CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

The petroleum industry has created economic boom for many countries and at the same time exposed them to environmental and socio-economic problems. The environmental impacts associated with exploration and exploitation of crude oil has remained a concern and has been major area of research in the last three decades. Pollution caused by the refined products such as engine oil and diesel have not been given adequate attention (Ikhajiagbe *et al*., 2013). There are several components of the oil, including solvents and detergents added during the blending process, aliphatic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs) distilled from crude oil, and heavy metals from engine wear. These are either toxic in themselves or can combine with products of combustion to generate toxic materials, carcinogens and endocrine disruptors (Pimda and Bunnag, 2012).

Engine oil is a complex mixture of hydrocarbons and other organic compounds, including some organometallic constituents (Abioye *et al*., 2010). It contains hundreds of aliphatic, branched and aromatic hydrocarbons, most of which are toxic to living organisms (Jain *et al*., 2011). Used oil is any petroleum or synthetic oil that has been used and as a result of such use is contaminated by physical or chemical pollutants (US EPA, 2011). Used engine oil contains numerous toxic substances, including PAHs, which are known to be carcinogenic and mutagenic. Indiscriminate disposal of used engine oil into gutters, water drains, open vacant plots and farm lands is a common practice in Nigeria especially by automobile technicians (Okonokhua *et al*., 2007). Heavy metals such as Vanadium (V), Lead (Pb), Aluminium (Al), Nickel (Ni) and Iron (Fe) usually below detectable limits in unused lubricating oil have been reported to give high values (ppm) in used oil (Adams *et al*., 2014). These metals may be

retained in soils in the form of oxides, hydroxides, carbonates, exchangeable cations, and/or bound to organic matter in soil (Okonokhua *et al*., 2007). There is a growing global concern because of the numerous health risks to animals and humans following exposure (Adams *et al.*, 2014).

Used engine oil renders the environment unsightly and constitutes a potential threat to humans, animals and vegetation (Edewor *et al*., 2004). Fat soluble components may accumulate in the organs of animals and may be enriched in the food chain, even up to humans (Mackay and Fraser, 2000). Prolonged exposure to high concentration of used engine oil may cause the development of liver or kidney disease, possible damage to the bone marrow and an increased risk of cancer (Lloyd and Cackette, 2001; Mishra *et al*., 2001). In the long term, toxic and carcinogenic compounds can cause intoxication, diseases, cell damage, developmental disorders and reproduction problems (Jain *et al*., 2011). In addition to toxic effects, oil products can affect plant and animals physically. A thick layer of petroleum oil inhibits the metabolism of plants (such as photosynthesis and respiration) and suffocates them. Destruction of plants affects the whole food web and decreases the natural habitat of numerous species (Helsinki Commission (HELCOM), 2003).

Diesel oil is a common product of crude oil distillation with a very complex composition. It consists mainly of low molecular weight alkanes and polycyclic aromatic hydrocarbons (PAHs) (Bona *et al*., 2011). The fate of the latter compounds in nature may be of great human health importance, since PAHs have been considered toxic for plants and carcinogenic to humans (Reynoso-Cuevas *et al*., 2008; Bona *et al*., 2011). In case of an uncontrolled industrial leakage, diesel oil and its constituents may act as a persistent water and soil pollutant. According to Nogueira *et al*. (2011), petroleum compounds can decrease the

availability of water, oxygen and nutrients in the soil, which in consequence, may decline the rate of seed germination.

Gasoline (petrol) is the most commonly used transport fuel. It is a highly volatile hydrocarbon derived from crude oil. Accidental spills of gasoline and other petroleum products in the fuel distribution terminals are a common cause of soil and water systems contamination (Vieira *et al*., 2007). Benzene, toluene, ethylbenzene and xylenes (BTEX compounds) present in petrol are highly toxic and harmful to human health; among these, benzene is a known carcinogenic substance (Liu *et al*., 2015; Tohon *et al*., 2015). The presence of ethanol in petrol can increase the solubility of BTEX dissolved in the soil and groundwater, thereby hindering its natural biodegradation by increasing the persistence of these compounds in the environment (Leal *et al*., 2017).

Studies on the effects of metals on organic pollutant biodegradation are not extensive, but the available few demonstrate that metals have the potential to inhibit pollutant biodegradation (Ekpenyong *et al.,* 2007). These metals however, can inhibit various cellular processes and their effects are often concentration-dependent and also vary in their individual toxicity (Talley, 2006). The impacts of metals (Cadmium, Nickel, Zinc, Mercury and Chromium) on litter decomposition, methanogenesis, acidogenesis and biomass generation have all been studied (Ekpeyong *et al*., 2007; Manyi-Loh *et al*., 2013). Olaniran *et al*. (2009) reported the impact of zinc, lead, copper and manganese on crude oil biodegradation by a *Micrococcus* sp. and a *Pseudomonas* sp. Biodegradation measured by microbial growth, was reduced most by zinc and least by manganese.

The acidity of the soil is an important soil parameter. Extreme pH of soils may have a negative influence on the ability of microbial populations to degrade hydrocarbons (Lim, 2006). Many studies have shown that pH mediates metal toxicity (Franklin *et al.,* 2000; Olaniran *et al*., 2013). Increasing the pH reduces the toxicity of nickel to a variety of different organisms, including bacteria (*Serratia marcescens*), filamentous fungi (*Arthrobotrys conoides, Penicillium vermiculatum, Rhizopus stolonifer*), and yeast (*Cryptococcus terreus*) (Olaniran *et al*., 2013). Under mildly basic conditions (pH 8.5), much of the nickel may not be bioavailable because it forms complexes with various ligands. More commonly, increasing pH has been shown to increase the toxicity of zinc, copper, and uranium to certain algal species (Franklin *et al.,* 2000) and of cadmium to various bacteria (*B. subtilis, E. coli, M. luteus, S. bovis*), actinomycetes (*Micromonospora chalcea, Nocardia corallina, Streptomyces flavovirens*), and fungi (*Saccharomyces cerevisiae, Schizosaccharomyces octosporus*) (Olaniran *et al*., 2013).

There have been reports on metal stimulation of biodegradation processes under favourable environmental conditions of pH, temperature and aerobiosis (Ekpeyong *et al*., 2007), but Sandrin and Maier (2003), observed that such stimulatory effects of metals on biodegradation occurred only when consortia, but not single microbial cultures were used for degradation processes. They argued that stimulation was a result of differential toxicity effects of the tested metals.

Oil-contaminated soils are of environmental concern because they are unsuitable for agricultural and recreational uses and are potential sources for surface and ground water contamination. The PAH components of the oil have very low water solubility and often tightly bound to soil particles. Oil-polluted soil could also become unsuitable due to a

reduction in the level of available plant nutrients or a rise to a toxic level of elements such as manganese (Lale *et al*., 2014). It is therefore important that various means by which these pollutants are removed from the environment be carefully considered. This is even more important considering the fact that there are several remediation technologies, some of which are controversial, particularly when they involve physical and chemical methods. These chemical and physical methods which are the most widely used procedures for clean-up are neither entirely simple nor environmentally favourable (Ikhajiagbe *et al*., 2013). This therefore underscores the need for environmental friendly approaches to remediation.

Biodegradation of petroleum hydrocarbons involves the participation of diverse taxonomic groups of microorganisms particularly bacteria and fungi, but the relative importance of bacteria versus fungi in aquatic hydrocarbon degradation is a matter of controversy (Ekpenyong *et al*., 2007). *Penicillium, Aspergillus, Rhodotorula, Candida, Saccharomyces, Sporobolomyces* and *Geotrichum* are fungal genera frequently encountered in oil-impacted aquatic systems (Ijah and Antai, 2003). Fungi are especially well-suited to polynuclear aromatic hydrocarbon (PAH) degradation relative to other bacterial decomposers for a few reasons. They can degrade high molecular-weight polynuclear aromatic hydrocarbons (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. Also, they can function in the very low-oxygen conditions that occur in heavily PAH-contaminated zones (Fernandez-Luqueno *et al*., 2010).

Enzymes are biological catalysts that facilitate the conversion of substrates into products by providing favourable conditions that lower the activation energy of the reaction (Karigar and

Rao, 2011). Fungal enzymes especially, oxidoreductases, laccase and peroxidases have prominent application in removal of PAH contaminants either in fresh, marine water or terrestrial environments. 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 in fungi receives a considerable attention for their enzyme secretion in response to hydrocarbon contaminated sites.

Microbial surfactants (biosurfactants) are a structurally diverse group of amphipathic surface-active molecules synthesized by a variety of bacteria, yeasts and filamentous fungi (Christofi and Ivshina, 2002). Biosurfactant producing microorganisms are naturally present in the oil-contaminated soil. Oil-contaminated environments contain large amount of hydrocarbons (both aliphatic and aromatic hydrocarbons). Microorganisms exhibit emulsifying activity by producing biosurfactants often mineralizing them or converting them into harmless products. They are more active and less toxic than chemical surfactants which are difficult to remove or degrade from the environment. Biosurfactants can be used in handling industrial emulsions, control of oil spills, biodegradation and detoxification of industrial effluents and in bioremediation of contaminated soil (Kosaric, 2001).

Biodegradation of petroleum hydrocarbons produces as end products, innocuous substances including microbial biomass, carbon dioxide and water, which are all environment – friendly (Ekpenyong and Antai, 2007). The application of bioremediation capabilities of indigenous microorganisms to clean up pollutants is viable and has economic values (Bijofp, 2003). The major drawback of a bioremediation exercise is the relatively slow rates at which the process occurs. Heavy metals and pH, among other implicated factors, are on the frontline of this

limitation (Sandrin and Maier, 2002). The manipulation of pH (which curiously has not been well studied) has been suggested as a possible approach to reducing heavy metal toxicity to hydrocarbon-degrading microorganisms (Sandrin and Maier, 2003). Heavy metal ions exert a stronger inhibitory effect on biodegradation than the inhibitory effect caused by high concentrations of toxic organic compounds (Shukla and Cameotra, 2012). Therefore, it is very important to include the effect of heavy metal concentration in bioremediation studies.

1.2 Statement of the Problem

As the local consumption of engine oil in Nigeria increases due to the upsurge in the number of vehicles and other machines that make use of this lubricant, pollution from used (spent) engine oil has become a major environmental problem in Nigeria. The discharge of spent engine oil into gutters, watercourse, open vacant plots and farm-land are common practices among automobile technicians. These practices increase the incidence of oil contamination of agricultural soils, and render the soils unfit for agricultural and recreational activities as well as potential sources for surface and ground water contamination (Igwe-Ezikpe *et al*., 2009; Achuba and Peretiemo-Clark, 2008; Nwoko *et al*., 2007). Used motor oil contains numerous toxic substances, including polycyclic aromatic hydrocarbons, which are known to be carcinogenic and mutagenic. In addition, tiny pieces of metal from engine wear and tear, such as lead, zinc, iron, copper, cobalt and arsenic make their way into lubricants, further contributing to the health hazards of used engine oil following exposure. These elements if in excess will lead to translocation of plant tissues (Adams *et al*., 2014). Although some heavy metals at low concentrations are essential micronutrients for plants, at high concentrations, they could cause high metabolic disorder and growth inhibition for most plant species. This is coupled with the potential of some plants to bioaccumulate heavy metals make the presence of spent oil in soil worrisome. Soil contamination with spent engine oil that is rampant in

Nigerian environment has adverse effects for seasonal crops such as tomato (*Lycopersicum esculentum*) and maize (*Zea mays*) (Okonokhua *et al*., 2007).

It is therefore important that various means by which these pollutants are removed from the environment be carefully considered. The chemical and physical methods which are the most widely used procedures for clean-up are neither entirely simple nor environmentally favourable (Ikhajiagbe *et al*., 2013). This therefore underscores the need for environment friendly approaches to remediation.

1.3 Justification of the Study

Since contamination of soil and groundwater by the indiscriminate discharge of petroleum products has become a significant problem in Nigeria, several methods such as physical and chemical (physicochemical techniques) are already being used to clean up the contaminants, but most of them are costly and far away from their optimum performance. Chemical and thermal methods are both technically difficult, expensive and can degrade the valuable components of the soil (Tangahu *et al*., 2011). The physicochemical techniques also destroy all biological activities in the soil (Gaur and Adholeya, 2004). Excavation and subsequent disposal to landfill merely shifts the contamination problem elsewhere along with the hazards associated with the transportation of contaminated soil.

Bioremediation is inexpensive, naturally and socially acceptable and environmentally friendly. The technology under study will help minimize the prohibitive cost associated with soil remediation, prevent significant soil texture alteration, prevent the transfer of pollutants from one medium to another and ensure a healthier environment.

1.4 Aim of the Study

This study aims to investigate the impact of heavy metals on used engine oil degradation by fungi isolated from automobile workshops in Mgbuka-Nkpor, Nigeria.

1.5 Objectives of the Study

The objectives of the study are to:

i. Determine the physicochemical parameters of the soil samples
ii. Determine the level of heavy metals in used and unused engine oils
iii. Isolate and identify fungi from soil samples obtained from used engine oil-polluted soil
iv. Assess the used engine oil, diesel and petrol biodegradation potentials of the isolates
v. Assay for biosurfactant activity of the isolates in used engine oil, diesel and petrol
vi. Assay for activity of catalase, lipase and peroxidase of the isolates in used engine oil, diesel and petrol
vii. Determine the ability of both the pure and mixed culture of the isolates to degrade the PAH component of used engine oil, diesel and petrol
viii Determine the effects of varying concentrations of heavy metals on used engine oil degradation by the pure and mixed cultures of the isolates at different pH levels
ix Evaluate the effectiveness of the isolates in bioremediation of used engine oil contaminated soil using bioaugmentation technique.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Effect of Heavy Metals on Organic Pollutant Biodegradation

Forty percent (40%) of hazardous waste sites on the Environmental Protection Agency's (EPA) National Priority List (NPL) are co-contaminated with organic pollutants and heavy metals, and remediation of these sites pose a complex challenge because of the mixed nature of contaminants (Olaniran *et al.,* 2013). Co-contamination often causes a synergistic effect on cytotoxicity (Lin *et al.,* 2006), and the two components often must be treated differently (Hoffman *et al.,* 2005; Sandrin and Maier, 2003; Sandrin *et al.,* 2000). Such concerns have heightened the need for novel and advanced bioremediation techniques to effectively remove organic pollutants from a variety of co-contaminated environmental media including water, sediments and soil (An *et al.*, 2005; Duran and Esposito, 2002). Metals most frequently found at United States EPA Superfund sites are divided into two categories namely; *cationic* metals (metallic elements whose forms in soil are positively charged cations) and *anionic* compounds (elements whose forms in soil are combined with oxygen and are negatively charged).

The most common problem-causing cationic metals are mercury (Hg), cadmium (Cd), lead (Pb), nickel (Ni), copper (Cu), and zinc (Zn) and chromium (Cr), whereas the most common anionic compound is arsenic (As) (Natural Resources Conservation Services (NRCS), 2000). Common organic pollutants at these sites include petroleum, polycyclic aromatic hydrocarbons (PAHs), chlorinated solvents, herbicides and pesticides (Hoffman *et al.,* 2005; Amor *et al.,* 2001). Few reports have focused on the adverse effects of heavy metals on biodegradation in co-contaminated environments, under both aerobic and anaerobic conditions. These effects include extended acclimation periods, reduced biodegradation rates

and failure of the degradation of the target compound (Shukla and Cameotra, 2012). Complications of the effects of metal toxicity on organic pollutant biodegradation in co-contaminated soil and water environments stems from the fact that heavy metals may be present in a variety of chemical and physical forms, namely, soil adsorbed species, soluble complexed species and ionic solutes (Olaniran *et al.*, 2013). Further impediments arise due to the impact of environmental conditions such as pH, redox potential of the water phase as well as soil properties, including ion exchange capacity, clay type and content and organic matter content, on the physical and chemical state of the metals (Sandrin and Maier, 2003).

Metals play an integral role in the life processes of microorganisms. Some metals, such as calcium, chromium, cobalt, copper, iron, magnesium, manganese, nickel, potassium, sodium and zinc serve as micronutrients and are used for redox-processes; to stabilize molecules through electrostatic interactions; as components of various enzymes; and for regulation of osmotic pressure (Bruins *et al.*, 2000). Thus, metal ions may play important roles as "trace elements" in sophisticated biochemical reactions. Many other metals (e.g., silver, aluminium, cadmium, gold, lead and mercury), have no biological role and are nonessential and potentially toxic to microorganisms (Olaniran *et al.*, 2013). At higher concentrations these heavy metal ions form unspecific complex compounds within the cell, which leads to toxic effects, making them too dangerous for any physiological function. Toxicity of nonessential metals occurs through the displacement of essential metals from their native binding sites or through ligand interactions (Bruins *et al.*, 2000). For example, Hg^{2+}, Cd^{2+} and Ag^{2+} tend to bind to sulfhydryl (-SH) groups of enzymes essential for microbial metabolism, and thus inhibit the activity of the sensitive enzymes (Olaniran *et al.*, 2013). To have a physiological or toxic effect, most metal ions have to enter the microbial cell. Many divalent heavy metal cations (e.g., Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}) are structurally very similar. Also, the

structure of oxyanions such as chromate resembles that of sulfate, and the same is true for arsenate and phosphate. In such cases, these toxic metal cations may substitute for physiological essential cations within an enzyme rendering the enzyme non-functional (Olaniran *et al.,* 2013). Thus, to be able to differentiate between structurally very similar metal ions, the microbial uptake systems have to be tightly regulated.

Even though microorganisms have specific uptake systems, high concentrations of nonessential metals may be transported into the cell by a constitutively expressed unspecific system (Bruins *et al*., 2000; Olaniran *et al*., 2013). In addition, at high levels, both essential and nonessential metals can damage cell membranes; alter enzyme specificity; disrupt cellular functions; and damage the structure of DNA (Bruins *et al.,* 2000). Also, concentrations of elevated levels of heavy metals impose oxidative stress on microorganisms (Olaniran *et al*., 2013). Sandrin and Maier (2003) presented three inhibition patters of heavy metals (Fig. 2.1). In the first pattern (A), the inhibition of heavy metals is proportional to their concentrations. In pattern B, low concentrations of metals stimulate microbial activity while inhibition begins to show up at high metal concentrations. This phenomenon is usually found in the mixed consortia system. The stimulation at low metal concentrations can be attributed to the differential toxicity effects. Metals may select for a metal-resistant, functional population while inhibiting a metal-sensitive, non-functional population. Differential toxicity effects reduce competition for resource needed by the metal-sensitive, non-functional population, thus resulting in apparent stimulation. In pattern C, metals exhibit inhibition at low concentrations. However, inhibition, after a maximum level, becomes milder under higher metal concentrations. This may be the results of microbial community evolution of high metal-resistant microorganisms or more efficient detoxification mechanism induced at high metal concentrations.

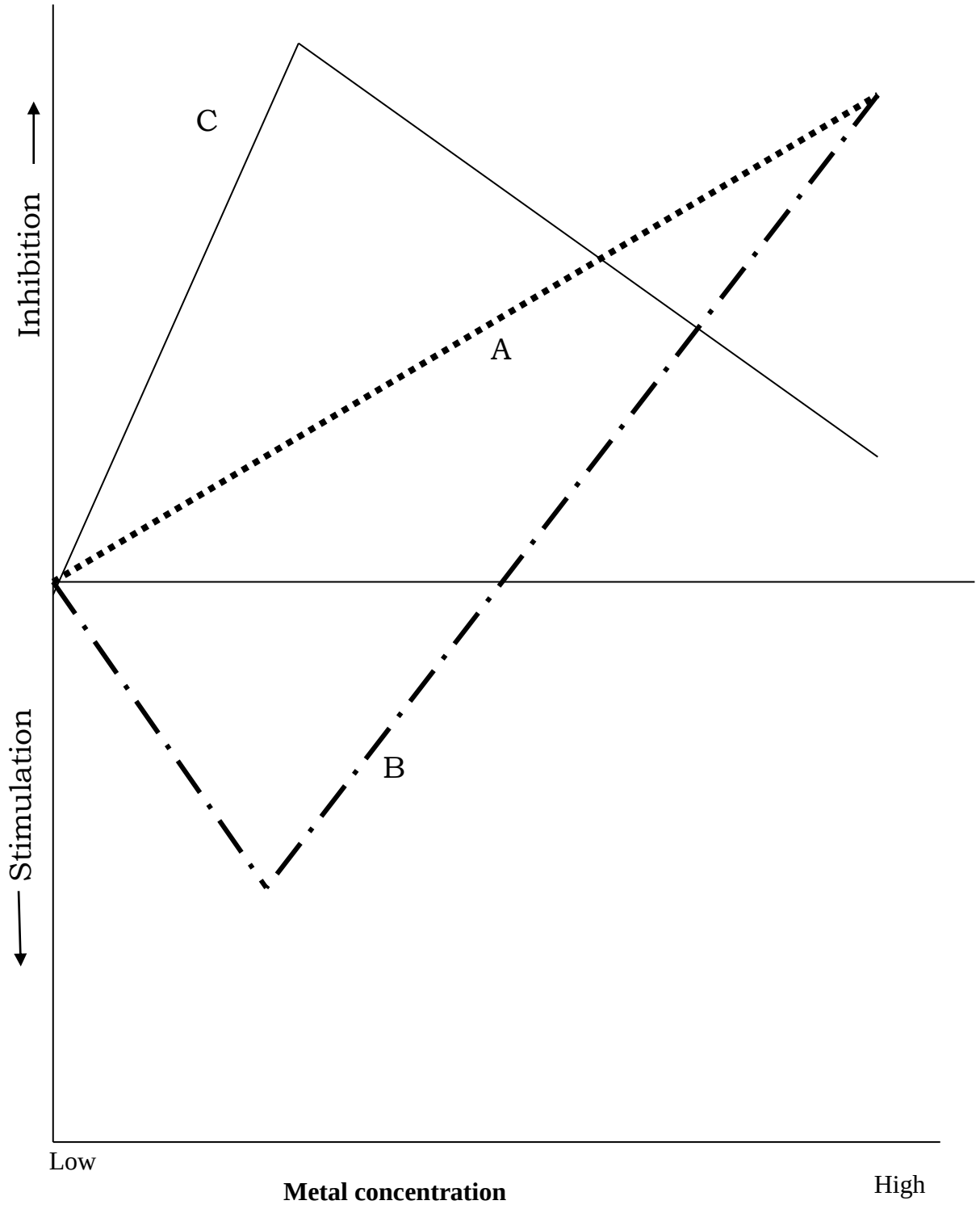

Fig. 2.1: Inhibition patterns of heavy metals (Sandrin and Maier, 2003).

Heavy metals may inhibit pollutant biodegradation through the interaction with enzymes directly involved in biodegradation or those involved in general metabolism. The ionic form

of the metal mediates inhibition of enzymes involved in pollutant degradation in heavy metal contaminated environments (Olaniran *et al*., 2013), indicating that metal toxicity is related to the concentration of bioavailable metal rather than the total or even total soluble metal concentration.

Wang *et al*. (2010) investigated the toxicity of Cu (II) and Ni (II) on aerobic granular sludge in sequencing batch reactors (SBRs). The reactors were operated with 5mg/L Cu(II) and Ni(II) for 26 days and then the metals concentration was increased to 15mg/L. Results show that biomass growth in the reactor was inhibited by 5mg/L of Cu(II), while Ni(II) stimulated biomass yield even under the concentration of 15mg/L. Fluorescent *in situ* hybridization (FISH) analysis of Principi *et al.* (2006) revealed dramatic decrease in the abundance of *β-proteobacteria* under metal addition, which comprised ammonia-oxidizing bacteria. Stasinakis *et al.* (2003) also demonstrated nitrifying bacteria the most sensitive parts in the microbial community. Heavy metal can influence phosphorus removal through affecting alkaline phosphatase activity (APA). Zn^{2+} and Cu^{2+} at the concentration of 5.0mM had an obvious inhibitive effect on the APA while Mn^{2+}, Co^{2+}, Pb^{2+} and Cr^{6+} ions stimulated the APA (Xie *et al.,* 2010).

Many researchers studied the effects of heavy metals on activated sludge system (Ong *et al.,* 2004; Ong *et al.*, 2005; Santos *et al.,* 2005; Sirianuntapiboon and Ungkaprasatch, 2007; Tsai *et al.,* 2006). However, results from these studies are difficult to be compared due to the various metal bioavailability caused by different operation modes, substrates compositions seed sludge sources and concentrations. The total organic carbon (TOC) removal efficiency of activated sludge process decreased from 98% to 88% in the presence of Ni(II) while the

same Ni(II) loading rate exerted slight influence on aerobic granules system (Ong *et al.*, 2004; Wang *et al.*, 2010).

Microorganisms can alternate their metabolic pathways or redistribute themselves to acclimate to metal toxicity. Viret *et al.* (2006) studied the influence of Zn (II) and Ni (II) on oxygen consumption of benthic microbial communities. The oxygen consumption at the surface was found to decrease from 90% to 60% after Zn (II) and Ni (II) spiking. However, the oxygen consumption zone was stretched, implying the migration of aerobe into inner space to avoid metal toxicity and/or the metabolism switch of facultative aerobic microorganisms to aerobic respiration which is more efficient than the original fermentation.

Heavy metals mainly exist in the forms of Me^{2+}, $MeSO_4$, and $MeCl^-$ at pH < 7, while $MeHCO_3^+$ and $MeCO_3$ are dominant at pH > 7 (Hietala and Roane, 2009). Sandrin and Maier (2002) reported that the ionic cadmium (Cd^{2+}) concentration at pH 4 was 44mg/L while it decreased to 4mg/L at pH 7. Metal bioavailability is enhanced under acidic conditions, thus increasing the potentional metal toxicity. Metal bioavailability is also influenced by redox potential. High redox potential (800 to 0 mV) favors metal solubility while low redox potential (0 to - 400 mV) immobilizes metal in precipitated forms (Hietala and Roane, 2009).

Microbial bioremediation affects the fate of heavy metals. Heavy metals cannot be degraded in microbial metabolism/co-metabolism process. Bioremediation of heavy metals is accomplished by the conversions between inorganic and organic forms or the inorganic valence changes through redox (Ramasamy *et al.*, 2007). At least four general approaches are involved in the bioremediation of heavy metals (Shuguang *et al.*, 2010, Fig. 2.2). The mechanism by which microorganisms act on heavy metals include biosorption (metal

sorption to cell surface by physiochemical mechanisms), bioleaching (heavy metal mobilization through the excretion of organic acids or methylation reactions), biomineralization (heavy metal immobilization through the formation of insoluble sulfides or polymeric complexes), intracellular accumulation, and enzyme-catalyzed transformation (redox reactions) (Lloyd, 2002). On the basis of energetic requirements, biosorption seems to be the most common mechanism (Haferburg and Knothe, 2007). It is the only option when dead cells are applied as bioremediation agent. However, systems with living cells allow more effective bioremediation processes as they can self-replenish and remove metals via different mechanisms (Malik, 2004). On the other hand, living cells shows higher sensitivity to environmental conditions and demand nutritional and energetic sources. Many genera of microbes like *Bacillus, Enterobacter, Escherichia, Pseudomonas* and also some yeasts and molds help in bioremediation of metal and chromium-contaminated soil and water by bio-absorption and bioaccumulation of chromium (Kotas and Stasicka, 2000). The heavy metal removal by the bacteria *Pseudomonas* was attributed to the cellular growth of these organisms (Ray and Ray, 2009).

Biosorption between positively charged heavy metals and negatively charged cell walls occurs commonly in the biological treatment system. Moreover, cell walls and extracellular polymeric substances (EPS) secreted by cells consist of complex substances, such as lipopolysaccharides, proteins and carbohydrates (Shuguang *et al.*, 2010). These chemicals contain abundant functional groups which provide sorption sites for metal binding and metal immobilization. Heavy metals can be accumulated within cells via membrane transport systems. Heavy metals are first bound to extracellular ligands and then get transported through cell wall with these ligands. Once inside the cell wall, metals are inactivated,

localized within intracellular structures, or participate in biochemical process (Shuguang *et al.,* 2010).

The principle of reductive biotransformation of heavy metals lies on the decrease of mobility and toxicity when metals are reduced to lower redox states. In the direct reduction process, metal-reducing microorganisms use the oxidized form of metals (such as Cr (VI), U (VI), and Tc (VII)) as electron acceptors and transform them into reduced species (Cr (III), U (IV), and Tc (IV), Fig. 2.2). Aerobic and anaerobic reduction of Cr (VI) to Cr (III) has been accomplished by a wide range of microorganisms (Kamaludeen *et al.,* 2003).

Reductive biotransformation also facilitates indirect metal immobilization. Metal-reducing and sulphate-reducing bacteria are usually involved in this process. Electrons extracted from the oxidation of organic compounds or hydrogen is used to reduce Fe (III), Mn (IV), and SO_4^{2-} to Fe (II), Mn (III), and H_2S. Heavy metals then interact with these reduced products to form separate or multicomponent insoluble species (van Hullebusch *et al.,* 2005). Indirect enzymatic reduction often happens in sedimentary and subsurface environments or in anaerobic wastewater treatment reactors. The most active reduced products are Fe (II) and H_2S. Fe (II) is used as electron donor in the reduction of Cr (VI) to Cr (III) by Fe-reducing bacteria, such as *Geobacter, Desulfuromonas, Shewanella,* and *Pelobacter* (Wielinga *et al.,* 2001). Heavy metals in up-flow anaerobic sludge bed (UASB) reactors are often precipitated by sulphide produced by sulphate biological reduction, which enables the simultaneous removal of heavy metal, sulphate, and organic pollutants (De Lima *et al.,* 2001; Sierra-Alvarez *et al.,* 2006).

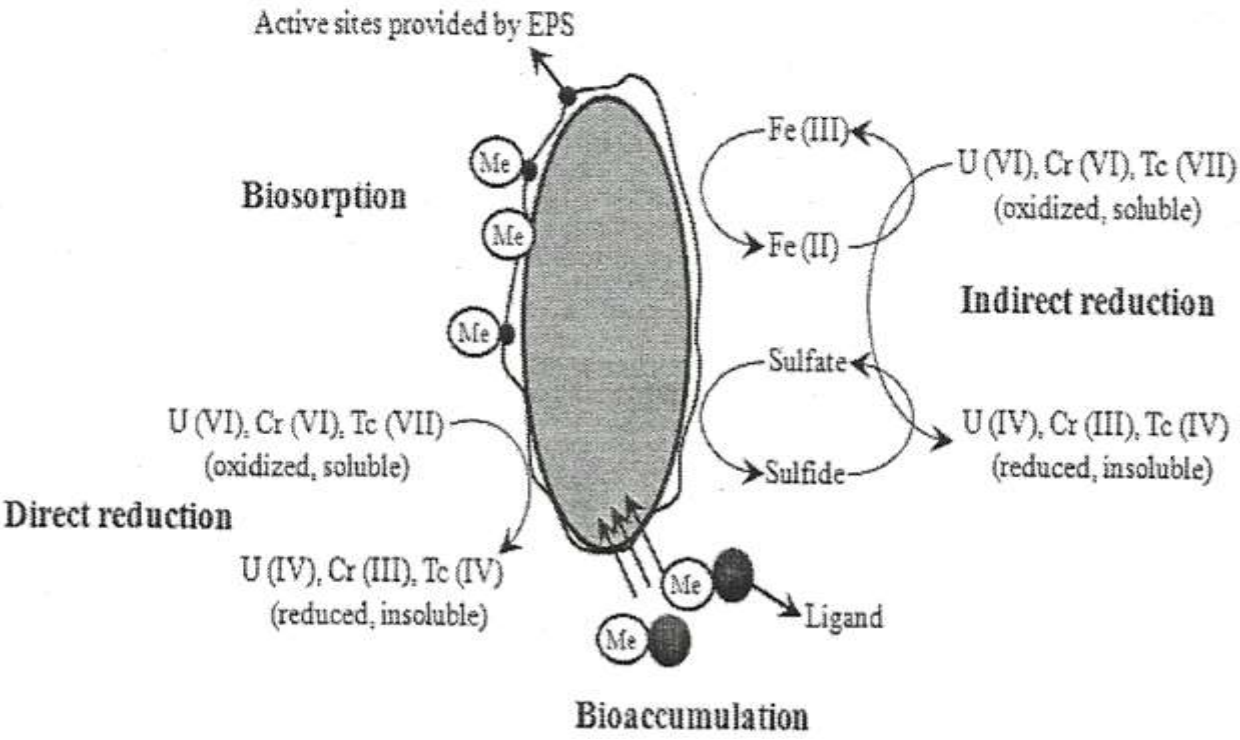

Fig. 2.2: Possible metal-microbe interactions (Shuguang *et al.,* 2010).

Various biosorbents, such as fungus, algae, bacteria, and activated sludge, have been used to remove heavy metals. The maximum adsorption capacities for Cd^{2+} of these biosorbents lie in a range of 22-153 mg/g, and those for Zn^{2+} and Cu^{2+} were 14-170 and 5.9-130mg/g dry biomass (Liu *et al.,* 2004). The adsorption capacity depends on various system parameters, such as pH, temperature, and ionic strength. Among them, pH is the most important factor by affecting the chemistry of both the biomass surface and heavy metals. The adsorption capacities of aerobic granules are usually enhanced at higher solution pH. For example, the biosorption capacities of aerobic granules increased from 20mg Pb^{2+}/g to 44mg Pb^{2+}/g when the solution pH was increased from 3.0 to 4.0 (Yao *et al.,* 2008). Cu (II) adsorbed by aerobic granules at pH 3 was 19.25mg/g and that at pH 5 was 36.72mg/g (Gai *et al.,* 2008). It is

believed that the biomass surface is protonized at low pH. The protonized ligands and metal cations will compete for binding sites. As the pH increases, more functional groups with negative charges becomes exposed, which results in the biosorption capacity enhancement (Gai *et al.,* 2008; Hawari and Mulligan, 2006). Higher temperature always favours the adsorption process through the increase in surface activity and kinetic energy of the solute (Sag and Kutsal, 2000). However, the influences of temperature are usually insignificant and operating the adsorption system at higher temperature is impractical.

Heavy metal ions are naturally found in soil but human activities are increasing unexpectedly the concentration of heavy metals in the soil, which is of great concern (Shukla and Cameotra, 2012). Heavy metals affect the ecosystem adversely, for example mercury at low concentrations represents a major hazard to microorganisms and other biota. Inorganic mercury has been reported to produce harmful effects at concentrations of 5µg/l in a culture medium (Boening, 2000; Orct *et al.,* 2006). Similarly other metals also have an adverse effect on microorganisms and ecosystem (Sobolev and Begonia, 2008). Heavy metals not only contaminate the soil but also restrict the microbial activity at the polluted site, consequently affecting the degradation of other organic pollutants (Mittal and Ratra, 2000).

Generally, heavy metals exert an inhibitory action on microorganisms by blocking essential functional groups, dislodging essential metal ions or transforming the active conformations of bio-molecules (Saurav and Kannabiran, 2011). After heavy metal ions entered into the cell, toxicity occurs through numerous biochemical pathways, which can be divided into five categories as follows (Harrison *et al.,* 2007).

1. Toxic metal species can bind to proteins in lieu of essential inorganic ions, thereby altering the biological function of the target molecule.

2. Heavy metals tend to react with thiol (SH) or disulfide groups destroying the biological function of proteins that contain sensitive S groups. By binding to these groups, metals can inhibit the functions or activity of many sensitive enzymes.
3. Certain transition metals can participate in catalytic reactions, known as Fenton-type reactions that produce reactive oxygen species (ROS). Collectively, these reactions place the cell in a state of oxidative stress, and increased levels of ROS damage DNA, lipids, and proteins through a range of biochemical routes.
4. The transporter-mediated uptake of toxic metal species might interfere with the normal transport of essential substrates owing to competitive inhibition.
5. Some metal oxyanions are reduced by the oxidoreductaseDsbB, which draws electrons from the bacterial transport chain through the quinone pool. In fact, certain toxic metal species starve microbial cells by indirectly siphoning electrons from the respiratory chain.

It has been found that heavy metals greatly affect the biodegradation of organic pollutants by interacting with microbial enzymes or their cell walls, by interfering with the microbial general metabolism or by interrupting the functioning of the enzymes participating in the degradation of hydrocarbons (Sobolev and Begonia, 2008; Merroun, 2007; Haferburg and Kothe, 2007; Sandri and Maier, 2003). Silver *et al.* (2007) reported the high metal tolerance of the strain *Burkholderia cepacia* JT50 for chromium and mercury, however a reduction in the degradation efficiency was observed at high metal concentrations. The study was carried out using mineral salt medium at pH 7.0 in which different chromium and mercury concentration were incorporated; however it was not clear from the study the actual metal speciation of the mercury and chromium ions during testing (Shukla and Cameotra, 2012). Tadama *et al*. (2015) showed that the anaerobic degradation of phenol, benzoate and their

chloro derivatives; 2-chlorophenol and 3-chlorobenzoate respectively slowed down in the presence of added heavy metal ions; Cd (II), Cu (II), Cr (VI) and Hg (II). However, the different metals showed different toxicity patterns to the organisms. The consortia showed susceptibility towards some specific metals for example 3-chlorobenzoate degradation was most sensitive to Cd (II) and Cr (VI), degradation of phenol and benzoate was sensitive to Cu (II) and Hg (II), however enhanced biodegradation of phenol and benzoate was observed on the addition of small amount of Cr (VI). Cu(II) was found to enhance the degradation rate of 2-chlorophenol and interestingly Hg (II) at 1.0 to 2.0ppm was found to enhance the degradation rate of 2-chlorophenol and 3-chlorobenzoate after an extended acclimation period indicating the adaptation of the consortia to mercury (Shukla and Cameotra, 2012).

Heavy metals are increasingly found in microbial habitats due to natural and industrial processes (Shukla and Cameotra, 2012). Thus, microorganisms have been exposed to metal polluted environments for long time, which has forced them to develop several mechanisms to tolerate, resist or detoxify these metal ions by efflux, complexation, or reduction of metal ions, to use them as terminal electron acceptors in anaerobic respiration (Dhanjal and Cameotra, 2010; Jaysankar *et al.,* 2008; Sobolev and Begonia, 2008; El-Deeb, 2009; Johncy *et al.,* 2010; Chaalal *et al.,* 2005; Patel *et al.,* 2006). However, toxicity of metals depends upon the extent to which it penetrates the microorganism cells (Shukla and Cameotra, 2012).

The concern over the persistence, disposition and presence of co-contamination of metals and polycyclic aromatic hydrocarbons in the environment (air, soil and water system) has increased, since these chemicals have been shown to be carcinogenic to humans and livestock. Their addition in soil has been known to inhibit soil respiration, nitrogen mineralization and nitrification (Lao *et al.,* 2005; Sobolev and Begonia, 2008; Nwuche and

Ugoji, 2008). Heavy metals have also been implicated in the reduction of degradation of vegetable materials and can potentially limit the biodegradation of organic contaminants in the environment (Sokhn *et al.,* 2001; Riis *et al.,* 2002; Atagana, 2010).

Field studies of metal contaminated soils have demonstrated that elevated metal concentrations can result in decreased microbial community size and thus their activity. This occurs either by complete inhibition of various metabolic activities like protein denaturation, inhibition of cell division, cell membrane disruption or the organisms develop resistance or tolerance to the elevated metals (Chaalal *et al.,* 2005; Zukauskaite *et al.,* 2008; Alisi *et al.,* 2009). The resultant effect of co-contamination is increased time span of remediation and increase in cost implication associated with carrying out effective remediation of contaminant.

2.2 Environmental Impact of Petroleum Hydrocarbon Pollution

The world depends on oil. Vast amounts is used, transported, processed and stored around the world. In 2003, the total world consumption of petroleum was over 13.1 billion liters per day. The United States Energy Information Administration Projects (as of 2006) world consumption of oil to increase to 98.3 million barrels per day ($15.63X10^6$ m^3 $day^{-1)}$ in 2015 and 118 million barrels day^{-1} ($18.8X10^6$ m^3 day^{-1}) in 2030 (Energy Information Administration (EIA), 2006). With such a huge consumption, oil spills are inevitable.

Incidence of environmental pollution due to high rate of petroleum related activities in the Niger Delta area of southern Nigeria and other oil producing areas of the world has been associated with frequent oil spills, especially through oil well blow outs, tanker accidents, bunkering, rupture of pipelines and sabotage. Disasters arising from such incidence results in

the discharge of crude oil into the environment affecting both soil, air and water bodies. This threatens human health and that of organisms that are dependent on the soil (Aboribo, 2001). Accidental release of hydrocarbons into the environment and its attendant detriments is not restricted to oil producing regions alone, but other areas which are also prone to the increasing risks and possibility of spills due to tanker accidents and leakage from ruptured pipelines networked across such areas (Akpoveta *et al.,* 2011).

The most notable oil spills at sea involve large tankers, such as Exxon Valdez, which spilled thousands of tones oil (Albaiges *et al.,* 2006). These oil spills can cause severe damage to sea and shoreline organism (Whitfield, 2003). Most responsible for the contamination are service stations, garages, scrap yards, waste treatment plants, sawmills and wood impregnation plants. Several studies have been carried out to examine the fate of petroleum in various ecosystems (Okoh, 2006; Jain *et al*., 2011).

Our seas, oceans and coastal zones are under great stress; and pollution particularly by petroleum oil, remains a major threat to the sustainability of planet Earth (Halpern *et al.,* 2008). An estimated 1.3million tons of petroleum enters the marine environment each year (National Research Council (NRC), 2003). Acute pollution incidents cause great public concern, notably 600,000 tons of crude oil released after the Deepwater Horizon explosion in the Gulf of Mexico (Crone and Tolstoy, 2010) and 63,000 tons from the Prestige oil tanker (International Tanker Owners Pollution Federation (ITOPF), 2006) off the coast of north-west Spain.

The fate of petroleum oil spilled at sea depends on both the prevailing weather and the composition of the oil; but its environmental impact is exacerbated on reaching the shoreline,

especially in low-energy habitats, such as lagoons and salt marshes. Acute pollution events can result in mass mortality; for example, more than 66% of total species richness (including polychaetes, mollusks, crustaceans and insects) was lost in the worst affected beaches following the Prestige spill (De La Huz *et al*., 2005). Hydrocarbons also contaminate the feathers and fur of marine birds and mammals, resulting in the loss of hydrophobic properties, leading to death from hypothermia (Mason, 2002), or lethal doses following ingestion of oil during preening.

The development of petroleum industry into new frontiers, the apparent inevitable spillages that occur during routine operations and records of acute accidents during transportation has called for more studies into oil pollution problems, which has been recognized as the most significant contamination problem (Snape *et al.*, 2001). Moreover, the impact of hydrocarbons, especially polycyclic aromatic hydrocarbons (PAHs), on wildlife and fisheries may be long-lasting; for example the Fisheries Exclusion Zone imposed after the Braer spill (McGenity *et al*., 2012) due to contaminated fish and shellfish, remained in place for over 6years. Chronic pollution can cause physiological or behavioural damage at sub-lethal concentrations; and genetic damage and decrease in both growth and fecundity have been observed in fish (Heintz, 2007). Deep-sea sediments and associated biota are also chronically affected by drilling, which deposits vast amounts of oil-contaminated drill cuttings on the seafloor (McGenity *et al*., 2012).

Oil is a natural, heterogeneous mixture of hydrocarbons, with potentially 20,000 chemical components (Marshall and Rodgers, 2004), consisting mainly of alkanes with different chain lengths and branch points, cycloalkanes, mono-aromatic and polycyclic aromatic hydrocarbons (McGenity *et al*., 2012). Some compounds contain nitrogen, sulfur and oxygen;

while trace amounts of phosphorus and heavy metals such as nickel and vanadium are also found (Van Hamme *et al.,* 2003). Its composition varies widely, and each oil component has different physico-chemical properties, including viscosity, solubility, as well as varying in its bioavailability and toxicity. Crude oil, released naturally from the geosphere to the biosphere (e.g. from cold seeps) (Suess, 2010) may supply up to half of the oil in the sea (National Research Council, 2003).Used engine oil renders the environment unsightly and constitutes a potential threat to humans, animals and vegetation (Edewor *et al.,* 2004). Fat soluble components may accumulate in the organs of animals and may be enriched in the food chain, even up to humans (Mackay and Fraser, 2000). In the long term, toxic and carcinogenic compounds can cause intoxication, diseases, cell damage, developmental disorders and reproduction problems (Jain *et al*., 2011). In addition to toxic effects, oil products can affect plant and animals physically. A thick layer of oil inhibits the metabolism of plants and suffocates them. Destruction of plants affects the whole food web and decreases the natural habitats of numerous species (Helsinki Commission (HELCOM), 2003).

Hydrocarbon compounds such as petroleum are essential for life. Since, they do not naturally occur in the forms most useful to humans, they can be hazardous. Fuel and lubricating oil spills have become a major environmental hazard to-date (Jain, *et al.,* 2011). The contamination of the environment with petroleum hydrocarbons poses serious problems for many countries. Although hydrocarbons are relatively stable molecules, their fuel value and presence in the environment for millions of years have led to the evolution of many microbes able to activate and use them as a major or sole source of carbon and energy, including at least 175 genera of Bacteria (Prince *et al.,* 2010). Several haloarchaeal genera (Al-Mailem *et al.,* 2010) and many Eukarya can grow on or transform hydrocarbons (Prince *et al*., 2010).

Pollution of the soil with petroleum derivatives is often observed in municipal soils around industrial plants and in areas where petroleum and natural gas are obtained (Adam *et al.*, 2002; Clark, 2003). The decontamination and cleanup of hydrocarbon polluted sites has increasingly received attention and interest (Akpoveta *et al.*, 2011). Crude oil is a known source of energy and income in the world, but its introduction into the environment poses a lot of pollution problems as it distorts the soil's originality, thus leading to loss of agricultural land. Considering the large quantity of oil going into the Niger Delta environment, especially farmlands, and the fact that the inhabitants of these areas are subsistent farmers, and also the seemingly inevitable consequences of oil spill, the need to clean up oil contaminated sites has become a key environmental issue. Due to the ability of certain microbes to mineralize hydrocarbon components into environmentally friendly species such as carbon dioxide and water, the potentiality of these microbes in breaking down hydrocarbons has gained growing attention in modern day research (Akpoveta *et al.*, 2011).

PAHs are usually found mixed with other organic pollutants (commonly petroleum and derived products) in contaminated sites, which may alter their fate and transport (Terry *et al.*, 2012). This is of particular relevance when considering aged or weathered oils, in which PAHs will be less bioavailable because they are more effectively partitioned within the residual oil phase (McGenity *et al*., 2012). PAHs, particularly, HMW PAHs, absorb strongly to mineral and their associated organic matter (Semple *et al.*, 2007), further diminishing their bioavailability. Owing to the low solubility and high levels of adsorption of PAHs, many microbes have evolved mechanism to access them more readily. For example some PAH-degrading microbes have high-affinity uptake systems that efficiently reduce the PAH concentration close to the cell surface, thereby enhancing diffusive flux (Bastiaens *et al.*, 2000; Harms *et al.*, 2010). Living on the mineral surfaces to which PAHs are adsorbed is

another strategy that reduces diffusion time (McGenity *et al*., 2012) by physically reducing the distance between cells and substrate. During such interactions, the nature of the cell surface is extremely important; for example the mycolic acids of mycobacteria and related Actinobacteria enhance cell-surface hydrophobicity which serves to encourage biofilm formation and uptake of lipophilic compounds into the cell (Wick *et al.*, 2002).

2.3 Bioremediation of Petroleum Hydrocarbon Contaminated Environment

Remediation refers to removing, degrading or transforming contaminants to harmless or less harmful substance. Additionally, it include methods that reduce mobility and migration of the contaminants, preventing their spreading to uncontaminated areas; toxicity of the contaminants remains unaltered, but the risk they pose to the environment is reduced (Jain *et al*., 2011). For the treatment of contaminated soil, physiochemical treatments have been used, but it is costly and environmentally unfriendly. The physicochemical treatments are incineration, thermal desorption, coker, cement kiln, solvent extraction and land filling etc. (Jain *et al.*, 2011). Incineration is a very effective treatment method, but it is costly and after burning, the soil has lost most of its nutritional value and structure. Land filling does remove the contaminants but only relocates the problems (Lageman *et al.*, 2005). Furthermore, in Europe, legislation requires reduction of the number of landfills. In 2004, the number of landfills untreated contaminated material in England and Wales was reduced from over 200 to only eleven (Environment Agency, 2006). In Finland, the number of landfills has decreased from 232 in 1999 to 80 in 2005 (Finnish Environment (FEI), 2006). As a result, the cost of dumping contaminated soils into landfills has risen considerably. It is therefore evident that new, innovative methods are needed to treat contaminated soils.

Microbial remediation of a hydrocarbon-contaminated site is accomplished with the help of a diverse group of microorganisms, particularly the indigenous bacteria present in soil. These microorganisms can degrade a wide range of target constituents present in oily sludge (Barathi and Vasudevan, 2001; Mishra *et al.,* 2001). Microbial biodegradation of pollutants has intensified in recent years as mankind strives to find sustainable ways to clean up contaminated environments (Diaz, 2008). Biodegradation of hydrocarbons by natural populations of microorganisms represent one of the primary mechanism by which petroleum and other hydrocarbon pollutants are eliminated from the environment. The effects of environmental parameters on microbial degradation of hydrocarbons, the elucidation of metabolic pathways, genetic basis for hydrocarbon dissimilation by microorganisms and the effects of hydrocarbon contamination on microbial communities have been the areas of intense interest and the subjects of several reviews (Jain *et al*., 2011).

Hydrocarbon degrading bacteria and fungi are widely distributed in marine, freshwater and soil habitats. Similarly, hydrocarbon degrading cyanobacteria have been reported (Lliros *et al.,* 2003; Chaillan *et al.,* 2004), although, contrasting reports indicated that growth of mats built by cyanobacteria in the Saudi Coast led to preservation of oil residues (Barth, 2003). Typical bacterial groups already known for their capacity to degrade hydrocarbons include *Pseudomonas* sp. *Marinobacter* sp., *Alcanivorax* sp., *Microbulbifer* sp., *Sphingomonas* sp., *Micrococcus* sp., *Cellulomonas* sp., *Dietzia* sp. and *Gordonia* sp. (Brito *et al.,* 2006). Molds belonging to the genera *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Amorphoteca* sp., *Neosartorya* sp., *Paecilomyces* sp., *Talaromyces* sp., *Graphium* sp. and the yeasts *Candida* sp., *Yarrowia* sp. and *Pichia* sp. have been implicated in hydrocarbon degradation (Chaillan *et al.,* 2004).

Petroleum oil degradation by bacteria can occur under both aerobic and anaerobic conditions (Okoh, 2006). It has been established that the first step in the aerobic degradation of hydrocarbons by bacteria is usually the introduction of molecular oxygen in the hydrocarbon. Prokaryotes convert aromatic hydrocarbons by an initial dioxygenase attack to trans-dihydrodiols that are further oxidized to dihydroxy products, example catechol in the case of benzene (Okoh, 2006) (Fig. 2.3). Eukaryotic microorganism use mono-oxygenases, producing benzene 1,2-oxide from benzene, followed by the addition of water, yielding dihydroxydihydrobenzene (Cis-dihydrodiol). This is oxidized in turn to catechol, a key intermediate in biodegradation of aromatic, which is then opened by *ortho* or *meta* cleavage, yielding muconic acid or hydroxymuconic semialdehyde, respectively (Jain *et al.*, 2011).

In the case of alkanes, aerobic alkane degraders use O_2 as a reactant for the activation of the alkane molecule. Oxidation of methane, which is a special case, renders methanol that is subsequently transformed to formaldehyde and then to formic acid (Fig. 2.4). This compound can be converted to CO_2 or assimilated for biosynthesis of multi-carbon compounds either by the ribulose monophosphate pathways, or by the serine pathway, depending on the microorganism considered (Lieberman and Rosenzweig, 2004).

In the case of n-alkanes containing two or more carbon atoms, aerobic degradation usually starts by the oxidation of a terminal methyl group to render a primary alcohol, which is further oxidized to the corresponding aldehyde, and finally converted into a fatty acid (Fig. 2.5). Fatty acids are conjugated to CoA and further processed by β-oxidation to generate acetyl – CoA (Van Hamme *et al.*, 2003; Wentzel *et al.*, 2007). In some cases, both ends of the alkane molecule are oxidized through ω-hydroxylation of fatty acids at the terminal methyl

group (the ω-position), rendering an ω-hydroxy fatty acid that is further converted into a dicarboxylic acid and processed by β-oxidation (Coon, 2005).

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Subterminal oxidation of n-alkanes has also been reported (Fig. 2.5) (Kotani *et al.,* 2006, 2007). The product generated a secondary alcohol, is converted to the corresponding ketone (Fig. 2.5), and then oxidized by a Baeyer-Villiger Monoxygenase to produce an ester. The ester is hydrolyzed by an esterase, generating an alcohol and a fatty acid. Both terminal and subterminal oxidation can coexist in some microorganisms.

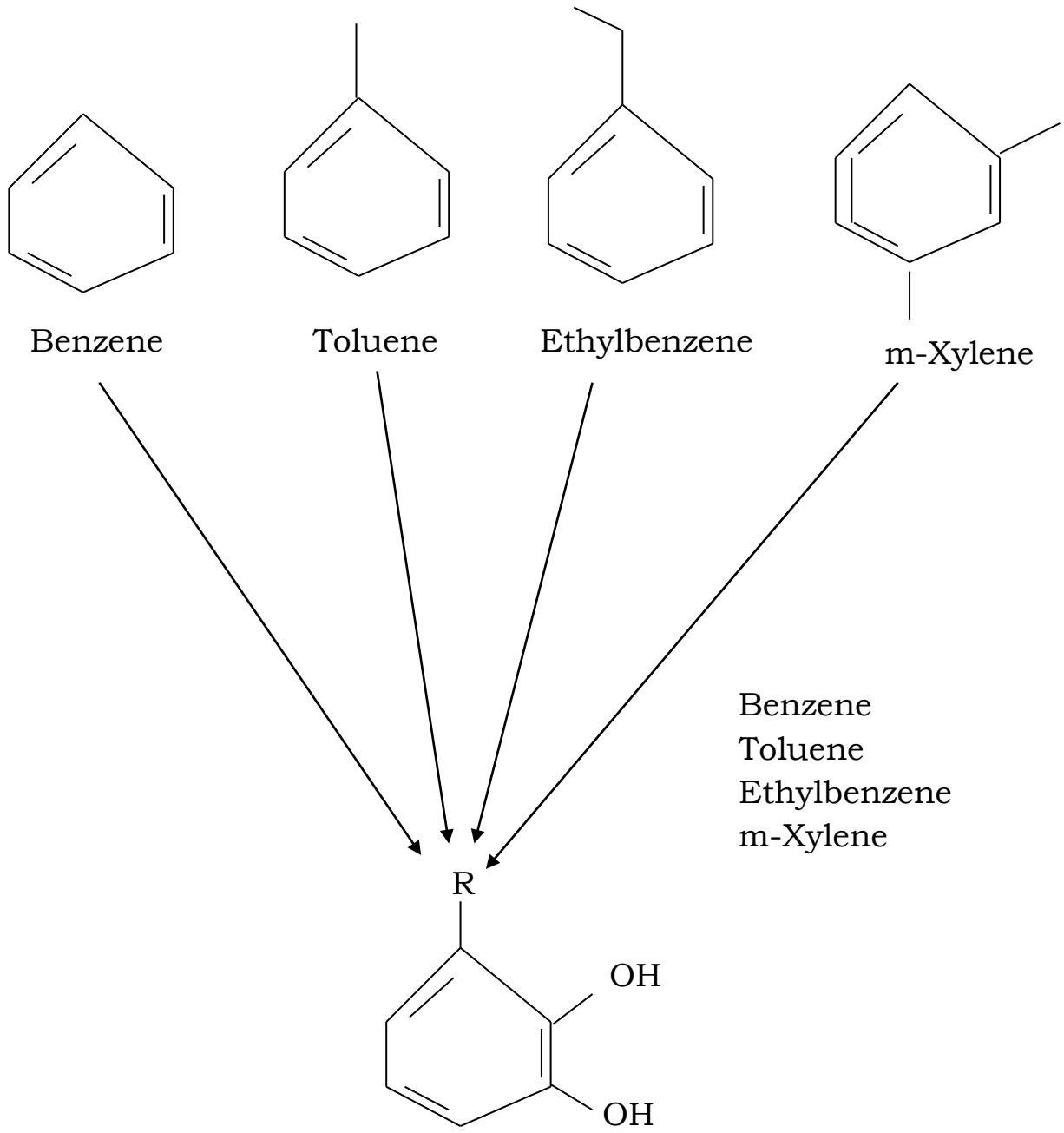

Fig. 2.3: Aerobic degradation of the BTEX compounds (Jain *et al.*, 2011)

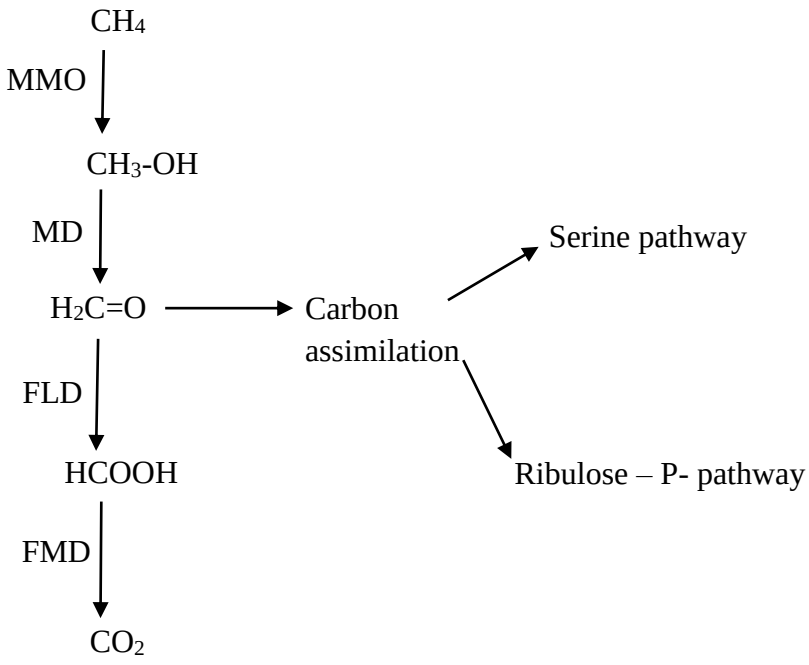

Fig. 2.4: Aerobic pathways for the degradation of methane (Rojo, 2009). MMO, methane monooxygenase; MD, methanol dehydrogenase, FLD, formaldehyde dehydrogenase; FMD, formate dehydrogenase.

In the subsurface, oil biodegradation occurs primarily under anoxic conditions, mediated by sulfate reducing bacteria (Okoh, 2006) or other anaerobes using a variety of other electron acceptors as an oxidant. Hydrocarbon biodegradation under anaerobic, denitrifying conditions also follows an oxidative strategy. In the presence of nitrate hydrocarbon substrates e.g., toluene, are metabolized to oxidized intermediates prior to further biodegradation (Jain *et al*., 2011). Anaerobic degradation of petroleum hydrocarbons in natural environments by microorganisms has been shown in some other studies to occur only

at negligible rates and its ecological significance has been generally considered to be minor (Jain *et al*., 2011). However, the microbial degradation of oxidized aromatic compounds such as benzoate and halogenated aromatic compounds such as the halobenzoates, chlorophenols and polychlorinated biphenyls (Jain *et al*., 2011) has been shown to occur under anaerobic conditions.

Severed bacterial strains able to use alkanes as carbon sources in the absence of O_2 have been described (Widdel and Rabus, 2001). These microorganisms use nitrate or sulfate as electron acceptor. Growth is significantly slower than that of aerobic alkane degraders. However, anaerobic degradation of alkanes also plays an important role in the recycling of hydrocarbons in the environment. For examples, Strain Bus5, a sulfate reducing bacteria that belongs to the genera *Desulfosarcina/Desulfococcus* cluster, assimilates only propane and butane (Kniemeyer *et al.,* 2007); *Azoarcus* sp. HxNI, a denitrifying bacteria, uses C_6-C_8 alkanes, while *Desulfobacterium* HdX3 metabolizes C_{12}-C_{20} alkanes (Widdel and Rabus, 2001). The metabolic pathways for anaerobic degradation of alkanes have been investigated for some strains. Two general strategies appear to be used (Fig. 2.6). One involves activation of the alkane at a subterminal position by addition of a fumarate molecule to the alkane, yielding an alkyl-succinate derivative. This reaction is believed to occur through generation of an organic radical intermediate, most likely a glycyl radical (Rabus *et al.,* 2001). The reaction product is subsequently, linked to CoA and converted into an acyl-CoA that can be further metabolized by β-oxidation. In the second reaction mechanism, which has been described only for propane, the fumarate molecule is added to one of the terminal carbon atoms of the alkane (Kniemeyer *et al.,* 2007).

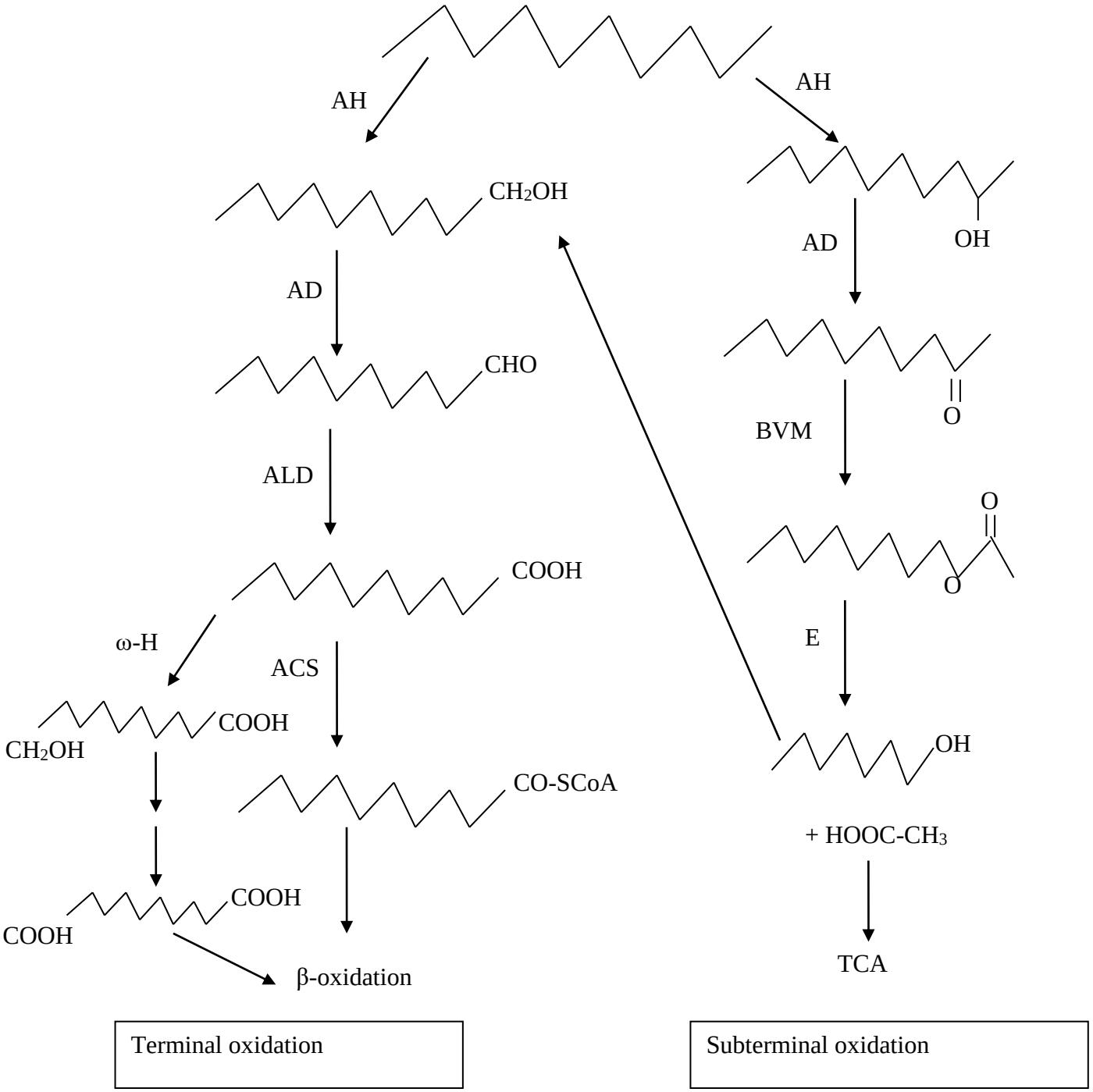

Fig. 2.5: Aerobic pathways for the degradation of larger n-alkanes by terminal and subterminal oxidation (Rojo, 2009). AH, alkane hydroxylase; AD, alcoholdehydrogenase; ALD, aldehyde dehydrogenase; ACS, acyl – CoA synthetase; ω-H, ω-hydroxylase; BVM, Baeyer-Villigermonooxygenase; E, esterase; TCA, tricarboxylic acids cycle.

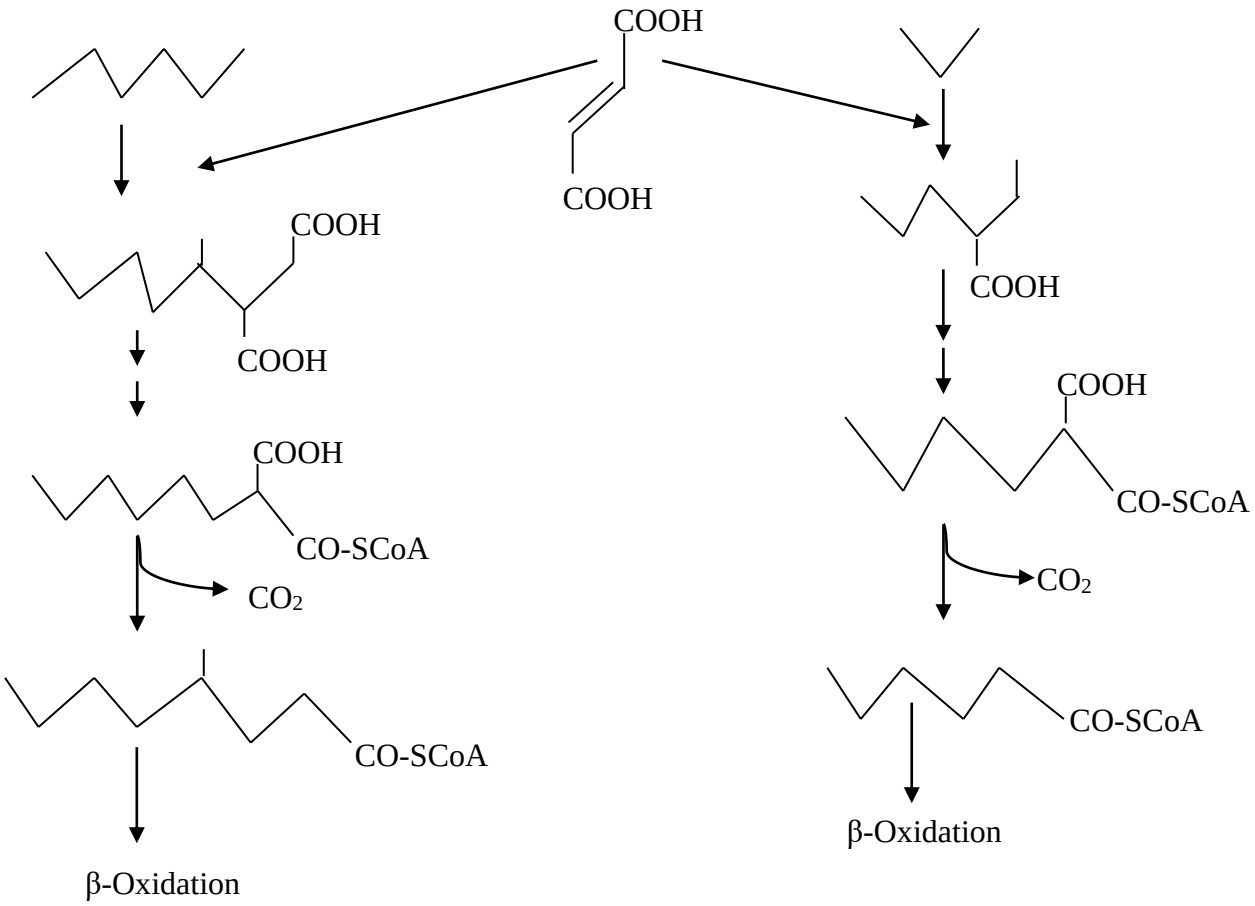

Fig. 2.6: Anaerobic degradation of alkanes (Rojo, 2009)

The various mixtures of an alkane-utilizing *Acinetobacter* sp. and a *Rhodococcus* sp., an alkyl-benzene-degrading *Pseudomonas putida*, and a phenanthrene-utilizing *Sphingomonas* sp. in an attempt to elucidate how alkane and aromatic degrading microorganism interact has been evaluated (Van Hamme *et al*., 2003). The degradation of Arabian light crude oil was monitored, and a combination of the *Acinetobacter* sp. and *Pseudomonas putida* was effective as a mixture of the four microorganism, degrading 40% of the saturates and 21% of the aromatics. Many bioremediation companies offer such mixed cultures for sale to cope with environmental pollution, but third-party testing of such products has not proved them to

be more effective than autochthonous microbial communities once additional nutrients and sorbents are removed (Van Hamme *et al*., 2003).

Biodegradation of multiple contaminants in the environment is a complex process that has been a source of major concern to environmental microbiologists and scientists (Owabor *et al.,* 2011). Microorganisms such as fungi and bacteria are the key agents of bioremediation as they have been very useful in effectively degrading a wide range of contaminants in the ecosystem. However, factors such as the characteristics, content and concentration of PAHs present, the physical, chemical and environmental conditions and the composition of the microbial population dictates the overall microbial degradation process (Rahman *et al.,* 2002; Tam *et al.,* 2002; Owabor and Agarry, 2009; Bogan *et al.,* 2003; Oleszczuk and Baran, 2003; Owabor and Irheren, 2006; Owabor and Osarumwense, 2008; Obahiagbon and Owabor, 2008; Owabor *et al.,* 2010). Cleaning areas of oil contamination is of interest because of the resultant threat of such contamination to the natural terrestrial ecosystem and the natural aquatic environment. Concerns have grown even more as researchers have discovered that microbial processes can now be used to effect cleanup of radioactive and metallic contaminants (Owabor *et al.,* 2011).

2.4 Factors Affecting Bioremediation

Successful application of bioremediation technology to contaminated systems requires knowledge of the characteristics of the site and the parameters that affect the microbial biodegradation of pollutants (Sabate *et al.,* 2004). The overall degradation rate of hydrocarbons biodegradation in soils are strictly limited by a variety of parameters (Rockne *et al.,* 2002). It is therefore necessary to understand the factors limiting microbial degradation in order to adopt appropriate methodology to optimize the process of degradation. The

various conditions and factors, which determine the rate of degradation of Pollutants, are documented (Oleszczuk and Baran, 2003; Jain *et al*., 2011).

2.4.1 Temperature

Temperature plays a very important role in biodegradation of petroleum hydrocarbons, firstly by its direct effect on the chemistry of the pollutants and secondly its effect on the physiology and diversity of the microorganism. Ambient temperature of an environment affects both the property of spilled oil and the activity of microorganisms (Venosa and Zhu, 2003). At low temperatures, the viscosity of the oil increases, while the volatility of toxic low-molecular weight hydrocarbons is reduced, delaying the onset of biodegradation (Jain *et al*., 2011).

Temperature affects the solubility of hydrocarbons (Okoh, 2006). Although hydrocarbon biodegradation can occur over a wide range of temperature, the rate of biodegradation generally decreases with decreasing temperature. Highest degradation rates generally occur in the range of 30-40^{0}C in soil environments, 20-30^{0}C in some fresh water environments and 15-20^{0}C in marine environments (Okoh, 2006). The biodegradation of hydrocarbons in psychrophilic environments have been also reported (Yumoto *et al.,* 2002; Delille *et al.,* 2004; Pelletier *et al.,* 2004).

It was observed that the uptake of cadmium was highly reduced at low temperatures (4^{o}c), while the degradation of hydrocarbons is favoured at higher temperatures (Shukla and Cameotra, 2012). However, there are a number of cold adapted microorganisms that can efficiently degrade petroleum hydrocarbons at low temperatures (Shukla and Cameotra, 2012). Cold-adopted microorganisms are able to grow and multiply even at O^{0}C and below. Their minimum, optimum and maximum temperature for growth are respectively 0-5, >15

and >20^0 for psychrotolerants and <0, <15 and <20^0C, respectively for psychrophiles. Among psychrotolerants, many have been proven to be hydrocarbon degraders, example, alkane degraders (Bej *et al.,* 2000; Ruberto *et al.,* 2005), aromatic degraders (Aislabie *et al.,* 2000; Baraniecki *et al.,* 2002; Grishchenkov *et al.,* 2003; Margesin *et al.,* 2005) and chlorophenol degraders (Mannisto *et al.,* 2001; Tiirola *et al.,* 2002). Cold-adapted microorganisms are widely distributed in nature with gram negative bacteria dominant (Jain *et al*., 2011). Cold-adapted strategies include the molecular adaptation of membrane lipid composition, enzyme activity and protein synthesis (Jain *et al*., 2011).

Hydrocarbon degraders are ubiquitous in most ecosystems. They comprise less than 0.1% of the microbial community in unpolluted environments but can constitute up to 100% of the cultivable microorganisms in hydrocarbon-polluted ecosystems (Graciella *et al*., 2011). The characteristics of many hydrocarbon degrading bacteria are being examined by the scientists for their possible use in bioremediation. Richmond *et al.,* (2001) studied the temperature effects on bacteria from benzene-contamined aquifer, Alaska using glutamate as a carbon source; they found that the overall microbial metabolic rates were higher at 25^0C than 10^0C.

2.4.2 Nutrients

The nutrient status of soil has direct impacts on microbial activity and biodegradation. To grow heterotrophic bacteria require in addition to an organic compound that serves as a carbon source, electron donor and a group of other nutrient elements. Many bacteria and fungi also require low concentrations of one or more amino acids and vitamins. Nitrogen and phosphorus are necessary for cellular metabolism and can be found in low concentrations in many soils, including Arctic soils (Mohn and Stewart, 2000).

Biodegradability is inherently influenced by the composition of the oil pollutant. For example, kerosene (consists of almost exclusively medium chain alkanes) is totally biodegradable. Similarly, crude oil is also biodegradable quantitatively but heavy asphaltic-naphthenic crude oil; only about 11% is biodegradable within a reasonable time period (Jain *et al.,* 2011) even if the conditions are favourable. Okoh (2002) reported that between 8.8 to 29% of the heavy crude oil Maya was biodegraded in soil microcosm by mixed bacterial consortium in 15 days, although major peak components of the oil was reduced by between 6.5 to 70% (Okoh, 2003). Also about 89% of the same crude oil was biodegraded by axenic culture of *Burkholderia capacia* RQI in shake flask (Okoh *et al.,* 2001) within similar time period. The petroleum biodegradation has been reported to be mostly enhanced in the presence of a consortium of bacteria species compared to monospecies activites (Ghazali *et al.,* 2004). Rahman *et al.* (2003) reported that the percentage of degradation by the mixed bacterial consortium decreased from 78 to 52% as the concentration of crude oil was increased from 1 to 10%.

Various reports on the effect of sunlight irradiation so far published have focused on the physico-chemical changes on intact crude oil other than to biodegraded crude oil (Okoh, 2006). Maki *et al.* (2005) reported that photo-oxidation increases the biodegradability of petroleum hydrocarbons by increasing its bioavailability and thus, enhancing microbial activities. In a related study, Trindade *et al.* (2005) assessed the bioremediation efficiency of a weathered and freshly contaminated soil. The additions of nutrients are necessary to enhance the biodegradation of oil pollutants (Choi *et al.,* 2002; Kim *et al.,* 2005). Pelletier *et al.* (2004) assessed the effectiveness of fertilizers for crude oil bioremediation in sub-Antarctic intertidal sediments over a one year and observed that chemical, microbial and toxicological parameters demonstrated the effectiveness of various fertilizers in a pristine

environment. In study using poultry manure as organic fertilizer in contaminated soil, increased biodegradation was reported but the extent of biodegradation was influenced by the incorporation of alternate carbon substrates or surfactants (Okolo *et al.,* 2005). Chaillan *et al.* (2006) reported that excessive nutrient concentration can inhibit the biodegradation activity and several authors have also reported the negative effect of a high NPK levels on the hydrocarbons biodegradation(Chaineau *et al*., 2005; Okoh, 2006) and more especially on the aromatics (Jain *et al*., 2011).

2.4.3 Effect of chemical composition of petroleum hydrocarbons

Petroleum hydrocarbons can be divided into four classes: saturates, aromatics, asphaltenes (phenols, fatty acids, ketones, esters and porphyrins) and resins (pyridines, quinolines, carbazoles, sulfoxides and arnides). Hydrocarbons differ in their susceptibility to microbial attack and ranked in the following order of decreasing susceptibility: n-alkanes > branched alkanes > low molecular weight aromatics > cyclic alkanes (Das and Chandran, 2011).

2.4.4 Bioavailability

Bioavailability is the amount of a substance that is physiochemically accessible to microorganisms. It is a key factor in the efficient biodegradation of pollutants. Chemotaxis or the directed movement of motile organism towards or away from chemicals in the environment is an important physiological response that may contribute to effective catabolism of molecules in the environment. In addition, mechanisms for the intracellular accumulation of aromatic molecules via various transport mechanisms are also important (Parales, 2008). It was demonstrated that growth on crystalline substrates (naphthalene) results in linear growth rates indicating that partitioning, example, solubilization of the substrate is rate limiting to biodegradation (Jain *et al*., 2011). Sharma *et al*. (2014) reported

that uptake of hydrocarbons most likely occurs by attachment than incorporation into the cytoplasmic membrane. Alternatively transport occurs by passive or facilitated diffusion in the presence of solubilizing agents; intracellular transport is probably coordinated with enzymatic oxidation.

Other methods for increasing bioavailability may also enhance the biodegradation of contaminants in a soil. For example, physical disruption of soil aggregates using sonication has been reported to increase biodegradation rates effectively in a land farm experiment (Jain *et al*., 2011). Soil constituents have significant impact on the bioavailability of contaminants. Leitao (2009) reported that mineralization rate of contaminants are lower in soils with a high organic matter content, which readily absorbs hydrophobic compounds. Soluble humic substances in particular humic and fulvic acids appear to be major binding sites. Their binding potential can be attenuated by mineral soil components, as well as pH and salt concentration (Saichek and Reddy, 2005). Weathering or the age of contamination may also affect bioavailability by physically trapping, hindering and/or slowing desorption of contaminants from the soil (Jain *et al*., 2011).

2.4.5 Physiochemical properties of the soil

Soils vary widely with regard to geology, hydrology, climate, fertility and other physical attributes. The most important physical and chemical property of the soil is determined by the composition of soil, organic matter and fraction of soil (Owabor and Ogunbor, 2007).

2.4.6 Oxygen

Oxygen is another important parameter because it determines the bacterial pattern of dissimilation and energy yielding process. Microbial utilization of aliphatic, cyclic and

aromatic hydrocarbons by bacteria and fungi require electron sink (Jain *et al*., 2011). In the initial attack, molecular oxygen is used as electron sink. In the subsequent steps too, oxygen is the most common electron sink. In the absence of molecular oxygen, further biodegradation of partially oxygenated intermediates may be supported by nitrate or sulphate reduction. Little or no hydrocarbon metabolism occurs in strictly anoxic sediments (Okoh, 2006).

2.4.7 Acidity or alkalinity (pH)

The acidity of the soil is an important soil parameter. Soil pH can be highly variable, ranging from 2.5 in marine environment to 11 in alkaline deserts. Most heterotrophic bacteria favour a pH 7.0 but fungi are more tolerant to acidic conditions. Therefore, extreme pH of soils would have a negative influence on the ability of microbial populations to degrade hydrocarbons. A doubling rate of biodegradation of gasoline in an acidic (pH 4.5) soil by adjusting the pH to 7.4 was reported (Sawadogo *et al*., 2014). Rates of biodegradation dropped significantly, however, when the pH was further raised to 8.5. Similarly, an optimal pH of 7.8, in the range 5.0 to 7.8 for mineralization of oily sludge in soil was observed (Jain *et al*., 2011).

pH affects metal toxicity to a great extent because metal ions could form complexes with the medium or buffered components or metal ions could precipitate as phosphates or sulphates, especially at pH ≥ 7.0 (Shukla and Cameotra, 2012). Tadama *et al.* (2015) replaced phosphate buffer by tris-HCl to avoid the precipitation of the metal ions. Anna and Zofia (2014) reported that the sensitive and resistant cells do not react in the same way to pH increase. Both types of cells accumulate more cadmium as the pH increases but the pattern of cadmium up taking was different.

At acidic pH, more protons (H^+) are available to saturate metal-binding sites. Therefore metals are less likely to form insoluble precipitates with phosphates when the pH of the system is lowered because much of the phosphates has been protonated (Olaniran *et al*., 2009). Under basic conditions, metal ions can replace protons to form other species, such as hydroxo-metal complexes. In some cases the hydroxo-metal complexes, such as those formed with cadmium, nickel, and zinc are soluble, while those formed with chromium and iron are insoluble. A small change in pH can decrease metal solubility and bioavailability by several orders of magnitude. For example, the solubility of cadmium was reduced 8.8 fold by an increase in pH from 6 to 7 in 1.3mM phosphate (Olaniran *et al.,* 2013). The dependence of metal bioavailability on pH varies between different metals. For example, a rapid decline in the concentrations of the free ionic species of copper and zinc in minimal media was observed at pH values higher than 5, while the free ionic form of cobalt remains prevalent until the pH value is higher than 8 (Sandrin and Hoffman, 2007).

Many studies have shown that pH mediates metal toxicity (Franklin *et al.,* 2000; Olaniran *et al*., 2013). Increasing the pH reduces the toxicity of nickel to a variety of different organisms, including bacteria (*Serratia marcescens*), filamentous fungi (*Arthrobotrys conoides, Penicillium vermiculatum, Rhizopus stolonifer*), and yeast (*Cryptococcus terreus*) (Olaniran *et al*., 2013). Under mildly basic conditions (pH 8.5), much of the nickel may not be bioavailable because it forms complexes with various ligands. It is also possible that the nickel was less toxic at a higher pH because some organisms may prefer basic environments to neutral or acidic environments. More commonly, increasing pH has been shown to increase the toxicity of zinc, copper, and uranium to certain algal species (Franklin *et al.,* 2000) and of cadmium to various bacteria (*B. subtilis, E. coli, M. luteus, S. bovis*),

actinomycetes (*Micromonospora chalcea, Nocardia corallina, Streptomyces flavovirens*), and fungi (*Saccharomyces cerevisiae, Schizosaccharomyces octosporus*) (Olaniran *et al*., 2013). Possible reasons for this occurrence may be due to cells being able to take up or adsorb more of the metal ions under high pH conditions (Sandrin and Maier, 2002). Also, various functional groups associated with the membrane of microorganisms would be protonated under acidic conditions, reducing the electrostatic attraction between the metal cations and the membrane. A third possibility is that metals are removed from the cell more efficiently under acidic conditions by efflux pumps that are driven by the proton motive force (Sandrin and Maier, 2002). Another possible explanation for increased toxicity at a higher pH is the formation of species that are more toxic, such as the hydroxo-metal species (Olaniran *et al*., 2013).

2.4.8 Effect of contact time

Contact time between microorganisms and pollutant influences the adaptation process of microorganisms. Depending on the residence time of microorganisms in the metal environment, it has been found that 10 to 90% of the microorganisms become metal resistant (Shukla and Cameotra, 2012). Tadama *et al*. (2015) reported the adaptation of bacterial consortia to Cd (II), Cu (II), Cr (VI) and Hg (II) after a comparative extended adaptation period. Yeom and Yoo (2002) evaluated the enzymatic adaptation of *Alcaligenes xylosoxidans* Y234 to heavy metal during degradation studies of benzene and toluene in the presence of heavy metal ions. They established that co-culturing is an effective method to reduce the metal inhibition effect. The reasoning is that, if one inhibitory metal ion to enzyme A is present in a system, then the inoculation of a microorganism which already has enough enzyme A through microbial adaptation or the addition of a compound inducing enzyme A production will offset the inhibition effect.

2.4.9 Effect of cell cycle

The relationship between the cell volume (as the volume of cells varies during the cell cycle) and the susceptibility to copper toxicity in *Saccharomyces cerevisiae* was investigated (Shukla and Cameotra, 2012). The correlation between the cell volume (by examining the forward angle light scatter) and the cell cycle stage (by examining the DNA content) indicated that the largest (the cells about to undergo mitosis) were most resistant to copper, and the smallest (newly divided cells) cells were also relatively resistant to copper. As the percent cell volume increased beyond 0 – 2%, Cu resistance started decreasing and approach to minimal when the percent cell volume reached to 38 – 40% of the maximum cell volume. Then the resistance towards copper started increasing with further increase in cell volume and reached to maximum at the stage when cells having 98 – 100% of the maximum cell volume.

2.4.10 Metal tolerant strains

Microorganisms can naturally exhibit the multifunctional properties of hydrocarbon degradation and metal resistance properties or microorganisms with hydrocarbon degradation capability can be genetically modified for metal resistance or vice – versa (Shukla and Cameotra, 2012). Ueki *et al.* (2003) identified two vanadium binding proteins and expressed them in *E. coli,* which showed the capability of accumulating twenty times more copper than their control strain. Marine bacteria are also known to have metal resistance capabilities (De *et al.,* 2003). Keramati *et al.* (2011) isolated multi metal resistant bacteria highly resistant to mercury. El-Deeb (2009) reported a natural strain exhibiting the properties of organic pollutant degradation and tolerance toward heavy metals. Yoon and Pyo (2003) reported the development and characterization of a strain highly effective in degrading phenol and resistant to the effect of heavy metal ions. In order to minimize the effect of heavy metals on

the hydrocarbon degradation capability of microorganisms, the following actions are recommended (Riis *et al.,* 2002);

1. Application of phosphate or sulfate compounds at the contaminated site to induce the precipitation of heavy metal ions.
2. Addition of metal chelators e.g. ethylene-diamine tetra-acetic acid (EDTA).
3. Increasing the pH of the soil. Addition of organic humus forming material.

Bioremediation processes have been shown to be an effective method that stimulates the biodegradation in contaminated soils (McLaughlin, 2001). Biodegradation of hydrocarbon-contaminated soils (exploits the ability of microorganism to degrade and/or detoxify organic contamination) has been established as an efficient, economic, versatile and environmentally sound treatment. Bioremediation involves the use of indigenous or introduced microorganism to degrade environmental contaminants (Obire and Nwaubeta, 2001).

2.4.11 Treatment Amendments

Many studies have been carried out to evaluate the ability of different chemical amendments to immobilize heavy metals in polluted environments. These additives include organic materials, phosphate rocks, iron and manganese oxides and oxy-hydroxides and waste by-products rich in these oxides as well as alkaline agents such as lime and zeolite (Shuman *et al.,* 2002; Basta *et al.,* 2001; Chen *et al.,* 2000; Hodson *et al.,* 2000). In general, these treatments prove to have an ameliorative effect on reducing the metal mobility or bioavailability. Cadmium sorption in three different minerals, vermiculite, zeolite and pumice was evaluated (Panuccio *et al.,* 2009). Zeolite and vermiculite reduced soluble cadmium concentrations by 90% and the metal sorbed on zeolite was mainly present in the non-exchangeable form (70%) at the lowest cadmium concentration (30-120µM). Furthermore, it

was reported that the percentage of cadmium sorption in zeolite and vermiculite was independent of the initial cadmium concentration, and the mineral sorption capacity was closely dependent upon pH. In particular, cadmium adsorption on pumice was raised from 20 to 90% with an increase in pH from 4 to 7.5 (Olaniran *et al.,* 2013). Phosphate amendments, in particular, have been given much attention for the treatment of Pb-contaminated environments (Shuman *et al.,* 2002; Chen *et al.,* 2000; Brown *et al.,* 2005; Chen *et al.,* 2003). Despite the well-documented ability of treatment amendments to reduce metal mobility and bioavailability, not much attention has been directed towards determining microbial endpoints after the treatment of contaminated environments. Brown *et al.* (2005) examined the effect of lime, phosphorus, red mud, cyclonic ashes, biosolids and water treatment residuals on the toxicity of cadmium, lead and zinc in an international inter-laboratory study. Each participating laboratory selected a common soil material, from mine wastes and common treatments. Nitrogen (N) transformation and a measure of the total soil microbial biomass were chosen as microbial endpoints. The N transformation test was designed to measure nitrate formation in soils after the addition of an organic substrate. The formation of nitrate is an indicator of microorganisms degrading the C-N bonds in the organic substrate and recycling nutrients within the soil. Of the amendments tested by the participating laboratories, phosphorus (P) added as either triple sugar phosphate or H_3P0_4 appeared to be the most effective (Olaniran *et al.,* 2013). Phosphorus addition to the soil resulted in reduced soil solution and extractable metals, reduced bioavailability of soil Pb, and increased microbial activity based on the two measures. These suggests that the use of treatment amendments may be an effective means to increase organic pollutant biodegradation in the presence of toxic levels of heavy metals (Olaniran *et al.,* 2013), however there were significant differences in efficacy within categories of amendments.

2.5 Microbial Surfactants (Biosurfactants)

Microorganisms synthesize surfactants known as biosurfactants, which have both hydrophobic and hydrophilic domains and are capable of lowering the surface tension and the interfacial tension of the growth medium. Biosurfactants are synthesized during the bacterial growth period on water-immiscible substrates, providing an alternative to chemically prepared conventional surfactants. In the case of the microbial degradation of hydrocarbons, microbial cells synthesize biosurfactants that solublizes oil droplets into the aqueous phase making easier the oil uptake by microbial cells (Ganesh and Lin, 2009), as shown in Figure 2.7.

Biosurfactants show different chemical structures: lipopeptides, glycolipids, neutral lipids, and fatty acids (Cameotra *et al*., 2010; Muthusamy *et al*., 2008). Biosurfactants are nontoxic, biodegradable biomolecules that have gained importance in the fields of enhanced oil recovery, environmental bioremediation, food processing, and pharmaceutical due to their unique properties. The economics of biosurfactants production is a bottle neck because of the low production efficiency and the high costs associated to the recovery and purification of biosurfactants (Shukla and Cameotra, 2012). To overcome these problems, the use of unconventional substrates together with biosurfactant producing bacterial strains or genetically modified strains have been suggested, which could make possible for biosurfactants to compete with synthetic surfactants (Makkar and Cameotra, 2002). Similarly, biosurfactants production costs could be reduced by using different production routes (development of economical engineering processes) aiming the increased biosurfactant production yield and the use of cost free or cost credit feed stock for microorganism growth and biosurfactant production (Gautam and Tyagi, 2006).

Another mechanism for increasing the bioavailability of these compounds is the production of biosurfactant (biological surface-active agents that have both hydrophilic and hydrophobic moieties). Some biosurfactants are known to inhibit certain microbes, while at the same time benefiting others by increasing the bioavailability of hydrophobic compounds that can serve as a carbon and energy source, thus acting as "common goods" (Diggle, 2010). Numerous studies have shown that production of biosurfactants, by either degrading or non-degrading microbes, is essential in enhancing the bioavailability of poorly soluble and adsorbed hydrocarbons (Pacwa-Plociniczak *et al.,* 2011; Perfumo *et al.,* 2010). Low-molecular-weight biosurfactant molecules are mostly glycolipids, including rhamnolipid, trehalose lipids and sophorolipids, or lipopeptides such as surfactin, gramicidin S, and polymyxin. High molecular weight EPS can also act as a biosurfactant, and represents a heterogeneous range of polymers composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixture of these biopolymers (Pacwa-Plociniczak *et al.,* 2011; Perfumo *et al.,* 2010). Biosurfactants preferentially partition at the interface between polar and apolar molecules (e.g. hydrocarbons and water), producing micro emulsions which in many cases enhance bioavailability and desorption of the hydrocarbon (Perfumo *et al.,* 2010).

Biosurfactants may also serve an antagonistic role-they are after all important virulence factors in many pathogens – and their effects will be dose – and species – dependent (Terry *et al.,* 2012). Rhamnolipid generally enhances hydrocarbon bioavailability and degradation (McKew *et al.,* 2007; Abalos *et al.,* 2004), but Shin *et al.,* (2005) reported that it inhibited degradation of phenanthrene by a two species consortium of *Sphingomonas* and *Paenibacillus* sp., even though in pure culture the rhamnolipid inhibited only *Sphingomonas* sp. It was therefore suggested that the increased stress caused by the solubilized phenanthrene, or the rhamnolipid in the presence of solubilized phenanthrene, was responsible for inhibition of *Paenibacillus* sp. It is also important to consider the potential

synergistic role of multiple biosurfactants. It was reported that biosurfactants produced by a pure strain did not emulsify crude oil, whereas those produced by the whole bacterial community did emulsify oil and led to rapid hydrocarbon degradation (McGenity *et al*., 2012). The extent to which such multi-species synthesis of biosurfactants may be coordinated remains to be discovered. Microbial, petroleum and clay interactions are important but very poorly understood. Chaerun *et al.* (2005), for example, showed that montmorillonite and kaolinite enhanced growth on heavy oil, acting as supports for microbes producing EPS, as well buffering pH. Degradation of adsorbed PAHs involves specific adaptations that are still not well understood, and some microbes specialize in accessing and degrading adsorbed PAHs (Bastiaens *et al.,* 2000).

The production of extracellular polymeric substances (EPS) has been shown to be an important mechanism in allowing attachment of *Pseudomonas putida to* solid PAHs (Rodrigues *et al.,* 2005). Vaysse *et al.* (2011) showed that *Marinobacter hydrocarbonoclasticus* exhibited a major change in the proteome of cells freshly detached from hexadecane compared with those attached to hexadecane. Their mobilization may be fuelled by intracellular wax esters accumulated while growing as a biofilm on hexadecane, and the dispersed cells demonstrated a high capacity to reattach to the n-alkane (Vaysse *et al.,* 2011). Thus, the ability to readily attach to hydrocarbons and then move to a new patch appears to be essential for many hydrocarbons – degrading bacteria. During this process the hydrocarbon surface will be modified by excreted microbial products, and would thus be expected to lead to colonization by a succession of different microbes (Terry *et al.,* 2012). Wouters *et al.* (2010) used differential fluorescence staining to analyze a model, three-species community on the surface of PAH crystals, which looks like a promising tool to investigate their interactions and succession on the hydrocarbon surface.

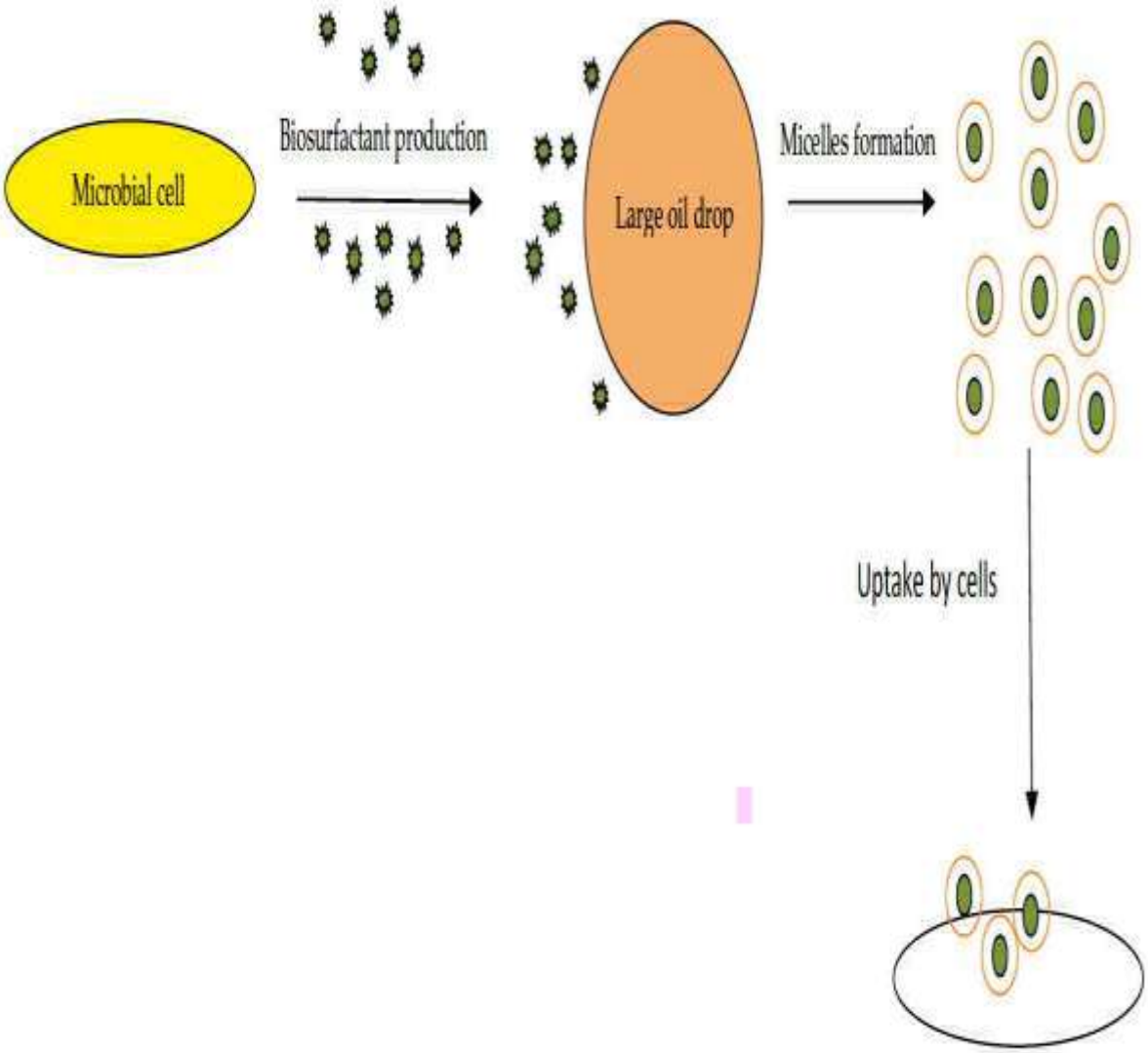

Fig. 2.7: Mechanism of oil solubilization by biosurfactants and uptake of solubilized oil by microbial cells (Shukla and Cameotra, 2012).

2.6 Microbial Enzymes in Bioremediation

2.6.1 Microbial oxidoreductases

The detoxification of toxic organic compounds by various bacteria and fungi and higher plants through oxidative coupling is mediated with oxidoreductases (Karigar and Rao, 2011). Microbes extract energy via energy-yielding biochemical reactions mediated by these enzymes to cleave chemical bonds and to assist the transfer of electrons from a reduced organic substrate (donor) to another chemical compound (acceptor). During such oxidation-reduction reactions, the contaminants are finally oxidized to harmless compounds.

The oxidoreductases participate in the humification of various phenolic substances that are produced from the decomposition of lignin in a soil environment. In the same way, oxidoreductases can also detoxify toxic xenobiotics, such as phenolic or anilinic compounds, through polymerization, copolymerization with other substrates, or binding to humic substances (Park *et al*., 2006). Microbial enzymes have been exploited in the decolorization and degradation of azo dyes (Vidali, 2001; Husain, 2006).

Chlorinated phenolic compounds are among the most abundant recalcitrant wastes found in the effluents generated by the paper and pulp industry. These compounds are produced upon the partial degradation of lignin during pulp bleaching process. Many fungal species are considered to be suitable for the removal of chlorinated phenolic compounds from the contaminated environments. The activity of fungi is mainly due to the action of extracellular oxidoreductase enzymes, like laccase, manganese peroxidase, and lignin peroxidase, which are released from fungal mycelium into their nearby environment. Being filamentous, fungi can reach the soil pollutants more effectively than bacteria (Rubilar *et al*., 2008).

Water polluted with phenolic compounds can be de-contaminated by plants with the help of enzymes exuded by their roots. The plant families of *Fabaceae*, *Gramineae* and *Solanaceae* are found to release oxidoreductases which take part in the oxidative degradation of certain soil constituents (Karigar and Rao, 2011). Phytoremediation of organic contaminants has been generally focused on three classes of compounds: chlorinated solvents, explosives and petroleum hydrocarbons (Duran and Esposito, 2000).

Microbial oxygenases belong to the oxidoreductase group of enzymes. They participate in the oxidation of reduced substrates by transferring oxygen from molecular oxygen (O_2) utilizing FAD/NADH/NADPH as a cosubstrate. Oxygenases are grouped into two categories; the monooxygenases and the dioxygenases on the basis of number of oxygen atoms used for oxygenation. They play a key role in the metabolism of organic compounds by increasing their reactivity or water solubility or bringing about cleavage of the aromatic ring. Oxygenases have a broad substrate range and are active against a wide range of compounds, including the chlorinated aliphatics. Generally the introduction of O_2 atoms into the organic molecule by oxygenase results in cleavage of the aromatic rings. Historically, the most studied enzymes in bioremediation are bacterial mono- or dioxygenases (Karigar and Rao, 2011). A detailed study of the role of oxygenases in biodegradation process is available (Arora *et al*., 2009; Fetzner, 2003).

Halogenated organic compounds comprise the largest groups of environmental pollutants as a result of their widespread use as herbicides, insecticides, fungicides, hydraulic and heat transfer fluids, plasticizers and intermediates for chemical synthesis. The degradation of these pollutants is achieved by specific oxygenases. Oxygenases also mediate dehalogenation

reactions of halogenated methanes, ethanes and ethylenes in association with multifunctional enzymes (Karigar and Rao, 2011).

Monooxygenases incorporate one atom of the oxygen molecule into the substrate. Monooxygenases are classified into two subclasses based on the presence of cofactor: flavin-dependent monooxygenases and P_{450} monooxygenases. Flavin-dependent monooxygenases contain flavin as prosthetic group and require NADP or NADPH as coenzyme. P_{450} monooxygenases are heme-containing oxygenases that exist in both eukaryotic and prokaryotic organisms. The monoxygenases comprise a versatile superfamily of enzymes that catalyzes oxidative reactions of substrates ranging from alkanes to complex endogenous molecules such as steroids and fatty acids. Monooxygenases act as biocatalysts in bioremediation process and synthetic chemistry due to their highly region-selectivity and stereoselectivity on wide range of substrates. Majority of mono-oxygenase studied previously are having cofactor, but there are certain monooxygenases which function independent of a cofactor. These enzymes require only molecular oxygen for their activities and utilize the substrate as reducing agent (Arora *et al*., 2010; Cirino and Arnold, 2002).

The desulfurization, dehalogenation, denitrification, ammonification, hydroxylation, biotransformation and biodegradation of various aromatic and aliphatic compounds are catalysed by monooxygenases. These properties have been explored in recent years for important application in biodegradation and biotransformation of aromatic compounds (Arora *et al*., 2010). Methane mono-oxygenase enzyme is the best characterized one, among monooxygenases. This enzyme is involved in the degradation of hydrocarbon such as substituted methanes, alkanes, cycloalkanes, alkenes, haloalkenes, ethers, and aromatic and heterocyclic hydrocarbons (Arora *et al*., 2010) (Fig. 2.8). Under oxygen-rich conditions,

mono-oxygenase catalyzes oxidative dehalogenation reactions, whereas under low oxygen levels, reductive dechlorination takes place. Oxidation of substrate can lead to de-halogenation as a result of the formation of labile products that undergo subsequent chemical decomposition (Jones *et al*., 2001).

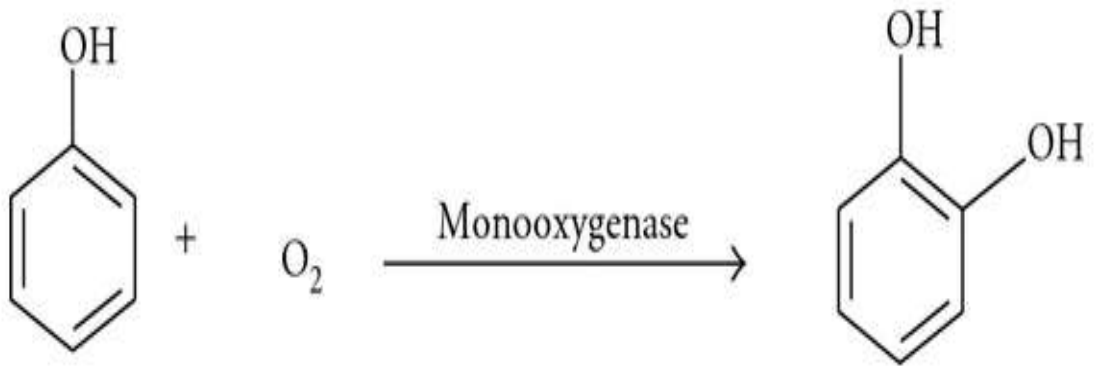

Fig. 2.8: Degradation of aromatic compound by monooxygenase (Arora *et al*., 2010).

Dioxygenases are multicomponent enzyme systems that introduce molecular oxygen into their substrate. Aromatic hydrocarbon dioxygenases belong to a large family of Rieske nonheme iron oxygenases. These dioxygenases catalyze enantiospecifically the oxygenation of wide range of substrates. Dioxygenases primarily oxidize aromatic compounds and therefore have applications in environmental remediation. All members of this family have one or two electron transport proteins preceding their oxygenase components. The crystal structure of naphthalene dioxygenase has confirmed the presence of a Rieske (2F – 2S) cluster and mononuclear iron in each alpha subunit (Dua *et al*., 2002).

The catechol dioxygenases serve as part of nature's strategy for degrading aromatic molecules in the environment. They are found in the soil bacteria and involved in the transformation of aromatic precursors into aliphatic products. The intradiol cleaving enzymes

utilize Fe (III), while the extradiol cleaving enzymes utilize Fe (II) and Mn (II) in a few cases (Arora *et al*., 2009) (Fig. 2.9).

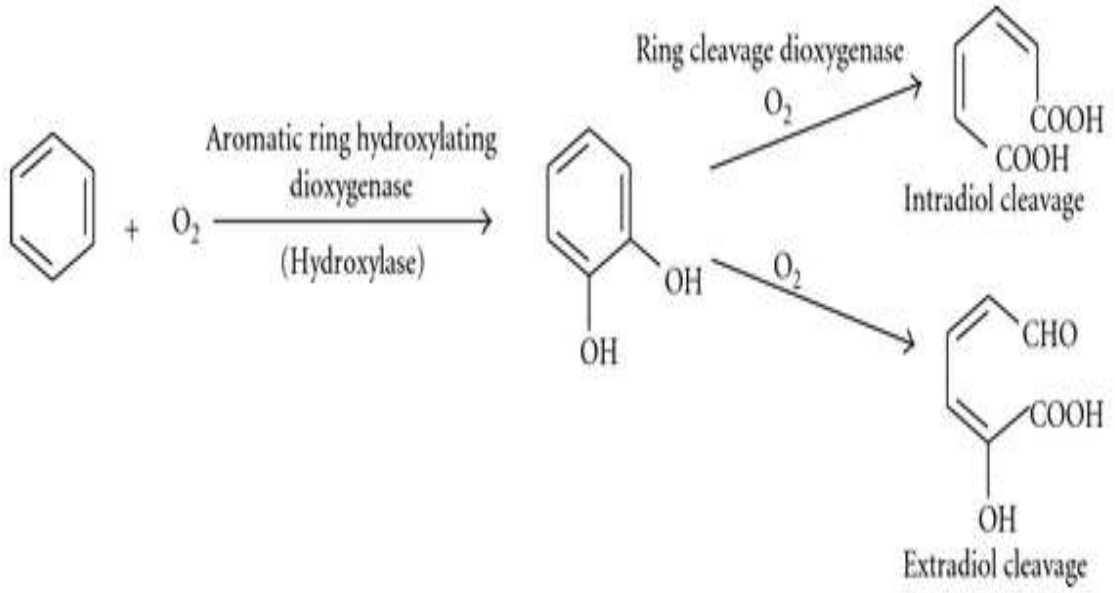

Fig. 2.9: Degradation of aromatic compound by dioxygenase (Arora *et al*., 2009).

Laccases (*p*-diphenol:dioxygen oxidoreductase) constitute a family of multicopper oxidases produced by certain plants, fungi, insects and bacteria, that catalyze the oxidation of a wide range of reduced phenolic and aromatic substrates with concomitant reduction of molecular oxygen to water (Mai *et al*., 2000). Laccases are known to occur in multiple isoenzyme forms each of which is encoded by a separate gene (Karigar and Rao, 2011), and in some cases, the genes have been expressed differently depending upon the nature of the inducer (Rezende *et al*., 2005).

Many microorganisms produce intra and extracellular laccases capable of catalyzing the oxidation of ortho and paradiphenols, aminophenols, polyphenols, polyamines, lignins and aryl diamines as well as some inorganic ions (Mai *et al*., 2000; Ullah *et al*., 2000; Rodriguez

and Toca, 2006). Laccases not only oxidize phenolic and methoxy-phenolic acids (Fig. 2.10), but also decarboxylate them and attack their methoxy groups (demethylation). These enzymes are involved in the depolymerization of lignin, which results in a variety of phenols. In addition, these compounds are utilized as nutrients for microorganisms or repolymerized to humic materials by laccase (Kim *et al*., 2002). Among the biological agents, laccases represent an interesting group of ubiquitous, oxidoreductase enzymes that show promise of offering great potential for biotechnological and bioremediation applications (Rao *et al*., 2010).

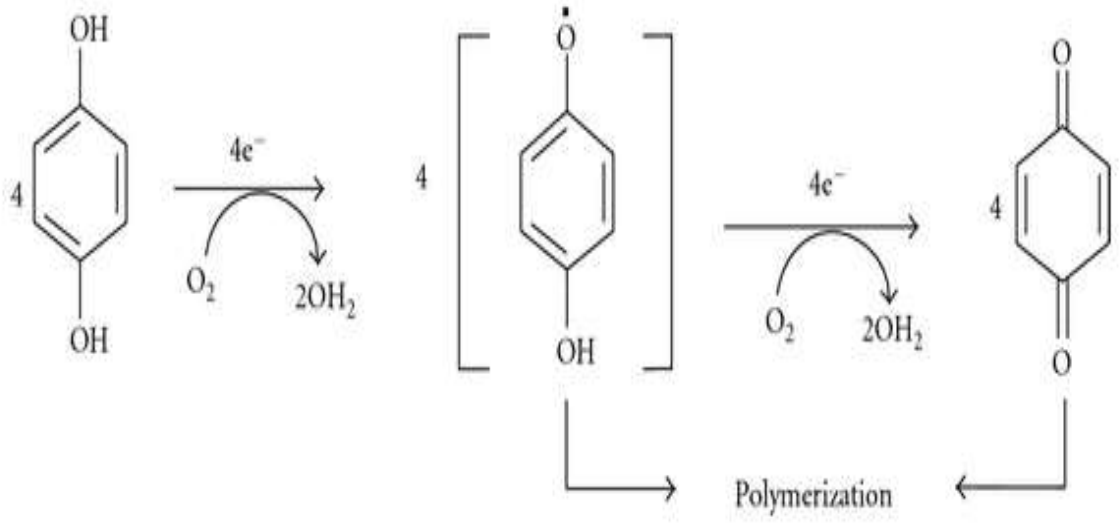

Fig. 2.10: General reaction mechanism for phenol oxidation by laccase (Dedeyan *et al*., 2000).

The substrate specificity and affinity of laccase can vary with changes in pH. Laccase can be inhibited by various reagents such as halides (excluding iodide), azide, cyanide and hydroxide (Karigar and Rao, 2011). Different laccases appear to have differing tolerance toward inhibition by halides, indicating differential halide accessibility. Laccase production is sensitive to the nitrogen concentration in fungi. High nitrogen levels are usually required to

obtain greater amounts of laccase (Karigar and Rao, 2011). Recombinant laccase can be produced by either homologous or heterologous means (Rao *et al*., 2010).

Peroxidases (donor: hydrogen peroxide oxidoreductases) are ubiquitous enzymes that catalyze the oxidation of lignin and other phenolic compounds at the expense of hydrogen peroxide (H_2O_2) in the presence of a mediator. These peroxidases can be haem and nonhaem proteins. In mammals, they are involved in biological processes such as immune system or hormone regulation. In plants, they are involved in auxin metabolism, lignin and suberin formation, cross-linking of cell wall components, defense against pathogens, or cell elongation (Hiner *et al*., 2002; Koua *et al*., 2009). Peroxidases have been classified into many types based on its source and activity. Among peroxidases, lignin peroxidase (LiP), manganese-dependent peroxidase (MnP) and versatile peroxidase (VP) have been studied the most due to their high potential to degrade toxic substances in nature (Karigar and Rao, 2011).

Lignin peroxidases are heme proteins secreted mainly by the white rot fungus during secondary metabolism. In the presence of cosubstrate H_2O_2 and mediator like veratryl alcohol, LiP degrades lignin and other phenolic compounds. During the reaction, H_2O_2 gets reduced to H_2O with the gaining of electron from LiP, which itself gets oxidized. The LiP (oxidized) with gaining an electron from veratryl alcohol returns to its native reduced state, and veratryl aldehyde is formed. Veratryl aldehyde then again gets reduced back to veratryl alcohol by gaining an electron from substrate. This results in the oxidation of halogenated phenolic compounds, polycyclic aromatic compounds and other aromatic compounds followed by a series of nonenzymatic reactions (Fig. 2.11) (TenHave and Teunissen, 2001). Lignin peroxidase (LiP) plays a central role in the biodegradation of the plant cell wall

constituent lignin. LiP is able to oxidize aromatic compounds with redox potentials higher than 1.4 V (NHE) by single-electron abstraction, but the exact redox mechanism is still poorly understood (Piontek *et al*., 2001).

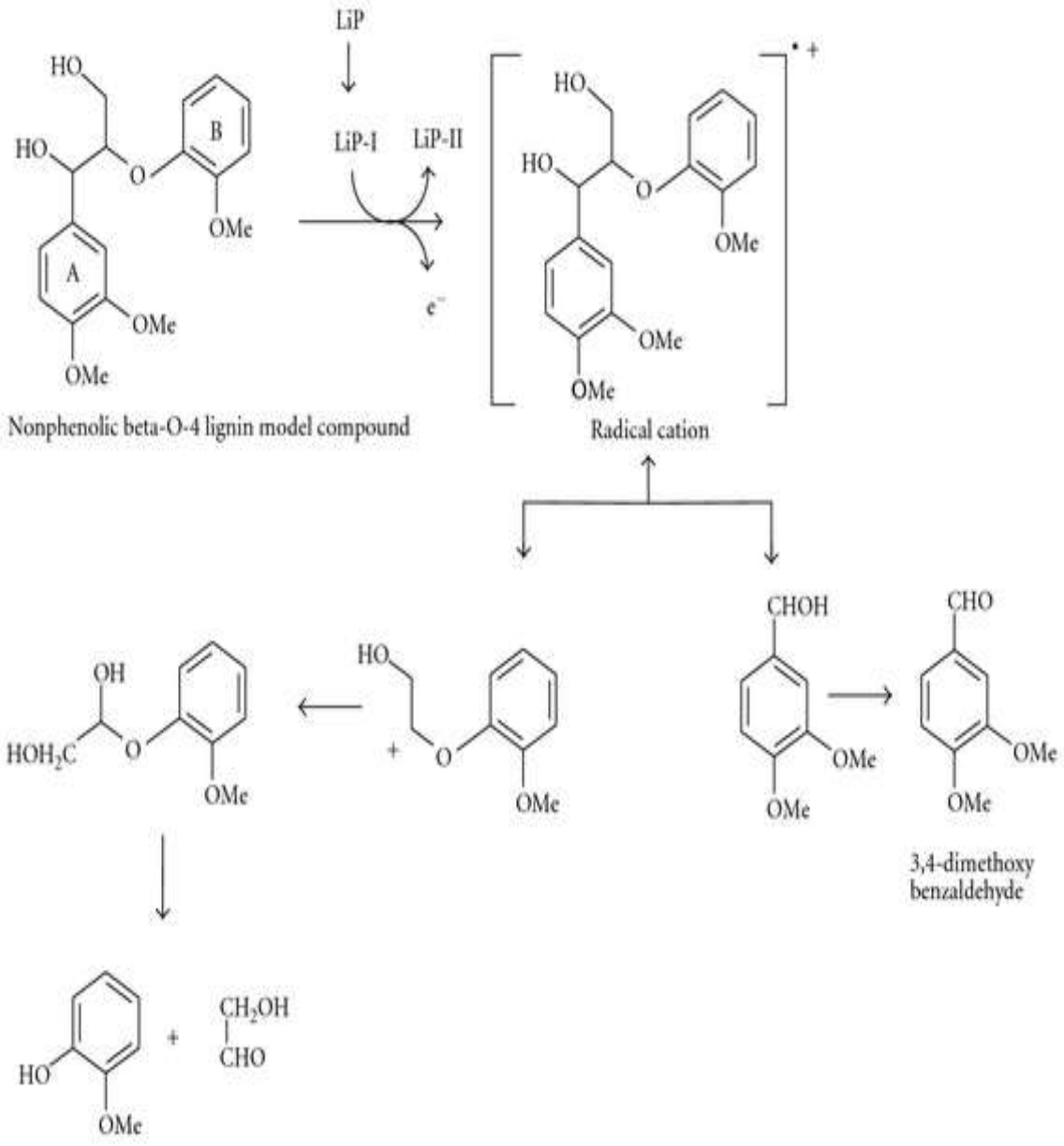

Fig. 2.11: Lignin peroxidase (LiP)-catalyzed oxidation of nonphenolic β-O-4 lignin model compound (Wong, 2009).

Manganese peroxidase (MnP) is an extracellular heme enzyme from the lignin-degrading basidiomycetes fungus, that oxidizes Mn^{2+} to the oxidant Mn^{3+} in a multistep reaction. Mn^{2+} stimulates the MnP production and functions as a substrate for MnP. The Mn^{3+}, generated by MnP, acts as a mediator for the oxidation of various phenolic compounds. The resulting Mn^{3+} chelate oxalate is small enough to diffuse into areas inaccessible even to the enzyme, as in the case of lignin or analogous structures such as xenobiotic pollutants (Fig. 2.12) buried deep within the soil, which are not necessarily available to the enzymes (TenHave and Teunissen, 2001).

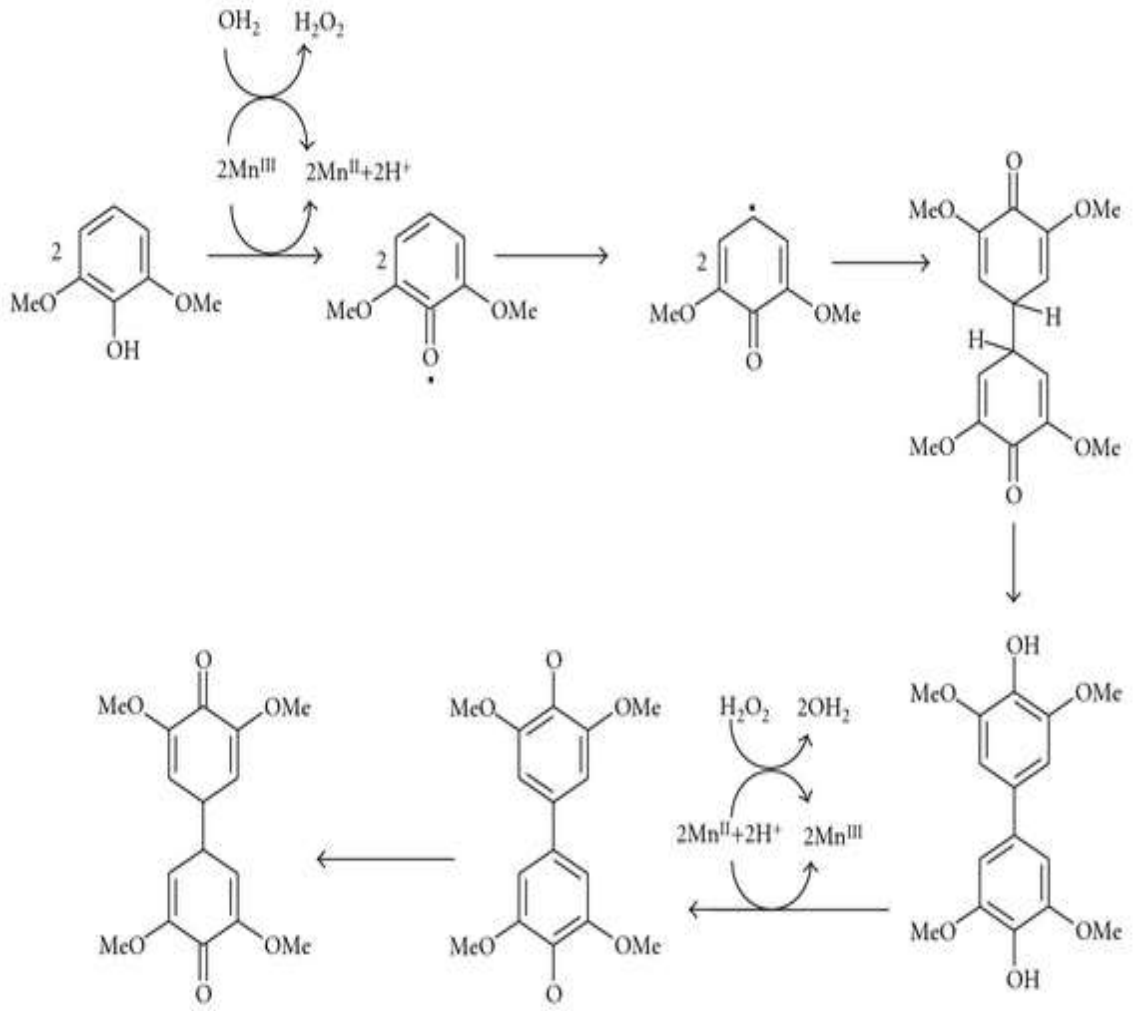

Fig. 2.12: Proposed mechanism for the oxidation of 2, 6-dimethoxyphenol by the MnP system (Karigar and Rao, 2011).

Versatile peroxidase (VP) enzymes are able to directly oxidize Mn^{2+}, methoxy benzenes, phenolic aromatic substrates like that of MnP, LiP and horseradish peroxidase. VP has extraordinary broad substrate specificity and tendency to oxidize the substrates in the absence of manganese when compared to other peroxidases. It has also been demonstrated that VP is able to oxidize both phenolic and nonphenolic lignin model dimmers (Ruiz-Duenas *et al*., 2007). Therefore, a highly efficient VP over production system is desired for biotechnological applications in industrial processes and bioremediation of recalcitrant pollutants (Wong, 2009; Tsukihara *et al*., 2006).

2.6.2 Microbial hydrolytic enzymes

Bacterial activity is the major process involved in the hydrolysis of organic pollutants (Karigar and Rao, 2011). Extracellular enzyme activity is a key step in the 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. Organochlorine insecticides such as DDT and heptachlor are stable in well-aerated soil but readily degrade in anaerobic environments (Vasileva-Tonkova and Galabova, 2003). Hydrolases also catalyze several related reactions including condensation and alcoholysis. Hydrolases belong to group 3 of enzyme class and may further be classified according to the type of bond hydrolyzed (Sanchez-Porro *et al*., 2003).

Extracellular hydrolytic enzymes such as amylases, proteases, lipases, DNases, pollulanases and xylanases have quite diverse potential usages in different areas such as food industry, feed additives, biomedical sciences and chemical industries (Sanchez-Porro *et al*., 2003). The hemicellulase, cellulase and glycosidase are of much importance due to its application in biomass degradation (Schmidt, 2006).

Lipase degrades lipids derived from a large variety of microorganisms, animals and plants. Recent works have shown that lipase is closely related with the organic pollutants present in the soil (Karigar and Rao, 2011). Lipase activity was responsible for the drastic reduction in total hydrocarbon from contaminated soil. Research undertaken in this area is likely to progress the knowledge in the bioremediation of oil spill (Riffaldi *et al*., 2006). Lipases have been extracted from bacteria, plant, actinomycetes and animal cell. Among these, microbial lipases are more versatile because of their potent application in industries. These enzymes can catalyze various reactions such as hydrolysis, interesterification, esterification, alcoholysis and aminolysis (Prasad and Manjunath, 2011).

Lipases are ubiquitous enzymes which catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. Lipolytic reactions occur at the lipid-water interface, where lipolytic substrates usually form equilibrium between monomeric, micellar and emulsified states. Lipases have been classified into two types on the basis of criteria such as (a) enhancement in enzyme activity as soon as the triglycerides form an emulsion and (b) lipases with a loop of protein (lid) covering on the active site (Sharma *et al*., 2011).

Triglyceride is the main component of natural oil or fat. This can hydrolyze consecutively to diacylglycerol, monoacylglycerol, glycerol and fatty acids. Glycerol and fatty acids are

widely used as raw materials, for instance, monoacylglycerol is used as an emulsifying agent in the food, cosmetic and pharmaceutical industries. The study made on triolein hydrolysis from *Candida rugosa* lipase in the biphasic oil-water system as proven to be effective (Hermansyah *et al*., 2007). The lipase adsorbs on to the oil-water interface in the bulk of the water phase. The lipase then breaks the ester bonds of triolein to produce consecutively diolein, monoolein and glycerol. During the catalysis, oleic acid is formed at each consecutive reaction stage. The glycerol formed is hydrophilic and thus dissolves into the water phase (Hermansyah *et al*., 2007) (Fig. 2.13).

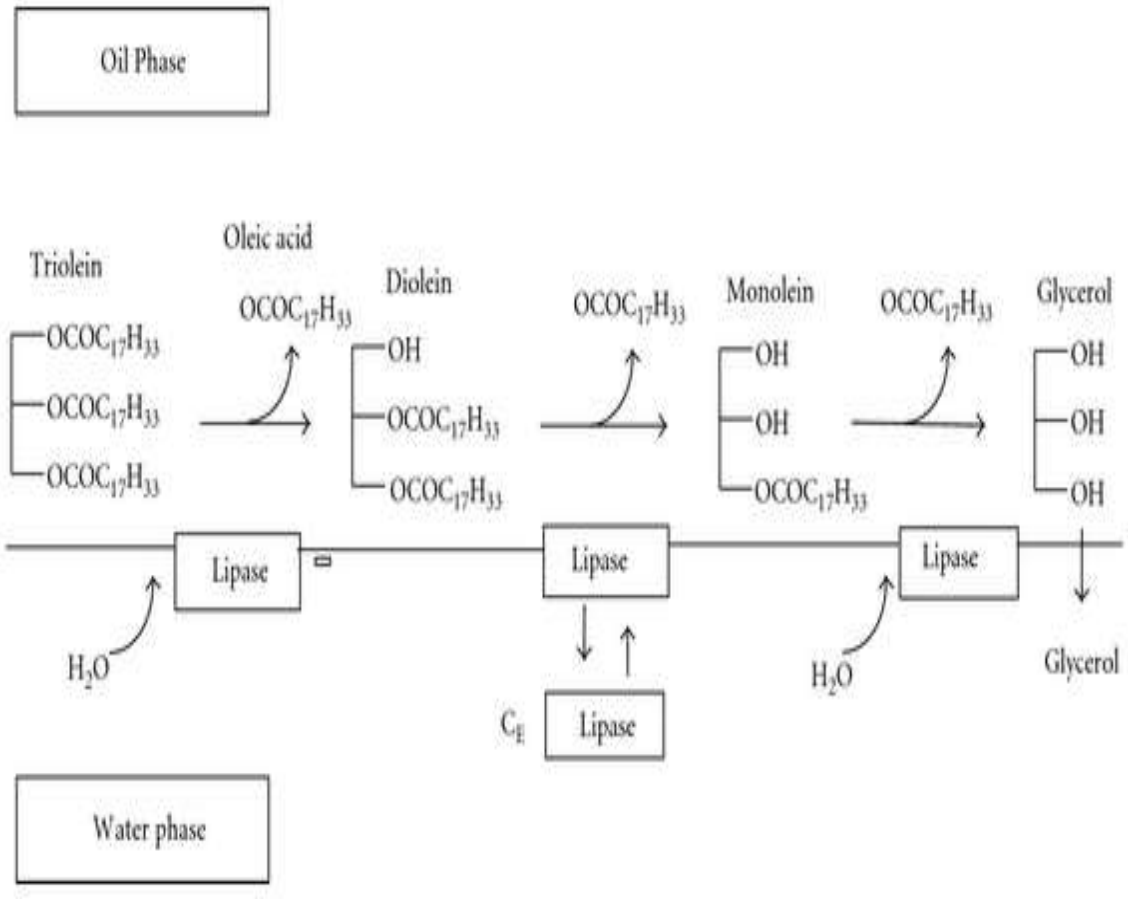

Fig. 2.13: Proposed mechanism for triolein hydrolysis by *Candida rugosa* lipase in biphasic oil-water system. C_E represents the enzyme concentration in the bulk of the water phase (Hermansyah *et al*., 2007).

Lipase activity was found to be the most useful indicator parameter for testing hydrocarbon degradation in soil (Riffaldi *et al*., 2006). Lipase is of much interest in the production of regiospecific compounds which are employed in pharmaceutical industry. Along with its diagnostic usage in bioremediation, lipase has many potential applications in food, chemical, detergent manufacturing, cosmetic, and paper making industries, but its production cost has restricted its industrial use (Sharma *et al*., 2011; Joseph *et al*., 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection of Samples

Soil samples were collected randomly using a pre-cleaned hand scoop at a depth of 0 – 3cm from 3 automobile workshops that had a heavy spillage of used engine oil (UEO) at Old Motor Spare Parts, popularly called Mgbuka-Nkpor (6º9'N 6º50'E), Nigeria. Replicate samples labeled for physicochemical analysis were also randomly collected accordingly from the automobile workshops in sterile containers. Uncontaminated soil samples were also collected randomly from a fallow plot of land about 100m from contaminated sites, and placed in a sterile container. Map of the sampling area is shown in Appendix III. The hydrocarbon (used engine oil) used in this work was collected directly from the engine of 911 Lorry (at Mgbuka-Nkpor) in a sterile container. Unused sealed engine oil (Tonimas Heavy Duty Engine Oil. SAE 40 API: SJ/CF-4) was purchased from a dealer at Mgbuka-Nkpor. Other hydrocarbons (diesel and petrol) used in this work were purchased from Nigerian National Petroleum Corporation (NNPC) Mega Station, Awka. The soil samples were transported in cold storage container to the Microbiology Laboratory of the National Agency for Food and Drug Administration and Control (NAFDAC), Agulu, for analysis.

3.2 Soil Physicochemical Analysis

Replicate samples labeled for physicochemical analysis were pooled together, carefully sorted out to remove stones and other unwanted debris, and homogenously mixed to obtain a composite sample. The samples were dried at ambient temperature (25ºC), pulverised in a porcelain mortar, sieved through a 2mm sieve, and transported in polythene bags to Springboard Research Laboratory, Awka for analysis.

The pH of the soil samples was determined with a pH meter (3505 Jenway, UK) (Haytham and Ibrahim, 2016). Nitrate, total nitrogen and total organic carbon (TOC) was determined using the standard method (Association of Official Analytical Chemists (AOAC), 2005). The moisture content was determined using the method described by Bahuguna *et al*. (2011). Phosphate content was determined using the ascorbic acid method (AOAC, 2005). Total Petroleum Hydrocarbon (TPH) was determined using the Spectrophotometric method (Akpoveta *et al*., 2011). The heavy metals (Zn, Pb, Cd and Cu) as well as elemental Na, Mg, K and Ca were determined using Varian AA240 Atomic Absorption Spectrophotometer (Hua *et al*., 2008) within 24 hour of sampling.

3.2.1 Determination of pH

Twenty grammes (20g) of the soil samples were measured into 100 ml beaker and 40 ml of distilled water was added into the beaker. The suspension was stirred with a glass rod at regular intervals for 30 minutes. The electrode of the pH meter was inserted into the partly settled suspension and the reading on the pH meter was noted and recorded accordingly.

3.2.2 Nitrate determination

Nitrate content was determined using the standard method (AOAC, 2005). Two grammes (2g) of the soil samples were weighed into a 50ml digestion flask and 20 ml of the concentrated acid mixtures (650 ml of nitric acid (HNO_3); 80 ml of perchloric acid ($HClO_4$) and 20 ml of sulphuric acid (H_2SO_4)) was added into the flask. The flask was heated until a clear digest was obtained. The digest was diluted with deionized water to the 50 ml mark. Fifty millilitres (50 ml) of the sample was transferred into a porcelain dish and evaporated to dryness on a hot water bath. Two millilitres of phenol disulphonic acid was added to dissolve the residue by constant stirring with a glass rod. One molar (1M) solution of sodium

hydroxide was added with stirring until the solution develops a yellow colour. The solution was then filtered into a Nessler's tube and made up to 50 ml with deionized water. The absorbance was read at 410nm using spectrophotometer after the development of colour. The absorbance of the standard was also measured and the concentration determined using the formula;

$$\text{Nitrate concentration (mg/L)} = \frac{\text{Absorbance of sample X Concentration of standard}}{\text{Absorbance of standard}}$$

3.2.3 Phosphate determination

The phosphate content was determined using the ascorbic acid method (AOAC, 2005). Two grammes (2g) of the soil samples were weighed into a 50 ml digestion flask and 20 ml of the concentrated acid mixtures (650 ml HNO_3; 80 ml $HClO_4$ and 20 ml H_2SO_4) was added into the flask. The flask was heated until a clear digest was obtained. The digest was diluted with deionized water to the 50 ml mark. One drop of phenolphthalein indicator was added, followed by the addition of 1 ml of sulfuric acid solution and 0.4 g of ammonium persulfate. The content of the flask was boiled until the total volume drops to 10 ml. When cooled, 1 drop of phenolphthalein indicator was added and the solution was neutralized to a faint pink colour with 1N sodium hydroxide, and then made up to the 50 ml mark with deionized water. The digested sample was now tested for phosphate concentration.

Fifty millilitres of the digested sample was filtered into an acid cleaned dry 125 ml Erlenmeyer flask. One drop of phenolphthalein indicator was added, and 5N sulphuric acid was gradually added until the red colour disappears. Eight millilitres (8.0 ml) of ascorbic acid reagent was added, mixed thoroughly and allowed to stand for 10 minutes for the development of blue colour. The absorbance was then measured at 880 nm. The samples

absorbance was measured along with the standard, and phosphate concentration determined using the formula;

$$\text{Concentration of sample} = \frac{\text{Absorbance of sample X Concentration of standard}}{\text{Absorbance of standard}}$$

3.2.4 Total Organic Carbon (TOC)

The TOC was determined using the Walkley-Black Chromic acid wet oxidation method (AOAC, 2005). One gramme (1g) of the soil samples was weighed into 250 ml Erlenmeyer flask and 10 ml of 1N potassium dichromate ($K_2Cr_2O_7$) was added and swirled gently to disperse the soil in the solution. Gradually, 20 ml of concentrated H_2SO_4 was added and swirled gently. A 200^{o}C thermometer was inserted and heated while swirling the flask and the contents over a gas burner and gauze until the temperature reaches 135^{o}C. The flask was set aside to cool slowly on an asbestos sheet in a fume cupboard. Two blanks without soil were also run in the same way to standardize the ferrous sulphate ($FeSO_4$) solution. When cool, the content of the flask was diluted to 200 ml with deionized water. Three drops of ferroin indicator was added and the content of the flask was titrated with 0.4N $FeSO_4$. As the end point was approached, the solution takes on a greenish colour, and then changes to a dark green. At this point, the ferrous sulphate was added drop by drop until the colour changes sharply from blue-green to reddish-grey.

One millilitre (1 ml) of 1N dichromate solution is equivalent to 3 mg of carbon. The percentage carbon was determined from the formula;

$$\text{Total Organic Carbon (\%)} = \frac{0.003g \text{ x } N \text{ x} 10mL \text{ x } (1\text{-}T/S) \text{ x } 100}{ODW}$$

Where;

N = Normality of Potassium dichromate solution

T = Volume of $FeSO_4$ used in samples titration (mL)

S = Volume of $FeSO_4$ used in blank titration (mL)

ODW = Oven-dry sample weight (g)

3.2.5 Moisture Content

The moisture content was determined using the method described by Bahuguna *et al*. (2011). Ten grammes (10g) of the soil samples were weighed (W1) and dried at 105^{o}C in hot air oven until a constant weight (W2) was obtained. The moisture content (W1 – W2) was estimated as mg of moisture/g of soil. The percentage (%) moisture content was then calculated using the formula; W1 – W2/W1 X 100.

3.2.6 Total Nitrogen

The micro-Kjeldahl method for protein determination (AOAC, 2005) was employed for total nitrogen determination. One gramme of the soil sample was weighed into the micro-Kjeldahl flask. To this were added 1 Kjeldahl catalyst tablet and 20 ml of concentrated H_2SO_4. These were set in the appropriate hole of the digestion block heaters in a fume cupboard. The digestion was left on for 4 hours after which a clear colourless solution was left in the tube. The digest was carefully transferred into 100 ml volumetric flask, thoroughly rinsing the digestion tube with deionized distilled water and the volume of the flask made up to the 100 ml mark with deionized water. Five millilitres (5 ml) portion of the digest was then pipetted to kjeldahl apparatus and 5 ml of 40% (w/v) NaOH added.

The mixture was then steam distilled and the liberated ammonia collected into a 50 ml conical flask containing 10 ml of 2% boric acid plus mixed indicator solution. The green

colour solution was then titrated against 0.01N HCl. At the end point, the green colour turns to wine colour, which indicates that all the nitrogen has been trapped as ammonium chloride. The percentage nitrogen was calculated by using the formula:

% N = Titre value x atomic mass of nitrogen x normality of HCl used x 4

3.2.7 Total Petroleum Hydrocarbon (TPH)

TPH was determined using the Spectrophotometric method (Akpoveta *et al.*, 2011). One gramme (1g) of soil sample was suspended in 10 ml of hexane and shaken for ten minutes using a mechanical shaker. The solution was filtered using a Whatmann 42 filter paper and the filtrate diluted by taking 1 ml of the extract into 9 ml of hexane in a test tube, up to 10^{-3} dilution. The absorbance of this solution was read at 450 nm, using n-hexane as blank, and the concentration read off from the standard curve obtained from known concentrations of unbiodegraded used engine oil. The experiment was done in triplicate and mean results obtained.

3.2.8 Heavy metal analysis of soil samples

Heavy metals (Zn, Pb, Cd and Cu) were analysed using Varian AA240 Atomic Absorption Spectrophotometer. One gramme of the soil samples was transferred into a 250 ml glass beaker, to which 20 ml of concentrated nitric acid was added and heated to boil till the volume was reduced to about 15 ml. Concentrated nitric acid was added in increments of 5 ml till all the residue was completely dissolved. The mixture was cooled, transferred to a 100 ml beaker and made up to 100 ml using deionized water. The sample was aspirated into the oxidizing air-acetylene flame. When the aqueous sample was aspirated, the sensitivity for 1% absorption was observed. The same process was also employed for elemental determination of Na, Mg, K and Ca.

3.3 Heavy Metal Analysis of Used and Unused engine oil

Heavy metal analysis of used and unused engine oil was conducted using Varian AA240 Atomic Absorption Spectrophotometer (Hua *et al*., 2008). Twenty millilitres of the sample was transferred into a 250 ml glass beaker, to which 5 ml of concentrated nitric acid was added and heated to boil till the volume was reduced to about 15 ml. Concentrated nitric acid was added in increments of 5 ml till all the residue was completely dissolved. The mixture was cooled, transferred to a 100 ml measuring cylinder and made up to 100 ml using deionized water. The sample was aspirated into the oxidizing air-acetylene flame. When the aqueous sample was aspirated, the sensitivity for 1% absorption was observed. The same process was repeated for diesel and petrol.

3.4 Isolation of Fungi with Used Engine Oil Utilizing Abilities

Used engine oil utilizing fungi were isolated from the soil samples obtained from automobile workshops (at Mgbuka-Nkpor, Nigeria) on mineral salts medium of Zajic and Supplission (1972), with composition (g/L); K_2HPO_4, 1.8; KH_2PO_4, 1.2; NH_4Cl, 4.0; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $FeSO_4.7H_2O$, 0.01 and agar, 15 g. Fifty micrograms per millilitre (50µg mL^{-1}) of each of penicillin G and streptomycin was incorporated into the medium to inhibit interfering bacteria. The medium pH was adjusted to 5.5. The whole preparation was autoclaved, distributed into sterile Petri dishes and allowed to solidify. Isolation of hydrocarbon utilizing fungi was done using vapour phase transfer method with used engine oil as carbon and energy source supplied from the lid of the plates (Hamamura *et al*., 2006; Quatrini *et al*., 2008).

One gramme of the homogenized soil sample was measured into 9 ml of sterile distilled water in a test tube and swirled gently. 1ml of the sample was pipetted and serially diluted up

to 10^{-3} dilution. 0.1ml of the sample from the 10^{-2} and 10^{-3} dilutions was transferred onto the surface of a freshly prepared mineral salt agar using the spread plate technique (APHA, 1998). A Whatman No. 1 filter paper saturated with sterilized used engine oil was placed inside the lid of the plates. The plates were incubated at 28^{o}C for 7 days. Each distinct colony on oil degrading enumeration plates was purified by repeated sub culturing onto the surface of a freshly prepared Sabouraud Dextrose Agar (SDA) (Merck, Germany) plates to obtain pure cultures of the isolates. The pure cultures were maintained on SDA slants.

3.5 Identification of the Isolates

The cultural characteristics of the pure isolates on SDA were noted, and the microscopic features were observed using the wet mount and the microslide culture technique with reference to the Manual of Fungal Atlas (Barnett and Hunter, 2000; Efiuvwevwere, 2000 and Watanabe, 2002).

3.5.1 Wet Mount Technique

A drop of lactophenol cotton blue (LPCB) was placed on the surface of a clean slide. With a sterile inoculating loop, the mycelial growth of the mold cultures and the colony of the yeast isolates were transferred onto the LPCB stain, pressing it gently so that it easily mixes with the stain and covered with a clean cover slip. The preparation was mounted on a microscope and observed under X 40 and X 10 objectives.

3.5.2 Microslide Culture Technique

With the help of a sterile blade, an agar block (6 x 6 mm) was cut out from an already prepared SDA and placed onto a sterile glass microscope slide. The slide was then placed inside a sterile Petri dish and the four sides of the agar block inoculated with the colony of the

fungal isolates. A sterile cover slip was placed on the surface of the agar block. Incubation was done without inverting the plate at 28^{o}C for 5 days. After incubation, the slide was removed from the Petri dish and the cover slip was also aseptically removed from the agar block. A drop of LPCB was placed onto a clean slide and the cover slip from the slide culture was then placed onto the LPCB. The slide was then examined under the light microscope. The agar block was removed from the slide culture and a drop of LPCB was added to the slide and covered with a clean cover slip. The culture was also examined under the light microscope.

3.6 Molecular Identification of the Potential Isolates

3.6.1 DNA Extraction

The isolates were aseptically transferred from Sabouraud Dextrose Agar slant to 1.5 ml Eppendorf tubes containing 0.5 ml of Sabouraud Dextrose broth for isolation of fungal DNA. The DNA was isolated according to the Automated Genomic DNA isolation-kit (Invitrogen). PCR amplification and sequencing in both directions of the ITS region was performed at Rudbeck Laboratory, Uppsala, Sweden.

3.6.2 PCR Amplification

Extracted DNA was amplified using a RoboCycler 96 temperature cycle. The primers and PCR conditions used are specified below. PCR amplification was carried out in two steps.

3.6.2.1 First-round Amplification

The universal primers used for fungal amplification were ITS1 (5’ TCC GTA GGT GAA CCT GCG G 3’), which hybridizes at the end of 18S rDNA, and ITS4 (5’ TCC TCC GCT TAT TGA TAT GC 3’), which hybridizes at the beginning of 28S rDNA (White *et al.*, 1990).

The 50 µl PCR mixtures contained 10 µl of DNA template, 6 µl of 25 mM $MgCl_2$, 5 µl of PCR buffer without $MgCl_2$; 200 µM each deoxynucleoside triphosphate (dNTP), 25 pmol of each primer, and 1 U of Taq DNA polymerase. Reactions involved 1 cycle at 95ºC for 5 minutes, followed by 35 cycles with a denaturation step at 95ºC for 30 s, an annealing step at 55°C for 1 min, and an extension step at 72°C for 1 min, followed by 1 cycle at 72°C for 6 mins.

3.6.2.2 Second-round Amplification

For the second amplification, the primers used were ITS86 (5' GTG AAT CAT CGA ATC TTT GAA C 3'), which hybridizes with the 5.8S rDNA region (Lott *et al*., 1998), and ITS4. Seminested PCR amplification mixtures contained 1 µl of first-round product in 50 µl of PCR reaction mixture (6 µl of 25 mM $MgCl_2$, 5 µl of PCR buffer without $MgCl_2$, 200 µM each deoxynucleoside triphosphate, 50 pmol of primer ITS4, 100 pmol of primer ITS86, and 1 U of *Taq* DNA polymerase). Reactions involved 1 cycle at 95°C for 5 min, followed by 30 cycles with a denaturation step at 95°C for 30 s, an annealing step at 55°C for 30 s, and an extension step at 72°C for 30 s, followed by 1 cycle at 72°C for 6 min.

3.6.3 DNA Sequencing of PCR products

Amplified DNA from PCR was purified using the Gene Clean II kit (Bio 101, Inc., Carlsbad, California) as specified by the manufacturer and directly cycle sequenced in both directions using the BigDye terminators Ready Reaction Kit (PE Applied Biosystems, Foster City, California) on an ABI Prism automated DNA sequencer (Model 377, version 2.1.1; Applied Biosystems Warrington, United Kingdom). The primers used were ITS4 and ITS86. Sequences were manually edited and aligned using BioEdit v 5.0.9, and matched with DNA

sequences from GenBank at NCBI using the expected value in the BLAST search function (Altschul *et al*., 1997).

3.6.4 Phylogenetic Analysis

A phylogenetic tree was constructed using the neighbor-joining method, in MEGA version 4.1. (Tamura *et al*., 2007). The topology of the distance tree was tested by resampling data with 1000 bootstraps to provide confidence estimates.

3.7 Screening Test for UEO Biodegradation Potentials of the Fungal Isolates

Each isolate was tested for used engine oil utilization capabilities in mineral salt broth medium. One hundred milliliters (100ml) of mineral salt broth supplemented with 1% (v/v) used engine oil was distributed into 250ml Erlenmeyer flasks. A loopful of a 24 hour pure cultures of the isolates were aseptically inoculated into the 250ml Erlenmeyer flasks. Uninoculated control flasks were also set up. The flasks were incubated in an Orbital Shaker (VWR DS2 – 500 – 2 Orbital Shaker) at 120 revolutions per minute (rpm) and temperature of 28^{o}C for 16 days. During the period of incubation, the pH and total viable counts (TVC) of the broth cultures were determined at time intervals as biodegradation indices (Rahman *et al*., 2002; Emtiazi and Shakarami, 2004). The residual hydrocarbon was also determined at time intervals using the Spectrophotometric method (Akpoveta *et al*., 2011) described earlier.

3.7.1 pH Determination

The pH of the culture fluids were determined at 0, 4, 8 12 and 16 days, by pipetting 5 ml sample from the Erlenmeyer flasks and the pH determined using a calibrated pH meter.

3.7.2 Total Viable Count Determination

The total viable counts of each fungal isolate was determined (at 0, 4, 8, 12 and 16 days) by aseptically pipetting 1 ml sample from the Erlenmeyer flasks and serially diluted up to 10^{-5} dilution. 0.1ml of the sample was pipetted from the 10^{-5} dilution and transferred onto the surface of freshly prepared Sabouraud Dextrose Agar (Merck, Germany) plates using the spread plate technique (APHA, 1998). Incubation followed immediately at 28^{o}C for 48 hours. Colonies formed were counted at the end of 48 hour incubation and expressed as colony forming unit per milliliter (Cfu/ml).

3.7.3 Residual Hydrocarbon Determination

The residual hydrocarbon was determined at 0, 4, 8, 12 and 16 days. One millilitre of the broth culture was pipetted into 50 ml Erlenmeyer flasks and 10 ml of n-hexane was added and shaked vigourously for 10 minutes. The content of the flasks was separated into a separating funnel and the organic layer was collected and measured at 450 nm in a Spectrophotometer. The concentration was then read off from a standard curve prepared using known concentrations of used engine oil (Appendix IV).

3.8 Determination of UEO, Diesel and Petrol Biodegradation of the Potential Isolates

The extent of biodegradation of used engine oil, diesel and petrol by four potential isolates namely *Candida tropicalis*, *Rhodosporidium toruloides*, *Fusarium oxysporum*, and *Aspergillus clavatus* were assessed using the Spectrophotometric method (Akpoveta *et al*., 2011). About 100 ml of mineral salt broth incorporated separately with 1% (v/v) sterile used engine oil, diesel and petrol, were prepared in 250 ml Erlenmeyer flasks. A loopful of a 24 hour pure cultures of the isolates were aseptically inoculated into the flasks. Uninoculated control flasks were also set up. Degradation study flasks as well as controls were incubated in

triplicate in an orbital shaker at 28ºC and 120 revolutions per minute (rpm) for 16 days. The amounts of hydrocarbon left after 16 days incubation was determined by extracting the residual oil with n-hexane (BDH Chemicals, England) in a separating funnel and noting their absorbance reading at 450nm, using n-hexane as blank. The concentrations were then read off accordingly, from the standard curves prepared using known concentrations of used engine oil, diesel and petrol (Appendices IV to VI). The experiment was done in triplicate and the mean result expressed as percentage (%) removal efficiency using the formula; Removal Efficiency = Initial concentration of oil before degradation – Final concentration of oil after degradation.

% Removal Efficiency = Removal Efficiency/ Initial concentration of oil before degradation X 100.

The standard curve of used engine oil was prepared by measuring different concentrations (0.5 to 5.0 g) of fresh used engine oil and diluting them up to 10^{-3} with n-hexane. From the 10^{-3} dilution, their corresponding absorbance was measured at 450nm using n-hexane as blank. Their absorbance was then plotted against concentrations and a straight line curve passing through the origin (Appendix IV) was obtained.

Different concentrations (0.5 to 5.0 g) of diesel was obtained and diluted to 10^{-1} with n-hexane. From the 10^{-1} dilution, their corresponding absorbance was measured at 450 nm using n-hexane as blank. Their absorbance was then plotted against concentrations and a straight line curve passing through the origin was obtained (Appendix VI). The same process was repeated for petrol.

3.9 Assay for Biosurfactant Activity of the Isolates

A 24 hour pure cultures of the two best potential isolates *Candida tropicalis* and *Aspergillus clavatus* were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of Mineral Salt broth and 1% (v/v) sterile used engine oil as carbon source. The flasks were incubated in an Orbital Shaker at 120 rpm for 7days at 28^{o}C. After 7 days incubation, the culture broth containing biosurfactant was separated from the culture media by centrifugation (at 3000rpm for 30 minutes at 4^{o}C) to get the culture supernatant. The supernatant was used for the determination of the emulsification index and oil spreading technique, while the fungal cells were harvested and used for the determination of the cell surface hydrophobicity in three different oils namely used engine oil, diesel and petrol.

3.9.1 Emulsification Index (E_{24})

Emulsification index (E_{24}) of culture sample was determined by mixing equal volume of substrate (used engine oil) and cell free broth supernatant in a test tube and vortexed for 2 minutes. The mixture was allowed to stand for 24 hour. E_{24} was calculated by dividing the height of the emulsion layer by the mixture total height and then multiplying by 100 (Tabatabaee *et al*., 2005; Sarubbo *et al*., 2007). The process was repeated with diesel and petrol.

3.9.2 Oil Spreading Technique

Fifty millilitres of distilled water was added to a large Petri dish (15 cm diameter) followed by addition of 20µL of used engine oil to the water surface and 10µL of culture supernatant. A clear zone was visible under light. The area of this circle was measured and calculated for Oil Displacement Area (ODA) using the following equation: $ODA = 22/7 (radius)^2 cm^2$

The triplicate assays from the same sample were measured (Sarubbo *et al*., 2007). The process was repeated for diesel and petrol.

3.9.3 Microbial Adhesion to the Hydrocarbon (MATH)

Microbial cell surface hydrophobicity was assessed by microbial adhesion to the hydrocarbon method (MATH) (Padmapriya *et al*., 2013). The harvested fungal cells were washed twice with PUM buffer (buffer salt solution, pH 7.0). The cells were again suspended in PUM buffer and absorbance (Ao) was measured at 600nm on a Lightwave Diode Array Spectrophotometer. Used engine oil (500µL) was added to 5ml of fungal suspension and vortexed for 2 minutes. The absorbance (A_1) of the aqueous phase was measured after 10 minutes. The percentage of the cell surface hydrophobicity (H %) was calculated using the following equation: $H\% = (Ao - A_1/Ao) \times 100$. The same process was repeated for diesel and petrol.

3.10 Assay for Enzyme Activity

A 24 hour pure cultures of the two isolates; *C. tropicalis* and *A. clavatus* were aseptically inoculated separately into 100 ml of Mineral Salts broth supplemented with 1% (v/v) sterile used engine oil, diesel and petrol, in 250 ml Erlenmeyer flasks. Uninoculated control flasks were also set up. The flasks were incubated in an orbital shaker at 120 rpm for 7 days at 28^{o}C. After 7 days incubation, the aliquots were centrifuged at 3000 rpm for 30 minutes at 4^{o}C and the supernatants (enzyme extract) were used for assay of extracellular enzymes such as catalase, lipase and peroxidase activities.

3.10.1 Determination of Catalase Activity

The activity of catalase (CAT) was determined according to Luck (1974) by measuring the decomposition of hydrogen peroxide (H_2O_2), and the change in absorbance at 240nm was followed every 60 seconds for 5minutes. The reaction mixture (3.0ml) contained 0.1ml of the supernatant (enzyme extract) in phosphate buffer (50mM phosphate buffer, pH 7.0) and 2.9ml of 30.0mM H_2O_2 in phosphate buffer. The reference reagent contained 0.1ml of phosphate buffer and 2.9ml of 30.0mM H_2O_2 in buffer. The molar extinction coefficient for H_2O_2 at 240nm of 43.6 $M^{-1}Cm^{-1}$ was used for the calculation. One unit of catalase was defined as the amount of enzyme that decomposes 1µmol of H_2O_2 per minute under standard assay conditions.

$$\text{Catalase Activity} = \frac{\Delta \text{ Absorbance x Total reaction volume}}{\text{Sample volume x Extinction coefficient}}$$

3.10.2 Determination of Lipase Activity

In the present investigation, one unit of lipase activity was defined as the amount of enzyme solution liberating 1µmole of p – nitrophenol per minute under standard assay conditions. The spectrophotometric method (Venkatesagowda *et al*., 2012), using P – nitrophenylPalmitate (P^{NPP}) as substrate was applied for rapid and routine measurement of the lipase activity. Enzyme or blank solution (480µl) was added to the reaction buffer (500µl) which has 50mM Tris-HCl and 30mM Triton x – 100. The content was incubated at 25^oC for 5 minutes and 10mM P^{NPP} in 2 – propanol (200µl) was added to the enzyme buffer solution and shaken well. The progress of the reaction was followed by monitoring the change in absorbance at 400nm every 60 seconds over a period of 5 minutes using Spectrophotometer (Genesys 10-S, USA). The molar extinction coefficient of p – nitrophenol (E = 18.2mM^{-1} Cm^{-1}) was used for the calculation of lipase activity.

$$\text{Lipase Activity} = \frac{\Delta \text{ Absorbance x Total reaction volume}}{\text{Sample volume x Extinction coefficient}}$$

3.10.3 Determination of Peroxidase (POD) Activity

The method proposed by Reddy *et al*. (1995) was adopted for assaying the activity of peroxidase. This method is based on the principle that in the presence of the hydrogen donor, pyrogallol or dianisidine, peroxidase converts H_2O_2 to H_2O and O_2. The oxidation of pyrogallol or dianisidine to a coloured product called purpurogallin can be followed spectrophotometrically at 420nm. To 3.0ml of pyrogallol solution, 0.1ml of the enzyme extract was added and the spectrophotometer was adjusted to read zero at 420nm. To the test cuvette, 0.5ml of H_2O_2 was added and mixed. The change in absorbance was recorded every 60 seconds up to 5 minutes in a spectrophotometer (Genesys 10-S, USA). The molar extinction coefficient of pyrogallol (E = 12 M^{-1} Cm^{-1}) was used for the calculation of peroxidase activity. One unit of peroxidase was defined as the change in absorbance per minute at 420nm.

$$\text{Peroxidase Activity} = \frac{\Delta \text{ Absorbance x Total reaction volume}}{\text{Sample volume x Extinction coefficient}}$$

3.11 Biodegradation of PAH components of the hydrocarbons by the isolates

A 24 hour pure cultures of each isolate; *C. tropicalis* and *A. clavatus* as well as their mixed culture (1:1 loopfuls) were inoculated into Mineral Salt broth (100ml in 250ml Erlenmeyer flask) containing 1% (v/v) sterile used engine oil. Uninoculated controls were also prepared accordingly. The flasks were incubated at ambient temperature of 28^{o}C at 120rpm for 16 days. The PAH components of the flask were determined prior to incubation and after 16

days incubation by Gas Chromatography at Springboard Research Laboratory, Awka, Nigeria, and compared with the control.

The specific procedure for the assay was as follows: 1ml of n-hexane extract of the samples (both degraded and undegraded used engine oil, diesel and petrol) was dissolved in 50ml of chloroform and transferred to a 100 ml volumetric flask and diluted to the mark. Most of the chloroform was allowed to evaporate at room temperature and 1ml of interesterification reagent (20vol% benzene and 55vol% methanol) was added. The samples were sealed and heated at 40^{o}C in a water bath for 30 minutes. After interesterification, the organic layer was extracted with n-hexane and water, so that the final mixture of the reagent, hexane and water, is in proportion of 1:1:1 (i.e., add 1ml of hexane and water to the reaction mixture). The mixture was shaken vigorously for 2 minutes and the top organic layer was transferred to a test tube for injection. When the instrument was ready, a 1µL sample was injected onto the GC column using proper injection technique.

The Buck 530 Gas Chromatography was equipped with a column oven, automatic injector, Mass spectrometer (Quadrupole Mass spectrometer, m/z 50 to m/z 400), HP 88 capillary column (30.0 m x 0.32 mm, film thickness 0.25µm) CA, USA. The analytic conditions of the chromatography were as follows: detector temperature, 250^{o}C, injector temperature, 220^{o}C, integrator chart speed, 2cm min^{-1}. Initial – final oven temperature was 70 – 280^{o}C/min, and a holding time of 2 – 5 minutes. Carrier gas was helium (99.999% or 5.0 grade purity) at 5 psi, and injection volume, 1µL. The chromatograph was then attached to an integrator.

The concentration of PAH components of the samples were shown by the peak heights of the chromatographic runs (Appendix VIII), and calculated by the integrator using a standard. The percentage (%) removal of PAH was calculated using the formula;

% removal of PAH = concentration of PAH in unbiodegraded UEO (control) – concentration of PAH in the test sample/concentration of PAH in unbiodegraded UEO (control) X 100.

The whole process was repeated for diesel and petrol.

3.12 Effect of varying concentrations of heavy metals on UEO degradation at different pH levels

The heavy metal salts employed in this study include: zinc chloride ($ZnCl_2$), lead trioxonitrate (V) $(PbNO_3)_2$, cadmium chloride ($CdCl_2$) and copper (II) tetraoxosulphate (VI) ($CuSO_4$). A weight of each of these metal salts (2.1, 1.6, 1.63, and 2.5 g, respectively) that gave a corresponding 1g of each of the respective metal was weighed and dissolved in 1000 ml of sterile deionized water. These were left to stand for 30 minutes to ensure complete dissolution. Working concentrations of 0.1, 1.0, 10, and 100mgL^{-1} of the respective metals were prepared by serial dilutions of the stock solution (Zhang and Crow, 2001) in mineral salt broth.

The effect of varying concentrations of Zn on UEO degradation at different pH was determined as described in Ekpeyong and Antai (2007). Tubes of mineral salt broth (MSB) (10ml) supplemented with 500µl of used engine oil as carbon source and different concentrations of zinc which included 0.1, 1, 10 and 100 mgL^{-1} were prepared, and the pH adjusted to 4.5, 5.5 and 6.5 levels, using concentrated orthophosphoric acid and 1M NaOH. The tubes were then inoculated with the pure as well as the mixed culture of the two isolates; *Candida tropicalis* and *Aspergillus clavatus*, and incubated in triplicate in an orbital shaker at 120rpm at ambient temperature of 28^{o}C for 16 days. Control tubes which included: control 1

(un-inoculated); MSB + UEO without inoculum and heavy metal, control 2 (inoculated); MSB + UEO + Inoculum without heavy metal, were also set up.

The residual hydrocarbon remaining in the tubes after 16 days was determined by Spectrophotometric method (Akpoveta *et al*., 2011), and compared with the control. The effect of heavy metals on used engine oil degradation, expressed as percentage inhibition (Ekpenyong *et al*., 2007) was determined using the formula;

% Inhibition = concentration of residual oil in test sample – concentration of residual oil in control 2/concentration of residual oil in test sample X 100.

The growth response of the isolates during utilization of used engine oil in the presence of varying concentrations of Zn at different pH was also determined by measuring the fungal biomass at 540 nm (Ramasamy *et al.,* 2011). The fungal cells were harvested by centrifugation (3000 rpm for 30 minutes), washed twice with phosphate buffer and resuspedded in 5 ml phosphate buffer. The absorbance was then measured at 540 nm. The whole process was also repeated for lead, cadmium and copper.

3.13 Evaluation of the effectiveness of the isolates in bioremediation of UEO contaminated soil using bioaugmentation technique

To evaluate the effectiveness of the isolates in bioremediation of UEO contaminated soil, soil microcosms were prepared in 250 ml Erlenmeyer flask using mineral salt medium as described in Alisi *et al.* (2009). One hundred grammes (100g) of the uncontaminated soil samples were added into 60ml of mineral salt broth in 250 ml Erlenmeyer flask. The slurries obtained were spiked with 5g of sterile UEO simulating a soil contamination corresponding to 50,000 mg kg^{-1} soil, and the pH adjusted to 5.5 using concentrated orthophosphoric acid and 1M NaOH. The heavy metals (Zn, Pb, Cd and Cu) content of the flasks was determined

using Varian AA240 Atomic Absorption Spectrophotometer (Hua *et al*., 2008) prior to incubation to access the level of heavy metals in the soil.

Bioaugmentation was carried out by inoculating 10 ml of standard inocula (OD = 1.0) of each of the pure and mixed culture of the best isolates (*C. tropicalis* and *A. clavatus*) into the flasks. Treatment with only soil and UEO served as control 1. Additional control 2 was also set up which contained autoclaved soil and sterile UEO to monitor abiotic loss of oil in the oil-contaminated soil. The flasks were incubated in triplicate in an orbital shaker at 120 rpm and 28^{o}C for 56 days. The total petroleum hydrocarbon (TPH) content of the flask was determined at 0, 14, 28, 42 and 56 days. Ten grammes of the soil sample from each microcosm were withdrawn into 250 ml Erlenmeyer flask. One hundred millilitres of hexane was added into the flask and shaken vigourously for 30 minutes in an orbital shaker at 180 rpm and the content of the flasks was filtered into a separating funnel. The organic layer was collected and diluted with hexane up to 10^{-3}. The absorbance of the 10^{-3} dilution was measured at 450 nm in a Spectrophotometer. The total petroleum hydrocarbon (TPH) remaining in the soil was estimated with reference to a standard curve derived from fresh used engine oil diluted with hexane. TPH data were fitted to the first-order kinetic model (Abioye *et al*., 2012); $C = Coe^{-kt}$, where C is the hydrocarbon content in soil (mg kg^{-1}) at time t, Co is the initial hydrocarbon content in soil (mg kg^{-1}), *k* is the biodegradation rate constant (day^{-1}), and *t* is time (day). The model estimated the biodegradation rate and half-life of hydrocarbons in soil relative to treatments applied. Half-life was then calculated from the model of Yeung *et al*. (1997). Half-life = ln (2)/k, where ln (2) is the natural logarithm of 2 (approximately 0.693).

The effectiveness of the isolates in remediation of used engine oil contaminated soil was also determined by calculating the net percentage loss of TPH in the oil contaminated soil, using the formula:

Net % loss = % loss of TPH in soil microcosm inoculated with the isolates - % loss of TPH of uninoculated soil microcosm (ie. control 1).

3.14 Statistical Analysis

Data were analysed and presented as mean ± standard deviation (SD) of three replicates. The Student's t-test was used to test the significance of difference between the mean values. Analysis of variance (ANOVA) was used to test significance of variations within and among the groups. When significant difference was indicated by ANOVA, the least significant difference (LSD) and Duncan multiple range test was used for pair-wise separation of the means. A statistical package for social sciences (SPSS) software was used for statistical analysis in this study and test for significance between means was implied at P = 0.05 level.

CHAPTER FOUR

4.0 RESULTS

4.1 Soil Physicochemical Characteristics

There were some observed differences in the physicochemical properties of the contaminated and the uncontaminated soil samples (Table 4.1). From the result, the nitrate, total nitrogen, total organic carbon (TOC), total petroleum hydrocarbon, lead, magnesium, zinc, cadmium and copper content of the contaminated soil are significantly higher ($P<0.05$) than that of the uncontaminated soil, while the pH, potassium, calcium and moisture content of the uncontaminated soil are significantly higher ($P<0.05$) than that of the contaminated soil (Table 4.1). However, phosphate, and sodium (Na) content of the contaminated and the uncontaminated soil are not different significantly ($P>0.05$).

Table 4.1: Physicochemical properties of the soil sample

Parameters	Contaminated soil	Uncontaminated soil	P-value
pH	6.850±0.312[a]	7.540±0.087[a]	0.021[*]
Nitrate (mg/L)	8.421±0.286[a]	7.763±0.104[a]	0.020[*]
Phosphate (mg/L)	6.855±0.110[b]	6.697±0.193[b]	0.286
Total Nitrogen (%)	2.688±0.016[a]	2.016±0.115[a]	0.001[*]
Total Organic Carbon (%)	0.174±0.006[a]	0.163±0.002[a]	0.031[*]
Moisture Content (%)	3.51±0.015[a]	6.79±0.012[a]	0.000[*]
Total Petroleum Hydrocarbon (mg/g)	180.0±1.044[a]	0.001±0.000[a]	0.000[*]
Potassium (ppm)	18.885±0.091[a]	19.855±0.152[a]	0.001[*]
Sodium (ppm)	9.576±0.120[b]	9.591±0.101[b]	0.840
Lead (ppm)	0.960±0.029[a]	0.240±0.010[a]	0.000[*]
Magnesium (ppm)	21.046±0.064[a]	16.481±0.029[a]	0.000[*]
Zinc (ppm)	8.628±0.087[a]	1.140±0.10[a]	0.000[*]
Cadmium (ppm)	1.762±0.011[a]	1.323±0.010[a]	0.000[*]
Copper (ppm)	2.579±0.008[a]	0.318±0.008[a]	0.000[*]
Calcium (ppm)	34.222±0.282[a]	73.863±0.360[a]	0.000[*]

Values are mean of three replicates ± Standard Deviation (SD).

Comparism of means along the rows; values followed by letter ‘a’ indicate significant difference at $P < 0.05$. Values followed by letter ‘b’ are not different significantly at $P < 0.05$.

4.2 Heavy Metal Characteristics of UEO, Diesel and Petrol

The result of the heavy metals analysis of used engine oil, diesel and petrol is presented in Table 4.2. Analysis of variance (ANOVA) indicates significant difference ($P < 0.05$) between the concentration of the heavy metals (Zn, Pb, Cd and Cu) in petrol, diesel, used and unused engine oil. However, the least significant difference (LSD) and Duncan multiple range test revealed that there was no significant difference in the level of Zn in petrol and diesel. Moreover, Pb and Cd were not up to detectable limit in diesel. Among the heavy metals analysed, Zn was predominantly found in used engine oil (Table 4.2).

Table 4.2: Heavy metal analysis of used engine oil, diesel and petrol

Samples	Zinc (ppm)	Lead (ppm)	Cadmium (ppm)	Copper (ppm)
Petrol	1.153±0.01[b]	0.014±0.001[a]	0.092±0.003[a]	0.073±0.007[a]
Diesel	1.149±0.004[b]	0.00±0.00[a]	0.00±0.00[a]	0.109±0.003[a]
Used engine oil	7.593±0.007[a]	0.040±0.001[a]	0.106±0.008[a]	0.119±0.004[a]
Unused engine oil	1.877±0.026[a]	0.008±0.002[a]	0.011±0.002[a]	0.018±0.026[a]

Values are mean of three replicates ± SD

Comparism of means along the columns; values followed by letter 'a' indicate significant difference at $P < 0.05$. Values followed by letter 'b' are not different significantly at $P < 0.05$.

4.3 Isolation and Identification of Fungi with Used Engine Oil Utilizing Abilities

A total of eight hydrocarbon utilizing fungi were isolated from soil samples obtained from used engine oil contaminated soil. The cultural and microscopic characteristics of the fungal isolates are presented in Table 4.3. The fungal genera identified were *Candida tropicalis*, *Rhodosporidium toruloides*, *Fusarium oxysporium*, *Aspergillus clavatus*, *Saccharomyces cerevisiae*, *Candida albicans*, *Microsporium gypseum* and *Trichophyton mentagrophytes*.

Table 4.3: Cultural and Microscopic Characteristics of the fungal isolates

Isolates	Colonial Morphology	Microscopic Observation	Suspected Organisms
G	White/creamy, smooth, soft and glabrous	Spherical to sub-spherical budding yeast-like cells	*Candida tropicalis*
H	Distinctive orange/red colony	Spherical to elongate budding yeast-like cells	*Rhodosporidium toruloides*
I	Purple/white and cone shaped colony	Branched conidiophores, smooth and rough conidia in pairs and chains	*Fusarium oxysporium*
J	Colonies are fast growing, flat and yellow-green to dark green in colour	Distinctive conidial heads with flask-shaped phialides arranged in whorls on a vesicle	*Aspergillus clavatus*
K	White/creamy smooth colonies	Large globose to ellipsoidal budding yeast-like cells.	*Saccharomyces cerevisiae*
L	Milky, waxy and butyrous texture	Small circular budding yeast cells	*Candida albicans*
M	Pale yellow-brown mycelium	Multiseptate, rough-walled, broadly spindle shaped macroconidia	*Microsporum gypseum*
N	Colonies are yellowish-brown and powdery	Smooth-walled, spherical and numerous microconidia produced in clusters	*Trichophyton mentagrophytes*

4.4 Molecular Identification of the Potential Isolates

To confirm the results of the cultural and microscopic identification of the isolates, the 18S rRNA gene sequence (Appendix VII) of the four isolates (G, H, I and J) that exhibited higher hydrocarbonoclastic potentials in the screen flasks were determined. Database comparism using BLAST program revealed that isolate G had a high similarity of 98 % with those of strain *Candida tropicalis* MCCC2E00325. However, isolates H, I and J had 100% similarity with those of strains *Rhodosporidium toruloides* K-1-8, *Fusarium oxysporium* F11 and *Aspergillus clavatus* ATCC 1007, respectively (Table 4.4). The expected values (E-value) for all the isolates are zero. The phylogenetic relationships of the sequence of the ITS region of the isolates (G, H, I and J) are shown in Figures 4.1 to 4.4. Based on the phylogenetic trees, the closest relatives of isolates G, H, I and J were respectively, *Candida tropicalis*, *Rhodosporidium toruloides*, *Fusarium oxysporium* and *Aspergillus clavatus*.

Table 4.4: Molecular identification of the potential isolates

Isolates	Closest Match	Percentage (%) identity	E-value
G	*Candida tropicalis* Voucher MCCC2E00352	98	0
H	*Rodosporidium toruloids* strain K-1-8	100	0
I	*Fusarium oxysporium* f.sp. Cumini strain F11	100	0
J	*Aspergillus clavatus* ATCC 1007	100	0

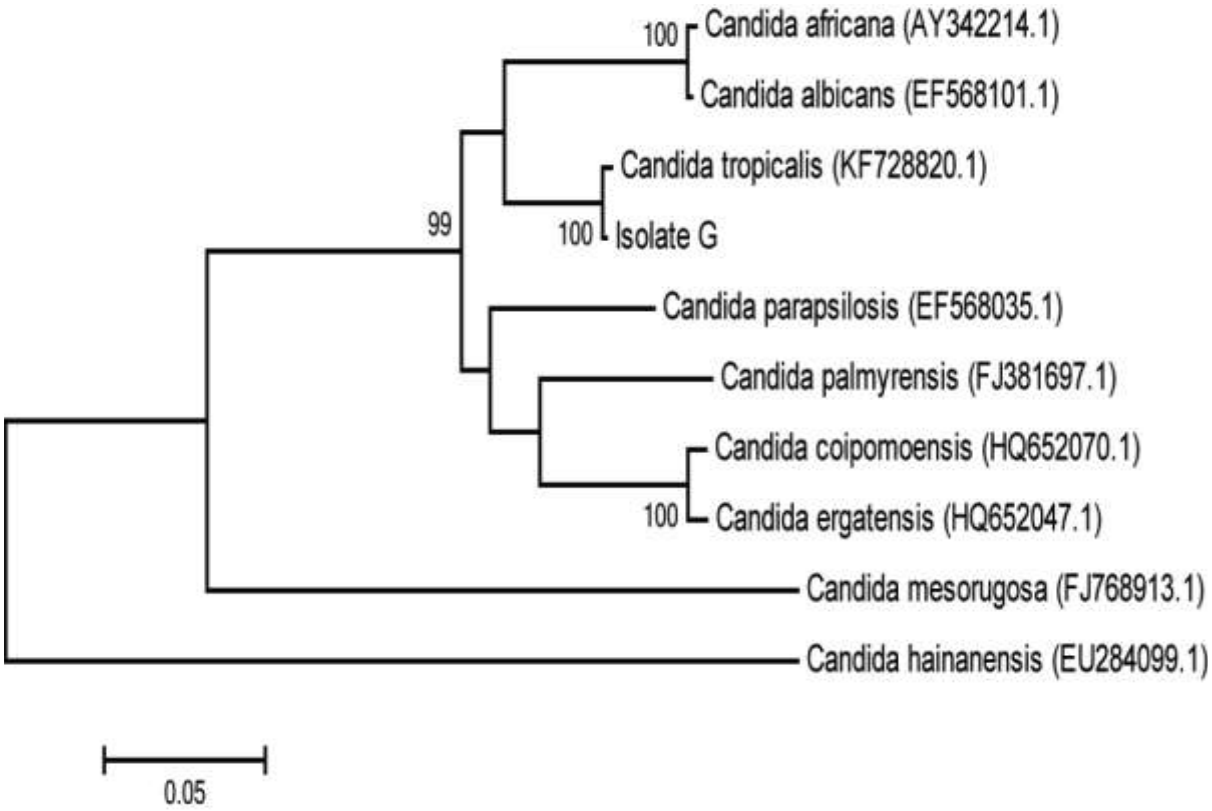

Fig. 4.1: Phylogenetic tree obtained by neighbor-joining analysis of the ITS sequences of isolate G (*Candida tropicalis*). Support values for neighbor-joining were established by boostrapping with 1000 replicates, and the scale bar represents 0.05 sequence divergence. *Candida hainanensis* was used as the outgroup.

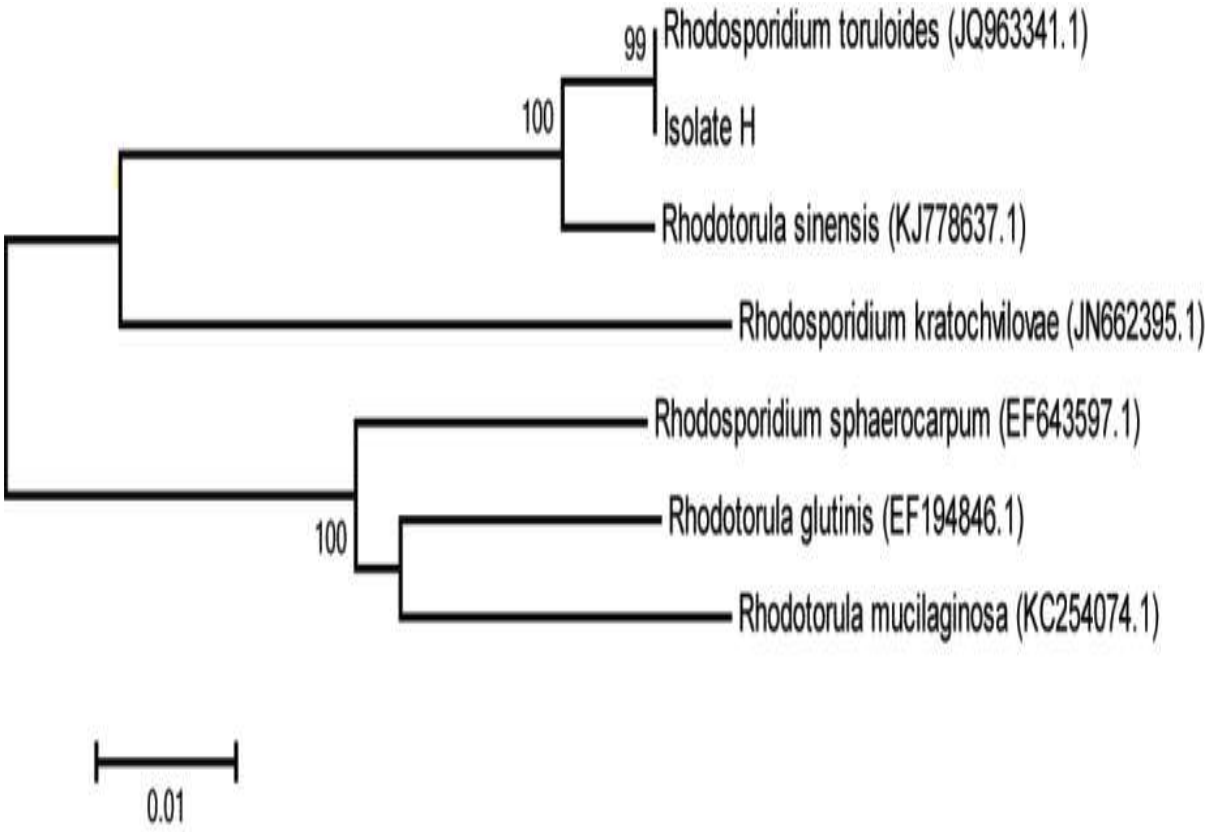

Fig. 4.2: Phylogenetic tree obtained by neighbor-joining analysis of the ITS sequences of isolate H (*Rhodosporidium toruloides*). Support values for neighbor-joining were established by boostrapping with 1000 replicates, and the scale bar represents 0.01 sequence divergence. *Rhodotorula mucilaginosa* was used as the outgroup.

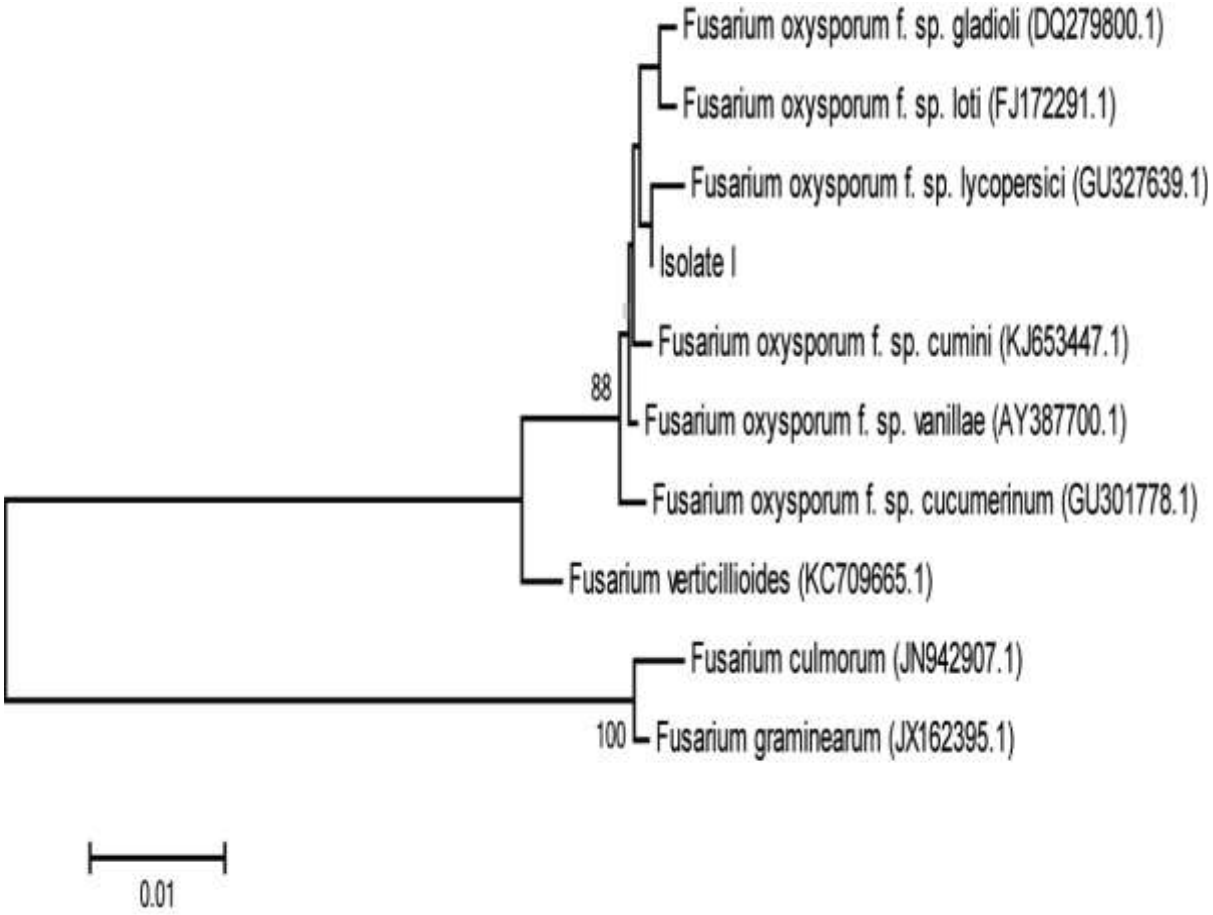

Fig. 4.3: Phylogenetic tree obtained by neighbor-joining analysis of the ITS sequences of isolate I (*Fusarium oxysporium*). Support values for neighbor-joining were established by boostrapping with 1000 replicates, and the scale bar represents 0.01 sequence divergence. The outgroup we used was *Fusarium graminearum*.

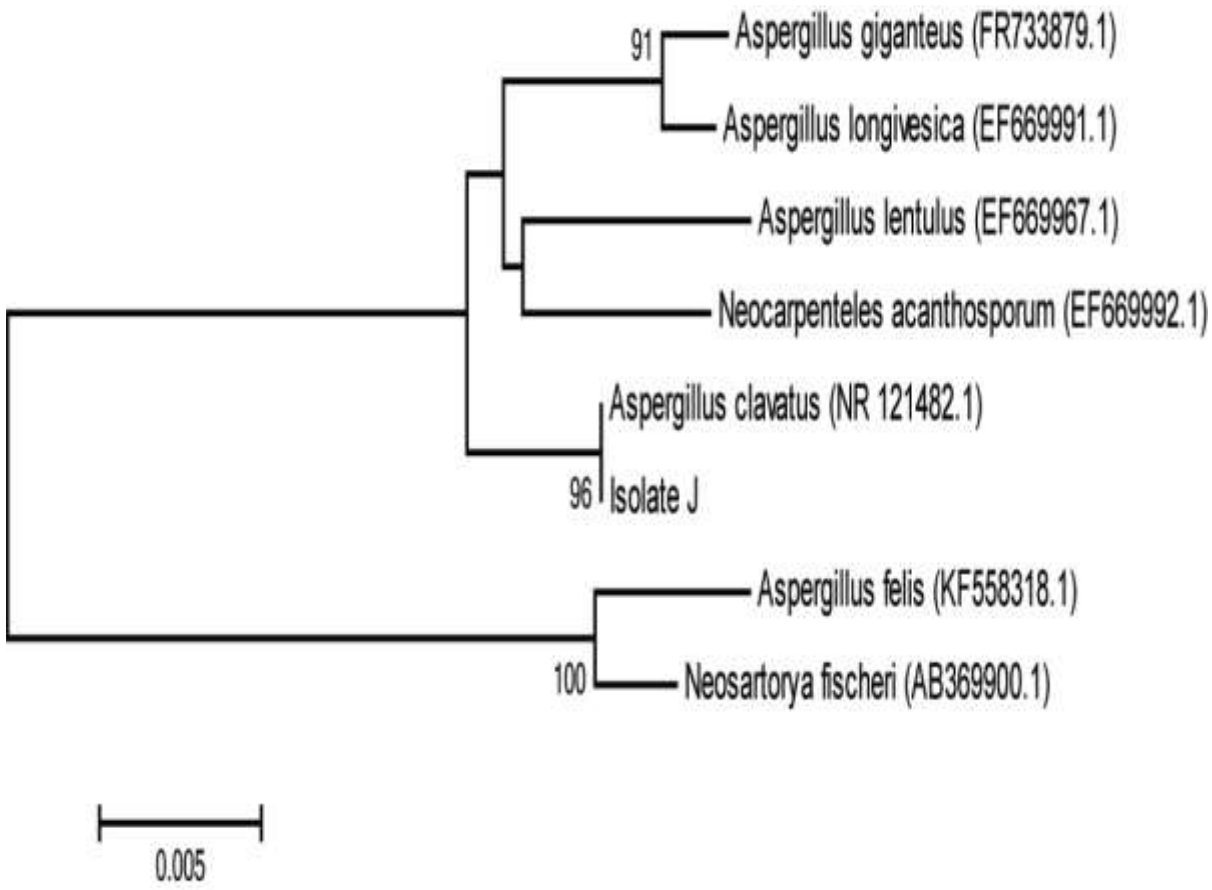

Fig. 4.4: Phylogenetic tree obtained by neighbor-joining analysis of the ITS sequences of isolate J (*Aspergillus clavatus*). Support values for neighbor-joining were established by boostrapping with 1000 replicates, and the scale bar represents 0.005 sequence divergence. The outgroup we used was *Neosartorya fischeri*.

4.5 Screening Test for UEO Biodegradation Potentials of the Fungal Isolates

The relationship between pH, TVC and oil loss are shown in Figures 4.5 to 4.7. Generally, a decreasing trend in pH was observed in the experimental flasks within the incubation period, with a concomitant decrease in hydrocarbon levels, as growth (TVC) increases. However, the decreasing trend in pH was more evident in the experimental flasks containing *Candida tropicalis*, *Rhodosporidium toruloides*, *Fusarium oxysporium* and *Aspergillus clavatus* (Fig. 4.5). Moreover, there was an increase in Total Viable Count from 0 to 12 days, with a slight decrease on the 16^{th} day (Fig. 4.6), in all the experimental flasks. The hydrocarbon losses were higher (> 50 %) in *C. tropicalis*, *R. toruloides*, *F. oxysporium* and *A. clavatus* containing test systems, while the hydrocarbon losses in *S. cerevisiae*, *C. albicans*, *M. gypseum* and *T. mentagrophytes* containing test systems were lower, (< 50 %) within the experimental period (Fig. 4.7). In the control flasks, no growth was observed within the experimental period, with no significant oil loss and pH change (Figs. 4.5 to 4.7). Based on these observations, *Candida tropicalis*, *Rhodosporidium toruloides*, *Fusarium oxysporium* and *Aspergillus clavatus* were selected for hydrocarbonoclastic potential studies.

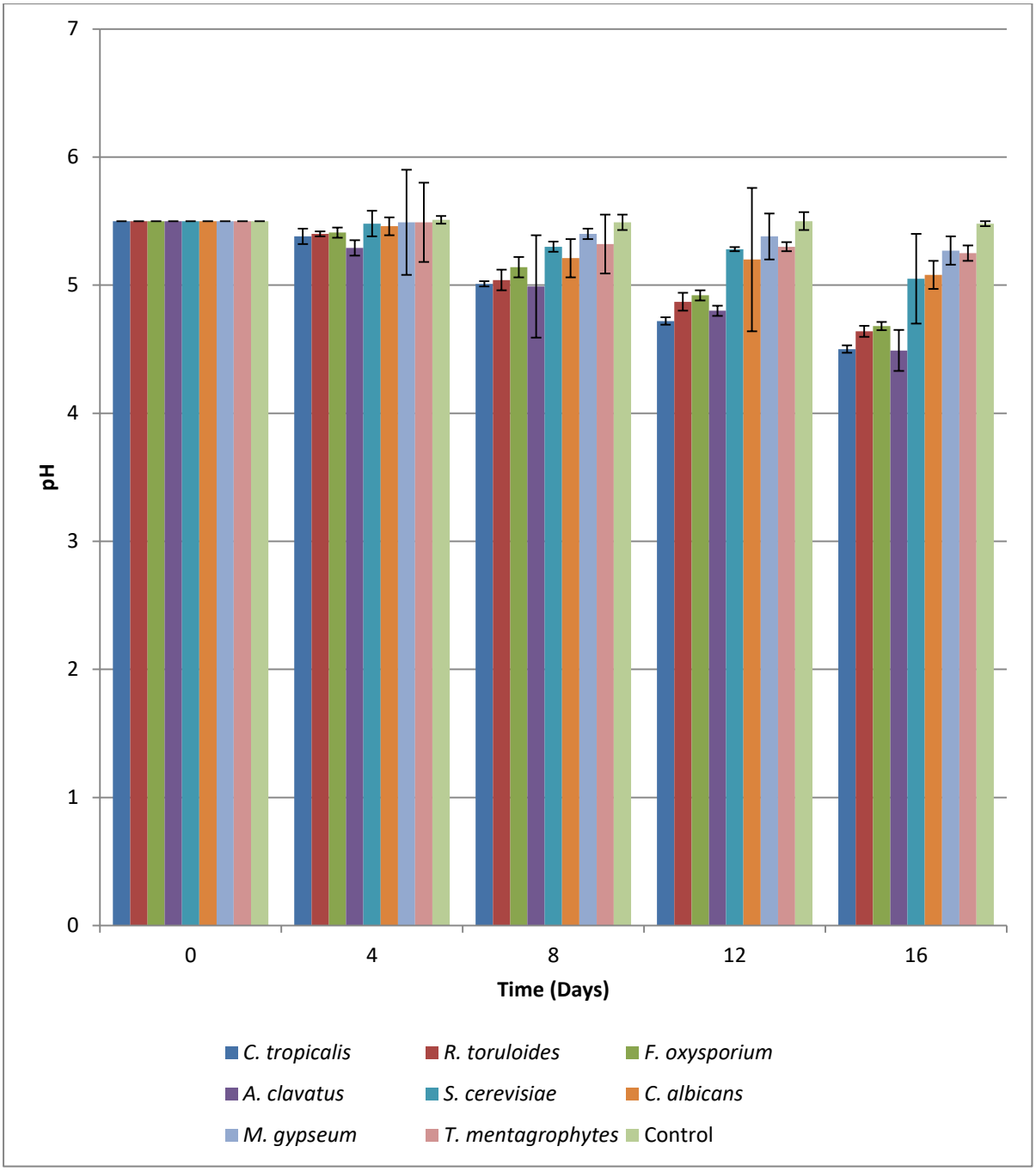

Fig. 4.5: Changes in pH with time during utilization of UEO by the isolates. Bars indicate the average of triplicate samples while the error bars shows standard deviation (±SD).

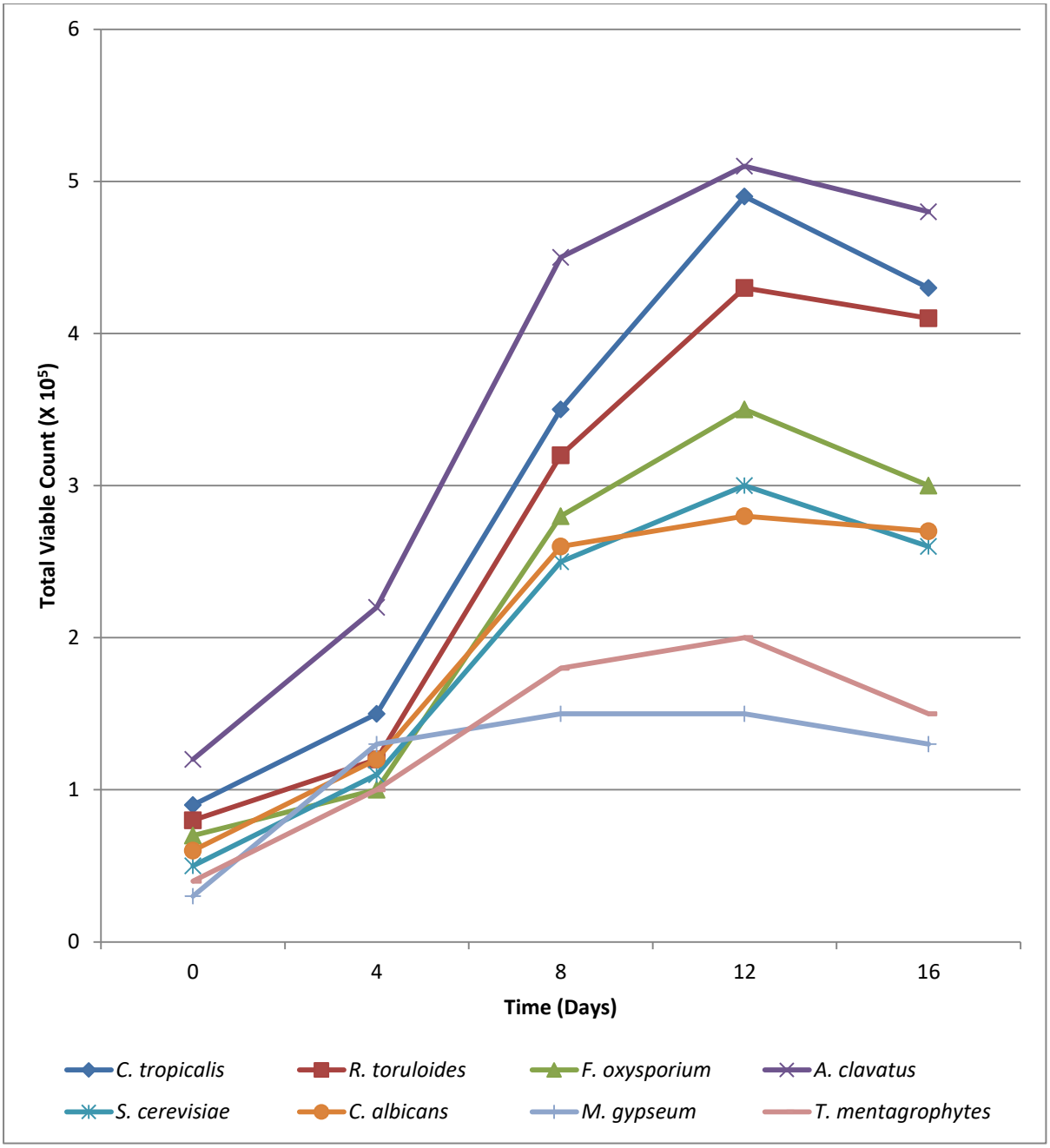

Fig. 4.6: Changes in total viable count with time during utilization of UEO by the isolates

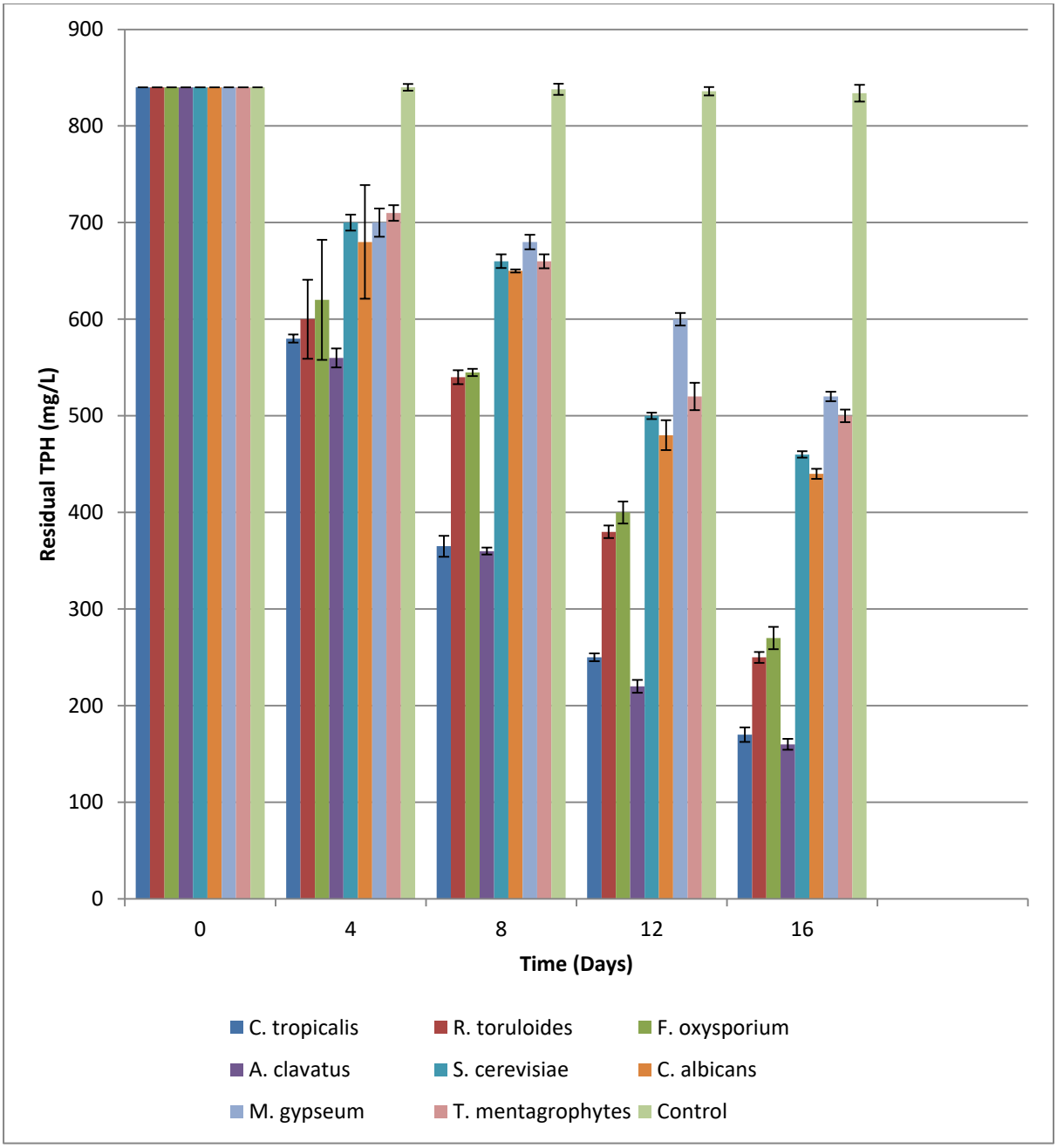

Fig. 4.7: Changes in total petroleum hydrocarbon with time during utilization of UEO by the isolates.

4.6 Determination of UEO, Diesel and Petrol Biodegradation of the Potential Isolates

The hydrocarbonoclastic potentials of the selected isolates revealed that *C. tropicalis* and *A. clavatus* achieved higher removal efficiency (%) of total petroleum hydrocarbon (TPH) in used engine oil, diesel and petrol during the experimental period, when compared to *R. toruloides* and *F. oxysporium* (Fig. 4.8). However, there were minimal abiotic losses in used engine oil, diesel and petrol (Fig. 4.8). Based on the above observations, *Candida tropicalis* and *Aspergillus clavatus* were selected for further studies.

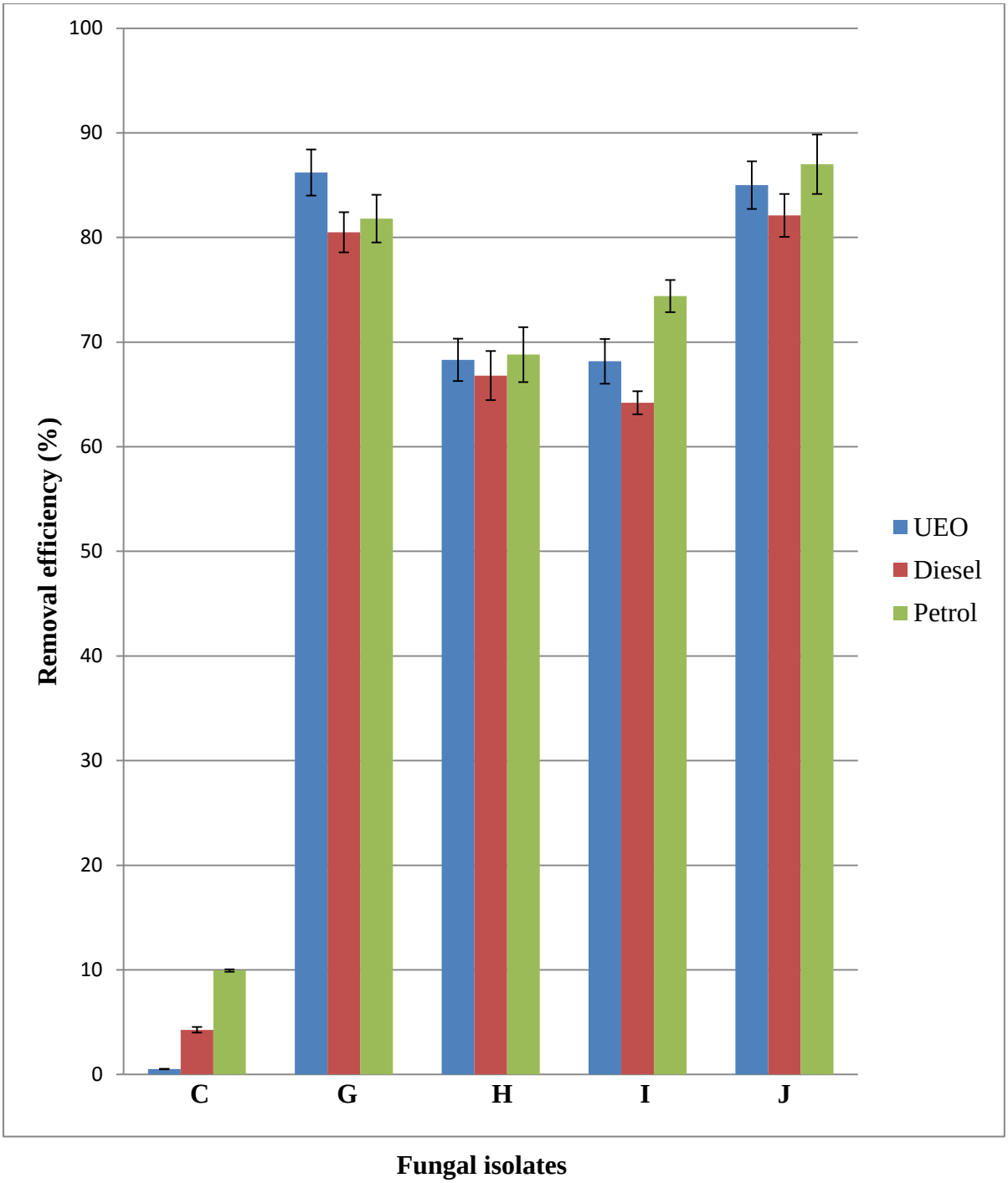

Fig. 4.8: Hydrocarbonoclastic potentials of some fungal isolates in used engine oil, diesel and petrol. C; control, G; *Candida tropicalis*, H; *Rhodosporidium toruloides*, I; *Fusarium oxysporium* and J; *Aspergillus clavatus*. Bars indicate the average of triplicate samples while the error bars show the standard deviation.

4.7 Assay for Biosurfactant Activity of the Best Isolates

The biosurfactant activity of the two best isolates (*Candida tropicalis* and *Aspergillus clavatus*) revealed that the isolates exhibited higher emulsification index (E_{24}) in petrol, when compared to their E_{24} in used engine oil and diesel (Fig. 4.9). However, highest E_{24} was achieved by *A. clavatus* in petrol.

In oil spreading technique, *Aspergillus clavatus* exhibited the highest oil displacement area (ODA) in petrol, followed by diesel and the least ODA was recorded in used engine oil. Similarly, *C. tropicalis* recorded the highest ODA in petrol, followed by used engine oil, and the least ODA was recorded in diesel (Fig. 4.10).

The microbial adhesion to the hydrocarbon (MATH) revealed that in the presence of petrol, *Candida tropicalis* and *Aspergillus clavatus* exhibited higher cell surface hydrophobicity than in the presence of used engine oil and diesel. However, highest cell surface hydrophobicity was achieved by *C. tropicalis* in petrol (Fig. 4.11)

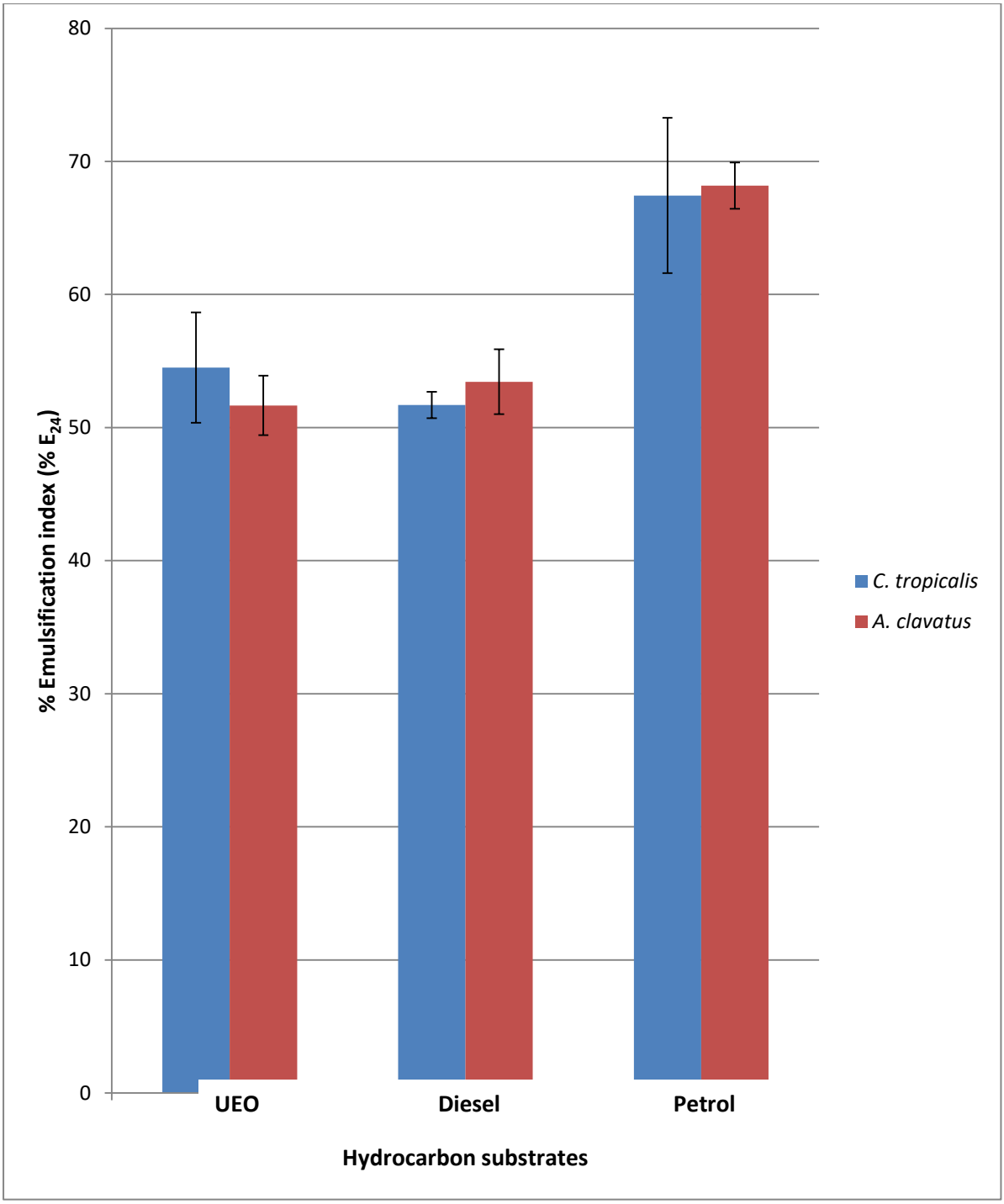

Fig. 4.9: Emulsification index (% E_{24}) of the isolates in UEO, diesel and petrol.

Bars indicate the average of triplicate samples while the error bars show the Standard Deviation.

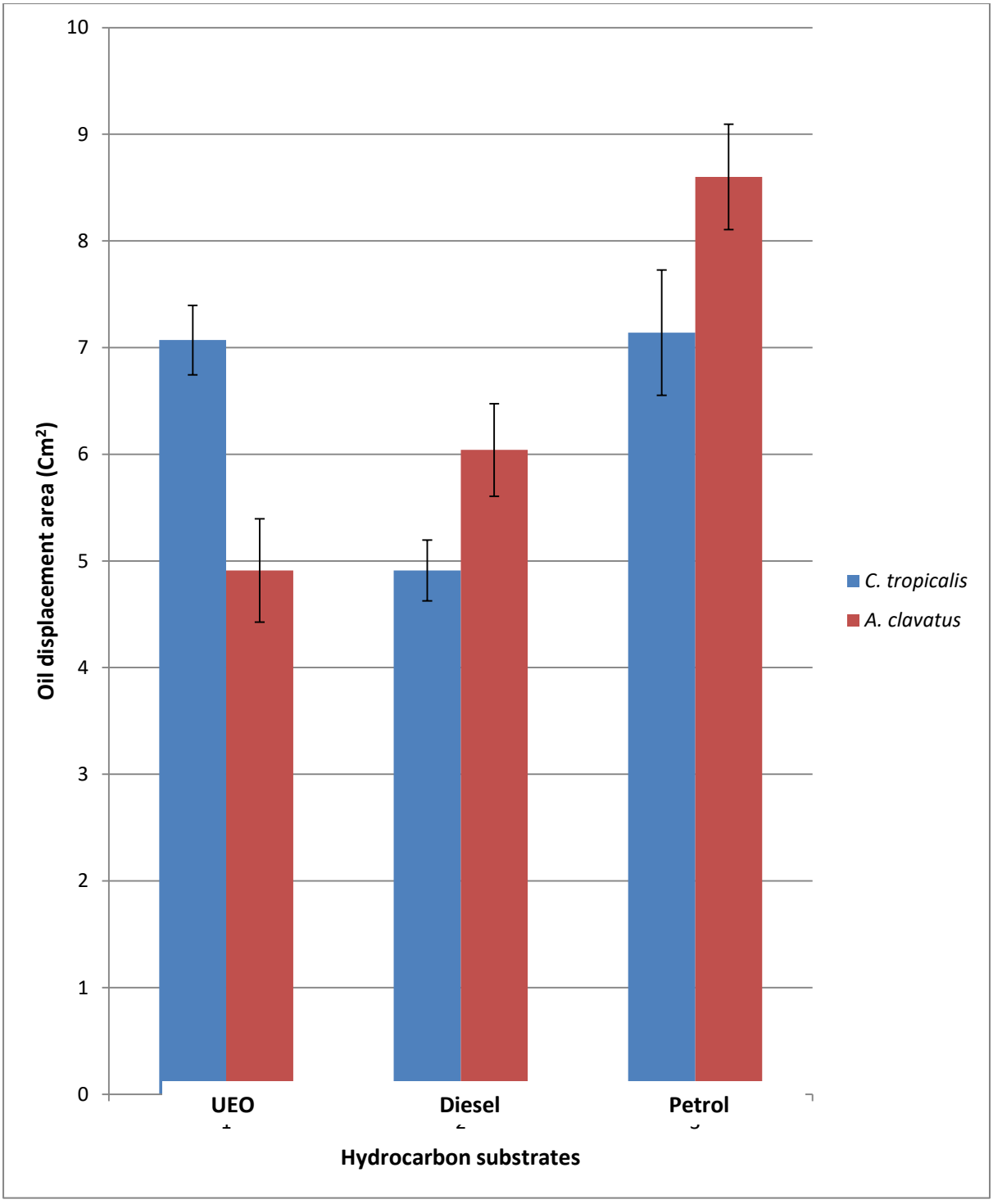

Fig. 4.10: Oil displacement area (ODA cm^2) of the isolates in UEO, diesel and petrol.

Bars indicate the average of triplicate samples while the error bars show the Standard Deviation.

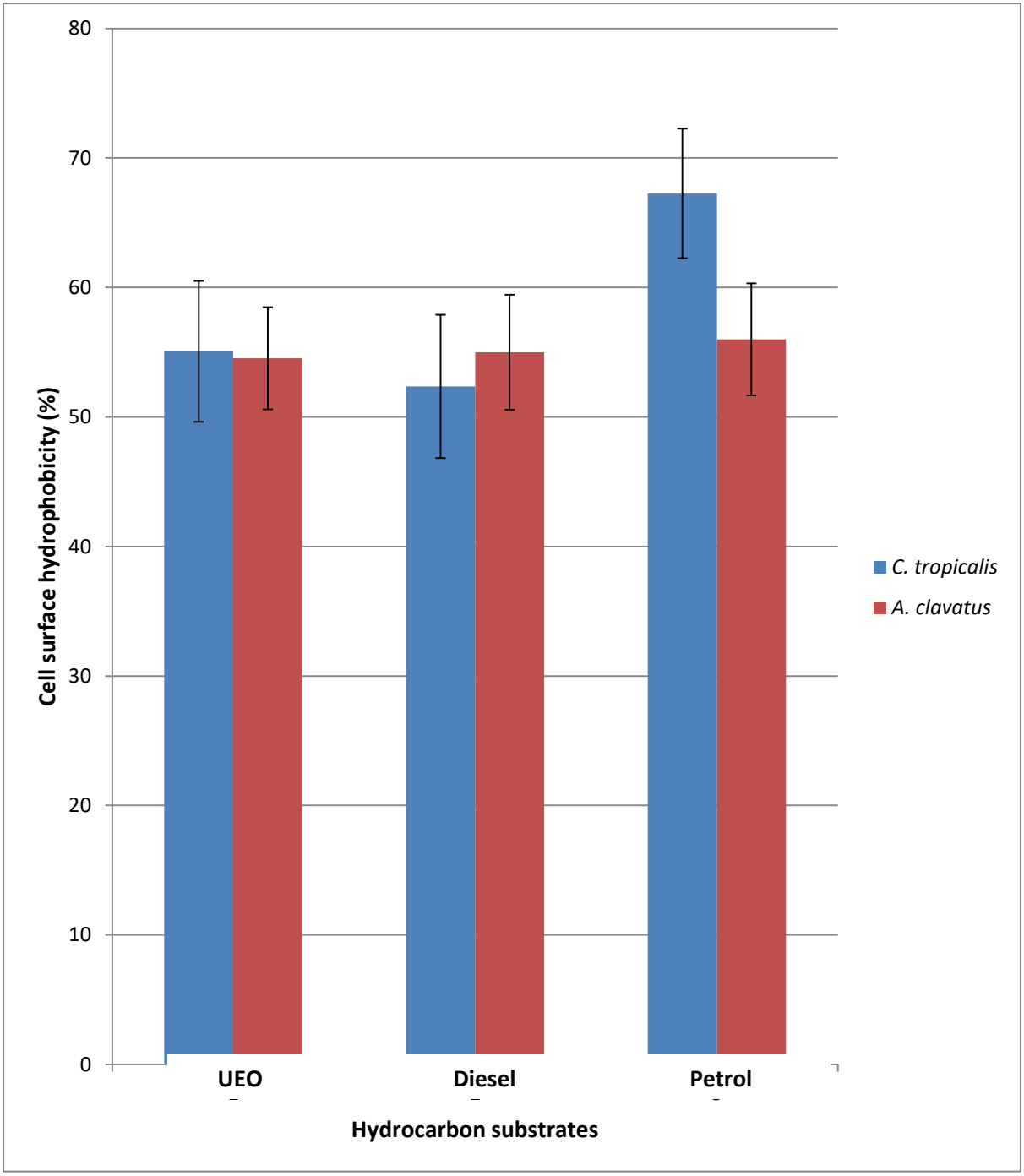

Fig. 4.11: Microbial adhesion to the hydrocarbon (MATH) assay of the isolates in UEO, diesel and petrol.

Bars indicate the average of triplicate samples while the error bars show the Standard Deviation.

4.8 Assay for Enzyme Activity

The results for the assay of extracellular enzymes (catalase, lipase and peroxidase) activity of the isolates (*Candida tropicalis* and *Aspergillus clavatus*) in used engine oil, diesel and petrol are presented in Tables 4.5 and 4.6. The isolates showed significant activity of catalase, lipase and peroxidase in the hydrocarbon substrates. Analysis of variance (ANOVA) indicates significant difference ($P < 0.05$) between the catalase, lipase and peroxidase activity of the isolates *C. tropicalis* and *A. clavatus* in the hydrocarbon substrates (Tables 4.5 and 4.6). However, the peroxidase activity of the isolates was higher when compared to the catalase and lipase activity in the hydrocarbon substrates (Tables 4.5 and 4.6). Moreover, Duncan multiple range test and least significant difference (LSD) revealed that no significant difference exists between the catalase and lipase activity of the isolate *C. tropicalis* in petrol as well as diesel (Table 4.5).

Table 4.5: Enzyme activity (Uml^{-1}) of *C. tropicalis* in used engine oil, diesel and petrol

Hydrocarbon Substrates	Catalase	Lipase	Peroxidase	F-value	P-value
Used engine oil	1.05 ± 0.036^{a}	2.96 ± 0.65^{a}	17.59 ± 1.38^{a}	317.979	0.000
Diesel	0.89 ± 0.02^{b}	1.18 ± 0.36^{b}	37.35 ± 0.70^{a}	6439.660	0.000
Petrol	2.50 ± 0.044^{b}	3.17 ± 0.89^{b}	36.81 ± 1.25^{a}	1467.200	0.000

Values are mean of three replicates ± SD

Comparism of means along the rows; values followed by letter 'a' indicate significant difference at $P < 0.05$, while values followed by letter 'b' are not different significantly at $P < 0.05$ level.

Table 4.6: Enzyme activity (Uml^{-1}) of *A. clavatus* in used engine oil, diesel and petrol

Hydrocarbon Substrates	Catalase	Lipase	Peroxidase	F-value	P-value
Used engine oil	9.68±1.48[a]	1.88±0.87[a]	15.83±1.43[a]	87.865	0.000
Diesel	4.50±0.36[a]	0.81±0.36[a]	30.86±1.07[a]	1723.495	0.000
Petrol	18.90±0.66[a]	11.66±1.27[a]	36.27±1.78[a]	276.925	0.000

Values are mean of three replicates ± SD

Comparism of means along the rows; values followed by letter 'a' indicate significant difference at $P < 0.05$ level.

4.9 Biodegradation of PAH components of the hydrocarbons by the isolates

The Gas Chromatographic analysis for the removal of the PAHs in used engine oil by the two best isolates: *Candida tropicalis* and *Aspergillus clavatus*, as well as the mixed culture of the organisms are presented in Table 4.7. Most of the PAH components of used engine oil were completely removed by the single and mixed culture of the isolates. *Aspergillus clavatus* and the mixed culture achieved 100% depletion of the PAH components: phenanthrene, fluoranthene, pyrene and benzo (k) fluoranthene, while *Candida tropicalis* recorded 100% depletion of phenanthrene and benzo (k) fluoranthene, 96.76% depletion of fluoranthene and 99.27% depletion of pyrene. However, *Candida tropicalis*, *Aspergillus clavatus* and the mixed culture achieved 100%, 90.28% and 96.38% removal of dibenzyl (a,h) anthracene respectively, as well as 94.09%, 71.82% and 87.09% removal of benzo (a) pyrene, respectively (Table 4.7).

Table 4.8 shows the Gas Chromatographic analysis for the removal of PAHs in diesel by *Candida tropicalis* and *Aspergillus clavatus* as well as their mixed culture. *Candida tropicalis* achieved 100% removal of the components: acenaphthene, acenaphthylene, phenanthrene, 1,2- benzanthracene, chrysene, benzo (k) fluoranthene, anthracene, naphthalene and fluorine, while benzo (a) pyrene and fluoranthene recorded 99.96% and 97.88% removal, respectively by *Candida tropicalis* in 16 days. However, *Aspergillus clavatus* and the mixed culture achieved 100% removal of acenaphthene, acenaphthylene, phenanthrene, benzo (k) fluoranthene, anthracene and naphthalene. Also, *Aspergillus clavatus* and the mixed culture, respectively recorded 98.71% and 80.75% removal of 1,2-benzanthracene, 100% and 92.74% removal of chrysene, 78.0% and 75.02% removal of benzo (a) pyrene, 95.12% and 89.94% removal of fluoranthene, 78.07% and 100% depletion of fluorene in 16 days (Table 4.8).

Table 4.9 indicates that most of the PAH components found in petrol were also completely removed by both the single and mixed culture of the two best isolates: *Candida tropicalis* and *Aspergillus clavatus*. *Candida tropicalis*, *Aspergillus clavatus* and the mixed culture achieved 100% depletion of benzo (k) fluoranthene, anthracene and naphthalene in 16 days. *Candida tropicalis* and *Aspergillus clavatus* achieved 100% depletion of fluoranthene, while the mixed culture recorded 87.89% removal of fluoranthene. However, *Candida tropicalis* and the mixed culture achieved 100% removal of fluorine, while *Aspergillus clavatus* recorded 76.88% removal of fluorine over a 16 day period. Moreover, *Candida tropicalis* and *Aspergillus clavatus* recorded 80.22% and 79.76% removal of benzo (a) pyrene respectively, while the mixed culture achieved 99.88% removal of benzo (a) pyrene in 16 days (Table 4.9). The chromatograms for the gas chromatographic analysis are presented (Appendix VIII).

Table 4.7: Degradation of PAHs in used engine oil by the isolates after16 days

PAHs component	Amount of PAHs in control flask (mg/ml)	Percentage (%) removal of PAHs in UEO		
		C. tropicalis	*A. clavatus*	Mixed culture
Phenanthrene	0.2651	100	100	100
Fluoranthene	0.1636	96.76	100	100
Pyrene	1.4344	99.27	100	100
Benzo (k) fluoranthene	0.2990	100	100	100
Benzo (a) pyrene	0.2338	94.09	71.82	87.09
Dibenzyl (a,h) anthracene	1.8603	100	90.28	96.38

% removal of PAH = concentration of PAH in undegraded UEO (control) – concentration of PAH in the test sample/concentration of PAH in undegraded UEO (control) X 100.

Table 4.8: Degradation of PAHs in diesel by the isolates after 16 days

PAHs component	Amount of PAHs in control flask (mg/ml)	Percentage (%) removal of PAHs in diesel		
		C. tropicalis	*A. clavatus*	Mixed culture
Acenaphthene	2.0038	100	100	100
Acenaphthylene	1.4797	100	100	100
Phenanthrene	0.0318	100	100	100
Fluoranthene	0.322	97.88	95.12	89.94
1,2 Benzanthracene	1.2432	100	98.71	80.75
Chrysene	0.8739	100	100	92.74
Benzo (K) fluoranthene	0.6331	100	100	100
Benzo (a) pyrene	1.5714	99.96	78.0	75.02
Anthracene	0.483	100	100	100
Naphthalene	0.114	100	100	100
Fluorene	0.3694	100	78.07	100

% removal of PAH = concentration of PAH in undegraded UEO (control) – concentration of PAH in the test sample/concentration of PAH in undegraded UEO (control) X 100.

Table 4.9: Degradation of PAHs in petrol by the isolates after 16 days

PAHs component	Amount of PAHs in control flask (mg/ml)	Percentage (%) removal of PAHs in petrol		
		C. tropicalis	*A. clavatus*	Mixed culture
Anthracene	0.0932	100	100	100
Fluoranthene	0.0112	100	100	87.89
Naphthalene	0.0543	100	100	100
Benzo (K) fluoranthene	0.0404	100	100	100
Benzo (a) pyrene	0.6040	80.22	79.76	99.88
Fluorene	0.0783	100	76.88	100

% removal of PAH = concentration of PAH in undegraded UEO (control) – concentration of PAH in the test sample/concentration of PAH in undegraded UEO (control) X 100.

4.10 Effects of varying concentrations of heavy metals on UEO degradation at different pH levels

The effects of varying concentrations of zinc on used engine oil degradation are presented in Figures 4.12 to 4.17. In the presence of 0.1 mg/l zinc, there was stimulation of used engine oil degradation in the media containing *C. tropicalis*, at all the pH levels. Higher stimulation was recorded at pH 5.5 when compared to pH 4.5 and 6.5. However, an increasing trend in inhibition of used engine oil degradation was recorded as the zinc concentration increased from 1.0 to 100 mg/l at all the pH levels (Fig. 4.12). Similarly, the optical density (OD) of the isolate *C. tropicalis* was higher in the presence of 0.1 mg/l zinc at all the pH levels, when compared to the control (Fig. 4.13). However, there was a decreasing trend in optical density of *C. tropicalis*, as the zinc concentration increased from 1.0 to 100 mg/l at all the pH levels (Fig. 4.13).

In the media containing *A. clavatus*, there was also stimulation of used engine oil degradation in the presence of 0.1 mg/l zinc at all the pH levels; however, the stimulation was more evident at pH 5.5 when compared to pH 4.5 and 6.5. An increasing trend in inhibition of used engine oil degradation was however observed as the zinc concentration increased from 1.0 to 100 mg/l at all the pH levels (Fig. 4.14). Similarly, the OD of *A. clavatus* was higher in the presence of 0.1 mg/l zinc at all the pH levels, when compared to the control. A decreasing trend in OD was however observed when the zinc concentration increased from 1.0 to 100mg/l within the experimental period (Fig. 4.15).

In the presence of 0.1 to 10 mg/l zinc, there was stimulation in used engine oil degradation in the media containing the mixed culture of the isolates; *C. tropicalis* and *A. clavatus*, at pH 5.5. There was also stimulation in used engine oil degradation in the presence of 0.1 to 1.0

mg/l zinc at pH 4.5 and 6.5. However, stimulation was maximum at pH 5.5 in the presence of 0.1 mg/l zinc (Fig. 4.16). The OD was higher in the presence of 0.1 to 10 mg/l zinc at pH 5.5, when compared to the control. The OD was also higher at pH 4.5 and 6.5, in the presence of 0.1 to 1.0 mg/l zinc, when compared to the control. However, highest OD was achieved in the presence of 0.1 mg/l zinc at pH 5.5. Moreover, a decreasing trend in OD was observed as the zinc concentration increased from 10 to 100 mg/l at 4.5 and 6.5 pH levels (Fig. 4.17).

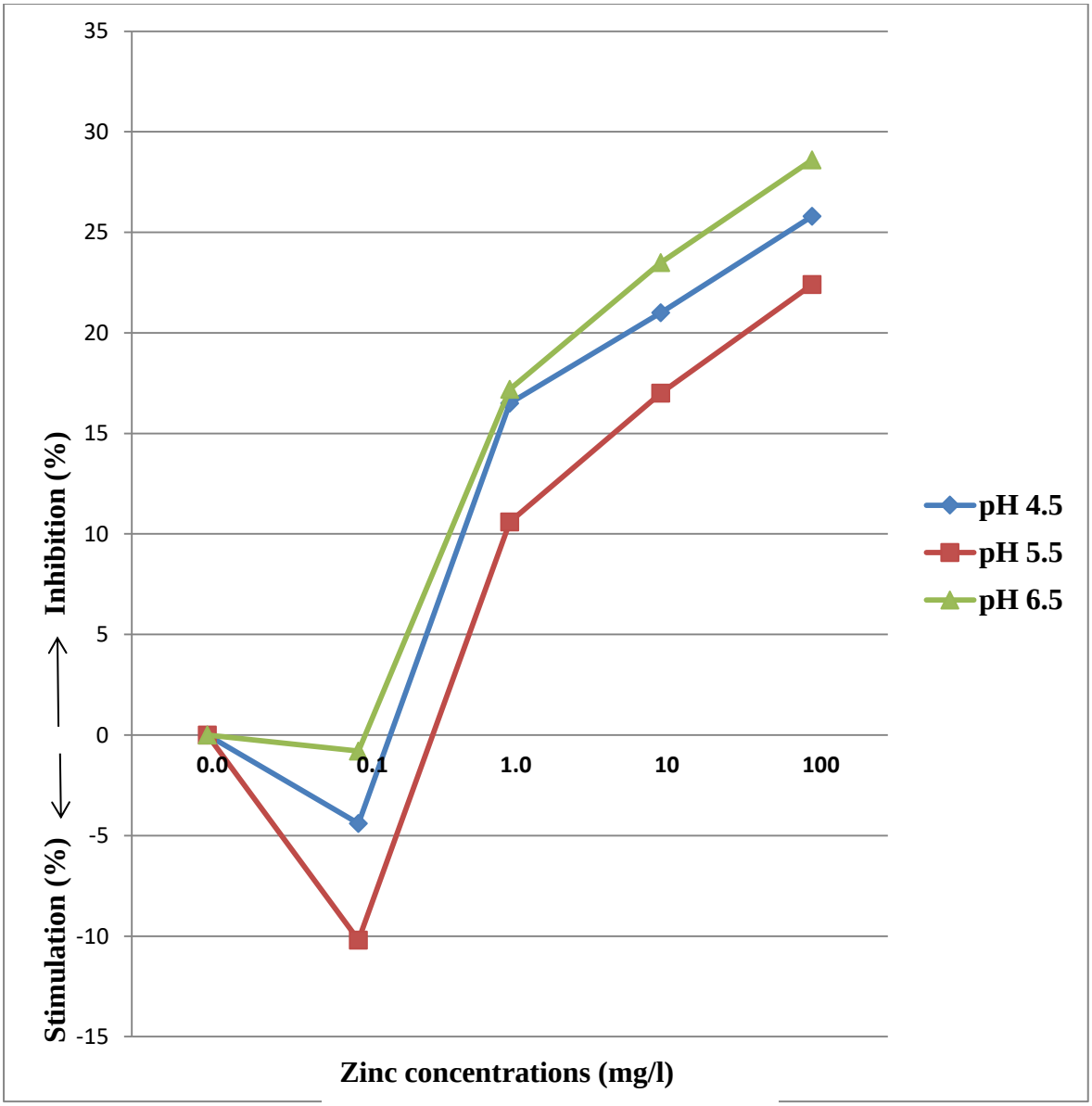

Fig. 4.12: Effect of varying concentrations of zinc on UEO degradation by *C. tropicalis* at different pH.

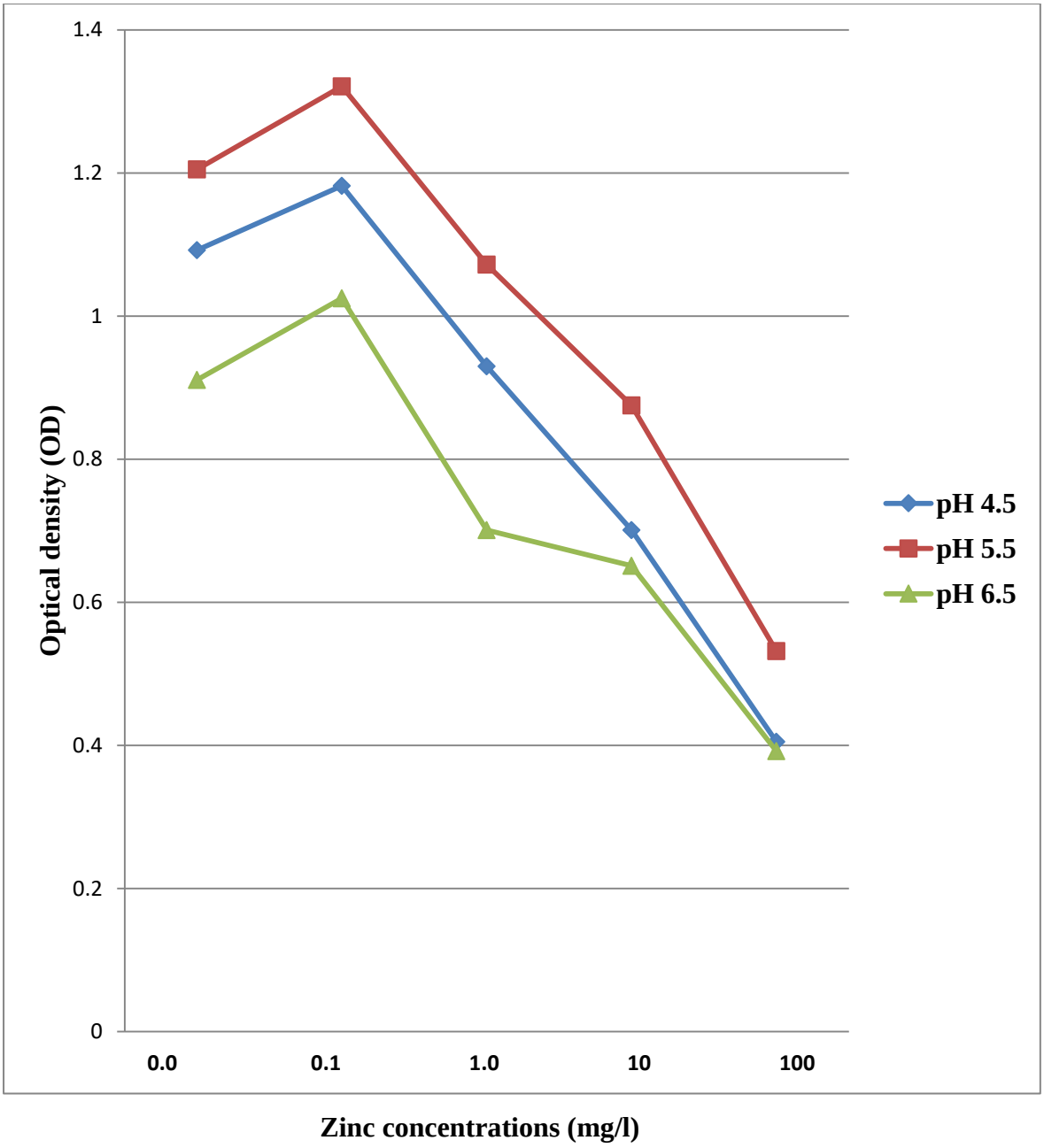

Fig. 4.13: Growth response of *C. tropicalis* during utilization of UEO in the presence of varying concentrations of zinc at different pH.

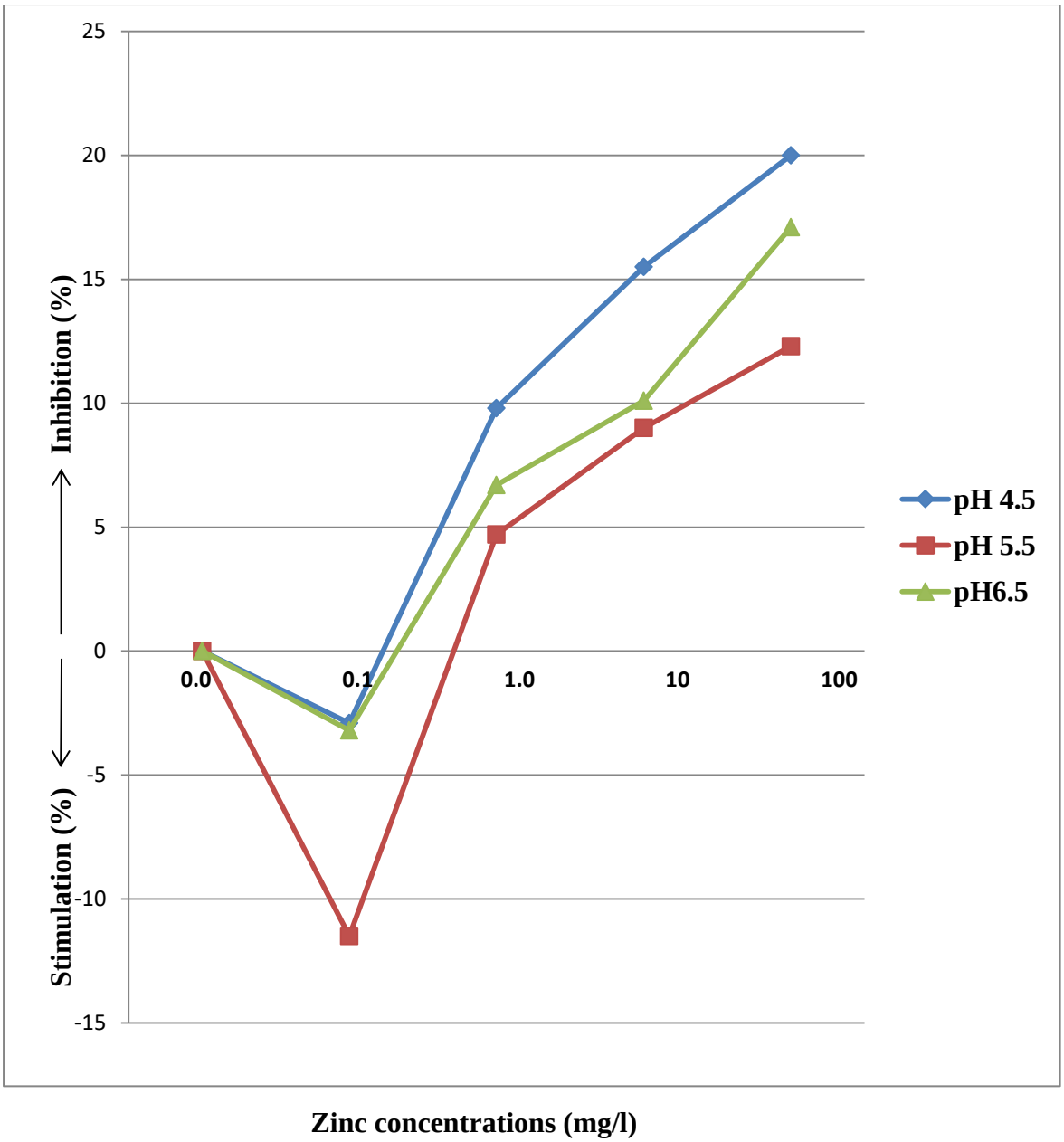

Fig. 4.14: Effect of varying concentrations of zinc on UEO degradation by *A clavatus* at different pH.

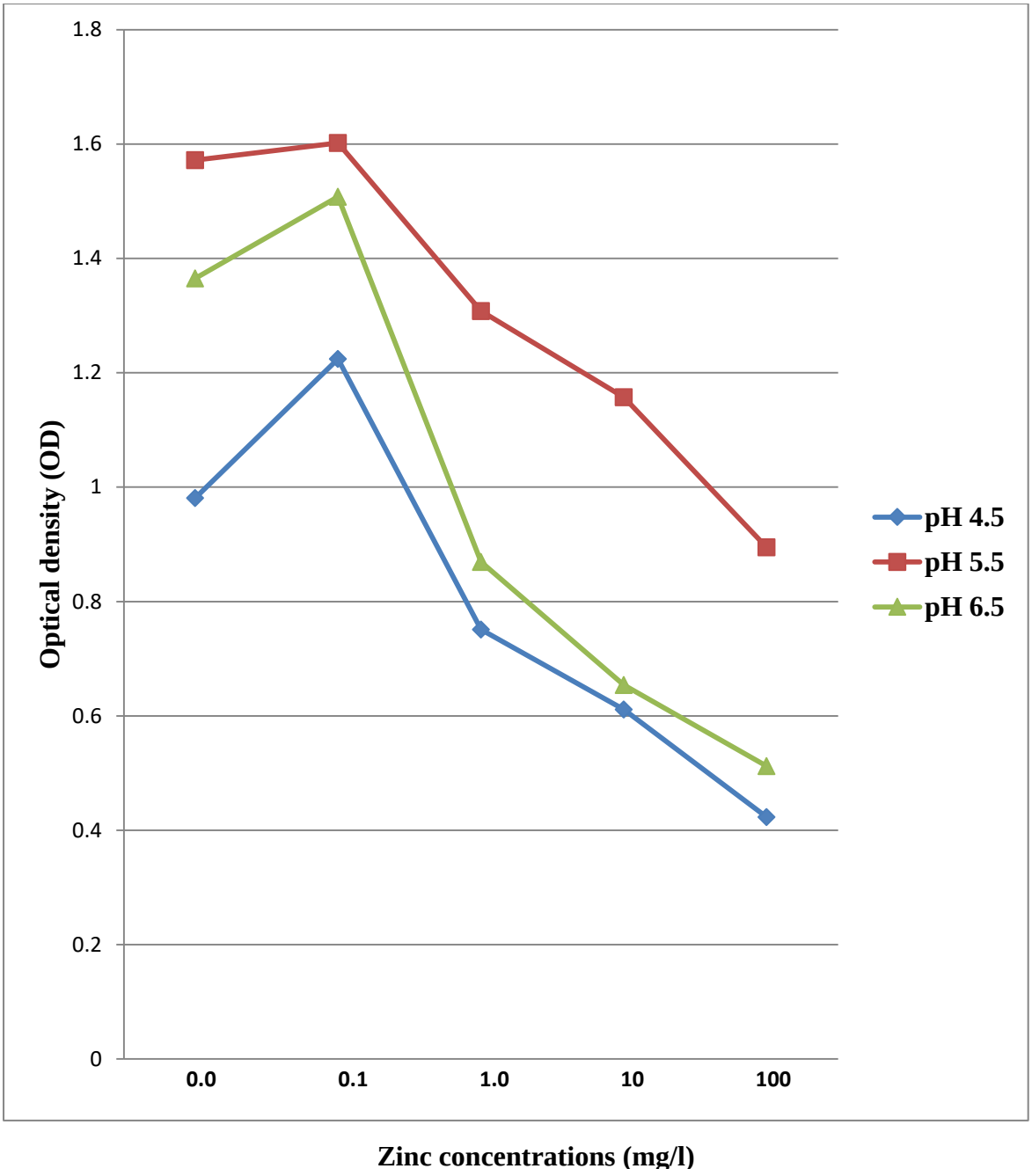

Fig. 4.15: Growth response of *A. clavatus* during utilization of UEO in the presence of varying concentrations of zinc at different pH.

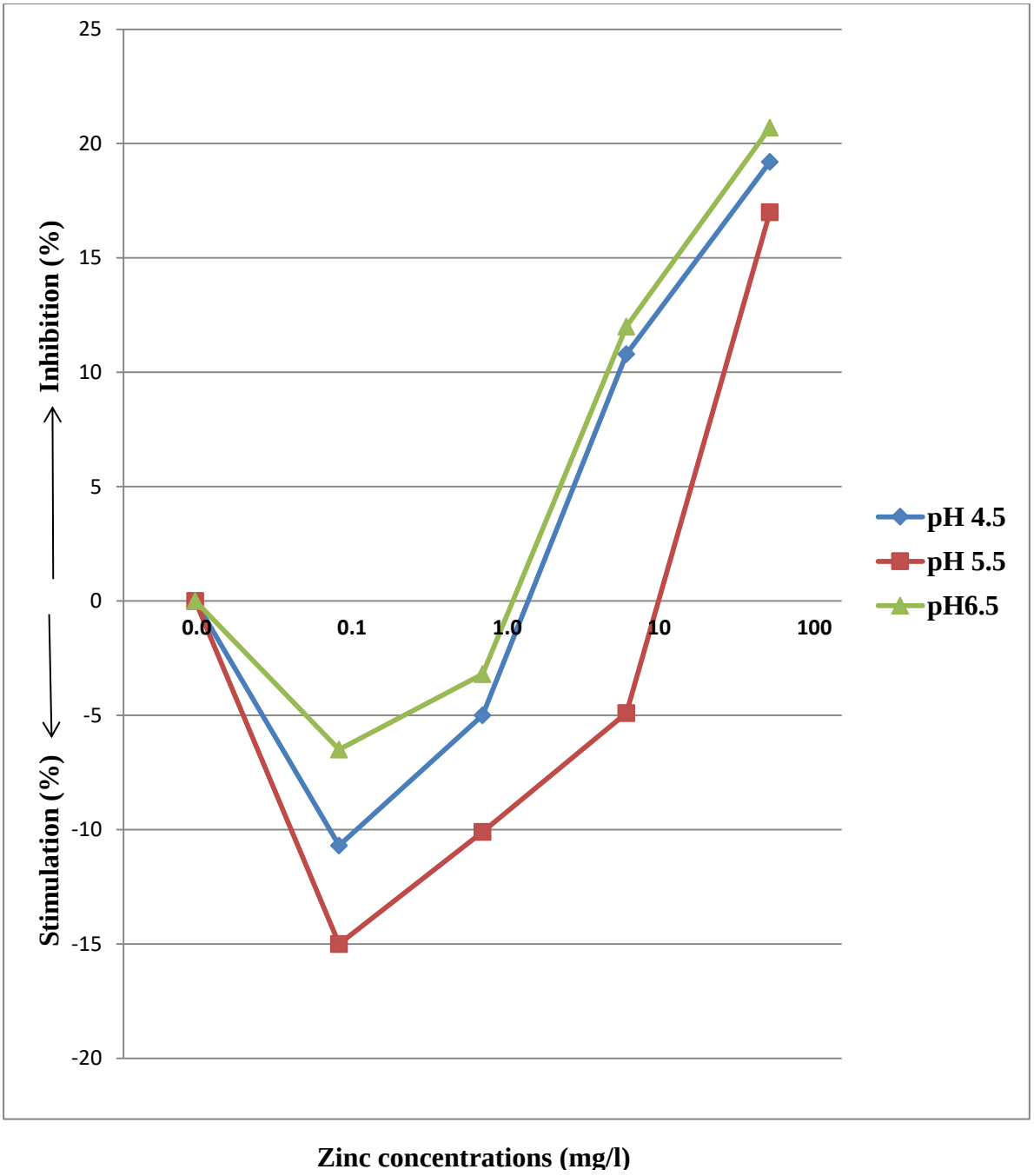

Fig. 4.16: Effect of varying concentrations of zinc on UEO degradation by the mixed culture at different pH.

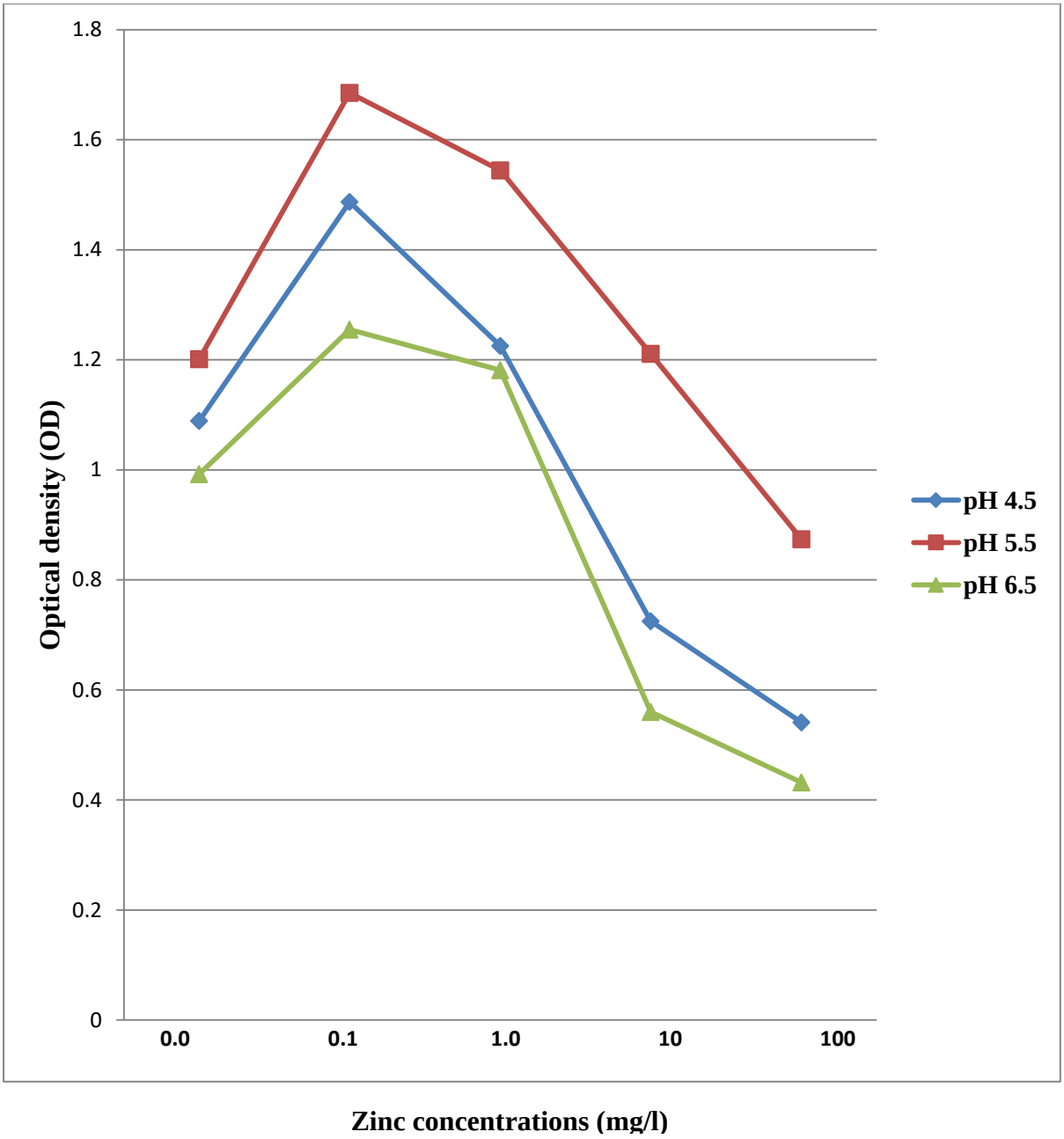

Fig. 4.17: Growth response of the mixed culture during utilization of UEO in the presence of varying concentrations of zinc at different pH.

The effects of varying concentrations of lead on used engine oil degradation at different pH levels are presented in Figures 4.18 to 4.23. In the presence of 0.1 mg/l Pb, there was stimulation in used engine oil degradation at pH 5.5, in the media containing *C. tropicalis*. At pH 4.5 and 6.5, an increasing trend in inhibition of used engine oil degradation was observed as the Pb concentration increased from 0.1 to 100 mg/l (Fig. 4.18). Similarly, the OD was higher in the media containing 0.1 mg/l Pb at pH 5.5, when compared to the control. A decreasing trend in OD was observed as the Pb concentration increased from 0.1 to 100 mg/l at pH 4.5 and 6.5 (Fig. 4.19).

In the media containing *A. clavatus*, stimulation in used engine oil degradation was observed in the presence of 0.1 to 1.0 mg/l Pb at pH 5.5. At pH 4.5 and 6.5, there was also stimulation in used engine oil degradation in the presence of 0.1 mg/l Pb, however, the stimulation was more evident at pH 5.5 (Fig. 4.20). Similarly, an increase in OD was observed in the presence of 0.1 to 1.0 mg/l Pb at pH 5.5, while a decreasing trend in OD was observed as the Pb concentration increased from 10 to 100 mg/l (Fig. 4.21). At pH 4.5 and 6.5, an increase in OD was observed in the presence of 0.1 mg/l Pb, when compared to the control, while a decreasing trend in OD was observed as the Pb concentration increased from 1.0 to 100 mg/l (Fig. 4.21).

In the media containing the mixed culture of the isolates; *C. tropicalis* and *A. clavatus*, there was stimulation in used engine oil degradation in the presence of 0.1 mg/l Pb at pH 5.5. However, there was an increasing trend in inhibition of used engine oil degradation as the Pb concentration increased from 1.0 to 100 mg/l. At pH 4.5 and 6.5, an increasing trend in inhibition of used engine oil degradation was observed in the presence of 0.1 to 100 mg/l Pb (Fig. 4.22). Similarly, an increase in OD was observed in the presence of 0.1 mg/l Pb at pH

5.5, when compared to the control, while a decreasing trend in OD was observed as the Pb concentration increased from 1.0 to 100 mg/l (Fig. 4.23). At pH 4.5 and 6.5, a decreasing trend in OD was observed in the presence of 0.1 to 100 mg/l Pb (Fig. 4.23).

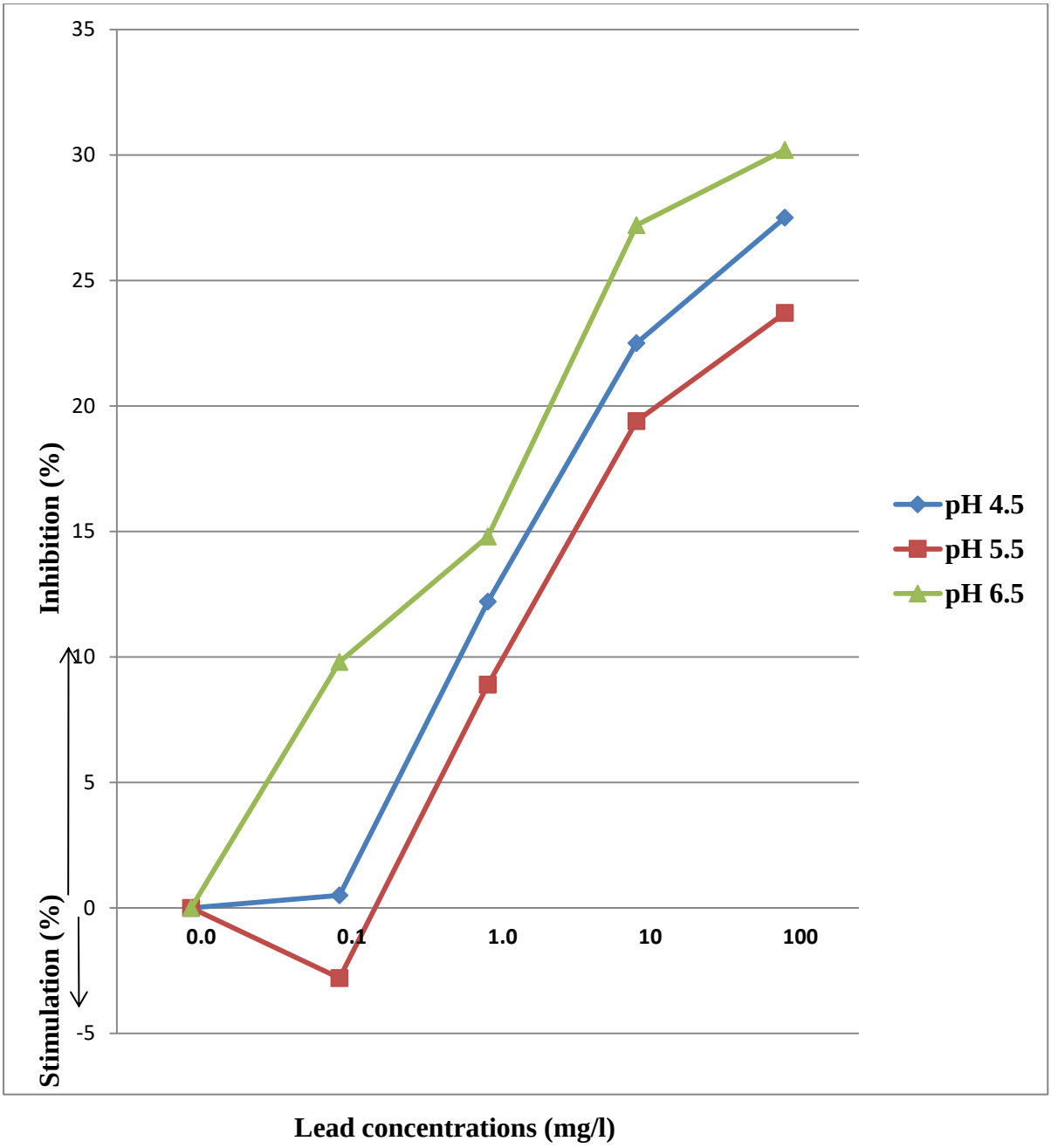

Fig. 4.18: Effect of varying concentrations of lead on UEO degradation by *C. tropicalis* at different pH.

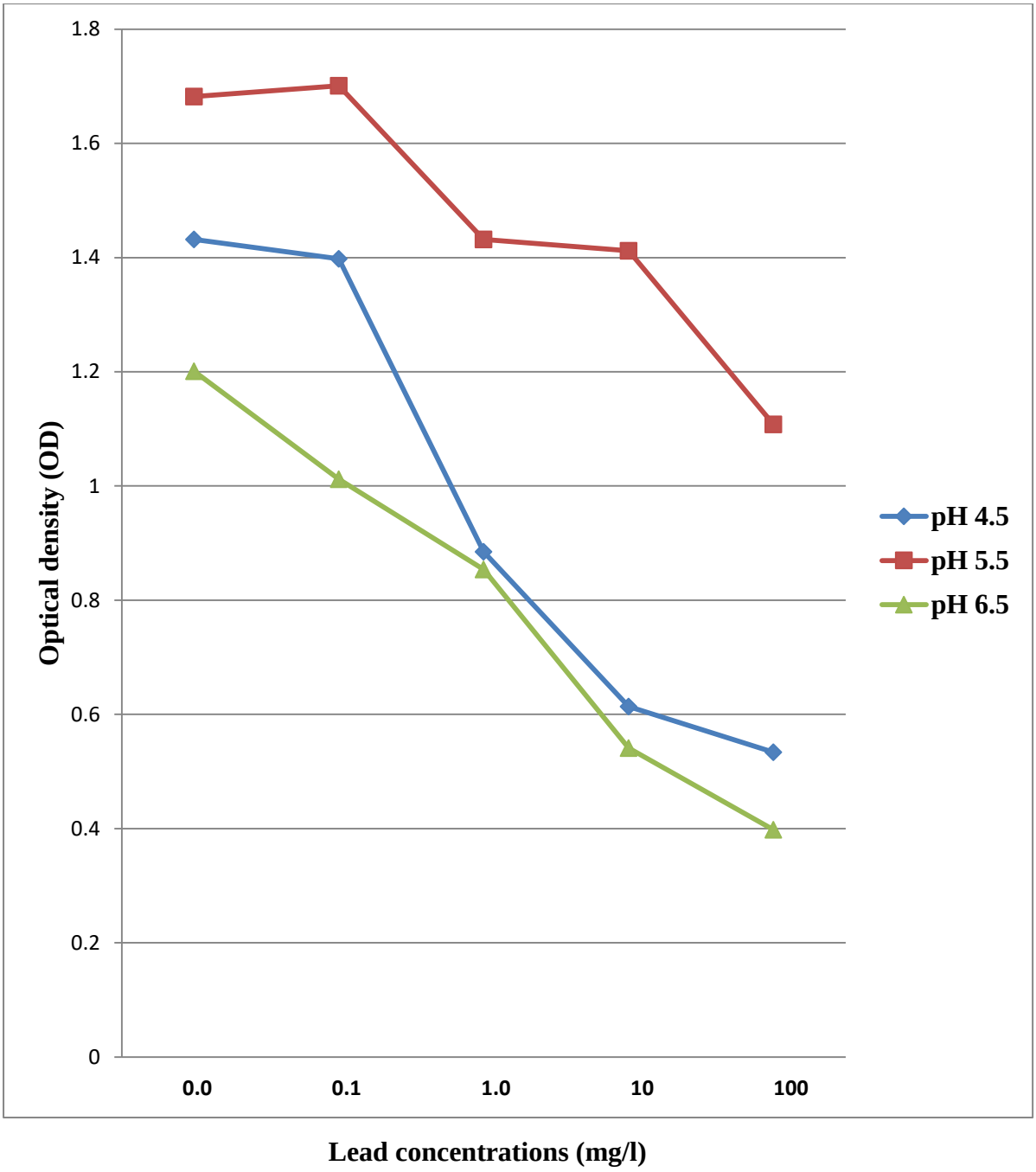

Fig. 4.19: Growth response of *C. tropicalis* during utilization of UEO in the presence of varying concentrations of lead at different pH.

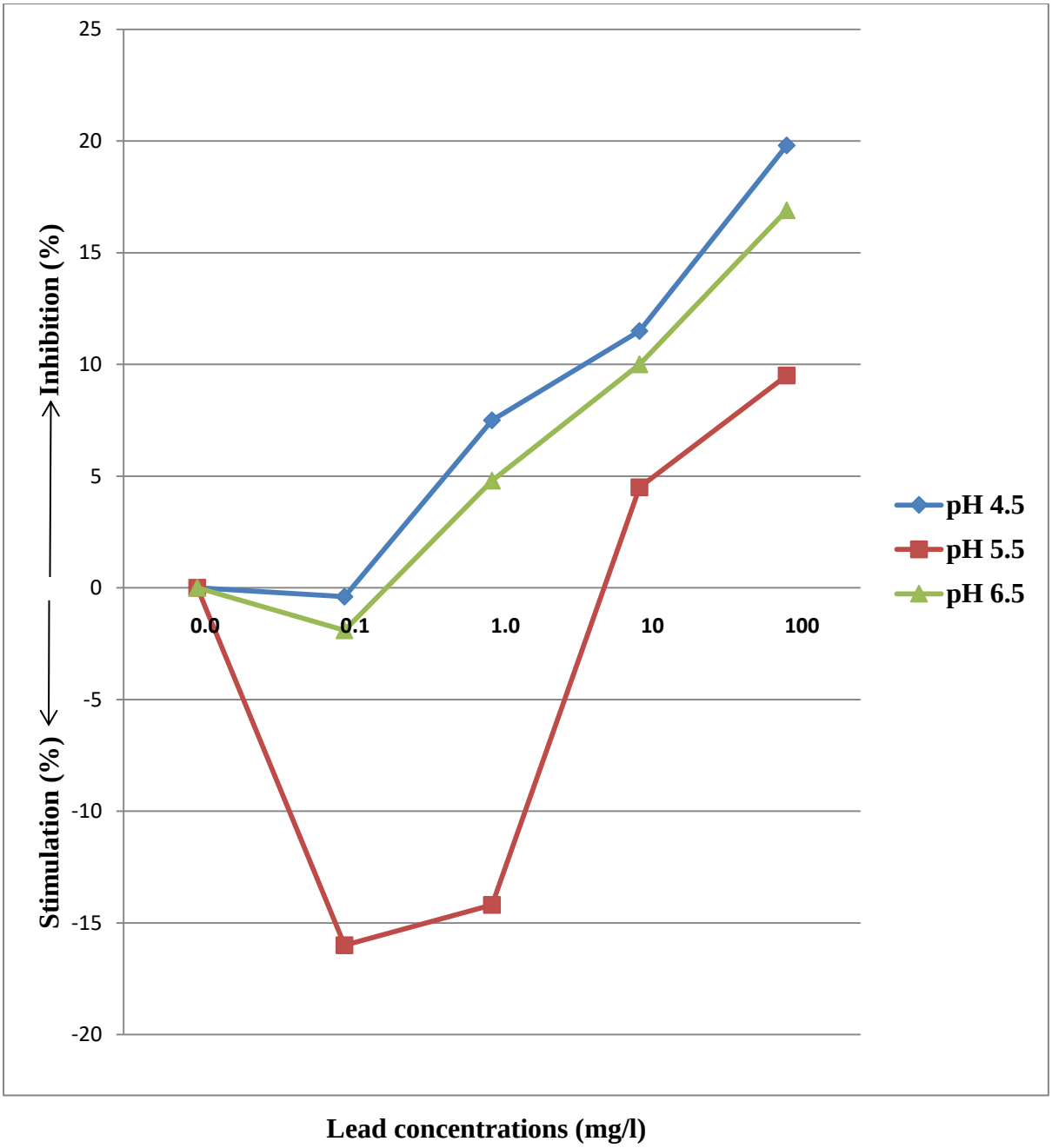

Fig. 4.20: Effect of varying concentrations of lead on UEO degradation by *A. clavatus* at different pH.

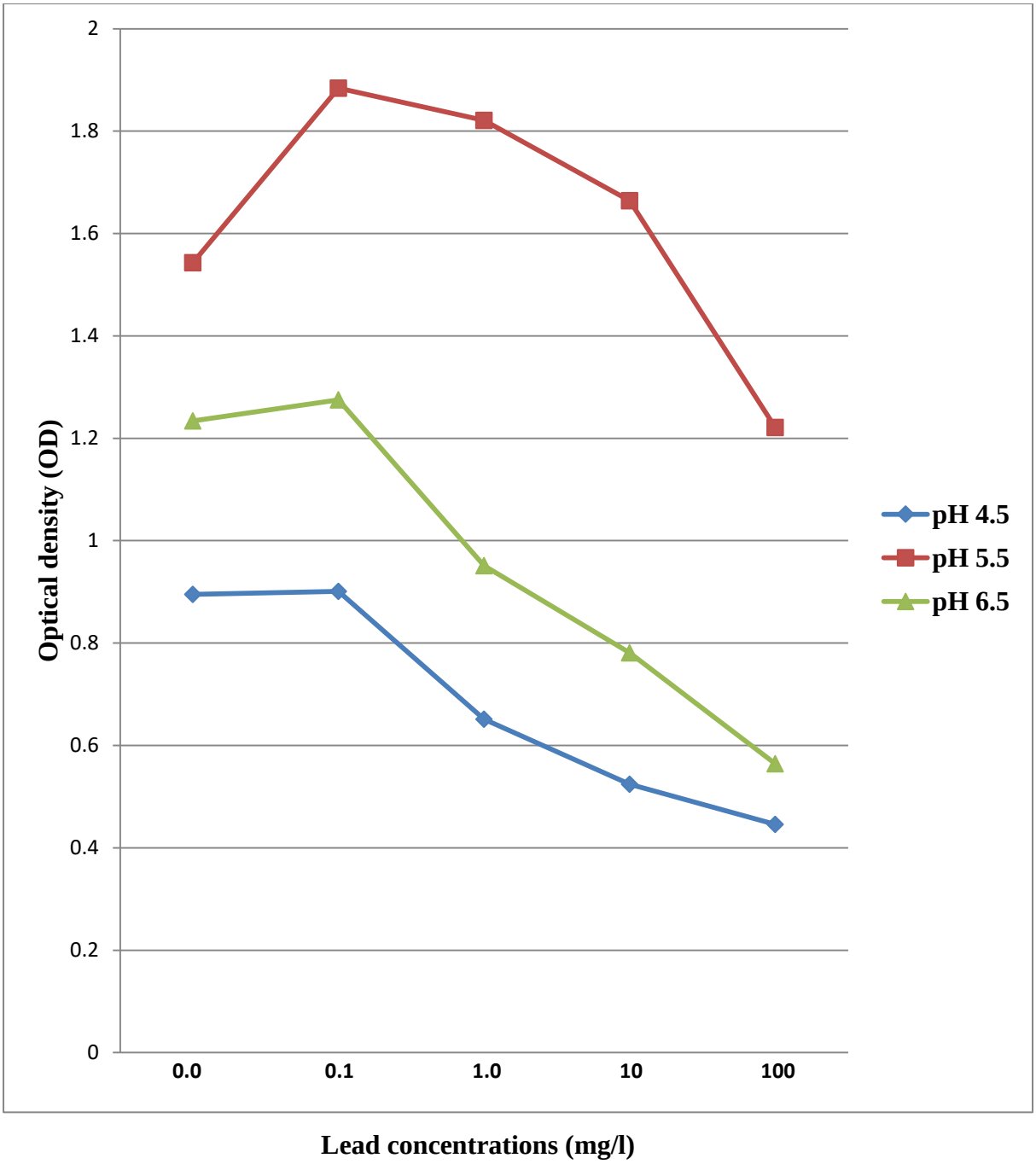

Fig. 4.21: Growth response of *A. clavatus* during utilization of UEO in the presence of varying concentrations of lead at different pH.

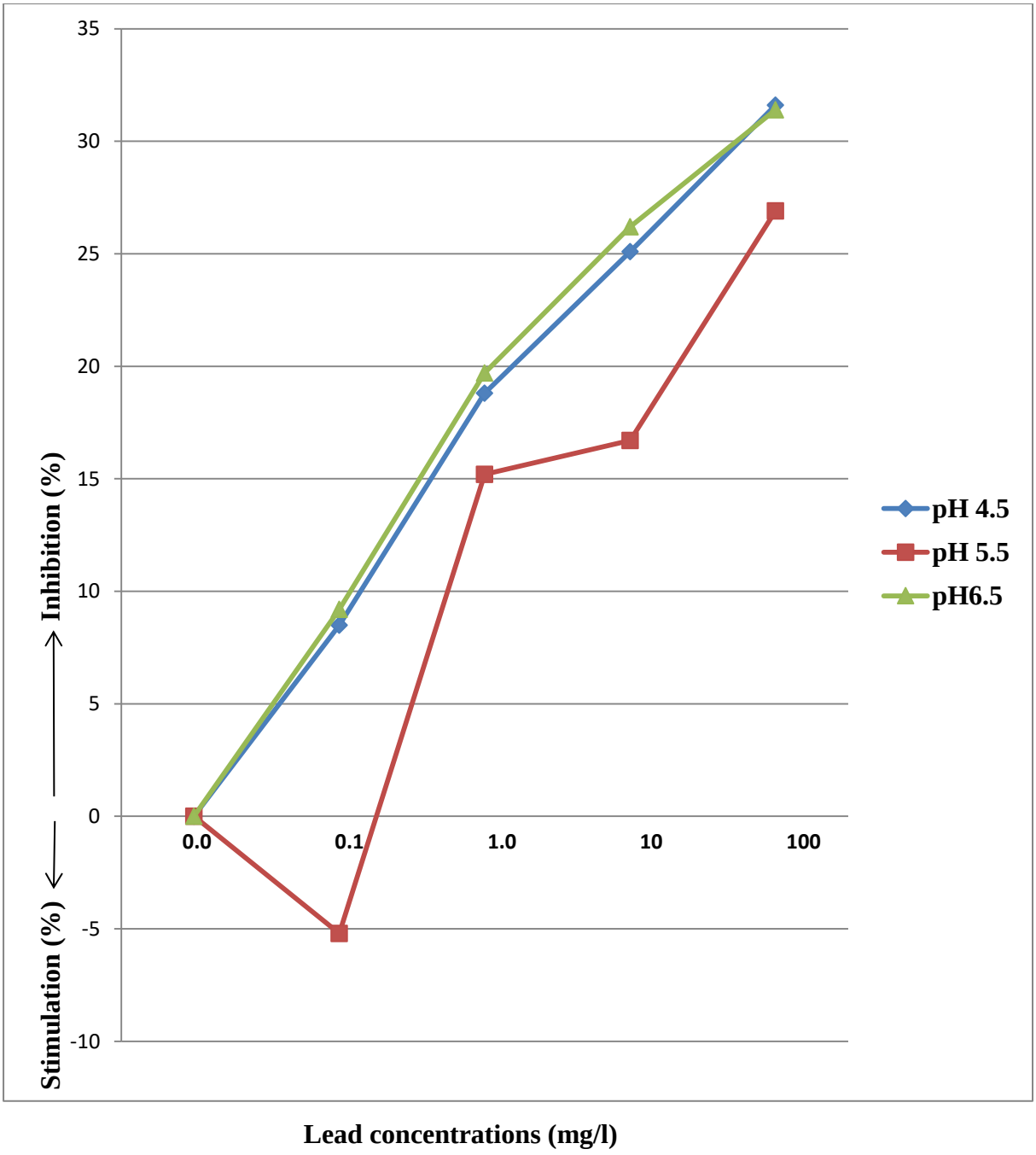

Fig. 4.22: Effect of varying concentrations of lead on UEO degradation by mixed culture at different pH.

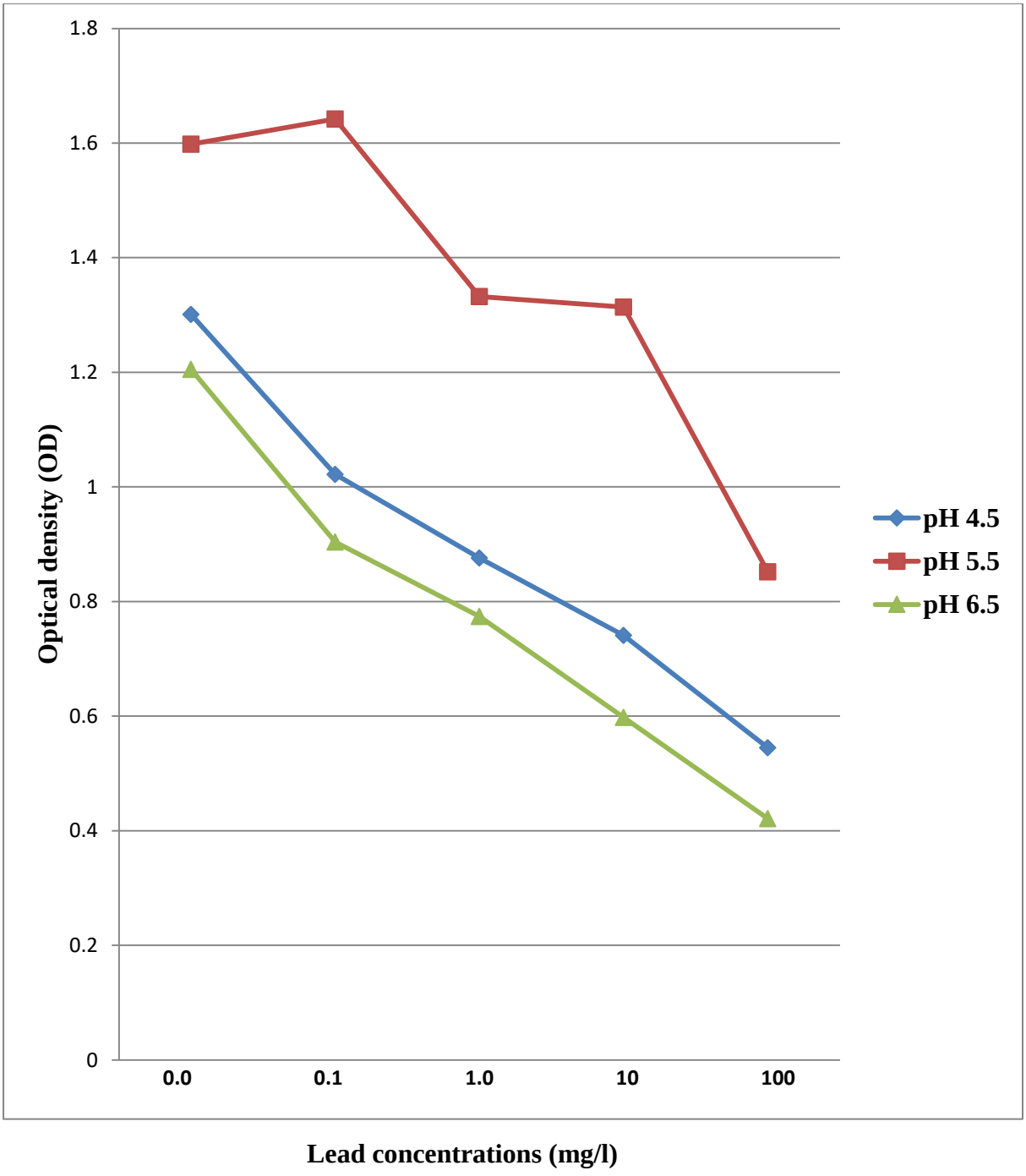

Fig. 4.23: Growth response of the mixed culture during utilization of UEO in the presence of varying concentrations of lead at different pH.

The effects of varying concentrations of cadmium on used engine oil degradation at different pH levels are presented in Figures 4.24 to 4.29. In the media containing *C. tropicalis*, there was stimulation in used engine oil degradation in the presence of 0.1 to 10 mg/l Cd at pH 5.5. However, maximum stimulation was recorded in the presence of 0.1 mg/l cadmium. An increasing trend in inhibition of used engine oil degradation was observed in the presence of 0.1 to 100 mg/l cadmium at pH 4.5 and 6.5 (Fig. 4.24). Similarly, the OD was higher in the presence of 0.1 to 10 mg/l Cd, at pH 5.5, when compared to the control (Fig. 4.25). However, maximum OD was recorded in the presence of 0.1 mg/l Cd at pH 5.5. At pH 4.5 and 6.5, a decreasing trend in OD was observed as the Cd concentration increased from 0.1 to 100 mg/l (Fig. 4.25).

There was also stimulation in used engine oil degradation in the media containing *A. clavatus*, in the presence of 0.1 to 10 mg/l Cd at pH 5.5, and maximum stimulation was achieved in the presence of 0.1 mg/l Cd. At pH 4.5 and 5.5, there was also an increasing trend in inhibition of used engine oil degradation in the presence of 0.1 to 100 mg/l Cd (Fig. 4.26). Similarly, the OD was higher at pH 5.5, in the presence of 0.1 to 10 mg/l Cd, when compared to the control. However, at pH 4.5 and 6.5, there was a decreasing trend in OD as the Cd concentration increased from 0.1 to 100 mg/l (Fig. 4.27).

In the media containing the mixed culture of the isolates, there was stimulation in used engine oil degradation in the presence of 0.1 to 1.0 mg/l Cd at pH 5.5, while an increasing trend in inhibition of used engine oil degradation was observed in the presence of 0.1 to 100 mg/l at pH 4.5 and 6.5 (Fig. 4.28). Similarly, the OD was higher in the presence of 0.1 to 1.0 mg/l Cd when compared to the control, at pH 5.5. Moreover, a decreasing trend in OD was also observed as the Cd concentration increased from 0.1 to 100 mg/l at pH 4.5 and 6.5 (Fig. 4.29).

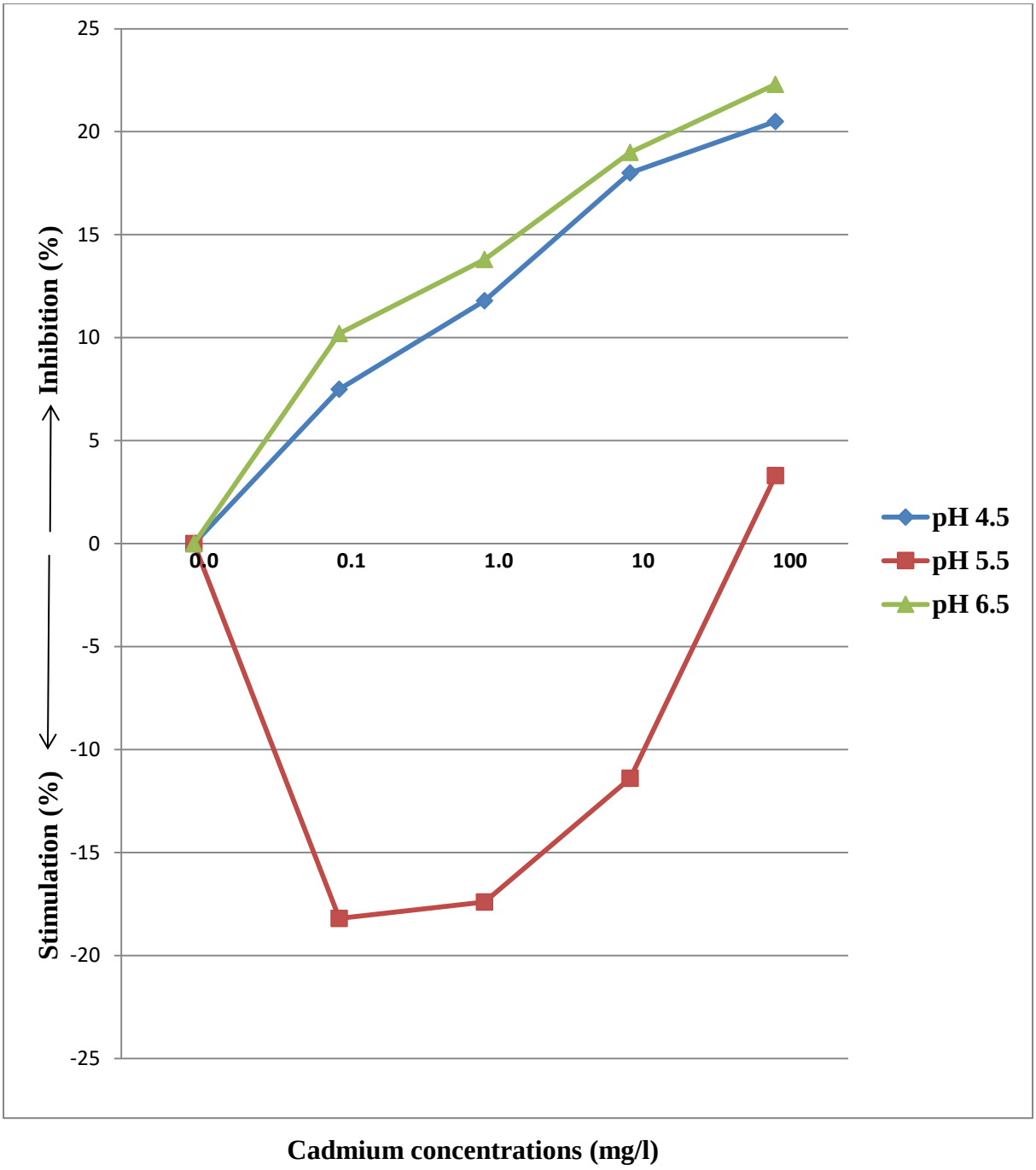

Fig. 4.24: Effect of varying concentrations of cadmium on UEO degradation by *C. tropicalis* at different pH.

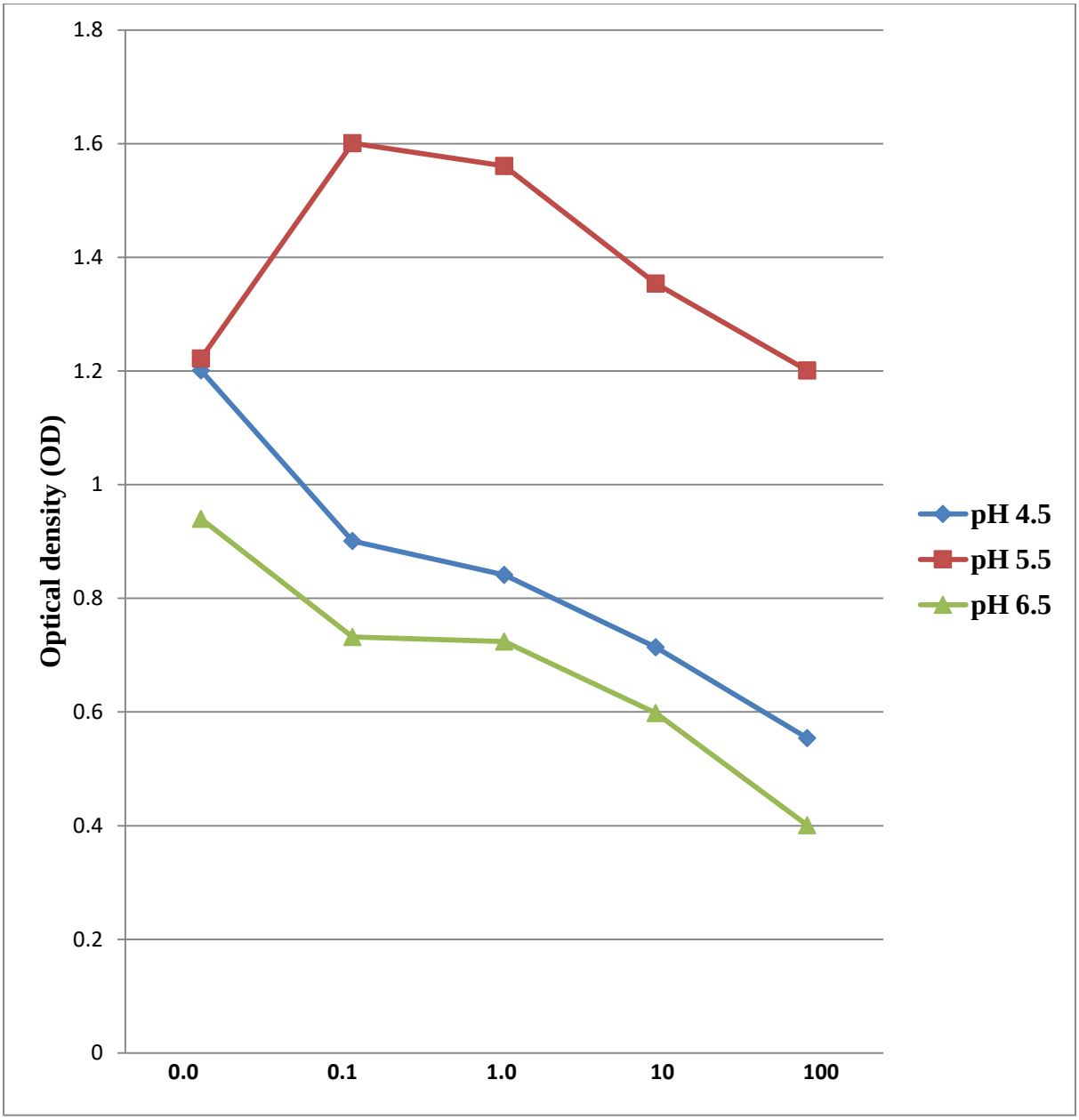

Fig. 4.25: Growth response of *C. tropicalis* during utilization of UEO in the presence of varying concentrations of cadmium at different pH.

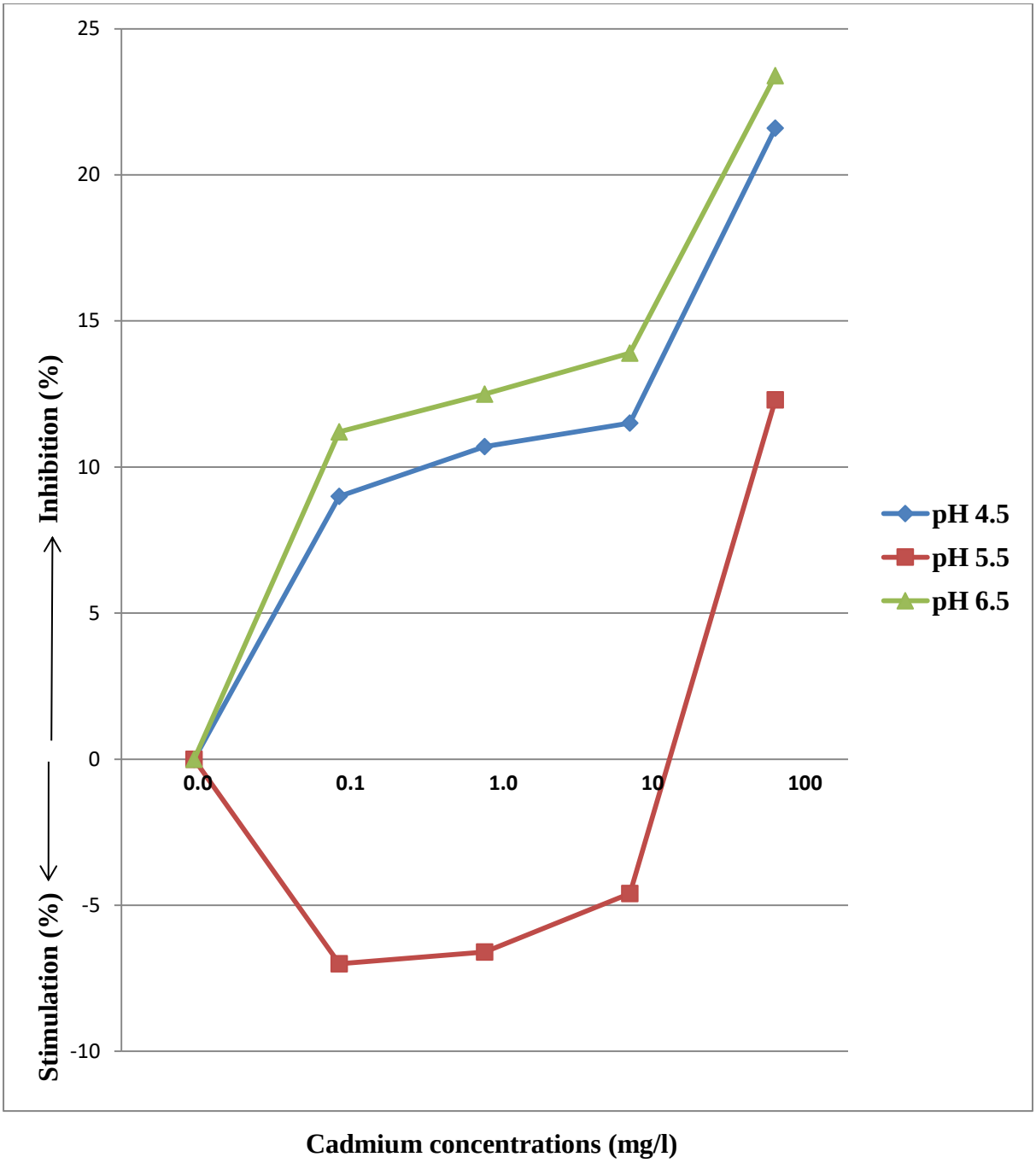

Fig. 4.26: Effect of varying concentrations of cadmium on UEO degradation by *A. clavatus* at different pH.

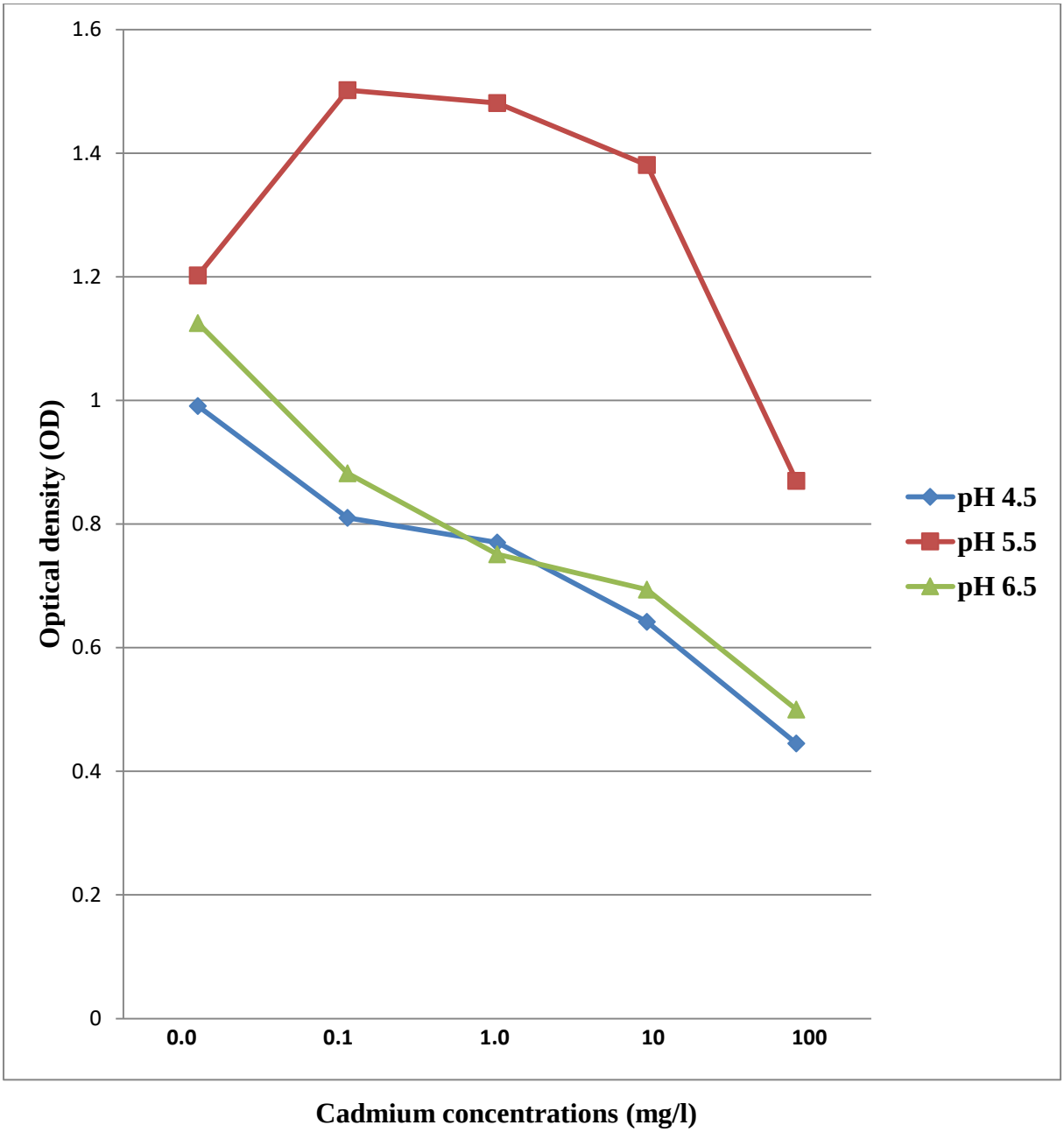

Fig. 4.27: Growth response of *A. clavatus* during utilization of UEO in the presence of varying concentrations of cadmium at different pH.

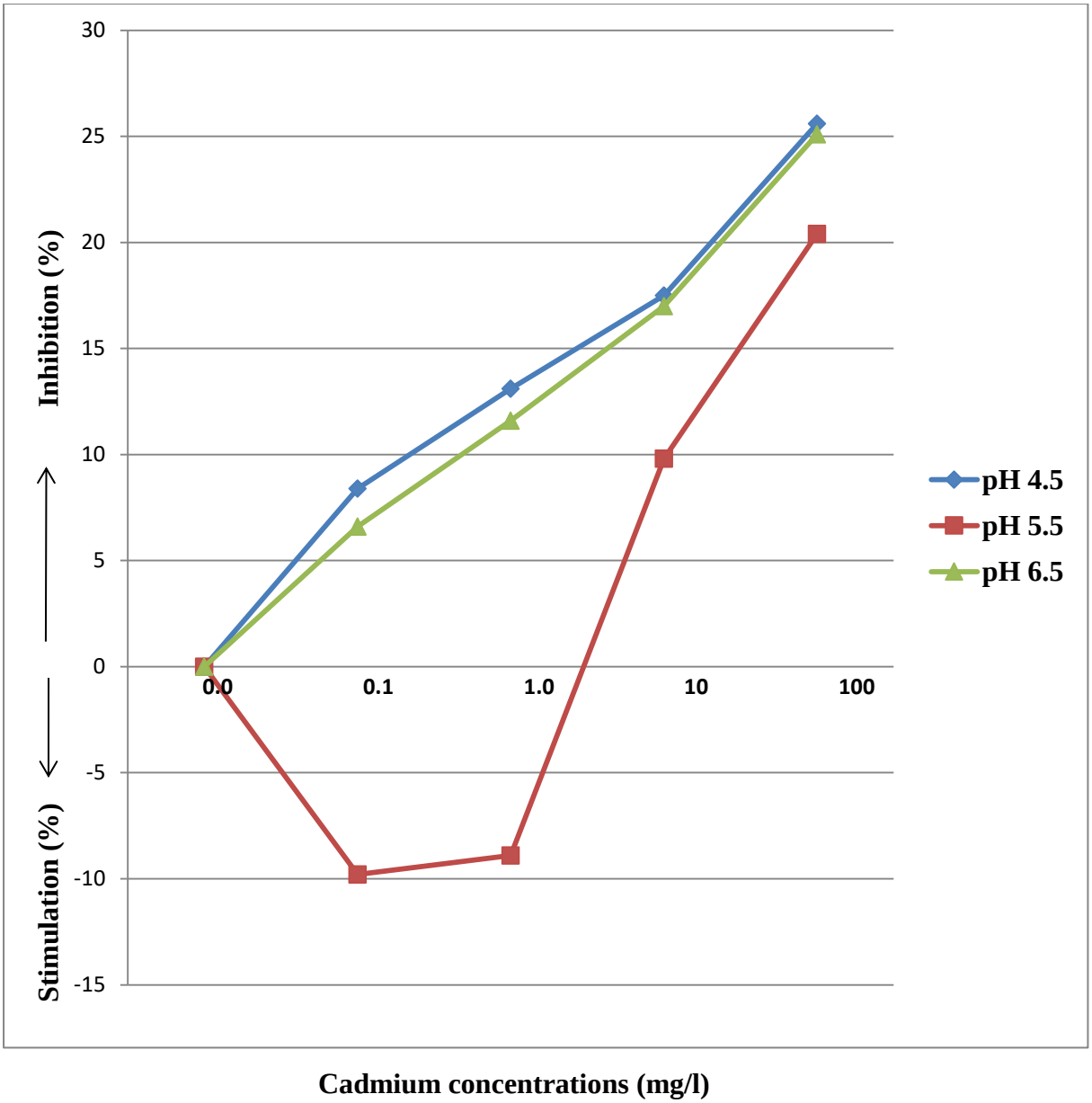

Fig. 4.28: Effect of varying concentrations of cadmium on UEO degradation by the mixed culture at different pH.

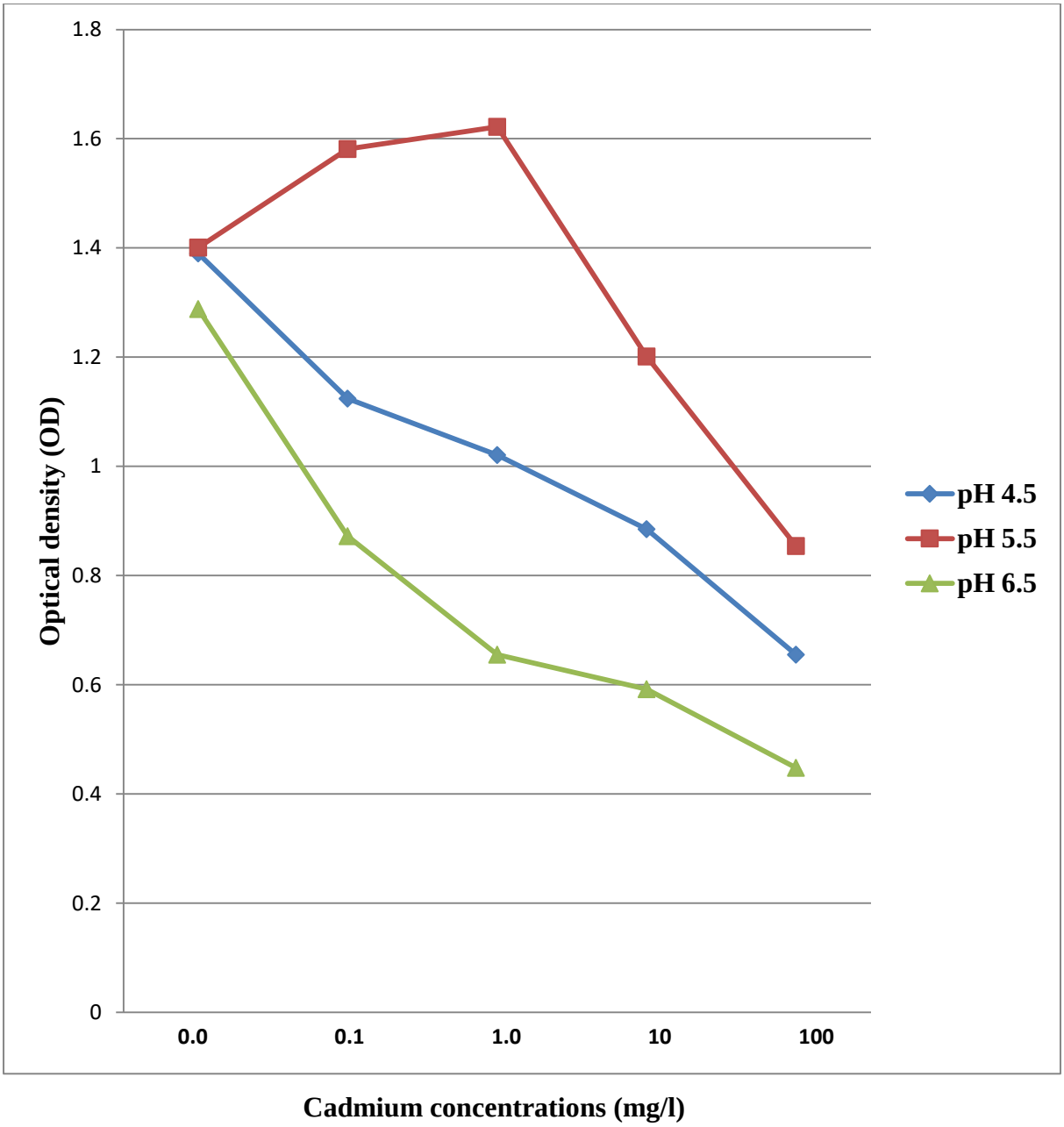

Fig. 4.29: Growth response of the mixed culture during utilization of UEO in the presence of varying concentrations of cadmium at different pH.

The effects of varying concentrations of copper on used engine oil degradation at different pH levels are presented in Figures 4.30 to 4.35. There was an increasing trend in stimulation of used engine oil degradation in the media containing *C. tropicalis*, in the presence of 0.1 to 10 mg/l Cu at pH 5.5. A stimulatory effect on used engine oil degradation was also observed in the presence of 0.1 mg/l Cu at pH 4.5. However, at pH 6.5, there was an increasing trend in inhibition of used engine oil degradation in the presence of 0.1 to 100 mg/l Cu (Fig. 4.30). The optical density was higher in the presence of 0.1 to 10 mg/l Cu, when compared to the control, at pH 5.5 (Fig. 4.31). The OD was also higher in the presence of 0.1 mg/l Cu at pH 4.5, when compared to the control. At pH 6.5, there was a decreasing trend in OD in the presence of 0.1 to 100 mg/l Cu (Fig. 4.31).

In the media containing *A. clavatus*, there was stimulation in used engine oil degradation in the presence of 0.1 to 100 mg/l Cu at pH 5.5. However, highest stimulation was recorded at 10 mg/l Cu at pH 5.5. At pH 6.5, there was also stimulation in used engine oil degradation in the presence of 0.1 mg/l Cu, while at pH 4.5, an increasing trend in inhibition of used engine oil degradation was observed in the presence of 0.1 to 100 mg/l Cu (Fig. 4.32). Similarly, there was increase in OD in the presence of 0.1 to 100 mg/l Cu at pH 5.5, when compared to the control (Fig. 4.33). Highest OD was recorded at 10 mg/l Cu at pH 5.5. At pH 6.5, the OD was higher at 0.1 mg/l Cu, when compared to the control, while at pH 4.5, a decreasing trend in OD was observed in the presence of 0.1 to 100 mg/l Cu (Fig. 4.33).

In the media containing the mixed culture of the isolates, there was stimulation in used engine oil degradation in the presence of 0.1 to 1.0 mg/l Cu at pH 5.5. There was also stimulation in used engine oil degradation at pH 4.5 and 6.5 at 0.1 mg/l Cu, while inhibition increased as the Cu concentration increased from 1.0 to 100 mg/l (Fig. 4.34). The OD was higher in the

presence of 0.1 to 1.0 mg/l Cu at pH 5.5, when compared to the control (Fig. 4.35). At pH 4.5 and 6.5, the OD was also higher in the presence of 0.1 mg/l Cu, when compared to the control, while a decreasing trend in OD was however observed as the Cu concentration increased from 1.0 to 100 mg/l (Fig. 4.35).

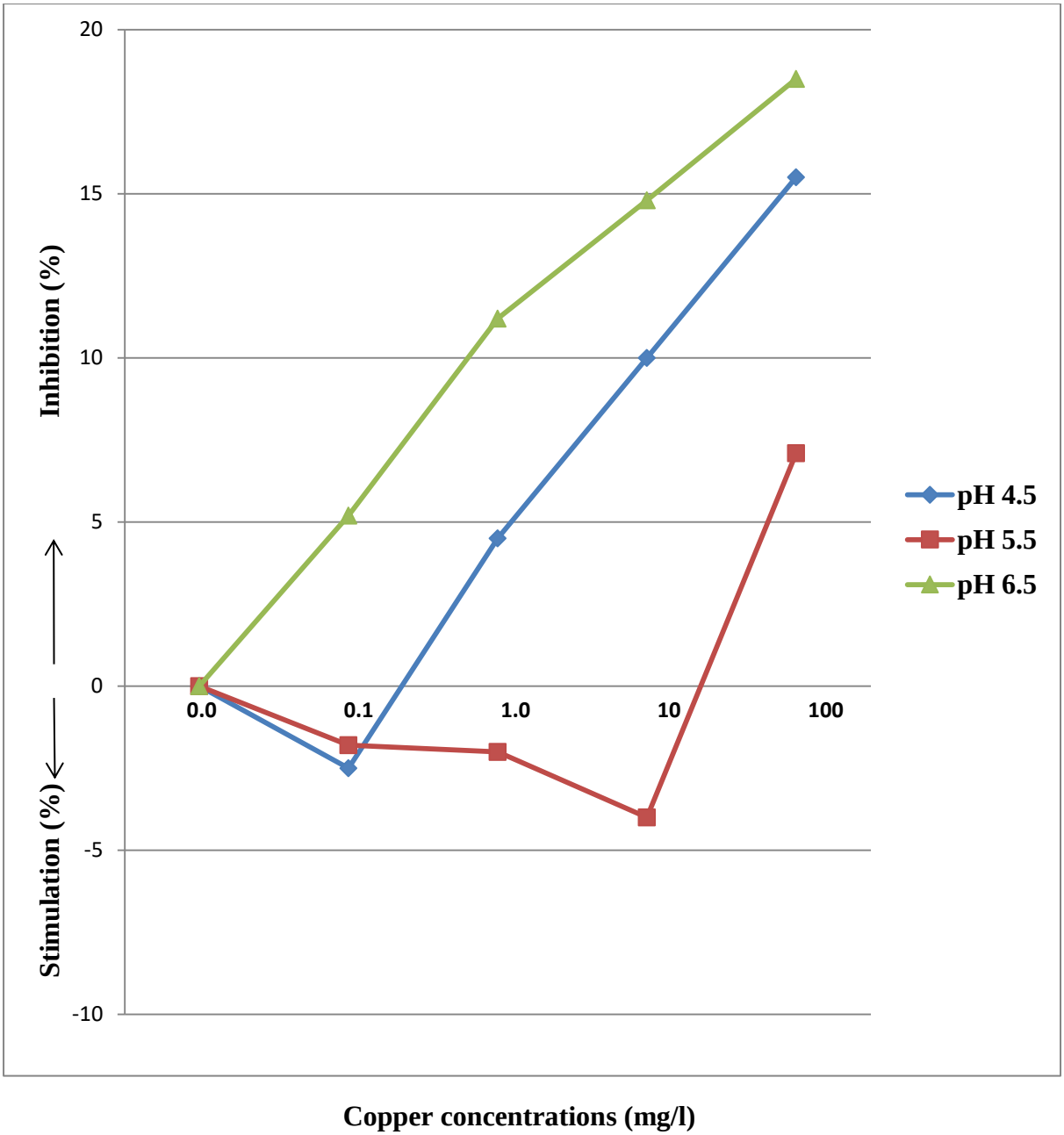

Fig. 4.30: Effect of varying concentrations of copper on UEO degradation by *C. tropicalis* at different pH.

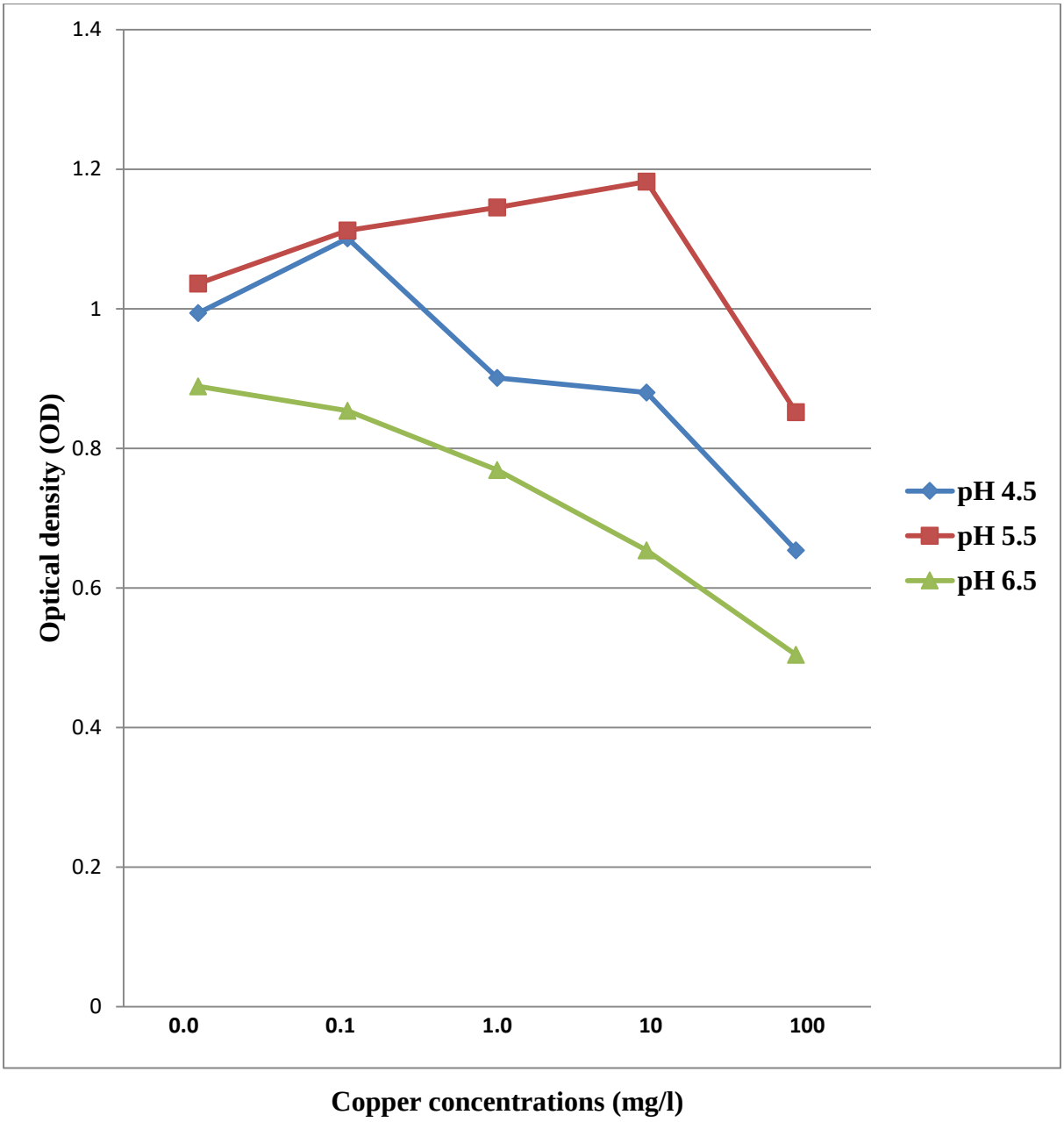

Fig. 4.31: Growth response of *C. tropicalis* during utilization of UEO in the presence of varying concentrations of copper at different pH.

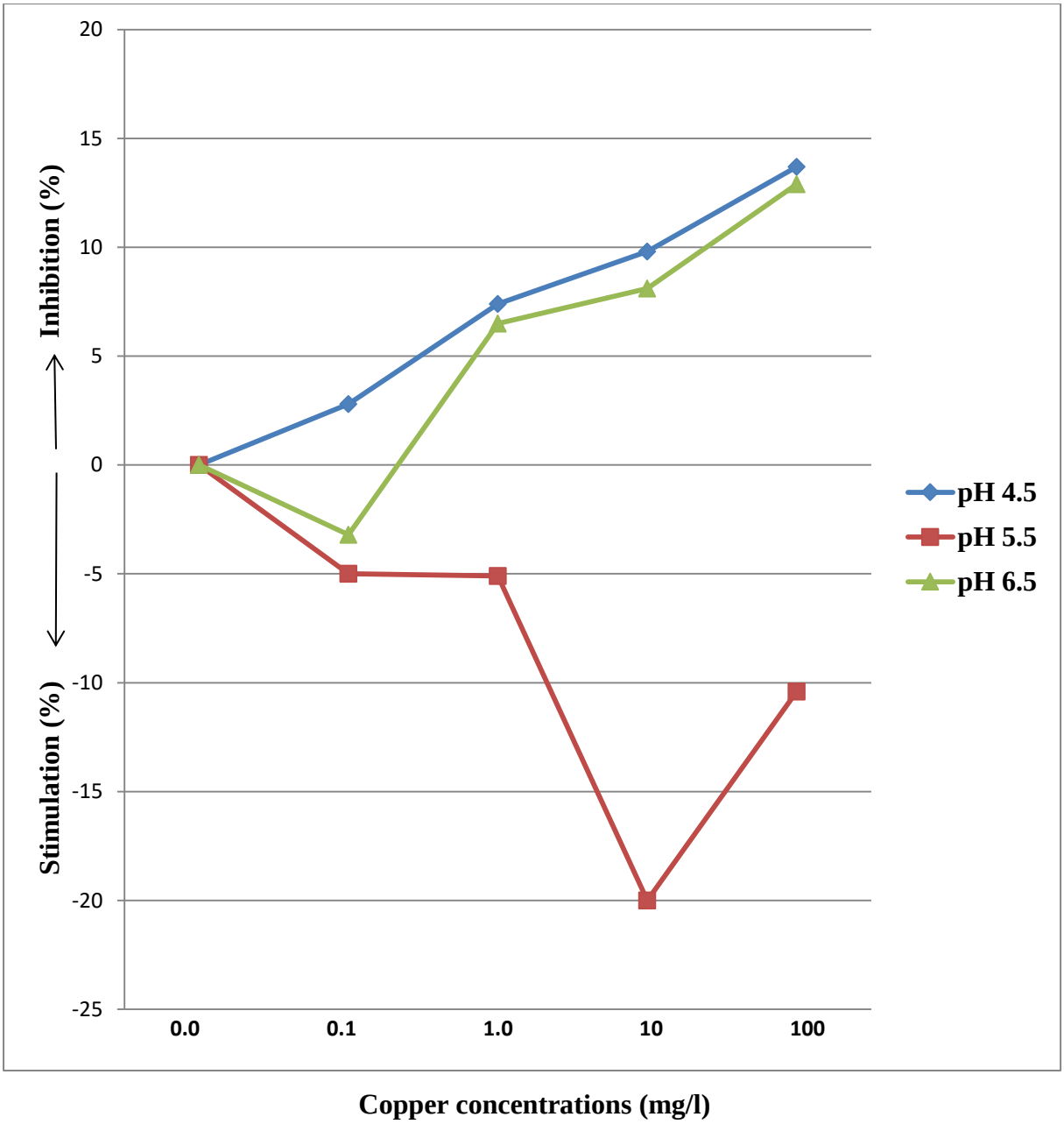

Fig. 4.32: Effect of varying concentrations of copper on UEO degradation by *A. clavatus* at different pH.

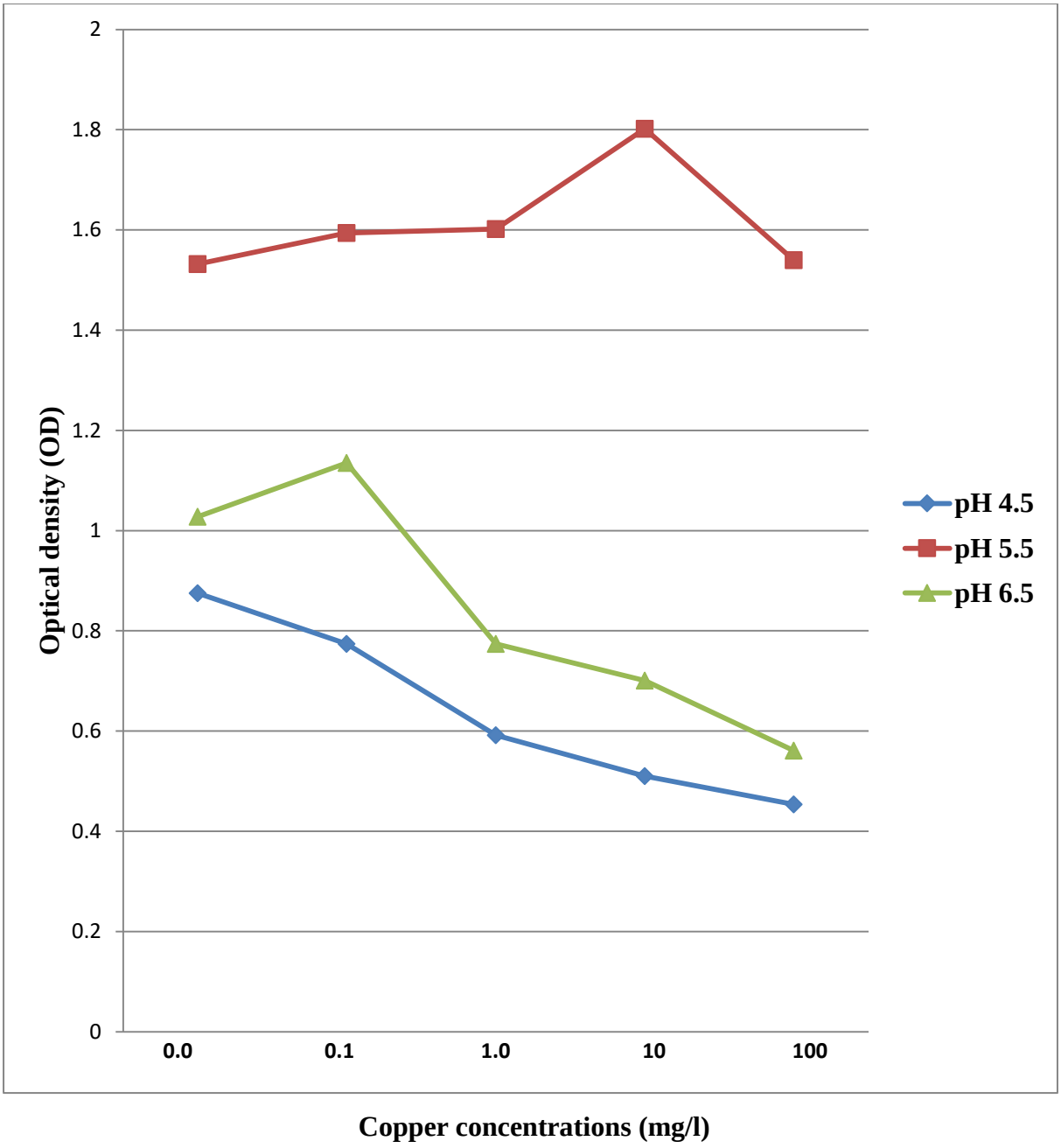

Fig. 4.33: Growth response of *A. clavatus* during utilization of UEO in the presence of varying concentrations of copper at different pH.

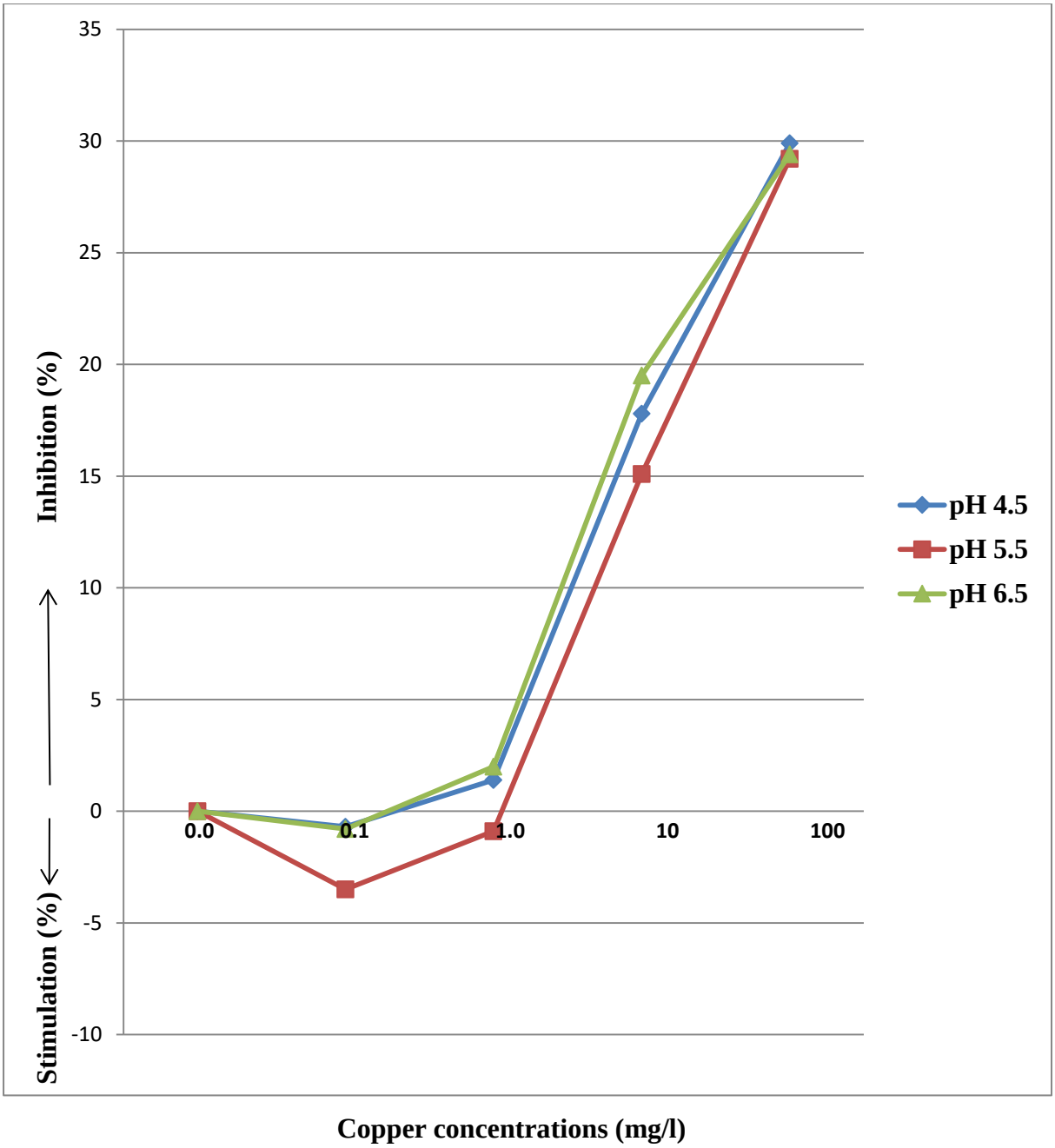

Fig. 4.34: Effect of varying concentrations of copper on UEO degradation by the mixed culture at different pH.

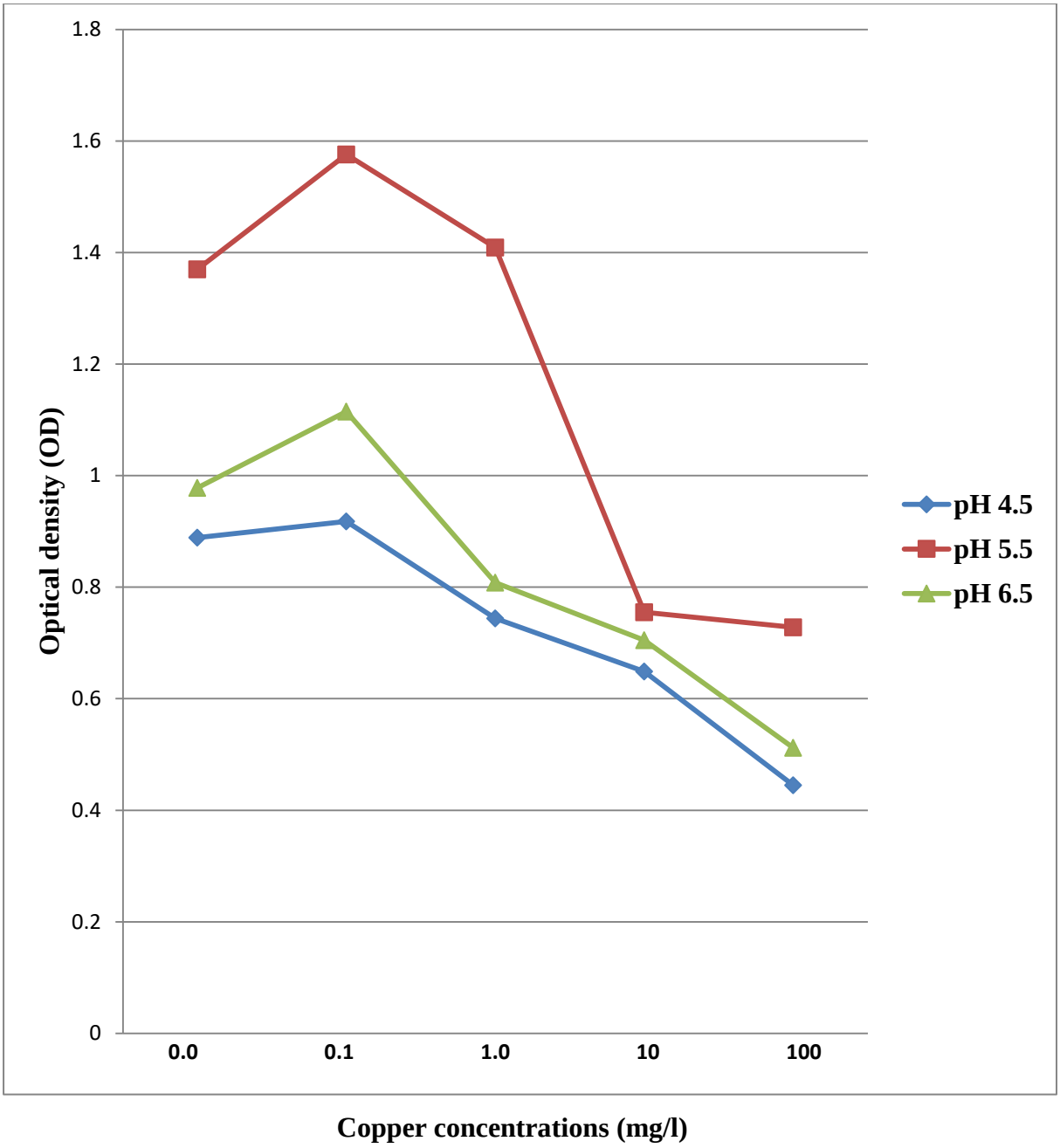

Fig. 4.35: Growth response of the mixed culture during utilization of UEO in the presence of varying concentrations of copper at different pH.

4.11 Effectiveness of the Isolates in Bioremediation of UEO Contaminated Soil Using Bioaugmentation Technique

4.11.1 Biodegradation of used engine oil contaminated soil

The level of biodegradation of used engine oil throughout the experimental period is shown in Figure 4.36. There was a rapid reduction in the TPH within the first 14 days of the study in the soil microcosm inoculated with the pure and mixed culture of the isolates compared to the uninoculated control. At the end of the experimental period (56 days), oil contaminated soil inoculated with the mixed culture of the isolates (*C. tropicalis* and *A. clavatus*) showed the highest reduction in concentration of used engine oil (95.42 %). This was followed closely by 90.63 and 90.42 % reduction in soil microcosm inoculated with the pure cultures of *A. clavatus* and *C. tropicalis*, respectively. Uninoculated control 1 showed 54.17 % reduction at the end of the experimental period. Moreover, there was 4.0 % abiotic loss in the autoclaved soil containing sterile used engine oil (i.e., control 2) at the end of the experimental period.

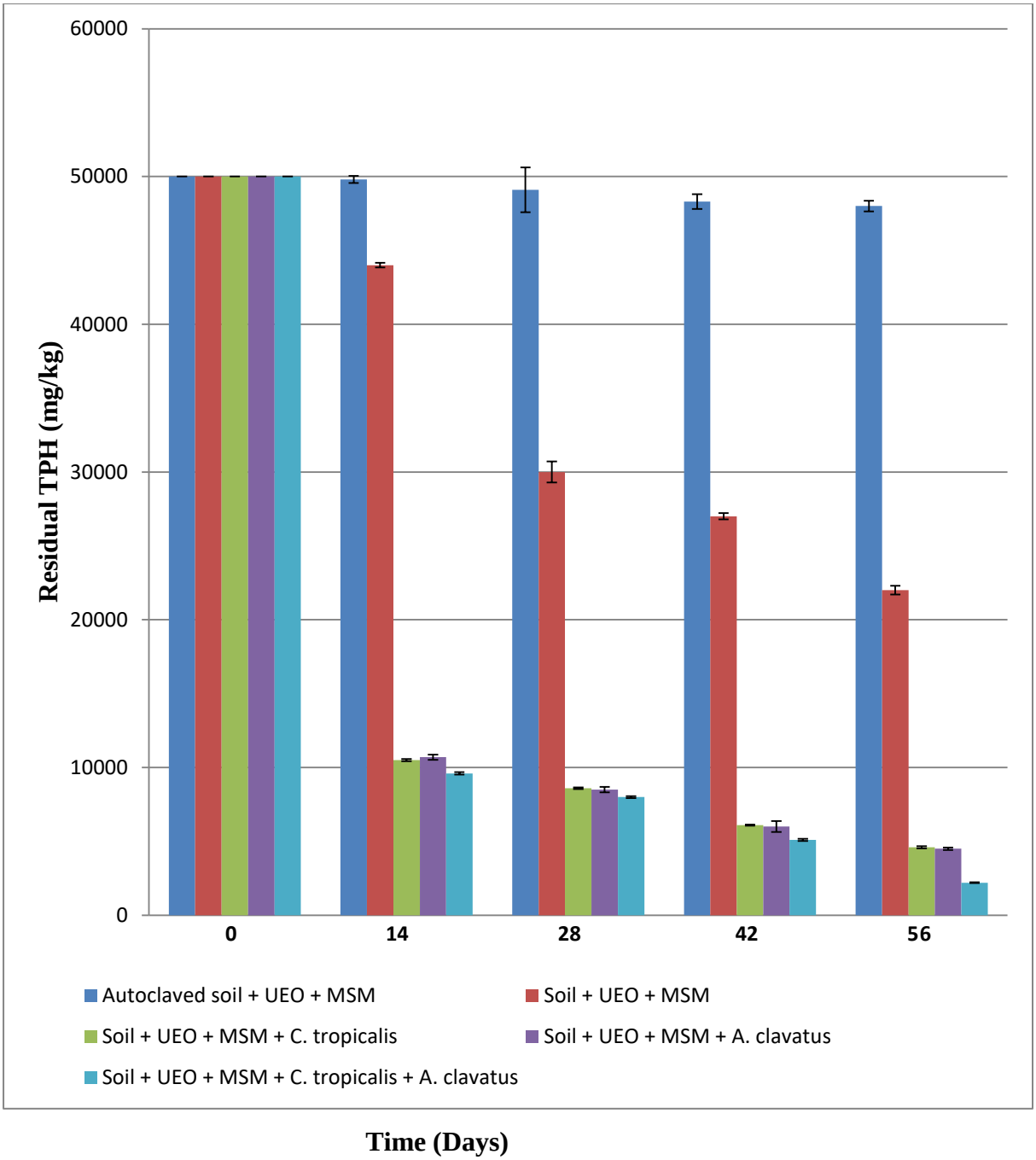

Fig. 4.36: Residual total petroleum hydrocarbon in soil during bioremediation

4.11.2 Net Percentage (%) Loss of TPH in Soil during Bioremediation

As shown in Table 4.10, the highest net percentage loss of TPH was observed at 14 day in the soil microcosm containing the pure and mixed culture of the isolates. However, it was observed that the net percentage loss of TPH in soil inoculated with both the pure and mixed culture of the isolates decreased from 14 day throughout the experimental period. Moreover, the net percentage loss in soil inoculated with the mixed culture of the isolates was higher throughout the experimental period (from 14 to 56 days), when compared to the single cultures.

Table 4.10: Net percentage (%) loss of TPH in soil during bioremediation

Microcosm set up	Time (days)				
	0	14	28	42	56
A	0	67.27±0.13	43.60±0.14	43.27±2.23	36.27±0.96
B	0	66.86±0.26	43.79±0.72	43.48±0.69	36.46±0.44
C	0	69.07±0.76	44.81±0.44	45.34±0.39	41.25±0.32

A = Soil + UEO + MSM + *C. tropicalis,* B = Soil + UEO + MSM + *A. clavatus,* C = Soil + UEO + MSM + *C. tropicalis* + *A. clavatus*. Net % loss = % loss of TPH in inoculated soil microcosm - % loss of TPH in uninoculated soil microcosm (control 1).

4.11.3 Biodegradation Rate Constant and Half-Life

Table 4.11 shows the biodegradation rate constant (k) and half-life ($t_{1/2}$) for the different soil microcosms within the experimental period. Soil inoculated with the mixed culture of the isolates showed the highest biodegradation rate (day^{-1}) and lowest half-life (days), while the uninoculated soil (control) showed the lowest biodegradation rate (day^{-1}) and highest half-life (days).

Table 4.11: Biodegradation rate and half-life of TPH in oil-polluted soil

Microcosm set up	Biodegradation constant (k) day^{-1}	Half-life ($t_{1/2}$) days
A	0.2295 ± 0.013^{b}	3.02
B	0.2303 ± 0.009^{b}	3.01
C	0.3013 ± 0.029^{b}	2.30
D	0.0553 ± 0.004^{b}	12.53

A = Soil + UEO + MSM + *C. tropicalis*, B = Soil + UEO + MSM + *A. clavatus*, C = Soil + UEO + MSM + *C. tropicalis* + *A. clavatus*, D = Soil + UEO + MSM. Values followed by letter b are different significantly at $P < 0.05$ level.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

The resulting weakly acidic pH in contaminated soil could be as a result of hydrocarbon contamination and the presence of hydrocarbon degrading fungi in the soil, which utilize the used engine oil. This result was similar to the findings of Osuji and Nwoye (2007), who reported that soil pH was reduced due to the presence of hydrocarbons that produce organic acids when acted upon by microorganisms. The findings of Akpoveta *et al*. (2011), who reported that hydrocarbons contain many free cations causing them to have properties of a weak acid, also supports this explanation. A reduction in pH implies increased acidity which is a problem for agricultural soils because many metal cations are more soluble and available in the soil solution at very low pH, including Cd, Cu, Hg, Ni, Pb and Zn (Akpoveta *et al*., 2014). Adenipekun (2008) observed a pH range of 6.65 – 7.15 in oil-contaminated soils. The optimum soil pH for efficient bioremediation has been shown to be 5.5 to 8.8 (Vidali, 2001).

Soils contaminated with petroleum products have been shown to have large increases in nitrogen and phosphate content (Ikhajiagbe *et al*., 2013b). This was in agreement with the findings made on nitrogen determination in this study, whereby the nitrogen content was significantly higher in contaminated soil than in uncontaminated soil, but was not in agreement with the findings on phosphate determination whereby the phosphate content of contaminated and uncontaminated soil showed no significant difference. However, the observation made on phosphate determination was in agreement with the findings of Akoachere *et al*. (2008), who reported that there was no significant difference in the nitrate and phosphate contents between soils contaminated with hydrocarbons and uncontaminated soil. Adenipekun and Fasidi (2005) reported a low value of nitrogen, potassium and

phosphorus reserve in petroleum hydrocarbon contamination. An increase in nutrient (nitrate, nitrogen, total organic carbon, magnesium) content observed in contaminated soil was probably due to the effect of contamination with hydrocarbons in the soil. Adenipekun (2008) reported a higher level of organic carbon in engine oil-contaminated soil compared with the uncontaminated soil. Adenipekun *et al*. (2011) also reported that the addition of crude oil to an ecosystem will enrich primarily the microorganisms capable of utilizing the hydrocarbons and secondary microorganisms capable of utilizing metabolites produced by the oil-utilizing microorganisms. The low moisture content observed in contaminated soil compared to uncontaminated soil was due to the presence of hydrocarbons and PAHs which cause an increase in soil hydrophobicity and therefore leading to decrease in the moisture holding capacity of soil (Balk *et al*., 2002).

The higher concentrations of heavy metals (Zn, Pb, Cu and Cd) observed in contaminated soil compared to the uncontaminated soil used in this study may be attributed to the co-contamination of the soil with used engine oil and heavy metal pollutants. This result was in agreement with the findings of Olaniran *et al*. (2009), who reported that rapid industrial and domestic activities have caused a concomitant increase in the quantities of metals that are being introduced into the environment, thus resulting in co-contamination of soil with organic and heavy metal pollutants. Used engine oil is also a source of these metals. This continues to present a more serious and complex problem, as the two contaminants must be treated differently (Sandrin and Maier, 2003; Roane *et al.*, 2001).

The higher level of heavy metals (Zn, Pb, Cd and Cu) found in used engine oil compared to fresh (unused) engine oil could be due to the use of the lubricant in car engine. As motor oil circulates through a car's engine, it picks up heavy metals such as arsenic, lead, cadmium, copper and zinc (Reisewitz and Martin, 2015). Haytham and Ibrahim (2016) also

demonstrated the presence of several heavy metals such as lead, nickel, manganese, copper and zinc in used engine oil. Heavy metals such as lead, vanadium, aluminium, nickel and iron usually below detectable limits in unused lubricating oil have been reported to give high values (ppm) in used oil (Adams *et al*., 2014). The higher concentrations of zinc observed in used and unused engine oil compared to other heavy metals studied may be attributed to the use of zinc as an additive in engine oil. Similar result of high concentration of zinc in used engine oil was reported by Haytham and Ibrahim (2016).

Some of the isolates obtained in this study have earlier been reported as hydrocarbon degraders (April *et al*., 2000). Akpoveta *et al*. (2011) reported the isolation of *Trichoderma* sp., *Penicillium* sp., *Rhizopus* sp., *Fusarium* sp. and *Aspergillus* sp. from crude oil-polluted soil. George-Okafor *et al*. (2009) reported the isolation of *Aspergillus* spp., *Syncephalastrum* spp., *Trichoderma* spp., *Neurospora sitophila*, *Rhizopus arrhizus* and *Mucor* spp., from petroleum-contaminated soil.

The decreasing trend in pH observed during the screening test in this study could be as a result of mineralization of hydrocarbons in used engine oil by the isolates. This was in agreement with the findings of Olajide and Ogbeifun (2010), who reported a decreasing trend in pH during hydrocarbon degradation by *Proteus vulgaris*. Sepahi *et al*. (2008) reported that microbial degradation of hydrocarbons often leads to production of organic acids, thus the organic acids probably caused the reduction in pH.

The increase in the growth (TVC) of the isolates in the media containing used engine oil could be attributed to the ability of the organisms to utilize UEO as a sole source of carbon. It could also be that the cultural condition was adequate for the growth of the organisms. This

was in agreement with the findings of Vanishree *et al*. (2014) who reported that several fungal isolates such as *Fusarium solani*, *Fusarium oxysporium*, *Trichoderma viride* and *Aspergillus niger* cultured in mineral salt medium (MSM) at pH 5.5 showed good growth. The decrease in the growth of the isolates on the 16^{th} day could be due to the decrease in pH level. It could be that the pH became too acidic for the organisms to thrive. It could also be as a result of decrease in substrate (UEO) used by the organisms as a sole source of carbon and energy. The findings of Obire and Nwaubeta (2002) who reported an initial gradual increase in the bacterial population following the application of petroleum hydrocarbons but a decline as the biodegradation progressed supports this explanation. Similarly, Akpoveta *et al*. (2011) reports that hydrocarbon degrading fungi increased within the first seven days from 2.16 x 10^4 cfu/g to 11.1 x 10^4 cfu/g and decreased progressively to 1.5 x 10^4 cfu/g within the next four weeks. A decrease in substrate will therefore result in a drop in the population of oil-degraders.

The inability of the isolates; *S. cerevisiae*, *C. albicans*, *M. gypseum* and *T. mentagrophytes* to achieve high oil loss in the experimental flask could be due to toxic effect of co-contamination of used engine oil and heavy metals. It could also be that they lack the vital enzymes for used engine oil degradation. Different species and different life stages of organisms have been demonstrated to have different susceptibilities to pollution (Vanishree *et al*., 2014).

Among the 4 isolates that showed high promise for hydrocarbon bioremediation potentials, *Candida tropicalis* and *Aspergillus clavatus* displayed the highest extent of biodegradation of used engine oil, diesel and petrol, thus they were selected for further studies. The high rate of hydrocarbon degradation by the two fungi could emanate from their massive growth and

enzyme production responses during their growth phases. This could be supported by the reports of Ekundayo (2014), which showed that extracellular ligninolytic enzymes of white rot fungi are produced in response to their growth phases.

In this study, it was observed that the isolates utilized comparatively less amount of diesel than petrol and used engine oil from the media. The higher rate of degradation observed in petrol compared to diesel may be due to the fact that petrol contains low concentrations of PAHs, thus does not resist microbial attack. The higher abiotic loss observed in petrol may also be a contributing factor.

The isolates; *Candida tropicalis* and *Aspergillus clavatus* showed biosurfactant producing ability through emulsification test, oil displacement assay and microbial adhesion to the hydrocarbon (MATH). The result seems quite promising and allows the identification of some adjustments that could be introduced for the scale up of bioremediation process. For instance, selection of biosurfactant producers in order to avoid the introduction in the environment of chemical surfactants, which are more toxic and non-biodegradable. Experimental results on the emulsification activity of biosurfactant obtained from *Candida tropicalis* and *Aspergillus clavatus* revealed that the samples were able to form a stable emulsions (E_{24} > 50%) with used engine oil, diesel and petrol for 24 hours. Similar results of E_{24} values were obtained from biosurfactants isolated from 30^{o}C and 45^{o}C growth culture (Namir *et al*., 2009). Thavasi *et al*. (2010) reported that biosurfactant produced from a substrate can emulsify different hydrocarbons to a greater extent, which confirmed its applicability against different hydrocarbon pollution.

In the oil displacement test, the supernatants obtained on centrifugation of the media in which the organisms (*C. tropicalis* and *A. clavatus*) were grown, produced visible clear zones in used engine oil, diesel and petrol (Appendix IX) when observed under light. This indicated that the two organisms were positive for biosurfactant production, as well as having bioremediation potential. The ability of *Candida* species to produce clear zone in oil spreading test has been reported elsewhere (Padmapriya *et al*., 2013).

The cell surface hydrophobicity (>52%) achieved in this study is an indication of the high affinity of the isolates to the hydrocarbon substrates (used engine oil, diesel and petrol), which confirmed their ability to utilize them as carbon sources. Kaczorek *et al*. (2011) reported a 31.4% adhesion to the hydrocarbon substrate, diesel oil, with *Pseudomonas alcaligens*. Padmapriya and Suganthi (2013) reported that *C. tropicalis* have higher hydrophobicity to hydrocarbon substrates than *Pseudomonas aeruginosa*.

Biosurfactants have gained importance in the fields of enhanced oil recovery, environmental bioremediation, food processing and pharmaceuticals owing to their unique properties such as higher biodegradability and lower toxicity. Interest in the production of biosurfactants has steadily increased during the past decade. However, large-scale production of these molecules has not been realized because of the low yields in the production processes and high recovery and purification costs (Padmapriya *et al*., 2013).

The higher activity of peroxidase observed in the hydrocarbon substrates, compared to catalase and lipase activity could be attributed to the relative affinity of each of the substrate for the microbial enzymes. High activity of peroxidase was also reported in the soil microorganisms in petroleum-polluted soils (Ugochukwu *et al*., 2008; Akubugwo *et al*.,

2009). Peroxidase enzymes play a crucial role in the degradation of PAHs (Steffen *et al*., 2002; Hofrichter *et al*., 2010).

The result of gas chromatographic analysis for PAHs degradation in used engine oil, diesel and petrol, which showed that the two isolates *Candida tropicalis* and *Aspergillus clavatus* as well as their mixed culture exhibited biodegradation efficiency above 70%, also confirmed their high degradation potential. The abilities of these organisms in oxidizing the polycyclic aromatic hydrocarbons (PAHs) can be attributed to the non-specific nature of their enzymes especially the peroxidases on degrading chemicals. The fact that both the pure and mixed cultures of the isolates were able to degrade PAHs very effectively suggests that the degradation of the aliphatic moieties could be easier and faster than the polycyclic aromatic moieties. The implication of these two organisms in hydrocarbon degradation from our results is similar to the findings of April *et al*. (2000). The inability of either the pure or mixed culture of the isolates to achieve a 100% depletion of Benzo(a)pyrene (BaP) in used engine oil, diesel and petrol in this study, could be attributed to the physical and chemical characteristics of the PAH. Numerous genera of microorganisms have been observed to oxidize PAHs (Juhasz and Naidu, 2000). While there is a great diversity of organisms capable of degrading the low molecular weight PAHs such as naphthalene, acenaphthalene and phenanthrene, relatively few genera have been observed to degrade the high molecular weight PAHs, such as BaP (Juhasz and Naidu, 2000). Bishnoi *et al*. (2008) reported that PAH adapted fungal strain *Phanaerochaete chrysosporium*, isolated from the soil of petroleum refinery, have the ability to degrade phenanthrene, anthracene, acenaphthene, fluoranthene and pyrene in sterilized as well as unsterilized soil in optimum conditions.

The inhibition of used engine oil degradation in the media containing both the single and mixed culture of the isolates; *C. tropicalis* and *A. clavatus,* at increasing concentrations (> 10 mg/l) of the heavy metals, could be attributed to the toxic effects of the metals, as reflected in their optical density. Several authors have reported the toxic effects of heavy metals to hydrocarbon degrading microorganisms at high concentrations (Al-Kadeeb, 2007; Olaniran *et al*., 2009; Wong *et al*., 2013). However, the stimulation in used engine oil degradation observed at low concentrations of zinc, at the different pH levels, could be attributed to the trace requirement of this metal by the hydrocarbon degrading enzyme systems of the fungal isolates. This result was in agreement with the findings of Owabor *et al*. (2011), who reported that some heavy metals (Cu, Zn, Cr, Ni and Fe) are essential for the growth of microorganisms in trace amounts, but they have however, been shown to be toxic at high concentrations. However, the higher stimulation in used engine oil degradation observed in the media containing the mixed culture of the isolates compared to the single cultures in the presence of Zn could be as a result of synergistic effect. Metal stimulation of organic biodegradation has been reported by Tadama *et al*. (2015), although in studies involving microbial consortia. Sandrin and Maier (2003) argued that such stimulation was a result of differential toxicity effect wherein a second population more sensitive to the metal stress competes with the population expressing the activity of interest. The decrease in optical density in the presence of high concentration of zinc implies inhibition of the growth of the organisms due to toxic effect of the heavy metal. Similar observations about toxic effect of higher concentration of heavy metals on growth of fungi have been reported (Malik, 2004).

The stimulation in used engine oil degradation by the single and mixed culture of the isolates; *C. tropicalis* and *A. clavatus*, in the presence of Pb at pH 5.5, suggests that pH 5.5 was more adequate for the growth of the organisms. However, stimulation was more evident in the

media containing *A. clavatus*, suggesting higher tolerance of the organism to Pb. Similar results were reported by Ezzouhri *et al*. (2009) and Iram *et al*. (2013), about different isolates of *Aspergillus* sp. having a high tolerance for Pb.

Despite the report that Cd was among the most toxic metal to microorganisms (Wong *et al*., 2013), these isolates; *C. tropicalis* and *A. clavatus* were able to grow well in the presence of up to 10 mg/l Cd at pH 5.5. The stimulation of used engine oil degradation with corresponding increase in the growth of the organisms at high Cd concentration was similar to the findings of Ramasamy *et al*. (2012), who reported maximum growth of *Aspergillus* sp. in the media containing 10 mg/l Cd. This may be attributed to the biosurfactant producing ability of the isolates. It is possible that biosurfactant secreted by the isolates acted as metal chelating agents, reducing the bioavailability of Cd and rendering the metal less toxic. Das *et al*. (2009), reported that a biosurfactant produced by *Bacillus circulans* was able to chelate positively charged lead and cadmium to the outer hydrophilic surface of biosurfactant, which consisted of anionic peptide head groups.

The stimulation in used engine oil degradation observed in the presence of high concentrations of copper may be attributed to the source of isolation of the organisms. This observation was in agreement with the findings of Iram *et al*. (2013), who reported that fungal populations isolated from heavy metal contaminated sites have the ability to resist higher concentrations of metals. However, the tolerance and the resistance of the isolates depended more on the fungus tested than on the site of its isolation, since all the isolates were obtained from the same source but exhibiting different levels of tolerance. This was evidenced by the higher level of tolerance to copper exhibited by *A. clavatus* in this study. Similar results were reported by Price *et al*. (2001), who showed that *Aspergillus* was able to

tolerate heavy metals as compared to other fungi. The most probable reason for the difference in resistance levels could be the variation in the mechanism of resistance (Ezzouhri *et al*., 2009; Sani *et al*., 2003). This variation may be explained by the development of tolerance or adaptation of the fungi to heavy metals.

The higher net percentage (%) loss of TPH in UEO observed in the soil microcosm inoculated with the mixed culture suggested that the isolates could co-exist with no adverse effect and possibly have a synergy, which may be responsible for the higher net % loss of oil observed in this study. The advantages of employing mixed cultures have been reported (Akoachere *et al*., 2008; Larik *et al*., 2016). Ghazali *et al*. (2004) reported that some species are able to remove the toxic metabolites that prohibit the activities of the other species. Then it is possible that the other species degrade complex compounds totally.

High biodegradation rate and low half-life observed in soil microcosm inoculated with the mixed culture of the isolates could be attributed to the high net loss (%) of TPH throughout the experimental period. However, the higher biodegradation rate and lower half-life observed in soil microcosm inoculated with both the single and mixed culture of the isolates compared to the uninoculated control could be due to the previous exposure of the isolates to soil co-contaminated with UEO and heavy metals. It was speculated that native strains of co-contaminated soil already shaped by selective pressure, could take advantage with respect to sensitive strains in accomplishing biodegradation, when heavy metals are present. This way, they could help to overcome an important limitation in bioremediation applications such as the co-occurrence of heavy metals which inhibit biodegradation process (Alisi *et al*., 2009).

5.2 Conclusion

This study has demonstrated the potential of the isolates; *C. tropicalis* and *A. clavatus* to utilize used engine oil, diesel and petrol as sole sources of carbon, as well as degrade the PAH components of the hydrocarbons.

The result of the effect of varying concentrations of heavy metals on used engine oil degradation at different pH levels affirmed that the response of the isolates to heavy metals depend on the metal tested, its concentration in the medium, the isolate considered and pH. However, from this study, it was concluded that the level of heavy metals found in the soil contaminated with used engine oil may not have any negative impact on the ability of the isolates to degrade used engine oil, when adequate environmental conditions such as pH are maintained.

Bioaugmentation with the fungal isolates enhanced the remediation of used engine oil contaminated soil co-contaminated with heavy metals, as reflected in the biodegradation constant and half life of the total petroleum hydrocarbons observed in the soil microcosm containing the isolates, when compared to the uninoculated soil microcosm. This bioaugmentation strategy may contribute to overcome a critical bottleneck of the bioremediation technology. Finally, fungi such as *C. tropicalis* and *A. clavatus*, isolated from automobile workshops can efficiently facilitate the bioremediation of used engine oil contaminated soil co-contaminated with heavy metals.

5.3 Recommendations

Selection of microorganisms with biosurfactant producing ability for the scale up of bioremediation exercise is encouraged, in order to avoid the introduction to the environment of further chemicals, such as chemical surfactants, which are more toxic and non biodegradable. Selection of microorganisms with the ability to produce the specific enzymes involved in the biodegradative processes is encouraged. Moreover, suitable environmental conditions such as pH should be maintained during biodegradative studies.

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APPENDIX

APPENDIX I

MEDIA COMPOSITION AND PREPARATION

MINERAL SALT AGAR

Composition	g/l
K_2HPO_4	1.8
KH_2PO_4	1.2
NH_4Cl	4.0
$MgSO_4.7H_2O$	0.2
NaCl	0.1
$FeSO_4.7H_2O$	0.01
Agar	15.0
pH 5.5	

The components were weighed and dissolved in 1 liter of distilled water. Mycological agar (15g/L) was added to the solution where solid medium was required. They were sterilized by autoclaving at 121^{o}C for 15 minutes, allowed to cool to about 45^{o}C and used for analysis.

SABOURAUD DEXTROSE AGAR

Composition	g/l
Dextrose	40
Mycological peptone	10
Agar	15
pH 5.6	

65g of the agar powder was dissolved in 1 litre of distilled water and sterilized by autoclaving at 121^0C for 15 minutes. It was allowed to cool to about 45^0c and distributed into sterile plates or slants.

SABOURAUD DEXTROSE BROTH

Composition	g/l
Dextrose	20
Peptone, special	10
pH 5.6	

Suspend 30 grams in 1000 ml distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes. Mix well and dispense as desired.

MINERAL SALT BROTH

Composition	g/l
K_2HPO_4	1.8
KH_2PO_4	1.2
NH_4Cl	4.0
$MgSO_4.7H_2O$	0.2
NaCl	0.1
$FeSO_4.7H_2O$	0.01
pH 5.5	

The components were weighed and dissolved in 1 liter of distilled water. Mycological agar (15g/L) was added to the solution where solid medium was required. They were sterilized by autoclaving at 121^{o}C for 15 minutes, allowed to cool to about 45^{o}C and used for analysis.

APPENDIX II

REAGENTS' COMPOSITION

Mixed Indicator Solution

Composition	Percentage (%)
Bromocresol green	0.1
Methyl red	0.02
Ethanol	50

Diphenylamine Indicator

Composition	
Diphenylamine	0.5 g
Deionized water	20 ml
Sulfuric acid	100 ml

Lactophenol Cotton Blue

Composition	
Phenol	20.0 g
Cotton blue	0.05 g
Lactic acid	20.0 ml
Glycerol	40.0 ml
Distilled water	20.0 ml

PUM BUFFER

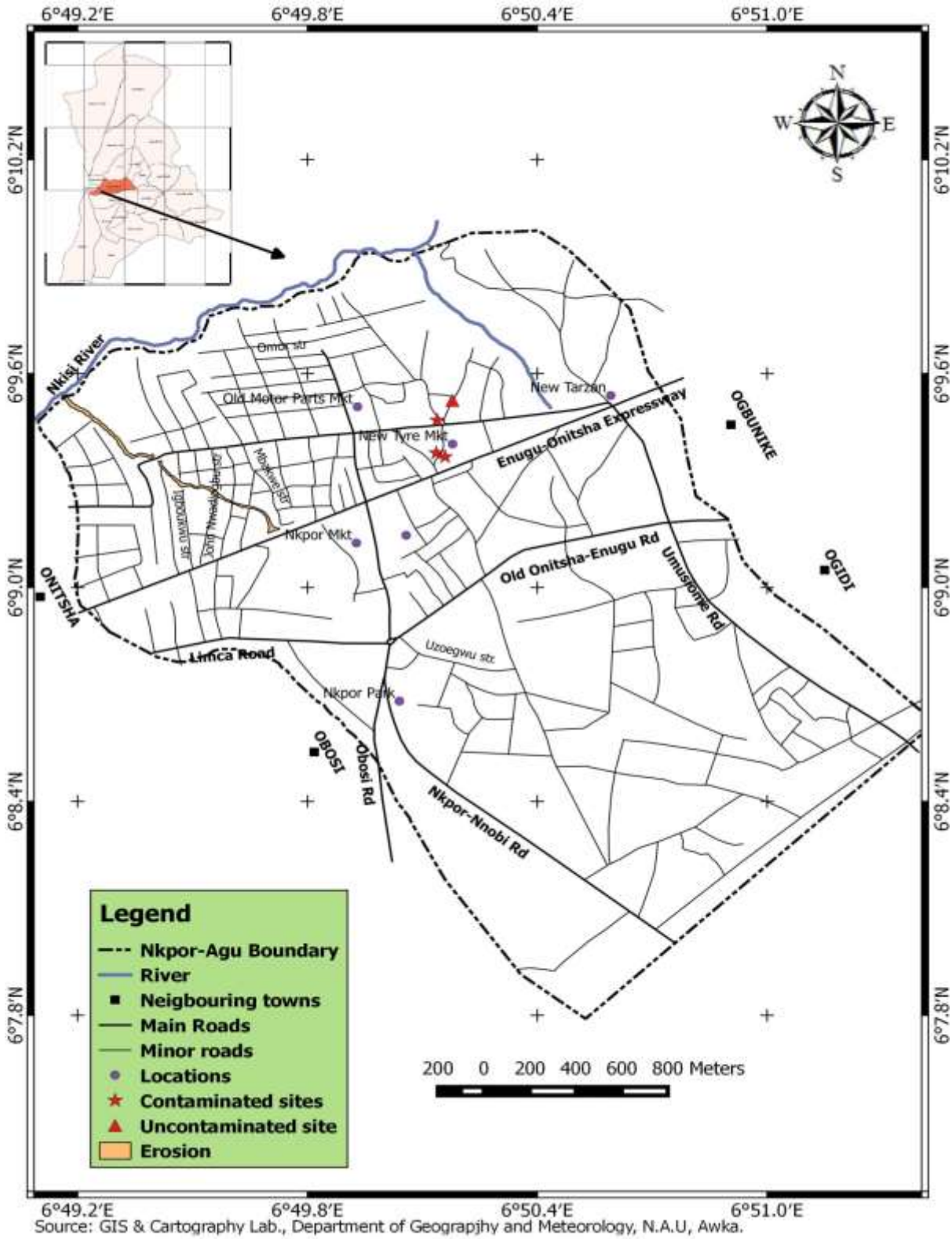
Composition	g/l
KH_2PO_4	7.26
K_2HPO_4	19.7
Urea	1.8
$MgSO_4.7H_2O$	0.2
pH 7.0	

Phosphate Buffer

Composition	
0.1M NaOH	6.8 ml
0.1M KH_2PO_4	50 ml
Deionized water	930 ml
pH 7.0	

APPENDIX III

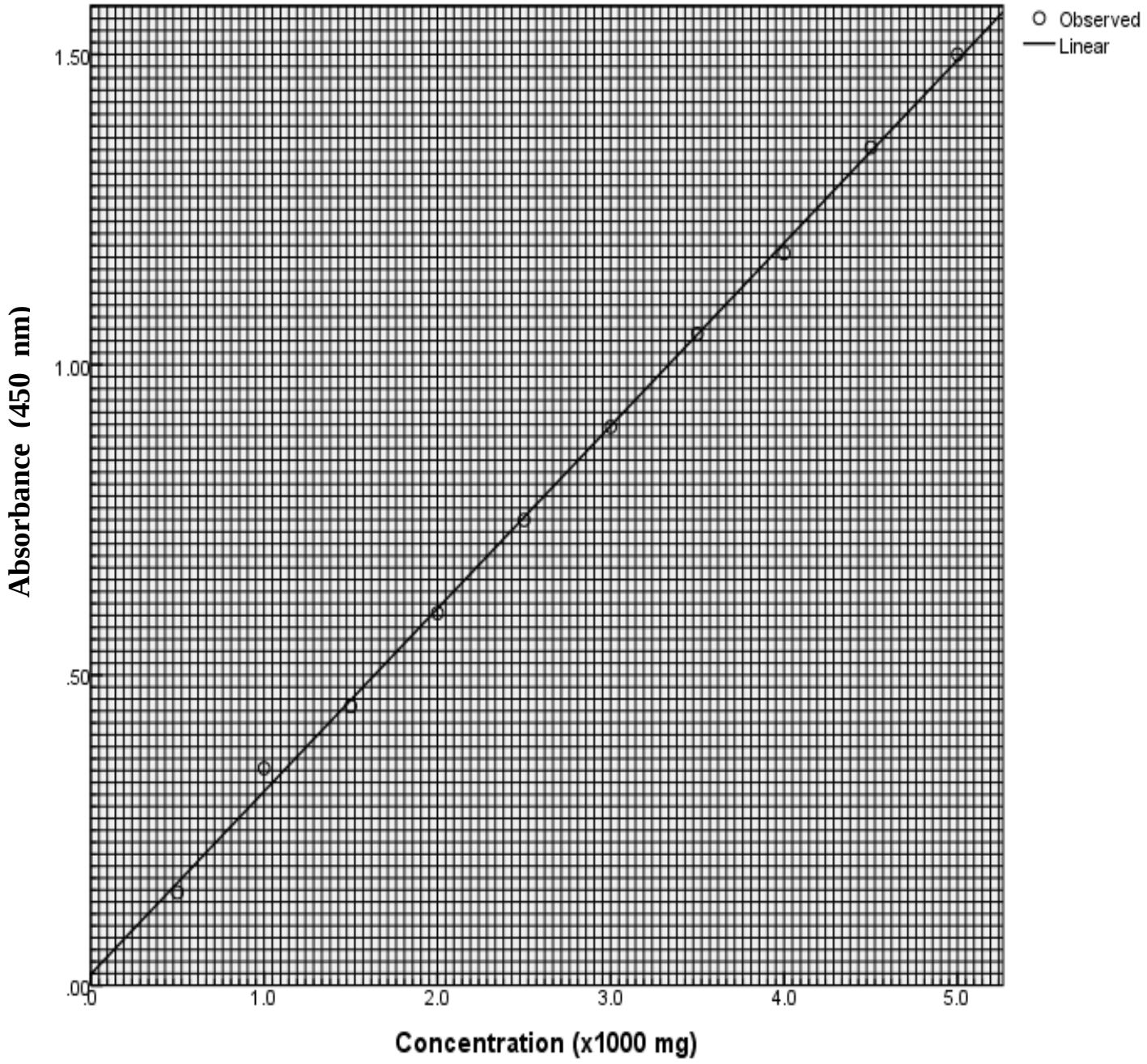
MAP OF THE SAMPLING AREA



Source: GIS & Cartography Lab., Department of Geograpjhy and Meteorology, N.A.U, Awka.

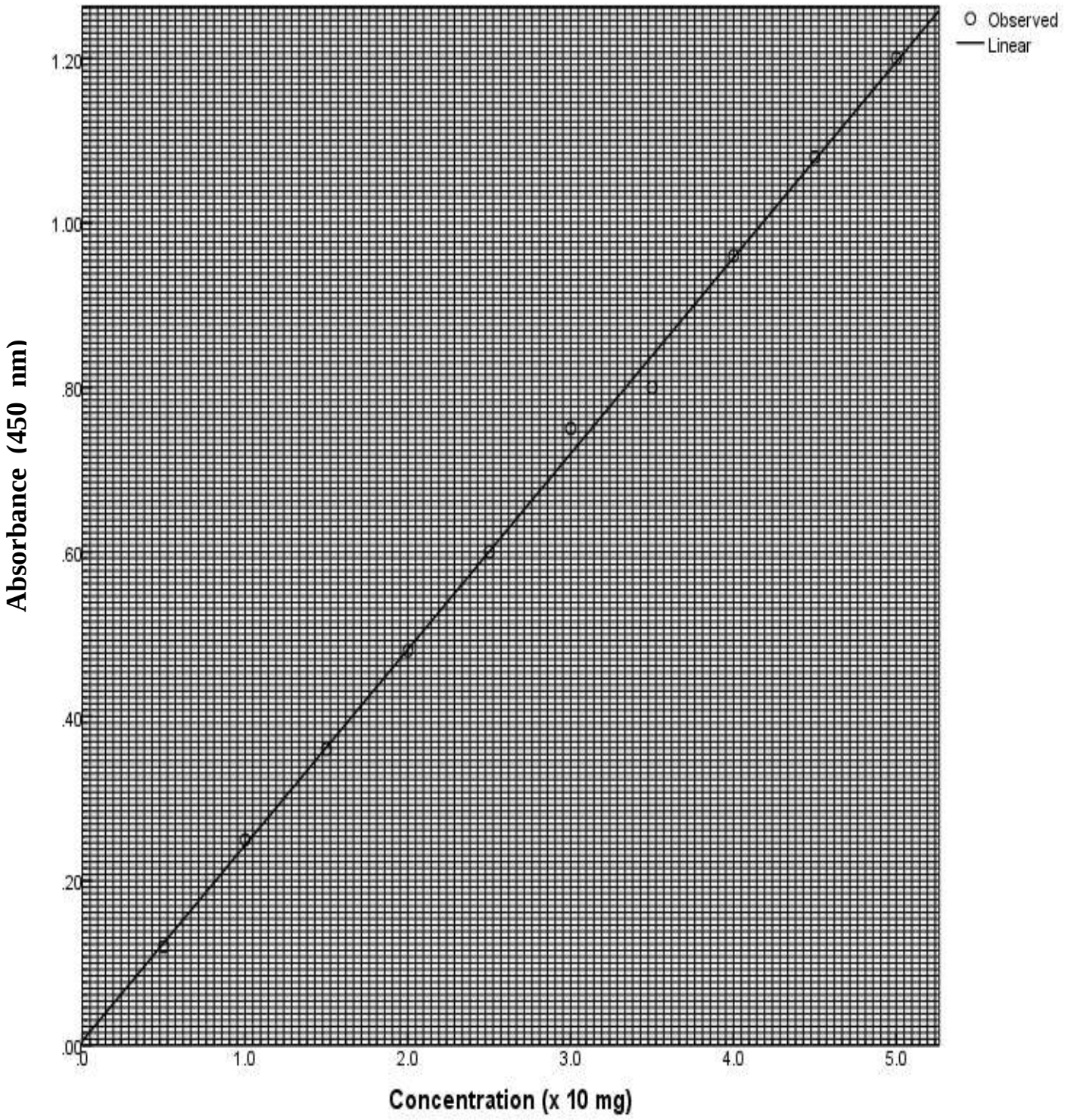
APPENDIX IV

STANDARD CURVE OF USED ENGINE OIL



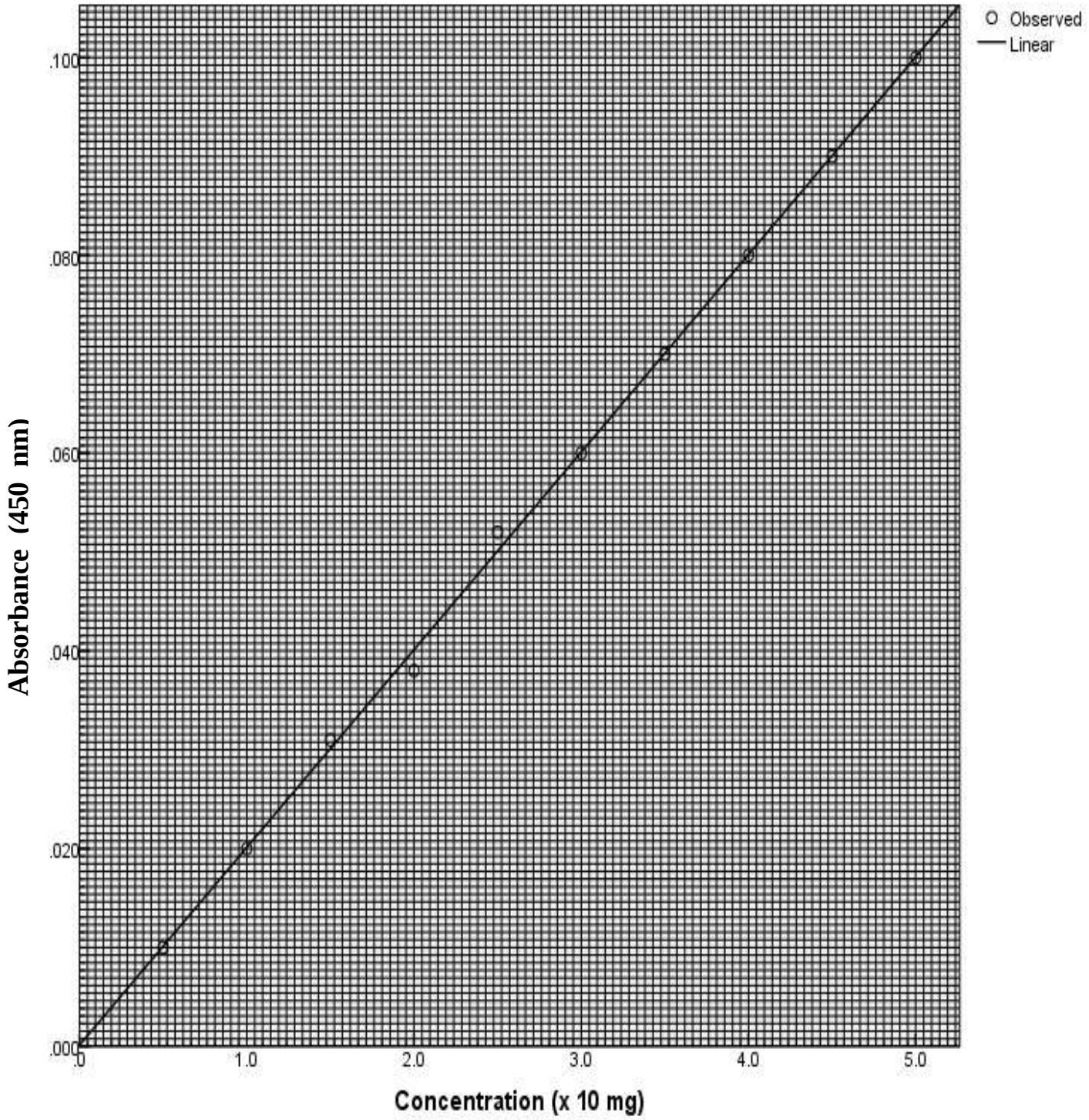
APPENDIX V

STANDARD CURVE OF DIESEL



APPENDIX VI

STANDARD CURVE OF PETROL



APPENDIX VII

GENE SEQUENCE OF THE IDENTIFIED ISOLATES

Hello Tony,

we have now sequenced the ITS of the four isolates and the results are attached. In short:

Isolate G: Candida tropicalis
Isolate H: Rhodosporidium toruloides
Isolate I: Fusarium oxysporum
Isolate J: Aspergillus clavatus

Please wait with submitting the sequences to GenBank (if you plan to) as I want to double-check the forward sequence of isolate G. I think that these results are OK for publishing, but if you want to I can make some phylogenetic trees of closely related species so that we can associate the species identification with some statistical support (bootstrap) instead of merely reporting % sequence identity.

What is your plans, do you plan for a manuscript? I will of course be willing to assist with the writing. Let me know if you have any questions.

With best regards,

Magnus

Species identification of four fungal isolates from the Niger delta

Four isolates, G (yeast), H (yeast), I (fungi) and J (fungi), were successfully grown and DNA extracted. PCR amplification and sequencing in both directions of the ITS region was performed.

Isolate G

Morphological characters indicated yeast of type Candida. The forward sequence chromatogram contained some background peaks, but the sequence is reliable. I will double-check this before publication.

>Isolate G ITS

GTAAAAGTGGTACCAAGGTTTCGGTAGGTGAACTTGGGGAAGGATCATTATTGATTTGTTTAATTGCACCACA
TGTGTTTTTTATTGAACAAATTTCTTTGGTGGCGGGAGCAATCCTACCGCCAGAGGTTATAACTAAACCAAACT
TTTTATTTACAGTCAAACTTGATTTATTATTACAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATC

GATGAAGAACGCAGCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTGAACGC
ACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTTCTCCCTCAAACCCCCGGGTTTGGT
GTTGAGCAATACGCTAGGTTTGTTTGAAAGAATTTAACGTGGAAACTTATTTTAAGCGACTTAGGTTTATCCAA
AACGCTTATTTTGCTAGTGGCCACCACAATTTATTTCATAACTTTGACCTCAAATCAGGGAGGAATACCCGCTG
AACTTAA

BlastN in NCBI:

Candida tropicalis voucher MCCC2E00325 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence, 98% identity, E-value = 0.

All top hits are towards Candida tropicalis isolates.

Isolate H

Morphological characters indicated yeast of type Rhodotorula. Perfect sequences.

>Isolate H ITS

TGAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAGTGAATATTAGGGTGTCCA
ACTTAACTTGGAACCCGACCCTCACTTTCTAACCCTGTGCATTTGTCTTGGGTAGTAGCTTGCGTCGGCGAGCG
AATCCCATTTCACTTACAAACACAAAGTCTATGAATGTAACAAATTTATAACAAACAAAACTTTCAACAACGGA
TCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATTCAGTGAAT
CATCGAATCTTTGAACGCACCTTGCGCTCCATGGTATTCCGTGGAGCATGCCTGTTTGAGTGTCATGAATTCTT
CAACCCACCTATTTCTTAGTGAATCAGGCGGTGTTTGGATTCTGAGCGCTGCTGGCCTCACGGCCTAGCTCGCT
CGTAATGCATTAGCATCCGCAATCGAACTTCGGATTGACTCGGCGTAATAGACTATTCGCTGAGGATTCTGGT
CTCTGACTGGAGCCGGGTGAGATTAAAGGAAGCTACTAATCCTCATGTCTATCTTGAGATTAGACCTCAAATC
AGGTAGGACTACCCGCTGAACTTAAGCAT

BlastN in NCBI:

Rhodosporidium toruloides strain K-1-8 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence, 100% identity, E-value = 0.

Isolate I

Morphological characters indicated ascomycete of type Microsporidium. Perfect sequences.

>Isolate I ITS

GGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTTACAACTCCCA
AACCCCTGTGAACATACCACTTGTTGCCTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGA
GGACCCCTAAACTCTGTTT

CTATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA
TGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC
ATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACAGCTTGGTGTT
GGGACTCGCGTTAATTCGCGTTCCCCAAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAAC
CCTCGTTACTGGTAATCGTCGCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGG
AATACCCGCTGAACTTAA

BlastN at NCBI:

Fusarium oxysporum f. sp. cumini strain F11 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence, 100% identity, E-value = 0.

Most top hits are towards Fusarium oxysporum.

Isolate J

Morphological characters indicated ascomycete of type Aspergillus. Perfect sequences.

>Isolate J ITS

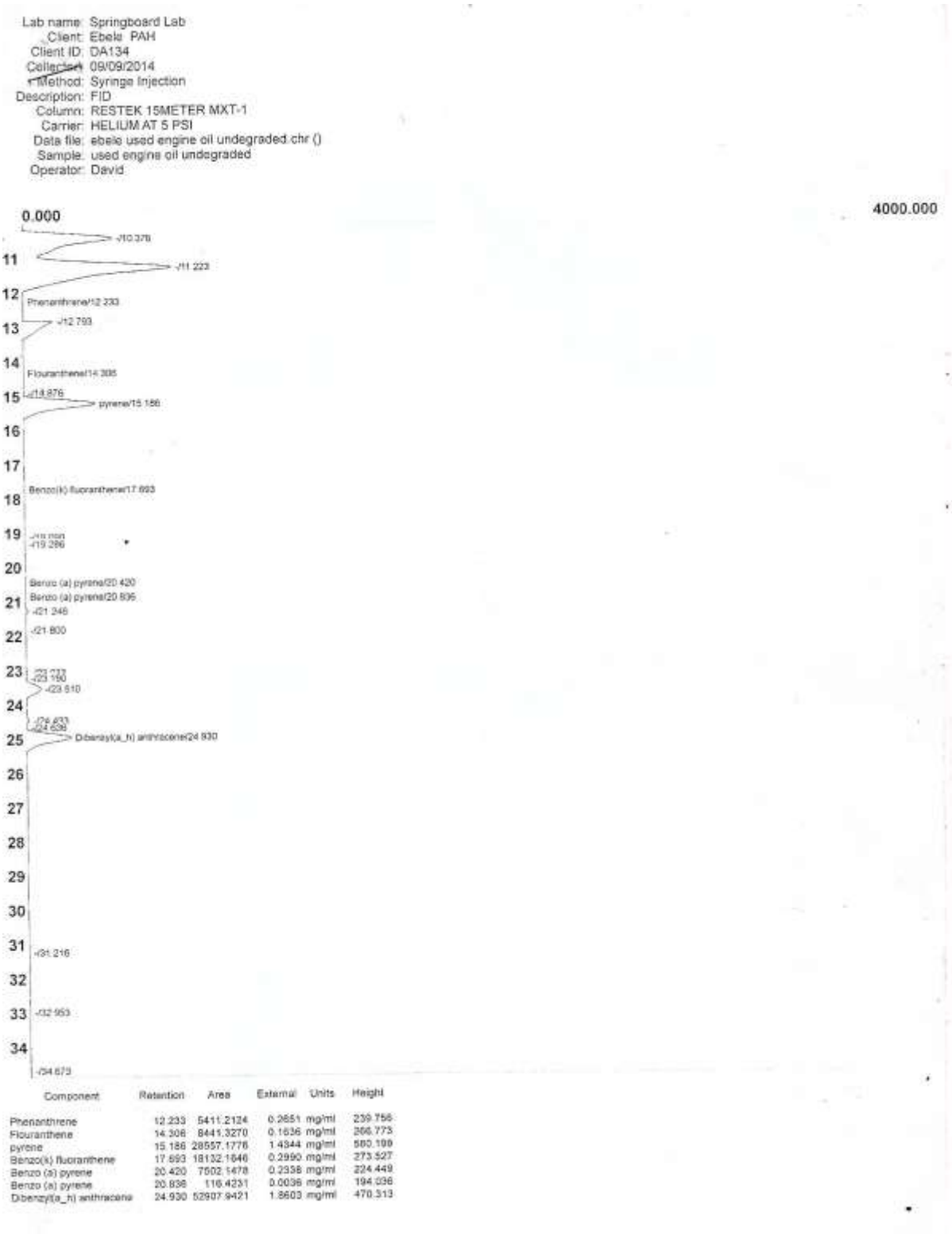
AGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGCGGGCCCTCTGGGT
CCAACCTCCCACCCGTGTTTATCGTACCTTGTTGCTTCGGCGGGCCCGCCGTCTTCGGACGGCCGCCGGGGAG
GCCTCCGCGCCCCCGGGCCCGCGCCCGCCGAAGACCACAACATGAACTCTGTTCTGAAGTTTTGCAGTCTGAG
TTGATTATCATAATCAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAA
ATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTAT
TCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCCCCCGTCCCCGG
TTTCCCGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACC
CGCTCTTGTAGGGCCGGCCGGCGCCTGTCGACACCAACCCAATTTTTCTAAGGTTGACCTCGGATCAGGTAGG
GATACCCGCTGAACTTAA

BlastN at NCBI:

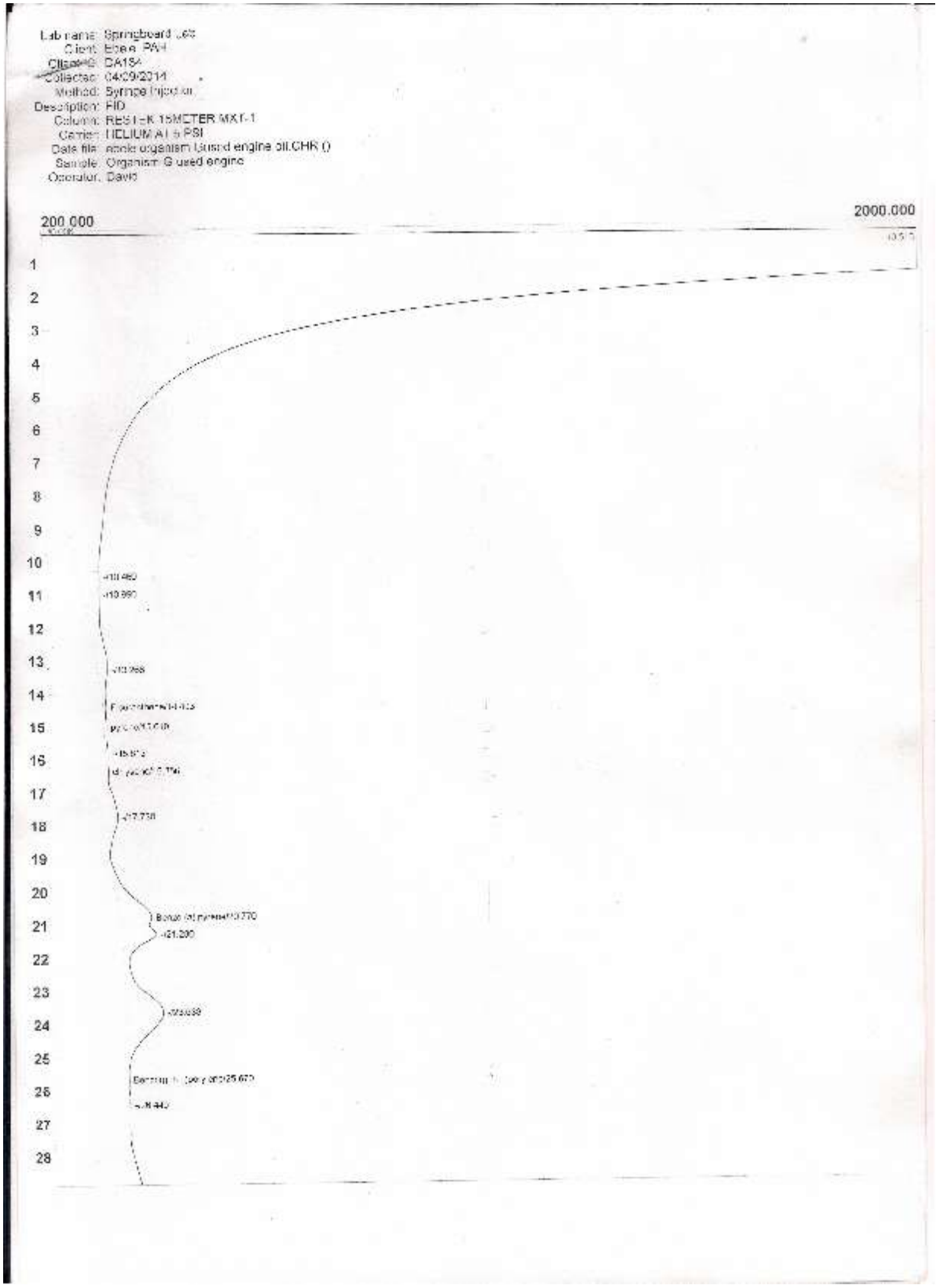
Aspergillus clavatus ATCC 1007 ITS region; from TYPE material, 100% identity, E-value = 0.

APPENDIX VIII

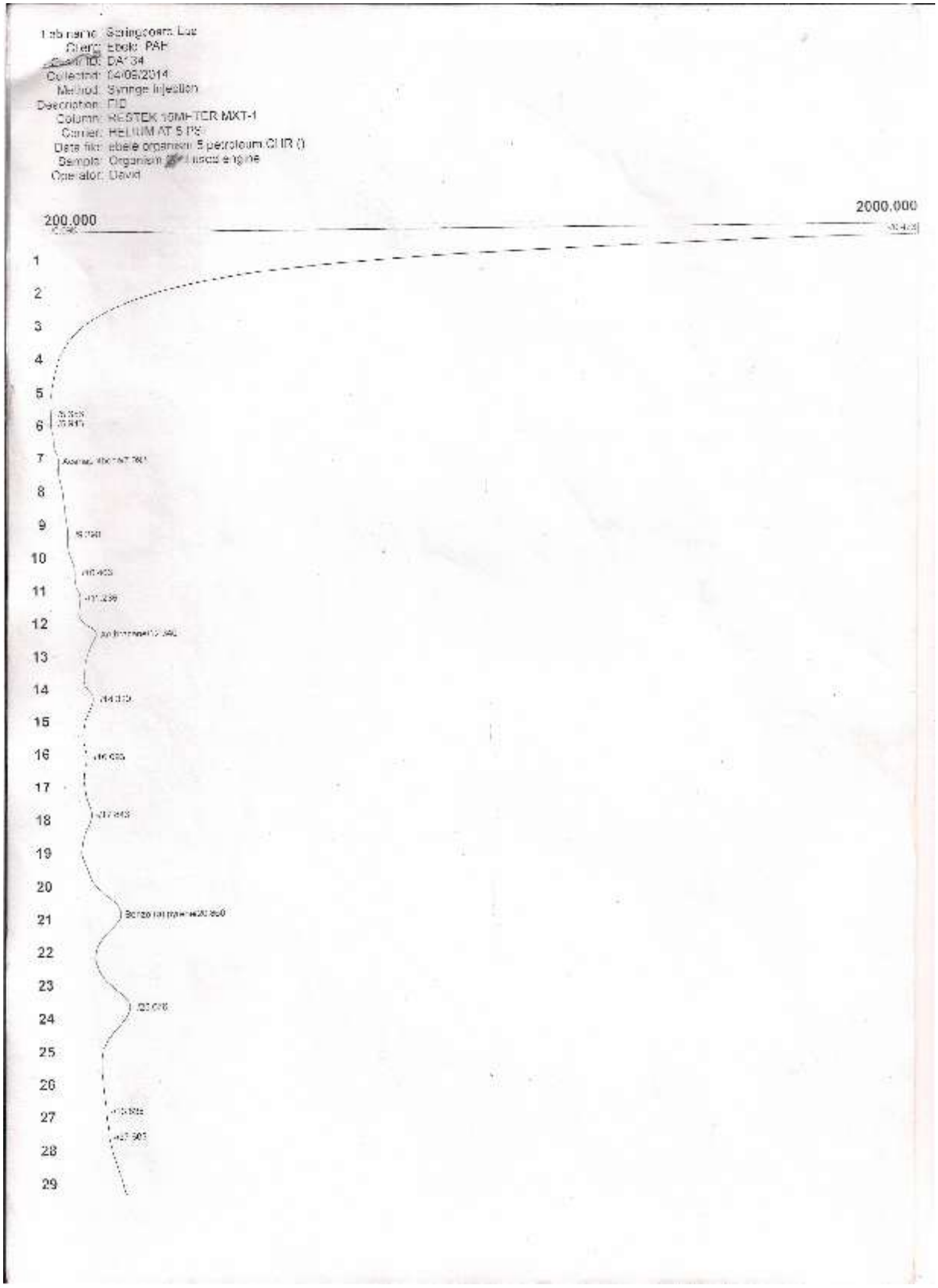
THE CHROMATOGRAM OF THE GAS CHROMATOGRAPHIC ANALYSIS



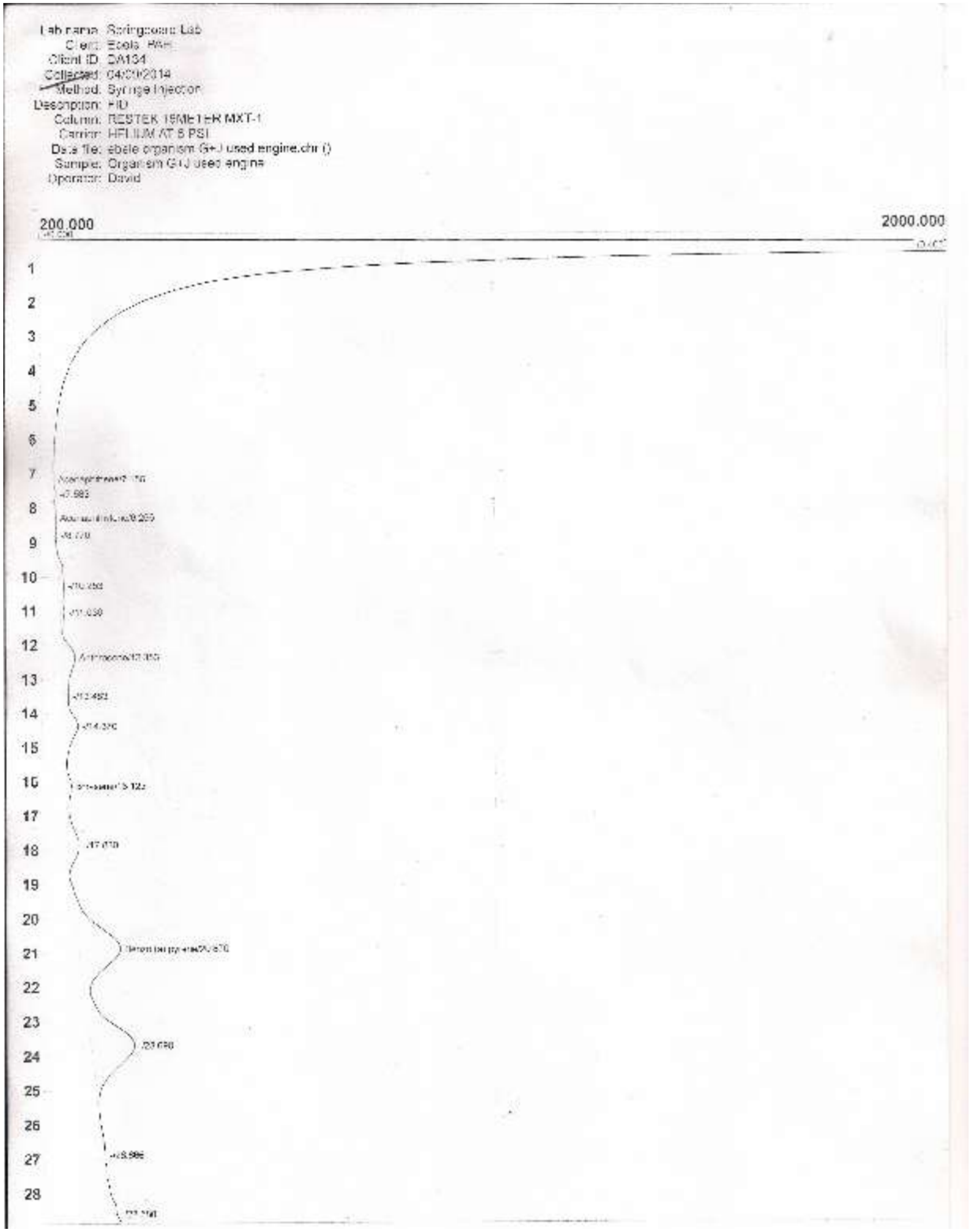
Gas chromatogram of PAHs present in undegraded used engine oil (control).



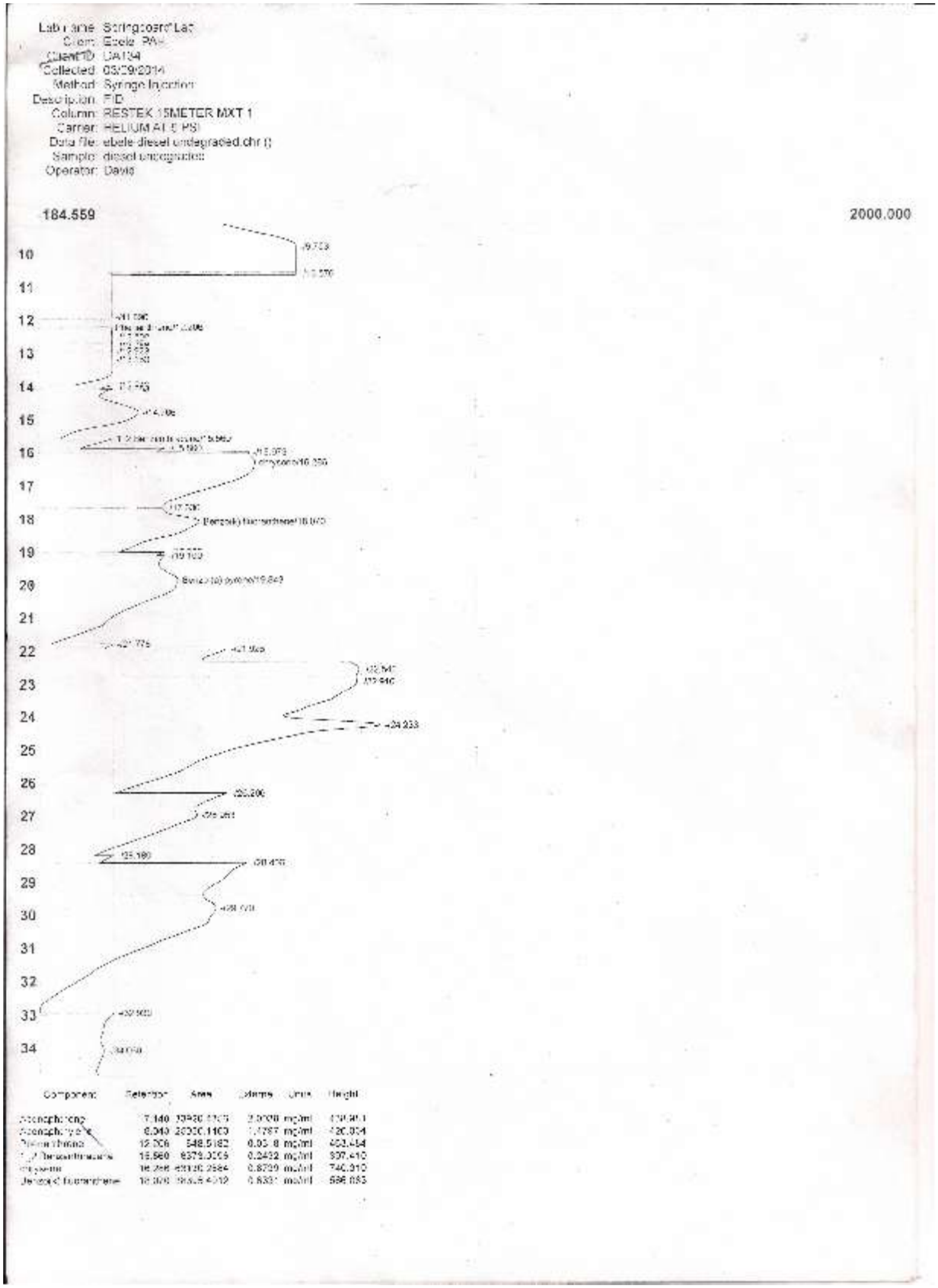
Gas chromatogram of PAHs removal in used engine oil after 16 days in the presence of *Candida tropicalis*.



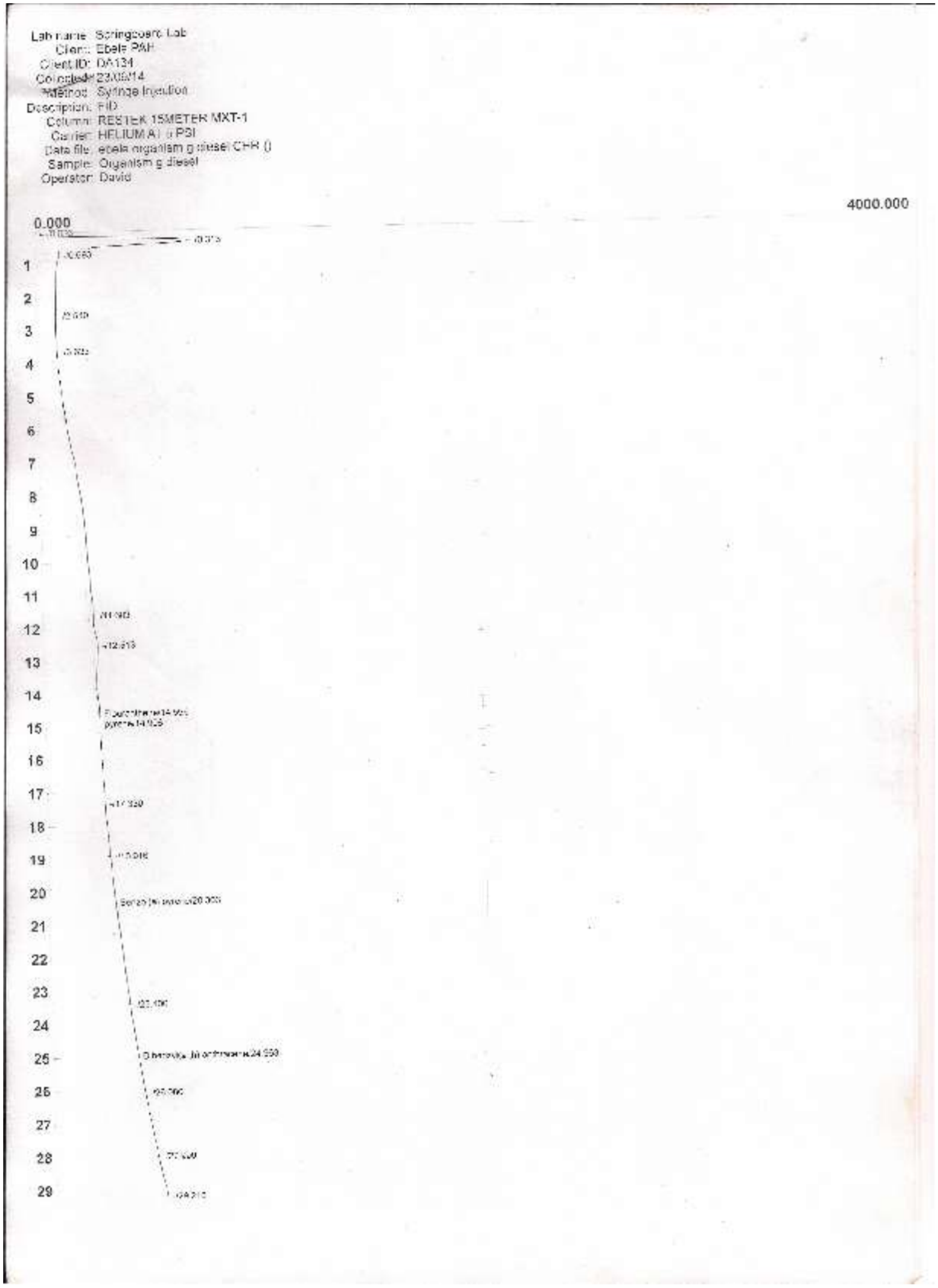
Gas chromatogram of PAHs removal in used engine oil after 16 days in the presence of *Aspergillus clavatus*.



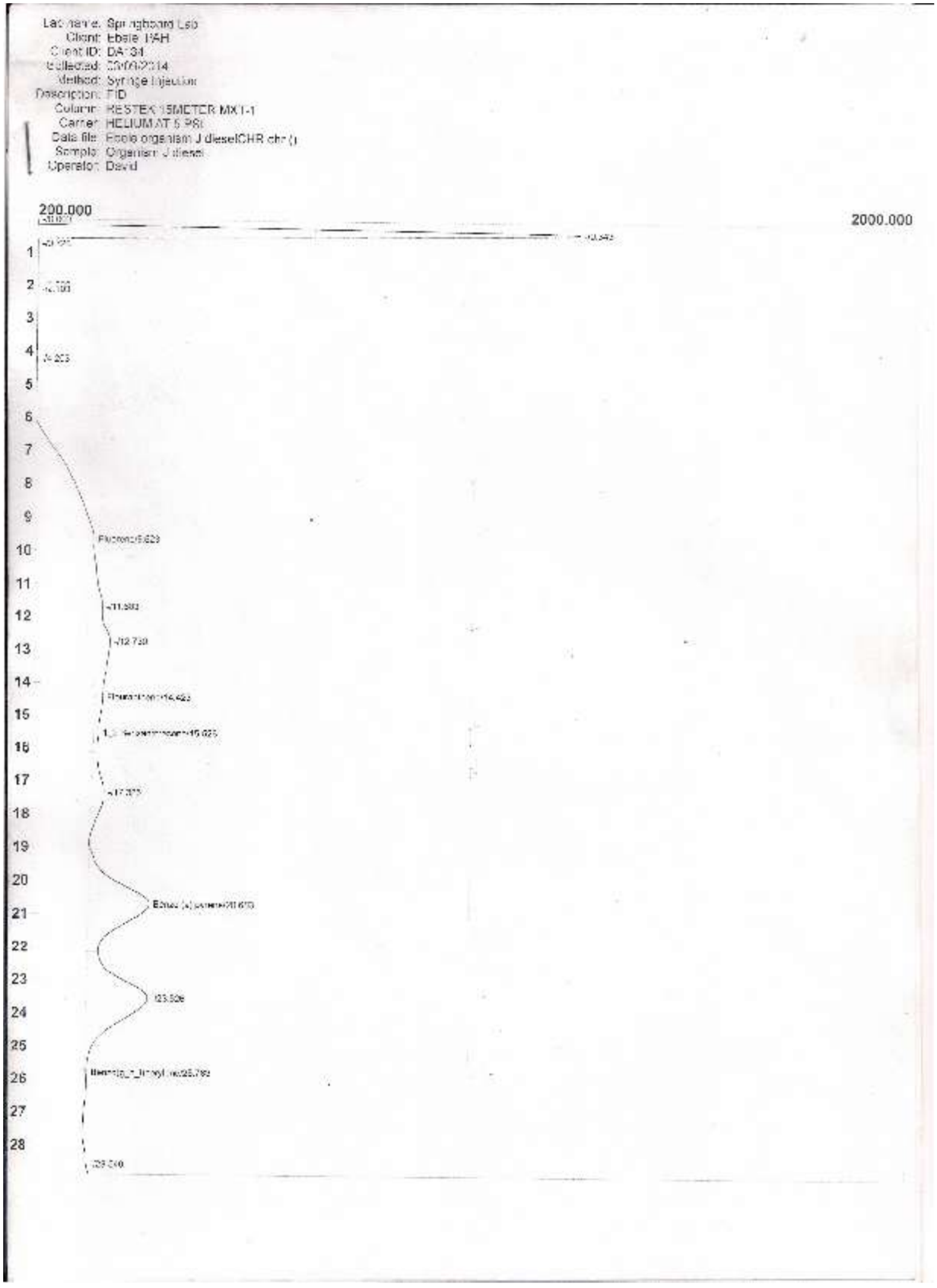
Gas chromatogram of PAHs removal in used engine oil after 16 days in the presence of the mixed culture of *Candida tropicalis* and *Aspergillus clavatus*.



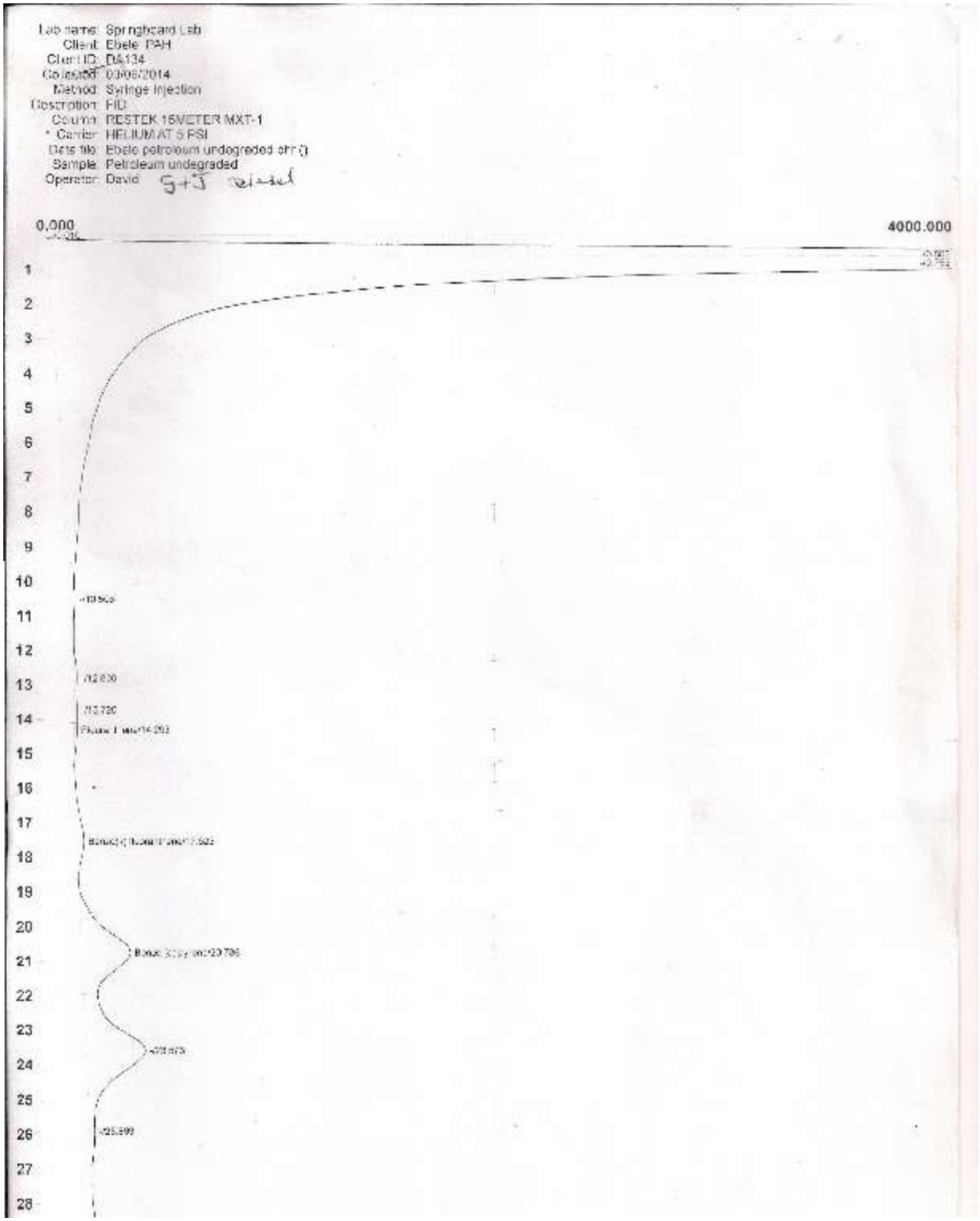
Gas chromatogram of PAHs present in undegraded diesel (control).



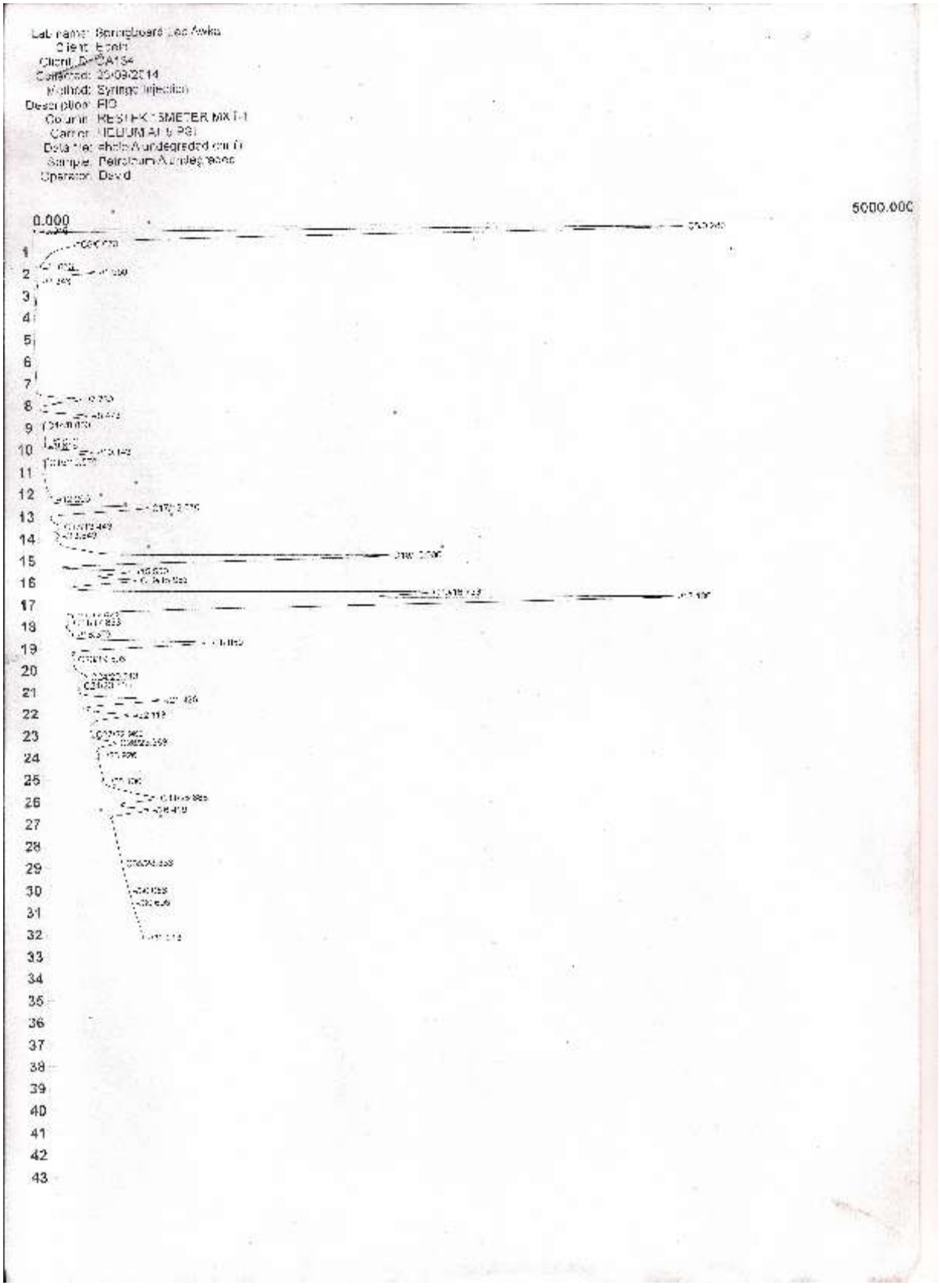
Gas chromatogram of PAHs removal in diesel after 16 days in the presence of *Candida tropicalis.*



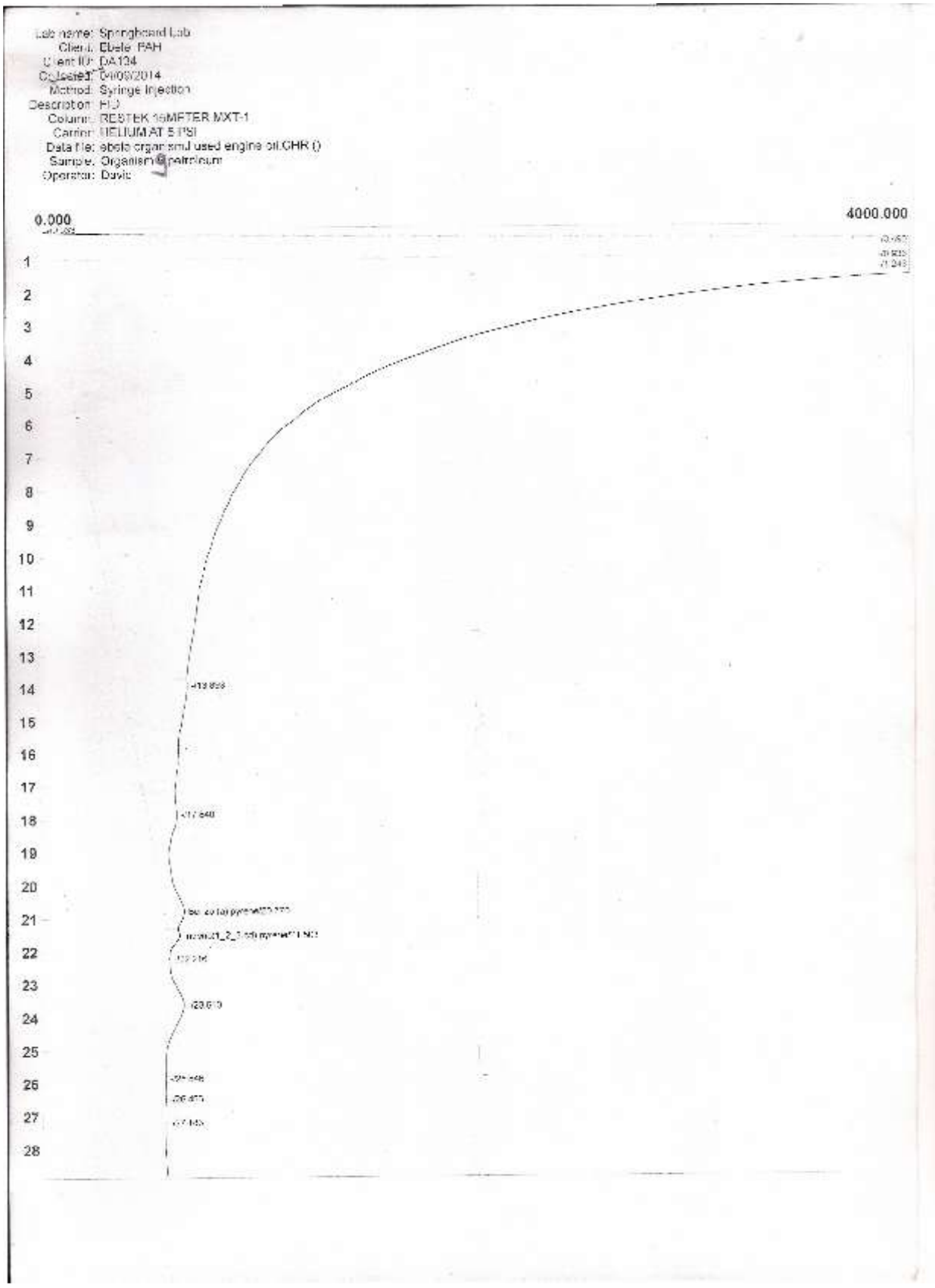
Gas chromatogram of PAHs removal in diesel after 16 days in the presence of *Aspergillus clavatus*.



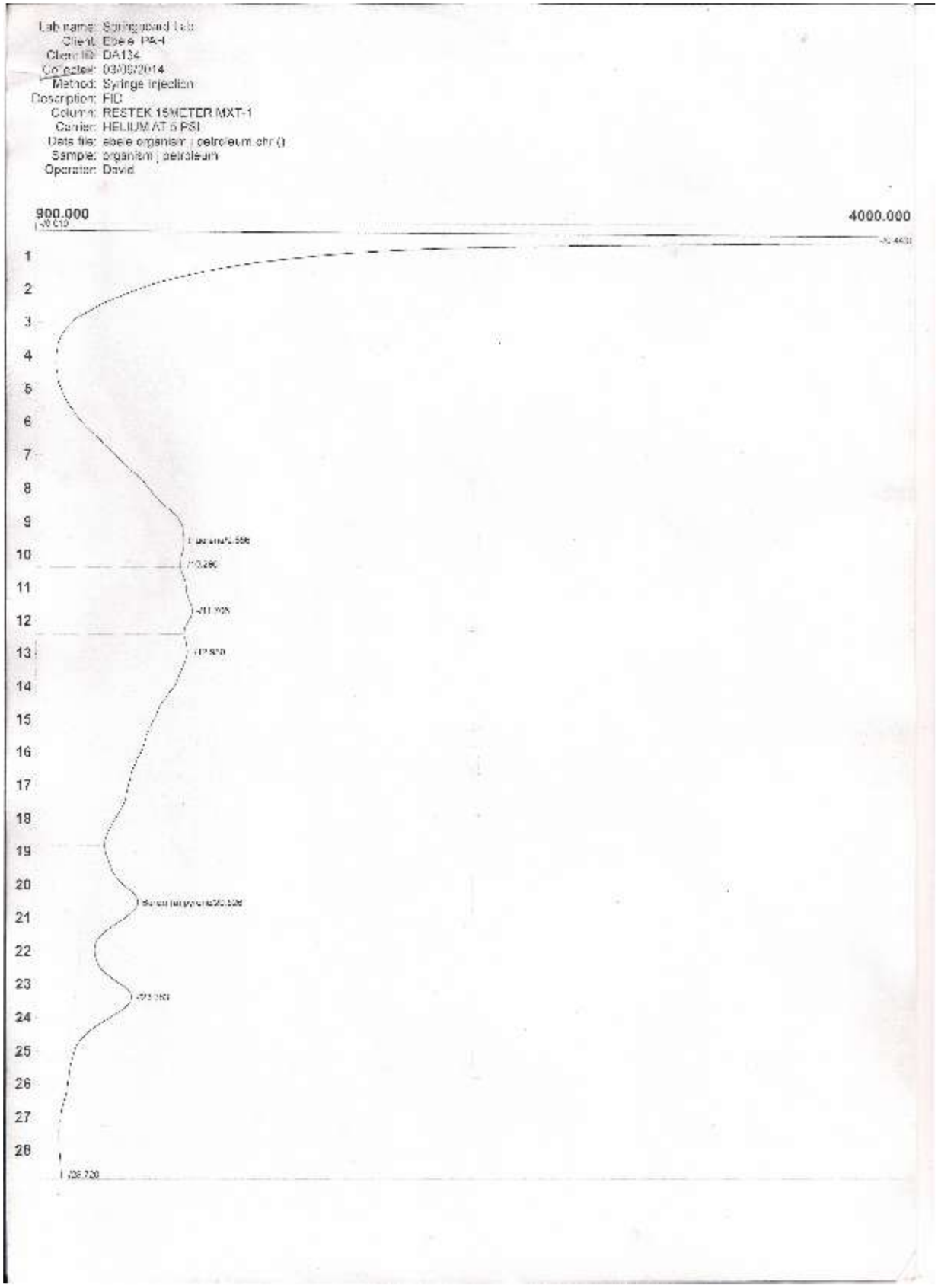
Gas chromatogram of PAHs removal in diesel after 16 days in the presence of the mixed culture of *Candida tropicalis* and *Aspergillus clavatus*.



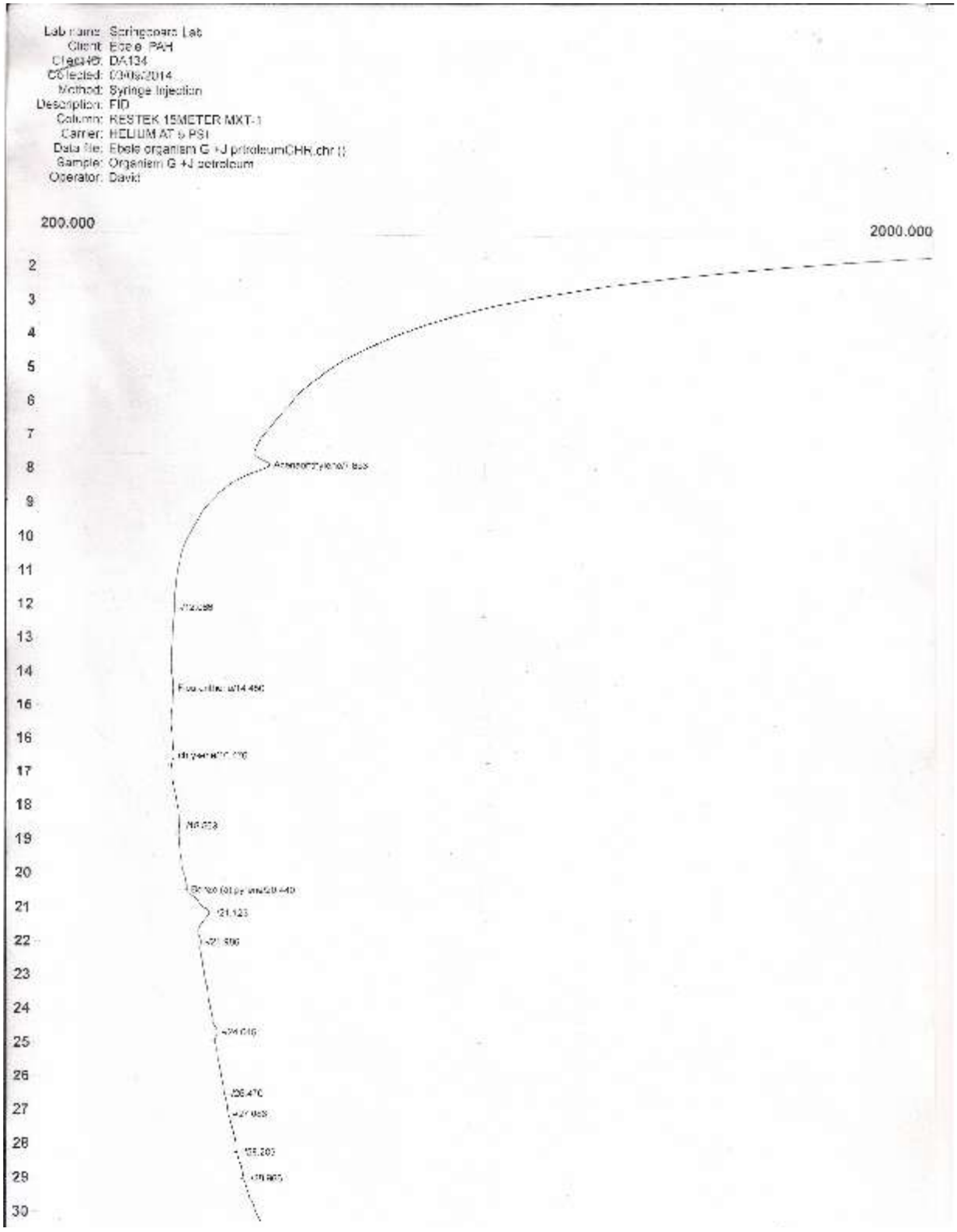
Gas chromatogram of PAHs present in undegraded petrol (control).



Gas chromatogram of PAHs removal in petrol after 16 days in the presence of *Candida tropicalis*.



Gas chromatogram of PAHs removal in petrol after 16 days in the presence of *Aspergillus clavatus.*



Gas chromatogram of PAHs removal in petrol after 16 days in the presence of the mixed culture of *Candida tropicalis* and *Aspergillus clavatus*.

APPENDIX IX

ZONE OF CLEARANCE IN OIL DISPLACEMENT AREA (ODA) OF THE ISOLATES IN USED ENGINE OIL, DIESEL AND PETROL.

Plate 1: Zone formation in used engine oil by biosurfactant producing fungi *Candida tropicalis* in oil spreading technique.

Plate 2: Zone formation in used engine oil by biosurfactant producing fungi *Aspergillus clavatus* in oil spreading technique.

Plate 3: Zone formation in diesel oil by biosurfactant producing fungi *Candida tropicalis* in oil spreading technique.

Plate 4: Zone formation in diesel oil by biosurfactant producing fungi *Aspergillus clavatus* in oil spreading technique.

Plate 5: Zone formation in petrol by biosurfactant producing fungi *Candida tropicalis* in oil spreading technique.

Plate 6: Zone formation in petrol by biosurfactant producing fungi *Aspergillus clavatus* in oil spreading technique.