatG. *Journal of the American Chemical Society,* **127**:13428ï 13442.

Golka, K., Kopps, S. and Myslak, Z.W. (2004). Carcinogenicity of azo colorants: influence of solubility and bioavailability ï a Review. *Toxicology Letters*, **151**(1): 203-210.

Govere, E.M., Tonegawa, M., Bruns, M.A., Wheeler, E.F., Heinemann, P.H., Kephart, K.B. and Dec, J. (2005). Deodorization of swine manure using minced horseradish roots and peroxidase. *Journal of Agricultural and Food Chemistry*, **53**: 4880-4889.

Govere, E.M., Tonegawa, M., Bruns, M.A., Wheeler, E.F., Kephart, K.B., Voigt, J.W. and Dec J. (2007). Using minced horseradish roots and peroxidase for the deodorization of swine manure: A pilot scale study. *Bioresource Technology*, **98**: 1191-1198.

Guarantini, C. C. and Zanoni, M. V. (2000). Textile dyes. *Química Nova,* **23**: 71ï 75.

Halpin, B., Pressey, R., Jen, J. and Mondy, N. (1989). Purification and characterization of peroxidase isoenzymes from green peas (*Pisum sati*V*um*). *Journal of Food Science*, **54**: 644-649.

Hamid, H. (2009). Potential applications of peroxidases. *Food chemistry*, **115**: 1177-1186.

Haraguchi, H., Mochida, Y., Sakai, S., Masuda, H., Tamura, Y., Mizutani, K., Tanaka, O. and Chou, W.H. (1996). Protection against oxidative damage by dihydroflavonols in *Engelhardtia chrysolepis. Bioscience, Biotechnology and Biochemistry,* **60**: 945-948.

Harayama, S. (1997). Polycyclic aromatic hydrocarbon bioremediation design. *Current Opinion in Biotechnology*, **8**: 268-273.

Harford-Cross, C.F., Carmichael, A.B., Allan, F.K., England, P.A., Rouch, D.A. and Wong, L.L. (2000). Protein engineering of cytochrome P450cam (CYP101) for the oxidation of polycyclic aromatic hydrocarbons. *Protein Engineering*, **13**: 121-128.

Henriksen, A., Welinder, K.G. and Gajhede, M. (1998). Structure of barley grain peroxidase refined at 1.9-A resolution. A plant peroxidase reversibly inactivated at neutral pH. *Journal of Biology and Chemistry,* **273**: 2241ï 2248.

Hiner, A.N. (1995). Comparative study of the inactivation of wild-type, recombinant and two mutant horseradish peroxidase isoenzyme c by hydrogen peroxidase and m-cloroperoxybenzoic acid. *European Journal of Biochemistry,* **234**:506ï 512.

Hiraga, S., Sasaki, K., Sasaki, H., Ohashi, Y. and Matsiu, H. (2001). A large family class III plant peroxidases. *Plant Cell Physiology*, **45** (5): 462-468.

Hoch, H., Adam, W., Fell, R., Saha-Moller, C.R. and Schreier, P. (1997). Horseradish peroxidase ï a biocatalyst for the one-pot synthesis of enantiomerically pure hydroperoxides and alcohols. *Journal of Molecular Catalysis A,* **117**:321ï 328.

Hochman, A. and Goldberg, I. (1991). Purification and characterization of a catalase-peroxidase and a typical catalase from the bacterium *Klebsiella pneumonia*. *Biochimica et Biophysica Acta,* **1077** (3): 299-307.

Hosoya, Y. (1960). Turnip peroxidase: purification and physicochemical properties of multiple components in turnip peroxidase. *Biochemistry,* **47**:369ï 381.

Howes, B.D., Feis, A., Raimondi, L., Indiani, C. and Smulevich, G. (2001). The critical role of proximal calcium ion in the structural properties of horseradish peroxidase. *Journal of Biology and Chemistry,* **276**: 40704ï 40711.

Hu, Y., Wu, J., Luo, P. and Mo, Y. (2012). Purification and partial characterization of peroxidase from lettuce stems. *African Journal of Biotechnology*, **11**(11): 2752-2756.

Hunger, K. (1994). On the toxicology and metabolism of azo dyes. *Chimia*, **48**: 520-522.

Husain, Q. (2010). Peroxidase mediated decolorization and remediation of wastewater containing industrial dyes: a review. *Reviews in Environmental Science and Biotechnology*, **9**:117ï 140.

Huystee, R.B. and McManus, M.T. (1998). Glycans of higher plant peroxidases: recent observations and future speculations. *Glycoconjugate Journal,* **15**:101ï 106.

Igarashi, K., Uchida, Y., Murakami, N., Mizutani, K. and Masuda, H. (1996). Effect of astilbin in tea processed from leaves of *Engelhardtia chrysolepis* on the serum and liver lipid concentrations and on the erythrocyte and liver antioxidative enzyme activities of rats. *Bioscience, Biotechnology and Biochemistry*, **60**: 513-515.

Jauregui, J., Valderrama, B., Albores, A. and Vazquez-Duhalt, R. (2003). Microsomal transformation of organophosphorus pesticides by white rot fungi. *Biodegradation*, **14**: 397-406.

Kalsoom, U., Ashraf, S.S., Meetani, M.A., Rauf, M.A. and Bhatti, H.N. (2013). Mechanistic study of a diazo dye degradation by soybean peroxidase. *Chemistry Central Journal*, **7**(93): 1-10.

Karla, B. and Gross, R.A. (2002). HRP-mediated polymerization of acrylamide and sodium acrylate. *Green Chemistry,* **4**:174ï 178.

Kennelly, J.C., Aidan, S.A. and Martin, C.N. (1984). Reduction to benzidine is not necessary for the covalent binding of a benzidine azodye to rat liver DNA. *Toxicology,* **32**(4): 315-324.

Kirby, N., Mullan, G. and Marchant, R. (1995). Decolourisation of an artificial textile effluent by *Phanerochaete chrysosporium*. *Biotechnology Letters*, **17:** 761-764.

Kjaersgard, I.V.H., Jespersen, H.M., Rasmussen, S.K. and Welinder, K.G. (1997). Plant *Molecular Biology*, **33**: 699-708.

Kookana, R.S., Baskaran, S. and Naidu, R. (1998). Pesticide fate and behavior in Australian soils in relation to contamination and management of soil and water: a review. *Australian Journal of Soil Research*, **36**: 715-764.

Kopsell, D.A., Kopsell, D.E., Lefsrud, M.G., Curran-Cellentano, J. and Dukach, L. (2004). Variation in lutein, b-carotene, and chlorophyll concentrations among *Brassica oleracea* cultigens and seasons. *Horticultural Science,* **39**:361ï 364.

Koua, D., Cerutti, L., Falquet, L., Sigrist, C.J., Theiler, G., Hulo, N. and Dunand, C. (2009). PeroxiBase: a database with new tools for peroxidase family classification. *Nucleic Acids Research*, **37**: 261-266.

Kunishima, N., Fukuyama, K., Matsubara, H., Hatanaka, H., Shibano, Y. and Amachi, T. (1994). Crystal structure of the fungal peroxidase from *Arthromyces ramosus* at 1.9 A ę resolution. Structural comparisons with the lignin and cytochrome c peroxidases. *J Molecular Biology*, **235**:331ï 344.

Kurilich, A.C. and Juvik, J. (1999). Quantification of carotenoid and tocopherol antioxidants in *Zea mays*. *Journal of Agricultural and Food Chemistry,* **47**:1948ï 1955.

Kusznierewicz, B., Bartoszek, A., Wolska, L., Drzewiecki, J., Gorinstein, S. and Namies´nik, J. (2007). Partial characterization of white cabbages (*Brassica oleracea var. capitata f. alba*) from different regions by glucosinolates, bioactive compounds, total antioxidant activities and proteins. *LWT Food Science and Technology*, **41**:1ï 9

Kvaratskhelia, M., Winkel, C. and Thorneley, R. N. F. (1997). Purification and characterization of a novel class III peroxidase isoenzyme from tea leaves. *Plant Physiology*, **114**: 1237.

Lavania, M., Chauhan, P.S., Chauhan, S.V., Singh, H.B. and Nautiyal, C.S. (2006). Induction of plant defence enzymes and phenolics by treatment with plant growth-promoting rhizobacteria *Serratia marcescens* NBRI1213. *Current Microbiology*, **52**: 363ï 368.

Lee, H. C. and Klein, B. P. (1990). Classification of green pea peroxidases by preparative isoelectric focusing. *Journal of Food Biochemistry,* **14**:137-141.

Lee, M. Y. and Kim, S. S. (1994). Characteristics of six isoperoxidases from Korean radish roots. *Phytochemisry*, **35**: 287-290.

Lee, T.T. (1977). Role of phenolic inhibitors in peroxidase-mediated degradation of indole-3-acetic acid. *Plant Physiology,* **59**(3): 372-375.

Leelakriangsak, M. and Borisut, S. (2012). Characterization of the decolorizing activity of azo dyes by *Bacillus subtilis* azo-reductase AzoR1. *Songklanakarin Journal of Science and Technol*ogy, **34** (5): 509-516.

Li, H., Li, Y., Cao, H., Li, X. and Zhang, Y. (2011). Degradation of pentachlorophenol by a novel peroxidase-catalyzed process in the presence of reduced nicotinamide adenine dinucleotide. *Chemosphere,* **83**: 124ï 30.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry,* **193**:265-275.

Mackova, M., Ferrib, E.N., Demnerova, K. and Macekc, T. (2001). Quantitative chemiluminescent detection of plant peroxidases using a commercial kit originally designed for blotting assays. *Chemical Journal*, **95**: 130-132.

Maddhinni, V.L., Vurimindi, H.B. and Yerramilli, A. (2006). Degradation of azo dye with horse radish peroxidase (HRP). *Journal of Indian Institute of Science*, **86**: 507ï 514.

Mader, M. and Fuss, R. (1982). Role of peroxidase in lignification of tobacco cells. *Plant Physiology*, **70**:1132ï 1134.

Mathews, M.C., Summers, C.B. and Felton, G.W. (1997). Ascorbate peroxidase: a novel antioxidant enzyme in insects. *Archives of Insect Biochemistry and Physiology,* **34**: 57ï 68.

McCauley, L.A., Langley, R., Robson, M.G. and Rohlman, D. (2006). Studying health outcomes in farmworker populations exposed to pesticides. *Environmental Health Perspectives*, **114**: 953-960.

Mdluli, K.M. (2005). Partial purification and characterization of polyphenol oxidase and peroxidase from marula fruit (*Sclerocarya birrea* subsp. Caffra). *Food Chemistry,* **92**: 311-323.

Meizler, A., Roddick, F. and Porter, N. (2011). A novel glass support for the immobilization and UV-activation of horseradish peroxidase for treatment of halogenated phenols. *Chemical Engineering Journal*, **172**: 792ï 798.

Missall, T. A. & Lodge, J. K. (2005). Thioredoxin reductase is essential for viability in the fungal pathogen, *Cryptococcus neoformans. Eukaryotic Cell,* **4***:* 487ï 489.

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Sciences*, **7**: 405ï 410.

Mudd, J.B. and Burris, R.H. (1959). Inhibition of peroxidae-catalyzed oxidation. *The Journal of Biological chemistry,* **234** (12): 3281-3285.

Nawaz, K.,Hussain, K., Choudary, N., Majeed, A., Ilyas., U.,Ghani, A., Lin, F., Ali, K., Afghan, S., Raza, G., Lashari, M.I. (2011). Eco-friendly role of biodegradation against agricultural pesticides hazards. *African Journal of Microbiology Research,* **5**(3): 177-183.

Nicell, J.A., Al-Kassim, L., Bewtra, J.K. and Taylor, K.E. (1993). Wastewater treatment by enzyme catalyzed polymerization and precipitation. *Water Resources*, **27**: 1629-1639.

Novotny, C., Rawal, B., Bhatt, M., Patel, M., Susek, V. and Molutoris, H. P. (2001). Irpex lactless and *Pleatotus ostreatus* for decolourization of chemically different dyes. *Journal of Biotechnology,* **89**: 113ï 122.

Nwanguma, B.C. and Eze, M.O. (1995). Heat sensitivity, optimum pH and changes in activity of sorghum peroxidase during malting and mashing. *Journal of the lnstitute of Brewing*, **101**: 275-276.

Ong, S.T., Keng, P.S., Lee, W.N., Ha, S.T. and Hung, Y.T. (2011). Dye waste treatment. *Water*, **3**: 157-176.

Onsa, G.H., bin Saari, N., Selamat, J. and Bakar, J. (2004). Purification and characterization of membrane-bound peroxidases from *Metroxylon sagu*. *Food Chem*istry, **85**: 365-376.

Panchuk, I.I., Volkov, R.A., and Schöffl, F. (2002). Heat stress- and heat shock transcription factor-dependent expression and activity of ascorbate peroxidase in *Arabidopsis*. *Plant Physiology,* **129**: 838ï 853.

Park, C., Lee, M., Lee, B., Kim, S-W., Chase, H.A., Lee, J. and Kim, S. (2007). Biodegradation and biosorption for decolorization of synthetic dyes by *Funalia trogii. Biochemical Engineering Journal*, **36**(1): 59-65.

Park, J.B. and Clark, D.S. (2006). Deactivation mechanisms of chloroperoxidase during biotransformations. *Biotechnology and Bioengineering*, **93**:1190ï 1195.

Passardi, F., Penel, C. and Dunand, C. (2004). Performing the paradoxical: how plant peroxidases modify the cell wall. *Trends in Plant Science,* **9**(11):534-540.

Passardi, F., Bakalovic, N., Teixeira, F.K., Pinheiro-Margis, M., Penel, C. and Dunand, C. (2007). Prokaryotic origins of the peroxidase superfamily and organellar-mediated transmission to eukaryotes. *Genomic*, **89**: 567ï 579.

Petersen, J.F., Kadziola, A. and Larsen, S. (1994). Three-dimensional structure of a recombinant peroxidase from Coprinus cinereus at 2.6 A ę resolution. *FEBS Letters,* **339**:291ï 296.

Pielesz, A., Baranowska, I., Rybak, A. and Wğpchowicz, A. (2002). Detection and Determination of Aromatic Amines as Products of Reductive Splitting from Selected Azo Dyes. *Ecotoxicology and Environmental Safety*, **53**(1): 42-47.

Pineyro, M.D., Parodi-Talice, A., Arcari, T. and Robello, C. (2008). Peroxiredoxins from *Trypanosoma cruzi*: virulence factors and drug targets for treatment of Chagas disease? *Gene*, **408**: 45-50.

Pinheiro, H.M., Touraud, E. and Thomas, O. (2004). Aromatic amines from azo dye reduction: status review with emphasis on direct UV spectrophotometric detection in textile industry wastewaters. *Dyes and Pigments,* **61**(2): 121ï 139.

Platzek, T., Lang, C., Grohmann, G., Gi, U.S. and Baltes, W. (1999). Formation of a carcinogenic aromatic amine from an azo dye by human skin bacteria in vitro. *Human and Experimental Toxicology*, **18**(9): 552-559.

Poulos, T.L., Freer, S.T., Alden, R.A., Edwards, S.L., Skogland, U., Takio, K., Eriksson, B., Xuong, N., Yonetani, T. and Kraut, J. (1980). The crystal structure of cytochrome c peroxidase. Journal of *Biology and Chemistry,* **255**: 575ï 580.

Poulos, T.L., Edwards, S.L., Wariishi, H. and Gold, M.H. (1993). Crystallographic refinement of lignin peroxidase at 2Å. *Journal of Biology and Chemistry,* **268**:4429ï 4440.

Preethi, S., Anumary, A., Ashokkumar, M. and Thanikaivelan, P. (2013). Probing horseradish peroxidase catalyzed degradation of azo dye from tannery wastewater. *SpringerPlus*, **2**: 341-349.

Pulido, R., Bravo, L. and Saura-Calixto, F. (2000). Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *Journal of Agricultural and Food Chemistry,* **48**: 3396-3402.

Puppo, A., Rigaud, J., Job, D., Ricard, J. and Zeba, B. (1980). Peroxidase content of soybean root nodules. *Biochimica et Biophysica Acta,* **614**:303ï 312.

Rajaguru, P., Fairbairn, L.J., Ashby, J., Willington, M.A., Turner, S., Woolford, L.A., Chinnasamy, N. and Rafferty, J.A. (1999). Genotoxicity studies on the azo dye DirectRed 2 using the *in vivo* mouse bone marrow micronucleus test. *Mutation Research*, **444**: 175-180.

Ramanathan, K., Jonsson, B.R. and Danielsson, B. (2000). Thermometric sensing of peroxide in organic media. Application to monitor the stability of RBP-retinol-HRP complex. *Anal Chem* **72**:3443ï 3448.

Raven, E.L. (2003). Understanding functional diversity and substrate specificity in haem peroxidases: what can we learn from ascorbate peroxidase? *Natural Product Report,* **20**: 367-381.

Regelsberger, G., Jakopitsch, C., Furtmuller, P.G., Rueker, F., Switala, J., Loewen, P.C. and Obinger, C. (2001). The role of distal tryptophan in the bifunctional activity of catalase-peroxidase. *Biochemical Society Transactions,* **29**:99ï 105.

Rehman, K., Yaqub, M., Sheikh and Arshad, M. (1999). Extraction and evaluation of peroxidases from various vegetable sources. *International Journal of Agriculture and Biology*, **3**: 170-173.

Riera-Torres, M., Gutiérrez-Bouzán, C., Crespi, M. (2010). Combination of coagulation ï flocculation and nanofiltration techniques for dye removal and water reuse in textile effluents. *Desalination*, **252**:53ï 59.

Robinson, D.S. (1987). Scarvenging enzyme and catalases. In Biochemistry and Nutritional Value; Robinson, D. S., Ed.; Longman Scientific and Technical: Harlow, pp 459-465.

Robinson, D. S. (1991). Peroxidases and catalases in foods. In *Oxidative Enzymes in Food*, 1st ed, Robinson, D. S., Eskin, N. A. M., Eds.; Elsevier Applied Science, London, U.K., pp 1-37.

Rodrigo, C., Rodrigo, M., Alvarruiz, A. and Frigola, A. (1996). Thermal inactivation at high temperatures and regeneration of green asparagus peroxidase. *Journal of Food Protection*, **59**: 1065-1071.

Saboora, A., Parsiavash, l. and Moosavi-Nejad, Z. (2012). Purification and kinetic properties of guaiacol peroxidase in turnip (*Brassica napus var. okapi*) root during different growth stages. *Progress in Biological Sciences*, **2** (1): 76-86.

Saini, P.K., Webert, D.W. and Judkins, J.C. (1995). Role of sodium azide in reducing nonspecific color development in enzyme immunoassays. *Journal of Veterinary Diagnostic Investigation,* **7**:509-514.

Sakharov, I.Y., Vorobiev, A.C. and Leon, J.J. (2003). Synhesis of polyelectrolyte complexes of polyaniline and sulfonated polystyrene by palm tree peroxidase. *Enzyme Microbiology and Technology*, **33**: 661-667.

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):138ï 157.

Sasaki, K., Iwai, T., Hiraga, S., Kuroda, K. and Seo, S. (2004). Ten rice peroxidases redundantly respond to multiple stresses including infection with rice blast fungus. *Plant Cell Physiol* **45**: 1442ï 1452.

Schuller, D.J., Ban, N., van Huystee, R., McPherson, A. and Poulos, T.L. (1996). The crystal structure of peanut peroxidase. *Structure,* **4**: 311ï 321.

Shigeoka, S., Ishikawa, T., Tamoi, M., Miyagawa, Y., Takeda, T., Yabuta, Y. and Yoshimura, K. (2002). Regulation and function of ascorbate peroxidase isoenzymes. *Journal of Experimental Botany,* **53**: 1305ï 1319.

Silva, E., Lourenço, E. J. and Neves, V. A. (1990). Soluble and bound peroxidase from papaya fruit. *Phytochemistry,* **29**, 1051-6.

Silva, M.C., Torres, J.A., Corrêa, A.D., Junqueira, A.M., Amorim, M.T. and dos Santos, C.D. (2012). Obtention of plant peroxidase and its potential for the decolorization of the reactive dye Remazol Turquoise G 133%. *Water, Science and Technology*, **65**(4): 669-675.

Singh, J., Dubey, A., Diwakar, S.K., Rawat, S.K., Batra, N. and Joshi, A. (2010). Biochemical characterization of peroxidases from the fruits of *Mallus pumilus*. *International Research Journal of Biotechnology*, **1**(4): 50-58.

Smith, A.T. and Veitch, N.C. (1998). Substrate binding and catalysis in heme peroxidases. *Curr Opin Chem Biol* **2**:269ï 278.

Smulevich, G., Paoli, M. and Burke, J.F., Sanders, S.A., Thorneley, R.N. and Smith, A.T. (1994). Characterization of recombinant horseradish peroxidase C and three site-directed mutants, F41V, F41W, and R38K by resonance Raman spectroscopy. *Biochemistry,* **33**: 7398ï 7407.

Soltis, P.S. and Soltis, D. (1990). Isozymes in Plant Biology. Baltimore: Timber Press.

Stolz, A. (2001). Basic and applied aspects in the microbial degradation of azo dyes. *Applied Miocrobiology and Biotechnology*, **56**: 69ï 81.

Stahlmann, R., Wegner, M., Riecke, K., Kruse, M. and Platzek, T. (2006). Sensitising potential of four textile dyes and some of their metabolites in a modified local lymph node assay. *Toxicology*, **219**(1): 113ï 123.

Straus, W. (1971). Inhibition of peroxidase by methanol and by methanol-nitroferricyanide for use in immunoperoxidase procedures. *Histochemistry and Cytochemistry*, **19** (11): 628-688.

Sundaramoorthy, M., Kishi, K, Gold, M.H. and Poulos, T.L. (1994). The crystal structure of manganese peroxidase from *Phanerochaete chrysosporium* at 2.06-Aę resolution. *Journal of Biology and Chemistry,* **269**: 32759ï 32767.

Surekha, R.M., Lakshmi, P.K.L., Suvarnalatha, D., Jaya, M., Aruna, S., Jyothi, K., Narasimha, G., Venkateswarlu, K. (2008). Isolation and characterization of a chlorpyrifos degrading bacterium from agricultural soil and its growth response. *African Journal Microbiology Research,* **2**: 26-31.

Tams, J.W. and Welinder, K.G. (1995). Mild chemical deglycosylation of horseradsih peroxidase yields a fully active, homogeneous enzyme. *Analytical Biochemistry,* **228**:48ï 55.

Taurog, A. (1999). Molecular evolution of thyroid peroxidase. *Biochimie*, ***81***(5): *557-562.*

Theorell, H. (1942). The preparation and some properties of crystalline horse-radish peroxidase. *Ark Kemi Mineral Geology,* **16**:1ï 11.

Thongsook, T. and Barrett, D.M. (2005). Purification and partial characterization of broccoli (*Brassica oleracea Var. Italica*) peroxidases. *Journal of Agricultural and Food Chemistry*, **53**: 3206-3214.

Thorpe, G. H. and Kricka, L. J. (1986). Enhanced chemiluminescent reactions catalyzed by horseradish peroxidase. *Methods in Enzymology,* **133**: 331-338.

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

Torres, E. and Ayala, M. (2010). Biocatalysis Based on Heme Peroxidases: Peroxidases as a potential Industrial biocatalysts. Springer Heidelberg Dordrecht, London, New York, pp 8-22.

Tsuboy, M.S., Angeli, J.P., Mantovani, M.S., Knasmüller, S., Umbuzeiro, G.A. and Ribeiro, L.R. (2007). Genotoxic, mutagenic and cytotoxic effects of the commercial dye CI Disperse Blue 291 in the human hepatic cell line HepG2. *Toxicology in Vitro*, **21**(8): 1650-1655.

Ueda, H. and Ogawa, H. (1999). Glycobiology of the plant glycoprotein epitope: structure, immunogenicity and allergenicity of plant glycotopes. *Trends in Glycoscience and Glycotechnology,* **11**:413ï 428.

Umbuzeiro, G.A., Freeman, H., Warren, S.H., Kummrow, F. and Claxton, L.D. (2005). Mutagenicity evaluation of the commercial product C.I. Disperse Blue 291 usingdifferent protocols of the *Salmonella* assay. *Food and Chemical Toxicology,* **43**(1): 49-56.

Valderrama, B. (2010). Deactivation of hemeperoxidases by hydrogen peroxide: focus on Compound III. In: Torres, E. and Ayala, M. (Eds), Springer Heidelberg Dordrecht, London New York. Pp 291-314.

Van Loon, L.C., Rep, M. and Pieterse, C.M. (2006). Significance of inducible defencerelated proteins in infected plants. *Annual Review of Phytopathology,* **44**: 135ï 162.

Vera, P., Torneo, P. and Conejero, V. (1993). Cloning and expression analysis of a viroid-induced peroxidase from tomato plants. *Molecular Plant Microbe-Interactions,* **6**: 790ï 794.

Veitch, N.C. and Smith, A.T. (2001). Horseradish peroxidase. *Advance Inorganic Chemistry,* **51**: 107ï 162.

Veitch, N.C. (2003). Structural determinants of plant peroxidase functions. *Phytochemistry Reviews*, **3**: 3-18.

Veitch, N.C. (2004). Horseradish peroxidase: a modern view of a classic enzyme. *Phytochemistry,* **65**:249ï 259.

Wada, N., Kinoshita, S., Matsuo, M., Amako, K., Miyake, C. and Asada, K. (1998). Purification and molecular properties of ascorbate peroxidase from bovine eye. *Biochemical and Biophysical Research Communications*, **242**(2): 256-261.

Wagner, M. and Nicell, J.A. (2002). Detoxification of phenolic solutions with horseradish peroxidase and hydrogen peroxide. *Water Research,* **36**: 4041ï 4052.

Walker, R. (1970). The Metabolism of Azo Compounds: A Review of the Literature. *Food and Cosmetics Toxicology,* **8**(6): 659-676.

Ward, W.W. and Swiatek, G. (2009). Protein purification. *Current Analytical Chemistry,* **5**(2): 1-21.

Weber, R., Johnston, P., Forter, M., Hollert, H., Heinisch, E., Holoubek, I., Lloyd-Smith, M., Masunaga, S., Moccarelli, P., Santillo, D., Seike, N., Symons, R.,

Torres, J.P., Verta, M., Varbelow, G., Vijgen, J., Watson, A., Costner, P., Woelz, J., Wycisk P. and Zennegg, M. (2008). Dioxin- and POP-contaminated sitescontemporary and future relevance and challenges: overview on background, aims and scope of the series. *Journal of Environmental Science and Pollution Research*, **15**: 363-393.

Weisburger, J.H. (2002). Comments on the history and importance of aromatic and heterocyclic amines in public health. *Mutation Research*, **506**: 9ï 20.

Welinder, K.G. (1976). Covalent structure of the glycoprotein horseradish peroxidase (EC 1.11.1.7). *FEBS Letters,* **72**:19ï 23.

Welinder, K.G. and Gajhede, M. (1993). Structure and Evolution of Peroxidases. In: Welinder, K.G., Rasmussen, S.K., Penel, C., Greppin, H. (Eds.), Plant Peroxidases: Biochemistry and Physiology. University of Geneva, Geneva, pp. 35ï 42.

Xialing, C. and Lin, M. (2009). Horseradish peroxidase catalyzed free radical cannot free move in reaction solution. *Journal of Biochemical Technology*, **1**(4):92-95.

Yang, B.Y., Gray, J.S.S., Montgomery, R., (1996). The glycans of horseradish peroxidase. *Carbohydrate Research,* **287**: 203ï 212.

Young, S.A., Guo, A., Guikema, J.A., White, F.F. and Leach, J.E. (1995). Rice cationic peroxidase accumulates in xylem vessels during incompatible interactions with *Xanthomonas oryzae pv. oryzaae. Plant Physiology,* **107**: 1333ï 1341.

Zahn, J.A., Hatfield, J.L., Do, Y.S., DiSpirito, A.A., Laird, D.A. and Pfeiffer, R.L. (1997). Characterization of volatile organic emissions and wastes from a swine production facility. *Journal of Environmental Quality*, **26**: 1687-1696.

Zhang, G. and Nicell, J.A. (2000). Treatment of aqueous pentachlorophenol by horseradish peroxidase and hydrogen peroxide. *Water Research,* **34**:1629ï 1637.

Zollinger, H. (1991). *Color chemistry: syntheses, properties and applications of organic dyes and pigments*. 3nd ed., Wiley ï VCH, New York, USA.

APPENDICES

1.0. Preparation of solutions

1.1. Preparation of buffer solution

1.1.1. Phosphate buffer (0.0.5M) pH 6.0

di-sodium hydrogen orthophosphate (Na_2HPO_4) in 1000ml of distilled water

Molecular weight of Na_2HPO_4= 142g

1M of 1000ml= 142g/mol

0.1M Na_2HPO_4= 0.1 x 142g/mol = 14.2g

14.2g of Na_2HPO_4 was dissolved in distilled water and stirred with magnetic stirrer until a homogenous solution was formed. The solution was titrated against phosphoric acid till the required pH was obtained at room temperature. Then distilled water was added to make it up to 1000ml

1.1.2. Tri's ($C_6H_{11}NO_3$) buffer (0.05M), 100ml volume, pH 8.0, 8.5, 9.0

Molecular weight= 121.14g

1M= 121.14g/mol

0.05M = 0.05x121.14 =6.057g

1000ml= 0.05M = 6.057g

Therefore 100ml= $6.057 \times \frac{100}{1000} = 0.61g$

0.61g of Triȍs was dissolved in distilled water and stirred with magnetic stirrer until a homogenous solution was formed. The solutions were titrated against HCl untill the required pHs were obtained at room temperature. Then distilled water was added to make each solution up to 100ml.

1.1.3. Acetate (CH_3COONa) buffer (0.05M), volume of 100ml, pH 3.5, 4.0, 4.5, 5.0, 5.5

Molecular weight of CH_3COONa = 82.03g

1M CH_3COONa = 82.03g/mol

0.05M CH_3COONa = 0.05x82.03= 4.10g

1000ml = 0.05M = 4.10g

Therefore, 100ml = $4.10 \times \frac{100}{1000} = 0.41g$

0.41g of acetate was dissolved in distilled water and stirred with magnetic stirrer until a homogenous solution was formed. The solutions were titrated against HCl until the required pHs were obtained at room temperature. Then distilled water was added to make each solution up to 100ml.

1.2. Preparation of Hydrogen peroxide solution (0.01M to 2.4M)

The stock H_2O_2 was labeled 30%, specific gravity =1.10g, molecular weight= 34g

Using the formula;

$$volume = molarity \times molecular\ weight \times \%assay \div specific\ gravity$$

For 0.01M,

$$volume = 0.01 \times 34 \times \frac{100}{30} \div 1.1 = 1.03ml$$

$$1000ml \equiv 1.03ml(of\ the\ Stock) \equiv 0.01M$$

$$\therefore 100ml \equiv 1.03 \times \frac{100ml}{1000ml} = 0.1ml$$

Therefore, to prepare 0.01M of H_2O_2 in 100ml, 0.1ml of H_2O_2 from the stock was dissolved in 20ml of distilled water and was made up to 100ml mark using distilled water.

1.3. Preparation of 0.1% O-dianisidine solution (0.04M)

Molecular weight of O-dianisine = 244.3g/mol

$$\therefore 1M \equiv 244.3g \equiv 1000ml$$

$$0.04M \equiv 244.3g \times \frac{0.04M}{1M} = 9.772g$$

$$0.04M \equiv 9.772g \equiv 1000ml$$

$$\therefore 50ml = 9.772g \times \frac{50ml}{1000ml} = 0.489g$$

Therefore to prepare 0.04M of O-dianisidine, 0.489g of O-dianisidine was dissolved in little volume of methanol and made up to 50ml mark with methanol. The mixture was then filtered with Whatman filter paper. The filtrate was used as the O-dianisidine solution.

1.4. Preparation of Vat dye solution

Vat Green 9 and Vat Orange 11 were prepared by dissolving 7g of the dye pigment, 3g of sodium hydrosulphite salt and 3g of NaOH in 200ml of boiling water and was made up to 2000ml

Azo Brilliant Black, Azo trypan Blue, Azo Blue 5, Azo Citrus Red 2, Azo Yellow 6, Azo Pink and Azo Purple were prepared by dissolving 0.5g of the dye pigment in 500ml of distilled water.

1.5. Preparation of the Component Reagents For Protein Determination

The reagents used were prepared as follows;

Solution A: 2g of Na_2CO_3 and 0.4g NaOH were dissolved in distilled water and make it up to 100ml with distilled water.

Solution B: 0.5g of sodium potassium tartarate and 0.25g of $CuSO_4$ were dissolved in distilled water and make it up to 50ml with distilled water.

Solution C: 1N folin ciocalteau (phenol reagent) in 1:1 dilution with distilled water.

Solution D: 0.02g of bovine serum albumin (BSA) dissolved in 100ml of distilled water. Concentration of the BSA is 0.02g/100ml (0.2mg/ml).

Solution E: 50ml of solution A + 1ml of solution B

Protein Standard Curve, Using Bovine Serum Albumin (BSA)