ASSESSMENT OF THE CAPACITY OF *ASPERGILLUS FLAVUS* AND *TRICHODERMA* SP IN THE REMOVAL OF HYDROCARBONS AND HEAVY METALS FROM RAW REFINERY EFFLUENT

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BY

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AUGUST, 2017

DECLARATION

I hereby declare that the work in this dissertation entitled, **"Assessment of the Capacity of *Aspergillusflavus* and *Trichoderma*sp in the Removal of Hydrocarbons and Heavy Metals From Raw Refinery Effluent"** was carried out by me in the Department of Microbiology, Ahmadu Bello University, Zaria, Nigeria under the supervision of Prof. D.A. Machido and Dr. M.B Tijjani. The information derived from this literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

Zainab AZEEZ _____ _____

Signature Date

CERTIFICATION

This dissertation entitled "ASSESSMENT OF THE CAPACITY OF *ASPERGILLUS FLAVUS* AND *TRICHODERMA* SP. IN THE REMOVAL OF HYDROCARBONS AND HEAVY METALS FROM RAW REFINERY EFFLUENTS"meets the regulations governing the award of Master of Science (Microbiology) degree of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to JESUS CHRIST, the Faithful One and to My Beloved MrMomoh Sati Joseph.

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ABSTRACT

This study assessed the capacity of two fungi *Aspergillusflavus*and*Trichoderma*sp.individually and synergistically to remove hydrocarbon, cadmium (Cd), lead (Pb), and nickel (Ni), from broth cultures charged with raw refinery effluents. Raw effluent from refinery were collected in 200 ml sample bottle and transported to the laboratory for isolation of *Aspergillusflavus*and*Trichoderma*sp. using Potato Dextrose Agar (PDA). The physicochemical qualities of the raw refinery effluent were investigated using standard methods. The effluent contained very high concentrations of oil and grease (26.42 mg/l), COD (171.2 mg/l), Dissolved solids (592.20 mg/l), Conductivity (866 µs/cm) and phosphate (8.1 mg/l), but low in sulphate(39), nitrate(0.01) and pH(7.52) which were within the permissible limit. Biochemical oxygen demand (BOD) was determined at intervals of five days for twenty days to assess the capacity of *Aspergillusflavus*and *Trichoderma* sp. to remove hydrocarbon from raw refinery effluent using the modified Winkler method. It was observed that the amount of hydrocarbon removed increased from day 0 to day 20. The concentrations of the three metals in the raw refinery effluents and tissues of the test fungi were determined both before and after the mycoremediation studies using Microwave Plasma Atomic Emission Spectrophotometer (MP-AES AGILENT 4200). Both the percentage removal as well as the potential of the test isolates to bioaccumulate the metals in their tissues were calculated following standard procedures. It was observed that the two isolates tested could remove from 18 to 29% of Cd, 87 to 95% of Ni and 49 to 79% of Pb. *Trichoderma* sp. proved to be the most efficient in the removal of the three metals from raw refinery effluent while *Aspergillusflavus* was consistently the least efficient. It was also observed that co-culture of*Aspergillusflavus*and *Trichoderma* sp. proved to be more efficient when compared to *Aspergillusflavus* alone but less efficient when compared to

Trichoderma sp. alone. *Trichoderma*sp had the highest potential to bioaccumulate the metals than *Aspergillusflavus*. It was therefore concluded that*Aspergillusflavus*and *Trichoderma*sp. could be employed in the removal of hydrocarbons, Cd, Ni and Pb from heavy metal polluted effluents generated by petroleum refineries and other petro-chemical industries.

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LIST OF ABBREVIATIONS

HC- Hydrocarbon

HCs- Hydrocarbons

PAH- Poly Aromatic Hydrocarbons

PCB- Polychlorinated Biphenyls

TCE- Trichlororinated Biphenyls

MMO- Methane Monoxygenase

MTBE- Methyl Tertiary Butyl Ether

FMENV- Federal Ministry of Environment

EPA- Environmental Protection Agency

KRPC- Kaduna Refinery and Petrochemical Company

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CHAPTER ONE

1.0 INTRODUCTION

With the rapid increase in human population worldwide, there is an increased demand for petroleum products such as diesel, petrol, kerosene and other industrial chemicals (Chakrabarty *et al*., 1998). However, refinery and petrochemical plants generates solid waste and sludge which act globally as environmental pollutant (Smita *et al.,* 2012). Petroleum refinery effluents are wastes originating from industries primarily engaged in refining crude oil and manufacturing fuels, lubricants and petrochemical intermediates (Harry, 1995). The effluents are composed of oil and grease along with many other toxic organic and inorganic compounds. These effluents are a major source of aquatic environmental pollution (Wake, 2005).

Reports from several investigations have shown that hydrocarbons and heavy metals constitute an important group of pollutants often found in effluents released from refineries and other petrochemical plants, common example of such compounds include paraffin's (methane, propane, isobutene), nephthenes (cyclohexane, dimethyl cyclopentane) and aromatics (benzene, toluene, xylenes) (Atlas and Philip, 2005; Benson *et al.*, 2007; Hargrave *et al.,* 2000 and Lee *et al.,* 2000). These hydrocarbons and heavy metals are biopersistent, bioaccumulative and can cause deleterious effects to aquatic fauna and flora as well as to humans (Benson *et al.*, 2007). Heavy metals have been shown to pose significant problems to human health. They are said to contaminate food sources in the environment (i.e. through soil, water and air). Excess loading of hazardous wastes has also led to scarcity of potable water and pollution of soil, thus limiting crop production (Hargrave *et al.,* 2000; Lee *et al.,* 2000). Metals may be accumulated, concentrated and magnified within food chains, causing organisms at higher trophic levels to become contaminated with high concentrations of chemical pollutants and metal contaminants than their preys (Hargrave *et al.,* 2000; Lee *et al.,* 2000). Effluents from refinery operations do contain

toxic and hazardous materials that settle in rivers as part of the bottom sediment. They pose health hazards to the urban population that depends on the water as source of supply for domestic uses (Rahman *et al.*, 2005). Metal toxicity appears in metabolic processes like nitrogen fixation, nitrogen reduction, irregularities on enzyme synthesis (Nwuche and Ugoji, 2008). The treatment of effluent generated by industrial activity is a major concern for plant operators and in particular those of refineries and petrochemical units (Arpita *et al.*, 2014). Industrial effluents are conventionally treated using a variety of hazardous chemicals for pH correction, sludge removal, colour and odour removal. Extensive use of chemicals for effluent treatment results in huge amount of sludge which forms the so called hazardous solid waste generated by the industry and finally disposed of by depositing them in landfills, the conventional methods are inefficient but effluents are readily degradable by microbes, hence hydrocarbons can be removed (Gupta and Mukerji, 2001)

Microorganisms have been used to remove organic matter and toxic chemicals from domestic and industrial waste discharges (Gupta and Mukerji, 2001; Varjani and Srivastava, 2015). In 2011, Nilanjana and Preethy reviewed the action of microorganisms on hydrocarbons. Among the microorganisms, fungi can produce a wide range of enzymes that facilitate the breakdown of recalcitrant compounds such as phenolic compounds, dyes and hydrocarbons, among others, through non-specific oxidation reactions (Giraud *et al.*, 2001; D'Annibale *et al.*, 2004; Jaouani *et al.*, 2005). Fungi can also be explored for removal of heavy metals because of their significant physiological and morphological characters (Vimala and Das, 2009). Fungi have a potential application in protection of environment from heavy metals toxicity and recovery of precious metals (Vimala and Das, 2009). The removal of hydrocarbon by fungi is achieved through the production of extra-cellular and intracellular enzymes which catalyze various reactions

(Paszezynski and Crowford, 2000). Fungi take up metals actively (bioaccumulation), passively (biosorption) and by transformation (biologically catalyzed redox reaction and solubilisation). In biosorption process, microbes uptake metal ions only onto the cell surfaces, whereas intracellular sequestration of metal ions takes place in bioaccumulation and transformation processes by living organisms (Sag and Kutsal, 2000). Fungi also have a greater resistance to inhibitory compounds typical of all petroleum refinery effluents (Van Hamme and Sigh, 2003; Ayenimo *et al.*, 2005; Emoyan *et al.*, 2006; Dugal and Gangawane, 2012). The hyphal growth of fungi provides a greater protection to their sensitive organelles. The larger surface area of fungi acts like an adsorption layer of extra-polysaccharide matrix, protecting them from inhibitory compounds (Dugal and Gangawane, 2012). Moreover, fungi are eukaryotes, having considerably more genes than bacteria, which further make them more versatile in tolerating inhibitory compounds (Guest and Smith, 2002). The higher number of genes in fungi imparts greater reproductive selectivity, which might result in better environmental adaptations (Bennett and Lasure, 1991). In the process of removing organic carbon from the refinery effluent, microorganisms consume hydrocarbons as a carbon source, organic carbon is being removed, carbon dioxide and water are released as by-products (mineralization). This research studied the removal of hydrocarbons and heavy metals from raw refinery effluents using *Aspergillus flavus* and *Trichoderma* sp.

1.1 Statement of Research Problem

Refining of petroleum produces large amounts of effluents that are toxic and about 5-10% of inputs are released as effluents often without adequate treatment (Emoyan *et al.*, 2006; Agalino

and Eyinla, 2009; Al-Malack and Muhammad, 2013). When effluent are released into the environment, terrestrial and aquatic sites that serve as recipients of raw and partially treated refinery effluents could be contaminated. Such contaminated habitats lose their capability to support both plant and animal life and thus constitute public health and socio-economic hazards as well as pose serious aquatic toxicity problems (Okerentugba and Ezeronye, 2003). Petroleum oil is a serious threat to the ecology. Hydrocarbons contain substances that are toxigenic, carcinogenic and mutagenic to the flora and fauna found in the ecosystem (Al-Malack and Muhammad, 2013). The kinetics of fungal processes in the removal of hydrocarbon and heavy metals, especially *Aspergillus flavus* and *Trichoderma* sp. are more novel and not so exhaustively developed and thus it is necessary to establish a better understanding of fungal systems to gain acceptance as a wastewater treatment technology (Fernandez-Luqueno *et al.,* 2010).

The problem of heavy metal pollution is the lack of reliable, efficient and cost effective method of waste treatment that ensures their removal. Most of the physicochemical methods of waste treatment available are fraud with many problems (Ahluwalia and Goyal, 2007; Wang and Chen, 2009). Importantly, these methods have been shown to be inefficient in the removal of heavy metal ions from refinery effluents (Ajao *et al.,* 2014).

1.2 Justification for the Research

Reports from several investigations have shown that hydrocarbons constitute an important group of pollutants often found in effluents released from refineries and other petrochemical plants (Atlas and Philip, 2005; Benson *et al.,* 2007; Nwachukwu, 2000). In recent times, use of fungi for removing hydrocarbons from raw refinery effluents is considered more prolific, when compared to other methods of remediation, since the metabolic activities of these

microorganisms lead to complete breakdown of organic compounds into non-toxic compounds potentially ending in their mineralization. *Aspergillus flavus* and *Trichoderma* sp. have been listed amongst fungi isolated from Kaduna raw refinery effluents (Machido *et al.*, 2014). The populations of microorganisms found in polluted environment will degrade petroleum differently and at different rates than microorganisms in relatively unpolluted environment (Obire and Putheti, 2009)and these organisms have earlier been reported as hydrocarbon bio-degraders by Oudot *et al.*, (1998); April *et al.*,(2000) and Uzoamaka *et al.*, (2009). These organisms also produce laccase, cellulase and xylanase enzymes (Sathiyavathi and Parvatham 2011). According to Pradub and Wattanachai (2012), *Trichoderma* sp showed high efficiency of enzyme activity for biodegradation compared to other fungi isolated in their studies, and this contributes significantly in improving the effluent quality at the point of discharge into the environment. Pigment and extracellular polymers located in *Aspergillus flavus* can reduce the toxic effect of heavy metals.

Application of microorganisms for effective removal of hydrocarbon contamination from effluent has been considered by several workers since other methods lead to production of toxic compounds and these techniques are non-economic also.

1.3 Aim of the Research

The aim of the study was to assess the capacity of *Aspergillus flavus* and *Trichoderma* sp. in the removal of hydrocarbons and heavy metals from raw refinery effluents.

1.4 Objectives of the Research

The specific objectives were to:

1. determine the physicochemical properties of the raw refinery effluents.
2. isolate and identify *Aspergillus flavus* and *Trichoderma* sp. from refinery effluents.

3. assess individually and as co-culture, the capacity of *Aspergillus flavus* and *Trichoderma* sp. in the removal of hydrocarbons from the raw effluents.
4. assess individually and as co-culture, the capacity of *Aspergillus flavus* and *Trichoderma* sp. in the removal of heavy metals from the raw effluents

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History of the Nigerian Petroleum Industry

Oil was discovered in Nigeria in 1956 at Oloibiri in the Niger Delta after half a century of exploration. The discovery was made by Shell British Petroleum (Shell-BP), at the time the sole concessionaire. Nigeria joined the ranks of oil producers in 1958 when its first oil field came on stream producing 5,100 barrel per day (bpd). After 1960, exploration rights in onshore and offshore areas adjoining the Niger Delta were extended to other foreign companies. (Oluwande *et al.,* 1993).

In 1970, the end of the Biafran war coincided with the rise in the world oil price and Nigeria was able to reap instant riches from its oil production. Nigeria joined the Organisation of Petroleum Exporting Countries (OPEC) in 1971 and established the Nigerian National Petroleum Company (NNPC) in 1977; a state owned and controlled company, which is a major player in both the upstream and downstream sectors (Oluwande *et al.,* 1993).

Following the discovery of crude oil by Shell D'Arcy Petroleum, pioneer production began in 1958 from the company's oil field in Oloibiri in the Eastern Niger Delta. By the late sixties and early seventies, Nigeria had attained a production level of over 2 million barrels of crude oil a day. Although production figures dropped in the eighties due to economic slump, 2004 saw a total rejuvenation of oil production to a record level of 2.5 million barrels per day. Current development strategies are aimed at increasing production to 4 million barrels per day by the year 2010 (Oluwande *et al.,* 1993). Petroleum production and export play a dominant role in Nigeria's economy and account for about 90% of her gross earnings. This dominant role has

pushed agriculture, the traditional mainstay of the economy, from the early fifties and sixties, to the background (Oluwande *et al.,* 1993).

2.2 Refining of Petroleum

Petroleum is a complex mixture of organic liquids called crude oil and natural gas, which occurs naturally in the ground and was formed millions of years ago. Crude oil is a mixture of hydrocarbons with different boiling temperatures it varies from oilfield to oilfield in colour and composition, from a pale yellow low viscosity liquid to heavy black 'treacle' consistencies (Australian Institute of Petroleum ACT, 2600).

Crude oil and natural gas are extracted from the ground, on land or under the oceans, by sinking an oil well and are then transported by pipeline and/or ship to refineries where their components are processed into refined products. Crude oil and natural gas are of little use in their raw state; their value lies in what is created from them: fuels, lubricating oils, waxes, asphalt, petrochemicals and pipeline quality natural gas (Australian Institute of Petroleum ACT, 2600).

An oil refinery is an organised and coordinated arrangement of manufacturing processes designed to produce physical and chemical changes in crude oil to convert it into everyday products like petrol, diesel, lubricating oil, fuel oil and bitumen (Australian Institute of Petroleum ACT, 2600).

As crude oil comes from the well, it contains a mixture of hydrocarbon compounds and relatively small quantities of other materials such as oxygen, nitrogen, sulphur, salt and water. In the refinery, most of these non - hydrocarbon substances are removed and the oil is broken down into its various components and blended into useful products (Australian Institute of Petroleum ACT, 2600) .

There are many methods of treatment available to sites that have been contaminated. Some of these treatment methods are ex-situ while others are in-situ. Ex-situ processes require removing the soil or water from the site and transporting the waste to a treatment facility. In-situ processes take place with minimal disruption of the contaminated area (Australian Institute of Petroleum ACT, 2600).

2.3 Refineries and the Environment

Air, water and land can all be affected by refinery operations. Refineries are well aware of their responsibility to the community and employ a variety of processes to safeguard the environment. The processes described in this text are those used by the Shell refinery at Geelong in Victoria, but all refineries employ similar techniques in managing the environmental aspects of refining (Halen, 2005).

Preserving air quality around a refinery involves controlling sulphur oxides, hydrocarbon vapours and smoke and smells emission. Sulphur enters the refinery in crude oil feed, crude's may contain up to 5 per cent sulphur. To deal with these refineries incorporate a sulphur recovery unit. Many of the products used in a refinery produce hydrocarbon vapours. The escapes of vapours to atmosphere are prevented by various means. Floating roofs are installed in tanks to prevent evaporation and so that there is no space for vapour to gather in the tanks. Where floating roofs cannot be used, the vapours from the tanks are collected in a vapour recovery system and absorbed back into the product stream. In addition, pumps and valves are routinely checked for vapour emissions and repaired if a leakage is found. Smoke is formed when the burning mixture contains insufficient oxygen or is not sufficiently mixed (Halen, 2005). Modern furnace control systems prevent this from happening during normal operation. Smells are the

most difficult emissions to control and the easiest to detect. Refinery smells are generally associated with compounds containing sulphur, where even tiny losses are sufficient to cause a noticeable odour (Halen, 2005).

Aqueous effluents consist of cooling water, surface water and process water. The majority of the water discharged from the refinery has been used for cooling the various process streams. The cooling water does not actually come into contact with the process material and so has very little contamination. The cooling water passes through large "interceptors," which separate any oil from minute leaks etc., prior to discharge. Rainwater falling on the refinery site must be treated before discharge to ensure no oily material washed off process equipment leaves the refinery. This is done first by passing the water through smaller "plant oil catchers", which each treats rainwater from separate areas on the site, and then all the streams pass to large "interceptors" similar to those used for cooling water. The rainwater from the production areas is further treated in a Dissolved Air Flotation (DAF) unit. This unit cleans the water by using a flocculation agent to collect any remaining particles or oil droplets and floating the resulting flock to the surface with millions of tiny air bubbles. At the surface, the flock is skimmed off and the clean water discharged. Process water has actually come into contact with the process streams and so can contain significant contamination. This water is treated in the "sour water treater" where the contaminants (mostly ammonia and hydrogen sulphide) are removed and then recovered or destroyed in a downstream plant. The process water, when treated in this way, can be reused in parts of the refinery and discharged through the process area rainwater treatment system and the DAF unit. Any treated process water that is not reused is discharged as Trade Waste to the sewerage system. This trade waste also includes the effluent from the refinery sewage treatment plant and a portion of treated water from the DAF unit (Halen, 2005).

The refinery safeguards the land environment by ensuring the appropriate disposal of all wastes. Within the refinery, all hydrocarbon wastes are recycled through the refinery slops system. This system consists of a network of collection pipes and a series of dewatering tanks. The recovered hydrocarbon is reprocessed through the distillation units. Wastes that cannot be reprocessed are either recycled to manufacturers (e.g., some spent catalysts can be reprocessed), disposed of in Environmental Protection Agency (EPA)-approved facilities off-site or chemically treated on-site to form inert materials, which can be disposed of in land-fill within the refinery. Waste movements within the refinery require a "Process liquid, Sludge and Solid waste disposal permit". Wastes that go off-site must have an EPA "Waste Transport Permit"(Halen, 2005).

2.4 Environmental Impacts of Refinery Effluents

While the petroleum refinery and petrochemical industries are most desirable for national development and improved quality of life, the unwholesome and environmentally unacceptable pollution effects of the wastes from these industries are a cause for worry. This is because in the process of converting crude oil into petroleum products (liquefied petroleum gas, naphtha, kerosene, diesel oil and residual oil) and petrochemical products (polypropylene, polyethylene), wastes of different kinds are generated. These wastes can be broadly categorized into oily materials, spent chemicals, spent catalysts and other residuals. These wastes are released to the environment in the form of gases, particles and liquid effluents (liquid consisting of surface runoff water, sanitary wastewater, solid waste and sludge) (World Bank, 1998). The wastewater released from the refineries are characterized by the presence of large quantity of crude oil products, polycyclic and aromatic hydrocarbon, phenols, metal derivatives, surface active substances, sulphides, naphthalene acids and other chemicals (Suleimanov, 1995).

The appropriate disposal of effluents generated in oil refineries has been of great concern because of the speed and intensity of the environmental damage they can provoke. Effluents from industrial processes in oil refineries can pollute the environment, representing a danger to all forms of life when not disposed of appropriately. It becomes the source of pollution for surface and subsurface water, soil and air. Wastewater deteriorates not only the quality of soil, crop and environment but are also directly harmful to the human, animal and aquatic lives. Unplanned discharges of industrial wastewater degrade the quality of food crops. The total land irrigated with raw or partially diluted wastewater has been estimated to be about 20 million hectares in fifty countries, which is approximately 10% of total irrigated land (Bruinsma, 2003).

As a result of ineffectiveness of purification systems, wastewater may become seriously dangerous, leading to the accumulation of toxic products in the receiving water bodies with potentially serious consequences on the ecosystem (Beg *et al.,* 2003; Aghalino and Eyinla, 2009). The uncontrolled disposal of wastes into water renders water unsafe for economic use, recreational use and poses a threat to human life and it is also against the principle of sustainable development. Water-borne diseases and water-caused health problems are mostly due to incompetent management of water resources. Safe water for all can only be assured when access, sustainability and quality can be guaranteed. Urban areas generally have a higher coverage of safe water than rural areas. Even within the urban area, there are variations in the quality of water as much of the water get contaminated in many different ways, through industrial effluent and untreated municipal sewage (Oluwande *et al.,* 1993; Atubi, 2009a). Kuehn *et al.,* (1995) observed that refinery effluent contaminated with aromatic hydrocarbons produces poor health and lethal toxicity in fishes and two species of *Tilapia*. Onwumere and Oladimeji (1990) earlier demonstrated accumulation of heavy metals with accompanying histopathology in *Oreochromnis*

niloticus exposed to treated petroleum refinery effluent from the Kaduna Refining and Petrochemical Company. These and other studies agree that petroleum refinery effluents pose a serious problem to both aquatic and human life forms.

Drinking contaminated water can cause various diseases such as typhoid and paratyphoid fevers, dysentery, cholera and other intestinal disorders (Adeyemi, 2004). According to Gore (1993), human beings are made up of water, in roughly the same percentage as water in the surface of the earth. Our tissues and membranes, brains, and hearts, our sweat and tears, all reflect the same recipe for life. Water is essential for the development and maintenance of the dynamics of every ramification of the society (United Nations Development Program, 2006). Water is indeed life and thus is the most important natural resource, without which life would be non-existent. Availability of safe and reliable source of water is an essential prerequisite for sustained development (Asonye *et al*., 2007).

Oil in the aquatic environment may be damaging in a variety of ways. These may involve changes in the composition of aquatic communities that affect their ability to survive, permanent damage and, in some cases, massive mortalities. Odour, taste and colour are present in oil polluted water. Oil pollution of water also constitutes a potential health risk to humans who use such water for domestic and drinking purposes and consume fish found therein (Helmer, 2006).

2.5 Biodegradation

Biodegradation is defined as the biologically catalyzed reduction in complexity of chemical compounds (Alexander, 1994). Indeed, biodegradation is the process by which organic substances are broken down into smaller compounds by living microbial organisms (Marinescu *et al.,* 2009). When biodegradation is complete, the process is called "mineralization". However, in most cases the term biodegradation is generally used to describe almost any biologically

mediated change in a substrate (Bennet *et al.,* 2002). So, understanding the process of biodegradation requires an understanding of the microorganisms that make the process work. The microbial organisms transform the substance through metabolic or enzymatic processes. It is based on two processes: growth and cometabolism. In growth, an organic pollutant is used as sole source of carbon and energy. This process results in a complete degradation (mineralization) of organic pollutants. Cometabolism is defined as the metabolism of an organic compound in the presence of a growth substrate that is used as the primary carbon and energy source (Fritsche and Hofrichter, 2008). Several microorganisms, including fungi, bacteria and yeasts are involved in biodegradation process. Algae and protozoa reports are scanty regarding their involvement in biodegradation (Das and Chandran, 2011). Biodegradation processes vary greatly, but frequently the final product of the degradation is carbon dioxide (Pramila *et al.,* 2012). Organic material can be degraded aerobically, with oxygen, or anaerobically, without oxygen (Fritsche and Hofrichter, 2008; Mrozik *et al.,* 2003). Biodegradable matter is generally organic material such as plant and animal matter and other substances originating from living organisms, or artificial materials that are similar enough to plant and animal matter to be put to use by microorganisms. Some microorganisms have the astonishing, naturally occurring, microbial catabolic diversity to degrade, transform or accumulate a huge range of compounds including hydrocarbons (e.g. oil) polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), radionuclides and metals (Leitão, 2009)

Accepted disposal methods of incineration or burial insecure landfills can become prohibitively expensive when amounts of contaminants are large. Mechanical and chemical methods generally used to remove hydrocarbons and heavy metals from contaminated sites have limited effectiveness and can be expensive. Biological methods harness natural processes in order to

meet today's demands for cleaning and waste elimination without the use of potentially-harmful chemicals. The biodegradation of these compounds is often a complex series of biochemical reactions and is often different when different microorganisms are involved.

2.5.1 Approaches to Biodegradation of Hydrocarbons

There have been several approaches to treat soil and water contaminated with hydrocarbons:

2.5.1.1 ***Phytoremediation*****:** This strategy makes use of plants for bioremediation, but is outside the scope of this review.

2.5.1.2 ***Use of microbial consortium***: This involves use of multiple microbial species together rather than relying on catabolic capacity of any single species. Consortium can be in form of microbial biofilms, where production of certain biosurfactants can enhance oil degradation by increasing its bioavailability. Biofilms may be single-species or multi-species. Some organisms apply behavioural strategies such as adhesion and biofilm formation to acquire carbon and energy from hydrophobic organic compounds (HOCs) contained in marine aggregates. When more than one type of organisms is present at the contaminated site, some engage in co-metabolism (fortuitous metabolism), wherein a microorganism transforms the given compound without being able to grow on it or derive energy from it; this transformed compound can be used as a growth substrate by another microorganism present in the same environment or in a mixed culture. Co-metabolic transformations may gradually lead to recycling of recalcitrant compounds on which no individual microbial culture can grow.

2.5.1.3 ***Stimulation of anaerobic degradation using alternative electron acceptor***: Anaerobic degradation can be used as an alternative where aerobic conditions cannot be maintained. Several alternative electron acceptors have been proposed for use in anaerobic degradation, including nitrate, sulphate, iron (Fe^{3+}), and carbon dioxide (*Slonczewski and Foster, 2011).*

2.5.1.4 ***Nutrient augmentation (biostimulation)***: Nutrients are very important ingredients for successful biodegradation of hydrocarbon pollutants especially nitrogen, phosphorus, and in some cases iron (Nilanjana and Preethy, 2011). Some of these nutrients could become limiting factor thus affecting the biodegradation processes. Assimilation of hydrocarbons may be done by microorganisms already present in an environment, albeit at a slow pace. However, relying solely on this small population of naturally-present organisms is not sufficient for effective bioremediation in most cases. Natural environments are often deficient in nutrients. Microbial growth rate can be accelerated to enhance soil remediation by providing exogenous supply of nutrients, especially nitrogen and phosphorous to the contaminated soil. Other nutrients present in limiting concentrations can also be added. This approach is known as nutrient augmentation. Hydrocarbons degradation in contaminated soil can be enhanced by applying surfactants (along with other nutrients) to soil, which makes hydrocarbons more easily available to microorganisms.

2.5.2 Co-metabolic Biodegradation

Co-metabolism is the process by which a contaminant is fortuitously degraded by an enzyme or cofactor produced during microbial metabolism of another compound. Typically, there is no apparent benefit to the microorganisms involved. Bioremediation strategies that use electron donors that only stimulate a specific group of microorganisms that can degrade the contaminants of concern are ideal for many applications. Many electron donors used as amendments for bioremediation can broadly stimulate many members of the indigenous microbial community, most of which do not have the ability to degrade or completely degrade the contaminants of concern. Indeed, this often creates problems of excess biomass (e.g., plugging the aquifer around the injection site), incomplete degradation of contaminants, transformation of contaminants to

more recalcitrant or toxic daughter products, higher costs (amendment/ contaminant) and inability of the amendment to stimulate biodegradation at low contaminant concentrations. Co-metabolic bioremediation enables remediation strategies that stimulate biodegradation of the contaminants at contaminant concentrations that are well below the concentration that could be of carbon or energy benefit to the degrader. Thus, co-metabolic bioremediation has the added advantage of allowing scrubbing of environmental contaminants down to undetectable concentrations, e.g., parts per trillion. Co-metabolic bioremediation has been applied both aerobically and anaerobically to a wide variety of contaminants in different environments. The first mention of co-metabolic bioremediation was by Wilson and Wilson in 1985 and was later defined by McCarty (1987). Co-metabolic bioremediation has been used in the field for more than 20 years on some of the most recalcitrant contaminants, e.g., chlorinated alkenes, polyaromatic hydrocarbons, halogenated aliphatic and aromatic hydrocarbons, methyl tertiary butyl ether (MTBE), explosives, dioxane, polychlorinated biphenyls (PCB) and pesticides.

Microorganisms are versatile in their ability to exist in a variety of habitats and live in hostile environments having a wide range of pH, temperature, heavy metal concentrations, oxygen concentrations, barometric pressures, salinity and radiation. Under these diverse conditions, a number of microbial types have been isolated that co-metabolize contaminants and their daughter products. Ensley (1991) demonstrated a linkage between trichloroethylene (TCE) degradation and aromatic metabolism in *Pseudomonas cepacia* G4, *P. mendocina* and *P. putida*. Ensign *et al.* (1992) reported that pure cultures of *Xanthobacter* sp. co-metabolized TCE with the utilization of propylene as a substrate using the enzyme alkene monooxygenase. The aerobic co-metabolic degraders are dependent upon oxygenases, e.g., methane monooxygenase (MMO), toluene dioxygenase, toluene monooxygenase, and ammonia monooxygenase. These enzymes are

extremely strong oxidizers, e.g., methane monooxygenase is known to degrade over 300 different compounds.

2.5.3 Biodegradable Organic Substances

The contaminants that may be degraded via bioremediation can be subdivided into five main categories (Hickey and Smith, 1996):

- Organic solvents
- Polyaromatic hydrocarbons (PAH) (creosote oily wastes)
- Halogenated aromatic hydrocarbons
- Pesticides
- Munitions wastes

Bioremediation is generally viewed as a new technology; however, microorganisms have been used for the treatment and transformation of waste products for at least 100 years. Municipal wastewater systems are based on the use of microorganisms in a controlled and engineered environment (King *et al.*, 1992).

2.5.4 Advantages of Bioremediation

There are many advantages to the use of bioremediation for treatment of hazardous waste sites (Alexander, 1994):

- Bioremediation is an environment friendly approach and is therefore accepted by the public as a remedy in the treatment of contaminants
- Biological systems are often less expensive than conventional treatment
- Bioremediation can be done on site and site disruption is minimal
- It eliminates waste and also eliminates the chance of future liability associated with treatment and disposal of contaminated material

- The microorganisms involved in the degradation of contaminant increases in their number till the contaminant is absent. After the degradation of the contaminant, the microbial population itself decreases naturally
- Bioremediation transforms the toxic substances to harmless products such as CO_2 (utilized by plants in photosynthesis), H_2O and fatty acids and can be coupled with other treatment techniques to form a treatment terrain

One drawback of bioremediation is that only a selected few bacteria and fungi act on a broad range of organic compounds. To date, no organism is known that is sufficiently versatile that it will destroy a large percentage of the natural chemicals present.

Another drawback to bioremediation is that it requires a large amount of time to obtain detectable results. However, this is only a draw back if time is a constraint on the clean-up of the site.

2.6 Bioremediation by Fungi

Some fungi exude extracellular enzymes, which allow for digestion of energy sources in their surroundings and further diffusion of these molecules through the substrate towards the fungus (Mai *et al.*, 2004). Fungi possess these decomposing abilities to deal with wide array of naturally-occurring compounds that serve as potential carbon sources. Hydrocarbon pollutants have similar or analogous molecular structures, which enable the fungi to act on them as well (Fernandez-Luqueno *et al.*, 2010). Fungi attack plastic polymers as well; these come in a wide range of structures as lignin and are acted upon by different fungi species for different polymers. This decomposing ability is perhaps even more impressive than PAH decomposition. Fungi have been found to be effective in degradation of PAHs as well as bioremediation of toxic metals as

well, which are commonly found in the same polluted sites and can reduce the effectiveness of some degradative microorganisms.

2.6.1. Role of Enzymes in Bioremediation

Oxidoreductase constitutes an important class of enzymes that are involved in humification of phenolic substances produced from decomposition of lignin in soil. They are also capable of detoxification of xenobiotics such as phenolic or anilinic compounds through polymerization or copolymerization with other substrates (Park *et al.*, 2006). Activity of fungi is seen due to action of extracellular oxidoreductase enzymes, like laccase, manganese peroxidase and lignin peroxidase, released from fungal mycelium. Fungi are more efficient in reaching soil pollutants as compared to bacteria (Rubilar *et al.*, 2008).

An important group of ubiquitous oxidoreductase enzymes that have displayed potential for biotechnological and bioremediation applications are laccases. Pollutants are degraded by specific oxygenases, which mediate dehalogenation reactions of halogenated methanes, ethanes and ethylenes along with multifunctional enzymes. Extracellular enzyme activity is a key step in degradation and utilization of organic polymers, since only compounds with molecular mass lower than 600 daltons can pass through cell pores (Vasileva-Tonkova and Galabova, 2003). Hydrolytic enzymes disrupt major chemical bonds in the toxic molecules and results in the reduction of their toxicity. This mechanism is effective for the biodegradation of oil spill and organophosphate and carbamate insecticides. The most useful indicator for testing hydrocarbon degradation in soil is lipase activity. Though its production cost is high, lipase enzyme plays very important role in food, chemical, detergent manufacture, cosmetic and paper making industries (Sharma *et al.*, 2011).

2.6.2 Fungi for Bioremediation of Hydrocarbon Pollutants

Fungi are the decomposers in the global cycle of life and death. They are usually there to do the work when anything--animal, plant or even non-living object, is ready to be broken down again into its inorganic constituents. Fungi are found in soil, in fresh and sea waters, inside the bodies of plants and animals, and travelling through the air as spores. While they often are found functioning together with bacteria and an array of microorganisms, it is fungi that can especially handle breaking down some of the largest molecules present in nature (Fernandez-Luqueno *et al.*, 2010). Their growth patterns facilitate the investigation of a wide range of environments in search of energy sources. Fungi can either grow in a multicellular form, with the somatic mycelium extending minute root-like structures through the substrate; or it can be present as unicellular yeast. Some fungi exude extracellular enzymes which allow for digestion of energy sources in their surroundings and further diffusion of these molecules through the substrate towards the fungus (Mai *et al.,* 2004). If fungi occupy the niche of decomposers, then what is their particular relationship with the ever-increasing quantity of stuff that humans have manufactured and left to rot? One of the largest categories of inputs to the environment in need of decomposition is man-made hydrocarbon waste. Large reserves of hydrocarbons that were previously stored deep underground are being brought to the surface, altered and used. A majority of the pollution that occurs now involves fossil fuels, whether it is the exhaust and byproducts of spent fuel or the accumulated polymer plastics made from these same hydrocarbons. Fossil fuels are composed of polycyclic aromatic hydrocarbons (PAHs) as well as shorter carbon molecules. Many PAHs are naturally occurring (Prenafeta-Boldu *et al.,* 2004) in plants and animals; these were the raw ingredients that first decomposed to form fossil fuel reserves. PAHs are building blocks of life and they are very common on the planet. However, the

accumulation and chemical alteration of these PAHs are following a pattern now dominated by the actions of humans (Prenafeta-Boldu *et al.,* 2004). PAHs form when large amounts of PAHs are extracted, refined and transported, and contamination of the environment occurs frequently all over the world. Burning fossil fuels, manufacturing gas and coal tar, wood processing, fuel burning kitchen stoves and incinerating wastes are some of the ways PAHs escape into the environment. This is some of the most widespread pollution in the world, and because of the hydrophobic nature of PAHs, they can easily accumulate in fatty tissue and spread throughout the food chain. Seven of the sixteen PAHs listed as pollutants by the environmental protection agency (EPA) are carcinogenic, teratogenic, and mutagenic (Prenafeta-Boldu *et al.*, 2004). Fungi possess these decomposing abilities to deal with the array of naturally-occurring compounds that serve as potential carbon sources. Hydrocarbon pollutants have similar or analogous molecular structures, which enable the fungi to act on them as well (Fernandez- Luqueno *et al.*, 2010). Furthermore, Peng *et al.* (2008) reported that the genes responsible for PAH degradation are present as many homologous loci within the genome, which provides a particularly large pool of mutation and rearrangement possibilities within that gene family.

In addition, microorganisms have within them a number of other stress responses that generate more phenotypic and genetic diversity so that they can deal with a new environmental stress (Fernandez-Luqueno *et al.,* 2010). In one study, sexual reproduction in soil fungi was found to increase under ecologically stressful conditions (Grishkan *et al.*, 2003). This shift in reproduction increases genetic diversity, and it can be seen at different levels within the fungal community. At the species level, the sexual ascomycetes increased in their relative abundance to asexual fungi as stress in the environment increased. At the level of the individual, they found the sexual fungi spending more time in a teleomorphic state (sexual ascospore state) versus an anamorphic

asexual state when stress levels were higher. They hypothesized that residence of the fungi in an extreme environment that is often changing leads to selection for the ability to adapt. This is expressed through selection for genetic diversity and individuals' responses to stress such as increased sexual reproduction (Grishkan *et al.,* 2003).These observations point to fungi's ability to adapt to changing environment (Gautam *et al.,* 2006). Abilities that have not been found yet in fungi relating to the degradation of pollutants could be evolving right now in some much polluted pocket of soil.

Fungi are especially well-suited to PAH degradation relative to other bacterial decomposers for a few reasons. They can degrade high molecular-weight PAHs, whereas bacteria are best at degrading smaller molecules (Peng *et al.,* 2008). They also function well in non-aqueous environments where hydrophobic PAHs accumulate; a majority of other microbial degradation occurs in aqueous phase. They can also function in the very low-oxygen conditions that occur in heavily PAH contaminated zones (Fernandez-Luqueno *et al.,* 2010). A review of different studies by Fernandez-Luqueno *et al.* (2010) yielded a list of over 51 fungal species or specie groups that are successful at degrading different PAHs. A wide variety of fungi have evolved effective mechanisms to attack specific PAHs. One reason for this ability lies in the similarity between lignin, a long, aromatic family of molecules that is present in wood and PAHs. Lignin is one of the main components of woody tissue in all vascular plants along with cellulose and hemicellulose. It has been described as the cement in woody tissue that adds strength and flexibility to cellulose. This is the substance that gives trees the strength to grow taller towards the light and provides the crunch of vegetables (McCready, 1991). Fungi produce extracellular enzymes to degrade lignin, which cannot pass through the cell walls of microorganisms. This process of degradation is called mineralization, and the end product is carbon dioxide. Since

lignin is comprised of many different aromatic rings in long varied chains, the fungal enzymes for mineralization are non-specific and frequently can also mineralize PAHs (Mai *et al.,* 2004). These basidiomycetes have at least two pathways. One pathway is the cytochrome 450 system, much like the system in mammal livers, which break down large molecules into metabolites; however, many of these metabolites are toxic themselves. The lignin extracellular degradation pathway is preferable because the metabolites are fully broken down into carbon dioxide.

Litter-decomposing basidiomycete fungi also have substantial ability to degrade PAHs. Although their performance is not as high as typical white-rot fungi, litter-decomposing fungi are native to the soil environment in which most PAH contamination is found, and so their long-term ability to exist and function in PAH-contaminated soil could be greater than that of the wood-inhabiting white-rot fungi (Steffen *et al.,* 2002).

Fungi have an astonishing potential to clean up contaminated environments. After looking at the list of fungi that can degrade different PAHs, one could imagine that there is a fungus out there to degrade every type of persistent pollutant, and each one only has to be found. Polluted sites can naturally become incubators for the few species that can consume the pollutant. This is welcome.

2.6.2.1 *Enzymes Involved in Hydrocarbon Biodegradation*

Over the years, investigators have characterized pathways of microbial degradation of hydrocarbons and enzymes involved. Cytochrome P450 alkane hydroxylases constitute a super family of ubiquitous heme-thiolate monooxygenases, which play an important role in the microbial degradation of oil, chlorinated HCs and fuel additives (Van Beilen and Funhoff, 2005). The diversity of alkane oxygenase systems in prokaryotes and eukaryotes that actively participate in the degradation of alkanes under aerobic conditions like cytochrome P450

enzymes, integral membrane di-iron alkane hydroxylases (e.g. *alkB*), soluble di-iron methane monooxygenases and membrane-bound copper containing methane monooxygenases has been discussed by Van Beilen and Funhoff (2005).

2.6.3 Mechanisms of Metabolism of Petroleum Hydrocarbons

Hydrocarbon-degrading fungi have been known for more than five decades, but our understanding of mechanisms involved in the catabolic breakdown of hydrocarbons is still incomplete. Certain aspects of fungal metabolism of hydrocarbons are similar to the metabolism of both higher eukaryotic organisms and bacteria (Smith and Rosazza, 1974). Like bacteria, fungi participate in the transformation reactions and assimilate hydrocarbons as the sole source of carbon and energy for growth, resulting in the formation of carbon dioxide. The fungal mechanisms of metabolism of petroleum hydrocarbon have been discussed by many researchers and divided into four categories.

2.6.3.1 *Aliphatic Hydrocarbons*

*T*he most thoroughly studied aliphatic hydrocarbons are *n*-alkanes of C10 to C20 that can be metabolized rapidly. Methyl alkanes may also be metabolized but with less growth. The degradation of long-chain alkanes greater than C24 can occur in complex mixtures, but no major studies are known to support fungal growth. Kremer and Anke (1997) listed the alkane-assimilating genera of fungi.

As with most microorganisms, the most common major pathway of alkane metabolism is monoterminal oxidationto the corresponding alcohol, aldehyde and fatty acid. This involves a mixed function of alkane monoxygenase, nicotinamide adenine dinucleotide (NAD)-dependent alcohol and aldehyde dehydrogenases. The initial mode of attack is at the site of the terminal methyl group by alkane monoxygenase involving the insertion of molecular oxygen and an

electron transfer system. This array involves the combination of cytochrome P450 as the terminal oxidase and nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450-reductase as the electron transfer component. The following dehydrogenase steps to long-chain alcohols and aldehydes yield fatty acids. Fatty acids are also generated due to alkane assimilation to function as building blocks (Brakemeier *et al*., 1995). Further catabolism involves the initial activation of fatty acids to acyl-CoA ester via an acyl-CoA synthetase and β-oxidation and yields to acetyl-CoA. The acetyl- CoA can either act as an anabolic precursor or is further catabolized to CO2 via a tricarboxylic acid cycle. The possibility exists of terminal oxidation to the corresponding fatty acids followed by β-oxidation for alkane mineralization in marine fungi (Singer and Finnerty, 1984). The slow alkane utilization by marine fungi indicates a lack of constitutive enzymes for the initial oxidation, and this requires further investigation.

Although a monoterminal pathway is most commonly encountered, certain species possess alternative pathways. Some yeasts and species of *Aspergillus*, *Botrytis* and *Penicillium* can oxidize both terminal methyl groups of aliphatic hydrocarbons to produce long-chain dicarboxylic acid, which is known as diterminal oxidation. This oxidation may occur either simultaneously or the ω-methyl group is oxidized after formation of the fatty acids. Another mechanism is subterminal oxidation, which is initiated in the formation of a secondary alcohol, followed by oxidation to the corresponding ketone and ester, which cleaves to yield both a primary alcohol and acetate. Subterminal oxidation is noted in species of *Aspergillus*, *Cladosporium*, *Penicillium*, *Verticillium*, *Cunninghamella echinulata*, *Fusarium lini*, *Mortierella isabellina* and *Rhizopus nigricans* (Hoffmann and Rehm, 1976a; Hoffmann *et al*., 1977; Yi and Rehm, 1982; Kremer and Anke, 1997). Subterminal oxidation can occur in various positions, with formation of a mixture of secondary alcohols whose further metabolism yields the

corresponding primary alcohols and organic acids. Further metabolism of alcohol employs steps of dehydrogenation and β-oxidation. A scheme of the various steps involved in alkane oxidation by Mucorales has also been proposed (Pelz and Rehm, 1973; Hoffmann and Rehm, 1976b).

2.6.3.2 *Aromatic Hydrocarbons*

The metabolism and transformation of aromatic hydrocarbons by fungi have been well established during the past two decades. Several species of fungi have shown the ability to assimilate a wide range of aromatic hydrocarbons. The oxidation of aromatic hydrocarbons is initiated with an epoxidation to arene oxides by cytochrome P450-dependent monoxygenases. Several metabolic pathways are recognized for the degradation of aromatic hydrocarbons (Manuelada *et al*., 2004).

2.6.3.3 *Co-oxidation of Hydrocarbons*

In certain cases, fungi can initiate breaking or modifying complex hydrocarbons despite nonoccurrence of growth. Some degree of partial conversion may occur in the presence of an alternative substrate acting as a source of both carbon and energy. Few fungi can metabolize cycloalkanes. The metabolism of cycloalkanes in fungi appears to proceed via cooxidation. Studies of fungi capable of growth on cyclic aliphatics are rare. Cyclohexane has no terminal methyl groups and is assimilated by a mechanism similar to subterminal oxidation (Perry, 1979). This mechanism involves the formation of dicarboxylic acid, which is subsequently metabolized by β-oxidation. The possibility of cooxidation of alkanes by lignicolous marine fungi is correlated, owing to much better growth on glucose with hydrocarbons (Kirk and Gordon, 1988).

2.6.3.4 *Uptake of Hydrocarbons*

The uptake of hydrocarbons involves penetration of insoluble substrate into a cell with a variety of mechanisms. One of the most acceptable mechanisms is via the transport process. Yeasts accumulate fatty acids within cell material, and filamentous fungi show intracellular aggregation of non-assimilated alkanes during conditions of excessive carbon. At the cell surface, penetration can occur through pores and channels, and hydrocarbon moves towards the plasmalemma. If the initial enzymes are present in the plasmalemma, the aldehydes or fatty acids may enter cytoplasm as a result of activation of hydrocarbons, but the cytochemical location of enzymes does not agree with such a mechanism. Membrane-bound, electron-dense inclusion bodies (vesicles) in *Clado*sp*orium resinae* have been detected during growth on alkanes (Cooney *et al*., 1980). Lindley and Heydeman (1986) described dodecane uptake by whole cells of *C. resinae* in two stages. Here, passive adsorption of the hydrocarbon to the outer cell surface followed by a mechanism that obeys Michaelis-Menten saturation kinetics. This two-stage uptake mechanism is similar to that of the yeast *Candida tropicalis* (Kappeli and Fiechter, 1981). This yeast produces a fatty acid complex that emulsifies the hydrocarbon and thus results in an induced uptake of pure hydrocarbon. The alkane solubilization is indicated by an aqueous phase during transport by *Candida lipolytica* (Goma *et al.,* 1973).

Three steps of alkane metabolism are stated: adsorption to the cell surface, movement through the rigid cell wall via pores or channels, followed by alkane movement via pinocytosis to microbodies and other sites of oxidation (Singer and Finnerty, 1984). They also show the production of extracellular products in *C. lipolytica*, which induces both emulsification and uptake of hydrocarbons. The exact nature of biochemical mechanism of alkane metabolism remains unknown, but the available knowledge suggests the involvement of the physicochemical nature of the cell wall. In eukaryotic microorganisms, hydrocarbon catabolism is associated with

distinct and induced ultrastructural characteristics (Fukui and Tanaka, 1979). The most important changes include the appearance of crystalline peroxisomes in yeasts and fungi. During alkane degradation by *Candida* and other species, peroxisomes are induced in large proportions of the cell volume and in high numbers are similar to methylotrophic yeasts and unlike *Saccharomyces cerevisiae*. The intracellular distribution of various biochemical pathways is located in diverse microbodies (peroxisomes, microsomes and mitochondria) associated with alkane metabolism In *C. Tropicalis* (Tanaka *et al.,* 1982).

2.7 Oxidation of Petroleum Hydrocarbons by Fungal Enzymes

Little is known of the enzymatic oxidation of petroleum hydrocarbons, and this topic is emerging due to regio- and stereoselectivity and mild physiological conditions. Enzymatic oxidation can take the upper hand when success is not achieved with chemical catalysts. Faber (1997) discussed biocatalytic oxidation reactions of alkanes, alkenes, aromatics and heteroatoms. In general, these biotransformations are carried out by microbial cultures. Choloroperoxidase (CPO) has been employed for the enantio-selective epoxidation of alkenes and olefins (Dexter *et al*., 1995). Laccase is well documented with a mediator to oxidize certain aromatic compounds. This is called *mediated oxidation*. Laccase from the *Trichoderma* sp. is employed for the oxidation of hydrocarbon (Sathiyavathi and Parvatham, 2011). This oxidation is a two-step process: (1) the enzyme catalyzes the oxidation of primary substrate, the mediator; and (2) the oxidized mediator oxidizes the secondary substrate, the hydrocarbon.

2.8 Cytochrome P450 Enzyme Systems

During the past decade, the role of cytochrome P450 enzymes in several complex fungal bioconversions has been established (van den Brink *et al*., 1998). Cytochrome P450-mediated

bioconversions in filamentous fungi involve assimilation of long-chain alkanes and polycyclic aromatic hydrocarbons (PAHs). A well-studied example of alkane assimilation involving P450 process is carried out by species of *Candida* such as *C. apicola*, *C. maltosa* and *C. tropicalis*. These bioconversions are terminal hydroxylation of *n*-alkanes and ω-hydroxylation of fatty acids. The monoxygenase system required for these reactions comprises several cytochrome P450s induced by alkanes (Seghezzi *et al*., 1991; Scheller *et al*., 1996). Comparable bioconversion reactions have also been examined in *Yarrowia lipolytica* (van Dyk *et al*., 1994). Certain fungal cytochrome P450-encoding genes for alkane assimilation have been identified and reviewed (van den Brink *et al*., 1998). Eight genes of a large cytochrome P450 gene family, *cyp52*, in species of *Candida* associated with *n*-alkane assimilation have been identified. Substrate specificity is different for each gene that reveals the capability of yeast to modify a range of different *n*-alkanes (Seghezzi *et al*., 1991; Scheller *et al*., 1996). Cytochrome P450 reductase (CPR)-encoding genes are also identified in *Aspergillus flavus*, *A. Parasiticus, A. niger*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *C. tropicalis*, and *Candida maltosa*. The P450 overproduction in fungi has a potential use in the enhancement of bioremediation of oil contaminants in the environment.

2.9 Factors Affecting Metabolism of Petroleum Hydrocarbons

Various factors influencing the fungal degradation of petroleum hydrocarbons have been recognized. These factors are divided into three categories: (1) physicochemical factors that include the physical nature, solubility, size and concentration of the hydrocarbon, oil-water interface and volatility, (Leahy and Colwell, 1990; Sabate *et al*., 2004; Beskoski *et al.*, 2011). (2) environmental factors such as temperature, pH, light, salinity, oxygen level, nutrients, soil/sediment type, etc. (Chaudhry *et al*., 2005; Rojo, 2009; Beskoski *et al*., 2011; Chandra *et al.*,

2013) and (3) Population density, genetic composition, distribution and adaptation of the fungal community in the polluted site(Baldwin *et al.*, 2003; Rocha *et al*., 2011; Meckenstock *et al.*, 2016.

2.9.1 Physical nature of the hydrocarbons

The physical nature of hydrocarbons has a great effect on the process of biodegradation.

The hydrocarbon-degrading yeasts and fungi act primarily at the oil-water interface. However, yeasts and fungi can be found growing over the entire surface of an oil droplet, and growth does not occur within oil droplets in the absence of entrained water. The movement of emulsion droplets through a water column allows the uptake of oxygen, nutrients and oil to fungi(Chaudhry *et al*., 2005; Rojo, 2009; Beskoski *et al*., 2011; Chandra *et al*., 2013),.

2.9.2 Temperature

Based on temperature, hydrocarbon degradation can occur under three conditions: psychrophilic, mesophilic and thermophilic. In general, most fungi are mesophilic in isolation, growth and reproduction. Temperature is essential for the growth requirements of certain yeasts and fungi along with petroleum as a substrate. Low temperatures generally retard the rates of volatilization of low-molecular-weight hydrocarbons, some of which are toxic to yeasts and fungi. Fungi also show a propensity to withstand dry environments and high temperatures and thus appear to be suitable for the remediation of these contaminated areas. The effects of temperature are also considered with other factors, such as the quality of the hydrocarbon mixture and the composition of the fungal population. Temperature influences diesel oil biodegradation by the psychrotrophic yeast *Yarrowia lipolytica* in a mineral medium and in soil (Margesin and Schinner, 1997). Abiotic loss of diesel oil increases with incubation time and with temperature and is lower in a mineral medium than in soil. This amounts to a loss of 20 to 45% (5000 mg/kg

soil dry weight) in soil and 15 to 27% in liquid media after 30 days at 4 to 30°C. Higher biodegradation activity is detected in liquid culture than in soil at all temperatures. *Y. lipolytica* was shown to degrade about 20% of the diesel oil in soil. The degradation activity was highest at 10 to 15°C after 5 days and at 4°C after 10 days. Benzene toluene ethylene and xylene (BTEX) degradation by *Phanerochaete chryso*sp*orium* was higher at 25°C than at 37°C (Yadav and Reddy, 1993).

2.9.3 pH

Several fungi grow well at pH levels of 4 to 5 and yeasts at 3 to 4 and are more tolerant of acidic conditions, where it is difficult for bacteria to thrive. *Trichoderma* sp grow well at pH level of 5.0 (Ainon *et al.*, 2012)

2.9.4 Oxygen

Fungi are both aerobic and anaerobic but grow well under aerobic conditions. Oxygen is necessary for the mineralization of hydrocarbons in estuarine sediments. The rates of hydrocarbon degradation are reduced with decreasing oxygen reduction potential. Hydrocarbons persist in reduced sediments for longer periods than in aerated surface layers. The initial steps in the catabolism of aliphatic, cyclic and aromatic hydrocarbons by fungi involve oxidation of the substrate by oxygenases and molecular oxygen. Thus, aerobic conditions are necessary for the oxidation of hydrocarbons in the environment. Substantially, greater degradation of all BTEX compounds occurs in static than in shaken liquid cultures (Yadav and Reddy, 1993). Negligible rates of biodegradation of hydrocarbons occur in anaerobic environments.

2.9.5 Nutrients, Dispersants and Biosurfactants

Little is known about petroleum degradation in the presence of nutrients by yeasts and fungi. Low nitrogen levels, low pH, low moisture content and inadequacy of certain nutrients favour

the development of fungi. In oil slicks, a proportion of carbon is readily available for yeast growth within a limited area (Bento and Gaylarde, 2001). Since nitrogen and phosphorus components are essential for incorporation into yeast or fungal biomass, the availability of these nutrients within the hydrocarbon location is important. In many cases, the supply of nitrogen and phosphorus depends on the diffusion in the oil slick. The degradation of hydrocarbons can be accelerated by the addition of specific urea-phosphate, N-P-K fertilizers, and so on, for fungal growth. Fungal cells usually contain less nitrogen than bacterial cells and thus fungi can act favourably in ecosystems that have low nitrogen content. Many fungal isolates grow equally well in laboratory diesel-water systems with or without an additive (Bento and Gaylarde, 2001). However, the composition of fungal cells can be represented empirically by $C_{10}H_{17}O_6N$. Dispersants have demonstrated a positive effect on rates of degradation by dissolution and emulsification of hydrocarbons. Fungal levels in analytical freshwater ponds are enhanced significantly after the addition of oil-dispersant mixtures. Some dispersants are toxic and inhibitory to yeasts and fungi. The use of natural biosurfactants produced by yeasts or fungi has a marked potential in such biospheres (Lindley, 1994). The chemical nature of biosurfactants produced by yeasts appears to be that of glycolipids. The physical properties of such compounds play an important role in petroleum degradation. The major action of these biosurfactants is to increase the available surface area of the hydrocarbon phase for uptake transport by fungi.

2.10 Biochemical Oxygen Demand (BOD)

Biochemical oxygen demand (BOD) is the amount of dissolved oxygen needed by aerobic biological organisms in a body of water to break down organic material present in a given water sample at certain temperature over a specific time period. The term also refers to a chemical procedure for determining this amount. This is not a precise quantitative test,

although it is widely used as an indication of the organic quality of water (Clair *et al.*, 2003). The BOD value is most commonly expressed in milligrams of oxygen consumed per litre of sample during 5 days of incubation at 20 °C and is often used as a robust surrogate of the degree of organic pollution of water. BOD can be used as a gauge of the effectiveness of wastewater treatment plants (Clair *et al.*, 2003).

In the presence of free oxygen, aerobic organisms use the organic matter found in wastewater as "food". The BOD test is an estimate of the "food" available in the sample. The more "food" present in the waste, the more Dissolved Oxygen (DO) will be required. The BOD test measures the strength of the wastewater by measuring the amount of oxygen used by the organism as they stabilize the organic matter under controlled conditions of time and temperature. The BOD test is used to measure waste loads to treatment plants, determine plant efficiency (in terms of BOD removal), and control plant processes. It is also used to determine the effects of discharges on receiving waters. A major disadvantage of the BOD test is the amount of time (5 days) required to obtain the results.

2.11 Chemical Oxygen Demand (COD)

The Chemical oxygen demand (COD) test is an empirical test that is a measure of the potential oxygen consumption of waste waters. Chemical oxygen demand (COD) is an indicative measure of the amount of oxygen that can be consumed by reactions in a measured solution. It is commonly expressed in mass of oxygen consumed over volume of solution which in SI units is milligrams per litre (mg/L) (Clair *et al.*, 2003). A COD test can be used to easily quantify the amount of organics in water. The most common application of COD is in quantifying the amount of oxidizable pollutants found in surface water (e.g. lakes and rivers) or wastewater. COD is useful in terms of water quality by providing a metric to determine the effect an effluent will

have on the receiving body much like biochemical oxygen demand (BOD). The basis for the COD test is that nearly all organic compounds can be fully oxidized to carbon dioxide with a strong oxidizing agent under acidic conditions. For the analysis of COD, samples are oxidised by heating the water in vials with sulphuric acid and potassium dichromate. Mercuric sulphate is added to suppress chloride interference. The dichromate is reduced to chromate during the digestion and the chromate produced is measured colorimetrically (Clair *et al.,* 2003).

2.12 Fungal Biosorption of Heavy Metals

The subtle and intricate process of removal of metals from aqueous solution has been known to the world for more than 5000 years, since Moses sweetened water using wood (Eccles, 1995). But microorganisms have been used to treat waste liquids since the end of the nineteenth century. However, the removal and/or recovery of metals from liquids or streams have received most attention during the past three decades. Global industrialization is of great concern as a result of the release to the environment of toxic and persistent heavy metals that cause deleterious ecological effects and pose a serious threat to animals and humankind. The releases also lead to the mobilization of metals through leaching. Several industries—electroplating, electronic circuit production, steel and nonferrous processes, chemical and pharmaceutical, and others—discharge a variety of metal-laden wastewaters into the environment. Coal-fired power plants also produce large quantities of metal releases at several points during combustion. Release of industrial wastewater due to acid mine drainage over a large area is another big problem. In the United States, 389 of 703 National Priority List sites contain toxic metal contaminants and at least 100000 such sites are estimated in the European Union (Schmitt and Stitcher, 1991; Wilmoth *et al*., 1991). Government agencies have developed metal discharge standards in a number of countries to regulate such releases and contaminants in wastewaters entering waterways and

sewerage systems. Microorganisms have the ability to bind metals from aqueous solution. This phenomenon is known as biosorption, and the microorganisms responsible for the process are considered biosorbents. A wide variety of living and dead biomass of bacteria, algae, fungi and plants is capable of sequestering toxic metals from waste streams. This is the foundation of biosorption technology, which offers a promising and economical alternative for the treatment of discharges of a wide variety of metal-containing industrial effluents. A plethora of literature exists on the biosorption of metal ions by bacteria, algae, fungi and plants. Conservative estimates of new biosorbents in the North America environmental market amount to $27 million per year (Volesky, 2001). Yeasts and fungi are unique in metal biosorption, and this process is known as mycosorption. The fungal biomass used in mycosorption is termed mycosorbent. Mycosorption is a topic of great interest for researchers all over the world (Paknikar *et al*., 1998; Tobin, 2001; Malik, 2004).

2.12.1 Biosorption and Bioaccumulation of Heavy Metals

The process of biosorption and bioaccumulation of metals by microorganisms is not new. The accumulation of metals by fungi has received more attention in recent years because of its applications in environmental protection and recovery of metals. The biological removal of metals from solutions can be divided into three categories: (1) biosorption of metal ions on the surface of fungi, (2) intracellular uptake of metal ions, and (3) chemical transformation of metal ions by fungi. Living fungal biomass is required in the last two categories. Non-living fungal biomass does not depend on requirements for growth, metabolic energy and transport. In addition, nonliving biomass shows a strong affinity for metal ions due to the lack of protons produced during metabolism. The problem of toxicity of metals does not affect this type of biomass, which is seen as one of the major advantages of biosorption. Fungal biomass can be

generated as a waste by-product of large-scale industrial fermentation and is pretreated by washing with acids and/or bases before final drying and granulation. All these factors contribute to reducing the final cost of the process. Biosorption is a pseudo-ion-exchange process in which metal ion is exchanged for a counter ion in the biomass or resin. In general, the filamentous fungi possess higher adsorption capacities for heavy metal removal. Aquatic fungi are also known to accumulate heavy metals. Uptake of metals was described by Michelot *et al*. (1998) and a tentative approach related to mechanisms of bioaccumulation in mushrooms was projected. The marine fungi, *Corollospora lacera* and *Monodictys pelagic*, have been found to accumulate lead and cadmium extracellularly in mycelia (Taboski *et al*., 2005). Biosorption involves a number of external factors (e.g., type of metal, ionic form in solution and the functional site) and tends to be exothermic. Other factors, such as pH, temperature, biomass concentration, type of biomass preparation, initial metal ion concentration and metal characteristics as well as concentration of other interfering ions, are also important in evaluating the extent of biosorption. Biosorption and recovery can be intensified in the presence of stirring induced by magnetic field (Gorobets *et al*., 2004).

2.12.2 Mechanisms of Fungal Biosorption of Heavy Metals

Biosorption consists of several mechanisms that differ according to the fungal species used, the origin of the biomass and its processing. These mechanisms include ion exchange, chelation, adsorption, crystallization and precipitation, followed by ion entrapment in inter- and intrafibrillar capillaries, spaces of the polysaccharide material and diffusion through the cell wall and membranes of fungi (Kuyucak and Volesky 1988; Ercole, *et al.* 1994). Cell walls of fungi are composed of chitins, chitosans and glucans and also contain proteins, lipids and other polysaccharides. Yeast cell walls consist mainly of glucans and an outer layer of mannoprotein.

Precipitation can occur in the cell wall components. The biomass usually contains a larger number and variety of functional groups or sites than those in monofunctional group ion-exchange resins. These sites include carboxyl, sulfate, phosphate, hydroxyl, amino, imino, sulfonate, imidazole, sulfydryl, carbonyl, thioether and other moieties. Certain fungal species are more effective and selective than others in removing particular metal ions from solution. The mechanisms of fungal biosorption can be divided into two categories: metabolism-independent and metabolism-dependent. The first category employs live or dead biomass, and the second category transforms the metal internally coupled with the production of extracellular metabolites. The mechanisms of fungal biosorption are a topic of great interest to many authors; however, significant differences are recognized in the biosorbent mechanisms of fungal biomass. Electron microscopy, x-ray energy diffraction analysis and infrared (IR) spectroscopy are also used to study binding mechanisms. The amino group of the cell walls of *Rhizopus nigricans* is involved in Cr(VI) binding from solution and from wastewater (Bai and Abraham, 2002). Chemical modification increases the number of active binding sites on the surface area that enhances the chromium adsorption capacity. The amine functional groups on the cell walls of *Mucor* also contribute to the removal of chromium from tanning effluent (Tobin and Roux, 1998). Fourier transform infrared (FTIR) spectroscopic analysis reveals the involvement of —COOH groups of acetone-washed yeast biomass in lead biosorption (Ashkenazy *et al*., 1997). The —COOH groups also contribute to the binding sites of metals in the cell walls of *Mucor rouxii* (Gardea-Torresdey *et al*., 1996). Electron microscopy, x-ray energy diffraction analysis and IR spectroscopy reveal three hypotheses of the mechanism of lead uptake (Zhang *et al*., 1998). NaOH-treated and NaOH-untreated biomass establish that biosorption occurs in the chitin structure of cell walls. Electron microscopy reveals the localization of nickel on the cell surface

of *Rhizopus* sp. (Mogollon *et al*., 1998). Transmission electron microscopy (TEM) shows that Pb(II) is associated in the cell wall and membrane after 3 minutes and cytoplasm after 2 hours in *Saccharomyces cerevisiae* (Suh *et al*., 1998). A three-step mechanism of Pb(II) accumulation is advocated. The first step is metabolism-independent, the second step is metabolism-dependent, and the third step is metabolism-dependent or independent after 24 hours. Two mechanisms govern the removal of Cr(VI) from the aqueous solution by dead biomass of *Aspergillus niger* (Park *et al.,* 2005). During mechanism I, Cr(VI) is reduced directly to Cr(III) by contact with the biomass. Mechanism II consists of three steps: the binding of Cr(VI) to positively-charged groups in the cell wall, reduction of Cr(VI) to Cr(III) by adjacent functional groups and release of Cr(III) by electron repulsion. Fungi can remove both soluble and insoluble metals from solution and can leach metals from solid wastes. Fungi produce protons, organic acids, phosphatases and other metabolites for solubilization. Many heterotrophic fungi produce organic acids that assist in solubility and complexing of metal cations. Several fungi are known to produce large amounts of different kinds of acids that assist in metal leaching purposes (Park *et al.,* 2005).

2.12.3 Biosorption by Filamentous Fungi

Different types of biomass have been tested for their metal-binding capacities under various conditions. Selection of biomass depends on the sequestering ability of the metal ions. Some types of sorbent have a broad range with no specificity; others are specific for certain metals (Park *et al.,* 2005). Fungal biosorbents have been described by several researchers. The characteristics of the fungal biomass for suitability in the process are (1) biosorption efficiency and metal uptake capacity as well as affinity for metal ions, (2) specificity for metal ions, (3) flocculation and sedimentation efficiency, (4) desorption efficiency, and (5) preservation of the

biosorption capacity. Fungal melanins of filamentous fungi also contribute to the removal of metals, and their interactions with metals have been explained by Fogarty and Tobin (1996). The first step in biosorption is the choice of metals. Uranium is given the highest consideration, due to great interest by the nuclear industry. Mercury, lead and cadmium are next, due to strong toxic environmental effects. Chromium is highly toxic and must be reduced to a trivalent state that is more amenable to removal. Copper is used in many applications and is also increasing in the environment. Little is known as to the removal of radium, thorium, strontium and neodymium by filamentous fungi. Metal oxalates are also produced by a wide range of fungi, including mycorrhizal and lichenicolous fungi. The white-rot fungi, *Bjerkandera fumosa*, *Phlebia radiata*, and *Trametes versicolor* and the brown-rot fungus, *Fomitopsis pinicola* produce oxalate crystals in high levels on ZnO, $CO_3(PO_4)_2$ and $CaCO_3$. In brown-rot fungi, induction of oxalic acid is related to copper tolerance (Green and Clausen, 2003).

Brown-rot fungi can maintain high concentrations of oxalic acid during leaching of metals from the treated wood (Humar *et al*., 2004). One-third of the isolates of soil fungi are able to solubilize at least one toxic metal compound, ZnO, $Co_3(PO_4)_2$, and $Zn_3(PO_4)_2$, and 10% solubilize all the three (Sayer *et al*., 1995). In *Penicillium simplicissimum*, adsorption of zinc is accompanied by the production of citric acid (Franz *et al*., 1991). The culture filtrate of *Aspergillus niger* can render the solubility of 18% Cu, 7% Ni, and 4% Co, and these amounts are enhanced by the addition of HCl (Sukla *et al*., 1992). Fe (III) is solubilized by a low-molecular-weight chelating compound known as the ferrichrome (Crichton, 1991). The structure of the hyphae in the form of hard compact pellets is altered due to a manganese deficiency (Schreferl *et al*., 1986). *Penicillium janthinellum* F-13 on different media reduces Al toxicity, but tolerance of the high external concentration of Al appears to be due to a different mechanism (Zhang *et al*., 2002).

2.12.4 Advantage of Fungi over Bacteria in Bioremediation

Fungus is considered as an efficient candidate for potential degradation of polyaromatic hydrocarbons for the following reasons:

- Among the eukaryotes, fungi are considered the most ubiquitous owing to their capacity to grow using a wide range of hydrocarbons (Kari *et al*., 2003; Ulfig *et al.*,2003) in the presence of high levels of toxic heavy metal ions (Ulfig *et al.*,2003; Ayenimo *et al.,* 2005; Shankar *et al.,* 2007; Bako *et al.,* 2002). Fungi have a greater resistance to inhibitory compounds than bacterial species
- The fungal hyphae provide a greater protection to their sensitive organelles. The larger surface area of fungi acts like an adsorption layer of extra-polysaccharide matrix, protecting them from inhibitory compounds (Ulfig *et al.*,2003; Ayenimo *et al.,* 2005; Shankar *et al.,* 2007; Bako *et al.,* 2002).
- Fungi contain a group of extracellular enzymes that facilitate the biodegradation of recalcitrant compounds such as phenolic compounds, dyes and polyaromatic hydrocarbons(PAH), among others, through non-specific oxidation reactions (D'Annibale *et al.,* 2004*;* Giraud *et al.,* 2001; Jaouni *et al.,* 2005). By contrast, the bacterial cell produces target-specific enzymes for degrading contaminants (Chrost and Siuda, 2002; Riser-Robert, 1998) and potential hydrolytic enzymes, which can penetrate and degrade the hydrocarbons-contaminated soil (Balaji and Ebenezer, 2008). Fungal enzymes, especially oxidoreductases, laccase and peroxidases have prominent application in removal of PAHs contaminants either in fresh, marine water or terrestrial habitats (Balaji *et al.,* 2005). However, lipases have been significantly less studied on

bioremediation of PAHs (Haritash and Kaushik, 2009). Fungi isolated from oil spill environment can reduce oil pollution (Chaillan *et al.,* 2004; Das and Chandran 2011). Nevertheless, interest on fungi receives a considerable attention for bioremediation of hydrocarbon-contaminated sites associated fungi for enzyme secretion (to remove hydrocarbons from the environment).

- Fungi are eukaryotes, having considerably more genes than bacteria, which further make them more versatile in tolerating inhibitory compounds (Guest and Smith, 2002).The higher number of genes in fungi imparts greater reproductive selectivity, which might result in better environmental adaptations (Bennett and Lasure, 1991).

2.12. 5 *Trichoderma* sp. as Agent of Bioremediation

One of the most potent group of fungi used widely for bioremediation is *Trichoderma* sp. In addition, they are known to enhance plant growth and development. The genus *Trichoderma,* occurs in diverse habitats and is a genetically diverse genus, filamentous in nature with agricultural importance (Ahamed and Vermette 2008; Contreras-Cornejo *et al.* 2009; Lorito *et al.* 2010), being a potent bio-control agent, plant growth promoter and plays an important role in improvement of soil fertility, disease suppression and composting (Contreras-Cornejo *et al.* 2009; Lorito *et al.* 2010). *Trichoderma* sp. has also been reported to be a producer of organic acids such as gluconic acid, fumaric acid and citric acid, which decrease soil pH and allow phosphate dissolution as well as dissolution of macro- and micronutrients such as iron, manganese and magnesium, which are necessary for plant metabolism. *Trichoderma* sp is characterised by its ability to modify the rhizosphere microflora of plants through the intensive colonisation of roots (Ociepa, 2011). It has also wide applications in enzyme production, paper, pulp and food industries (Ahamed and Vermette 2008; Singh and Singh, 2009) as well as in

remediation of soil and water pollution and biofilm preparation in the field of nanotechnology (Vahabi *et al.* 2011). *Trichoderma* exhibits resistance to many agrochemicals, thus it is used as a tool for integrated pest management. It is a potent producer of hydrolytic and industrially important enzymes, like amylase from *T. harzianum*, cellulases from T. *reesei* (Ahamed and Vermette 2008) 1,3 β glucanases from *T. harzianum, T. koningii* (Monteiro and Ulhoa 2006), and chitinases from *T. aureoviridae* and *T. harzianum* is well established. *Trichoderma* sp. are found to be highly resistant to a wide range of toxicants viz, heavy metals, organometallic compounds, tannery effluents and harmful chemicals like cyanide (CN) (Saba, 2016). This makes them an important fungal genus to be explored as a genetic resource to employ in bioremediation of toxic pollutants.

2.12.6 Role of *Trichoderma* sp. in Bioremediation

Many studies and reports have signified the role of *Trichoderma* in bioremediation and environmental cleanup. Based on bibliographical studies, there is little report about Cd accumulation ability of *Trichoderma* species. The biomass of soil fungi including *Trichoderma* plays an important role in the bioremediation of contaminated soils and can be applied in integrated pest management and phytoremediation. Moreover, it can remove and concentrate the various ions such as Pb, Cd, Cu, Zn and Ni, and sorption was widely recognised as the main mechanism of uptake (Yazdani *et al.*, 2009; Srivastava *et al.*, 2011). Asha *et al.*, (2012) concluded that *T. viride* can be successfully used for bioremediation of cadmium and lead from aqueous media. Therefore, bio-removal carried out by these fungi could serve as an economical mean of treating effluents and the polluted water areas charged with toxic metallic ions. Hence, it was concluded that *T. viride* has affinity to tolerate high concentration of applied heavy metals.

2.13 *Aspergillus flavus*

Aspergillus is a large genus composed of more than 180 accepted anamorphic species (Pitt *et al.*, 2000), with teleomorphs described in nine different genera (Pitt, *et al.*, 2000). The genus is subdivided into 7 subgenera, which in turn are further divided into sections (Klinch, 2002). As with fungi in general, *Aspergillus* taxonomy is complex and ever evolving. The genus is easily identified by its characteristic conidiophore, but species identification and differentiation is complex, for it is traditionally based on a range of morphological features. Macromorphological features, which are considered include conidial and mycelial colour, colony diameter, colony reverse colour, production of exudates and soluble pigments, presence of sclerotia and cleistothecia. Micromorphology characterization is mainly dependent on seriation, shape and size of vesicle, conidia and stipe morphology, presence of Hülle cells and morphology of cleistothecia and ascospores (Klinch, 2002). Furthermore, all these morphological features have to be determined under standardized laboratory conditions (Okuda *et al.*, 2000) by trained mycologists in order to obtain an accurate identification. Several *Aspergillus* taxonomic keys and guides are available (Klinch, 2002). Most species are adapted for the degradation of complex plant polymers but they can also dine on substrates as diverse as dung, human tissues and antique parchment. In the ecosystem, different substrates are attacked at different rates by consortia of organism from different kingdoms. Aspergillus and other moulds play an important role in this consortium because they are adept at recycling starches, cellulose, hemicellulose, pectins and other sugar polymers. Some aspergilli are capable of degrading more refractory compounds such as fats, oils, chitin and karatin. Maximum decomposition occurs when there is sufficient nitrogen, phosphorus and other essential inorganic nutrients

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection and Handling of Effluent Sample

The study area is Kaduna Refinery and Petrochemical company (KRPC) located in Chikun Local Government Area, Kaduna State. The wastewater treatment plant (WWTP) is located at the west end of the company, behind the NNPC Clinic, from the main entrance to the refinery. Raw effluent samples were collected from the effluent site of the Kaduna Refinery and Petrochemical Company (KRPC) within the refinery and it is called the untreated effluent sample channel, which retains the raw effluent for before flowing into the waste oil retention pond for treatment. Effluent sample were collected in new plastic 20litre container from untreated waste water channel for physicochemical analysis and mycoremediation studies. The samples were collected by lowering the container 30cm deep into the well mixed section of sampling point and allowed to overflow before withdrawing. Another 200ml sample were collected into sterile sampling bottles at sampling point for microbiological analysis. The body of the plastic container and bottles were rinsed with clean water. After collection, the samples were transported to the laboratory Department of Microbiology, Ahmadu Bello University for analysis. All samples were analyzed without delay in order to avoid microbial deterioration of the samples.

3.2 Determination of Physicochemical Properties of the Effluents

The physicochemical properties of the effluents were determined, so as to assess the composition of the effluent. The physical parameters analysed included: electrical conductivity, turbidity, temperature, dissolved solids and suspended solids. The chemical parameters were: pH, Dissolved Oxygen (DO), Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Total solids (TS), Oil and grease, Nitrate, Phosphates, and Sulphates, by following

methods of APHA (2012), while the heavy metals (lead (Pb), nickel (Ni), and Cadmium (Cd)) were analysed using microwave plasma atomic absorption spectrophotometry (MP-AES AGILENT 4200), by following methods of APHA (2012) .

3.2.1 Determination of pH, Temperature and Electrical Conductivity

The pH, temperature and electrical conductivity of the raw effluent were determined at the point of collection using the HANNA combo tester (H198130, Denver, USA). Following the manufacturer's instructions, the electrodes connected to each meter was submerged in a plastic beaker containing the sample. The values read for the pH, temperature and electrical conductivity were noted and recorded.

3.2.2 Determination of Total Solids

The measuring cylinder, funnels, dishes and beakers was washed with detergent solution and rinsed with distilled water and was placed in an oven to dry at 105ºC and allowed to dry, it was then transferred to desiccators to cool. Weighing of the dishes was done with the analytical balance. The sample was mixed and stirred, 30ml was measured with a measuring cylinder and poured into each dish, and placed on the water bath and evaporated to dryness, then transferred to the oven at 105ºC to dry before placing in the desiccators to cool. The weight of the dry dishes and content was obtained using the analytical balance B g\l. TS was determined as follows (APHA, 2012):

$$\text{Total Solids} = \frac{B - A \times 10^6}{\text{Volume of sample}}$$

Where B = weight of content + dish (g), A = weight of dish (g)

3.2.3 Determination of Total Dissolved Solids

Effluent sample was stirred with a magnetic stirrer and fifty millilitres (50ml) was taken into a glass fiber filter with applied vacuum. It was then washed with three successive ten millilitres volume of distilled water, allowing complete drainage between washings and suction was

continued for three minutes until filtration was completed. Total filtrate with washing was transferred to a weighed evaporating dish and evaporated to dryness on a steam bath. Evaporated sample was dried in the oven ay 180 ± 2^0C for 1h, cooled in a dessicator and weighed. A total dissolved solid was calculated using the formula:

$$TDS(mg/l) = \frac{(A-B)\times 1000}{\text{Volume of sample (ml)}}$$

A= weight of dried residue + dish (mg)

B= weight of dish (mg)

3.2.4 Determination of Suspended Solids

To determine the weight of suspended solids, filter paper was thoroughly washed with distilled water and transferred into an oven at 105^0C and allowed to dry, the filter paper was then cooled in a dessicator, the cooled filter paper was weighed on the analytical balance and the weight recorded as initial weight W1(in grams).with clean dry hands, the filter paper was folded in to a funnel shape and 50 ml of the effluent sample poured to strain off the effluent to retain the suspended solid, the filter paper was then transferred to the oven to dry at 105^0C.finally the filter paper plus the suspended solids were transferred to desiccators to cool before weighing (W2). The weight of suspended solids was determine as follows(APHA,2002)

$$\text{Suspended Solids (mg)} = \frac{W_2 - W_1 \times 10^6}{\text{Volume of sample}}$$

Where W_2 = weight of filter paper + suspended solid (mg), W_1 = weight of filter paper (mg)

3.2.5 Determination of Dissolved oxygen and Biological oxygen demand

The Azide modification of the Winkler method was used to determine the dissolved oxygen (DO) and biological oxygen demand (BOD). A 2ml of manganese sulphate solution was added to the

sample in a 300ml BOD bottle and then 2ml alkali-iodide-azide reagent was added well below the surface of the liquid. The BOD bottle lid was used to close the bottle, with care to exclude air bubbles and then mixed by inverting the bottle several times. After the precipitate settled, leaving a clear supernatant above, the BOD bottle was shaken again. When settling produced a clear supernatant (one third the total volume), 2ml of concentrated H_2SO_4 was added and the lid was used to close the bottle again. The contents were mixed by gentle inversion and then 200 ml was pipetted and titrated against 0.025N $Na_2S_2O_3.H_2O$ to a pale straw colour endpoint after which2ml starch solution was added. Titration was continued to the first disappearance of the blue colour. The iodine was measured with standard sodium thiosulphate solution and was interpreted in terms of dissolved oxygen.

On the other hand, the BOD test was carried out by measuring an initial dissolved oxygen (DO) reading and a second reading after five days of incubation at ambient temperature. Afterward, similar treatment was done for sample after 5 days of incubation at room temperature. The BOD was determined by subtracting the initial DO from the final value to obtain the BOD_5 (Radojavic and Bashkin, 1999). The BOD is expressed in milligrams per litre of DO using the following equation:

DO (mg/L) of first bottle - DO (mg/L) of second bottle = BOD_5 (mg/L)

3.2.6 Determination of Chemical Oxygen Demand

Anti-bumping granules were inserted into influx flask and 10 ml of the sample was measured into the flask and then 1ml of 20% m/v mercuric sulphate solution was added and swirled to mix. A 5 ml of 0.021M potassium dichromate was added and using a dispensing pipette, 15 ml of 1%m/v silver sulphate was added. The content in the flask was fitted to a condenser and heated gently for 2 hrs, after which the flask was removed to cool for approximately 10 minutes and

then 25 ml of distilled water were added. Two drops of ferroin indicator were added to the content in the flask and the residual dichromate were titrated with standardized ferrous ammonium sulphate

The end point was reached when the blue colour disappeared and the solution became colourless and the COD was determined using the equation

COD = (A - B) ×1000(mg/l) a × volume of sample used

Where: A = Volume of 0.0125N potassium trioxosulphate for blank

B = Volume of 0.0125N potassium trioxosulphate for sample

a = ml of sodium trioxosulphate required for 10 ml of potassium permanganate

3.2.7 Determination of Nitrate Content

Cadmium reduction method was used to analyse the concentration of nitrate in the sample. Cadmium metal reduces nitrates in the sample to nitrite, nitrite ion reacts in an acidic medium with sulfanilic acid to form an intermediate diazonium salt. The salt forms a complex with gentisic acid to form an amber coloured solution. The optical density of the sample was measured at 430nm using a spectrophotometer (HACH DR 2800). Result shows in mg/L NO_3 N

3.2.8 Determination of Sulphate Content

One hundred millilitres (100ml) of the sample was dispensed into a 50ml beaker. The accu vac-ampul was filled with the effluent sample and the tip was kept immersed while the ampul fits completely. The ampul was closed and inverted severally to mix undissolve powder and so as not to affect accuracy. A five minutes reaction time starts and the blank was prepared, while the sample cell was filled with ten millilitres of the effluent sample. After the timer expires the accu vac ampul was inserted into the cell holder and the zero button was pressed, the display shows 0mg/l SO_4^{2-}, within five minutes after the timer expires, the prepared effluent sample in the accu

vac ampul was inserted into the cell holder and the button “READ” was pressed, result shows in mg/l SO_4^{2-} .

3.2.9 Determination of Phosphate Content

The phosphate content in the effluent was measured using a HACH 2800 spectrophotometer. A 25 ml distilled water and sample were measured as blank and 1ml of ammonium molybdovanadate reagent was added to sample and the blank and was swirled to mix properly for 3min. The blank was used to auto zero and readings was taken in mg/l.

3.2.10 Determination of Oil and Grease

A hundred millilitres (100ml) of effluent sample was introduced into a separating funnel and 5ml of concentrated hydrochloric acid was added and then 10 ml of petroleum ether was added into the separating funnel and weighed (A). It was then agitated for 2 minutes and drained off. An additional 20 ml of petroleum ether were added and agitated. It was then put in an oven at 45^0C $\pm 2^0$C for 30 minutes and then transferred to a desiccator before weighing (B). The oil and grease content was determined as follows:

$$\text{Oil/Grease (mg/l)} = \frac{\text{A -B} \times 1000\text{ml}}{\text{Volume of sample (mg/l)}}$$

Where, A and B are the initial and final weight of the flask and its contents respectively.

3.2.11 Determination of Turbidity

Turbidity was measured with a Partech Model DRT 100B Turbimeter. The turbimeter was connected to a light source, the cuvette was removed and rinsed with distilled water, and then with the raw effluent. The raw effluent was poured into the cuvette to the meniscus of the red mark, and then the cuvette was placed in the cuvette section and closed. The test button on the meter was pressed and reading was noted and recorded.

3.2.12 Determination of Heavy Metal Content of the raw effluent

The concentration of heavy metal (lead, nickel and cadmium) in the sample was analyzed using the microwave plasma atomic absorption emission spectrophotometer (Model MP-AES AGILENT 4200) following the modified method of APHA (2012). The instrument's settings and operational conditions was carried out in accordance with manufacturer's specifications by calibrating with analytical grade metal standard stock solution. Five millilitres (5ml) of each sample was digested in a beaker by adding 37.5ml of nitric acid and 12.5ml of hydrochloric acid, heated to almost dryness and topped up to 50ml with distilled water. The digested sample was filtered to remove any insoluble material that could clog the atomizer. The filtrate was then analyzed for heavy metals using the MP-AES.

3.3 Determination of Pb, Ni and Cd contents of the raw wastes effluents

To be able to determine the amounts of Pb, Ni and Cd removed by the fungal isolates at end of the Mycoremediation experiment, the initial concentrations of the metals in the raw effluents were determined. This was done by digesting triplicate 5ml batches of the effluent samples using 7.5ml of nitric acid and 2.5ml of hydrochloric acid. The mixture was heated to near dryness and the volume made up to 20ml with distilled water. The digest was filtered to remove particulate materials that could clog the atomizer. The concentrations of Pb, Ni and Cd in the filtrates were then determined using Microwave Plasma Atomic Emission Spectrophotometer (MP-AES AGILENT 4200).

3.4 Microbiological Analysis of the Raw Refinery Effluent Sample

3.4.1 Isolation of *Aspergillus flavus* and *Trichoderma* sp

The 200 ml of the raw refinery effluent was allowed to stand in the sample container at room temperature on a disinfected laboratory work bench for 24 hour to concentrate the sample by

sedimentation. The supernatant was discarded leaving the sediment to 10ml volume followed by rigorous shaking manually to resuspend the sediments. Five (5) millilitres of the sample was measured into sterile centrifuge tubes in duplicate and centrifuged at 3000 rpm for 15 minutes to further concentrate the fungal propagules present in the samples. The supernatants were decanted to 2mls volume and shaken vigorously manually, to resuspend the sediments in the 2ml volume. A 0.1ml aliquot of the suspension was inoculated on freshly prepared potato dextrose agar (PDA) supplemented with 7.5% NaCl and 50µg/l of chloramphenicol to suppress bacterial growth and spread using sterile bent glass rod.

All inoculated plates were incubated aerobically at ambient laboratory conditions in disinfected dark cupboard for 7days. The primary culture plates were examined for evidence of fungal growth. The fungal cultures were subcultured on similar medium without antibiotics until pure colonies were obtained. Fungal colonies observed were identified into types based on their cultural characteristics. These included the colour of the surface and reverse sides and texture of the colonies. The distinct types were isolated into plates of potato dextrose agar to obtain pure isolates. Pure isolates were stored on slants of potato dextrose agar for identification.

3.4.2 Identification of the Fungal Isolates:

All the isolates obtained from the various samples analyzed, were identified based on their culture morphology and microscopic characteristics. Colour and texture of the fungal isolates were observed and recorded. For the microscopic characteristics, small portion of the growing region was mounted on grease free slide with a drop of lacto phenol cotton blue, covered with a cover slip, which was observed by microscopy using x10 objective lens and then x40 objective lens. Criteria such as presence or absence of septation, presence of foot cell at the base of conidiophores, chlamydospores and structures of asexual fruiting bodies, production of micro

and / or macroconidia and other relevant characteristics were used to identify the isolates to generic levels with reference to appropriate taxonomic guides (Barnette and Hunter, 1999; Klinch, 2002; Larone, 2002; Nagamani *et al.,* 2006; Hakeem and Bhatnagar, 2010; Thippaswamy *et al.,* 2012).

3.5 Determination of the Capacity of Isolates in Removal of Hydrocarbon From Raw Refinery Effluents

This was achieved by using a clean 4L plastic container to simulate the environment where the raw effluent was taken from. Five (5) plastic containers weresetup,3 L of raw effluents was measured in all. The first contained the raw, unsterilized refinery effluents, which served as the negative control; the second contained the raw but autoclaved effluents, which served as positive control. The contained the autoclaved raw effluents seeded with 10 ml broth of *Aspergillus flavus*. The fourth contains the autoclaved raw effluents seeded with 10 ml broth *Trichoderma* sp. The fifth contains the autoclaved raw effluents seeded with *Aspergillus flavus* and *Trichoderma* sp. All non-seeded tanks served as the control (Plate I). All the five (5) plastic container were incubated under ambient conditions and the setup were disturbed manually at interval of 12 hours to allow the fungi propagules to be evenly distributed and also enhance the breakdown of hydrocarbon into droplets, thereby providing increased surface area to accelerate biodegradation. Prior to inoculation, adequate amount of ammonium phosphate was prepared following standard procedure and added equally to each of the set ups to serve as a source of nitrogen and phosphorus. A 350 ml subsamples were withdrawn from each setup for biochemical oxygen demand (BOD) analysis by siphoning using a pipette and the effluent was released from the pipette very gradually to the BOD bottle, avoiding any turbulence (which would add oxygen to the sample) and was determined on five (5) occasions for 25 days using the modified Winkler method at day 5, 10, 15, 20 and 25.

The difference between the initial value of BOD and subsequent BOD's obtained from each gallon was used to indicate the amount of hydrocarbon removed and also showed the capacity of *Aspergillus flavus* and *Trichoderma* sp individually and as a coculture in the removal of hydrocarbon from the raw refinery effluents.

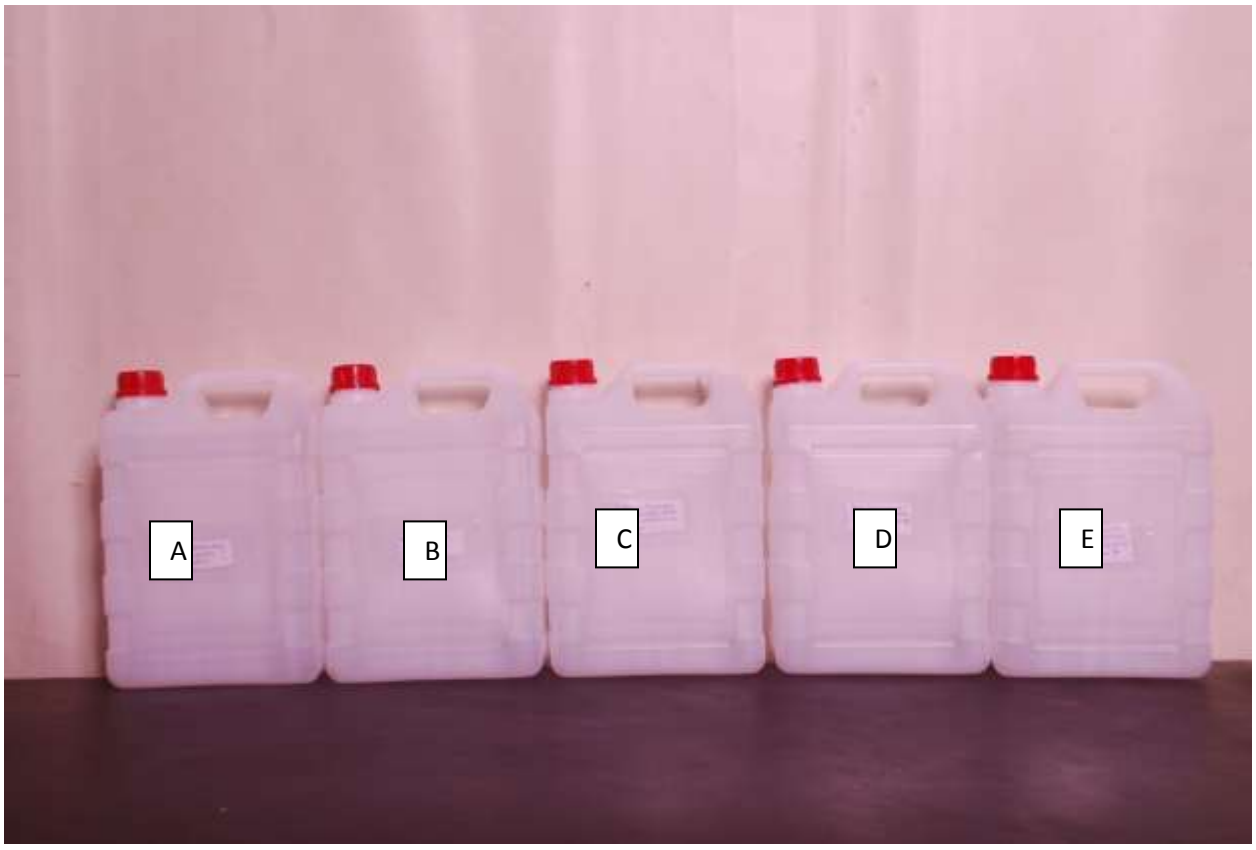

Plate 1: Experimental Setup for Removal of Hydrocarbon

A=Unsterilized raw effluents

B=Autoclaved raw effluents

C=Autoclaved raw effluents seeded with *Aspergillus flavus*

D=Autoclaved raw effluents seeded with *Trichoderma* sp

E=Autoclaved raw effluents seeded with *Aspergillus flavus* and *Trichoderma* sp

3.6 Determination of Removal of Pb, Ni and Cd by the Fungal Isolates

The capacity of the fungal isolates to remove these metals during growth in broth cultures charged with the raw effluent were evaluated by inoculating each isolate into duplicate flasks containing 100ml of freshly prepared potato dextrose broth (PDB) charged with autoclaved raw effluents in the ratio of 4:1. Each flask was similarly prepared in duplicate of PDB without the organism to serve as control. All flasks were incubated on a rotatory shaker (120rpm) at room temperature for 7 days. The 7 days old cultures were filtered through pre-weighed filter paper (Whatman No.1) to separate the mycellial mass from the spent broth cultures. The residual amounts of Cd, Ni and Pb in the filtrates were then determined using MP-AES. The metal removal capacities of the fungal isolates were calculated and expressed in percentage using the formula:

$$\text{Metal removal} = \frac{(X - Y)}{X} \times 100$$

Where X =Initial concentrations of the metal ions and Y = Final concentration of concentration of metal ions in solution after the sorption process respectively.

The harvested mycelia mass on the filter paper were rinsed repeatedly with distilled water to remove loosely bound metal ions and dried in an oven at 70^0 C for 18 hours. The dry weights of the harvested mycelia biomass were determined using sensitive top loading balance. The metal uptake by the test isolates were calculated using the formula below in accordance with Joshi *et al.* (2012).

$$Q = \frac{V(C_i - C_f)}{W} \times 1000 \text{ (mg/g)}$$

Where, Q = Amount of metal taken up and accumulated in the fungal tissues (mg/g),

C_i = concentrations of the metal ions in fungal tissue before the experiment,

C_f = concentrations of the metal ions in the fungal tissues after the experiment,

V = Total working volume,

W = Dry weight of the fungal biomass harvested.

3.7 Data Analysis

Results obtained were interpreted following descriptive statistics and presented in tables and plates

CHAPTER FOUR

4.0 RESULTS

4.1 Physicochemical Properties of Raw Refinery Effluent

Results of the physicochemical analysis of the raw refinery effluents generated by Kaduna refinery revealed that the effluent contained high levels of oil and grease, dissolved oxygen (DO), biochemical oxygen demand (BOD), chemical oxygen demand (COD) which were above the permissible limits set by the Federal Ministry of Environment Nigeria (FMENV, 1995) (Table 4.1). It was also noted that the effluent had much higher electrical conductivity and phosphate contents than the allowable limits. However, the pH and levels of sulphate and nitrate were found to be within the permissible limits (Table 4.1).

Results of analysis also revealed that the raw refinery effluents contained Cd (0.009 mg/l), Ni (0.104 mg/l) and Pb (0.210 mg/l). These concentrations are 3, 1.5 and 21 times respectively higher than values considered permissible for environmental safety (Table 4.1).

Table 4.1 Physicochemical Properties of Raw Refinery Effluent Obtained From KRPC

Physicochemical Parameter	Values	Permissible Limit (FMENV, 1995)
pH	7.52	6.0-9.0
Temperature (°C)	31.9	40
Electrical Conductivity (µS/cm)	866	400
Turbidity (NTU)	43	500
Dissolved Solids (mg/l)	592.20	500
Total solids	598.7	
Suspended Solids (mg/l)	6.530	
Phosphates (mg/l)	8.1	5
Sulphates (mg/l)	39	50
Nitrate (mg/l)	0.01	10
Oil and grease (mg/l)	26.42	10
Dissolved oxygen (mg/l)	290	10
Biochemical Oxygen Demand (mg/l)	130	50
Chemical Oxygen Demand (mg/l)	171.2	80
Cadmium (mg/l)	0.009	0.003
Nickel (mg/l)	0.104	0.070
Lead (mg/l)	0.210	0.010

4.2 Detection of *Aspergillus flavus* and *Trichoderma* sp in Raw Refinery Effluents

Results of microscopic and macroscopic characteristics of *Aspergillus flavus* and T*richoderma* sp isolated from the raw refinery effluent showed that *Aspergillus flavus* and T*richoderma* sp. were actually present in the raw refinery effluent. The result is shown in Table 4.2 and Plate II-VI

Table 4.2 Macroscopic and Microscopic Characteristics of *Aspergillus flavus* and T*richoderma* Species Isolated From the Raw Refinery Effluent

Colony colour on PDA	Colony Reverse colour on PDA	Growth Pattern	Microscopic characteristics	Inferences
Yellow-green	Brownish	Rapid Growth Granular, tuft of White becoming ash with time	Single step sterigmata Hyaline and coarsely Rough conidiophores	*Aspergillus flavus*
White and scattered Greenish patches which Spread from the centre to the margin	Yellowish	Fast growing; wooly becoming compact in time	Septate hyphae, short conidiophores which are flask shaped and clustered together at the end of each phialides	*Trichoderma* sp.

4.3 Capacity of *Aspergillus flavus* and *Trichoderma* sp in the Removal of Hydrocarbon from Raw Refinery Effluent

Results obtained from the mycoremediation studies indicated that *Aspergillus flavus* and *Trichoderma* sp both individually and as co-culture have great potential for application in the removal of hydrocarbon from raw refinery effluent. It was noted that *Aspergillus flavus, Trichoderma* sp and their co-culture removed 80, 74 and 89% of the biodegradable hydrocarbon present in the raw effluent over a period of 20 days respectively (Table 4.3) However, the co-culture of *Aspergillus flavus and Trichoderma* sp proved to be most efficient in the removal of hydrocarbon from the raw refinery effluent within the same period with 89% removal efficiency when compared to *Aspergillus flavus* (80%) *and Trichoderma* sp. (74%). It was also noted that the percentage of hydrocarbon removed increased with increase in period of incubation (Table 4.3).

Table 4.3 Performance of *Aspergillus flavus* and *Trichoderma* sp in the Removal of Hydrocarbon from Raw Refinery Effluent.

Treatment	Amount of Organic Carbon Removed				
	At initial	After 5 days (%)	After 10days (%)	After 15days (%)	After 20days (%)
Raw Effluent	130	30 (23)	81 (62)	90 (69)	100 (77)
Sterile Effluent	130	20 (15)	22 (17)	30 (23)	38 (29)
Sterile Effluent +*Aspergillus flavus*	130	56 (43)	78 (60)	100 (77)	104 (80)
Sterile Effluent + *Trichoderma* sp	130	64 (49)	70 (54)	86 (66)	96 (74)
Sterile Effluent+*Aspergillus flavus*+ *Trichoderma* sp	130	56 (43)	60 (46)	96 (74)	116 (89)

4.4 Performance of *Aspergillus flavus and Trichoderma sp* in the Removal of Cd, Ni and Pb from Raw Refinery Effluent

Results obtained from the mycoremediation studies indicated that the isolates of *Trichoderma* sp proved to be the most efficient in the removal of Cd, Ni and Pb ions from broth cultures charged with raw refinery effluents. *Trichoderma* sp. was found to remove 29%, 87% and 79% of Cd, Ni and Pb ions respectively while *Aspergillus flavus* was found to remove 28%, 87% and 62% of Cd, Ni and Pb ions from the broth culture respectively (Table 4.4). The co-culture of *Aspergillus flavus and Trichoderma* sp. was the least efficient being able to remove only 18%, 95% and 49% of Cd, Ni and Pb ions respectively from the solution (Table 4.4). However, co-culture of these fungi proved to be most efficient in the removal of Ni ions with 95% removal efficiency when compared to *Aspergillus flavus and Trichoderma* sp. alone. Both the individual and co-cultures of *Aspergillus flavus* and *Trichoderma* sp proved to be very efficient in the removal of Ni but not Cd from the effluent. Individual and co-cultures of these fungi also gave promising results as means of removing Pb from the effluent.

Table 4.4: Performance of *Aspergillus flavus* and *Trichoderma* sp. in the Removal of Cadmium, Nickel and Lead from Raw Refinery Effluent

	Amounts of Metal ion Removed (%)					
	Cd		**Ni**		**Pb**	
Test fungi	Initial	Final (%)	Initial	Final (%)	Initial	Final (%)
Aspergillus flavus	0.009	0.0025 (28)	0.104	0.0905(87)	0.210	0.1302 (62)
Trichoderma sp		0.0026 (29)		0.0905 (87)		0.1659 (79)
Co-culture of *A. flavus*+*Trichoderma* sp		0.0016(18)		0.0988 (95)		0.1029 (49)

4.5 Potentials of Fungal Isolates Tested to Bioadsorption of the Target Heavy Metal ions

This parameter was assessed with the view to determining the quantity of Cd, Ni and Pb adsorbed per gram of biomass of *Aspergillus flavus* and *Trichoderma* sp. individually and as co-culture. It was observed that Cd was poorly adsorbed by all the cultures tested. *Aspergillus flavus* adsorbed less Ni and Pb than *Trichoderma* sp and their co-culture (Table 4.5 and 4.6). *Trichoderma* sp. on the other hand, proved to be the most efficient in the adsorption of Pb followed by co-culture of the two fungi.

Table 4.5: Quantities of Cadmium, Nickel and Lead adsorbed by *Aspergillus flavus* and *Trichoderma* sp From Raw Refinery Effluent

	Concentration of Metal ion Adsorbed (mg/g of Biomass)		
Test fungi	Cd	Ni	Pb
Aspergillus flavus	0.00060	0.00958	0.01503
Trichoderma sp.	0.00035	0.01278	0.02544
Aspergillus flavus + *Trichoderma* sp	0.00064	0.01477	0.02388

CHAPTER FIVE

5.0 DISCUSSION

The results of the analysis indicated that the Refinery wastewater was grossly polluted, and that if not properly treated before being discharged into receiving water bodies could be a major cause of pollution. Results from this study showed that temperature of the untreated effluent was within the set down standard and therefore suitable for lives in aquatic environment. It is an indication that fungi tolerate range of temperature within the environment from which they were taken and also depends upon the season and time of sampling. The temperatures found in the present study can influence the amount of dissolved oxygen that is available to aquatic organisms. The value of temperature obtained was in agreement with the report of Ezeonuegbu *et al.* (2015), whose results is 31.7^0C but higher than those of similar work carried out in Morocco by El-Guamri and Belghyti (2006) whose values were between 17.3 and 23.2°C.

Electrical conductivity (EC) estimates the amount of dissolved solids or the total amount of dissolved ions in water (Nwaichi and James 2012). Deionised water has very low electrical conductivity and hence very low TDS. Electrical conductivity (866μs/cm) was found to be far above the permissible limit (400μS/cm) set by Federal Ministry of Environment (FMENV), Nigeria (FMENV, 1995). The high level of electrical conductivity could be due to the high concentration of organic and inorganic compounds (dissolved ions) from the various chemicals used in primary distillation process by the refinery plant and it implies that the effluent should be properly treated to lower electrical conductivity to appreciable levels, because the purer the water, the lower will be its conductivity. According to Yapo *et al.,* (2012) this value showed a strong mineralization of discharges. The results obtained may be due to the strong mineralization of organic load.

Total dissolved solids (TDS) are the total amount of mobile charged ions, including minerals, salts or metals dissolved in a given volume of water. This high value of dissolved solid obtained in this study indicates a high waste assimilation capacity. The high value of dissolved solids may be attributed to prolonged accumulation of the effluent in the sewer without proper dilution. The value of dissolved solids (592.2 mg/l) obtained in this study exceeds the permissible limits, therefore, indicating pollution of the effluent, but is higher than those of similar work performed in the Port Harcourt Refinery Effluent by Otukunefor and Obiukwu (2005) whose value was 383.6 mg/l and also in Kaduna Refinery Effluent by Ezeonuegbu *et al.,* (2015) whose value was 405.67 mg/l. However, Ajao *et al.* (2014) reported high total dissolved solids which was above the permissible limit in Kaduna Refinery effluent and is in agreement with findings in this study.

The results indicated a pH level of 7.52, which falls within the permissible limits. Therefore, this wastewater would have no adverse impact on lives and flora of receiving environment Yapo *et al.* (2012) reported that the alkaline nature of refinery effluent maybe due to the presence of soluble organic and inorganic alkalis. In another study, Ademoroti (1983) found the same results in Nigeria on effluents in Ibadan, also Marcus and Ekpete (2014) found the same result in receiving waterbody from an oil refinery in Rivers state. However the value obtained in this study is lower than those of similar work done in Port Harcourt Refinery effluent by Otukunefor and Obiukwu (2006) whose value is 7.93.

Phosphate is an essential nutrient to plant life, but when found in excess quantities, stimulates excessive plant growth such as algal bloom (Marcus and Ekpete, 2014). The higher phosphate level in the untreated effluent in this study may account for algal bloom observed in many discharge points and the attendant increase in high chemical oxygen demand and biological oxygen demand. These results are much higher than those obtained in similar works carried out

in Nigeria by Ekweozor *et al*., (2001); Ajao *et al.*, *(*2014) and in Cameroon by Endamana *et al*., (2003). This implicates that the effluent as an important source of this anion, high concentration are associated with the effect of eutrophication, a likely consequence of prolonged exposure (Marcus and Ekpete, 2014).

Dissolved oxygen is one of the most important parameters used for water quality control. The effect of waste discharge on a surface water source is largely determined by the oxygen balance of the system and its presence is essential in maintaining biological life within a system (DFID, 1999). Its correlation with water body gives direct and indirect information, e.g. microbial activity, photosynthesis, availability of nutrients, stratification etc (Premlata, 2009). The dissolve oxygen recorded in this study indicates much depletion may have occurred, caused by oxygen consuming chemicals from the refinery.

An indication of organic oxygen demand content of wastewater can be obtained by measuring the amount of oxygen required for its stabilization either as BOD or COD. BOD is the measure of the oxygen required by microorganisms whilst breaking down organic matter. The values of BOD (130mg/l) and COD (171.2mg/l) indicated a significant input of organic substances (Morrison *et al*., 2001). They also indicate that less oxygen is available for the living organisms in the wastewaters. This may be as a result of escape of organic matter from the biological treatment plant. BOD test is useful in determining the relative waste loading and higher degree therefore indicates the presence of large amount of organic pollutant and relatively higher level of microbial activities with consequent depletion of oxygen content. This is in agreement with the work of Ajao *et al*. (2014). The level of chemical oxygen demand (COD) was greater than the permitted limit. This is in agreement with the works of Fatoki *et al*. (2003); Morrison *et al*. (2001) and Ajao *et al*. (2014). These high COD and BOD concentrations observed in this

effluent might be due to the use of chemicals which are organic or inorganic and are oxygen demand in nature.

The concentration of oil and grease in raw refinery effluent (26.42mg/l) was found to be higher than the maximum permissible limit of 10 mg/l for the effluent. Joel and Amajuoyi (2009) reported that the high level of contamination with oil and grease poses a great concern and long term threat to all forms of life. Oil and grease is sticky in nature; they tend to aggregate, clogging drain pipes and sewer lines, causing unpleasant odour and corroding sewer lines under anaerobic conditions (Xu and Zhu, 2004). They also interfere with unit operations in municipal wastewater treatment plants because they float as a layer on top of the water. They also stick onto pipes and walls consequently blocking strainers and filters (Xu and Zhu, 2004). This finding is in agreement with the work of Ajao *et al*. (2014).

The most highly oxidized form of nitrogen compounds is commonly present in surface and ground water because it is the end product of aerobic decomposition of organic nitrogeneous matter. Unpolluted natural waters usually contain only minute amounts of nitrite (Jaji *et al*., 2007). The raw refinery effluent contains nitrite ion in the amount of 0.01 mg/L which is below detectable unit and within the permissible limit of 10mg/l set by FMENV. This result is in agreement with the work of Ajao *et al*. (2014)

The concentrations of Cd (0.009mg/l), Ni (0.104mg/l) and Pb (0.210mg/l) recorded for the effluent used in this study were at levels that are many times higher than values considered permissible for environmental safety (FMENV,1995). For instance, concentrations that are 3, 1.5 and 21 times respectively, higher than values considered permissible for environmental safety (FMENV, 1995). High level of these metals observed in this study may have originated from corrosion products of equipment and pipes, chemical additives, feedstock and catalysts used

(Ezeonuegbu *et al.,* 2014) and could pose significant risk to public health especially when large volume of the effluent is continuously pumped into the environment. In humans, it leads to neurological dysfunction, skin dermatitis and pulmonary odema (Borba *et al*., 2006; Deng *et al*., 2006; Ansari and Malik, 2007). Similar observations have been reported by Bako *et al.* (2002), Ayenemo *et al.* (2005) and Emoyan *et al.* (2006).

Pure cultures of *Aspergillus flavus* and *Trichoderma* sp. were isolated from the raw refinery effluent and was identified with reference to appropriate taxonomic guides (Nagamani *et al*., 2006). As revealed by the findings made in this study, *Asp**ergillus flavus* and *Trichoderma* sp. have the capacity to grow in the raw refinery effluent. This tends to suggest that these fungal genera have the capacity to withstand the harsh environment in the refinery effluent (Edwards and White, 1999; Ulfig *et al.,* 2003). In such environments, growth of fungi strongly suggests that, the genera present are capable of growth on hydrocarbons (Atlas and Bartha, 1992; Cerniglia *et al.,* 1992; Ulfig *et al.,* 2003). In addition, fungal flora of such environment needs to be able to withstand the direct toxicity of heavy metal ions often present at high levels in the effluents and sites impacted by the effluents (Wuyep *et al.,* 2007; Emoyan *et al.* 2006; Adewuyi and Olowu, 2012). Fungi indigenous to refinery effluent can be used for bioremediation (Musa *et al.,* 2015). The ability of the fungi to survive the toxic effects of polycyclic aromatic hydrocarbons (PAHs) (Kari *et al.,* 2003) would be an added ecological advantage. The ability of the fungi to secrete a wide range of extracellular enzymes into their growth environments have been advanced as an explanation for their capacity to grow on a wide range of carbon sources (Kari *et al.,*2003). On the other hand, resistance to high levels of toxic heavy metals has been attributed to the capacity of fungi to bioconvert (David and Jay, 2009), bioabsorb (Shankar *et al.,*

2007; Nilanjana *et al.*,2008; Ashok *et al.,* 2010) or bioaccumulate (David and Jay, 2009; Martins *et al.*,2010) the metal ions.

These results were similar to Uzoamaka *et al*. (2009) who reported that eight indigenous fungal isolates from petroleum-contaminated soils that showed potentials for hydrocarbon biodegradation included *Aspergillus niger, Aspergillus flavus, Trichoderma* sp., *Rhizopus* and *Mucor* sp. and Machido *et al.* (2014) also reported that *Aspergillus flavus,* and *Trichoderma* sp were amongst the fungal flora isolated from raw refinery effluent. This observation agrees with earlier report by Ulfig *et al.*, (2003).

Biological oxygen demand (BOD) is a measure of the oxygen used by microorganisms to decompose organic wastes. If there is a large quantity of organic waste in the water supply, there will also be a lot of organisms present working to decompose this waste. In this case, the BOD level will be high. When BOD levels are high, dissolved oxygen (DO) levels decrease because the oxygen that is available in the water is being consumed by the organisms. Since less DO is available in the water, fish and other aquatic organisms may not survive. As the waste is consumed or dispersed through the water, BOD levels will begin to decline. Nitrates and phosphates in a body of water can contribute to high BOD levels. High level biological oxygen demand of 130mg/l and 350mg/l was observed in both the raw effluent and sterile effluent respectively. The BOD level was reduced in each of the setup. The amount of hydrocarbon removed in each setup ranged from 29-89%. Co-culture of *Aspergillus flavus* and *Trichoderma* sp. proved to be more efficient in the removal of hydrocarbon from the effluent. The highest efficiency noted could be due to the ability of *Aspergillus flavus* and *Trichoderma* sp to secrete enzymes useful in hydrocarbon removal. Also, the pH and temperature also could be attributed to the high removal efficiency noted in this setup, while the lowest removal efficiency was obtained

in setup B, which corresponds to 29%. Although, setup A had removal efficiency, which corresponds to 77%, this high removal efficiency noted in A could be due to the effect of mixed population of microorganisms as the effluent was unsterilized.

The results of mycoremediation experiments conducted in this study revealed 18% to 29% removal of cadmium, 87% to 95% removal of nickel and 49% to 79% removal of lead *Aspergillus flavus* could remove 28% of Cd, 87% of Ni and 62% of Pb, *Trichoderma* sp could remove 29% of Cd, 87% of Ni and 79% of Pb and as co-culture, the two isolates tested could remove 18% of Cd, 95% of Ni and 49% of Pb from raw refinery effluent. *Trichoderma* sp. proved to be the most efficient in the removal of the three metals in raw refinery effluent while *Aspergillus flavus* was consistently the least efficient. It was also noted that as a co-culture *Aspergillus flavus* and *Trichoderma* sp. proved to be more efficient when compared with *Aspergillus flavus* alone but less efficient when compared with *Trichoderma* sp. alone. *Trichoderma* sp had the highest potential to bioaccumulate the metals than *Aspergillus flavus*. The differences may be ascribed to the intrinsic ability of *Trichoderma* sp., its chemical composition of cell wall leading various types of interactions of metals with the fungi (Gadd, 1993). It was therefore concluded that *Aspergillus flavus* and *Trichoderma* sp. could be employed in the removal of Cd, Ni and Pb from heavy metal polluted effluents generated by petroleum refineries and other petro-chemical industries.

Aspergillus flavus and *Trichoderma* sp. have the capacity to remove heavy metal ions from broth cultures charged with raw refinery effluent. These observations agree with the reports of several earlier investigators (Machido *et al.,* 2015; Say *et al.,* 2003; Ashok *et al.,* 2010; Nirmal *et al.,* 2010; Joshi *et al.,* 2011; Dwivedi *et al.,* 2012; Kumar *et al.,* 2012) which demonstrated the capacity of filamentous fungi to remove heavy metal ions from liquid cultures. Though several

mechanisms have been proposed by which heavy metal ions are removed from solution by these investigators, results obtain from this investigation strongly suggest that bioaccumulation to be the dominant mechanism (Table 4.3). This assertion lends support from the reports of Volesky (2001) and Ledin *et al.* (1996) which stated that fungi belonging to genera of *Aspergillus*, *Penicillium* and *Fusarium* have high capacity for bioaccumulation of heavy metals. However, other mechanisms such as biosorption (Wuyep *et al.,* 2007; Nilanjana *et al.,* 2008; Ashok *et al.,* 2010) and bioconversion (David and Jay, 2009), have been shown to contribute significantly in the removal of heavy metals from polluted effluents by fungi during mycoremediation process.

The observations made in this study imply that *Aspergillus flavus* and *Trichoderma* sp could provide an efficient means by which refinery waste effluents may be rendered free of heavy metal pollutants prior to their release into the environment.

The observations made in this study strongly suggest that direct release of untreated waste effluents generated by petroleum refining operations could result in heavy metal pollution of the recipient sites. This assertion lends support from the reports of Ayenimo *et al*. (2005) and Emoyan *et al*., (2006), which provided evidence of heavy metal pollution of two rivers that serve as dump sites for effluents from two refineries in Nigeria. Such reports also tend to emphasize the need for methods of waste treatments that would ensure the removal of heavy metals from such effluents prior to their release into either aquatic or terrestrial environments. Such a step is a pre-requisite towards averting the many hazards associated with exposure to heavy metal pollution (Wahab, 2000; Balaji *et al.*, 2005). Thus far, biological approaches that exploit the potentials of various categories of microorganisms appear to hold great promise and are being vigorously investigated.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

From the results obtained in this study, the following conclusions were drawn:

1. Petroleum refinery generates large volume of effluents as a result of the use of chemicals and water and discharge of untreated effluent, which contains high levels of Conductivity, Phosphate, Oil and grease, Dissolve solids (DS), Dissolved oxygen (DO), Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Pb, Ni and Cd than are considered safe for direct discharge into the environments and can affect the standard of living organism in the environment in a negative way and should therefore, be freed of these pollutants prior to their release into either aquatic and/or terrestrial sites.

2. *Aspergillus flavus* and *Trichoderma* sp. were isolated from raw refinery effluent. This is an indication that effluent contaminated with hydrocarbons and heavy metals can be exploited by oil-degrading organisms.

3. *Aspergillus flavus* and *Trichoderma* sp were found to be effective in the removal of Hydrocarbon and Heavy metals and could be potential biosorbent for the removal of Hydrocarbons, Ni and Pb.

6.2 Recommendations

1. There is need for proper treatment of raw effluent to meet standard guidelines for wastewater discharge with regards to physicochemical parameter such as EC, COD, BOD, TDS, Phosphates, oil and grease, Cd, Ni and Pb.
2. All fungal isolates tested obtained from bioremediation studies should be further explored to develop appropriate technologies applicable as an alternative treatment system for industries before discharging their effluents to appropriate channels.
3. There is need to carry out biodegradation in the already contaminated sites, where effluents are discharged without any other treatment.

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APPENDIX

Plate IIa

Plate IIb

Plate II: Surface (IIa) and Reverse (IIb) cultural appearance of *Aspergillus flavus* on PDA after five days incubation

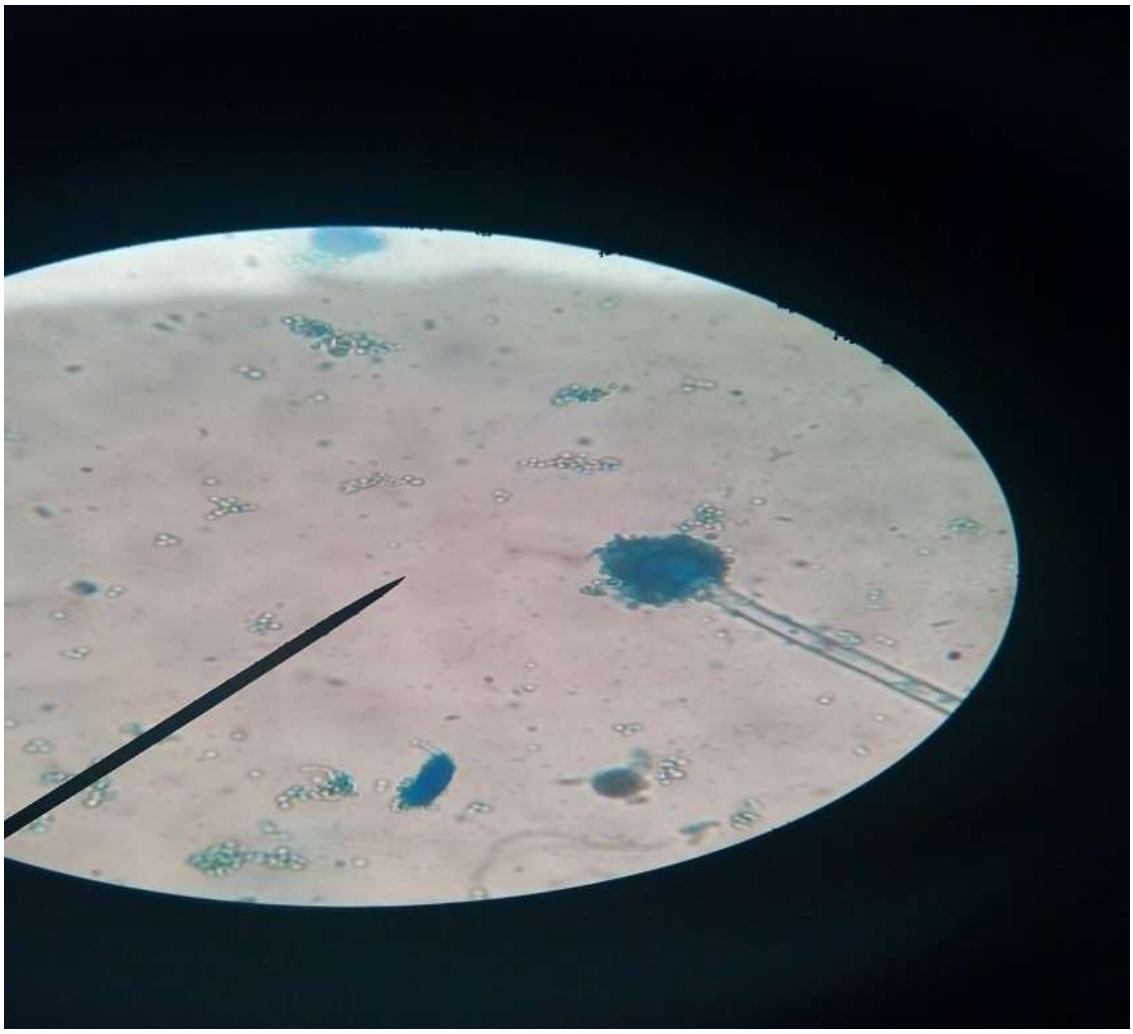

Plate III: Microscopic appearance of *Aspergillus flavus* (X40)

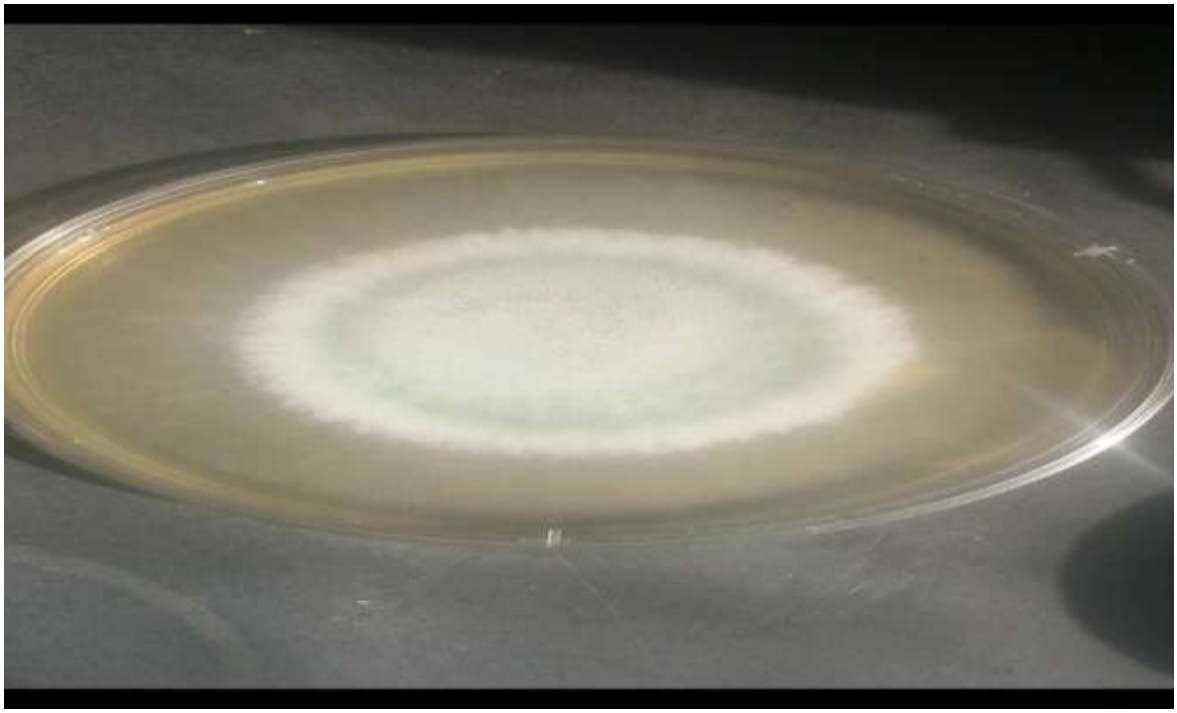

Plate IVa

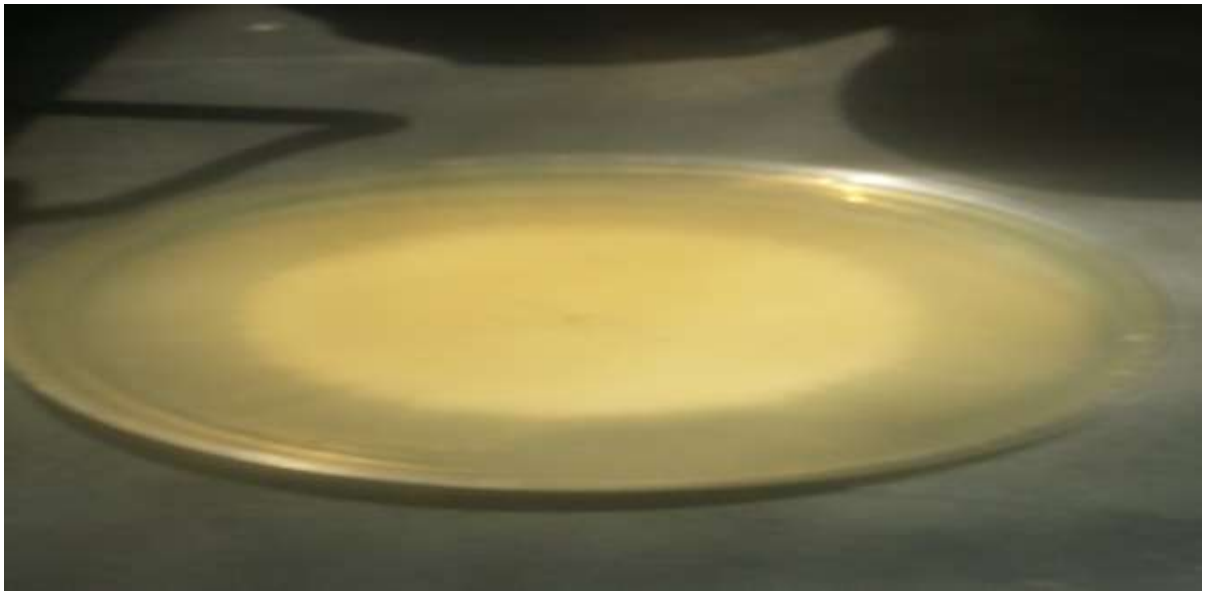

Plate IVb

Plate III: Surface (IIIa) and Reverse (IIIb) cultural appearance of *Trichoderma sp* on PDA

Plate V: Microscopic appearance of *Trichoderma* sp (X40)