BIODEGRADATION OF HYDROCARBONS BY PEROXIDASE FROM DI-CULTURE OF *Rhizopus stolonifer* AND *Saccharomy cescerevisiae* ISOLATED FROM CRUDE OIL-POLLUTED SOIL

BY

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CERTIFICATION

This is to certify that this work, "Biodegradation of hydrocarbons by peroxidase fromdi-culture of *Rhizopus stolonifer* and *Saccharomyces cerevisiae* isolated from crude oil-polluted soil", was carried out by FERDINAND PASCHALINE UDOKA (20164994648) in partial fulfilment for the award of the degree of M.Sc. in Environmental Health Biology in the Department of Biotechnology of the Federal University of Technology, Owerri.

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DEDICATION

To God almighty who has been my strength in all and the reason I smile while burning my midnight candle

And

To my family, friends and all my well-wishers for all the support so far throughout the journey.

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ABSTRACT

This study was carried out to evaluate the biodegradation of hydrocarbons by peroxidases obtained fromdi-culture of *Rhizopus* and *Saccharomyces* spp. isolated from crude oil-polluted soil. Crude oil-polluted soil samples were collected from loading jetty site at Onne. The physicochemical analysis of the soil samples revealed that it was acidic (pH 5.02) with conductivity of 723. Dissolved mineral contents of the contaminated soil included Cl, Mg, K, Ca and $P0_3^-$. Heavy metals identified in the contaminated soil included Fe, As, Cu and Pb with higher values compared to the control soil. Hydrocarbon degrading peroxidase was produced from the fungal di-cultures isolated from the polluted soil using standard microbiological and biochemical techniques. Additionally, molecular identification using primers targeting the 18S RNA of extracted DNA confirmed the presence of *Rhizopus stolonifer* and *Saccharomyces cerevisiae*. Assessment of emulsification potentials of the isolated fungi showed that both organisms displayed greater potentials in drop collapse and emulsification index. Fourteen days pilot study carried out on the isolated fungi optimized for peroxidase production showed day eight (8) as the day of maximum enzyme production in the fermentation media. Optimum operational conditions for enzyme substrate reaction for peroxidase activity from the fungi were evaluated and observed at pH (4.5) and temperature (50^{o}C) for the contaminated soil as compared to the control which was 6.0 and 40^{o}C respectively. Peroxidase was stable in the presence of the metal ions (0.02-0.05 M concentrations) and in all the pH ranges, but Pb^{2+} was found to have inhibitory effect. At pH 4.5, the enzyme showed the maximum stability strength. Peroxidase retained 78% of its activity for the 180 min pre-incubation time in the presence of the metal ions, except in Pb^{2+} (90% loss of activity). Calcium ionshowed more stabilization strength than all other metal ions. The enzyme retained 50% of its activity for 180 min in the absence of metal ions in all the pH ranges. The analysis of kinetic parameters showed that the enzyme had K_M of 1.8 mM and V_{MAX} of 20.3 μ/mol/min against the 5mM of O-dianisidine substrate. Peroxidase incubated with the crude oil in a basal mineral medium showed strength of utilization of the carbon catenation chains in all the optimized parameters. Peroxidase from the fungal di-culture showed optimal degradative ability at acidic pH range with the peak at 5.5, with the residual oil given as 15-20% v/v. Evaluation of biodegradation by the peroxidase showed that the total weight of degraded oil was 4.02g on day 12 of 20. The weight of oil degraded increased with increase in enzyme concentration with about 80% efficiency of degradation. Increase in concentration of crude oil caused no significant difference in the weight of oil degraded. This study has shown that peroxidase has a great potential in the biodegradation of hydrocarbons present in crude oil polluted soil and is of high eco-toxicological relevance as regards to environmental remediation. However, the present work was mainly laboratory based but assessment of the effectiveness in the field would be of future benefit.

Key words: Bio-degradation, heavy metals, peroxidase, *Rhizopus*sp., *Saccharomyces* sp., pH, temperature, stability.

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND INFORMATION

Environmental pollution from petroleum and petrochemical derivatives is recognized as one of the most serious problems in developing countries due to their growing population (Riskuwa-Shehu and Ijah, 2016). Oil pollution has made a huge impact in all spheres of life including human health risk, damage to the ecosystem, disruption of lives and means of livelihood, health hazards to aquatic animals, risk to food security, loss of aquatic plants, depletion of fish population, and damage to wildlife in general. Furthermore, it causes pollution of the air leading to other illnesses and poverty.

NAS (2003), reported that response of organisms to petroleum hydrocarbons can be manifested at four levels of biological organization: (1) biochemical and cellular; (2) organismal, including the integration of physiological, biochemical and behavioral responses; (3) population, including alterations in population dynamics; and (4) community, resulting in alterations in community structure and dynamics. Impairment of behavioural, developmental, and physiological processes may occur at concentrations significantly lower than acutely toxic levels; such responses may alter the long-term survival of affected populations. Thus, the integration of physiological and behavioural disturbances may result in alterations at the population and community levels.

Crude oil is a naturally occurring complex mixture of hydrocarbon and non-hydrocarbon compounds which at appropriate concentrations possesses a measurable toxicity towards living systems. The toxicity of crude oil or petroleum products varies widely, depending on their composition, concentration, environmental factors and on the biological state of the organisms at

the time of the contamination (Singh and Chandra, 2014). Hydrocarbons are any class of organic compounds composed only of carbon (C) and hydrogen (H) elements. They are the principal constituents of petroleum, they serve as fuels, lubricants and raw materials for the production of plastics, rubbers, fibers, solvents, explosives and industrial chemicals. The name petroleum covers both naturally occurring unprocessed crude oil and petroleum products that are refined from crude oil. Petroleum and petroleum products are highly complex and varied mixtures. Petroleum is defined as any mixture of natural gas, condensate, and crude oil. Crude oil can consist of thousands of individual compounds with hydrocarbons representing from 50 to 98 percent of the total weight of crude oil (Sheetal, 2012). It contains hundreds or thousands of aliphatic, branched and aromatic hydrocarbons (Chikere *et al.,* 2009), most of which are toxic to living organisms (ATSDR, 2010).

Microorganisms have been considered as treasure of useful enzymes. There is a great variation between various genera as to their ability to produce a particular enzyme which varies with the particular growth medium and pH. Recently the potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms. Microorganisms especially fungi have high tolerance of the toxicity of hydrocarbons due to their physiology and adaptations to such variations in the environment and have developed the mechanism for the elimination of oil from polluted environments. Fungus is considered as an efficient candidate for potential degradation of hydrocarbons. Hundreds of different species of fungi inhabit the soil, especially near the soil surface where aerobic conditions prevail. Such fungi are active in degrading a wide variety of biological materials present in the soil (Saranraj and Stella, 2013). The role of fungi in biodegradation process of petroleum products and the most common fungi have been recorded as bio-degraders. Fungi isolated from oil-spill environments can reduce oil pollution (Balaji *et al.,*

2013; Das and Chandran, 2011). Therefore, interest in fungi and associated enzyme secretion has received a considerable attention for bioremediation of hydrocarbon contaminated sites, to remove hydrocarbons from the environment.

The super-family of heme peroxidases from plants, fungi and bacteria is a group of enzymes that utilize hydrogen peroxide to oxidize a second (reducing) substrate often aromatic oxygen donor. Peroxidases catalyze various oxidative reactions in which electrons are transferred to peroxide species (often H_2O_2) and substrate molecules are oxidized. These enzymes have been found in all living organisms, involved in a variety of biological processes (Florian and Anton, 2015). Peroxidase forms part of the defense system of living organisms against radical-mediated peroxidation of unsaturated lipids. They are ubiquitous in nature and are involved in various physiological processes in microorganisms. Plant peroxidases are among the widely studied peroxidases; peroxidases from microorganism especially fungal strains are still gaining ground. Hiraga *et al. (*2001) reported that peroxidases produced by microorganisms are better than those from plants as microorganisms show a higher doubling time than plants and such peroxidases show high substrate specificity. Studies have suggested that peroxidases play a role in suberization, cross-linking of cell wall structural proteins, hormonal catabolism, anti-oxidation against oxidation from exogenous and endogenous radicals and self–defense against pathogens and senescence (Hiraga *et al.*, 2001).

Currently, industrial application of peroxidases in chemistry, pharmacology and biotechnology (environmental) is well developed. Peroxidases are used in waste treatment in order to remove aromatic phenols and amines from aqueous solutions in the presence of hydrogen peroxide. In this treatment, phenolic compounds are polymerized in the presence of hydrogen peroxide through a radical oxidation-reduction mechanism (Li *et al*., 2013). During the treatment process, as the

hydrogen peroxide concentration increases, an irreversible mechanism-based inactivation process becomes predominant and it leads to the degradation of the heme, the release of iron and the formation of two fluorescent products (Rodriguez-Lopez *et al*., 1997).

1.2. PROBLEM STATEMENT

Enormous quantities of noxious pollutants have been released into our ecosystem over the last few decades. Hydrocarbon contamination in the environment is a very serious problem whether it comes from petroleum, pesticides, or other toxic organic matter. Environmental pollution caused by petroleum is of great concern because petroleum hydrocarbons are toxic to all forms of life. It renders the environment unsightly and constitutes a potential threat to humans, animals and vegetation; its impacts on the environment are mainly negative. This is due to its toxicity which contributes to all forms of pollution such as land pollution, air pollution, acid rain and many types of illnesses in humans. Petroleum pollution also causes climate change due to increased green-house gas emissions which occur mainly during extraction, transportation, refinement and consumption phases.

Fat soluble components may accumulate in the organs of animals and may be enriched in the food chain, even up to human beings (Edewor *et al*., 2014). Prolonged exposure and high oil concentration may cause the development of liver or kidney diseases, possible damage to the bone marrow and an increased risk of cancer (Mishra *et al*., 2012). In the long term, toxic and carcinogenic compounds can cause intoxication, diseases, cell damage, developmental disorders and reproduction problems (ATSDR, 2009). In addition to toxic effects, oil products can affect plants and animals physically. A thick layer of oil inhibits the metabolism of plants and suffocates

them, this can lead to the destruction of plants which affects the food web and decreases the natural habitat of numerous species.

1.3. OBJECTIVES OF STUDY

Aim:

The main objective of this study is to evaluate the biodegradation of hydrocarbons by peroxidases obtained from *Rhizopus* and *Saccharomyces* spp. isolated from crude oil-polluted soil.

The specific objectives include:

(i) To determine some chemical parameters of the contaminated and uncontaminated soil samples;

(ii) To Isolate and identify species of *Rhizopus* and *Saccharomyces* from the crude oil-polluted soil using standard microbiological and biochemical techniques;

(iii) To carry out emulsification studies on the identified isolates and to characterize *Rhizopus* and *Saccharomyces* species using molecular techniques;

(iv) To extract peroxidase from the the fungal spp. via submerged fermentation and to purify the crude enzyme extract via ammonium sulphate precipitation (ASP), dialysis and gel filtration;

(v) To characterize the purified extract by determining the optimal pH, optimal temperature and effects of substrate concentrations using O-dianisidine as the standard substrate and to

carry out stability studies on the purified enzyme extract using varying concentrations of metal ions;

(vi) To carry out hydrocarbon biodegradation studies on the purified enzyme using gravimetric method and optimize physicochemical parameters such as pH, enzyme concentrations, crude oil concentration and incubation time during the biodegradation studies.

1.4. JUSTIFICATION OF STUDY

Several methods are being used for the degradation of hydrocarbons of various nature from the environment including chemical precipitation, ion exchange, electrochemical treatment, membrane technologies and adsorption on activated carbon (Matheickal and Yu, 1999). These methods have significant demerits such as high chemical and energy requirements, hazardous sludge formation, low efficiency when recalcitrant hydrocarbon concentration is below 100 mg/l and high cost at large scale (Marin-Rangel *et al.*, 2012; Mishra *et al.*, 2012). The search therefore for new cost-effective and nature friendly biotechnologies for the removal of hydrocarbons from polluted environment has been directed towards enzymic biosorption/biodegradation. This involves the use of microbial proteins (enzymes) produced from a biological material has emerged as a promising method, with advantages such as high efficiency even at low pollutant concentrations, low cost, no additional nutrient requirement, easy operation and without detrimental effect to the environment (Jiménez- Cedillo *et al.*, 2013; Manzoor *et al.*, 2013; Mishra *et al.*, 2012). Microorganisms generally have been exploited in most bioremediation of crude oil polluted environments and little work has been done using enzymes in cleaning crude oil polluted

environments. Peroxidases, with its great working mechanisms is exploited in this study, to estimate its potentials in degradation of hydrocarbons from petroleum polluted sites.

1.5. SCOPE OF STUDY

This present study was limited to assessing the efficacy of peroxidase produced from *Rhizopus and Saccharomyces* spp. in the biodegradation of hydrocarbon from petroleum polluted soil. The study does not include the environmental health (life cycle assessment), toxicology of the polluted soil or the chemistry of the hydrocarbon content of the petroleum and their mode of interaction.

CHAPTER TWO

LITERATURE REVIEW

2.1 CRUDE OIL: SOURCE OF POLLUTION IN NIGERIA

Crude oil is basically a hydrocarbon compound which is naturally available beneath the soil. The word petroleum is derived from a Latin word Petraoleum which means rock-oil. Petroleum (liquid)/oil consist of a mixture of liquid hydrocarbon compounds, these hydrocarbon compounds are made up of hydrogen and carbon in different proportions some other elements like sulphur, oxygen and nitrogen are also present in trace amount. Crude oil is formed in naturally occurring geological deposits formed from organic decomposition products of ancient plant and animal under high temperature and pressure. These petroleum hydrocarbons are the major sources of surface and groundwater contamination (Singh and Chandra, 2014).

The most common contaminant in the environment is crude oil and its derivatives (sometimes containing heavy metals). Due to their wide spread occurrence and severe risks they pose to human health and water bodies (surface as well as ground), they require intense remediation practices at the contaminated sites. Strictly speaking, contamination is strongly correlated with the degree of industrialization and intensity of chemical usage. All hydrocarbon compounds derived from petroleum sources are generally described as total petroleum hydrocarbons (TPHs). Fuels such Crude oil and refined fuel spills from tanker ship accidents have caused severe damages to ecosystems in many parts of the world. The quantity of oil spilled during accidents has ranged from a few million gallons to several hundred thousand gallons as petrol, diesel, kerosene, and lubricating oils/greases all come under the category of TPHs (Maddela and Scalvenzi 2017).

The contamination caused by petroleum hydrocarbon leads to various health threats which includes carcinogenic and neurotoxic effects. Crude oil contamination of soils has various toxic effects on growth and reproduction of soil bacteria (Ibuot and Bjhaiya, 2013) and consequently would be capable of inhibiting the growth or output of agricultural plants. In the stand point of geotechnical aspects, crude oil contamination of soil could increase the composition of carbon in the soil and as a result increases the organic matter content of the soil which might be a disadvantage to construction soil (Okonkwo *et al*., 2018). Therefore to reduce the hazardous effect of petroleum hydrocarbon, their control and treatment strategies are required. Consequently, there is a pressing need to clean-up the soil of the areas contaminated by crude oil spill.

Nigeria is very rich in crude oil especially in the Niger-Delta region and one of the largest producers in the Africa. The Niger Delta region is the hub of oil production in Nigeria. Nigeria experiences devastating polluted environment in various forms due to oil and gas activities in such areas. These activities by the oil and gas industries has led to various forms of land degradation and environmental pollution as a result of regular oil spillage. More often crude oil spill has been a common occurrence in the course of exploitation and production. The frequency of occurrence of crude oil spill in Nigeria has been on the increase.

There are many ways of petroleum hydrocarbon contamination some of which includes pipelines and oil wells leakages, storage facilities' failure, wrong methods of disposal of petroleum wastes and accidental oil spills (Singh and Chandra, 2014). The causes of pipeline damage and leakage ranges from material defects, pipe corrosion, accidents and contact with ship anchors in the offshore operations to vandalism of pipeline onshore. Soil pollution by petroleum hydrocarbons (PHs) is mainly due to oil drilling, waste disposal (oil and fuel dumping), and accidental spilling as may occur during activities. Crude oil has a paramount economic importance because it is the

main source of transport and heating fuels, plastics, solvents, and many other chemical. Maritime navigation transports crude oil from the producing to the consuming countries, and tanker accidents, associated to dangerous navigation areas and bad weather conditions, caused some of the most relevant marine pollution issues worldwide. Despite that, land-based sources are the main global input of oil in the sea. In the event of oil spilled in open sea, it is a priority to contain and recover the oil to avoid it reaching the coastline, where more important ecological and economic impacts are expected (Beiras, 2018).

Polycyclic aromatic hydrocarbons (PAHs) are more persistent and toxic than alkanes and are responsible for most ecotoxicological problems associated with oil spills. Those PAHs with lower molecular weight are more soluble and acutely chronic to marine fauna, while those with higher molecular weight may be carcinogens. They accumulate in sediments and invertebrates but can be metabolized by fish and mammals. Oil spills typically cause large mortalities of seabirds, and changes in benthic community structure characterized by mortalities of herbivores, excessive proliferation of macroalgae, and loss of ecological diversity. This ecological imbalance usually takes several years to recover (Beiras, 2018). Crude oil contamination is one of the major environmental concerns and it has drawn interest from researchers and industries. Therefore there is the need to find a faster and effective means for the cleaning-up process of the crude oil contaminated soils.

2.2 Chemical Composition and Properties of Crude Oil

The major elements of crude petroleum oils are carbon (85%-90%) and hydrogen (10%-14%) and the rest are non-hydrocarbon elements, such as sulfur (0.2%-3%), nitrogen (< 0.1%-2%) and oxygen (1%-1.5%), and organo-metallic compounds like lead, vanadium, arsenic, nickel and other metals in traces in parts per million or parts per billion concentration. Inorganic salts of sodium chloride, magnesium chloride and other mineral salts are also accompanied with crude oil from field-wells either owing to formation water or water and chemicals injected during drilling and production operations (Moustafa and Mori, 2012; Chandhuri, 2010).

Typically, conventional oils contain 67-97% saturates and aromatics, 2-33% resins and <0.1-12% asphaltene; and heavy oils contain 24-64% saturates and aromatics, 14-39% resins and 11-45% asphaltene (Santos *et al*., 2014). Heavy oils contain substantial amounts of resins and asphaltenes in addition to asphaltogenic acids/compounds, diamondoids and derivative, heavy aromatic hydrocarbons (PNA/PAHs), mercaptans, metal carbenes/organometallic and wax (Chandhuri, 2010; Akmaz *et al.,* 2011). Asphaltene increases crude oil viscosity and tendency to form emulsions, polymers, and coke (Bachmann *et al.,* 2014). Resins and asphaltenes play a major role in shaping the colloidal nature of heavy oils; hence, the industry's interest in these heavy oil fractions in particular. Resins and asphaltenes contain numerous heteroatoms as they usually encompass 30-60% of the total sulphur, 70-90% of total nitrogen and 80-90% of the total vanadium and nickel present in a crude oil (Gholami *et al*., 2013). Resins and asphaltenes mainly consist of naphthenic aromatic hydrocarbons with alicyclic chains which are the hardest to degrade. Asphaltenes are the heaviest and most polar components of petroleum fluids (Goual, 2012) as reported in Al-Sayegh1 *et al*. (2016).

Crude oil comprises hydrocarbons that are mainly grouped into paraffins (*e.g.* alkanes), olefins (*e.g.* alkenes), naphthenes (*e.g.* cycloalkane) and aromatics (*e.g.* benzenes) in different proportions. Heavy crude oil contains significant amounts of complex hydrocarbons, such as polynuclear aromatics (PNA) (*e.g.* polycyclic aromatic hydrocarbons - PAH), alkyl-aromatics, heteroatoms and metal contents, which are more difficult to process. Common hetero-atoms in hydrocarbons are sulfur, oxygen, nitrogen, and metallic atoms (Chandhuri, 2010; Akmaz *et al.,* 2011).

2.3 Effects of Crude Oil Pollution in the Environment; Aquatic and Terrestrial Environment

Aromatic hydrocarbons are abundant in crude oil and represent the most dangerous fraction. Low-boiling aromatics (benzene, toluene, xylene) can cause acute poisoning to many lung-breathing invertebrates and vertebrates, including man. Mogaji *et al*. (2018) reported in a study on the effects of crude oil exploration on fish and fisheries of Nigerian ecosystem, that as little as 0.1 ppm of crude oil can seriously affect fish, amphibians, crustaceans, and plankton. Crude oil has the potential to kill quickly by coating aquatic lives and interfering with gas exchange necessary for life. The contaminating impact has longer and perhaps more severe effects on benthic organisms.

Crude oil exploration poses grave danger to the biodiversity of the aquatic ecosystem. Oil toxicity also poses danger within 48 hours after a spill on different species of birds by reducing their fur insulating ability and damaging their water repellent nature. A serious threat posed by oil-related pollution is the impact on underground waters through seepage of crude oil, which results in underground water pollution. The polluted groundwater recharges aquatic ecosystems (streams, rivers, well, etc.) contaminating these water bodies in the process as well as increasing the cases

of water-borne diseases. The fish species in these environments are forced to migrate to safer areas that are free of crude oil pollution. This results in dwindling catch and loss of income to fisherfolks. (Mogaji *et al*., 2018)

When oil is spilled into an aquatic environment, it can harm organisms that live on, around, and under the water surface by both chemical toxicity and by coating and smothering wildlife. This has both short-term and long-term effects on all parts of the marine food web, including long-term damage to breeding and migration habitats that affects future generations of marine life. Short-term effects vary with the type of environment, amount of oil, effect of waves and weather, and type of oil: light, medium or heavy.

Also, oil spills are associated with heavy metals in the environment and these metals can inhibit various cellular process and its effects depends on the concentration and individual toxicity.

2.4 Heavy Metals.

These are metals with relatively high atomic mass which reflect in their atomic weights; Arsenic, beryllium, cadmium, chromium, lead, manganese, mercury, nickel, and selenium are some of the metals. (These are called 'heavy' because of their high relative atomic mass). They take part in bio-geochemical reactions and are transported between compartments by natural processes, the rate of which are at times greatly altered by human activities (Ajiboye *et al*., 2011). They persist in nature and can cause damage or death in animals, humans and plants even at very low concentrations.

2.4.1. Heavy Metal Entry Routes in the Environment

Heavy metals enter plant, animal and human tissues through air inhalation, diet and manual handling (Bánfalvi, 2011). Motor vehicle emissions are a major source of airborne contaminants including arsenic, cadmium, cobalt, nickel, lead, antimony, vanadium, zinc, platinum, palladium and rhodium (Di Maio, 2001). Water sources (groundwater, lakes, streams and rivers) can be polluted by heavy metals leaching from industrial and consumer waste. Metals can be mobilized by natural weathering processes such as erosion or dissolution, or as a direct result or side effect of human activities (Ajiboye *et al.*, 2011).

Plants are exposed to heavy metals through the uptake of water; animals eat these plants; ingestion of plant- and animal-based foods are the largest sources of heavy metals in humans (Duffus, 2002). Absorption through skin contact, for example from contact with soil, is another potential source of heavy metal contamination (Dyer, 2009). Toxic heavy metals can bio-accumulate in organisms as they are hard to metabolize (Emsley, 2011).

Once in the natural aquatic system metals can undergo a variety of transformations including in dissolved speciation, precipitation and oxidation/ reduction. All of these processes can drastically alter the mobility of the metals. The total concentration of dissolved metal species in water can be orders of magnitude greater than the concentration of free aquo metal due to the formation of soluble complexes with organic and inorganic ligands. The strength of complexes is affected by the identity of the atom involved and stereochemical factors (Ajiboye *et al*., 2011). Since heavy metals have a propensity to accumulate in selective body organs (such as brain and liver) their prescribed average safety levels in food or water are often misleadingly high (Ajiboye *et al*., 2011).

Heavy metals carried down by effluents are usually mentioned and have been the major threats for fish consumers and the effects on the contamination of fishing products becomes a serious issue to be addressed (Ajiboye *et al*., 2011). The metal contaminants in aquatic systems usually remain either in soluble or suspension form and finally tend to settle down to the bottom or are taken up by the organisms (Reddy *et al*., 2007). Heavy metal contamination may have devastating effects on the ecological balance of the recipient environment and a diversity of aquatic organisms (Ashraj, 2005; Vosyliene and Jankaite, 2006).

Fishes being one of the main aquatic organisms in the food chain may accumulate large amounts of certain metals (Mansour and Sidky, 2002). Among animal species, fishes are the inhabitants that cannot escape from the detrimental effects of these pollutants (Olaifa *et al*., 2004). Moreover, it is worthy to note that these heavy metals accumulate in various tissues and organs of fish species, which in turn may enter into the human system through consumption of the potential fishes causing serious health hazards.

Heavy metals from oil spills can bind to vital cellular components, such as structural proteins, enzymes, and nucleic acids, and interfere with their functioning (Evanko and Dzombak, 1997). Symptoms and effects can vary according to the metal or metal compound, and the dose involved. Broadly, long-term exposure to toxic heavy metals can have carcinogenic, central and peripheral nervous system and circulatory effects.

For human beings, typical presentations associated with exposure to any of the "classical (Finch *et al*., 2015) toxic heavy metals, or chromium (another toxic heavy metal) or arsenic (a metalloid), are shown in the Table 2.1

Table 2.1: Acute and Chronic Toxicities of Different Heavy Metals to Various Human Organs and Tissues.

ELEMENTS	Acute exposure usually a day or less	Chronic exposure usually a month or more
Cadmium	Pneumonitis(lung inflammation)	Lung cancer (lung Osteomalacia (softening of bones) Proteinuria (excess protein in urine; possible kidney damage) Stomatitis (inflammation of gums and mouth)
Mercury	Diarrhea Fever Vomiting	Nausea Nephrotic syndrome (nonspecific kidney disorder) Neurasthenia (neurotic disorder) Parageusia (metallic taste) Pink Disease (pain and pink discoloration of hands and feet) Tremor
Lead	Encephalopathy dysfunction) Nausea Vomiting	Brain Anemia Encephalopathy Foot drop/wrist drop (palsy) Nephropathy (kidney disease)
Chromium	Gastrointestinal hemorrhage (bleeding) Hemolysis (red blood cell destruction) Acute renal failure	Pulmonary fibrosis (lung scarring) Lung cancer
Arsenic	Nausea Vomiting Diarrhea Encephalopathy Multi-organ effects Arrhythmia Painful neuropathy	Diabetes Hypopigmentation/Hyperkeratosis Cancer

(Gilbert and Weiss, 2006)

2.5.0. Polycyclic Aromatic Hydrocarbon: Sources

PAHs are ubiquitous in nature as a consequence of synthesis in terrestrial vegetation, microbial synthesis, and volcanic activity, but quantities formed by these natural processes are small in comparison with those produced from forest and prairie fires and anthropogenic sources. Anthropogenic activities associated with significant production of PAHs include: coke production in the iron and steel industry; catalytic cracking in the petroleum industry; the manufacture of carbon black, coal tar pitch, and asphalt; heating and power generation; controlled refuse incineration; open burning; and emissions from internal combustion engines used in transportation (ATSDR, 2009).

Thus, the formation of PAHs in the environment is due to an endogenous synthesis by microorganisms, algae, and macrophytes which provide natural background, and to a second process which is connected to man-controlled high-temperature (>700^{o}C) pyrolysis of organic materials, to open burning, and to natural volcanic activities. The discovery in fossil fuels of complex mixtures of PAHs spanning a wide range of molecular weights has led to the conclusion that, given sufficient time (i.e., millions of years), pyrolysis of organic materials at temperatures as low as 100^{o}C to 150°C can also lead to production of PAHs. Other sources of PAHs include petroleum spills, oil seepage and diagenesis of organic matter in anoxic sediment (Edokpayi *et al.*, 2016).

Some PAHs are shown in Figure 2.1

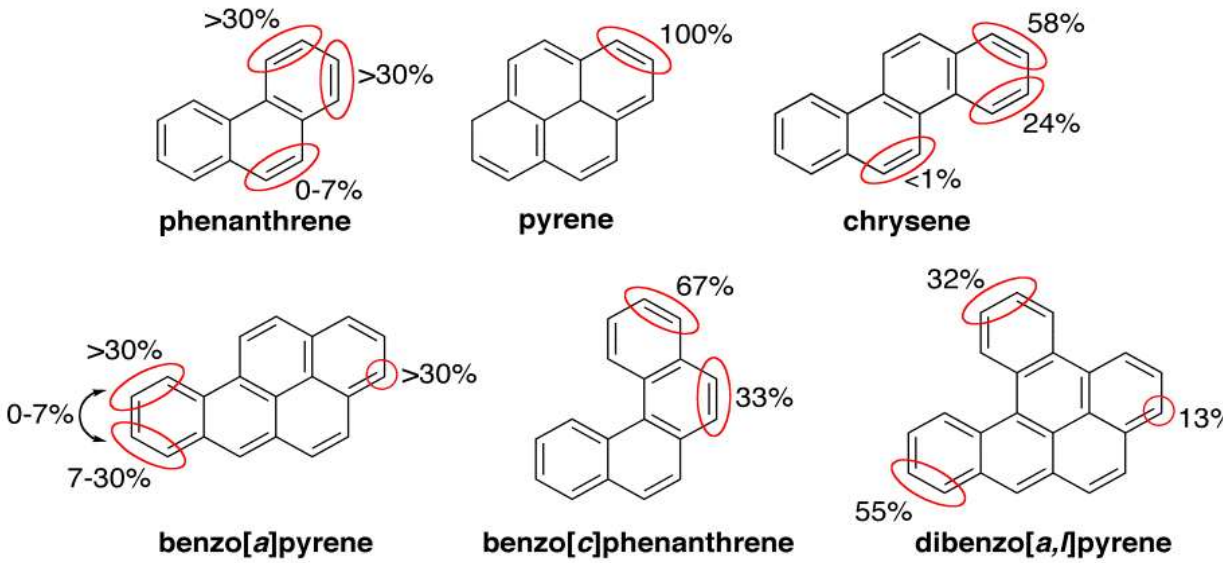


Figure: 2.1 Structures of some Polycyclic Aromatic Hydrocarbons. (Pampanin and Sydnes, 2013)

2.5.1. Environmental Implications of Polycyclic Aromatic Hydrocarbons

Concern about PAHs in the environment is due to their persistence and to the fact that some are known to be potent mammalian carcinogens, although environmental effects of most non-carcinogenic PAHs are poorly understood (Edokpayi *et al.*, 2016). Synthesis of PAHs by microorganisms and volcanic activity and production by man-made high temperature pyrolytic reactions and open burning seemed to be balanced by PAH destruction via photodegradation and microbial transformation. With increased industrial development and increased emphasis of fossil fuels as energy sources, the balance has been disturbed to the extent that PAH production and introduction into the environment greatly exceeds known PAH removal processes.

When released into the atmosphere, PAH compounds will become associated with particulate materials. The highly reactive PAHs photo-decompose readily in the atmosphere by reaction with ozone and various oxidants; Smaller atmospheric particulates containing PAHs are easily inhaled and may pose special problems, as yet unevaluated, for airborne organisms such as birds, insects, and bats. PAHs are moderately persistent in the environment and can bio-accumulate. The concentration of PAHs found in fish and shell fish are expected to be much higher than in the environment from which they are taken (Igwe and Ukaogo, 2015).

Various parameters may modify chemical and photochemical transformation of PAHs in the atmosphere, including light intensity, concentration of gaseous pollutants (0_3, NOx, SOx), and chemico-physical characteristics of particulates or substrates into which the PAHs are adsorbed; depending on these variables, the half-life of benzo(a)pyrene in the atmosphere varies from 10 min to 72 days. PAHs are sparingly soluble in water and therefore have an affinity for sediment, soil and biota. When found in air and water, the PAHs compounds are generally found adsorbed to particulate matter. Thus, although most are emitted in to the atmosphere, sediments and soils are the major environmental sinks for these compounds (Igwe and Ukaogo, 2015).

Much of the PAHs released into the atmosphere eventually reach the soil by direct deposition or by deposition on vegetation. The PAHs may be adsorbed or assimilated by plant leaves before entering the animal food chain, although some adsorbed PAHs may be washed off by rain, chemically oxidized to other products, or returned to the soil as the plants decay. PAHs assimilated by vegetation may be translocated, metabolized, and possibly photodegraded within the plant. In some plants growing in highly contaminated areas, assimilation may exceed metabolism and degradation, resulting in an accumulation in plant tissues. In water, PAHs may either evaporate, disperse into the water column, become incorporated into bottom sediments, concentrate in aquatic

biota, or experience chemical oxidation and biodegradation. The ultimate fate of those PAHs that accumulate in sediments is believed to be biotransformation and biodegradation by benthic organisms. The rates of degradation vary and generally decrease with increasing numbers of aromatic rings (Igwe and Ukaogo, 2015). Animals and microorganisms can metabolize PAHs to products that may ultimately experience complete degradation. Those in the soil may be assimilated by plants, degraded by soil microorganisms, or accumulated to relatively high levels in the soil. High PAH concentrations in soil can lead to increased populations of microorganisms capable of degrading the compounds.

2.6 Total Petroleum Hydrocarbons (TPH)

Total petroleum hydrocarbons (TPH) is a term used to describe a large family of several hundred chemical compounds that originally come from crude oil. TPH is a mixture of chemicals, but they are all made mainly from hydrogen and carbon, called hydrocarbons. Scientists divide TPH into groups of petroleum hydrocarbons that act alike in soil or water. These groups are called petroleum hydrocarbon fractions. Each fraction contains many individual chemicals. Petroleum hydrocarbon products are mixtures of over 250 hydrocarbon compounds. The various product mixtures produced by the manufacturers are based upon physical and performance-based criteria and not specific formulae. As a result, the product compositions can vary depending upon, in part, the crude oil refined to produce the product, the type of product, the season of the year, and any performance additives (ATSDR, 2010).

Petroleum hydrocarbon products are also subject to changes in composition once they are released into the environment. The lower molecular weight hydrocarbons are generally more volatile and water-soluble than are the higher molecular weight hydrocarbons. Some of the lower molecular weight hydrocarbons are also more subject to microbial decomposition and the degradation products might include compounds not originally found

in the product (ATSDR, 2010). It is not practical to identify and quantify all of the individual compounds contained in a particular hydrocarbon fuel or oil. The fractionation approach addresses these complications by dividing the hydrocarbon mixture into several fractions that are sufficiently homogeneous with respect to physical and chemical properties.

Petroleum products are complex mixtures of hundreds of hydrocarbon compounds, ranging from light, volatile, short-chained organic compounds to heavy, long-chained, branched compounds (Chikere *et al*., 2009). The exact composition of petroleum products varies depending upon (1) the source of the crude oil (crude oil is derived from underground reservoirs which vary greatly in their chemical composition) and (2) the refining practices used to produce the product. During the refining process, crude oil is separated into fractions having similar boiling points (ATSDR, 2010). These fractions are then modified by cracking, condensation, polymerization, and alkylation processes, and are formulated into commercial products such as naphtha, gasoline, jet fuel and fuel oils. The composition of any one of these products can vary based on the refinery involved, time of year, variation in additives or modifiers, and other factors. The chemical composition of the product can be further affected by weathering and/or biological modification upon release to the environment (ATSDR, 2010).

2.7 Crude Oil Biodegradation

Biodegradation is degradation caused by enzymatic process resulting from the action of cells (Vert *et al*., 2012). Crude oil biodegradation process, with its minimal energy need and environmentally friendly approach, presents an opportunity for bioremediation and as well for enhanced oil recovery to utilize heavy oil resources in an efficient manner. Biodegradation entails crude oil utilization as a carbon source for microorganisms that in turn change the physical properties of

heavy crude oil by oxidizing aromatic rings, chelating metals and severing internal bonds/chains between molecules. Biodegradation does not necessarily lower quality of crude oil as there are cases where quality was improved. Through the utilization of single microorganisms and consortia, researchers were able to biodegrade single pure hydrocarbon components, transform heavy crude oil fractions to lighter fractions, remove heavy metals and reduce viscosity of crude oil.

Crude oil spills that occur during transportation and storage operations, has intensified the oil pollution problems. The persistence of oil may continue even for decades after decontamination. The biodegradability efficiency of the oil components generally decreases in the following order: n-alkanes, branched-chain alkanes, branched alkenes, low molecular weight n-alkyl aromatics, monoaromatics, cyclic alkanes, polycyclic aromatic hydrocarbons (PAHs) and asphaltenes (Tyagi *et al*., 2011). n-Alkanes which have short hydrocarbon chains are easier to biodegrade than these which have long chains. Crude oil contains a large number of non-hydrocarbon components and any alteration in their qualitative or quantitative composition may significantly alter the characteristics of the crude oil. Crude oil biodegradation entails utilization of the crude oil as a substrate for the introduced microbial population and alteration of physical properties through bio-products (Bachmann *et al.*, 2014). The general microbial mechanism for accessing petroleum hydrophobic substrates are interfacial accession by direct contact of the cell with the hydrocarbon and biosurfactant-mediated accession by cell contact with emulsified hydrocarbons. Biosurfactants are surface-active chemicals produced by microorganisms in order to adsorb, emulsify, wet, or disperse or solubilize water-immiscible material to use them as a food source Biosurfactants enhances substrate bio-availability of non-aqueous phase liquids (NAPL), PAHs and other degraded products by increasing oil surface area (Das and Chandra 2011; Joshi and Desai, 2010).

One important requirement is the presence of microorganisms with the appropriate metabolic capabilities. If these microorganisms are present, then optimal rates of growth and hydrocarbon biodegradation can be sustained by ensuring that adequate concentrations of nutrient and oxygen are present and one's ability to maintain conditions that favor enhanced oil biodegradation rates in the contaminated environment (Das and Chandra 2011).

2.8 Use of Enzymes in Biodegradation and Detoxification of Hydrocarbons and Other Environmental Toxicants.

Enzymes are biological catalysts that facilitate the transformation of substrates into products by providing favorable conditions that lower the activation energy of the reaction. The functional diversity of these enzymes and its specificity in reactions has led to increased interest in the production process and field application studies of enzymes (Nair and Jaayachandran 2017). Degradation of petroleum components by microorganisms is possible due to the presence of enzymes secreted by them. The complex hydrocarbons can be degraded by those microorganisms which possesses high enzymatic activity (Singh and Chandra, 2014).

The presence of catabolic genes and enzymes in microorganisms, allowes them to utilize hydrocarbons as carbon and energy source. Chromosomal or plasmid DNA is the sites where the gene locates which is responsible for the production of enzyme required in the degradation of petroleum hydrocarbon (Singh and Chandra, 2014).The degradation of petroleum hydrocarbons can be mediated by specific enzyme system. Other mechanisms involved are (1) attachment of microbial cells to the substrates and (2) production of biosurfactants (Das and Chandra 2011). The type and yield of metabolic products can be controlled largely by modifications of environmental

conditions and nutrients. The increase in the rate of substrate transformation may be obtained by controlling enzymatic or regulatory step of the metabolic pathway and by the elevation of the activity of the rate-limiting protein.

Several studies have reported that enzymes play major role in detoxification and biodegradation of various contaminants in the environment (Bhunia *et al*., 2002; Chen *et al*; 2005; Bhatti *et al*., 2012; Dubrovskaya *et al*., 2017 etc.). Enzymes such as oxidoreductase (laccases and cytochrome-P450 mono-oxygenase (CYPs)) are being exploited for degradation of PAHs (Al-Sayegh *et al.,* 2016) others include oxygenases, deoxygenases and hydrolases. The distribution of enzyme in different cell parts produced by a microbial consortium and the degradation of naphthalene, phenanthrene, pyrene and crude oil by the osmotic shock method has been reported. It was observed that metabolic efficiency of periplasmic, cytoplasmic and extracellular enzymes secreted by a microbial consortium differed according to the utilized substrate (Al-Sayegh *et al.,* 2016).

Microbial enzymes like peroxidase and laccase has been reported for use in the treatment of heavy oil. Enzymes like hydrolase and oxidoreductase breaks a covalent bond in their substrate adding a water molecule in the process. The enzyme active site attaches to the oil and catalyze it, so larger oil droplets are broken into smaller ones allowing more oil release and improved mobility and permeability (Al-Bahry *et al.,* 2016). The initial intracellular attack of organic pollutants is an oxidative process and the activation as well as incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases and peroxidases.

Peroxidases are oxidative enzymes with the capacity to oxidize several recalcitrant compounds. They are characterized by various biotechnological potentials spanning different industries including textiles, paper and pulp, chemicals, water and cosmetics as reported by (Draelos, 2015; Taboada-Puig *et al,* 2015). They are ubiquitous enzymes that catalyse the oxidation of lignin and

other phenolic compounds at the expense of hydrogen peroxide (H_2O_2) in the presence of a mediator. These peroxidases can be heam or non-heam protein (Chandrakant and Shwetha 2011).

The detoxification of toxic organic compounds by various bacteria and fungi through oxidative coupling is mediated by oxidoreductases such as peroxidase. During such oxidation-reduction reactions, the contaminants are reduced to harmless or less harmful compounds. Hamid *et al*, (2016) reported in his review, that peroxidase are involved in the manufacturing of many aromatic complexes, elimination of phenolics complexes from waste water and peroxides from foods, beverage and industrial wastes. However, its isolated form, peroxidases are among the most useful enzymes in biotechnology, since several industrial processes can be performed by this kind of enzyme, such as soil detoxification and bioremediation of waste waters contaminated with phenols, cresols and chlorinated phenols (Pinto *et al*., 2015).

Various studies have shown that peroxidases from various sources especially microbial sources have remarkable properties in the degradation of varying concentration of crude oil (Ehiosun and Usman, 2018; Feltrin *et al.*, 2017a; Feltrin *et al*., 2017b; Neelam and Shamsher, 2013) and detoxification of polluted environment based on their ability to catalyze the reduction of peroxides and the oxidation of a variety of organic and inorganic compounds. However, little is known on the characteristics of peroxidase produced from fungi species especially from hydrocarbon populated soils

2.9 Peroxidases.

Peroxidases (PODs) are known to occur in different tissues and the pattern of expression and properties of these peroxidases vary among them. Peroxidases are heme-containing oxidoreductases (EC 1.11.1.7) that reduce peroxides, mainly hydrogen peroxide, to water and

subsequently oxidize small molecules, often aromatic oxygen donors (Florian and Anton, 2015). Plant peroxidases contain two calcium ions (Ca^{2+}), which are essential for the structural and thermal stability of the enzyme as well as its *in vitro* activation during analysis *(*Manu and Prasada Rao, 2009*)*. Peroxidases are also widely used in clinical laboratories, industries and in environmental conservation.

2.9.1 Functional Roles of Peroxidases

Most reactions catalyzed by peroxidase especially horseradish peroxidase can be expressed by the following equation, in which AH_2 and ^0AH represent a reducing substrate and its radical product respectively. Typical reducing substrates include aromatic phenols, phenolic acids, indoles, amines and sulfonates.

The catalytic cycle of peroxidase showing various reacting activated species of electrophiles intermediate is shown in Figure 2.2.

$H_2O_2 + 2AH_2 + POD \rightarrow 2H_2O + 2^0A$……Reaction 1

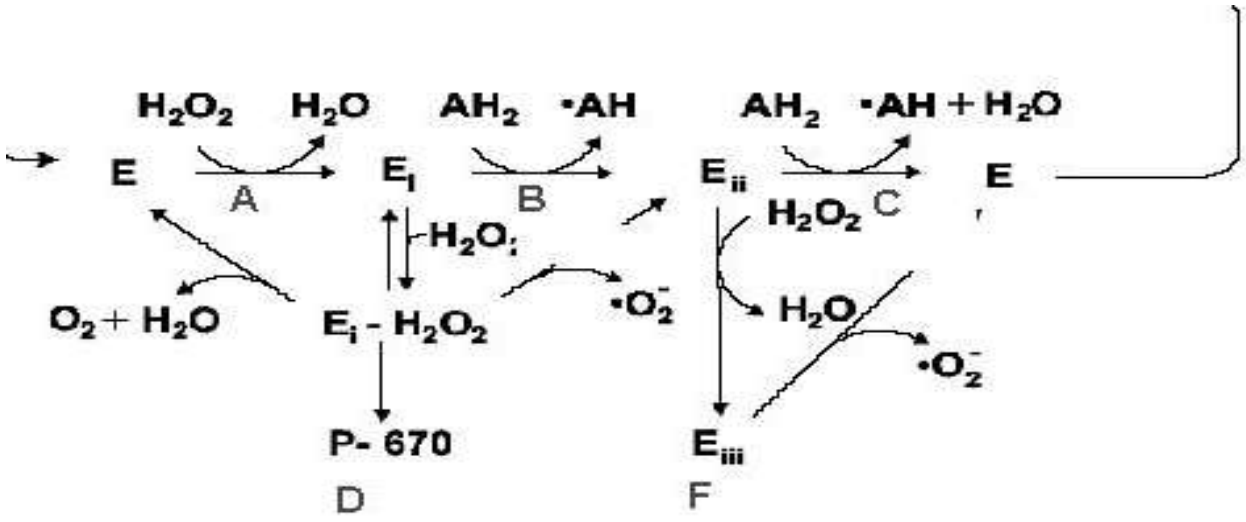


Figure 2.2: Catalytic Cycle of Peroxidase (Villalobos and Buchanan, 2002)

During the catalytic cycle of peroxidase as shown in Figure 2.2, the ground state enzyme undergoes a two electron oxidation by H_2O_2 forming an intermediate state called compound I (E). Compound I (E) will accept an aromatic compound (AH_2) in its active site and will carry out its one-electron oxidation, liberating a free radical (^0AH) that is released back into the solution, converting compound I (E) to compound II (Ei). A second aromatic compound (AH_2) is accepted in the active site of compound II (Ei) and is oxidized, resulting in the release of a second free radical (^0AH) and the return of the enzyme to its resting state, completing the catalytic cycle (Figure 2.2). The two free radicals (^0AH) released into the solution combine to produce insoluble precipitate that can easily be removed by sedimentation or filtration.

Various side reactions that take place during the reaction process are responsible for the enzyme inactivation (E) or inhibition (Eii) leading to a limited lifetime, but this form is not permanent since compound III (Eii) decomposes back to the resting state of peroxidase. Some peroxidases, like horseradish peroxidase (HRP), lead to a permanent inactivation state (P-670) when H_2O_2 is present in excess or when the end-product polymer adheres to its active site, resulting in permanent inactivation by causing changes in its geometric configuration (Villalobos and Buchanan, 2002).

2. 10 The Structure of Peroxidase

2.10.1 The Description of Horseradish Peroxidase (HRP)

Horseradish peroxidase comprises a single polypeptide of 308 amino acid residues, the sequence of which was determined by Welinder (1992). The N-terminal residue is blocked by pyroglutamate and the C-terminus is heterogeneous with some molecules lacking the terminal residue Serine (Ser) 308.

There are four disulphide bridges between cysteine residue 11-91, 44-49, 97-301, and 177-209 and a buried bridge between Aspartate (Asp) 99 and Arginine (Arg) 123. Nine potential N-glycosylation sites can be recognized in the primary sequence from the motif Asn-X-ser/Thr (Asn-Asparagin, where' X' represents an amino acid residue, Thr-Threonine) and of these, eight are occupied. A branched heptasaccharide accounts for 75 to 80% of the glycans, but the carbohydrate profile of horseradish peroxidase C (HRP C) is heterogeneous (Florian and Anton 2015). These invariably contain two terminals of GlcNAC (N-Acetylglucosamine) and several mannose residues. A further complication is the variation in the type of glycan present at any of the glycosylation site. The total carbohydrate content of the HRP C is somehow dependent on the source of the enzyme and value of between 18 and 22% typically.

The heme component of the horseradish peroxidase C is illustrated below:

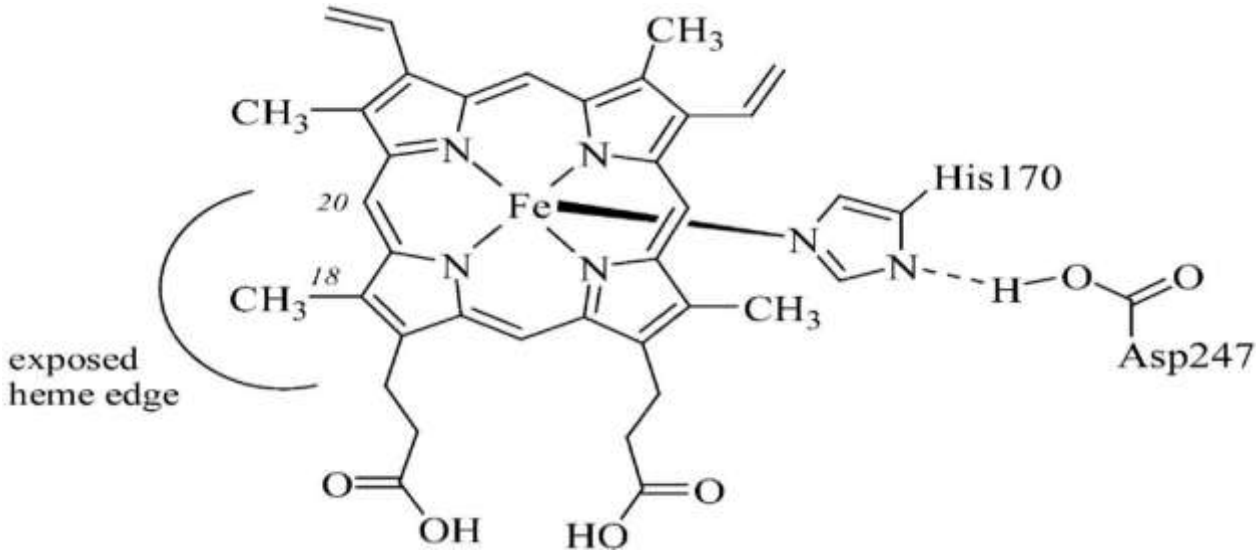


Figure 2.3: **Heme Component (tetra pyrole ring with an iron center) of Horseradish Peroxidase Isoenzyme C (HRP C)** (Veitch, 20

Legends: His-Histamine, Asp-Aspartate

As observed in Figure 2.3, His170 forms coordinate bond to heme iron. Asp242 carboxylate side-chain helps to control imidazolate character of His170 ring. His170, alanine (Ala) mutant undergoes heme degradation. When hydrogen peroxide is added and compound I and compound II are not detected, imidazole can bind to heme iron in the artificially created cavity but full catalytic activity is not restored because the His170 imidazole complex does not maintain a five coordinate state (His 42 also binds to Fe). Aromatic substrates are oxidized at the exposed heme edge but do not bind to heme iron.

The calcium ions component of Horseradish peroxidase C is shown in Figure 2.4

Distal O-donor **Proximal 0-donor**

Figure 2.4: Calcium ions Component of HRP C (Veitch, 2004)

For the distal o-donors: Asp43, Asp50, Ser52, (side chain) Asp43, Val46, Gly48 (carbonyl), one structural water.

For the proximal o-donors: Thr171, Asp222, Thre225, Asp230, (side chain) Thr171, Thre226, Ile228 (carbonyl). (Val- Valine, Gly-Glysine, Ile-Isoleucine)

I. Structural water of distal calcium site hydrogen bonded to Glu64 which is itself hydrogen bonded to Asn70 and thus connect to the distal heme pocket

II. Distal and proximal Ca^{2+} ions are both seven-coordinate.

III. On calcium ions loss, enzyme activity decreases by 40%

The carbohydrate component of HRP C is presented in Figure 2.5

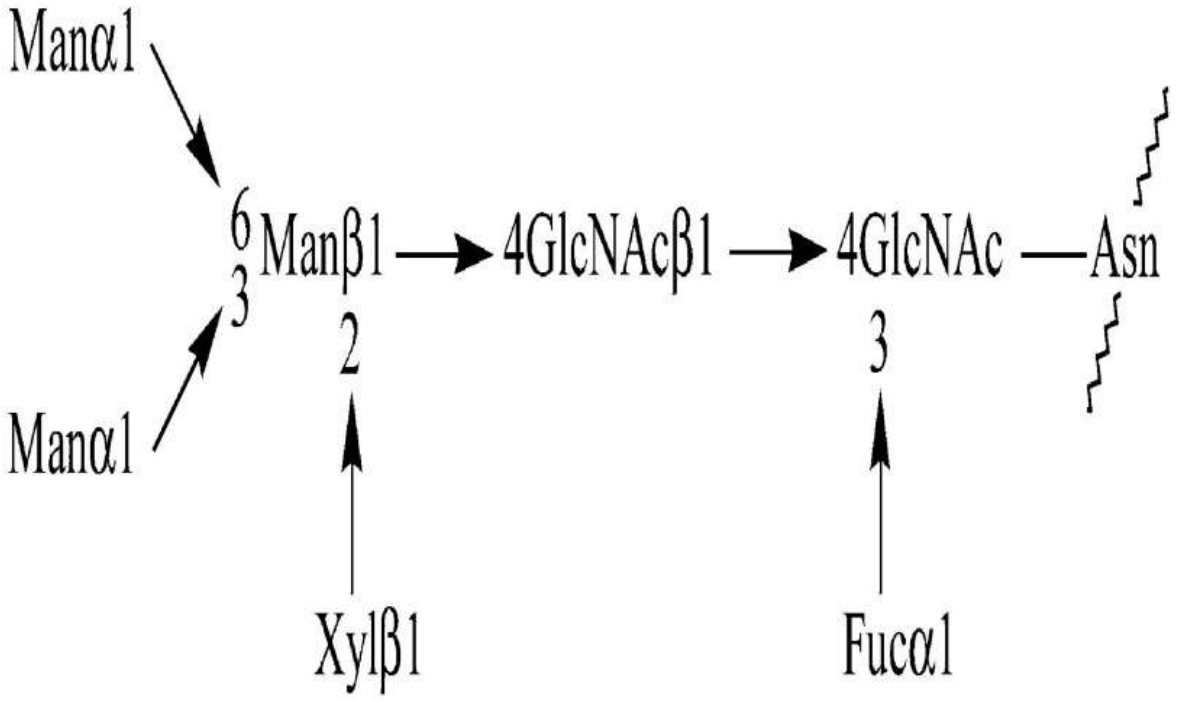


Figure 2.5: Carbohydrate Component of HRP C (Veitch, 2004)

Legends: Man-Mannose, Xyl- Xylose, Fuc- Fructose, Asn- Asparagine.

From Figure 2.5 above:

I. The site of glycosylation are in loop regions of the structure, at Asn57, Asn13, Asn158, Asn186, Asn198, Asn214, Asn255 and Asn268.

II. The major glycan is shown here, there are also minor glycans of the form Man_m $GlcNA_{c2}$

The key amino acid residues with the amino acyl positions in the heme-binding region are shown in Figure 2.6

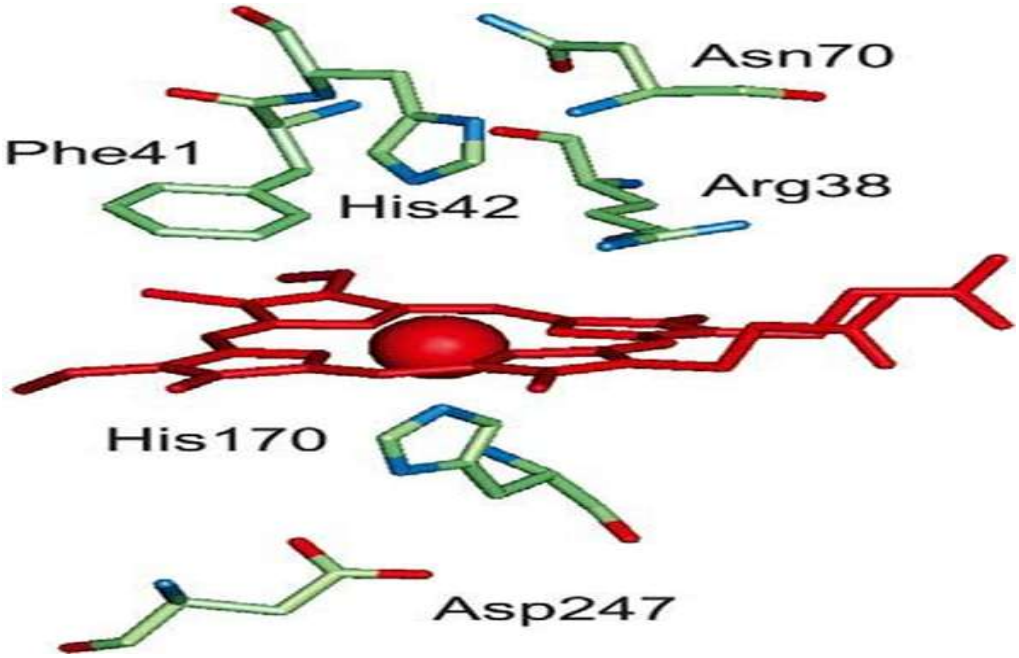


Figure 2.6: Key Amino Acid Residues in the Heme-binding Region of HRP C. (Veitch, 2004)

Amino acid residues and their functions

Arg38 Essential role in (i), the formation and stabilization of compound I, (ii) binding and stabilization of ligands and aromatic substrates (e.g. benzhydroxamic acid, ferulate etc.).

Phe41 Prevent substrate access to the ferryl oxygen of compound I.

His42 Essential role in (i), compound I formation (accept proton from H_2O_2) (ii) binding and stabilization of ligands and aromatic substrates.

Asn70 Maintains basicity of His42 side-chain through Asn70-His42 couple (hydrogen bond from Asn70 amide oxygen to His42 imidazole NH).

Pro139 Part of a structural motif, -Pro-X- Pro- (Pro139 (Proline)-Ala140-Pro141 in HRP C), which is conserved in plant and microbial peroxidases

HRP C contains two different types of metal centre, iron lll protoporphyrin IX (usually referred to as the heme group) and two calcium atoms. Both are essential for the structural and functional integrity of the enzyme. The heme group is attached to the enzyme at His170 (the proximal histidine residue) by a coordinate bond between, the histidine side-chain atom and the heme iron atom. The second axial coordination site (on the so called distal side of the heme plane) is unoccupied in the resting state of the enzyme, but available to hydrogen peroxide during enzyme turnover (Figure 2.6). Small molecules such as carbon II oxide, cyanide, fluoride and azide bind to the heme iron atom at the distal site giving six-coordinate peroxidase complexes. Some bind only in their protonated forms, which are stabilized through hydrogen bonded interaction with the distal heme pocket amino acid side-chain of Arg38 (the distal arginine) and the His42 (the distal histidine) (Figure 2.6).

The two calcium binding sites are located at positions distal and proximal to the heme plane and are linked to the heme-binding region by a network of hydrogen bonds. Each calcium site is seven-coordinate with oxygen-donor ligands provided by a combination of amino acid side-chain carboxylate (Asp), hydroxyl group (Ser, Thr), backbone carbonyls and a structural water molecules (distal site only). Loss of calcium results in decrease in both enzyme activity and thermal stability and two subtle changes in the heme environment that can be detected spectroscopically (Howes *et al.*, 2001).

The ribbon representation of the three–dimensional (3-D) x-ray crystal structure of HRP C is shown in Figure 2.7

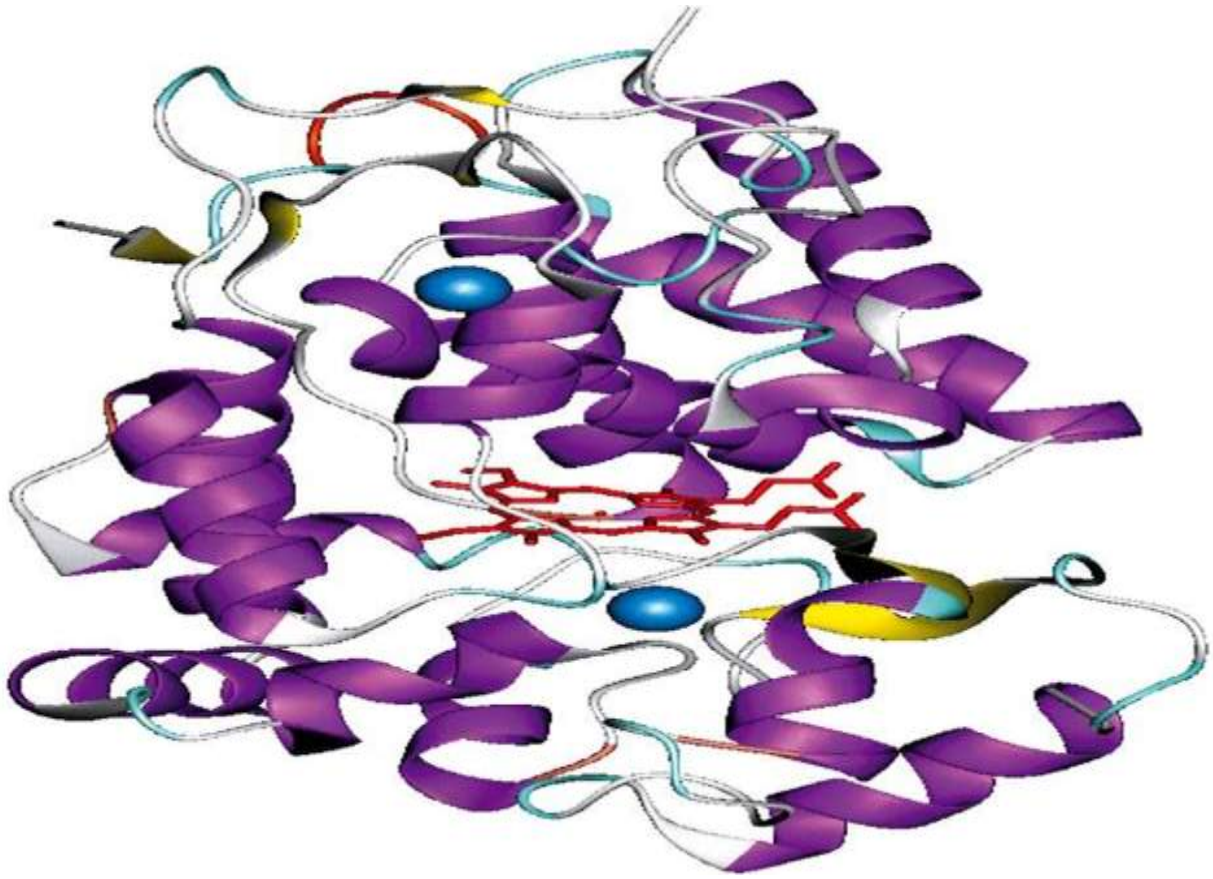


Figure 2.7: Three–dimensional Representation of the X-ray Crystal Structure of HRP C (Brook haven accession code IH5A). (Veitch, 2004)

The 3-D structure of the HRP:

Purple ribbons represent- α helicase, yellow arrows twined with the purple ribbons - β-plated sheets; blue spheres – calcium ions stabilized to the pocket catalytic sites embedded inside the cleft. The heme is located in the centre of the molecule (C, white, O, red; N, blue; Fe, Magenta)

2.10.2 Three-dimensional Structure of Peroxidase

The first solution of the three-dimensional structure of HRP C using X-ray crystallography appeared in the literature relatively. The recombinant enzyme used as the source of crystals and heavy atom derivatives was produced by expression in *Escherichia coli* in non-glycosylated form (Florian and Anton 2015). Previous attempts to obtain suitable crystals for diffraction were frustrated partly by the heterogeneity of plant HRP C preparations comprising multiple glycoforms.

The structure of the enzyme is largely α-helical, although there is also a small region of β-sheet (Figure 2.6). There are two domains, the distal and proximal, between which the heme group is located. These domains probably originated as a result of gene duplication, a proposal supported by their common calcium binding sites and other structural elements. Nowadays, horseradish peroxidase regains interest due to its broad applicability in the fields of medicine, life sciences, and biotechnology in cancer therapy, biosensor systems, bioremediation, and biocatalysis (Florian and Anton 2015).

2.11 The Mechanism of Oxidation of Peroxidase

2.11.1. Mechanisms of oxidation of indole-3-acetic acid with peroxidase

One of the most interesting reactions of peroxidase (HRP C) occurs with the plant hormone, indole-3-acetic acid (IAA) as shown in Figure 2.8 below. In contrast to most peroxidase–catalysed reactions, this takes place without hydrogen peroxide, hence the use of the term 'indole acetic acid oxidase' to describe this activity of HRP C in the older literature. More recent studies of the reaction at neutral pH indicate that it is not an oxidase mechanism that operates, but rather a peroxidase mechanism coupled to a very efficient branched-chain process in which organic peroxide is formed (Dunford and Jones 2010). The reaction is initiated when a trace of the indole-3-acetate cation radical is produced. The major products of indole-3-acetic acid oxidation include indole-3-methanol, indole-3- aldehyde and 3-methylene-2-oxindole, the latter most probably as a result of the non-enzymatic conversion of indole-3-methylhydroperoxide. Conflicting theories have been proposed to explain the mechanism of reaction at lower pH (Dunford and Jones 2010), in the formation of ferrous enzyme, compound III and hydroperoxyl radicals must also be accounted for. The physiological significance of IAA metabolism by HRP C and other plant peroxidases is still an area of active debate. Thus peroxidase expression in plant tissues at different stages of development must reflect a balance between the priorities of defense and growth.

The mechanism proposed for the formation of 3—methyl-2-oxindole from horseradiperoxidase (HRP C) and Indole-3-acetic acid is presented in Figure 2.8

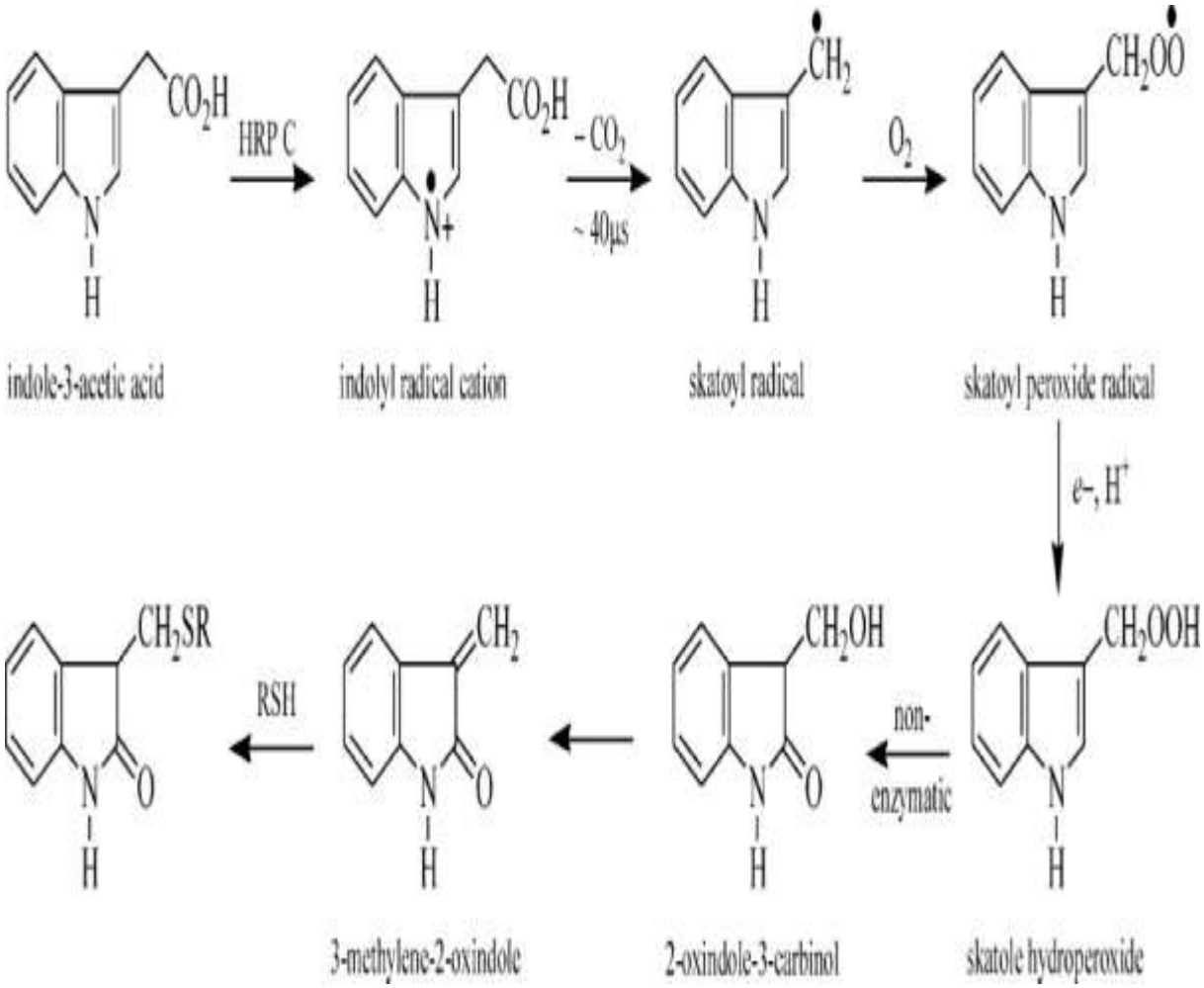


Figure 2.8: A Mechanism Proposed for the Formation of 3-methylene-2-oxindole from Horseradiperoxidase (HRP C) and Indole-3-acetic acid (Folkes *et al.*, 2002).

R represents a cellular nucleophile (e.g. sulphydryl groups of enzymes or histone)

2.11.2. Mechanism of Oxidation of Small Phenolic Substrates (Ferulic acid) with Peroxidase

Ferulic acid (3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid, FA) is a phenolic cinnamic acid derivative that is abundant in nature and known to act as an *in vivo* substrate for peroxidases. FA enhances the rigidity and strength of plant cell walls by cross-linking with pentosans, arabinoxylans, and hemicelluloses, thereby making the cell walls less susceptible to enzymatic hydrolysis during germination. The compound is a dibasic acid that exhibits an extended resonance stabilization of the phenolate anion, hence slightly increasing its acidity relative to phenol. The level of FA and its derivatives seems to be positively correlated with protection of the plant against insects, fungal, viral and avian attacks. In plants, FA is thought to arise from the conversion of cinnamic acid and frequently it is esterified to hydroxyl groups of polysaccharides flavonoids, hydroxycarboxylic acids and plant sterols. The initial step in the biosynthesis of lignin is the enzymatic dehydrogenation of monolignols to produce phenoxyl radicals. The radicals can link up to form dimers, trimers, and higher oligomers. There are other potential applications of ferulic acid from natural sources (Kumar and Pruthi, 2014).

Laccases and plant peroxidases have been proposed to be the *in vivo* generators of the phenoxyl radicals. Peroxidase oxidation of compounds with a syringyl group can be enhanced by esters of 4-coumaric acid and FA. For these reasons, it is of interest to study the interactions between the cell wall component of ferulic acid and the well characterized horseradish peroxidase C. Peroxidases catalyze the oxidative coupling of phenolic compounds using H_2O_2 as the oxidizing agent as shown.

The reaction is a three-step cyclic reaction by which the enzyme is first oxidized by H_2O_2 and then reduced in two sequential one-electron transfer steps from reducing substrates, typically a small molecule phenol derivative (the charges of heme propionates are ignored in scheme 1).

HRP C $[(Fe\ (III))\ Porph^{2-}]^{+} + H_2O_2 \rightarrow HRPC[(Fe(IV)=O)Porph^{0-}]^{0+} + H_2O$ ……Reaction 2

Native state Compound I

HRP C $[(Fe(IV)=O)Porph^{0-}]^{0+} + AH \rightarrow HRPC[(Fe(IV)=O)Porph^{2-}] + H^{+} + A^{0}$ …….Reaction3

Compound II

HRP C$[(Fe(IV)=O)Porph^{2-}] + H^{+} + AH \rightarrow HRPC[(Fe(III))Porph^{2-}]^{+} + H_2O + A^{0}$……Reaction4

SCHEME 1: Reactions 2–4

The oxidized phenolic radicals polymerize with the final product depending on the chemical character of the radical, the environment, and the peroxidase isoenzyme used (Frias *et al.*, 1991). The oxidation of native enzyme by H_2O_2 is well understood, and numerous experiments have confirmed the general catalytic mechanism for this step first proposed by Poulos and Kraut, (1980). The oxidation of phenolic substrates (reactions 3 and 4) is less well understood, but a histidine (His42 in HRPC) and an arginine (Arg38 in HRPC) (Rodriguez-lopez *et al*., 1997) have been shown to contribute significantly to enhance the rate of substrate oxidation.

The proposed mechanism for substrate oxidation in microbial and plant peroxidases are shown in Figure 2.9

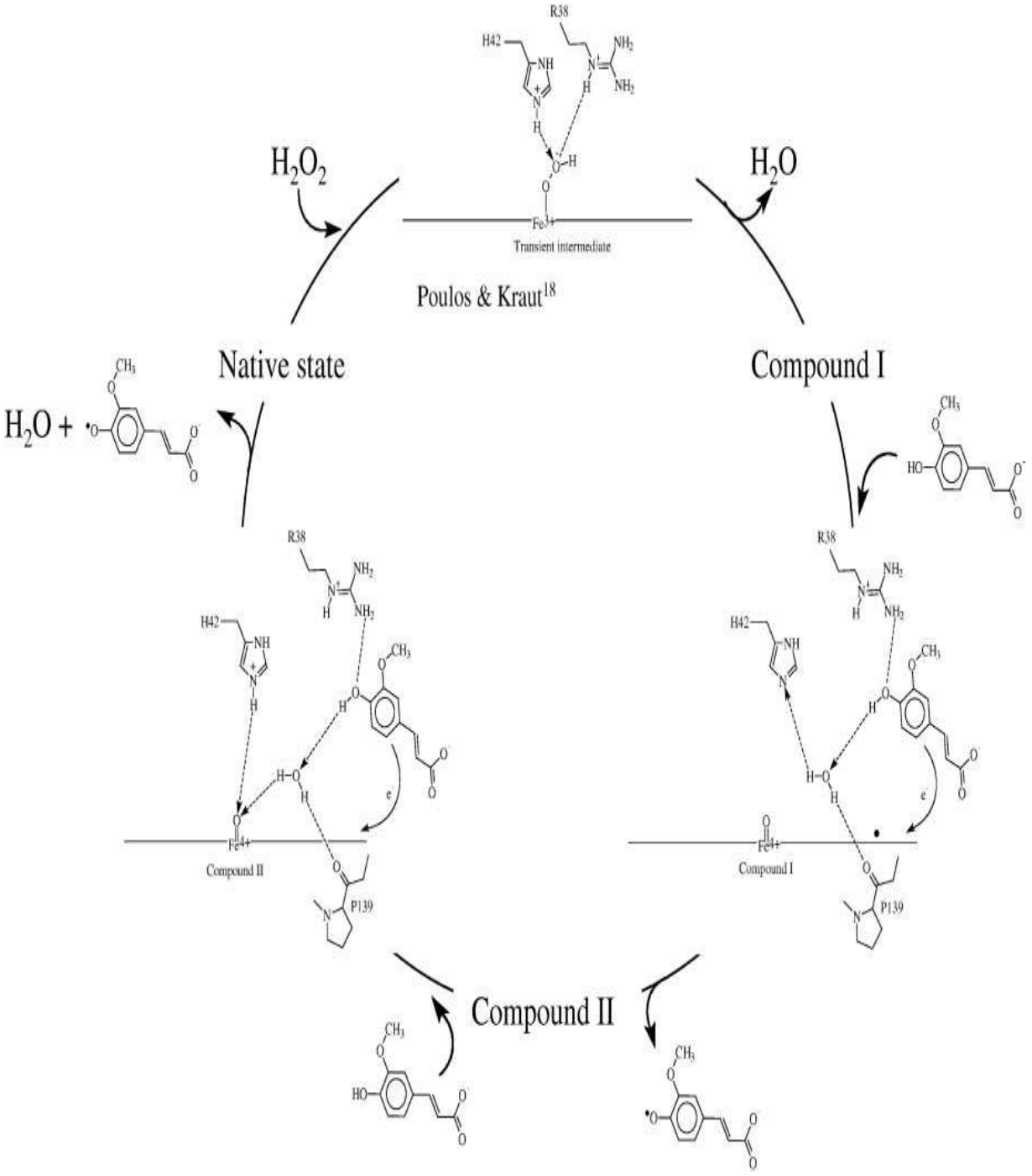


Figure 2.9: Proposed Mechanism for Substrate Oxidation in Microbial and Plant Peroxidases (Poulos and Kraut, 1980)

First, the active site arginine (Arg38 in HRPC) donates a hydrogen bond to the phenolic oxygen of the reducing substrate. This hydrogen bond will assist proton transfer from the phenolic oxygen to active site histidine (His42 in HRPC) through an active site water molecule held in position by the backbone oxygen of a conserved proline residue (Pro139 in HRPC). The electron is transferred to the heme group via the C-18 methyl-C-20 heme edge. Then compound II reduction is assisted by a similar proton transfer. The proton can be transferred to the ferryl oxygen through the active site water molecule situated equidistant between the distal histidine and the expected position of the ferryl oxygen of compound II, regenerating the resting state enzyme and a water molecule (Henriksen *et al.*, 1999)

2.12 Sources of Peroxidases

Peroxidases, a class of enzymes in animals, plants and microorganisms, catalyze oxido reduction between H_2O_2 and various reductants. Peroxidases fall into three major super families according to their primary sequence: animal, microorganism and plant peroxidases (Table 2.2).

Table 2.2: Classification of Peroxidases

CLASSES SUPERFAMILY	(EC NUMBER) MEMBER (PEROXIDASE)	ORIGIN	MOLECULAR WEIGHT(kDa)
Animal peroxidase	Eosinophil peroxidase (EC1.11.1.7)	Animal	50-75
	Lacto peroxidase (EC1.11.1.7)	Animal	78-85
	Myclo peroxidase (EC1.11.1.7)	Animal	79-150
	Thyroid peroxidase (EC1,11.1.9)	Animal	90-110

CLASSES SUPERFAMILY	(EC NUMBER) MEMBER (PEROXIDASE)	ORIGIN	MOLECULAR WEIGHT(kDa)
	Prostaglandin endoperoxidase (EC1.14.99.1,partial)	Animal	115-140
Catalase	Catalase (EC 1.11.1.6)	Animal, Plant, Fungus and Yeast	140-530
Microbial/Plant Peroxidase	Cytochrome C peroxidase (EC 1.11.1.6)	Bacterium and Yeast	32-63
	Catalase peroxidase (EC1.11.1.6)	Bacterium and Fungus	150-240
	Ascorbate peroxidase (EC1.11.1.11)	Plant	30-58
	Manganese-dependent peroxidase (EC1.11.1.13)	Fungus	43-49
	Ligninase (EC1.11.1.14)	Fungus	40-43
	Peroxidase (EC1.11.17POX)	Plant	28-60
			Hiraga *et al*., 2001

2.12.1. Microbial Peroxidases

Peroxidases produced from microbial sources such as bacteria (*Bacillus sphaericus, Bacillus subtilis, Pseudomonas* sp., *Citrobacter* sp., *Cyanobacteria* (*Anabaena* sp.), fungi (*Candida krusei, Coprinopsis cinerea, Phanerochaete chrysosporium),* Actinomycetes *(Streptomyces* sp., *Thermobifida fusca*), and yeast are used in decomposition of pollutants, production of animal feedstock, and raw materials for the chemical, agricultural, paper industries, textile dye degradation, paper-pulp industry for lignin degradation, dye de-colorization, sewage treatment, and also as biosensors.

2.12.1.1. Fungal Enzymes

All enzyme classes, apart from ligases, are represented among the industrial enzymes. Fungi have been and continue to be an important source of enzymes, both in terms of their role as a source for biological diversity and as a host for production of industrial enzyme products. Fungal enzymes especially, oxidoreductases, laccase and peroxidases have prominent application in removal of PAHs contaminants either in fresh, marine water or terrestrial. According to Balaji *et al.,* (2013), previous studies on degradation of polycyclic aromatic hydrocarbons by fungal strains are: cold-adapted bacteria and yeast effects of surfactants, imperfective fungi *Penicillium* sp., presence of non-ionic surfactants, white-rot fungus *Anthracophyllum discolor*, potential of mushroom cultivation substrate.

Saccharomyces spp. are genus of fungi that includes many yeasts. They are considered very important in food production. They are unicellular and saprophytic. One example is *Saccharomyces cerevisiae*, which is used in making wine, bread, beer, and for human and animal health. It is known as the brewer's or baker's yeast and has been instrumental to winemaking, baking, and brewing since ancient times. It is believed to have been originally

isolated from the skin of grapes (one can see the yeast as a component of the thin white film on the skins of some dark-colored fruits such as plums; it exists among the waxes of the cuticle). It is one of the most intensively studied organisms in biology and molecular biology, it is the microorganism behind the most common types of fermentation.

Scientific Classification using International Code for Nomenclature: (Turland *et al*., 2018)

Kingdom: Fungi

Division: Ascomycota

Subdivision: Saccharomycortina

Class: Saccharomycetes

Subclass: Saccharomycetidae

Order: Saccharomycetales

Family: Saccharomycetaceae

Genus: *Saccharomyces*

Rhizopus spp. are genus of common saprophytic fungi on plants and specialized parasites on animals. They are found on a wide variety of organic substrates. The widespread genus includes at least 8 species (Abe *et al*., 2010).

Scientific Classification using International Code for Nomenclature: (Turland *et al*., 2018)

Kingdom: Fungi

Division: Zygomycota

Subdivision: Zygomycotina

Class: Zygomycetes

Order: Mucorales

Family: Mucoraceae

Genus: *Rhizopus*

2.12.1.2. Why the Di-cultures?

The biological technique of using naturally occurring microorganisms in the environment has also been well utilized in the biodegradation of polluted sites. A single species can metabolize only a limited range of hydrocarbon substrates. Instead, a consortium of many different bacterial and/or fungal species, with broad enzymatic capacities, can degrade the maximum amount of contaminant. So far, according to Al-sayegh *et al,* (2016) several studies focused on the microbial degradation of PHs, these studies reported that the microorganisms possess specific enzyme systems that enable them to degrade and utilize hydrocarbons as their sole carbon and energy sources.

Rhizopus and Saccharomyces spp. have the ability to co-exist in harmful conditions, they are non-fastidious, have cross specie interaction and they complement each other and using a monoculture may require additional nutrient. Fungal Isolates from polluted soil are able to use hydrocarbon present in that area as substrates for growth. This can be achieved probably by releasing extracellular enzymes and acids which are capable of breaking down the recalcitrant hydrocarbon molecules by dismantling the long chains of hydrogen and carbon, thereby transforming petroleum into simpler forms or products that can be absorbed for the growth and nutrition of fungi.

Mixed fungal di-cultures have proven to be better degraders when compared to single fungal isolates. Also, a single microbe does not possess the enzymatic capability to degrade all or even most of the organic compounds in a polluted soil but mixed microbial communities possess powerful biodegradable potential because the genetic information of more than one organism is necessary to degrade the complex mixtures of organic compounds present in contaminated areas (Duniya *et al*., 2018).

Despite huge potentials of microorganisms to degrade organic compounds under favorable conditions, no single species of microorganism can degrade all components of a given oil (Esin and Ayten, 2011)

2.12.2 Methods of Production of Microbial Peroxidases

Generally, enzymes from microbial sources are secreted and produced in a bioreactor otherwise known as fermenter through fermentation. Basically there are two approaches to this method:

- Solid state fermentation
- Submerged fermentation (Swetha *et al*., 2006)

The two mentioned methods above are suitable for production of fungal peroxidases.

2.12.2.1 Solid State Fermentation.

Just as the name suggests solid state fermentation can be generally defined as the growth of micro-organisms, often fungi, in a moist solid substrate with negligible free water. The solid substrate provides either support or nutrients or both for the enzyme and the microbe from which the enzyme is secreted (Ashok, 1992). Solid-state fermentation processes are also of considerable promise on the pollution front. This technique is generally confined to the process involving fungi. In a wider sense several peroxidases have been isolated from strains of *Saccharomyces* under solid state fermentation using agricultural by-products (Vijayaraghavan *et al*., 2011). However, successful bacterial growth in solid state fermentation has been recorded in many natural fermentations and this is limited to the genus *Bacillus* (Babu and Satyanarayana, 1995).

2.12.2.2 Submerged Fermentation

This has long been utilized in peroxidase production. This involves the growth of organism in a synthetic medium such as nutrient broth, soluble starch as well as other components which enhance the secretion and production of peroxidase from the microbe (Swetha *et al.,* 2006).

2.13 Substrates

The enzyme peroxidase has two substrates, both of which must be present in order for the reaction to occur. One of the substrates is hydrogen peroxide, the other may vary, and depending on the cell or tissue the enzyme is found. The various substrates that can react with peroxide and their respective products are shown in Table 2.3.

Table 2.3: Substrates that can react with Peroxide and their Respective Products.

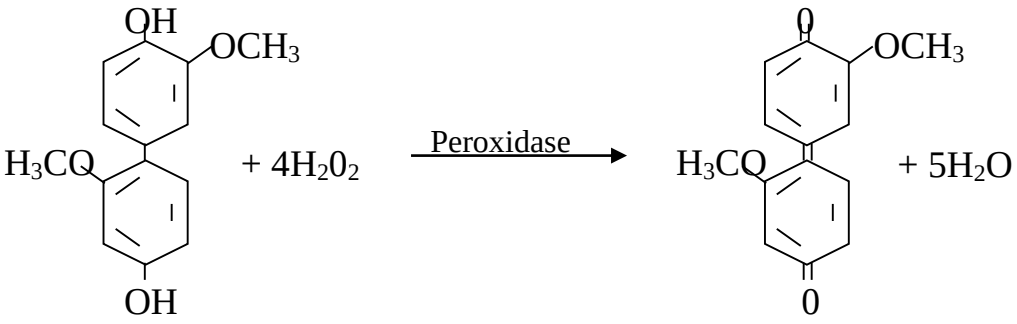
Substrate	Product
Pyrogallol	Purpurogallin
Guaiacol	Tetraguaiacol quinone
Benzidine	*o*-Quinonediamide
Catechol	*o*-Quinone
Hydroquinone	Quinhydroine
Tyrosine	Yellow solution
o-Cresol	Milky precipate
m - Cresol	Flesh-coloured solution
p-Cresol	Green-solution
o-Dianisidine	Vivid purplish red

The general reaction is as shown in reaction 5:

$$H - R - O - H + H - O - O - H \rightleftharpoons R = O + 2H - O - H \quad \text{.....Reaction 5}$$

Oxygen atom is transferred to an acceptor molecule, which for example is the organic molecule guaiacol. This reaction is facilitated by the enzyme peroxidase, which is found in many plant tissues. Peroxidase is more in horeseradishes than in turnips. Hydrogen peroxide and guaiacol are both substrates.

The structure of reduced and oxidized guaiacol is presented in Figure 2.10



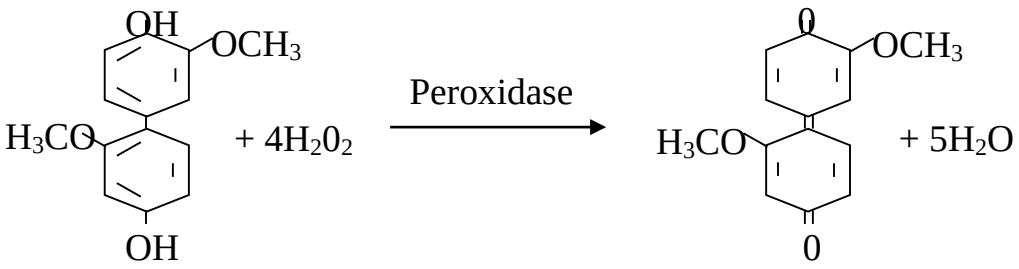
Reduced guaiacol (clear colour) oxidized tetraguiacol (brown).

Figure 2.10: Structure of Reduced and Oxidized Guaiacol (Venmwal, 2006).

The guaiacol peroxide system is convenient because guaiacol changes from colourless to brown colour as it is oxidized as shown in Figure 2.10. The functional peroxidase will make a test tube containing reduced guaiacol and hydrogen peroxide turn increasingly brown over time. This change to brown colour is influenced by three substances. These substances: hydrogen peroxide, guaiacol and peroxidase must be present in the test tube. Guaiacol is a phenolic natural product first isolated from guaiac resin and the oxidation of lignin. Guaiacol is readily oxidized by the haem iron of peroxidase including the peroxidase of cyclooxygenase (COX) enzymes. It therefore serves as a reducing co-substrate for COX reactions.

The one electron oxidation product of guaiacol is a dimer absorbing at 470 nm with an extinction coefficient of 26,600. Two moles of guaiacol are oxidized for each mole of hydrogen peroxide reduced by peroxidase. The resulting guaiacol chromophore can be used for the colorimetric determination of hydrogen peroxide activity.

Another substrate that can be used in place of guaiacol for plant peroxidase is *o*-dianisidine. *o*-dianisidine is not normally used because of its carcinogenic nature. The specific reaction of o-dianisidine is shown in Figure 2.11



Reduced *o*-dianisidine (Light orange) **Oxidised *o*-dianisidine (Bright orange)**

Figure 2.11: Structure of Reduced *o*-dianisidine and Oxidized *o*-dianisidine (Venmwal, 2006).

Hydrogen peroxide and reduced o-dianisidine are the substrates, water and oxidized *o*-dianisidine are the products as shown in Figure 2.10. In other cells, its function is to convert the potentially harmful peroxide into the non-toxic compound, water.

2.14 Factors that Affect Peroxidase Activity

2.14.1 pH

This is a measure of hydrogen ion activity of a solution and is defined as the negative logarithm of the hydrogen ion concentration. The rate of a chemical reaction or the enzyme activity is greatly influenced by the structure of the enzyme, in other words, a change in the structure of the enzyme affects the rate of reaction. When pH of a particular medium changes, it leads to alteration in the shape of the enzyme. pH level also affects the charge properties of the enzyme. Within a narrow pH range, changes in the structural shapes of the enzymes may be reversible. But for a significant change in pH levels, the enzyme may undergo denaturation. Since enzymes are proteins, they are very sensitive to changes in pH. Each enzyme has the optimum pH range where it is most active. Amino acid side chains contain groups, such as –COOH and $–NH_2$ that readily gain or lose H^+ and eventually enough side chain will be affected so that the enzyme is disrupted. This is the summary of the effect of pH and on a combination of these factors:

(1) The binding of the enzyme to substrate,
(2) The catalytic activity of the enzyme
(3) The ionization of the substrate, and
(4) The variation of protein structure.

2.14.2 Temperature

Temperature is one of the critical factors affecting enzyme-catalysed reactions. Like other chemical reactions, the rate of an enzyme-catalyzed reaction increases with modest increase in temperature. This is true only over a strictly limited range of temperature. When the temperature of a reaction is raised, there is sufficient energy to overcome the energy barrier and so cause an increase in the number of collisions between the enzyme involved and its substrate. These result in an increase in the rate of the reaction to reach its maximum activity. Beyond optimum

temperature, every further increase in temperature introduces vibrational energy that weakens the three-dimensional structure of the enzyme. Once the hydrogen bonds and hydrophobic bonds holding the native structure together are broken or disrupted, the enzyme is denatured and the reaction stops. The temperature range over which an enzyme is stable and catalytically active depends on the temperature of the cell in which the enzyme is found.

As temperature increases, the rate of reaction also increases, as is observed in many chemical reactions. However, the stability of the protein also decreases due to thermal degradation. Holding the enzyme at a high enough temperature for a long period of time may cook the enzyme or inactivate it. It was observed that the maximum temperature for peroxidase activity was between 30^{o}C and 70^{o}C in most vegetables and fruits that have been studied (Majed and Mohammad, 2005). Inactivation temperature of peroxidase has been reported to be 95^{o}C in soybean seed coat peroxidase, 81.5^{o}C in horseradish peroxidase C and that of *Coprinus cinereus* peroxidase (a class II POD from the fungus *Coprinus cinerus* with similar activity) is 65^{o}C (McEldoon and Dordick, 1996). That of litch POD was 90^{o} C for 10 min and 100^{o}C for 1 min.

2.14.3 Inhibition and Inhibitors of Peroxidase

Many substances alter the activity of an enzyme by combining with it in a way that influences the binding of the substrate or its turnover number. Substances that reduce an enzyme‘s activity in this way are known as inhibitors. Many inhibitors are substances that structurally resemble their enzyme’s substrate but either does not react or reacts very slowly compared to the substrate.

Inhibition of enzymes decreases yield of products and finally the effectivity of the process. There exists two prepositions concerning the mechanism of enzyme inactivation. The first one hypothesizes intermediates (radicals), a formation that reacts with active centre of enzyme (Chang

et al., 1999). Following the second hypothesis, microparticles adsorb enzyme (Masuda *et al.*, 2001). However, the intrinsic mechanism of inactivation of absorbed enzyme is not understood.

2.14.4. Inhibitor of Peroxidases/Peroxidase suppressor

Horseradish peroxidases are inhibited by thiol type inhibitor: mercaptoethanol (MCE) and mercaptoacetic acid (MCA). Mercaptoethanol (MCE) is a more potent inhibitor than mercaptoacetic acid using 4 – aminoantipyrine as a substrate.

Other inhibitors are p-aminobenzoic acid, sodium azide (NaN_3), cyanide, cyclopropanone, L-cystine dichromate ethylenethiourea, hydroxylamine, sulfide, sulfite, vanadate and divalent anions including Cd, Co, Cu, Fe, Mn, Ni, Pb (Zollner, 1993).

2.15 Inactivation of the Enzyme: Peroxidase

The enzymes have a region (called the substrate binding site, the active site or the catalytic site) that is complementary in size, shape and chemical nature to the substrate molecule. Today, it is recognized that the active site, rather than a rigid geometrical cavity, is a very specific and precise spatial arrangement of amino acid residues R-groups that can interact with complementary groups on the substrate (Segel, 1993). Three main processes have been considered to be involved in the inactivation of peroxidase.

(1) Dissociation of prosthetic (heme) group from the holoenzyme (active enzyme system);
(2) Conformational change in the apoenzyme (protein part of the enzyme); and/or
(3) Modification or degradation of the prosthetic group (Lemos *et al.*, 2000).

2. 15.1. Inactivation of Peroxidase by Hydrogen Peroxidase

The function of all the forms of peroxidase in plants is thought to be the scavenging of the H_2O_2 that is continuously generated in cells (Veitch, 2004). For instance, in the chloroplasts of photosynthetic organisms superoxide (O_2^{o-}) is formed when insufficient CO_2 is available to balance electrons being generated by the photosystems; these excess electrons then reduce O_2 to O_2^{o-}. Additionally, in the mitochondria the electron transport chains can also produce O_2^{o-}. In both cases superoxide dismutase converts O_2^{o-} into H_2O_2 which POD or catalase can then remove. The first step in the catalytic cycle of POD 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). In formal terms, compound I is two oxidising equivalents above the resting state. The first one-electron reduction step requires the participation of a reducing substrate and leads to the generation of compound II, an Fe(IV) oxoferryl species that is one oxidising equivalent above the resting state. Both compound I and compound II are powerful oxidants, with redox potentials estimated to be close to +1 V. The second one-electron reduction step returns compound II to the resting state of the enzyme. Reaction of excess hydrogen peroxide with the resting state enzyme gives compound III, which can also be prepared by several (Veitch, 2004), other routes (Dunford and Jones 2010) that lead to the degradation of heme, the release of iron and the formation of two fluorescent products and inactivation of the enzyme. This intermediate is best described as a resonance hybrid of iron (III)-superoxide and iron (II)-dioxygen complexes. A high-resolution crystal structure of

95% pure compound III shows dioxygen bound to heme iron in a bent conformation (Berglund *et al.,* 2002).

The mechanistic model of the reaction of peroxidase with H_2O_2 in the absence of other substrate is presented in Figure 2.12

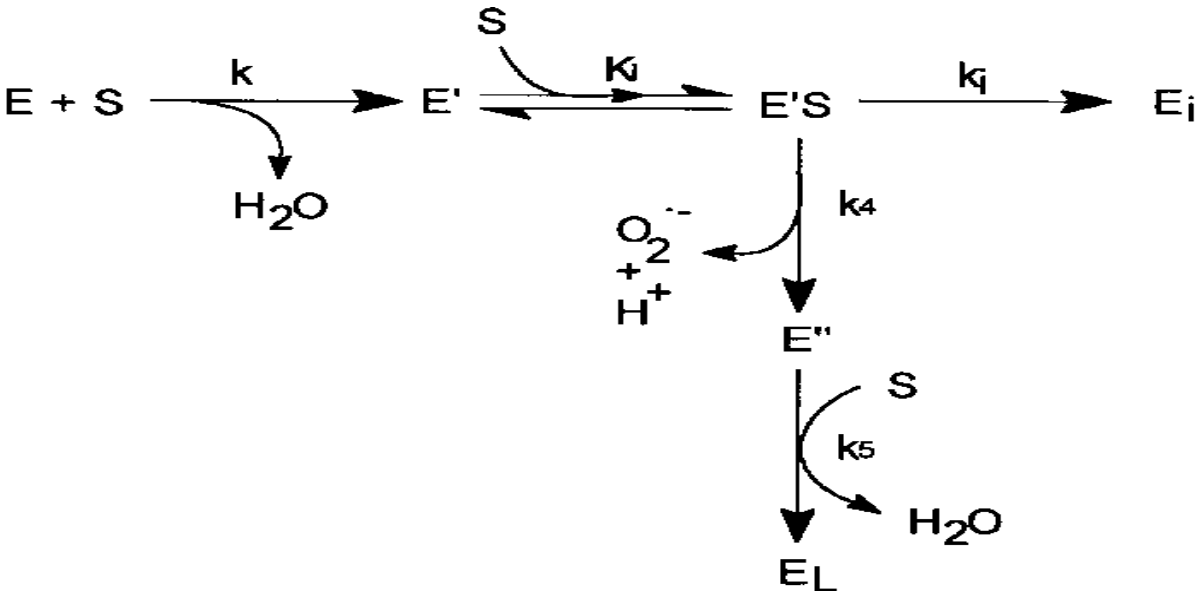


Figure 2.12 Mechanistic Model of the Reaction of Peroxidase with H_2O_2 in the Absence of other Substrates (Hiner *et al*., 2002)

E is native ferric peroxidase. *S* is H_2O_2. *E* ' and E'' are the enzyme intermediates, compounds I, and II, respectively. *E'S* and E_L are complexes between the respective intermediates and H_2O_2, [compound IH_2O_2], and [compound IIH_2O_2]. *Ei* is inactive peroxidase. E_L is also a peroxidase species with modified specificity for reducing substrate.

2.16 Biotechnological Applications of Peroxidase Bio-catalysis in Management of Environmental Pollutants

Recently, peroxidases have been used as reagents for organic syntheses and bio-transformations, as well as in coupled enzyme assays, chemiluminescent assays and immunoassays.

I. Oxidative decarboxylation of auxin (IAA), a plant hormone that affects many physiological processes by PODs (from tobacco and HRP). PODs induce IAA inactivation, thereby offering new potential for target cancer therapy. Studies reported that IAA is cytotoxic to human tumor cells in the presence of POD (Zia *et al.,* 2011). The mechanism of toxicity involves 3-methylene-2-oxindole which is generated through IAA oxidation. Many other substituted indole-3- acetic acid derivatives have been tested for cytotoxicity in combination with HRP C in an attempt to place relationship between structure and activity on a predictive level. No simple correlation was found between levels of cytotoxicity of indole derivatives and their reactivity towards compound I; for example 5-fluoroindole- 3-acetic acid is more cytotoxic towards tumour cells than IAA but less effective as a reductant of compound I (Folkes *et al.,* 2002*).*

Other factors such as the pKa of the indolyl radical cation and rates of decarboxylation and radical fragmentation may also be significant. One of the most cytotoxic indoles identified from *in vitro* screening is 6-chloroindole-3-acetic acid, a derivative with potential as a pro-drug for targeted cancer therapies mediated by HRP C (Rossiter *et al.,* 2002). The challenge now is to develop strategies to evaluate and implement this promising system *in vivo*. Indeed the combination of HRP C and indole-3-acetic acid or its derivatives offers several advantages for future antibody-, gene- or polymer-directed enzyme pro-drug therapies (Wardman, 2002). Among the favourable properties of HRP C are its good stability at 37^{o}C, high activity at

neutral pH, lack of toxicity and the ease with which it can be conjugated to antibodies and polymers. Furthermore, evidence available at present suggests that IAA does not show any adverse side effects in humans. The fact that peroxide is not required as a co-substrate for the reaction with HRP C is also a significant advantage.

II. Some applications of HRP in small-scale organic synthesis include N- and o-dealkylation, oxidative coupling, selective hydroxylation and oxygen-transfer reactions:

III. Peroxidase-catalysed oxidative coupling of methyl-(E)-sinapate with the syringyl lignin-odel compound,1-(4-hydroxy-3,5-dimethoxyphenyl) ethanol yielded a novel spirocyclohexadienone together with a dimerization side-product

IV. Coupling of catharanthine and vindoline to yield α-3, 4, - anhydrovinblastine. This reaction, catalysed by HRP, offers potential interest as it is a semisynthetic step in the production of the anti-cancer drugs vinblastine and vincristine from *Catharanthus roseus* (Vidali, 2001; Veitch, 2004).

V. Peroxidases have also shown an action on tyrosine, both as free amino acid and in peptides or proteins. After one electron oxidation and subsequent deprotonation, dityrosines and higher oligomers are produced.

VI. Ferulic acid and tyrosine are subject to peroxidase-mediated oligomerization. Such peroxidase-mediated hetero-coupling could provide an explanation for the occurrence of protein-carbohydrate complexes in plant cell walls and the incorporation of ferulic acid and other hydroxycinnamic derivatives into lignin and suberin tissues on a protein template. Recent studies have further explored the mechanism of hetero-adduct formation of GYG (Gly-Tyr-Gly) and FA. (Ahn *et al.,* 2002).

VII. Reactive oxygen species (ROS) generated through abiotic and biotic stresses trigger programmed cell death (PCD) in mammalian cells, yeast and plants *(*Delannoy, 2005*)*. In plants and yeasts the PCD is induced by Bax proteins that cause organelle dysfunction by their localization onto the outer mitochondrial membranes and formation of ion channels. Several enzymes have been reported to suppress Bax-induced cell death such as peroxidase, ascorbate peroxidase, peroxidase with glutathione transferase and phospholipid hydroperoxide glutathione peroxidase *(*Chen *et al.*, 2004).

VIII. Many studies (El Agha *et al.* 2008; 2009; Osman *et al.* 2008; Majdalany, 2008) have suggested an association of plant peroxidases with production and scavenging of hydrogen peroxide, porphyrin metabolism, senescence and organogenesis, indicating that PODs have diverse functions (Hiraga *et al.*, 2001) for the exploitation and valorization of crude POD from cheap vegetable sources.

2.16.1 The Use of Peroxidase as Detoxifiers during Waste Water Treatment

Although the use of enzyme in the waste water treatment was first proposed in the 1930s only as late as in the 1970s the concept of environmental biocatalysts that is, application of enzymes to destroy target pollutants was established. Enzyme may transform pollutant to diminish their toxicity, to increase water solubility and its subsequent removal from the industrial waste stream.

Industrial pollution has been a major factor causing the degradation of the environment around us, affecting the water we use; its quality and human health is directly related issues. Improved quality and increased quantity of water would bring forth health benefits. Safe water eliminates the infective agents associated with water-borne diseases; availability of greater quantity of water can improve health by allowing improved personal hygiene. Water pollution causes industrial waste

products to release into lakes, rivers, and other water bodies that make marine life no longer hospitable. Peroxidases have been applied to the bioremediation of waste waters contaminated with phenols, cresols, and chlorinated phenols (Ong *et al.*, 2011). Peroxidases have been applied and were shown to be able to remove a variety of phenols and aromatic amines from an aqueous solution and to decolorize phenolic compounds and amines in industrial effluents. It was shown that phenols are effectively removed by treatment with horseradish peroxidase in the presence of a coagulant. However, peroxidase quickly becomes inactivated during the reaction, and the coagulant prevents peroxidase inactivation and reduces the amount of peroxidase required for phenol removal.

Figure 2.13 below is the reaction system involved in the production of hydroxyl radical by white rot fungi during quinone redox recycling.

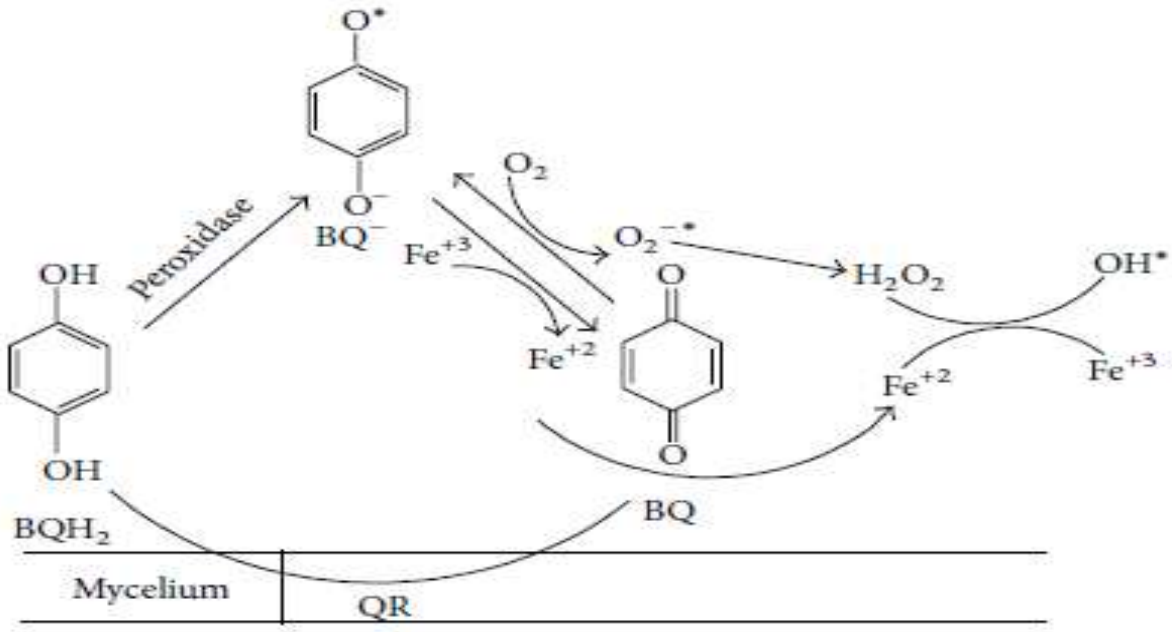


Fig. 2.13: Reaction Scheme involved in the Production of Hydroxyl Radical by White rot Fungi via Quinone redox cycling (Gómez-Toribio *et al*., 2009).

1,4- benzoquinone (BQ) is reduced by quinone reductase (QR) producing hydroquinone (BQH_2), which is oxidized by any of the lignin modifying enzymes to semiquinones (BQ^-).The production of superoxide anion radicals (O_2^-) by BQ^- autoxidation is mainly catalyzed by Fe^{3+} that is reduced to Fe^{2+}. Fenton's reagent formation is accomplished by O_2-dismutation to H_2O_2.

2.16.2 De-colorization of Synthetic Dyes.

Peroxidase bio-catalysis has been implicated in the management of recalcitrant environmental pollutants such as synthetic dyes. Wastes from dyes represent one of the most challenging groups of pollutants considered as xenobiotics that are not easily biodegradable. These dyes are mostly used in textile dyeing, paper printing, color photography, and as additive in petroleum products. When these synthetic dyes are discharged into industrial effluents they cause environmental pollution. Textile industries play a vital role in the economic increases in most countries of the world as Nigeria represent. Water is one of the major products of nature used enormously by human beings, and it is not unnatural that any growing community generates enormous waste water or sewage (Gopi *et al.*, 2012). To achieve the biodegradation of environmentally hazardous compounds, white rot fungi appear as a valuable alternative. The capability of oxidation is based on the ability of white rot fungi to produce oxidative enzymes such as laccase, manganese-peroxidase, and lignin peroxidase (Tien *et al.*, 2008). These oxidases and peroxidases have been reported as excellent oxidant agents to degrade dyes (Kirby *et al.*, 2005).

Several bacterial peroxidases have been used for decolorization of synthetic textile dyes. Removal of chromate Cr (VI) and azo dye Acid Orange 7 (AO7) using *Brevibacterium casei* under nutrient-limiting conditions has been studied. AO7 was used as an electron donor by the reduction enzyme of *B. casei* for the reduction of Cr (VI). The reduced chromate Cr (III) complexed with the oxidized

AO7 formed a purple intermediate (Ng *et al.,* 2010). Decolorization of different azo dyes by *Phanerochaete chrysosporium* RP 78 under optimized conditions was studied (Gopi *et al.,* 2012) by reaction mechanism via azo dye. Peroxidase was produced under aerobic conditions as a secondary metabolite in the stationary phase. *Bacillus* sp. VUS isolated from textile effluent contaminated soil showed capability for degrading a variety of dyes (Dawkar *et al.,* 2008).

The production of ligninolytic peroxidases directly oxidizing aromatic compounds has been described in fungi (Krishnaveni and Kowsalyla, 2011). Other peroxidases were detected in microorganisms responsible for the biodegradation of industrial dyes together with lignin peroxidase (Pomar *et al.,* 2002). An edible macroscopic fungi *Pleurotus ostreatus* produced an extracellular peroxidase that can decolorize remazol brilliant blue and other structurally different groups including triarylmethane, heterocyclic azo, and polymeric dyes. Bromophenol blue was decolorized best (98%), while methylene blue and toluidine blue O were least decolorized 10% (Gopi *et al.,* 2012). HRP was found to degrade industrially important azo dyes such as remazol blue. This dye contains at least one aromatic group in its structure making it a possible substrate of HRP (Bhunia *et al.,* 2002).

2.16.3 Degradation of Polychlorinated Biphenyls (PAHs) Pesticides.

Pesticides comprise a broad range of substances most commonly used to control insects, weeds, and fungi. Pesticide exposure in humans is associated with chronic health problems or health symptoms which include; neurologic deficits, memory disorders, respiratory problems, dermatologic conditions, cancer, depression, miscarriages and other birth defects. Biological decomposition of pesticides is the most important and effective way to remove these compounds from the environment. Microorganisms have the ability to interact, both chemically and physically,

with substances leading to structural changes or complete degradation of the target molecule (Dawkar *et al.,* 2008). Peroxidases extracted from some fungal species have great potential to transform several pesticides into harmless form(s).

White rot fungi have been implicated in the transformation of organophosphorus pesticides and transformation of several organophosphorus pesticides by chloroperoxidase from *Caldariomyces fumago* (McCauley *et al*., 2006). PAHs are composed of two or more fused aromatic rings and are components of crude oil, creosote, and coal. Most of the contamination by PAHs originated from the extensive use of fossil fuels as energy sources. Peroxidases and phenol oxidases can act on specific PAH's by transforming them to less toxic or products easier to degrade. PAHs are oxidized by peroxidases such as lignin peroxidase (Gopi *et al.*, 2012) and manganese-peroxidase (Dawkar *et al.*, 2008).

2.16.4. Peroxidase as Biosensors in Environmental and Clinical Implications.

Biosensors have been defined as analytical devices which tightly combine bio-recognition elements with physical transducers for detection of the target compound. Several biosensors have been developed for relevant environmental pollutants. Biosensors can be useful, for example, for the continuous monitoring of a contaminated area (Ahammad, 2013). They may also present advantageous analytical features, such as high specificity and sensitivity (inherent in the particular biological recognition bioassay. H_2O_2 is considered the mediator of the biochemistry of cellular pathology and maybe involved in the etiology of aging and progressive neurodegenerative diseases such as Parkinson's disease (Dawkar *et al.*, 2008). Due to its crucial role in neurochemistry, determination of the concentration of H_2O_2 has been a considerable interesting research field. Electrochemical methods based on peroxidase biosensors have proved to be significantly

advantageous to the biosciences due to their direct real-time measurements and capability for practical applications (Song *et al.,* 2011). A novel third generation biosensor for hydrogen peroxide was constructed by cross-linking HRP onto an electrode modified with multiwall carbon nanotubes (Xu *et al.,* 2011). At the same time, biosensors offer the possibility of determining not only specific chemicals but also their biological effects, such as toxicity, cytotoxicity, genotoxicity, or endocrine disrupting effects, that is, relevant information that in some occasions is more meaningful than the chemical composition (Xu *et al.,* 2011). Enzymatic biosensors are based on the selective inhibition of specific enzymes by different classes of compounds, with the decrease in activity of the immobilized enzyme in the presence of the target analyte as the parameter that is frequently used for quantification. A novel myoglobin-based electrochemical biosensor based on a nano composite prepared from multi walled carbon Nano tubes that were coated with ceria nanoparticles has been developed (Qui *et al.,* 2010). Another application of whole-cell biosensors is the determination of the biological oxygen demand (BOD).

Pesticides (herbicides, fungicides and insecticides) are widely used in agriculture and industry around the world due to their high insecticidal activity (Xu *et al.,* 2011). Biosensors are potentially useful as they detected pesticides quickly and have been active in the research area for some years. Another valuable HRP-based biosensor was developed in which polyvinyl pyrrolidone (PVP) nanofibers were spun with incorporation of the enzyme HRP. Scanning electron microscopy (SEM) of the spun nanofibers was used to confirm the non-woven structure which had an average diameter of 155 ± 34 nm (Ahammad, 2013). The HRP containing fibers were tested for their change in activity following electro spinning and during storage. A colorimetric assay was used to characterize the activity of HRP by reaction with the nanofiber mats in a microtiter plate and monitoring the change in absorption over time (Dai *et al*., 2012). Rapid and sensitive detection

methods are of utmost importance for the identification of pathogens related to health and safety. Peroxidase has been used in development of a nucleic acid sequence-based lateral flow assay which achieves a low limit of detection using chemiluminescence and enzymatic signal amplification (Wang *et al*., 2012).

CHAPTER THREE

MATERIALS AND METHOD

3.1. MATERIALS

3.1.1 Apparatus and Equipment

The following equipment were used in the course of this studies; FS 240 variant agilant atomic absorption spectrophotometer, nitrous oxidant gas, acetylene gas, air oxidant gas etc.

The entire instruments used were well calibrated and are in good working condition**.**

3.2 METHODS

3.2.1 Description of Sample Location

Contaminated soil samples were collected from a crude oil polluted site located at Eleme Jetty site particularly the crude effluent disposal of Onne, Eleme Local Government Area (LGA) (Latitude 5^{o} N, Longitude 7^{o} E), River State, Nigeria. Onne is situated beside Ogu creek, a side arm to Bonny River. It is part of the Odido clan in the Eleme LGA. It is the main base for offshore activity in that region and a large number of supply-vessels call at Onne every week. Onne loading jetty site is a suburb, surrounding and housing many oil drilling companies within Rivers state, most of the sites are seen with pipelines, loading oil tankers, disposal trucks and other anthropogenic activities. The soil is laden with much disposal of crude oil from anthropogenic activities. Uncontaminated soil samples were also collected from non-polluted site which was about 1.4 miles away from the polluted site free from oil activities.

3.2.2 Collection and Preparation of Soil Samples

Soil samples were collected from crude oil polluted soil at Onne oil jetty site Eleme, Rivers State. They were collected from the depths of 1-20 cm which is the top soil using auger. The unpolluted soil samples were collected from the nearest unpolluted soil area within a distance of about 1 miles away from the polluted site. They were put into a clean aseptic container and transferred to the laboratory. The collected soils were air dried and sieved using a 2mm sieve. Soil samples were sorted, the coarse particles were thrown away while the fine particles were used and for analysis.

3.2.3 Chemical Analysis of the Soil Samples

Soil samples from the polluted site prior to microbial isolations were subjected to various chemical soil profiling tests as described in the proceedings of Agency of Toxic substances and disease registry (ATSDR, 2010).

The following tests were carried out:

Soil pH

Soil pH was determined using pH meter. Five gram (5 g) of the soil sample were weighed out and mixed vigorously for 5 min with 50 ml of distilled water. After shaking, it was left standing, filtered off and the filtrate was used to determine the pH value using the pH meter (Model PHS-3C, Search Tech. instrument).

Soil Conductivity Test

This was carried out using the conductance meter from which the temperature of the soil was extrapolated. This was done by dipping the electrode (calibrated) of the meter into a solution of the soil (5 g in 10 ml of water) and kept standing for 5 min. The electrical conductivity was deduced from the meter reading.

Determination of Moisture Content

Oven -Dry Method

A plastic dish was washed thoroughly and dried in the oven at 110^{o}c. It was put inside the desiccator to cool and each dish weighed. The soil sample was mixed and the laboratory sample was put into the weighed dish. The weight of dish plus weight of undried sample (induplicate).

It was dried in the moisture oven at 70^{o}-80^{o}c for 2 h and at 100-135^{o}c (usually 105^{o}c) until weight was constant. The sample was cooled in the desiccators and the dry weight of sample plus dish was noted.

The moisture content was calculated as described in the equation below.

$$\% \text{ Moisture } = \frac{W_2 - W_3)}{(W_2 - W_1)} \, x \, 100 \qquad 3.1$$

Where;

W_1 = Initial weight of empty crucible

W_2 = Weight of crucible + soil sample before drying.

W_3 = Final weight of crucible + soil sample after drying.

Sulphate: (Gravimetric Method)

The soil was dissolved in 1:2 of solvent, 100 to 200ml of the sample, and filtered. HCL was added in drops 1:1 until acid litmus, three drops was added in excess and evaporated to 50 ml. The solution was boiled and Barium chloride solution added until all the sulphate is precipitated. It was digested on a water bath until the precipitate has settled. It was dried in a sintered-glass crucible to constant weight. The filtering equipment was connected to the vacuum pump and the precipitate filtered through sintered-glass crucible. It was washed a number of times with hot water until the filtrate is chloride-free ($AgNO_3$ test). The crucible precipitate was dried in an oven at 103- 105^{0} C to constant weight.

Know the weight of the precipitate alone by difference.

Calculation:

$$SO4\ (mg/kg) \ = \ \frac{mg\ BaSO4\ x\ 411.5}{ml\ sample\ (weight\ of\ the\ soil)} \qquad 3.2$$

Soil Mineral Contents

The following ions were determined from the crude oil polluted soil as follow: Cl^-, Ca^{2+}, $Mg^{2+}$and K^+.

Chloride ion (Cl^-)

The concentration of chloride ion was determined using the method according to Mohr (4500 B-Cl^-; Argentometric method (APHA, 1998; Sawyer *et al.,* 2000). Two test tubes were labeled blank and calibrator. Twenty-five grams of the soil were mixed with 100 ml of distilled water. Using the pH meter, the diluted mixture was vortexed and heated for 30 min to break up the carbon chains and then filtered off. The filtrate was then subjected to chloride ion examination as follows:

One millilitre of potassium chromate indicator was added to the filtrate of the contaminated soil; the mixture was then titrated against silver nitrate ($AgNO_3$). The silver nitrate was dropped until a brick red colour persists throughout the sample. The amount of silver nitrate used was recorded and Cl^- concentration calculated thus, concentration of chloride ion in both mixtures was deduced from the formula:

$$\text{Chloride ion Concentration mg/g} = \frac{A-B\ X\ MX70{,}900}{V\ Sample} \qquad 3.3$$

Where A = Titre value of the test sample

B = Titre value of the blank solution

M = Molarity of $AgNO_3$

V = Volume of pipetted sample

Total Hardness of the Soil Samples

The following procedure was used to determine total hardness:

Fifty ml of the sample were pipetted into a 250 ml conical flask and diluted to 100 ml using deionized water. Four ml (4 ml) of the buffer solution and 6 drops of mordant black II solution was added and titrated with EDTA solution, to a distinct blue endpoint (V ml).

Total hardness was calculates thus:

$CaCO_3$ content (mg/Kg) = $\frac{V\ X\ E\ (CaCO3)}{50}\ x\ 100$ 3.4

Where; V= ml titration for the sample

E= Calcium carbonate equivalent to 1 ml EDTA titrant.

Calcium Hardness

Calcium hardness was done following the procedure below:

Fifty ml of the test sample were pipetted into a 250 ml conical flask and dilute to 100 ml, preferably with deionized water. Two ml of 2 M NaOH solution and 6 drops of the Solochrome dark blue solution were added. The colour of the solution turned violet and its pH value 12.0. It was then titrated with EDTA solution, to a distinct blue endpoint (V ml).

$CaCO3\ content\ (mg/Kg)\ =\ \frac{TV\ X\ E\ (CaCO3)}{50} x\ 100$ 3.5

Tv = Titer value.

E= Equivalent weight of Calcium ion

Magnesium Hardness

Magnesium hardness was calculated from the determined total hardness and Calcium hardness Formula;

Magnesium (as mg/l) = (T– C) x 0.243 3,6

Where; T= total hardness mg/l (as $CaCO_3$)

C= Calcium hardness mg/l (as $CaCO_3$)

Potassium ion (K^+).

Briefly, 5 g of the soil was sampled out as described in the soil pH determination and dissolved in 250 ml of deionized water; the mixture was shaken and filtered off. Ten ml of the filtrate was added with a sachet of potassium and assayed for potassium concentration at wavelength of 610 nm. Potassium concentration was extrapolated from the absorbance read-off of the spectrophotometer.

Phosphate test

Phosphate was extracted using 0.5 M of sodium bicarbonate at soil to water ratio of 1:25**.** Phosphate was determined by amino acid method using HI83200 multi-parameter bench photometer at a wavelength of 525 mm. Ten ml of the sample was poured into two (2) separate sample cell. One was used for blank to zero the photometer and 10 drops of HI93717A-0 molyb date Reagent, then the content of one packet of HI 93717B-0 phosphate HR reagent B added to the cuvette. It was shaken gently to dissolve and was inserted into cell compartment and time for 5 min. At the end of the countdown, the READ button was pressed to display the result in mg/l of phosphate.

Heavy Metal Determination

Iron (Fe) using Spectrophotometer

Iron metal from the soil samples was identified using spectrophotometric assay method as described by Finch, *et al,* (2015). The following procedure was used:

Briefly, 10 g of the soil sample was mixed with 100 ml of distilled water, shaken for 30 min and filtered. Ten ml of the filtrate was pipetted into 25 ml volumetric flask to which 10 ml of hydroxylamine hydrochloric acid was added to. The mixture was shaken for 5 min and 5 ml of sodium acetate was added followed with 4 ml of phenanthroline reagent and 30 ml of deionized water. An aliquot of 1 ml was taken and added into the cuvette. Spectrophotometric assay of iron concentration was taken at wavelength of 510 nm.

Heavy Metal Analysis (Using Atomic Absorption Spectrophotometer)

Soil Digestion for Heavy Metal Analysis

Before the atomic absorption spectrophotometer analysis (AAS), briefly, a gram of sample was digested in 250 ml conical flask by adding 30 ml of aqua regia (HCl) and heated on a hot plate until volume remains about 7-12 ml. The digest was filtered using what-man filter paper and the volume made up to the mark in a 50 ml volumetric flask, and was then stored in a plastic container for AAS analysis.

Preparation of Working Solution.

Cd working solution: 100 ppm of Cd working solution was first prepared in 100 ml distilled water

Calculation

$$C_1V_1 = C_2V_2 \qquad 3.7$$

C_1 = concentration of the working solution = 100 ppm

V_1 = volume of distilled water used to prepare the working solution =100 ml

C_2 = concentration of zinc stock solution = 1000 ppm

V_2 = volume of stock that will be used. $\frac{1000 \text{ ppm X } 100 \text{ ml}}{1000\text{ppm}} = V_2$

V_2 = 10 ml of stock solution + 90 ml of distilled water

Arsenic working solution

100 ppm of As working solution was first prepared in 100 ml distilled water

Calculation

$$C_1V_1 = C_2V_2 \qquad 3.8$$

C_1 = concentration of the working solution = 100 ppm

V_1 = volume of distilled water used to prepare the working solution =100 ml

C_2 = concentration of zinc stock solution = 1000 ppm

V_2 = volume of stock that will be used.

$$\frac{1000\ ppm\ x\ 100\ ml}{1000\ \text{ppm}} = V2$$

V_2 = 10 ml of stock solution + 90 ml of distilled water

Sample Digestion

First, 2 g of the dried sample was weighed out into a digestion flask and 20 ml of the acid mixture (50 ml conc. HNO_3), was added that is; 30 ml per chloric acid, 20 ml conc. H_2SO_4. The flask containing the sample was heated until a clear digest is obtained. The digest was diluted with distilled water to the 25 ml mark. Appropriate dilutions was then made for each element.

Same preparations were carried out using Hg, Cu and Pb standard solutions

Principles of Atomic Absorption Spectrophotometer.

The working principles of atomic absorption spectrometer is based on the sample being aspirated into flame and atomized when the AAS light beam is directed through the flame into monochromator, and onto the detector that measures the amount of light absorbed by the atomized element in the flame. Since metals have their own characteristic absorption wave length a source

lamp composed of that element is used, making the method relatively free from spectral or radiational interferences. The amount of energy of the characteristic wavelength absorbed in the flame is proportional to the concentration of the element in the sample.

Total Organic Carbon Contents

The total organic carbon contents (TOC) was determined as follows:

Soil sample weighing 0.5g was mixed with 10 ml (0.5 M) of $K_2Cr_20_7$ and 20 ml of sulphuric acid and vortexed for 30 min. The mixture was diluted with 20 ml of water and 10 ml of phosphoric acid with 3-4 drops of ferroin indicator and titrated with 50 ml of ferrous ammonium sulphate (FAS).

$$TOC\ (mg)\ =\ \frac{V_b - Vs\ X\ 16{,}000}{\text{Vol. of sample used}} \qquad 3.9$$

Where Vb = Titre vol. of blank

Vs = Titre vol. of test solution

Total Petroleum Hydrocarbon Determination from the Soil Samples

Total petroleum hydrocarbon (TPH) described as the sum total of petroleum hydrocarbon in given bio system was determined according to the method described by Agency for Toxic Substances and Disease Registry (ATSDR, 2009).

Five gram (5 g) of the contaminated soil sample were weighed out and mixed together with 5 g of anhydrous sodium sulphate, 25 ml of n-hexane was added to the prepared colloidal sample and mixed thoroughly. To the standing solution, 25 ml of n-Hexane was again added to it, shaken very well. The resulting solution was later partitioned out using the separating funnel. The aqueous layer of the mixture was discarded and the solvent partition was used for spectrophotometric analysis at wavelength of 420 nm.

Total Organic Matter

Procedure was by ash determination.

Finely ground, dry sample (2.5 g) was weighed into a tared silica or porcelain crucible. The sample was charred on a bunsen flame inside fume cupboard, to drive off most of the smoke. Sample was transferred into a pre-heated muffle furnace at 550^{o}c and left at this temperature for 2 h or until a light grey ash results. When the residue turned black, it was moisten with a small amount of water dissolved salts, dried in an oven and the ashing process repeated. It was allowed to cool in the desiccators and reweighed.

Calculation:

$$\%\ Ash\ (dry\ basis) = \frac{Weight\ of\ ash}{\text{Weight of sample}}\ x\ 100 \qquad 3.10$$

$$= \frac{(W_3 - W_1)}{(W_2 - W_1)}\ x\ 100$$

TOM = 100 –%ASH

Where;

W_1 = Initial weight of empty crucible

W_2 = Weight of crucible + soil sample before drying and/or ashing.

W_3 = Final weight of crucible + ash.

3.2.4 Preparation of Buffer Solutions

Sodium acetate buffer

In preparing 0.05 M of sodium acetate buffer, 6.804 g of sodium acetate salt was dissolved in 1000 ml of distilled water. The pH was adjusted with the conjugate acid which is glacial acetic acid to pH ranging from 3.5-5.5.

Sodium phosphate buffer

To prepare 0.05 M of acetate buffer, 7.098 g of disodium hydrogen phosphate salts was dissolved in 1000 ml of distilled water. The pH was adjusted with sodium dihydrogen phosphate to pH ranging from 6.0-7.5.

Tris-HCl buffer

Tris buffer (0.05 M) was prepared by weighing 6.07 g of Tris (hydroxymethyl) amino methane and dissolved in 1000 ml of distilled water, the pH was adjusted with hydrochloric acid to pH ranging from 8.0-9.0.

All the prepared buffer solutions at corresponding pH were in the range of 0.5 units.

3.2.5. Microbial Isolation of Fungi from Soil Samples

Fungal isolation from the contaminated and uncontaminated soil (used as control experiment) was carried out according to the method described by Ezeonu *et al*. (2013).

For the isolation process in both experiments, 20 g of soil sample was weighed out into 40 ml of sterilized distilled water in a clean conical flask and was shaken vigorously. From the stock solution prepared, ten-fold serial dilutions were carried out and the 10^{-1} to 10^{-5} dilutions were plated on plate media (triplicate each).

Preparation of Media and Plate Pouring

Saboraud Dexterose Agar (SDA) was prepared according to the manufacturer's description which is 65 g in 1000 ml of distilled water. Five Petri dishes were displayed and 6.5 g of SDA was weighed out and dissolved in 100 ml of distilled water. Anti-bacterial agent (chloramphenicol) was added to the broth prepared.

Also Mineral Salt Agar (MSA) using the Bushnell-Hass formulation was prepared according to the recipe descriptions and solidified using the agar-agar gel with crude oil from the contaminated site as the sole carbon source.

Inoculations of Serially Diluted Soil Solution on the Prepared Plate and Sub Culturing

From the 10^{-4} to 10^{-6} fold dilutions, inoculations were carried out on the prepared SDA plates using a 1 ml insulin syringe and a glass rod spreader around a bursen flame. Streaks were made from each side of the plate, marking an initial point, with sterilization of the wire loop after each side has been completed. After inoculation, the inoculated plates were incubated for 3-4 days at room temperature (30^{o}C) for colony growth. All morphological contrasting colonies were purified by repeated streaking and sub-culturing on separate plates. This process was continued till pure fungal cultures were obtained.

On the mineral salt agar (MSA), 10^{-2} fold dilutions, inoculum was introduced on the culture plate as described above. Crude oil from the contaminated site was aseptically introduced into the prepared inoculated medium using sterilized filter paper with drops of the crude oil on it. The dropped filter paper was placed on the media and incubated for 5-6 days at 30^{0}C.

Storage of Pure Fungal Isolates

Pure fungal isolates were maintained on SDA slants as stock cultures in a bijou bottle and stored at 30^{o}C.

Morphological Features of the Isolated Fungi

Three day old pure cultures were examined. The color, texture, nature of mycelia or spores and growth patterns were also observed.

3.2.6. Biochemical Identification of the Pure Fungal Isolates

Biochemical properties of *Rhizopus* and *Saccharomyces* spp. were examined to characterize the fungi and these include:

Sugar test, Gelatin liquefaction, Urea hydrolysis, and Hydrogen sulphide hydrolysis.

Gelatin Liquefaction Test

The biochemical characteristics of. *Rhizopus and Saccharomyces* spp. strain was carried out using Urea Broth method (Davis, 1949) modified by Cappuccino and Sherman, (2004). Ten ml of gelatin protein were dispensed into each autoclaved test tube and fresh culture suspensions (3-5 day old) were inoculated into it. The culture medium was incubated using the orbital shaker at 25°C for 200 rpm and regularly checked for 1 week for sign of liquefaction.

Test for Hydrolysis of Urea

The urea medium was prepared according to the minimal Davis's Urea methods (Davis, 1949).

A wire-loop of culture suspension from 2-3 day old culture was introduced into the broth containing the liquid urea ($CONH_{2\,(2)}$ 3 mM). It was incubated using the rotatory shaker at 25°C for 200 rpm. The culture suspensions were diurnally checked for every 30 min and 2 h for up to 24 h for color change. It was further checked again after 36 h after introduction of the organism.

Fungal Identification

Fungal identification at this stage was done by microscopic slide mounting following these procedures:

The three day old pure cultures were used in preparing microscopic slides. A little bit of the mycelia was dropped on the slide and a drop of lacto-phenol blue was added to it. A cover slip was placed over it and examination performed under the light microscope at X40 magnification. Identification was carried out by relating features and the micrographs to "Atlas of mycology" by Barnett and Hunters (1972) and also curated by a mycologist.

Total Heterotrophic Count

Total heterotrophic biomass from both the SDA and the MSA was counted from the media plate and described as follow:

Total Colony forming Unit/g (TCFU/g) = Colony observed X Inverse of dilution factor X Inverse of volume of the organism(s) used.

3.2.7. Screening of Isolates for Bio-surfactant Production and Emulsification Studies

Prior to the screening for bio-surfactants, the isolates identified were inoculated into 10 ml of liquid broth medium each and incubated at 30^{o}C for 72 h. The culture medium was centrifuged at 3000 revolutions per minute (rpm.) for 30 min. The supernatant was collected and the cells discarded. The supernatant was used for the various bio-surfactant screening tests.

Emulsifying Assays

The following emulsification assays were carried out on the identified isolates as follows:

Drop Collapse Assay

The assay was carried out as described by Jain *et al*. (1991). A drop of the culture supernatant was placed carefully on an oil coated glass slide and observed after 1 min. If the drop of supernatant collapsed and spread on the oil coated surface, it signifies the presence of bio-surfactant. But if the drop remains after one minute, it showed the absence of surfactant. This test was simultaneously carried out on distilled water as control.

Oil Spread Assay

Using a micropipette, 10 µl of vegetable oil (coconut oil) was added to the surface of 40 ml of distilled water into a Petri dish to form a thin oil layer and 10 µl of the culture supernatant was gently dropped at the center of the oil layer. After one minute, if bio-surfactant is present in the supernatant, the oil would displace and a clear zone would be formed as described by Morikawa *et al.* (2000).

Emulsification Capacity

The emulsification capacity of bio-surfactant was developed by Cooper and Goldenberg (1987). Two ml of kerosene was added to 2 ml of the culture supernatant and the mixture was vortexed or shaken at high speed for 2 min. The mixture was left for 24 h; the height of the stable emulsion layer was measured. The same procedure was followed for both diesel and crude oil. The emulsion index E24 was calculated as the ratio of the height of the emulsion layer to the total height of liquid.

E24 = Height of the emulsion formed/ (H) total height of the liquid medium X 100%

3.2.8. Molecular Characterization of the Identified Fungal Genera (*Rhizopus* and *Saccharomyces* spp.)

This was done following the procedures:

Gene (DNA at the 18s ITS region) Extraction

During the extraction process, each of the microorganisms (both cultures) was grown aerobically by shaking (4000 g) at 30^{o}C for 3 days using 40 ml of mineral salt medium in a conical flask. After the aerobic shaking, fungal cells were filtered off, air-dried and lyophilized (reverse condensation) overnight. The genomic DNA of each sp. was extracted from the lyophilized cells by sonication as described by Sonne *et al.* (2007). The extracted genome was further purified using clean-up kit (Promega, Madison, WI) according to the manufacturer's protocol.

Polymerase Chain Reactions (PCR).

Primers were designed and authenticated from the protein data bank site (PDB) for the amplification of ITS region: ITS1 and ITS4 Primers as described in Abe *et al*. (2015).

All PCR amplification were performed in a total 50 ml of reaction mixture containing 100 ng of template DNA, 10 μmole of each primer, and 45 ml of Platinum PCR Super-Mix high Fidelity (Invitrogen, Carlsbad, CA). The reaction conditions for the amplification were as follows: initial denaturation at 94^{o}C for 2 min, 35 cycles of denaturation at 94^{o}C for 30 s, annealing at 55^{o}C for 30 s, and extension at 68^{o}C for 1 min. A final 5 min of chain elongation at 68^{o}C was carried out after cycling completion in a model 9700 thermal cycler (Applied Biosystems, Foster City, CA). In cycling condition, in addition to the conditions above, AmpiTaq Gold DNA polymerase (Applied Biosystems) and KOD-plus- ver . 2 (Toyobo, Osaka, Japan) were used. In the reaction with AmpliTaq Gold, the Platinum PCR SuperMix high Fidelity was replaced with a total 45 ml mixture consisting of 5 ml of 10AmpliTag Gold Buffer, 5 ml of Deoxynucleotide triphosphate (mM each), 3.5 ml of 25 mM $MgCl_2$ solution, and 2.5 U of AmpiTaq Gold DNA polymerase. Thermal cycling was performed with a 72^{o}C extension. In the reaction with KOD-plus- ver 2, the components of the mixture other than the template DNA were 15 pmole of each primer, 5 ml of 10 Buffer for KOD-plus-ver 2, 5 ml of deoxyribonucleotide triphosphate (2 mM each) 3 ml of 25 mM $MgCl_2$ solution, and 1 U of KOD-plus- . The reaction conditions were as follows: Initial denaturation at 94^{o}C for 2 min, 35 cycles of denaturation at 94^{o}C for 15 s, annealing at 55^{o}C foe 30 s and extension at 68^{o}C for 1 min. All PCR products were separated by electrophoresis following the following procedure:

1.5 % gel was prepared by measuring 1.5 g agarose in 150 ml of Tris Acetate EDTA (TAE) buffer, microwaved for 2 and half mins swirling at 30 secs intervals the solution heats, until the agarose is completely dissolved. The solution was allowed to cool at about 55^{o}C. Ethidium bromide 0.4 μg/mL and some buffer were added and the edges of the gel tray taped, the agarose solution poured slowly into the gel tray with the well comb in place. It was allowed to sit at room temperature for

up to 1 h until it has completely solidified. Loading buffer was added to each DNA sample, allowed to solidify and the agarose placed into the gel box and 1X TAE buffer used to fill it until it was completely covered. DNA ladder was loaded into the first lane of the gel followed by the DNA samples. The gel was ran at about 150 voltage until the dye line is about 80% way down. The gel was carefully removed from the gel box after turning off the power and disconnecting the electrodes and visualized with a UV trans-illuminator and photographed.

The separated genomic strands were sent to DNA sequencing laboratory where culture independent techniques were used to gun in the genes into an *E-coli* vector and subsequent sequencing of the cloned genome. The sequenced genes were blasted using the NCBI tools from where the maximum likely organism was given an ascension number. *Rhizopus stolonifer* DBOF141 and *Saccharomyces cerevisiae* CBS1171.

Table 3.1 shows the forward and reverse strands of the designed primer as gotten from the protein data bank base (PDB Cabohl data chat). Each strand of the forward and reverse strands have base pairs of 500 bp. and 650 bp longest chain.

Table 3.1: Primer Sequences for the Identified Genome of Strains of *Rhizopus* sp. and *Saccharomyces* sp.

Organism	Primer	Sequence (5^1 to 3^1)	Amplified length
Rhizopus sp.	ame 1F	CTATAAACATTAGCCTTATGAAATTCAGT	500 bp
	ame 2F	ACAGGTTAGCTTTAGCTTGCCTTT	
	ame 1R	TTTAGGCAGGTTTCCCCAA	
	ame 2R	GCAAGTGCTCTAGGGAAG	
Saccharomyces sp.	ame 2F	TCCGTAGGTGAACCTGCGG	650 bp
	ame 2R	TCCTCCGCTTATTGATATGC	

Cloning of PCR Products

The PCR products were purified using a Microspin S-400HR (GE Healthcare, Buckinghamshire, UK), and were used for ligation into pGME-T Easy (Promega). Ligation was conducted following the manufacturer's protocol. The ligation products were precipitated with ethanol and then used in the transformation of E-coli TOP 10 (Invitrogen). White colonies on an LB-ampicillin-Xgal-IPTG plate were chosen, and the molecular weights of the inserted DNA were checked by colony-direct PCR using M13 forward and reverse universal primers. The plasmids were extracted from the liquid culture and a plasmid with a different insert was used in sequencing analysis.

Sequence and Phylogenic Analyses

The sequencing reaction was performed using a BigDye O Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems), and was analyzed with an ABI PRISM 3100 Genetic Analyser (Applied Biosystems). The sequence data obtained from this study were deposited in the DDBJ/EMBL/Gen Bank data base under the accession numbers listed Clustal X (16 was used for the alignment of the sequences and Neighbor-joining clustering. PAUP ver. 4.0b10 software 17) was used for the parsimonious clustering of *Rhizopus stolonifer* and *Saccharomyces cerevisiae* strains.

3.2.9. Production of the Enzyme from the Di-culture of *Rhizopus and Saccharomyces* spp.

Fermentation Experiments

The isolates of interest were screened for peroxidase production before the fermentation studies. A wire loopful of the inoculate was innoculated in a basal mineral medium supplemented with O-dianisidine (standard substrate) prepared in 3% methanol and 0.2 m of phosphate buffer (6.0) and incubated for 3 days at room temperature.

Constitution of the Fermentation Broth

Submerged fermentation (SmF) technique was employed using a 250 ml Erlenmeyer flask containing 100 ml of sterile culture medium optimized for hydrocarbon degrading peroxidase with various optimized nutrients at favorable physical conditions. The flask was stoppered with aluminum foil and autoclaved at 121^{o}C for 15 min.

Inoculation of the broth

From the SDA slants, fresh plates were prepared and inoculated. Three days old cultures were used to inoculate the flasks. In every sterile flask, two discs of the respective fungal isolates were added using a cork borer of diameter 10 mm and then plugged properly. The culture was incubated for 14 days at room temperature (30^{o}C).

Harvesting of the Fermented Broth

At each day of harvest, flasks were selected from the respective groups and mycelia biomass separated by filtration. Each day, the filtrate was assayed for peroxidase activity and peroxidase extracellular protein concentration determined for the 14 days of fermentation.

Mass Production of Enzyme

After the 14 days of pilot submerged fermentation studies, the day of peak peroxidase activity (day 8) was chosen for mass production of enzyme from the respective fungal isolates.

Centrifugation of the Cell Filtrate

Filtrate from the cell biomass was subjected to centrifugation using a centrifuge at the spinning speed of 3500 rpm for 15 min. Afterwards, the separated supernatant from the pellet was simultaneously assayed for peroxidase activity as described by Mclellan and Robinson (1987) and modified by Eze *et al. (*2010)**.**

Peroxidase Assay Using *o*-Dianisidine as Substrate

Peroxidase activity was assayed using the modified method of Mclellan and Robinson (1987) and Eze *et al. (*2010). The change in absorbance at 460 nm due to the oxidation of *o*-dianisidine in the presence of hydrogen peroxide and enzyme extract at 30^{o}C were monitored using Jenway 6405 UV/VIS Spectrophotometer.

The standard assay solution contained 0.3 ml of 0.1% *o*-dianisidine, 0.2 ml of hydrogen peroxide, 2.4 ml of sodium phosphate buffer pH 6.5 and 0.1 ml of enzyme extract in total of 3.0 ml. One unit of enzyme activity was defined as the amount of enzyme that gave an absorbance change. = 0.1/min at 30^{o}C.

The readings were taken for every 30 s for 5 min.

The variables below were calculated as follows using the method of (Segel, 1993).

$$\text{Reaction rate} = \frac{\text{Change in absorbance (OD) at 460 nm}}{\text{Time internal}} \qquad 3.11$$

$$\text{Specific activity (Unit/ml)} = \frac{\text{Reaction rate}}{\text{Protein concentration}} \qquad 3.12$$

Where, Total units = unit/ml x total volume of enzyme.

$$\text{Percentage yield or recovery} = \frac{\text{Total unit of purified enzyme}}{\text{Specific activity of crude enzyme}} \qquad 3.13$$

$$\text{Purification fold} = \frac{\text{Specific activity of purified enzyme}}{\text{Specific activity of crude enzyme}} \qquad 3.14$$

Protein Concentration of the Crude Extract

Protein content was estimated by the method of Lowry *et al.* (1951).The standard protein used was bovine serum albumin (BSA).

Preparation of Reagent for Protein Standard Curve

Protein content was estimated by the method of Lowry *et al.* (1951). The standard used was bovine serum albumin. The reagent used was prepared as follows

I. Solution 1: 2% of Na_2CO_3 was dissolved in 0.1 N NaOH.

II. Solution 2: 1% of sodium potassium tartarate that is 1 g of sodium potassium tartarate dissolved in 100ml of distilled water. Also 0.5 g of Copper sulphate ($CuSO_4$) was dissolved in the 1% sodium potassium tartarate solution

III. Solution 3: 1 ml of solution 2 was dissolved in 50 ml of solution 1

IV. Solution 4: 1 N Folin Ciocalteau , 1:1 dilution of 1 N Folin Ciocalteau with distilled water was made

V. Solution 5: 0.50 g of bovine serum albumin (BSA) was weighed and dissolved in 100 ml distilled water

Measurement of Protein Content

I. Different concentrations of protein standard solution Bovine serum albumin (BSA) were prepared, by preparing a ratio of protein :water in the order :10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9,10:1 in eleven different test tubes

II. To each test tube, 5 ml of solution 3 was added (alkaline copper reagent) thoroughly mixed and left to stand for 5 min.

III. A known, 0.5 ml of solution 4 (diluted Folin Ciocalteau solution) was added to the test tubes rapidly and thoroughly mixed.

IV. Solution was left for 30 min to incubate and the absorbance read at 750 nm.

V. The final concentration of the protein was calculated using the equation $C_1V_1=C_2V_2$.

3.2.10. Purification of the Crude Enzyme Extracts from the Di-culture of *Rhizopus* and *Saccharomyces* spp.

The crude extracts was purified through: Ammonium sulphate precipitation, dialysis and gel filtration following the method of Ertan *et al*, (2012).

Ammonium Sulphate Precipitation

Test tubes numbering eight were used to form an ammonium sulphate precipitation profile. Peroxidase were precipitated with gentle stirring at 20-90% saturation of solid ammonium sulphate at intervals of 10% in each test tube. The ammonium sulphate crude enzyme solutions were allowed to stand at cold temperature of 4^{o}C for 30 h till the supernatant could be gently decanted off. The test tubes were centrifuged at 3500 rpm for 20 min. Precipitates from the individual percentage ammonium sulphate saturations were re-dissolved respectively in equal volumes of 0.1 M sodium phosphate buffer pH 6.5. Peroxidase activities of the precipitates were assayed to determine the percentage ammonium sulphate saturation that precipitated enzyme with maximum activity.

To the remaining volume of the enzyme extract, 75.52 g of the solid reagent that is ammonium sulphate $(NH_4)_2SO_4$ was added slowly to the enzyme extract in a beaker until it became 70% in saturation. This was stirred slowly for 1 h and then the solution was kept undisturbed at 4^{o}C for 48 h. Then the mixture was centrifuged at 4000 rpm for 30 min. The precipitated protein collected was re-dissolved in 0.1 M sodium phosphate buffer pH 6.5. The enzyme activity and protein

content were measured and determined. The remaining solution was kept and stored in the refrigerator.

Dialysis

The supernatant was discarded and precipitated extract was dialyzed by pouring the remaining precipitate in a dialysis bag suspended in a beaker containing ice pack, 0.01 M sodium phosphate buffer pH 6.5. The buffer in the beaker was continuously stirred with a magnetic stirrer and kept for 48 h. The buffer was changed every 12 h, the total volume of the dialyzed protein was measured and recorded. The enzyme activity and protein contents of the dialyzed extract was determined at 460 nm using *o*-dianisidine, as substrate and 750 nm for protein determination using (BSA) respectively. The remaining was stored in the freezer.

Gel Filtration

Sephadex G-75 was used for the gel purification of the enzyme. The column for the purification was prepared by first soaking 10 g of the gel in 0.1 M sodium phosphate buffer solution at pH of 6.5 overnight (12 h) for swelling up of the gel. The column tube (50 cm by 2.5 cm) for the gel filtration was gently mounted on the stand and swelled gel was gently and carefully poured in the column tube with gentle rocking, making sure that at each phase of pouring, the gel was well packed within the column tube before pouring another gel. After pouring the gel in the column it is was allowed to settle so as to pack homogenously until the bed height was reached. The packed gel was equilibrated with 0.1 M sodium phosphate buffer solution (pH 6.5) and 30 ml of the enzyme solution was introduced into the column. A total of 70 fractions were collected using 5 ml fraction tubes at a flow rate of 5 ml per approximate 20 min. The protein concentration of each fraction was determined using a spectrophotometer at wavelength of 280 nm. Peroxidase activity

of each fraction was also assayed using the spectrophotometer at 460 nm. The active fractions were pooled and stored at -4°C.

3.2.11. Characterization of the Purified Enzyme

This was done according to the method of Eze *et al*, (2010).

Determination of Optimum pH

The activity of peroxidase was examined within the pH range of 4.0 - 9.0 on a scale of 0.5 units using the following buffer salts: Sodium-acetate buffer (0.1 M, pH 4 – 5.5); sodium phosphate buffer (0.1 M, pH 6.0 – 7.5); Tris – HCl buffer (0.1 M, pH 8 – 9.0). The residual activity was then determined using o-dianisidine as the reducing substrate for the assay method.

Determination of Optimum Temperature

The optimum temperature was determined by incubating the enzyme at its optimal pH at various temperatures ranging from 30 to 70^{o}C in a water bath. Thereafter, the residual activity of the partially purified peroxidase was determined after incubating the enzyme at the various temperature ranges for 4 h in phosphate buffer at 6.0. After heat treatment, the enzyme solution was cooled and the residual activity assayed under standard assay conditions.

Determination of kinetic Parameters

The Michaelis-Menten kinetic parameters K_m and V_{max} was determined by measuring the peroxidase activity using various concentrations of O-dianisidine complex as substrate in the presence of hydrogen peroxide. The parameter values was obtained by curve fitting of the reciprocal plot of reaction rate versus substrate concentrations using the Line-weaver bulk plot transformation of Michealis-Menten rate equation.

3.2.12. Stabilization Studies of the Purified Enzyme

The purified extract was incubated with various concentrations of divalent metal ions (Ca^{2+}, Mg^{2+}, Co^{2+} and Pb^{2+}) varied up to 0.05 M in the assay mixtures singly at varying time interval, aliquot was drawn from the incubated sample and assayed for residual activity according to the method of Singh *et al*, (2012).

$$\% \text{ Residual activity} = \frac{At}{Ao} \times 100 \qquad 3.15$$

Where At is activity at present, Ao activity at time 0. The % residual activity was plotted against different times of incubation.

3.2.13. Biodegradation of Hydrocarbon by the Purified Enzyme

The hydrocarbon degradation was performed on the produced peroxidase from the di-culture of the fungal isolates after the emulsification screening assays using species with high emulsifying potentials based on their % E24 test. This was carried out using the gravimetric method as described by Ferrera cerrato *et al.* (2007) in Mbachu *et al.* (2016a).

Twenty g of the uncontaminated soil sample was dissolved in deionized water and intentionally polluted with 10% v/v Bonny light crude oil in a 250 ml conical flask and allowed to acclimatize for seven (7) days. Extraction was done using an organic solvent. A 200 ml reaction mixture containing crude oil, peroxidase, hydrogen peroxide and buffer solution was constituted and added into the extracted oil. Examination of hydrocarbon degradation was done in the sterile nutrient medium optimized for the degradation study which was supplemented in 5-20% v/v of petroleum hydrocarbon. Fifty ml of the medium was dispensed in 250 ml conical flasks. Given concentrations of the peroxidase (5-20% v/v) was mixed equally into the medium and incubated for 16 days at spinning rate of 180 rpm. The activity of the peroxidase was monitored and weight of hydrocarbon

degraded was estimated spectrophotometrically (OD) at 660 nm and gravimetrically (equivalent weight loss) at each of the varied parameter. This was carried out for a period of 20 days. Also, uncontaminated soil serving as the control contained the same without the addition of peroxidase.

Afterwards extraction was done using n-Hexane to determine the hydrocarbon remaining after the incubation. Twenty g of contaminated soil sample was dissolved in 40 ml of N-Hexane solvent in conical flasks. They were corked and shaken in a rotatory shaker at a speed of 3500 rpm. The contents were transferred to a separating funnel and extracted. Organic solution of the hydrocarbons with the hexane compounds formed an aqueous supernatant while the soil debris remained beneath the lower part. N-hexane was evaporated using a rotatory shaker while leaving the system uncorked under reduced pressure. Then the crude oil was left behind. The extraction was carried out twice to ensure complete recovery of oil. This was evaporated to dryness in a rotary evaporator under reduced pressure.

Gravimetric Analysis

Gravimetric method of determining percentage and rate of degradation of oil:

W”-W1= W2 3.16

W2 is the weight of residual crude oil after evaporation.

W1 is the weight of empty beaker

W” weight of the conical flask and the oil

Percentage Degradation

The percentage of oil degraded = weight of oil degraded/original weight of oil used x 100

Weight of oil degraded = original weight of oil used – Weight of residual oil obtained after evaporating the extract.

Rate of Degradation = weight of oil degraded/Time taken

Additionally, during the hydrocarbon biodegradation studies, the total petroleum Hydrocarbon (TPH) was also monitored and physicochemical parameters (pH, incubation days and crude oil concentrations) varied in the process. Two treatments were used which includes; test sample containing the contaminated soil treated with peroxidase and control containing contaminated soil but without any peroxidase. These treatments were tested in 5-20% v/v crude oil concentrations and their effects on TPH values noted.

3.2.14. Optimization of Physicochemical Parameters

The following physicochemical conditions were varied during the study for optimal determination of the peroxidase degrading ability:

pH, incubation days, peroxidase concentrations (%v/v) and crude oil concentrations (%v/v)

pH

Different pH ranging from 4.5- 8.0 were prepared using buffer salts of 1 M concentration. The mineral salt medium optimized for the enzyme degradation include: 5% crude oil, 0.5% phosphate salt, 5% enzyme suspension, deionize water 200 ml. These were incubated at 37^{O}C for 16 days.

Incubation Days

Different incubation days were optimized during the study ranging from 0-16 days. The mineral salt medium during the study include: 5% crude oil, 0.5% phosphate salt, 5% enzyme suspension, deionized water 200 ml. The cultivated media were incubated at pH 5.5 using acetate buffer.

Enzyme suspension concentrations (%v/v)

Peroxidase concentrations ranging from 0-20% (v/v) were varied during the degradation studies. The mineral salt medium during the study include: 5% crude oil, 0.5 % phosphate salt, deionized water 200 ml. The cultivated media was incubated at pH 5.5 using acetate buffer.

Crude oil concentrations (%v/v)

Crude oil concentrations ranging from 0 to 20% (v/v) were varied during the degradation studies. The mineral salt medium during the study include: 10% enzyme suspension, 0.5% phosphate salt, deionized water 200 ml. The cultivated media was incubated at pH 5.5 using acetate buffer.

3.2.15. Statistical Analysis

The results were presented in Figures and Tables. Graphs were plotted using Microsoft Excel (2010). Results were expressed as mean ± SD and Statistical Package for Social Sciences (SPSS, Model 3.1) was used to test for the statistical significance by employing the one way analysis of variance (ANOVA).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1. RESULTS

4.1.1 Soil Chemical Analysis

The results of the physiochemical properties of the crude oil contaminated and uncontaminated soil samples obtained from the Onne jetty site are shown in Table 4.1. The contaminated soil color was dark brown and the texture was rough and contained gravels and debris. The uncontaminated soil pH was at neutral pH range (7.2) however, the pH was acidic (5.02) in the contaminated soil. Temperature, conductivity and soil acidity were observed to be high in the contaminated soil compared to the control which had a higher moisture content. Dissolved mineral contents such as K, Cl, Mg, Ca ions were found in the uncontaminated soil sample but relatively less compared to that of the contaminated soil sample in concentration. Mineral contents of the contaminated soil showed strength in concentrations variably in the following order: Cl> Ca> K> Mg> PO_3 in both the contaminated and uncontaminated soil samples. Heavy metal identified in the uncontaminated soil sample include; Fe, Cu and Pb ions with Pb lowest in concentration. Heavy metal analysis also showed the following order of concentrations in the soil Fe> Cu> Pb> as in both the contaminated and the control experiments. Cd, and Hg were below detectable limit (BDL) in the contaminated and uncontaminated soil. Corresponding results from the uncontaminated soil showed a significant differing trend from the contaminated soil as the Table depicts. TPH, TOC and TOM were very high in the contaminated soil compared to the uncontaminated control experiment.

Table 4.1. Chemical Constituents of the Soil Samples from Onne Jetty Site.

Chemical Parameters	Contaminated	Uncontaminated
pH	5.02	7.2
Temperature (^{o}C)	33.0	24.5
Soil Conductivity (μS/cm)	723	398
Soil Moisture Content	12.43±0.1	15.40±0.12
Soil Acidity (SO_4)	11.2±0.01	1.06±0.1
Chloride ion (mg/g)	1151.614±0.36	393±0.18
Calcium (mg/g)	14.23±0.1	22.34±0.17
Potassium (mg/g)	12.52±0.45	7.42±0.22
Magnesium (Mg/g)	11.27±0.21	4.27±0.09
Phosphate (mg/g)	1.53±0.02	1.86±0.02
Iron (mg/g)	38.74±0.14	6.55±0.02
Cadmium (mg/g)	BDL	BDL
Mercury (mg/g)	BDL	BDL
Arsenic (mg/g)	0.45±0.04	BDL
Copper (mg/g)	12.59±0.13	4.28±0.03
Lead (mg/g)	9.69±0.04	0.23 ± 0.01
Total petroleum hydrocarbon (TPH) (mg/g)	1672 ±0.13	342.44 ±0.01
Total Organic Carbon (TOC) (mg/g)	10.64±0.1	2.85±0.02
Total Organic Matter (TOM) (mg/g)	7.23±0.1	2.78±0.01

BDL: Below Detectable Limit

4.1.2. Microbial Isolation and Identification of *Rhizopus and Saccharomyces* spp.

The petri-dishes presented in plate 4.1a and 4.1b shows the isolated organisms from the crude oil l. Each of the plates showed the growth pattern of the organisms on Sabouraud‘s Dextrose Agar (SDA).

Plate 4.2 and 4.3 shows isolates of *Rhizopus* and *Saccharomyces* spp. on culture plates

Plate 4.4 shows the microscopic view of *Rhizopus* sp. (left) and *Saccharomyces* sp. (right)

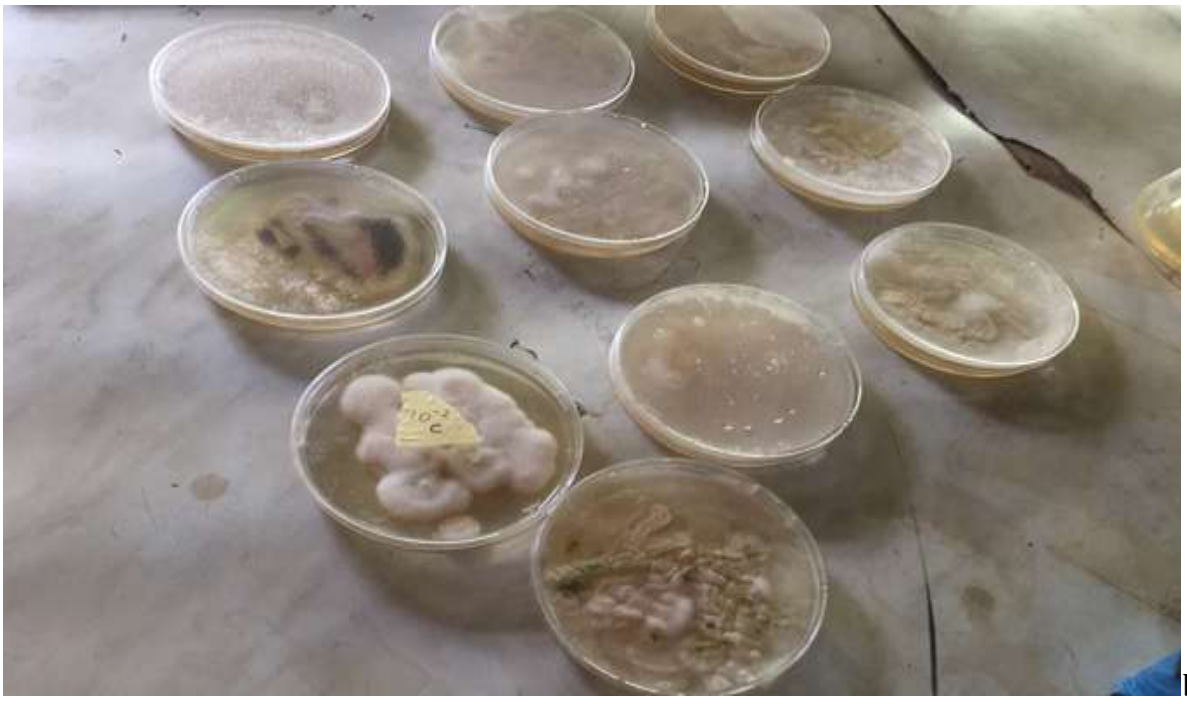
The results of culture based identification of the microorganisms present in the samples using Saboraund ‘s Dextrose Agar (SDA) are shown in Table 4.2. The Table enlists the basic microbial morphological features used for the general microbial identifications.

The morphological features of isolates from crude oil contaminated from Onne Jetty site, Eleme are presented in Table 4.2. The result obtained showed some organisms isolated from the crude oil contaminated site to be whitish, irregular in shape, having a fluffy filamentous mycelium, with a well-developed collumella like an umbrella shape, some were non-filamentous. However, those isolated from the uncontaminated soil were more colorful, with smooth conidia.

4.1.3: Biochemical Test of the Identified Fungal Isolates from the Contaminated Soil of Onne Oil Jetty Site.

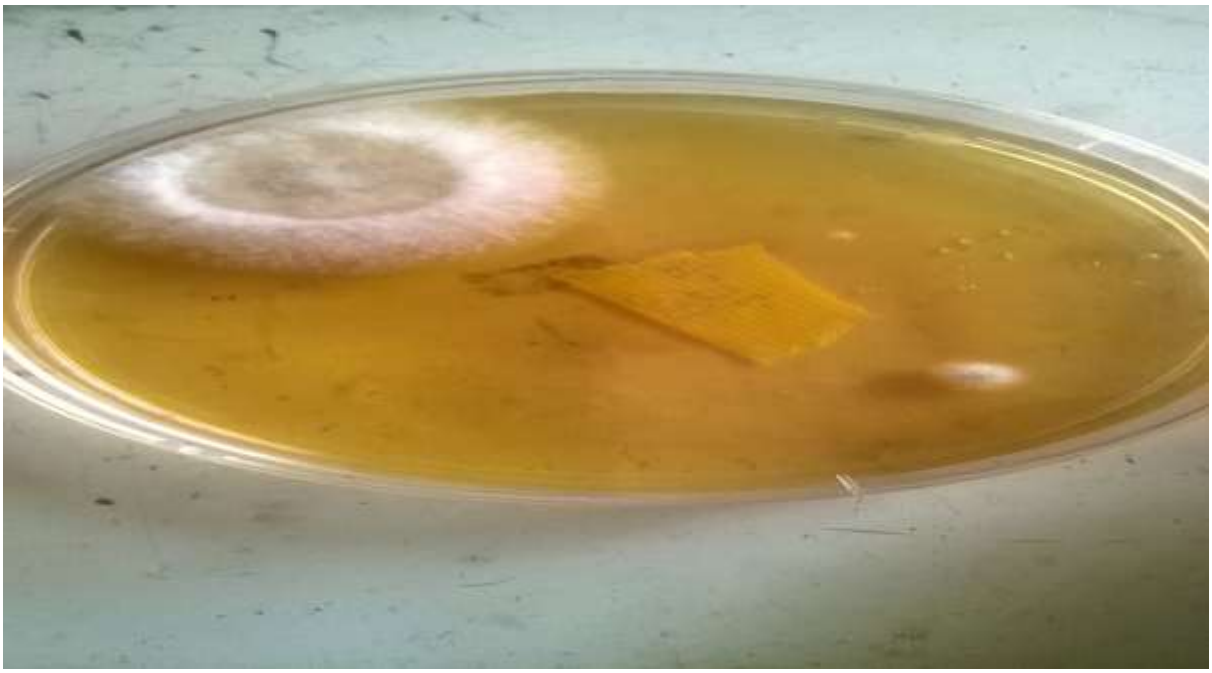
The results of the biochemical features of the organisms are shown in Table 4.3. The biochemical tests employed include sugar test (glucose, maltose, lactose and fructose), indole test, oxidase, starch hydrolysis and gelatin liquefaction. The test were considered based on the target fungal species (*Rhizopus* and *Saccharomyces* spp.)

a)



b)

Plate 4.1a and 4.1b: Organisms isolated from crude oil polluted soil of Onne oil Jetty site Eleme.



Plates 4.2: Isolated *Rhizopus* sp. on a culture plate

Plate 4.3: Isolated *Saccharomyces* sp. on a culture plate.

Plate 4.4: **The microscopic view of *Rhizopus* sp. (left) and *Saccharomyces* sp. (right) isolated from crude oil contaminated soil of Onne oil jetty site, Eleme.**

Table 4.2: Morphological Features of the Isolated Organisms from the Soil Samples

Sample	Color	Size	Elevation	Shape	TCFU/g	Texture	Morphological Characteristics	Suspected fungi
SDA Contaminated	Whitish	Above 7	Raised	Irregular	2.8×10^7	Fluffy Filamentous Mass Mycelium	Well developed collumela like an umbrella shape	*Mucor* sp.
SDA Contaminated	Creamy / Whitish	1.2	Raised	Spherical Regular	1.70×10^5	Smooth	Non Filamentous Drop-like Separated Colonies	*Saccharomyces sp*
SDA Uncontaminated	Creamy Greyish	1.4	Raised	Regular	3.0×10^8	Smooth		*Aspergillus* sp.
SDA Uncontaminated	Colorful	1.2	Raised	Irregular	2.8×10^7	Flat Filamentous Dark-brown Mycelium	Smooth conidia Conidiosphore, glubose vesicle	*Penicillium sp*

Table 4.3. Biochemical Characterization of the Fungal Isolates from the Contaminated Soil of Onne Oil Jetty Site.

Biochemical Test	Observation	Suspected fungus
Glucose	Acid/Gas Production	*Rhizopus/Saccharomyces* sp.
Maltose	"	*Rhizopus/Saccharomyces* sp.
Lactose	"	*Saccharomyces* sp.
Fructose	"	*Saccharomyces* sp.
Indole (Nitrogen Digestion) Test	-	*Saccharomyces* sp.
Oxidase Test	+	*Saccharomyces* sp.
Starch Hydrolysis	-	*Saccharomyces* sp.
Gelatin Liquefaction	-	*Saccharomyces* sp.

4.1.4. Total Fungal Population in the Contaminated and Uncontaminated Soil

The populations of the identified fungi on the culture plates using nutrient media and differential media (Bushnell Haas media) expressed in CFU/g in both the contaminated and uncontaminated soil samples shows appreciable number of fungi more in the crude oil contaminated soil than the uncontaminated soil sample.

The total heterotrophic count and the hydrocarbon degrading organisms obtained from the crude oil contaminated and uncontaminated soils of Onne oil Jetty site, Eleme are presented in Figure 4.1. Results obtained showed that total hydrocarbon content and hydrocarbon degraders were higher in contaminated soil compared to the uncontaminated soil. Major hydrocarbon degrading organisms were present in the crude oil contaminated soil with population counts of 6.9×10^8 and 1.15×10^7 for total heterotrophic count and hydrocarbon degrading fungi respectively. This showed higher population compared to the uncontaminated soil with population counts of 2.58×10^8 and 5.5×10^2 for total heterotrophic count and hydrocarbon degrading fungi respectively.

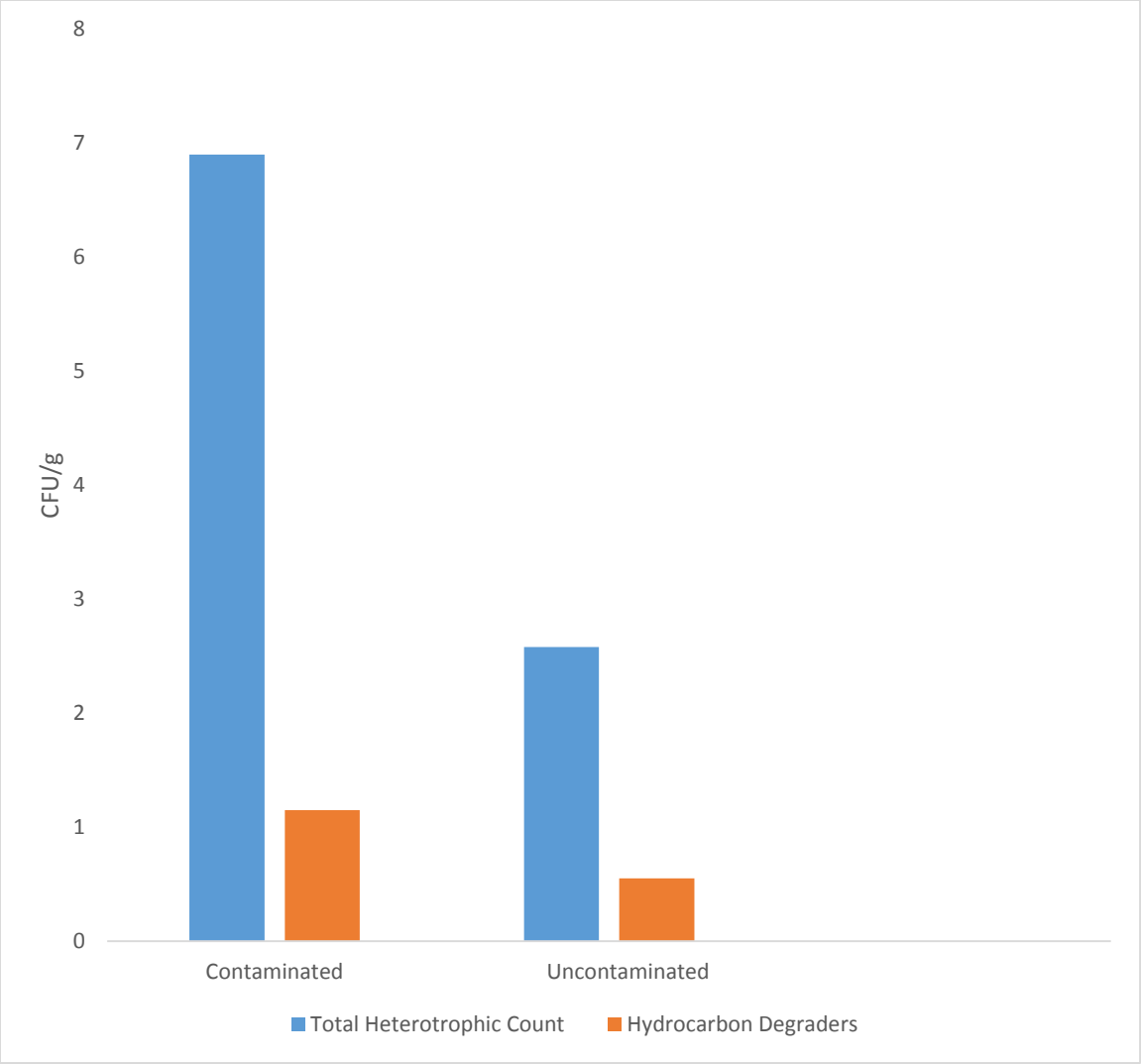


Figure 4.1: Soil Microbial Counts (CFU/g) Total Heterotrophic and Hydrocarbon Degrading Organisms from the Contaminated and Uncontaminated Soil.

4.1.5. Emulsification Screening Assay Result

The Tables (4.4 and 4.5) below show the various emulsification potentials of isolated organisms from the crude oil contaminated site.

The results obtained from Table 4.4 showed that *Rhizopus* sp. had a vigorous drop collapse compared to other organisms exposed to emulsification screening. However, *Rhizopus* and *Saccharomyces* spp. showed a clear zone diameter >3<6 (mm), compared to *Aspergillus* and *Penicillium* spp. which showed a clear zone diameter of >1<3 (mm). No clear zone diameter was seen in *Penicillium* sp. exposed to drop collapse test.

The result obtained in the emulsification screening of organisms using diesel, kerosene and crude oil as shown on Table 4.5, showed that *Rhizopus* sp. had the highest percentage value (56%) when exposed to crude oil compared to diesel and kerosene. *Saccharomyces* sp. had the highest percentage value (54%) when exposed to kerosene compared to other chemical substances used. However, *Aspergillus* sp. showed the least percentage value (10%) when emulsified with crude oil compared to the exposure to other compounds.

Table 4.4: Emulsification Screening Assay Result (Drop Collapse and Oil spread Plate)

ORGANISMS	DROP COLLAPSE	OIL SPREAD PLATE (mm)
Rhizopus sp.	++	++
Saccharomyces sp.	+	++
Aspergillus sp.	+	+
Penicillium sp.	-	+

KEY: + Drop-collapse: - No collapse, + slow drop collapse, ++ vigorous drop collapse
Oil spreading technique: - , no clear zone diameter, +, clear zone diameter >1<3 (mm), ++, clear zone diameter >3<6 (mm), +++, clear zone diameter >6 and < 9 (mm).

Table 4.5. The Emulsification Screening Assay (% E24) of Organisms Exposed to Diesel, Kerosene and Crude oil (%).

Organisms	Diesel + microbial suspensions (%)	Kerosene + microbial suspensions (%)	crude oil + microbial suspensions (%)
Rhizopus sp.	50	51.2	56
Saccharomyces sp.	46	54	42
Aspergillus sp.	39	36	10
Penicillium sp.	28	39	32

4. 1.6. Molecular Based Characterization of Strains of *Rhizopus and Saccharomyces* spp.

The agarose gel electrophoresis of the total DNA extracted from the organisms are shown in Plates 4.5 and 4.6.

The result of the gel electrophoresis picture of PCR amplified DNA sequences of *Rhizopus* sp. obtained from the crude oil contaminated soil extracted at the 18S ITS segment with the longest amplified base pair of 500-1000 bp. is presented on Plate 4.5.

The DNA fragment of IS1 and H1 were observed to be 500 base pair. Only one band which had very high intensity of light was observed in the gel picture.

The electrophoretogram of PCR amplified genomic DNA sequence of *Saccharomyces* sp. obtained from the crude oil contaminated soil extracted at the 18S ITS segment with the longest amplified base pair of 500-10200 bp. are shown on Plate 4.6.

The DNA fragments obtained from well 1-9 showed the DNA fragments to be equal and between 500-600 base pairs. The gel picture showed on DNA bands in the different wells (1-9). However, the DNA fragments showed high intensity of light in all the fragments except the DNA fragments of well 3 and 8. Also double bands or two DNA fragments were observed in well 5 compared to other wells. Again well 12-18 showed DNA fragments which were of the same base pairs (500) with high intensity of light. .No bands were observed in well 13, 15, 16 and 18.

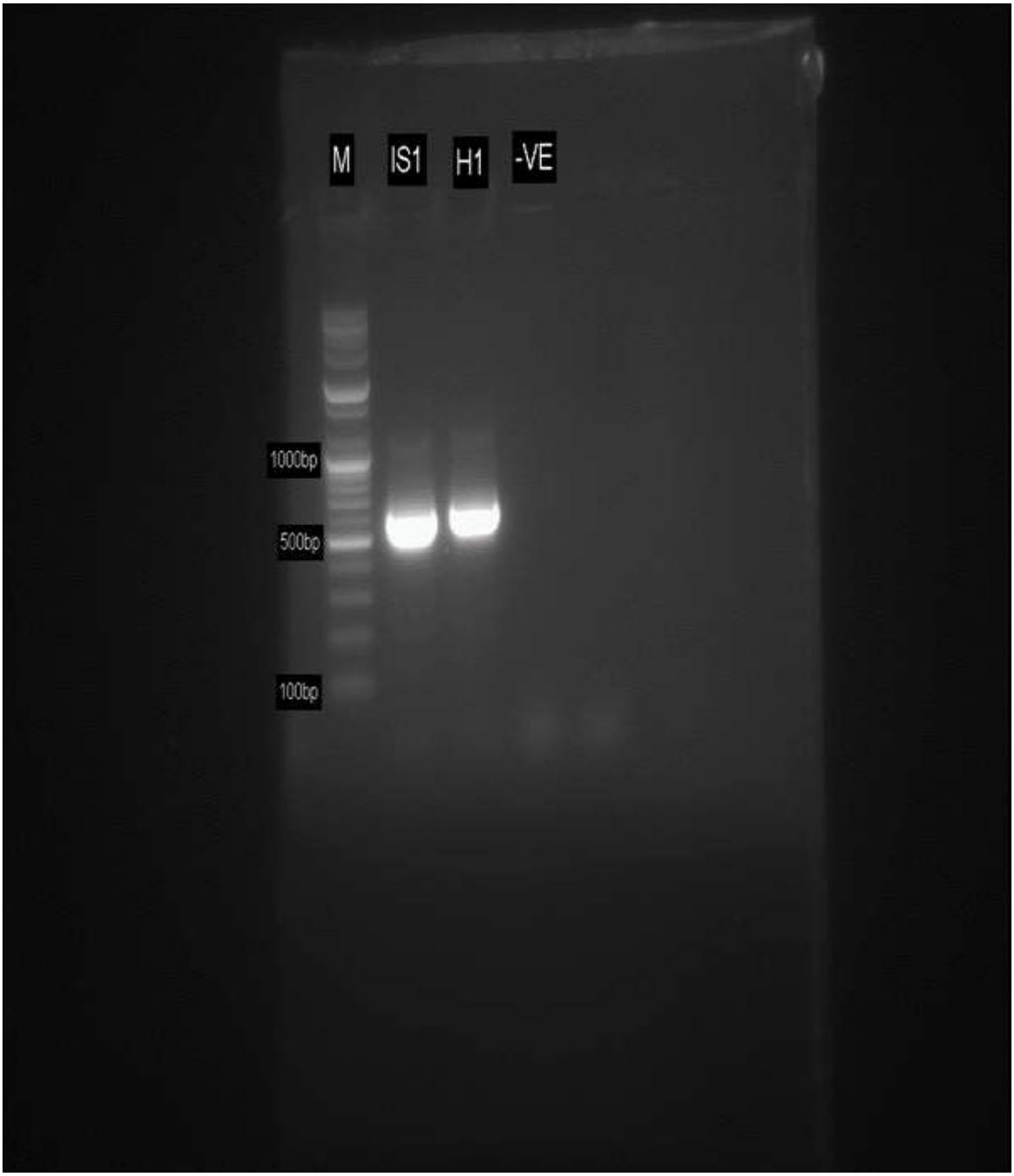


Plate 4.5: **Electrophoretogram of Genomic DNA of *Rhizopus* sp. from crude oil contaminated soil of Onne oil jetty site, Eleme viewed on a UV trans-illuminator.**

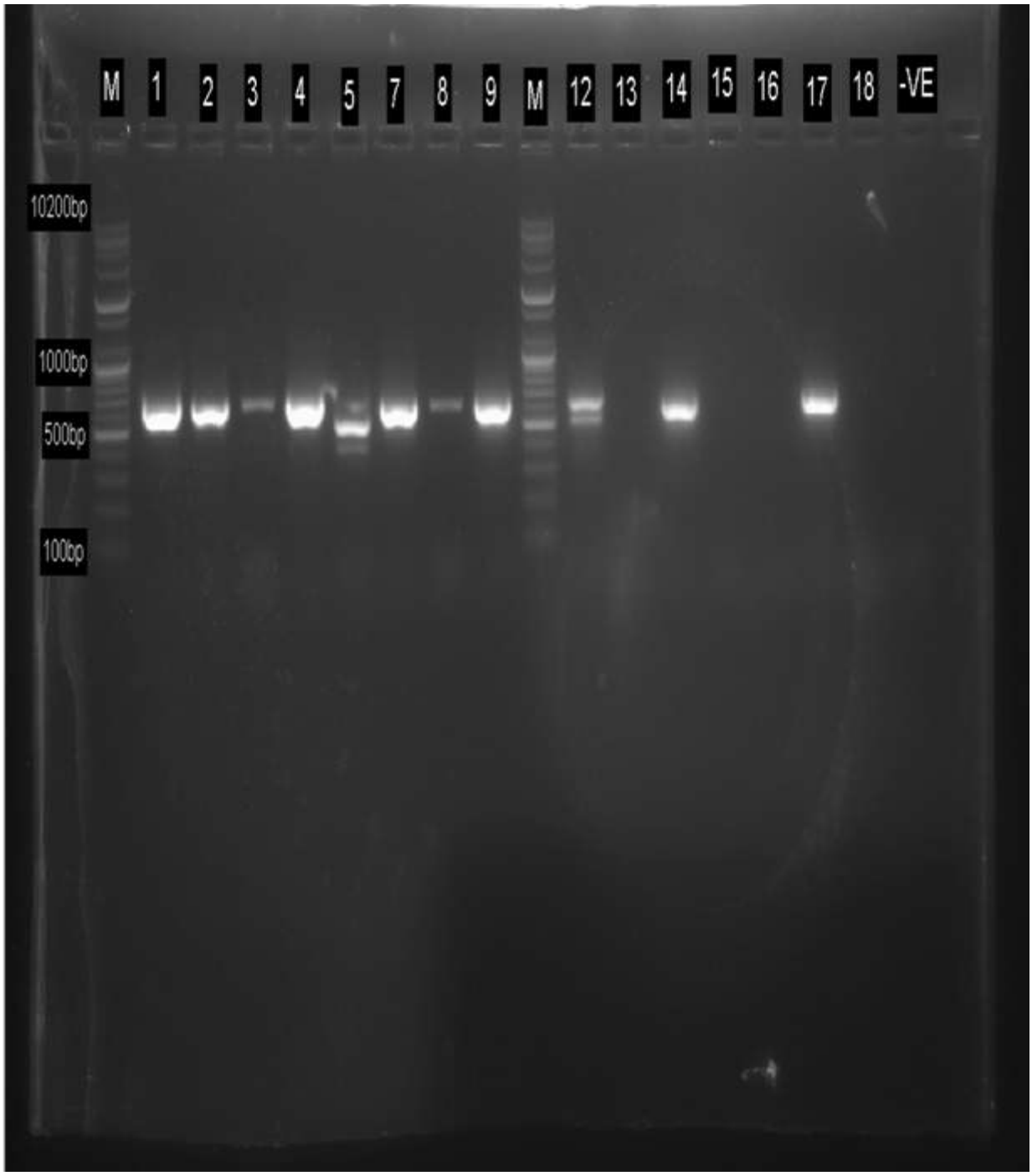


Plate 4.6: **Electrophoretogram of genomic DNA of *Saccharomyces* sp. obtained from crude oil contaminated soil of Onne oil jetty site, Eleme viewed on a UV trans-illuminator.**

4.1.7. Enzyme Production Activity

The effect of incubation days on the enzyme activity of peroxidase produced from the di-culture of *Rhizopus* and *Saccharomyces* spp. isolated from the crude oil polluted soil is presented in Figure 4.2.

The result obtained showed that day eight (8) was the peak in the enzyme activity in the test sample, as the enzyme production maintains a steady rise from day 0 of the incubation before reaching a regressive production pattern at days 5 and day 6 in the test sample and days 3, 4 and 5 in the control.

The peak of the enzyme activity produced from the di-culture of *Rhizopus* and *Saccharomyces* spp. is contrasting with the peak of the enzyme activity obtained from the control. The peak of the enzyme activity in the control was observed from the 6^{th} day incubation. However, decrease in enzyme activity was observed from the 10^{th} day of incubation which came to a stop at the 14^{th} day of incubation.

The effect of the incubation days on the protein concentration of the peroxidase produced from the di-culture of *Rhizopus* and *Saccharomyces* spp. is presented in Figure 4.3.

The results obtained showed the protein concentration in the test sample to be in a lag phase from the 1^{st} day to the 6^{th} day of incubation. However, the peak of the enzyme activity was observed on the 7^{th} and 6^{th} day of incubation in the test sample and control respectively. This contrasts with the peak of protein concentration recorded in the control. Both enzyme activity (Fig. 4.2) and protein concentration (Fig. 4.3) falls steadily after the 10^{th} day of incubation..

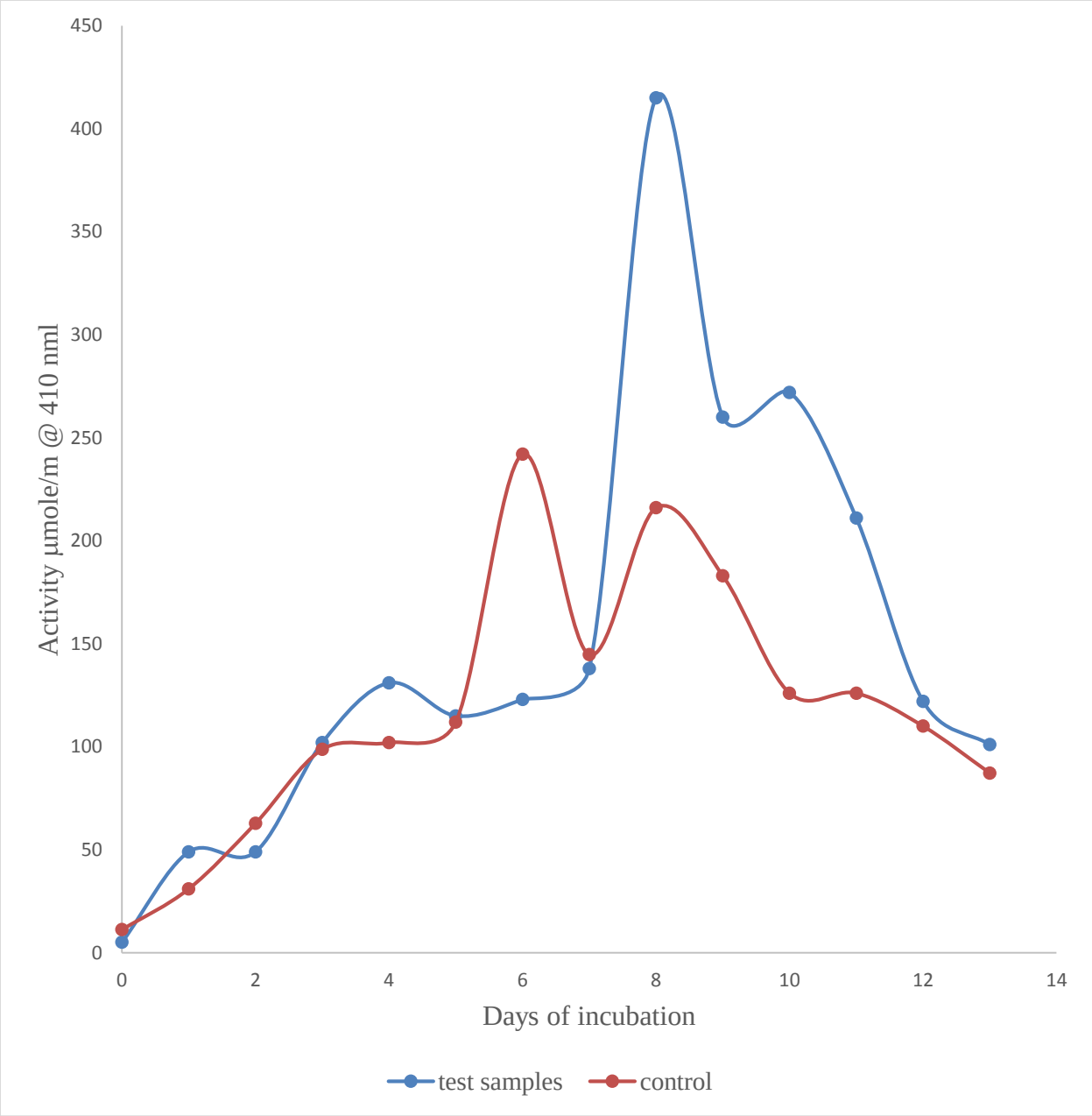


Figure 4.2.: Effects of incubation days on the enzyme activity of the peroxidase produced from the di-culture of *Rhizopus* and *Saccharomyces* spp.

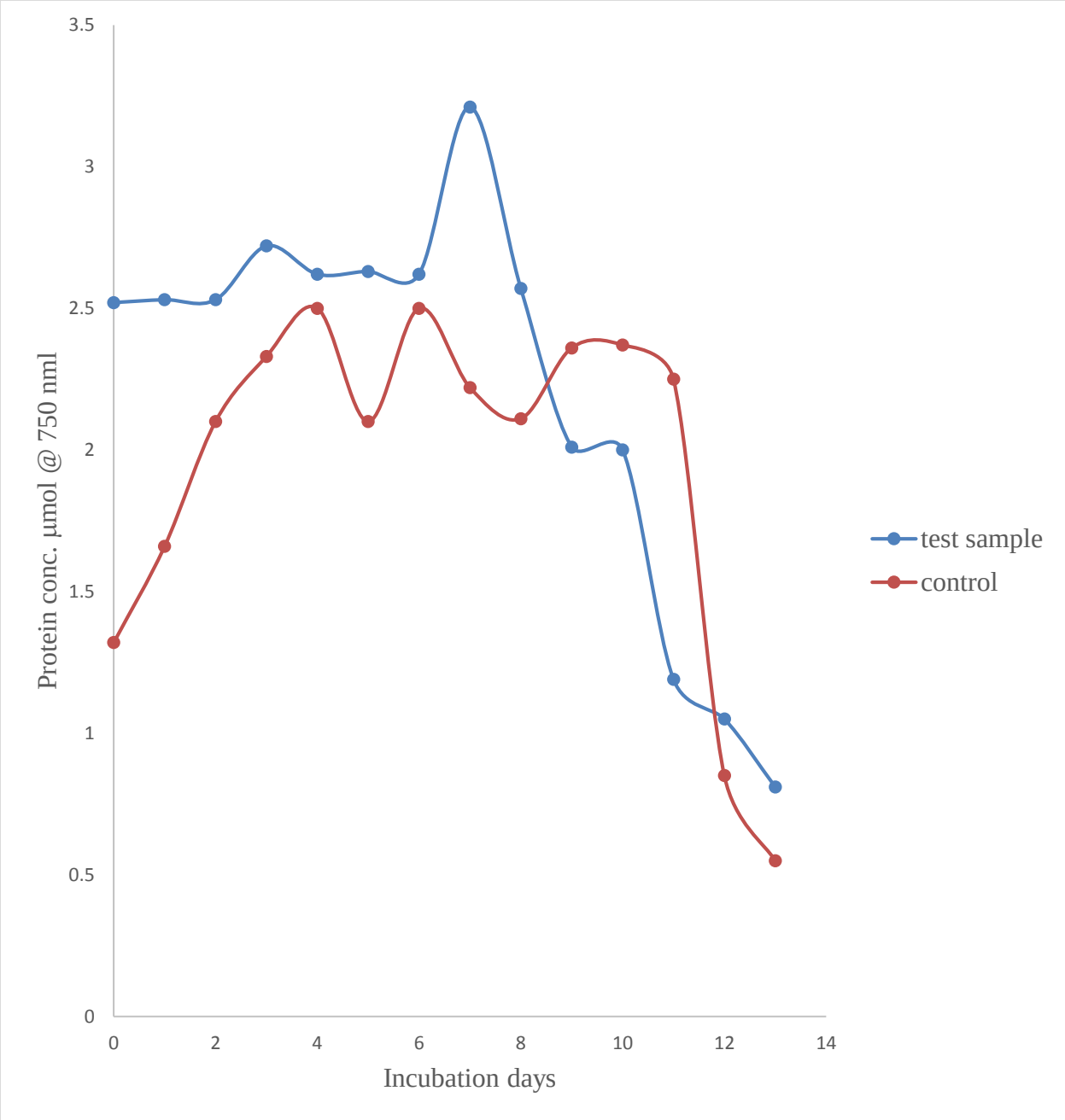


Figure 4.3: Effects of incubation days on the protein concentrations of the peroxidase produced from the di-culture of *Rhizopus* and *Saccharomyces* spp.

4.1.8. Purification of the Crude Enzyme.

The Ammonium sulphate precipitation profile of the peroxidase produced from the di-culture of *Saccharomyces* and *Rhizopus* spp. as presented in Figure 4.4.

It was observed from the result that 80% saturation of the salt precipitated most of the proteins from the aqueous solutions. The supernatant recorded the least concentration of the protein upon assaying at 50% saturation of the salt.

Purification via gel filtration was done using Sephadex G-75. The Gel elution profile of the protein extracts produced from the di-culture of *Rhizopus* and *Saccharomyces* spp. as shown in Figure 4.5.

The results observed from the elution profiles showed two peaks at fraction tubes number of 22-28 and 40-45 on both the enzyme activity and protein concentrations checked at 280 nm. Tubes 0-10 showed no presence of the protein from the elution column until fraction tubes from 11-20 and from 40-45.

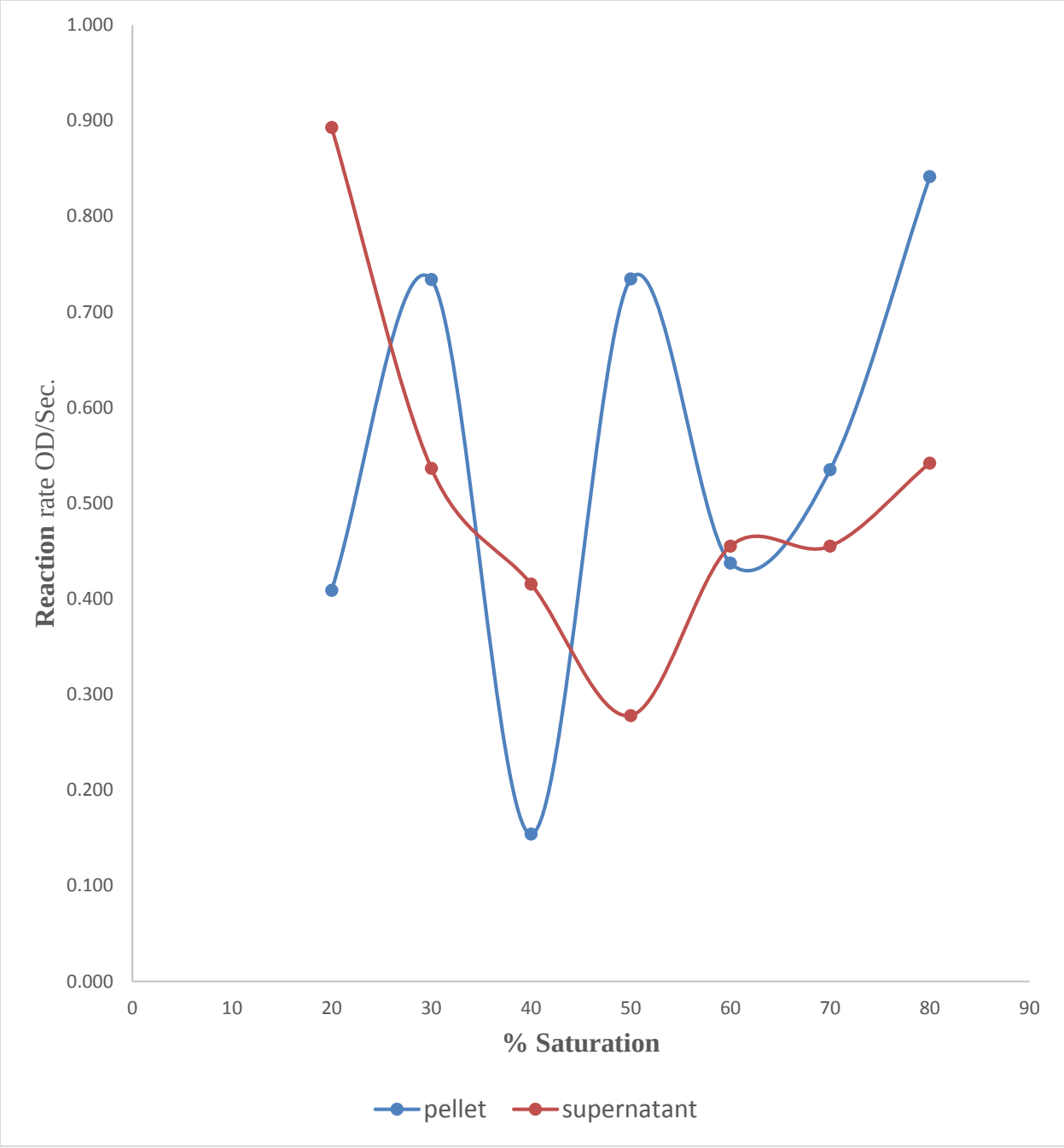


Figure 4.4.: Ammonium sulphate precipitation profile of the peroxidase produced from the di-culture of *Rhizopus* sp. and *Saccharomyces* sp.

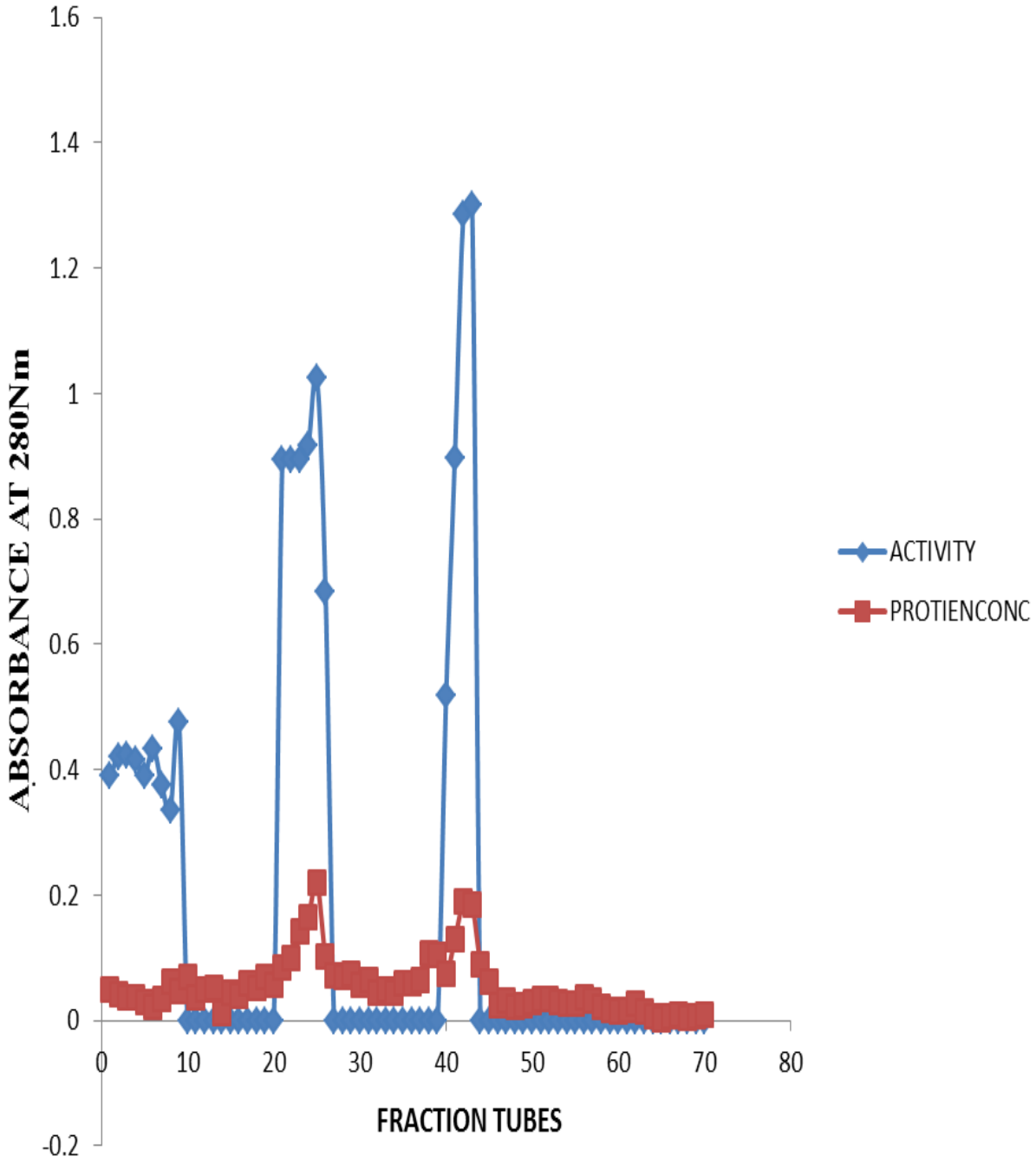


Figure 4.5: Gel elution profile of the protein extracts produced from the di-culture of *Rhizopus* sp. and *Saccharomyces* sp..

4.1.9: Characterization of the Purified Enzyme Extract via Optimum pH and Temperature Determination

The effect of pH profile activity of the peroxidase produced from the di-culture of ***Rhizopus* sp.** and *Saccharomyces* sp. is presented in Figure 4.6.

The result obtained from the pH activity profile depicts that the optimum pH of peroxidase upon profiling assay was found at optimum pH 4.5 and 6.0 for both the test sample and the control respectively. There was a steady decrease in enzyme activity after pH 6 in both the test sample and the control. These shows that peroxidase activity was pH dependent.

The effect of varying temperature ranges on the activity of the peroxidase produced from the di-culture of *Rhizopus* and *Saccharomyces* sp. is presented in Figure 4.7.

The result obtained showed the optimum temperature for the activity of peroxidase from the di-culture of fungi isolated from the contaminated and uncontaminated soil samples as 50^0C and 40^0C respectively. The activity of the enzyme began to decrease after the optimum range with a complete deactivation at 70^oC in both the test sample and control.

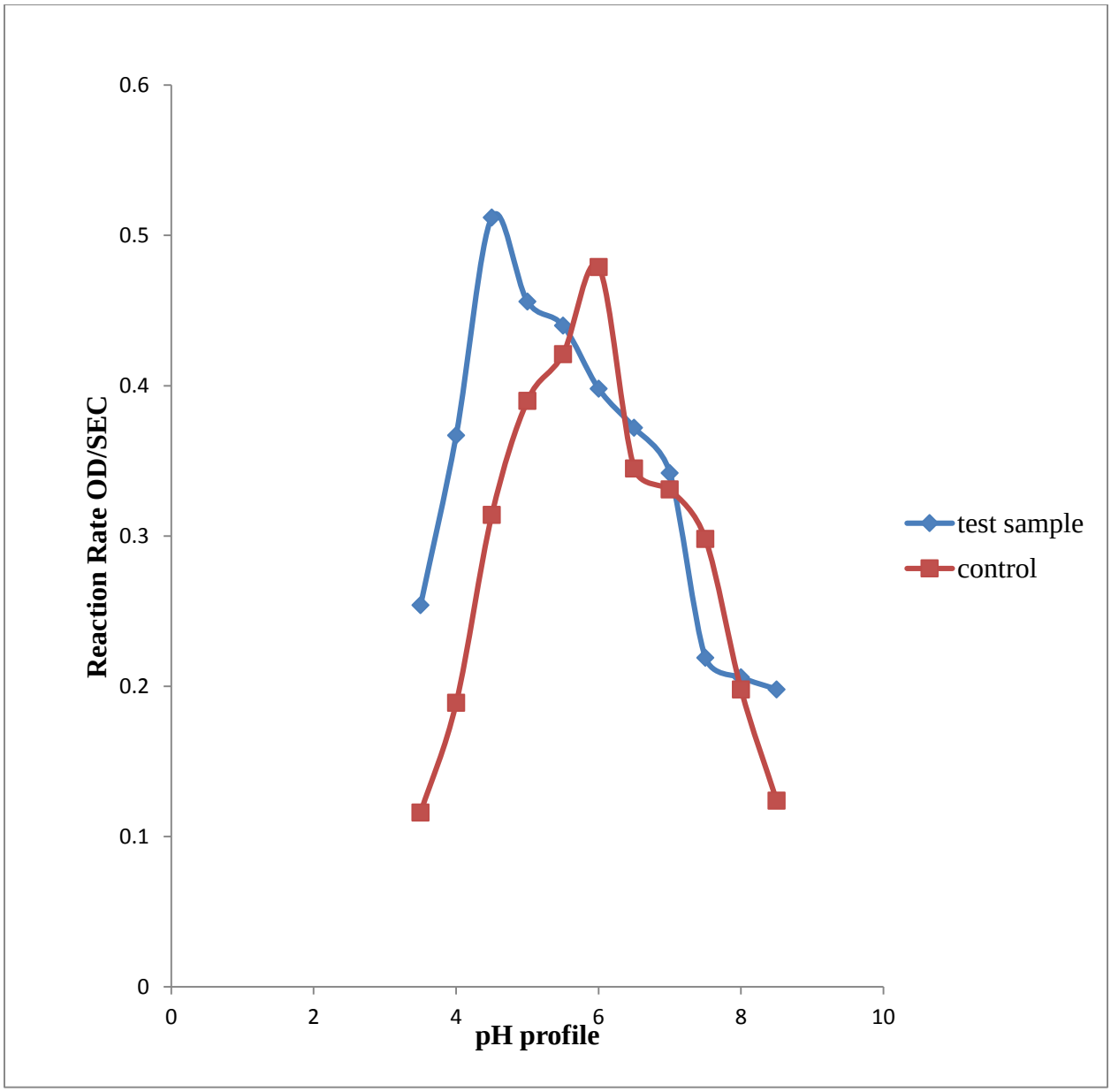


Figure 4.6. Effect of pH profile on the activity of the peroxidase produced from the di-culture of *Rhizopus* sp. and *Saccharomyces* sp.

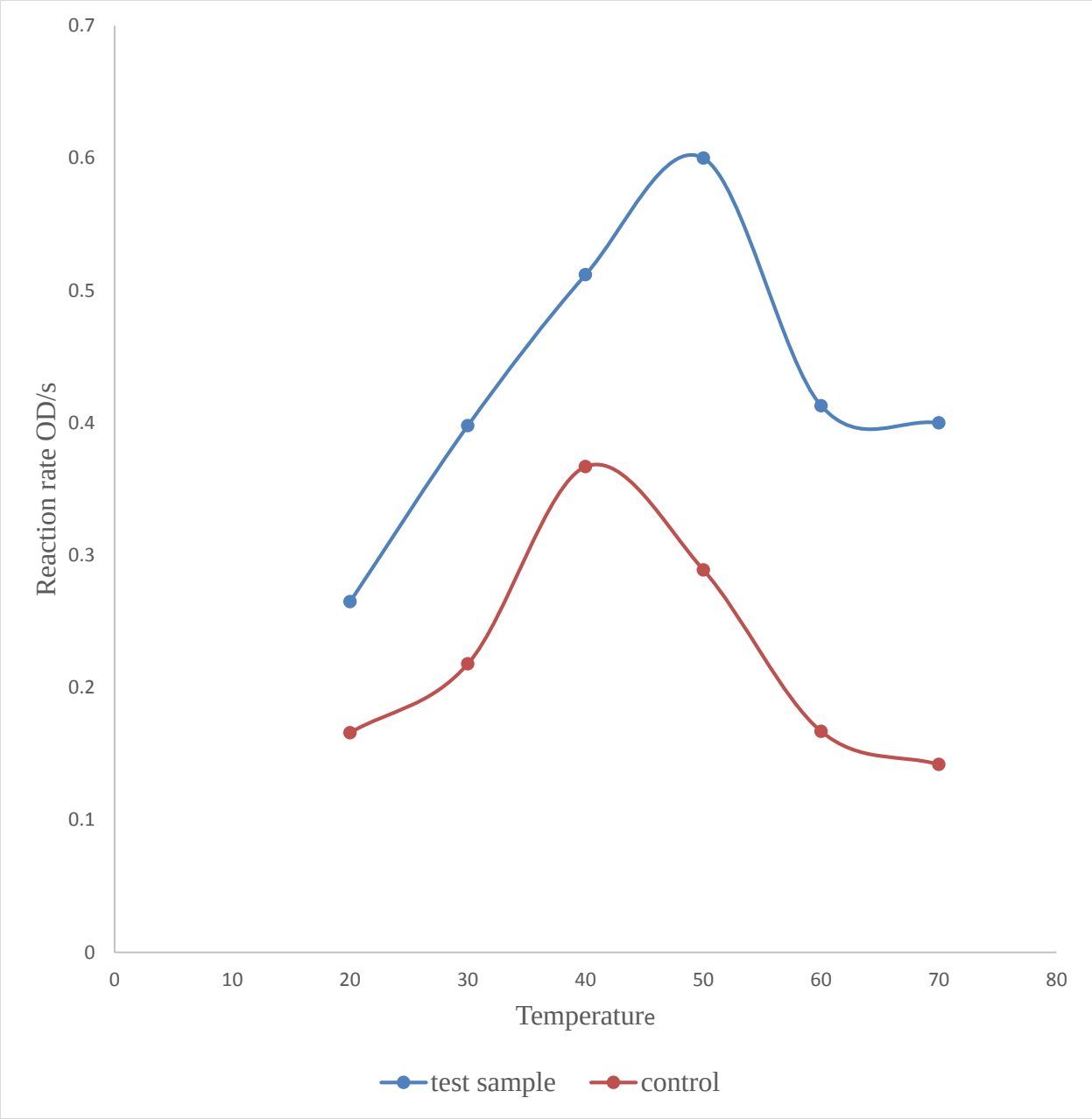


Figure 4.7. Effect of varying temperature ranges on the activity of the peroxidase produced from the di-culture of *Rhizopus* and *Saccharomyces* sp.

4.1.10. Effects of Substrate Concentration Using O-Dianisidine as Standard Substrate

Michaelis-Menten curve on the effect of o-dianisidine on the activity of the peroxidase produced from the di-culture of *Rhizopus* and *Saccharomyces* spp. is presented in Figure 4.8.

The results obtained showed the steady state kinetic studies of the enzyme using O-dinisidine as the substrate. The plot showed Km and Vmax obtained from the double reciprocal curve as 1.8 mM and 20 μmol/min respectively. The enzyme maintains steady rise in activity (U/ml) as the substrate concentration increases until the concentration reached 0.3 mM. At this point, further increase in substrate concentration does not lead to corresponding increase in the velocity of the reaction until it got to 0.5 mM.

The Lineweaver-Burke double reciprocal curve on the effect of o-dinisidine on the activity of the peroxidase produced from the di-culture of *Rhizopus* and *Saccharomyces* spp. is illustrated in Figure 4.9.

The Lineweaver-Burke double reciprocal curve which is the inverse of Michaelis-Menten curve as illustrated in the Figure 4.9 was deducted from the curve showing the kinetic constant. The result obtained showed that the reaction rate continued to increase with increase in concentration of the substrate

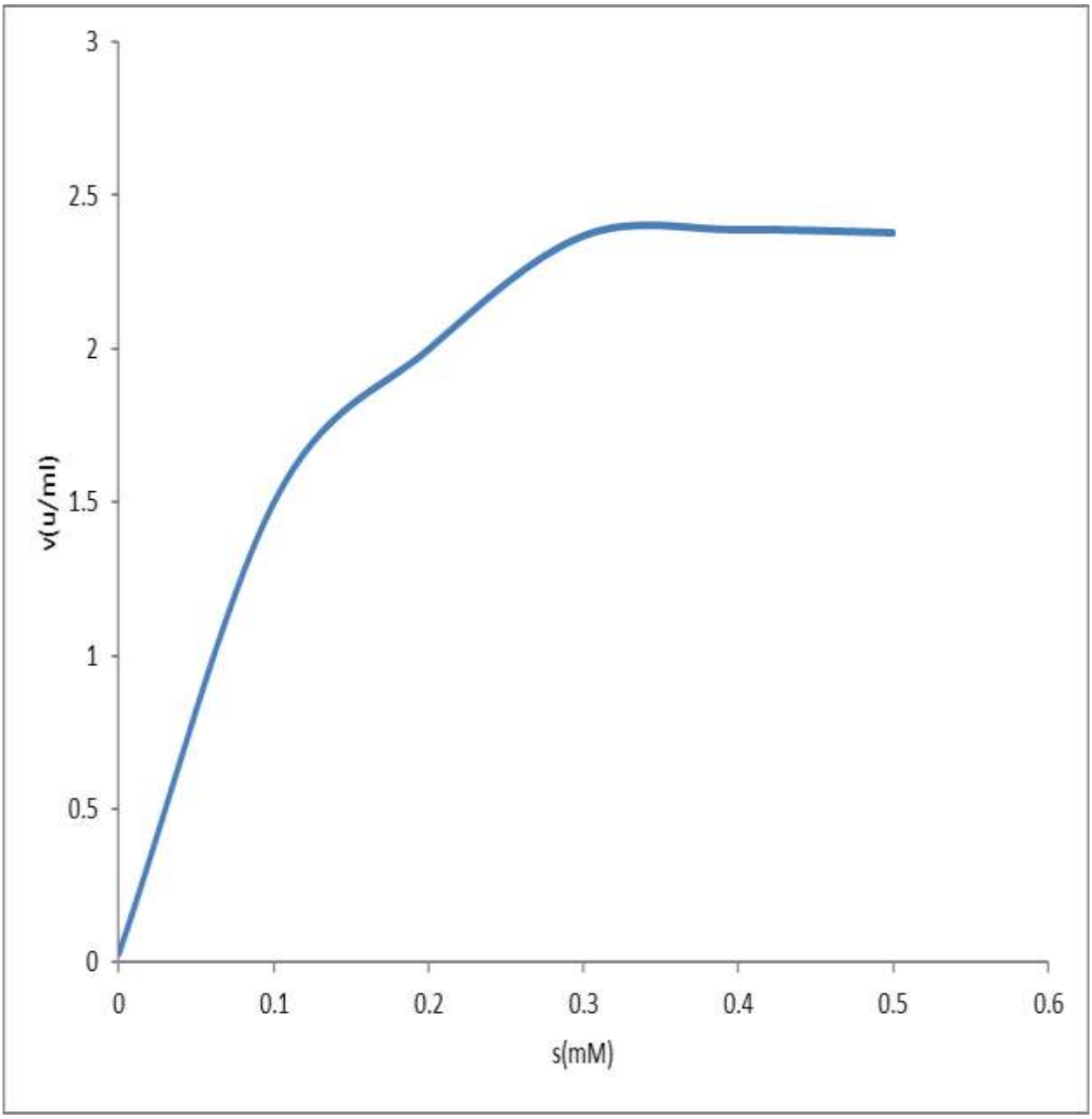


Figure 4.8. Michaelis-Menten curve on the effect of o-dianisidine on the activity of the peroxidase produced from the di-culture of *Rhizopus* and *Saccharomyces* spp.

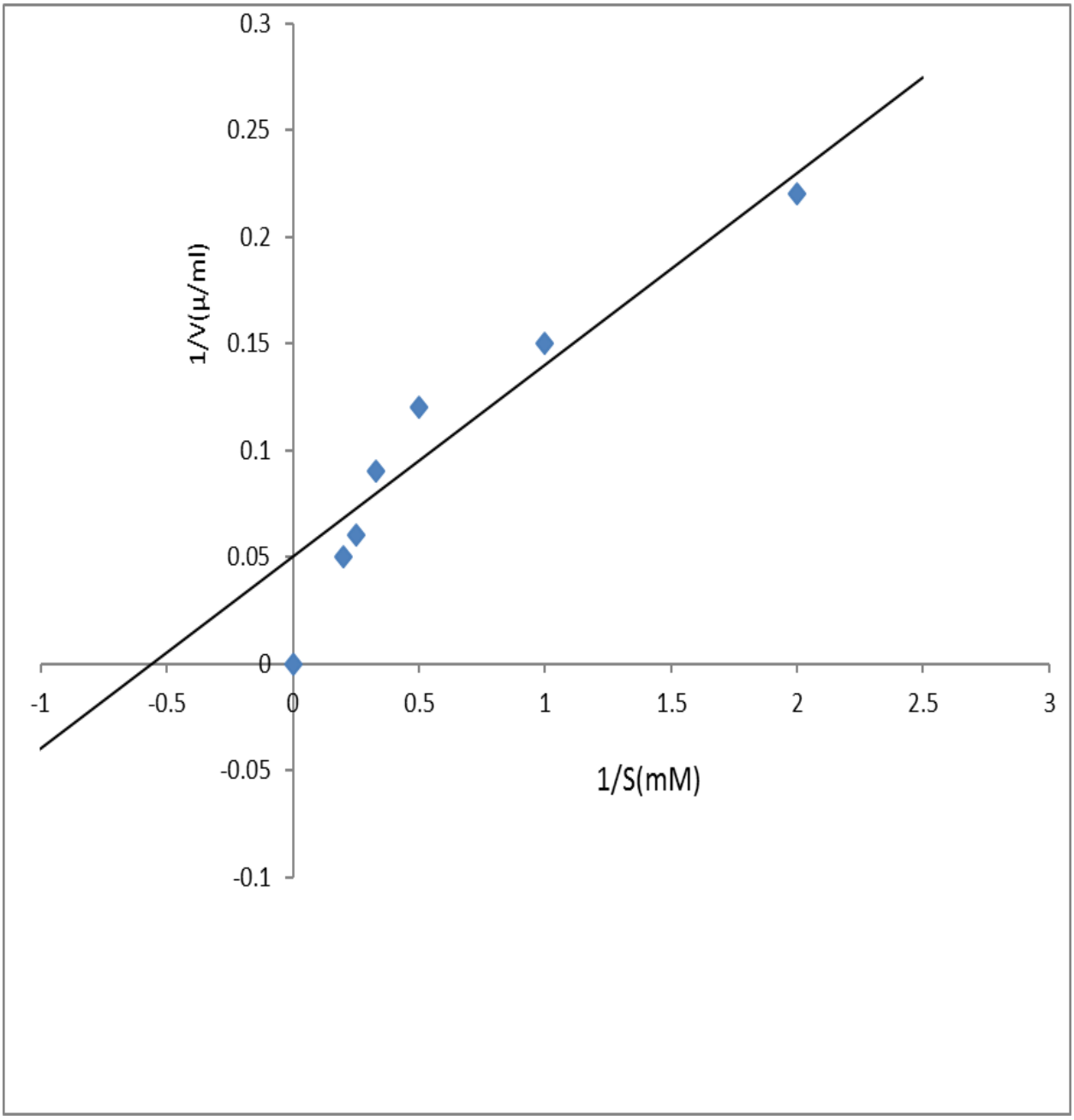


Figure 4.9. **Lineweaver-burke double reciprocal curve on the effect of o-dinisidine on the activity of the peroxidase produced from the di-culture of *Rhizopus* and *Saccharomyces* spp.**

4.1.11. Stability Studies on the Purified Enzyme Extract Using Varying Concentrations of Heavy Metals.

The pH (4.5) stability study on the activity of the peroxidase produced from the di-cultures of *Rhizopus* sp. *and Saccharomyces* sp. and incubated for 180 min as shown in Figure 4.10.

The pH 6.0 stability study on the activity of the peroxidase produced from the di-culture of *Rhizopus* and *Saccharomyces* spp. incubated for 180 min is shown in Figure 4.11

The effects of optimum pH 4.5 of the test sample (Fig. 4.10) and 6.0 of the control (Fig. 4.11) was stabilized using 0.02-0.05 M concentrations of Ca, Mg, Co and Pb ions. The enzyme was also stabilized in the presence and absence of the metal ions pre-incubated for 180 min. The result obtained clearly shows the enzyme maintained a steady time independent curve from time 0-90 min thereafter a biphasic decline was obtained from time 120-180 min. Pb was seen as a de-stabilizer to the enzyme during the study as there was a significant loss of enzyme activity. The stability study at the two pH ranges in the absence of metal ions showed decline in activity after 68 min pre-incubations.

The temperature 50^{o}C stability study on peroxidase produced from the di-culture of *Rhizopus* sp. and *Saccharomyces* sp. and incubated for 180 min. is presented in Figure 4.12.

The temperature 40^{o}C of the control stability study on the activity of the peroxidase produced from the di-culture of *Rhizopus* sp. and *Saccharomyces* sp. incubated for 180 min. is presented in Figure 4.13.

The effects of optimum temperature 50^{o}C of the test sample (Fig. 4.12) and 40^{o}C of the control (Fig. 4.13) was stabilized to monitor the activity of the peroxidase using varying concentrations of metal ions ranging from 0.02-0.05 M of Co, Mg, Pb and Ca ions. Stability effect was also assayed for in the presence and absence of the metal ions.

The result obtained showed the enzyme maintains a steady time independent curve from time 0-120 min thereafter a single phase decline was obtained from time 120-80 min. While Pb was seen as a de-stabilizer to the enzyme during the study as there was a significant loss in activity of the enzyme up to 80%, the stability study at the two temperatures in the absence of metal ions showed decline in activity after 90 min of pre-incubations.

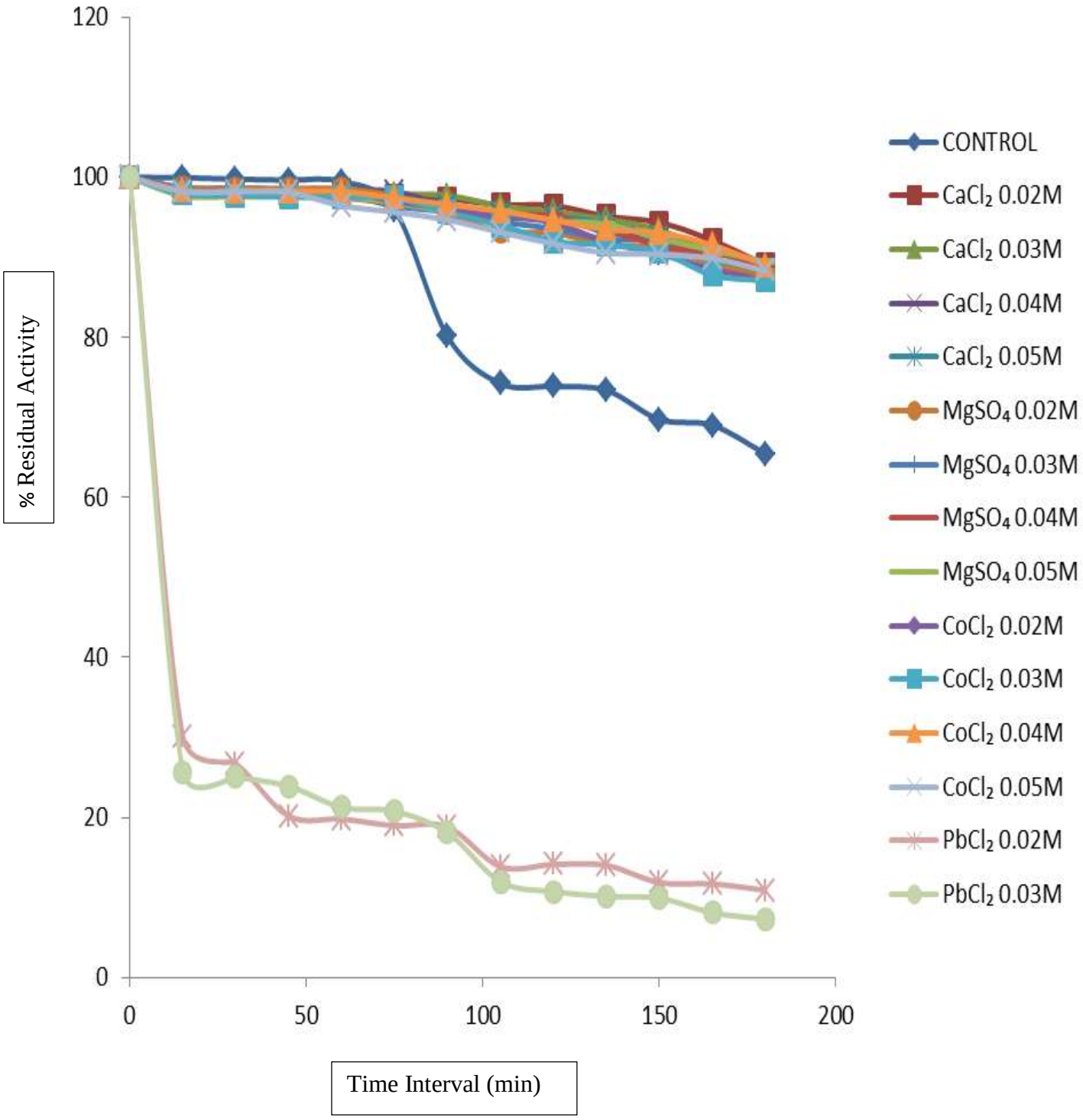


Figure 4.10: pH 4.5 stability study on the activity of the peroxidase produced from the di-culture of *Rhizopus* and *Saccharomyces* sp. incubated for 180 min

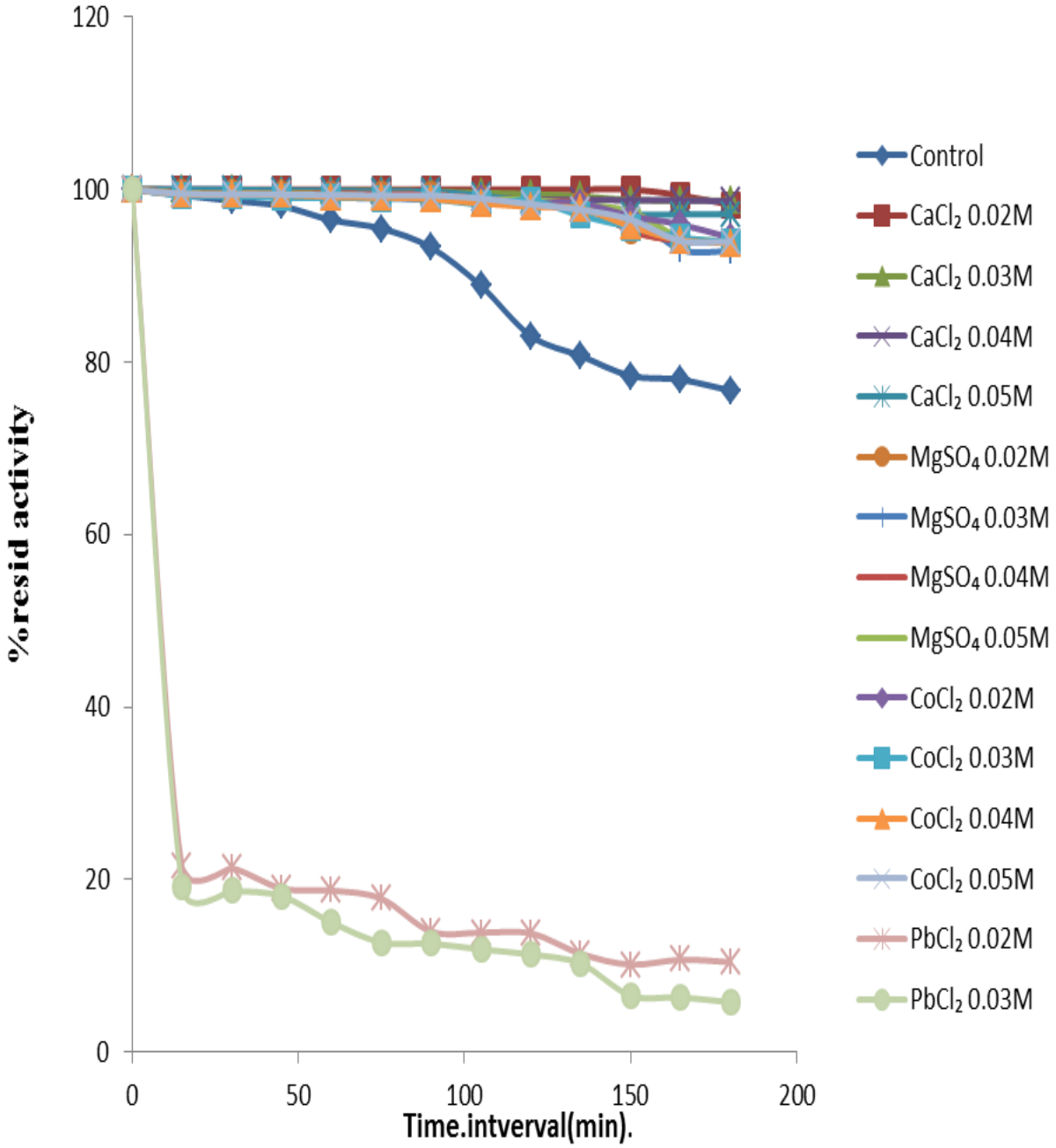


Figure 4.11: pH 6.0 stability study on the activity of the peroxidase produced from the di-culture of *Rhizopus and Saccharomyces* spp. incubated for 180 min

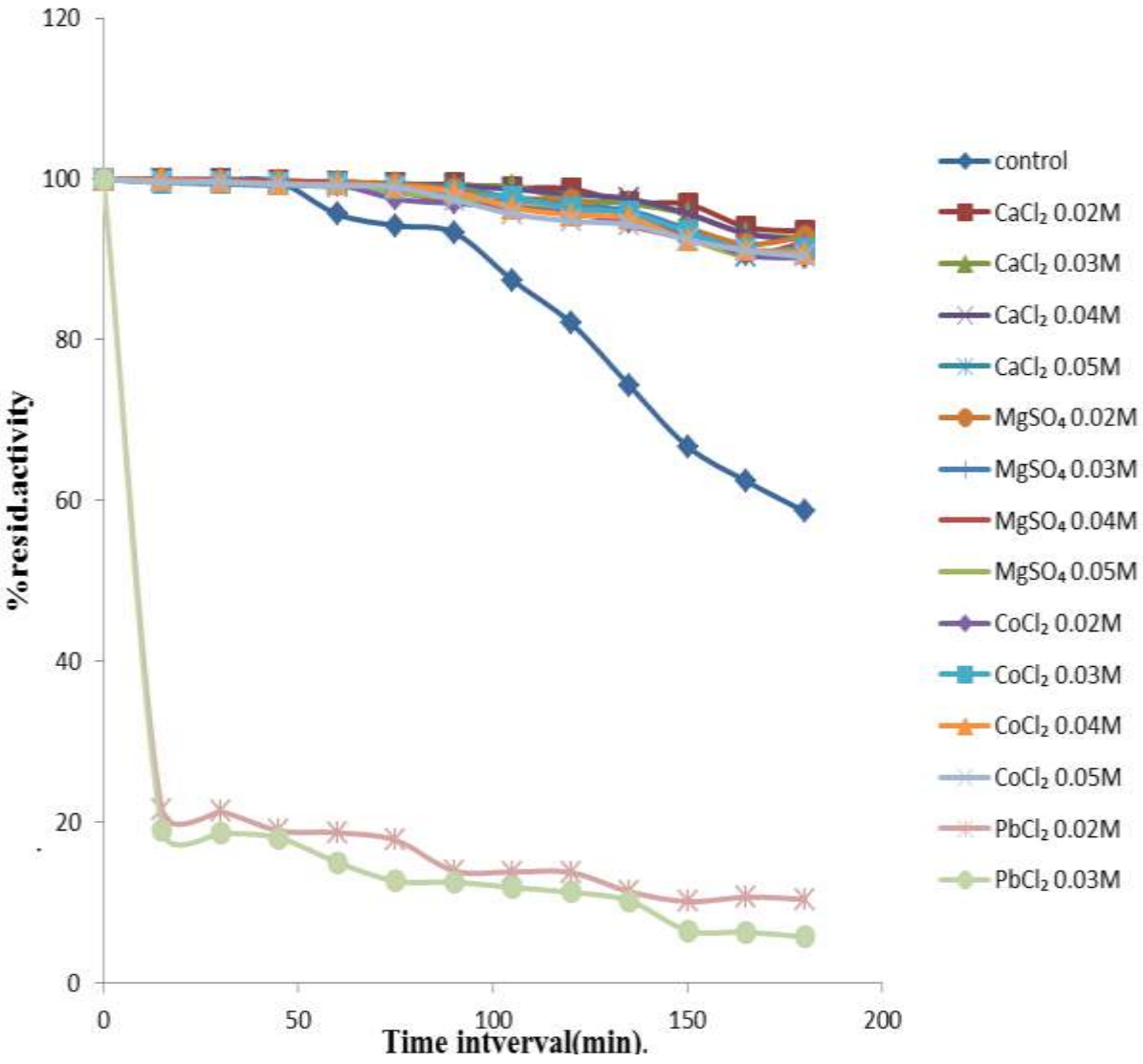


Figure 4.12: Temperature 50°C stability study on peroxidase produced from the di-culture of *Rhizopus* sp. and *Saccharomyces* sp. and incubated for 180 min.

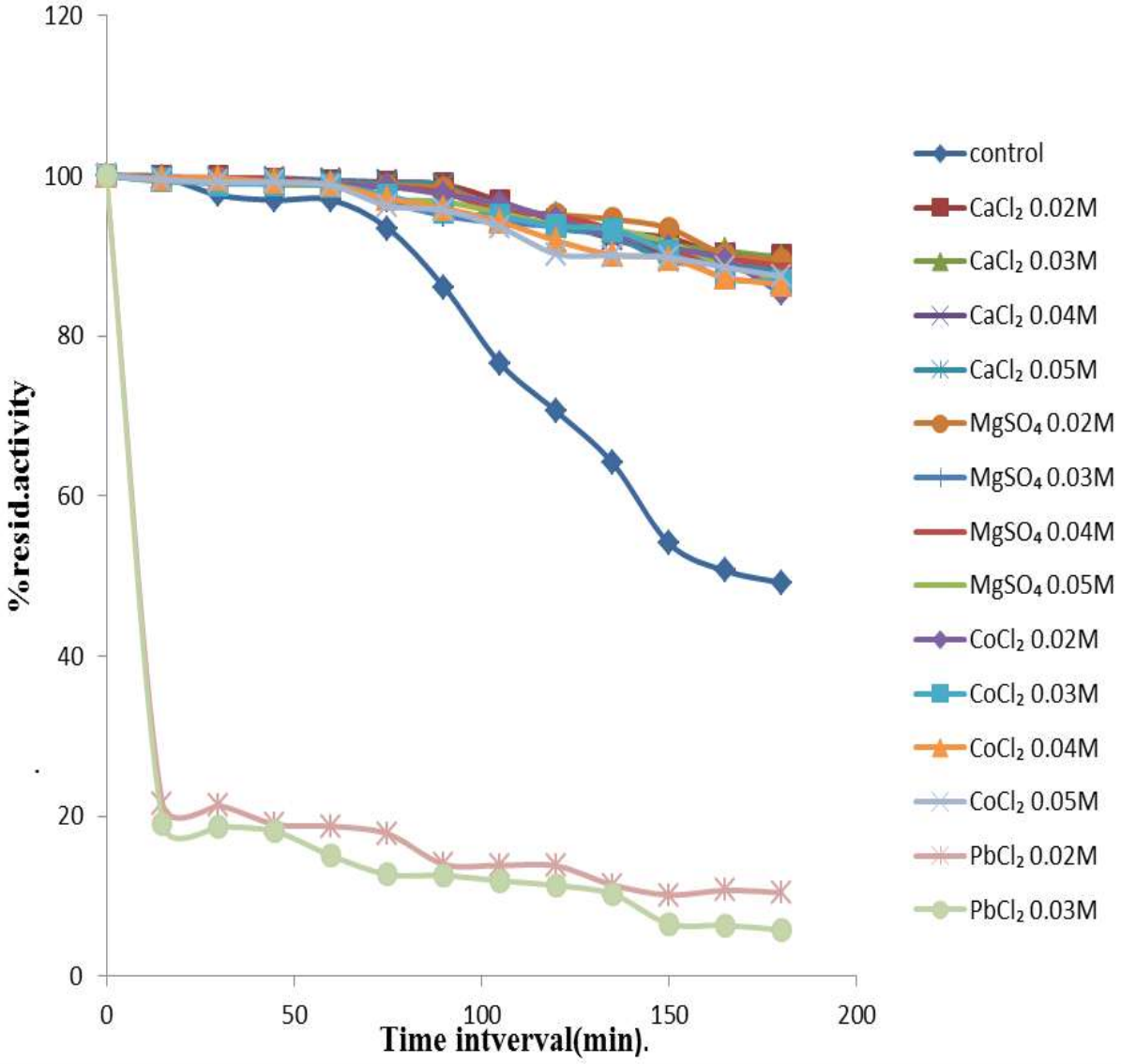


Figure 4.13: Temperature 40ºC stability study on the activity of the peroxidase produced from the di-culture of *Rhizopus* sp. and *Saccharomyces* sp. incubated for 180 min

4.1.12. Hydrocarbon Bio-degradation Studies on the Purified Enzyme and Optimization of the Physicochemical Parameters

The results of the optical density (OD) effect of pH on crude oil biodegradation at 10% (v/v) concentration of the peroxidase is presented in Figure 4.14

The results obtained showed that effective degradation of the crude oil hydrocarbons was optimum at pH 5.5 with 10% (v/v) concentration of the peroxidase at OD (610 nm) 0.72, the control experiment showed same trend at OD_{610} 0.28 nm. The enzymic degradation were seen less effective and much retarded at the neutral range (7.0, 7.5) before decreasing gradually at alkaline range (pH 8.0, 8.5) of the remediation system respectively. The control also fluctuated in the rest of the pH values.

The results of the effects of pH on weight loss of crude oil biodegradation at 10% (v/v) concentration of the peroxidase is presented in Figure 4.15

The results obtained showed the highest weight of the degraded crude oil in the test sample was given as 4.78 g at pH 6. The weight of crude oil degraded in the test sample showed great disparity compared to the control sample which showed little or no difference in the weight of oil degraded with values approximately between 0.3-0.4 g at all pH ranges. The weight of oil degraded varied in all pH ranges and reduced progressively after the optimum pH (6).

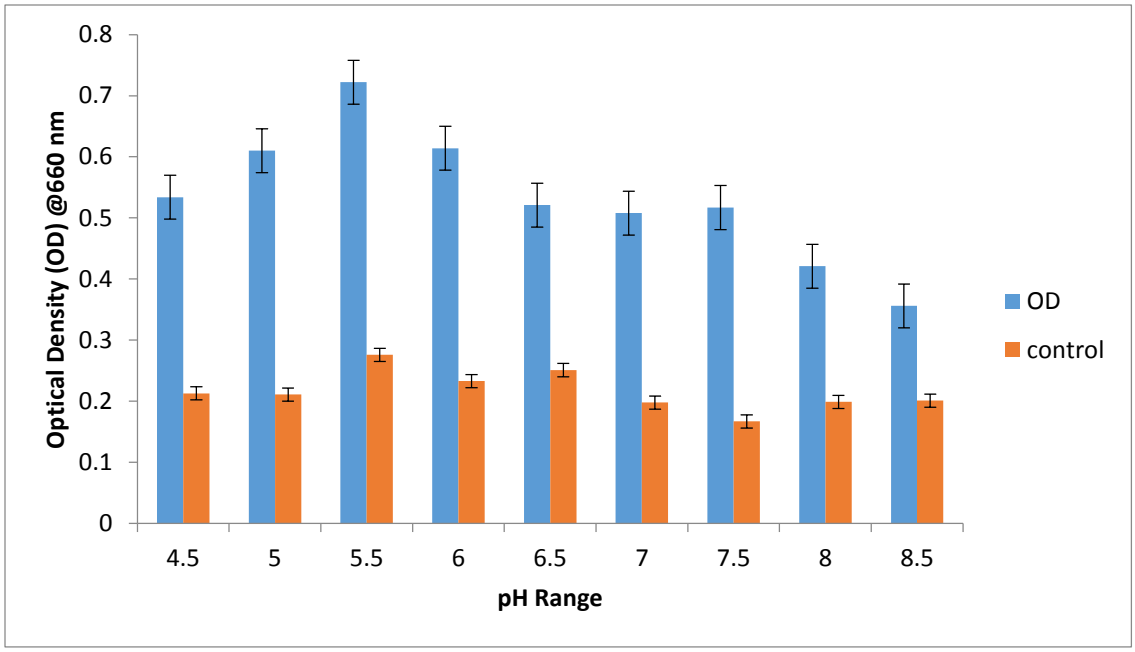


Figure 4.14: Optical density effect of pH on crude oil biodegradation at 10% (v/v) concentration of the peroxidase

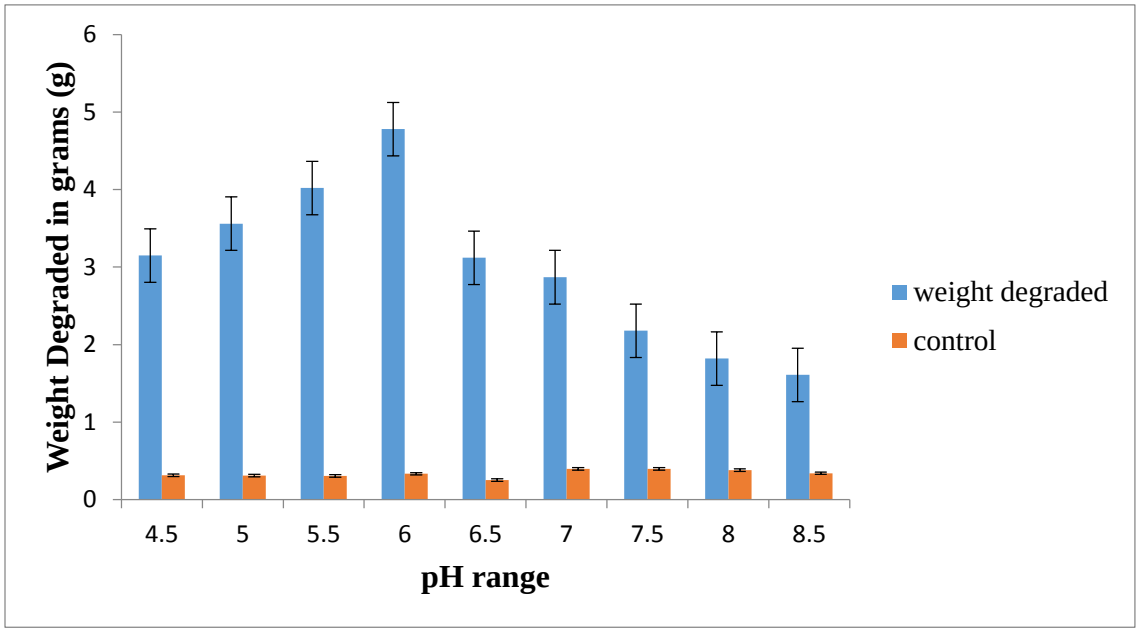


Figure 4.15: Effect of pH on weight loss of crude oil biodegradation at 10% (v/v) concentration of the peroxidase.

The Optical Density effect of incubation days on crude oil biodegradation at 10 %, (v/v) peroxidase incubated at pH 5.5 is presented in Figure 4.16.

The result obtained showed an optimal degradation of the crude oil hydrocarbons incubated for 20 days with 10% (v/v) of the peroxidase highest on day 12 with OD reading at approximately 0.25 nm. Day 8 of the incubation showed result similar to day 12 which was the peak day of peroxidase activity during incubation. Peroxidase activity was least at Incubation day 0, the control showed little or no difference with OD reading approximately between 0.3-0.24 nm. A decrease in activity was observed after the peak activity from Day 16-20 of the test sample.

Effect of incubation days on weight loss of crude oil biodegradation at 10% (v/v) peroxidase incubated at pH 5.5 is presented in Figure 4.17.

The result obtained showed the weight of crude oil degraded were higher in the test sample compared to the control. The study also showed that the weight increased as the incubation days progressed until day 20 which showed a slight drop in weight degraded in the test sample. The lowest weight (1.4 g) of crude oil degraded was obtained on day 1 of the incubation. The highest weight of degraded crude oil given as 4.19 g on day 16 of incubation in the test sample. It was also observed that from day 12 of incubation, the degradation though high, remained slightly stable. The weight of crude oil degraded in the control varied between 0.2-0.5 g with little or no difference, which contrasts compared to the weight degraded in the test sample.

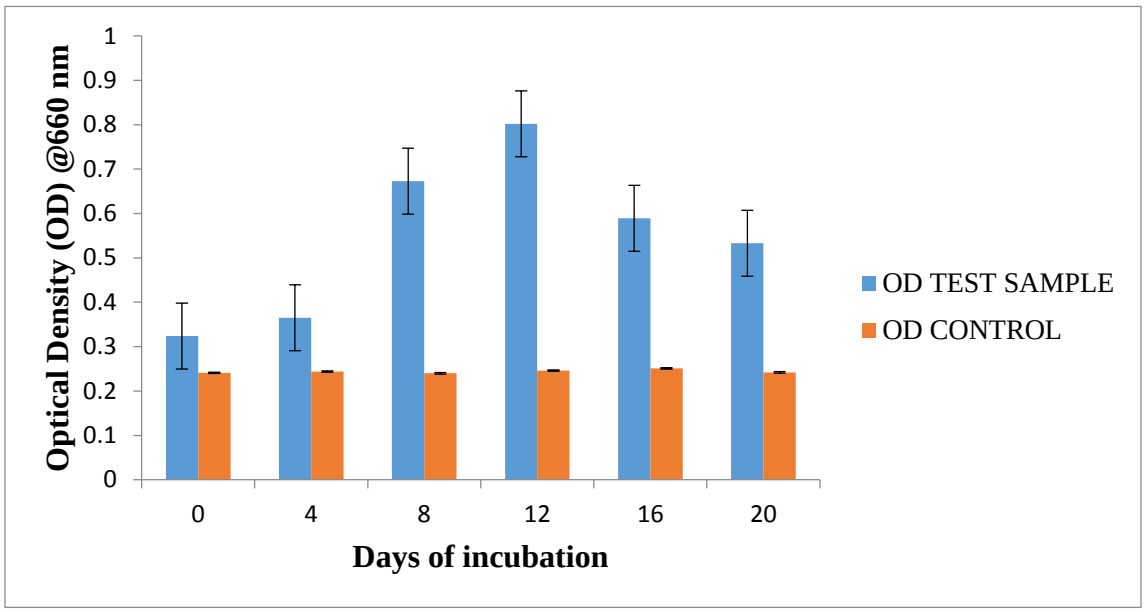


Figure 4.16. Optical Density effect of incubation days on crude oil biodegradation at 10% (v/v) peroxidase incubated at pH 5.5

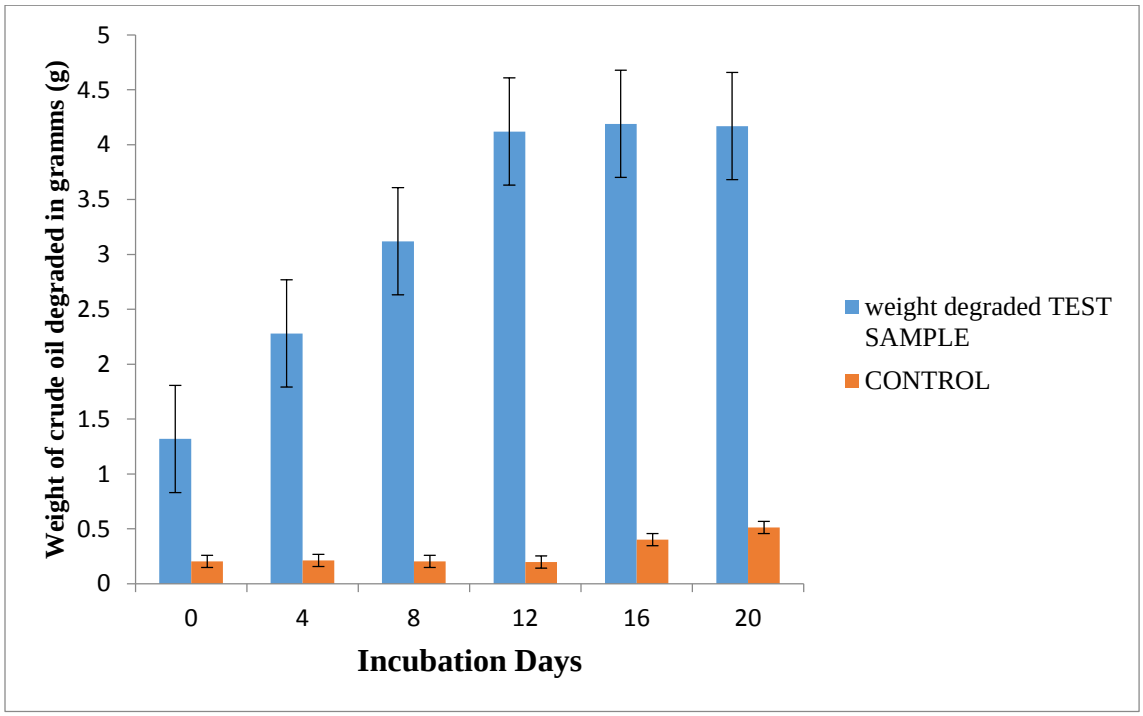


Figure 4.17. Effect of incubation days on weight loss of crude oil biodegradation at 10% (v/v) peroxidase incubated at pH 5.5

The Optical Density effects of varying concentrations of the crude oil (0-20% v/v) during the biodegradation at 10% v/v of the peroxidase incubated for 20 Days at pH 5.5 is presented in Figure 4.18.

The results obtained showed an optimal activity the peroxidase at varied crude oil concentrations, crude oil percentage increased with increase in activity of peroxidase in the test sample. Again, lowest activity was observed in the test sample at 5% v/v crude oil concentration with OD reading at approximately 0.4 nm. Peroxidase activity was highest at 15% v/v crude oil concentration before a slight decrease at 20 % v/v crude oil concentration in the test sample. However, the reverse was the case in the control experiment, there was decrease in peroxidase activity (OD) approximately between 0.3-0.4 nm as crude oil concentrations (0-20% v/v) increased. Highest activity was at 5% v/v while the lowest was at 20% v/v crude oil concentrations.

Effects of varying concentrations of the crude oil on weight loss during the biodegradation at 10% v/v of the peroxidase incubated for 20 Days at pH 5.5 is presented in Figure 4.19.

The result obtained showed the weight of crude oil degraded were higher in the test sample compared to the control. The study also showed that the weight increased as the crude oil concentration increased from 5-15% v/v in the test sample. The lowest weight (3.2 g) of crude oil degraded was observed in the 20% v/v crude oil concentration while the highest weight of degraded crude oil given as 4.28 g at 15% v/v crude oil concentration in the test sample. The weight of crude oil degraded in the control fluctuated between 0.1-0.2 g with little or no difference, which contrasts compared to the weight loss in the test sample.

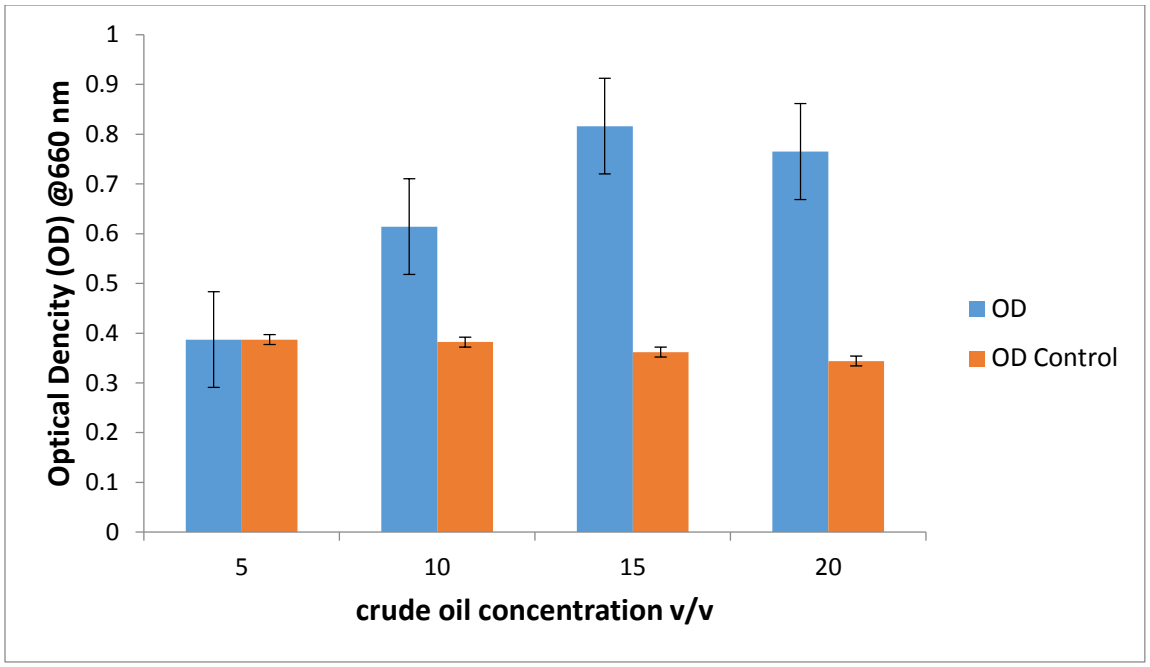


Figure 4.18: Optical Density effects of varying concentrations of the crude oil (0-20% v/v) during the biodegradation at 10% v/v of the peroxidase incubated for 20 days at pH 5.5.

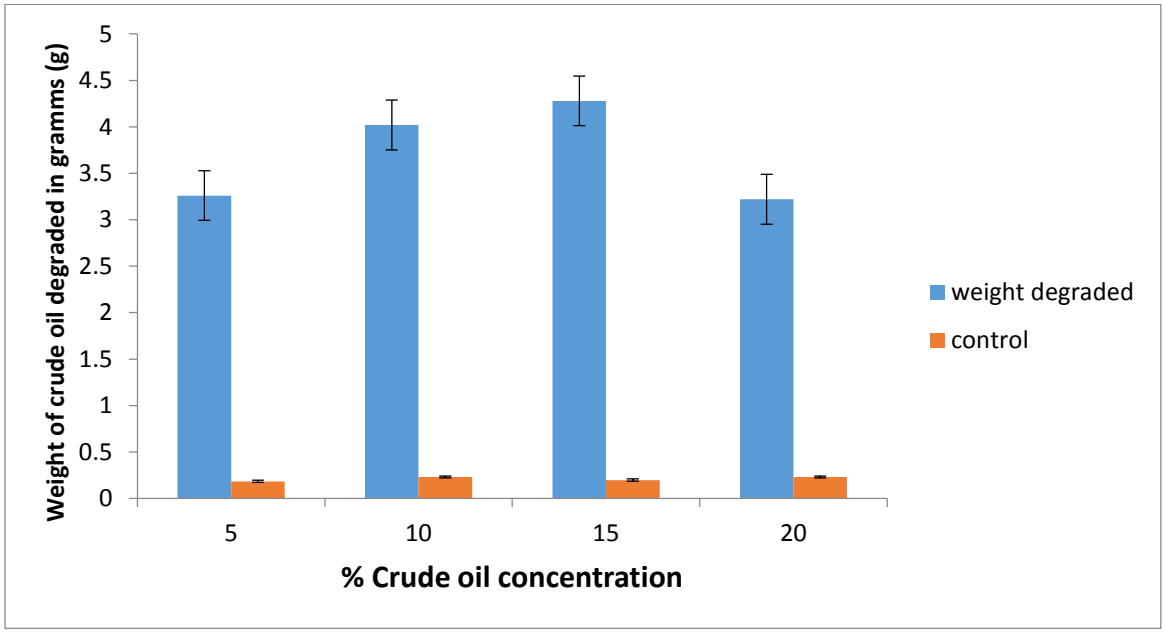


Figure 4.19: Effects of varying concentrations of the crude oil on weight loss during the biodegradation at 10% v/v of the peroxidase incubated for 20 Days at pH 5.5.

Optical Density (OD) effects of varying concentrations of peroxidase during the biodegradation at 10% v/v of the crude oil for 20 Days at pH 5.5 is presented in Figure 4.20.

The results obtained showed an optimal activity of the peroxidase at varied crude oil concentrations, crude oil percentage increased with increase in activity of varied peroxidase concentrations in the test sample. The lowest activity was observed in the test sample at 0% v/v crude oil concentration with OD reading at approximately 0.2 nm. Peroxidase activity was highest at 20% v/v crude oil concentration with OD reading at approximately 0.8 nm in the test sample.

Effects of varying concentrations of the peroxidase produced from the fungal di-culture on the weight loss during the biodegradation of 10% v/v of the crude oil is presented in Figure 4.21.

The result obtained showed the weight of crude oil degraded increased as peroxidase concentration (0-20% v/v) increased in the test sample. The lowest weight (0.2 g) of crude oil degraded was obtained on 0% v/v peroxidase concentration, while the highest weight of degraded crude oil given as 4.98 g at 20% v/v concentration.

It was observed that the rate of hydrocarbon degradation increased with increase in peroxidase concentration both in the OD reading (Fig. 20) and weight loss (Fig. 4.21). The enzyme activity increased with additional peroxidase.

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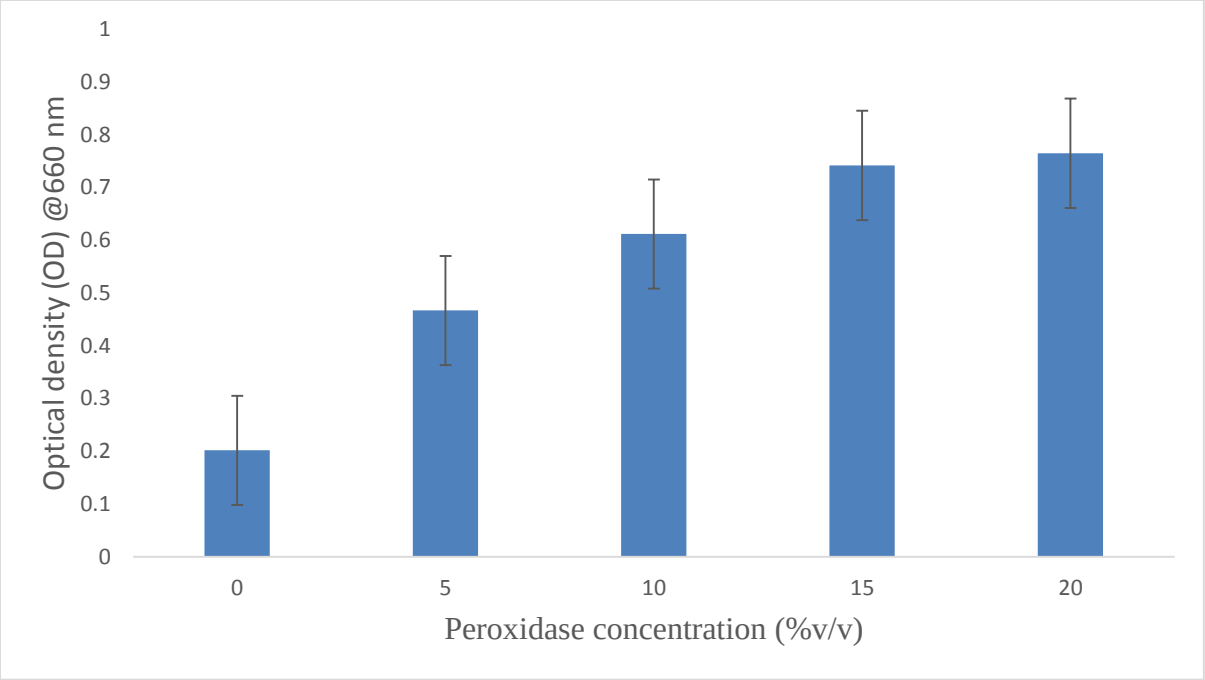

Figure 4.20: Optical Density (OD) effects of varying concentrations of peroxidase during the biodegradation at 10% v/v of the crude oil for 20 Days at pH 5.5.

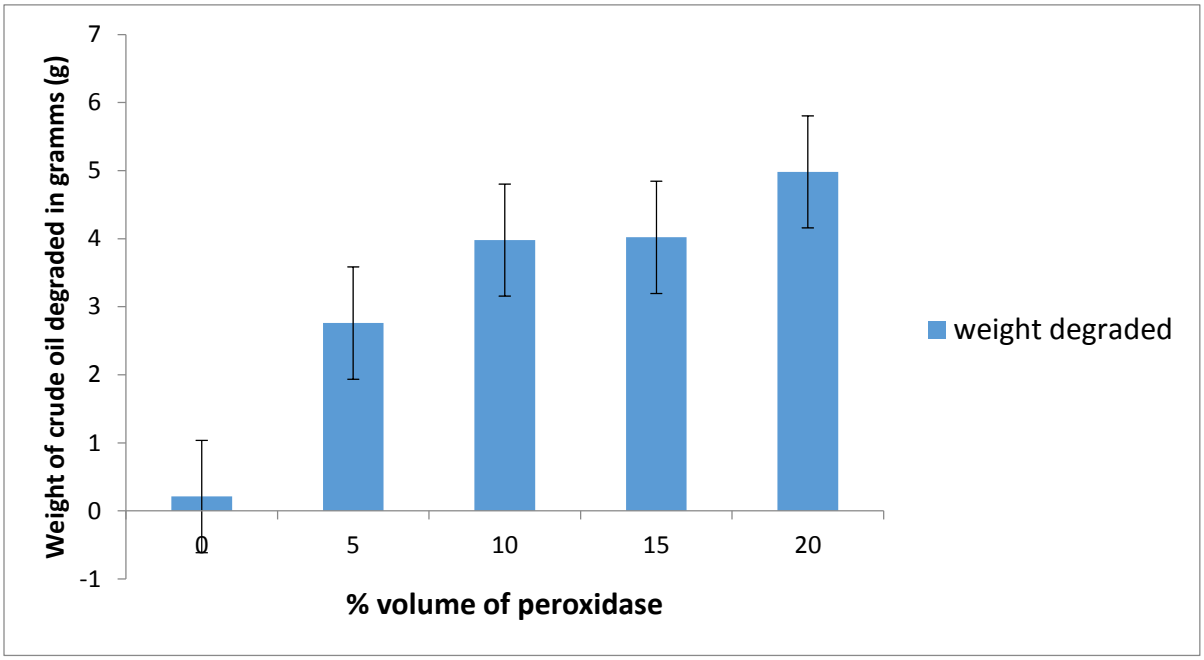

Figure 4.21: Effects of varying concentrations of the peroxidase produced from the fungal di-culture on the weight loss during the biodegradation of 10% v/v of the crude oil.

The effects of varied pH ranges on Total Petroleum Hydrocarbon (TPH) concentrations is presented in Table 4.6

The result obtained showed fluctuations in the TPH values as the pH increases in the test sample and control. However, pH 7.5-8.5 in the test sample maintained a steady rise with highest TPH concentration (1024 mg/kg) at pH 8.5, compared to the control TPH concentration (1671 mg/kg) was highest at pH 6. Lowest TPH values 531 mg/kg and 1622 mg/kg were recorded at pH 5.5 and 6.5 in the test sample and control respectively.

The effects of incubation days on Total Petroleum Hydrocarbon (TPH) concentrations is presented in Table 4.7

The result showed that during the 20 days of incubation, the TPH concentrations in the test sample maintained a progressive decrease as the days of incubation progressed (day 0-16) before a slight increase on day 20. However, TPH concentrations varied in the control samples with highest value recorded on incubation day 0 (1672 mg/kg) and lowest on incubation day 20 (1614 mg/kg). The test sample had its highest TPH concentration (1650 mg/kg) recorded on incubation day 0 and lowest on incubation day 16 (541 mg/kg).

Table 4.6: The effects of varied pH ranges on Total Petroleum Hydrocarbon (TPH) concentrations

pH	Test sample (mg/kg)	Control (mg/kg)
4.5	678	1667
5	702	1634
5.5	531	1656
6	609	1671
6.5	598	1622
7	892	1631
7.5	832	1623
8	933	1625
8.5	1024	1645

Table 4.7: Effects of incubation days on the amount of Total Petroleum Hydrocarbon (TPH) Concentrations.

Incubation days	Test sample (mg/kg)	Control (mg/kg)
0	1650	1672
4	1557	1632
8	1276	1632
12	876	1621
16	541	1617
20	587	1614

TPH values in the varied crude oil concentration (5-20% v/v) before treatment is presented in Table 4.8

Effects of 5 % -20% (v/v) crude oil concentrations on the Total Petroleum Hydrocarbon (TPH) of the treated test sample and control after the degradation is presented in Table 4.9

The results obtained from Table 4.8 showed that at 5%-20% v/v crude oil concentrations, TPH values before the degradation studies were given as 1649 mg/kg, 1659 mg/kg, 1669 mg/kg, and 1679 mg/kg respectively. There was increase in TPH concentrations as the as the crude oil concentrations increased from 5%-20% v/v. However, during the degradation studies, there was a progressive decrease (Table 4.9) in the TPH concentrations of the test samples in each of the crude oil concentrations (546 mg/kg, 552 mg/kg, 562 mg/kg, 564 mg/kg) respectively. Although, the TPH concentrations in the control experiment remained high (1640 mg/kg, 1648 mg/kg, 1660 mg/kg, 1667 mg/kg) in all the crude oil concentrations respectively compared to the test samples and the uncontaminated control (342 mg/kg) before the degradation studies.

Table 4.8: TPH values in the varied crude oil concentration (5-20% v/v) before treatment

Crude oil (v/v)	TPH concentration (mg/kg)
5%	1649
10%	1659
15%	1669
20%	1679

Table 4.9: Effects of 5%-20% (v/v) crude oil concentrations on the Total Petroleum Hydrocarbon (TPH) of the treated test sample and control after the degradation

	5%	10%	15%	20%
Test sample	546	552	562	564
Control	1640	1648	1660	1667

Table 4.10: Effects of 5%-20% (v/v) crude oil concentrations on the % loss Total Petroleum Hydrocarbon (TPH) of the treated test sample and control after the degradation

	5%	10%	15%	20%
% loss test sample	27.3	55.2	84.3	112.8
% loss control	82	164.8	249	333.4

4.2. DISCUSSION

The increasing trend of noxious environmental pollutants in our bio-system and possible biological technique of their removal have increased interest in the search for environmentally stable enzymes as possible alternative to other biological and chemical materials owing to their unique qualities. The present study assessed the biodegradation of hydrocarbons by peroxidase produced from di-culture of fungi (*Rhizopus* sp. and *Saccharomyces* sp.) isolated from a crude oil polluted site at Onne oil Jetty site Eleme, Rivers State.

4.2.1. Chemical Analysis of the Soil Samples

The results obtained from the soil chemical analysis of the crude oil contaminated soil at Onne oil jetty site at Eleme Rivers State showed that the soil contains relatively high hydrogen ion concentration (H^+) with pH of 5.02 showing an acidic range. This result is similar to the findings of Mbachu, (2016b) who reported that crude oil polluted soil are acidic in nature. The soil temperature and conductivity were high which is attributed to the nature of recalcitrant in the soil such as polycyclic aromatic hydrocarbons (PAHs) e.g. pyrene, naphthalene etc. that contain higher acidic contents as stated in the proceedings of the (ASTDR, 2010). The acidity of the soil could also be due to microbial metabolism. Low moisture content in the contaminated soil is similar to the finding of Kumari *et al,* (2016) who reported that this could be a limiting factor during biodegradation.

The values of the mineral elements in the crude oil polluted soil also fluctuated compared to the control. Higher concentration of ions of potassium (12.52 mg/g), phosphorus (1.53 mg/g), magnesium (11.27 mg/g) and chloride (1151.61 mg/g) revealed the level of pollution of the soil with the crude oil and other anthropogenic activities going on at the Onne oil jetty site Eleme, such

as farming, fishing, trading, truck loading, accidents involving tankers and oil storage facilities etc. The result showed a significant difference from the unpolluted soil sample which upon analysis showed a relative lower concentration of K, P, Mg and Cl ions in the following order respectively: 7.42, 1.86, 4.27, 393 mg/g. This result is similar to the findings of Chikere *et al.* (2009) in their study at Eleme petrochemical jetty port site reported a similar correlation of ion concentrations in the contaminated Eleme port soil. They revealed a higher concentration of the mineral ions in the following order 2.28, 1.84, 5.22 and 1789.22 mg/g respectively for potassium, nitrate, magnesium and chloride ions. This could be due to the presence of toxic metabolites and the unutilized nature of the mineral elements.

Heavy metal (Iron, Cadmium, Mercury, Arsenic, Copper and Lead) contents of the contaminated soil, showed that four (Fe, Ar, Cu, Pb) of six heavy metals were present. A greater proportion of Iron (Fe) in the soil with concentration of 38.74 mg/g, followed by Copper (Cu) with a concentration of 4.93 mg/g. Cadmium (Cd) and Mercury (Pb) were below detectable limits in both contaminated and uncontaminated samples. Arsenic (Ar) and Lead (Hg) were found in relatively lower concentrations of 0.45 and 2.29 mg/g in the contaminated sample; while Arsenic was found below detectable limits in the control sample. This correlates with the findings of Oparaji *et al.*, (2017) on the bioaccumulation of heavy metals in aquatic faunas and sediments at Eleme River, Port-Harcourt. Their results showed a higher proportion of Fe in all the tested species of aquatic fauna in the contaminated Eleme River while they reported Hg to be below detectable limits (BDL) in all the tested faunas and surrounding sediments.

Total petroleum hydrocarbon (TPH), Total organic carbon (TOC) content, and Total organic matter (TOM) content of the contaminated soil were observed at 234.64, 7.23, and 4.22 mg/g respectively. This showed a strong significant difference from the uncontaminated (control

experiments) which showed Total petroleum hydrocarbon (TPH), Total organic carbon content (TOC), and Total organic matter content (TOM) values of 34.2,.4.85 and 0.78 mg/g respectively. Mbachu *et al.* (2016b) reported a similar result in their research on microbial diversities in a spent engine oil polluted site with total petroleum hydrocarbon and total organic matter contents showing the highest in concentrations (4.06 and 5.21 mg/g respectively). The elevated values oof both TPH and TOC of the contaminated soil in comparison to the control is as a direct result of soil contamination with crude-oil deposits via related activities of the oil jetty. The increase in organic matter content in the contaminated sample could be attributed to the metabolic activities of microorganisms which used up the hydrocarbons for energy generation.

4.2.2. Microbial Isolation and Identification

The results obtained during the microbial isolation and identification showed that the soil samples from the crude oil polluted soil had the total heterotrophic and hydrocarbon utilizing fungi with colony forming unit per gram (CFU/g) of the contaminated soil as 6.9×10^8 and 1.15×10^7 while that of the uncontaminated was 4.58×10^8 and 5.5×10^2 respectively. The polluted site also had the highest percentage of heterotrophs that are hydrocarbon utilizing. This result is an indication that the site may be actively receiving hydrocarbons which in turn increased microbial activities. This study aligns with the work done by Chikere and Azubuike (2014), on the characterization of hydrocarbon utilizing fungi from hydrocarbon polluted sediments and water. The total heterotrophic count was higher than the hydrocarbon utilizing fungi and stated that the site was chronically polluted with hydrocarbons and possible presence of other carbon sources.

According to Adieze *et al*. (2003) and Abu and Ogiji (1996) in their study on hydrocarbon utilizing bacteria showed that bacteria with the ability to degrade a wide range of crude oil components exist ubiquitously in the environment and do appear to respond quite rapidly to the presence of petroleum hydrocarbons. It has also been reported that microbial communities exposed to hydrocarbons become adapted, exhibiting selective enrichment and genetic changes resulting in increased proportions of hydrocarbon degrading bacteria and bacterial plasmids encoding hydrocarbon catabolic genes (Leahy and Colwell, 1990).

The characterization and identification of the isolates shows that a total of four fungi isolates, belonging to three fungal families were obtained. These are; one species of *Saccharomyces, Penicillium, Rhizopus* and *Aspergillus*. Fungi are currently optimized active agents in petroleum degradation, and they work as primary degraders of spilled oil in the environment (Rahman *et al.,* 2002; Brooijmans *et al*., 2009). This correlates with the finding of Adieze *et al*. (2003) on microbial diversities during bioremediation of crude oil polluted site using culture dependent technique. Also, this finding is in line with the results obtained from the studies of Balaji *et al.* (2013) on enzymatic bioremediation of polyaromatic hydrocarbons (PAHs) by fungal consortia who recorded high fungal population from PAHs contaminated soil samples in which *Aspergillus, Mucor, Penicillium* and *Rhizopus,* were dominant.

The isolation of high number of certain oil-degrading microorganisms from an environment is commonly taken as evidence that those microorganisms are the most active oil degraders of that environment (Atlas and Bartha, 1998).

4.2.3. Emulsification Assay on the Identified Isolates

Upon three emulsification assays carried out on the isolated fungi, only two isolates showed higher emulsification potentials during the study (*Rhizopus* sp. and *Saccharomyces* sp).This suggests that these fungi utilized the crude oil as the sole carbon source without other supplement. Using drop collapse assay two (*Rhizopus* sp. and *Saccharomyces* sp.) out of the four isolates scored positive in drop collapse assay and also in oil displacement assay using the crude bonny light oil. Drops of cell free culture from *Penicillium* sp. remained intact and could not displace oil after one hour, this isolate is considered non-surfactant producer. *Aspergillus* showed a weak collapsing and displacement of the oil at the oil liquid interphase in both emulsification test conducted they may have utilized the hydrocarbon for the production of other metabolites. The spp. of *Aspergillus* and *Penicillium* generally showed less potentials.

The same trend was also seen during the oil spread plate assay on the isolated fungi. *Rhizopus* sp. and *Saccharomyces* sp. showed the highest strength in oil displacement in the presence of the crude oil. This according to Mbachu *et al*. (2016a) in their research reported that filamentous fungi have much ability to produce surface active compounds than the non-filamentous group. Emulsification activity of the culture isolates showed that *Rhizopus* sp. has the best emulsification activity with 51.2% and 56% index in the presence of kerosene and crude oil respectively. It was followed by *Saccharomyces* sp. with 54% and 48% emulsification activity for crude oil and kerosene. Also *Penicillium* sp. showed no positive reaction upon immersion on the oil water interface. *Aspergillus* still showed the same trend as observed in the drop collapse test. Therefore, the spp. of *Aspergillus* and *Penicillium* were eliminated from further studies.

4.2.4. Molecular Characterization of *Rhizopus* and *Saccharomyces* species.

Molecular indices of the identified fungi genera obtained from the crude oil polluted soil indicated from the gene extraction, that the species targeted by the specific primers used in this study were present in the sample. The presence of clear bands on the gels is an indication of the presence of well separated strands of DNA complementary to the target primers used in the study, hence this confirms the presence of the target organism. . The tested and accepted primers sent to Cabhol Protein Data Bank (PDB), was successfully amplified. This gave the highest number of the bands as shown in the result.

Results of the DNA sequencing analysis of the fungal isolates from the crude oil contaminated soil sample has shown that *Rhizopus stolonifer* and *Saccharomyces cerevisiae* were present with the ascension number DBOF141 and CBS1171 respectively.

Mbachu *et al*, (2016a) among his isolates from spent engine oil polluted site were *Aspergillus,* and *Saccharomyces cerevisiae*. Akpoveta *et al*., (2011) reported *Penicillium, Rhizopus* and *Aspergillu*s spp. from crude oil polluted soil in his study on microbial degradation and its kinetics in crude oil polluted soil. Also Chikere and Azubuike (2014), in their study on characterizations of hydrocarbon utilizing fungi from Hydrocarbon sediments and water reported *Penicillium, Rhizopus, Saccharomyces* and *Aspergillus* spp. among other fungi consortium.

4.2.5. Enzyme Extraction and Purification

The steady rise and fall during the 14 day pilot study for the extraction of peroxidase from the co-cultures of the emulsifying species of *Rhizopus* and *Saccharomyces* before the progressive decline towards day 13 of the enzyme activity, with protein concentrations following the same trend as in

the activity profile .This aligns with the report of Adefila *et al.* (2012) which stated that peroxidase and the larger family of the oxidases are specific house-keeping enzymes which are inductively secreted in any environment depending on the concentrations of recalcitrant in the medium.

Peroxidase which was produced from *Rhizopus* sp. and *Saccharomyces* sp. and mass-produced on 8th day was also precipitated using ammonium sulphate salts; further on it was desalted from the ammonium salts using the dialysis bag for 12 h at 37^0C using acetate buffer of pH 4.5. 80% of the ammonium sulphate salt gave the most efficient precipitation of the protein from the saturated fluid. Shen *et al*. (2012) reported 80% ammonium sulphate salt as the best saturation for precipitation of horseradish peroxidase from an under-utilized seed. Similar result was observed by Idesa and Getachew (2018) who reported 80% saturation of Ammonium sulphate precipitation of peroxidase from plant sources. However, this result is comparable to the report of Hamid *et al,* (2016) whose peroxidase extracted from wheat bran showed that peroxidase was precipitated efficiently with ammonium sulfate in saturation ratio of 70%. The purification profile of peroxidase from *Rhizopus stolonifer* and *S. cerevisiae* showed that the specific activity i.e activity per milligram protein of the enzyme increases at each purification step.

Upon dialysis of the precipitated enzyme, unwanted salt and proteins were removed. Following further purification by gel filtration and dialysis, the enzyme activity increased at each purification step. This is similar to the findings of Hamid *et al*. (2016) who reported a purification fold of 2.4, 3.4 and 5.1 with specific activities of 730 U/ml on a three-purification step using ammonium sulphate precipitation, ion exchange chromatography and gel filtration chromatography.

4.2.6. Characterization and Stability Studies on the Purified Enzyme Extract

Characterization and stabilization of purified enzymes under controlled optimum condition (pH and temperature) are key physiological factors to be considered for maximum enzyme activity and applications in industries, environment (Singh *et al*, 2014). The partially purified peroxidase, when examined at different pH ranges, showed that the partially purified enzyme was active over a broad pH range of 4.5-8.0 with maximum activity at pH 4.5. In addition, the peroxidase was relatively stable in the pH range of about 4.0-5.5 i. e the enzyme residual activity remained at about 65-76% of the maximum value at pH 4.5-5.5 after 180 min of pre-incubation of the enzyme at those pH ranges. This result is similar to that of Osuji *et al*. (2014) which showed peroxidase extracted from garlic to have an optimum pH of 5.0 with the highest percentage decolorization of dye at pH 4.5 and was stable at pH between 4.0 and 6.0. Also, another study by Bhatti *et al.* (2012) has shown peroxidase from *Raphanus sativus* to have an optimum activity at pH 4.0. The findings from this study agrees with findings of Pinto *et al*, (2015) who observed a broad range of pH 4.5 up to 7.5 for umbu peroxidase and its activity maintained at 70% levels. The pH curve also showed a bell-like shape distribution suggesting an involvement of two amino acid residues in catalysis.

Thermo stability analysis revealed that peroxidase had maximum stability at temperature of 50^{o}C, the significant drop at 70^{o}C suggests that temperature above that range can lead to denaturation and inactivation of the enzyme. This is similar to the report of Osuji *et al.*, (2014) and Bhatti *et al.*, (2012) which reported peroxidase to have an optimum activity at 55^{o}C. Also, the peroxidase retained 58% activity after 180 min of pre incubation at that temperature, these are comparable to the results of Singh *et al*. (2014) who reported 60^{o}C to be the maximum stability of its peroxidase isolated from *Streptomyces* sp. MSC702 and retains about 34.18% activity after 4 h of pre incubation. Shen *et al*. (2012) equally reported 60^{o}C to be the maximum stable range for

peroxidase from *Rhizopus microsporus* isolated from a refuse dump site and retained about 50% of activity after 60 min of pre incubation, Chen *et al*. (2005) reported 60^{o}C to be maximum stability temperature range for its peroxidase isolated from acid stable fungus which retained 90% activity at 70^{o}C. At other temperature ranges, the enzyme retained 55% activity at 70^{o}C and 50^{o}C after 180 min pre incubation period. At 30^{o}C and 40^{o}C the enzyme retained less than 50% activity. Chilaka *et al,* and Eze *et al,* (2010) reported a thermostable peroxidase from an oil bean plant with optimal temperature of 70^{o}C.

The effect of the substrate concentration on peroxidase activity increased with corresponding increase in substrate concentration until a saturation point of 5 mM for O-dianisidine, indicating that the active sites are saturated with the substrates. The Km from these results was 1.8 mM for hydrogen peroxide and Vmax value of 20 µ/ml/min for hydrogen peroxide. Similar to these results is the observation of Kim and Lee (2005) that reported Km value of 1.1 8 mM for o-dianisidine and 1.27 mM for hydrogen peroxide, with Vmax 0.032 u/ml/min for o-dianisidine and 0.138 u/ml/min for hydrogen peroxide, for peroxidase from *Raphanus sativus*. Also, Melda *et al*. (2010) reported that peroxidase from *Raphanus sativus* had Km values of 0.036 mM for guaiacol and 0.0084 mM for hydrogen peroxide, with Vmax values of 3512.23 u/ml/min and 38728.17u/ml/min respectively. Pinto *et al,* (2015) reported that peroxidases oxidize several different substrates in the presence of hydrogen peroxide. Peroxidase showed high affinity and catalytic efficiency towards the O-dianisidine substrate. From these reports it is evident that the nature of different reducing substrate affects the Km and Vmax of peroxidase.

Peroxidase showed a greater stability in the presence of different concentrations of heavy metal, this is in accordance with what Singh *et al*. (2014) reported that the large family of peroxidase and oxidases till date are metal-ion dependent and that Ca^{2+} are known to be a stabilizer for

peroxidase isolated from various microorganisms. However, Pb^{2+} in all concentrations inhibited the enzyme activity. The enzyme lost about 83-95% activity with $PbCl_2$ at concentrations of 0.02 - 0.03 M. Whereas $CaCl_2$, $MgS0_4$ and $CoCl_2$ stabilized the enzyme activity with best stability at $CaCl_2$, the degree of stabilization was proportional to the concentration of the metal ions in the reaction mixtures. In the presence of 0.02 M $CaCl_2$ the enzyme retained up to 90-95% activity at pH 5.0-5.5 and 78-88% at pH 3.5- 4.5. The stabilization in activity increased with increase in the concentration of the metal salts with 0.05 M concentrations of $CaCl_2$ conferring the highest stability. Other metal ions ($MgS0_4$ and $CoCl_2$) followed the same trend. Adefila *et al*. (2012) reported similarly in their work that Ca^{2+} and Mn^{2+} were potent activators of α-amylase. He reported that Cu^{2+} had an inhibitory effect on the enzyme activity. Pinto *et al*., (2015) reported a stimulatory effect of Ca^{2+} on peroxidase present in xilopodium exsudates of umbu plants, with Mg^{2+} having similar effect but at a lower concentration than that of Ca^{2+}, whereas Mn^{2+} displayed inhibitory effect. However, Pb^{2+} inhibited the activity of the enzyme Singh *et al*. (2014) reported an activation of α-amylase activity in the presence of varying concentrations of the salts of $CaCl_2$, $MgS0_4$, $MnCl_2$, $ZnCl_2$ and $FeCl_2$ and an inhibitory effect by Hg^{2+}, Pb^{2+} and Cu^{2+}. Singh *et al*. (2014) attributed this inhibition by these ions on the enzyme activity due to the presence of indole amino acids in the enzyme and also a reflection of competition between exogenous and protein associated cations.

In the presence of metal ions, the enzyme activity was enhanced and stabilized in all the temperature ranges i.e. 30-70^{o}C. Singh *et al*. (2014) reported the various ways by which metal ions affect enzyme catalysis, they stated in their work that metals modify the electron flow in the enzyme substrate reaction or change the orientation of the substrate with reference to the functional groups at the active sites. Singh *et al*. (2014) stressed that metal ions accept or donate electrons

and act as electrophiles, mask nucleophiles to prevent unwanted side reactions, binds enzyme and substrate by co-ordinate bonds holding the reaction groups in the required 3D orientation, and simply stabilize a catalytically active conformation of the enzyme. $CaCl_2$ and $MgS0_4$ salts show the highest activation potentials on the α-amylase activity at all the temperature ranges. In the presence of $CaCl_2$ the enzyme retained up to 87-89% activity at temperature range 30^oC- 40^0C and 90-93% activity at temperature range of 50^oC-60^0C and 89-90% at 70^0C. It is observed that the activity of the enzyme increases as the metal ions concentrations increases in all metal salts. MgS04 shows a promising strength in thermos ability of the enzyme while $CoCl_2$ follow suit. In the presence of Pb^{2+} the enzyme lost 90-95% activity in all the temperature ranges. These are in accordance with results of Adefila *et al*. (2012), Singh *et al*. (2014) and Shen *et al*. (2012) respectively which stated that Pb^{2+} and other heavy metals (Cu^{2+}, Hg^{2+} and Zn^{2+}) are like poison to enzymes and thus can inhibit their activity.

4.2.7. Hydrocarbon Biodegradation studies under Optimized Parameters using the Purified Enzyme

Biodegradation studies under optimized condition showed that hydrocarbon degradation was optimum at pH 5.5 and incubation day 12. Effective degradation was achieved as the incubation day progresses. The higher the concentration of peroxidase, the higher the degradation strength. This result is similar to the findings of Olajuyigbe and Ehiosun, (2016) who reported that specific activity of total peroxidase assayed over biodegradation period showed that peroxidase activity increased with degradation efficiency, suggesting that peroxidases play a key role in the crude oil biodegradation process. According to Mohsen *et al*, (2014) it has now been well established that the overall efficiency of oil spill bioremediation process relies on the development of optimal

conditions for enhanced oil bioremediation rates in contaminated media. Overall, 80% degradation was achieved in all optimized conditions. The result showed that optimized hydrocarbon degradation was achieved by providing adequate physicochemical conditions for the degradation. Fariba, (2018) evaluated the efficiency of peroxidase isolated from *Streptomyces albus* for crude oil bioremediation. The peroxidase showed 93% removal of bvenzopyrene from petroleum polluted soil. Lateef *et al.,* (2011) have also reported more than 90% degradation of Bonny light crude oil by two pseudomonas isolates but over a period of 21 days and 84.89 % for 5% crude oil concentration.

However, increase in concentration of crude oil caused increase in the equivalent weight loss during the degradation in 5-15% v/v peroxidase concentration and a reduction in 20% v/v, this is due to the fact that the contaminants have saturated the enzyme solution. The increase in peroxidase activity as crude oil concentration increased agrees with the report of Ehiosun and Usman, (2016) which suggest that total peroxidase was actively involved in the crude oil degradation. Effect of varied crude oil concentration depicts that peroxidase concentrations increases in strength of their biodegradation of crude oil hydrocarbons. This result illustrates the theory of Michealis Menten which shows that enzyme activity progresses as the substrate concentration increases.

Furthermore effective degradation was confirmed from the reduction in the amount of total petroleum hydrocarbon (TPH) concentrations in the varied crude oil concentrations (5-20% v/v), of the treated test sample and control experiment.

Peroxidases has proven to be useful in biodegradation of recalcitrant pollutants, a research done by Dubrovskaya *et al*., (2017) on the use of peroxidase from alfalfa roots stated that these peroxidase possess catalytic properties and participate in the degradation of polycyclic aromatic

hydrocarbons. They are able to transform various types of recalcitrant aromatic compounds. Studies have also shown that peroxidase are good degraders in optimum condition. Feltrin *et al.*, (2017a) on the characterization and application of the enzyme peroxidase to the degradation of the mycotoxin Deoxynivalenol (DON) confirmed that peroxidase extracted from rice bran can degrade DON in optimum condition. Feltrin *et al.*, (2017b) extracted peroxidase from rice bran and soy bean which showed 47% and 30% degradation and represented a greater upkeep in activity compared to the pure peroxidase.

The results from this present study on the degradation of the crude oil from Onne Oil jetty site using the peroxidase produced from the fungal di-culture (*Rhizopus stolonifer* and *Saccharomyces cerevisiae*) showed a very promising biodegradation ability of the house keeping enzyme and its ability to work in synergy during the process.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1. CONCLUSION

Crude oil pollution has ravaged and compromised the integrity of our environment both health wise and otherwise, and the recalcitrant nature of these pollutants in the environment has necessitated the need for the development of alternative treatment processes. Current approaches have been directed towards developing enzyme treatment systems. Enzymic technology is a promising trending technology in environmental remediation. Enzymes are vital elements in myriads of industrial processes without which modern biotechnology would have been impossible. Enzymes like peroxidase, can act on specific recalcitrant pollutants to remove them by precipitation or by transformation to other products. They can also change the characteristics of a given waste to render it more amenable to treatment. In this study, peroxidase produced from the di-culture of *Rhizopus stolonifer* and *Saccharomyces* cerevisiae showed significant dominance in the presence of carbon catenation chains of hydrocarbons.

The chemical analysis from this study was affected by the presence of hydrocarbon pollutants. The fungal di-culture *Rhizopus* and *Saccharomyces* spp. are ubiquitously present in the crude oil polluted soil as seen from the isolates. They are good degraders of these recalcitrant. The organisms also showed great emulsification potentials and as such, can produce surface active molecules in the presence of hydrocarbon recalcitrant and are able to act on the molecules by reducing the surface active tension between the organisms and the hydrocarbons.

The three-step purification of the crude extract gave a highly pure enzyme which is recommended industrially for environmental treatment purposes. The characterization of the enzyme contributed to the best physiological conditions for the enzyme activity which was at acidic pH range of 4.5. A highly stable thermophilic enzyme (50^{o}C) which is an environmentally implicated enzyme for industrial purposes was produced from this study. The stability process indicates its strong capability for bioremediation processes. The biodegradation studies under optimized conditions showed promising utility of peroxidase in clean-up exercises of the environment among other advantages such as; cost effectiveness and eco-friendly product upon eco-toxicological findings.

Based on these findings, the fungal di-culture (*Rhizopus* and *Saccharomyces* spp.) was isolated and peroxidase produced from it was efficient in the degradation of hydrocarbon contents of the petroleum polluted soil. Peroxidases among other vital biotechnological applications, are useful tools in environmental monitoring.

5.2. RECOMMENDATIONS

Among all the attributes of these enzymes (peroxidase) from the findings of this study, the following are recommended:

Peroxidase from di-culture of *Rhizopus stolonifer* and *Saccharomyces cerevisiae* are capable of and industrially recommended for environmental remediation and clean-up purposes particularly crude oil-polluted environment. However, this assessment concentrated on laboratory work, further studies are necessary to ascertain its effectiveness in the field.

More studies need to be directed towards classification of the type of peroxidase produced; and the dynamism of the activity of this enzyme especially from other microbial sources, this will help to elucidate pH and temperature dynamism of the enzyme activity and deduction of some of their optimized thermodynamic properties.

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APPENDIX

Microbial populations from the Contaminated and Uncontaminated soil Expressed in Colony Forming Units (CFU/g).

Heterotrophic Counts (CFU/g)	Uncontaminated	Contaminated Soil
Total Heterotrophic Counts	2.58×10^8	6.9×10^8
Hydrocarbon Degrading Fungi Counts	5.5×10^2	1.15×10^7

Preparation of Buffer Solutions

Sodium phosphate buffer

In preparing 1 M of sodium phosphate buffer of pH 6.5, 15.6 g of sodium dihydrogen phosphate (conjugate base) was dissolved in 100 ml of distilled water and 14.2 g of disodium hydrogen phosphate was dissolved in 100 ml of distilled water. The pH (6.0-7.0) was adjusted to 6.5 with the conjugate acid.

Acetate buffer

To prepare 1 M of acetate buffer, 13.6 g of sodium acetate was dissolved in 100 ml of distilled water. The pH (4.5-5.5) was adjusted with acetic acid.

Tris-HCl buffer

Tris-HCl buffer (1 M) was prepared by weighing 24.2g of Tris (hydroxymethyl) aminomethane and dissolving in 100 ml of distilled water, the pH (7.5-8.0) was adjusted with hydrochloric acid