CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Contamination of the environment is associated with hydrocarbon pollution most times due to the increasing demand for petroleum hydrocarbons and petroleum products globally. Pollution of the soil by hydrocarbons is hazardous to the ecosystem as it results in reduction of plant nutrients, bioaccumulation of pollutants in plants as well as bioaccumulation and biomagnification in animal tissues thus; leading to acute or chronic health effects, mutations and consequently, death (Adetitun *et al*., 2019). Prolonged exposure to high oil concentration may cause the development of liver or kidney diseases, possible damage to the bone marrow and increased risk of cancer (Mishra *et al*., 2001; Lloyd and Cackette, 2011).

Pollution problems have led to the exploration of many remedial approaches to facilitate the cleanup of the polluted soils (Ekanem and Ogunjobi, 2017). The commonly used techniques for soil remediation although expensive, include mechanical embedding, evaporation, dispersion, and washing. These can lead to incomplete degradation of contaminants (Das and Chandran, 2010). The removal of petroleum hydrocarbon pollutants from the environment by applying oleophilic microorganisms (oil loving individual isolate/consortium of microorganisms) is ecofriendly and economical.

Many microorganisms have the ability to utilize hydrocarbons as sole source of carbon, as energy for metabolic activities (Ekanem and Ogunjobi, 2017). The microbial utilization of hydrocarbons depends on the chemical nature of the compounds within the petroleum mixture and on environmental determinants (Adeline *et al*., 2009). The major microorganisms that consume petroleum hydrocarbons are known as fungi and bacteria. The quiescent qualities of microorganisms indicated as degrading agents of several compounds point to microbial treatment as being the most significant alternative for decreasing the ecological impact of oil spills (Facundo *et al*., 2001, Robert *et al*., 2003).

Petroleum hydrocarbons do not degrade easily. When hydrocarbon-containing contaminants spill on land, degradation by indigenous microorganisms progresses slowly due to inadequate nutrients and microorganism populations (Abed *et al.,* 2014), hence the need to focus on microbial enzymes is considered as alternative. Lipase-producing microorganisms have been found in diverse habitats such as contaminated with oil ,industrial wastes, vegetable oil processing factories, dairies, soil, oilseeds and decaying food, compost heaps, coal tips and hot springs (Mobarak-Qamsari *et al.,*2011).

Lipase are enzymes produced by many microorganisms such as bacteria, fungi, yeasts and actinomyces. Many microorganisms are known as potential producers of extracellular lipases, including bacteria, yeast, and fungi (Abada, 2008). Microbial lipases have gained special industrial attention due to their selectivity, great variety of catalytic activities, easy to manipulate genetically and capable of rapid growth on inexpensive media (Jinyong, 2014). Microbial enzymes are also more stable, convenient and safer than animal and plant enzymes (Veerapagu *et al.,* 2013).

Microbial lipases are one of the most important extracellular enzymes which have been the main focus of scientific research due to its huge biotechnological usage over the years (Ullah *et al*., 2015). Therefore, the objective of this study was to isolate, screen, characterize using morphological, biochemical and molecular methods and optimize appropriate lipase producing conditions for oil degrading bacterium that would be best suited to degrade benzene, phenol and hexane.

1.2. Biodegradation

Biodegradation is the process by which organic substances are broken down into smaller compounds by living microbial organisms (Marinescu *et al.,* 2009). Complete biodegradation is known as mineralization. Although, in most cases the term biodegradation is generally used to describe almost any biologically mediated change in a substrate (Bennet *et al.,* 2002).

The procedure of biodegradation requires an understanding of the microorganisms that make the process work. The microorganisms transform the substance through enzymatic processes or through metabolism. It is based on two processes: growth and co-metabolism. In growth, an organic pollutant is used as sole source of carbon and energy. This process results in a mineralization of organic pollutants. Co-metabolism is defined as the metabolism of an organic

compound in the presence of a growth substrate that is used as the primary carbon and energy source (Fristche and Hofrichter, 2008).

Several microorganisms, including fungi, bacteria and yeasts are involved in biodegradation process. Algae and protozoa reports are scanty regarding their involvement in biodegradation (Das and Chandran, 2011). Biodegradation processes vary greatly, but frequently the final product of the degradation is carbon dioxide (Pramila *et al.*, 201). Organic material can be degraded with or without oxygen (Fritsche and Hofrichter, 2008, Mrozik *et al.*, 2003).

The term biodegradation is often used in relation to ecology, waste management and mostly associated with environmental remediation (bioremediation) (Marinescu *et al.*, 2009). Bioremediation process can be divided into three phases or levels. First, through natural attenuation, contaminants are reduced by native microorganisms without any human augmentation. Second, bio-stimulation is employed where nutrients and oxygen are applied to the systems to improve their effectiveness and to accelerate biodegradation. Finally, during bio-augmentation, microorganisms are added to the systems. These supplemental organisms should be more efficient than native flora to degrade the target contaminant. A feasible remedial technology requires microorganisms being capable of quick adaptation and efficient uses of pollutants of interest in a particular case in a reasonable period of time (Seo *et al.*, 2009).

1.3. Bioremediation.

Microorganisms are widely distributed on the biosphere because their metabolic ability is very impressive and they can easily grow in a wide range of environmental conditions making it easy for microbial trophic diversity to be exploited for the biodegradation of contaminants (Leung, 2004). This process is called bioremediation and it is based on the ability of certain microorganisms to transform, modify, and utilize toxic pollutants to generate energy and biomass in the process.

Bioremediation is a well-organized microbiological system or a structured activity used to break down or transform contaminants into less or non-toxic forms of elements and compounds, rather than simply collecting and storing contaminants. Bioremediation is a microorganism mediated transformation or degradation of contaminants into nonhazardous or less-hazardous

substances. The employability of various organisms like bacteria, fungi, algae, and plants for efficient bioremediation of pollutants has been reported (Vidali, 2001; Leung, 2004).

The process of bioremediation mainly depends on microorganisms which enzymatically attack the pollutants and convert them to innocuous products. Bioremediators are biological agents used in bioremediation to repair contaminated sites. Bacteria, archaea, and fungi are typical major bioremediators (Strong and Burgess, 2008). As bioremediation can be effective only where environmental conditions permit microbial growth and activity, its application often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate.

Microorganisms are important tools for removing contaminants from soil, water and sediments mainly because it out performs other protocols for repair procedures. Microorganisms restore the original natural environment and prevent further contamination. The process of bioremediation is a very slow process and only certain species of bacteria and fungi have proven their ability as potent pollutant degraders. Many strains are known to be effective as bioremediation agents but only under laboratory conditions and the limitation of bacterial growth is under the influence of pH, temperature, oxygen, soil structure, moisture and appropriate level of nutrients, poor bioavailability of contaminants, and presence of other toxic compounds.

Bioremediation is an effective technique that takes advantage of the versatility of microbes to completely degrade petroleum compounds into innocuous end products. Apart from being environmentally friendly, the method is also cost effective for treatment of oil pollution compared to physicochemical methods (Geetha *et al.,* 2013).

Although microorganisms can exist in extreme environment, most of them prefer optimal condition a situation that is difficult to achieve outside the laboratory (Dua *et al*., 2002; Dana and Bauder, 2011).

Bioremediation is recognized as a cost-effective treatment technology for oil-contaminated soils (Cerqueira *et al.,* 2014) and is one of the most promising technology for environmental removal of petroleum contaminants. Application of bioremediation as a biotechnological process involving microorganisms to solve and eliminate many contaminant hazards through biodegradation from the environment. The terms bioremediation and biodegradation are rather interchangeable terms.

Petroleum is a diverse compound composed of saturated hydrocarbons. Due to its diversity, petroleum contains several constituents that are divided into different fractions based on their boiling points and petroleum products are divided into four classes: saturates, aromatics, resins and asphaltenes Benzene, phenol, naphthalene, camphor, diesel, kerosene, fuel, etc (Tebyanian *et al*., 2013).

The use of microbes has proven more efficient as they use these hydrocarbons as a carbon source to support growth and clean up polluted areas (Agbogidi *et al*., 2005). Additionally, some of the chemicals in fossil fuels tend to remain in the environment for a long period of time. These persistent contaminants have several health risks to humans, animals, and other living organisms. Petroleum-based products are a principle source of energy for industries and daily life, making them a vital commodity central to the global economy.

Studies have shown that petroleum-based products can primarily be eliminated from the environment by microorganisms such as bacteria, yeast, fungi and microalgae. The speed of degradation is affected by several physical, chemical and biological guidelines such as pH, temperature, nutrient and quantity of hydrocarbon (Santhini *et al*., 2009). Bacteria however play a major role in biodegradation of these hydrocarbon compounds. Some important microbial species with this potential are of the genera *Bacillus, Arthrobacter, Halononas, Pseudomonas, Klebsiella, Proteus* among others (Uzoamaka *et al.*, 2009). These microorganisms completely degrade or mineralize petroleum compounds into non-toxic end products that include carbon dioxide, water or organic acids and methane.

Most bioremediation systems operate under aerobic conditions, but anaerobic environments may also permit microbial degradation of recalcitrant molecules. Both bacteria and fungi rely on the participation of different intracellular and extracellular enzymes respectively for the remediation of recalcitrant and lignin and organo pollutants (Viadli, 2001).

Of importance does biodegradation which involves treating the petroleum pollutants with hydrocarbon degrading microorganisms possess many kinds of enzymes, notably lipases It has been shown to be a viable, relatively low cost, low-technology technique, break down the pollutants in a shorter time and its use is widespread (Frazar, 2011) Successful bioremediation techniques require the right combination of microbes, microbial enzymes and environmental conditions (Boopathy, 2000).

1.4. Degradation of hydrocarbons by microorganisms

A diverse group of micro-organisms (bacteria, fungi and algae) present in soil and aquatic environments possess enzymatic capabilities for complete mineralization of hydrocarbons (Kumar, 2013). The process of bioremediation may be aimed at achieving (a) mineralization, that is, complete oxidation of organic pollutants (b) biotransformation of organic contaminants into small, less toxic intermediates, or (c) reduction of compounds possessing highly electrophilic nitro- and halo- groups into less toxic forms by transfer of electrons from an electron donor usually a sugar or fatty acid to the contaminant.

1.5. Hydrocarbon degrading bacteria

Mandal and coworkers (2012) isolated 324 bacteria belonging to 110 different species from oil contaminated soils and crude oily sludge and these were found to efficiently degrade different fractions of total petroleum hydrocarbons. In a study conducted by Mahjoubi and co-workers, bacteria of the genera *Pseudomonas, Ochrabactrum, Bacillus, Agrobacterium, Stenotrophomonas, Brevundimonas, Gordonia, Acinetobacter, Achromobacter, Microbacterium, Sphingobium, Rhodococcus, Luteibacter, Kocuria* and *Novosphingobium* were isolated from oil contaminated environments (Mahjoubi *et al*, 2013). In another study, bacterial genera Gordonia, Burkholderia, Aeromicrobium, Mycobacterium, Dietzia, And Brevibacterium were isolated from petroleum contaminated soil (Chaillan *et al*, 2004).

1.5.2. Factors Affecting Petroleum Hydrocarbon Degradation

1.5.2.1. Temperature

Temperature is an important factor that effects on the biodegradation of petroleum hydrocarbon (Varjani *et al*., 2014b). Temperature effect overall biodegradation of oil by changing its chemical and physical nature in soil, moreover, rate of metabolism of hydrocarbon can be changed by microorganism and their community (Megharaj *et al*., 2011). Microorganisms that degrade petroleum hydrocarbon work at a specific range of temperatures such as thermophile (above 50ºC), mesophiles (15 ºC – 45 ºC) and psychrophiles (below 20 ºC). However, mostly microorganisms activated at mesothermal temperature range 20 – 35 ºC provide maximum degradation (Liu, 2020).

Slight biodegradation was recorded in the area of the Arctic sea ice as well as in the icy tundra, at insignificant values while the emission of heat is occurring throughout the operations of hydrocarbon degradation. It was reported by (Das and Chandran, 2011) that the lower of temperature from 25 °C to 5 °C leads to reduce the degradation values by around ten times. Moreover, mesophiles have more variety of organisms which use for degradation, so thermophiles and mesophiles are a good choice of microorganisms for degradation. The extreme temperature to sustain the existence of the microorganisms is 71 °C because there is no automatic burning, and the majority of petroleum compounds are burning when exceeding this temperature (Chandra *et al.,* 2013). Although, due to the volatilization of the hydrocarbon molecules, there is limited chance (Pathak and Jaroli, 2014).

1.5.2.2. Nutrients

Nutrients play important role in biodegradation such as iron, oxygen, nitrogen, and Phosphorus and are required to start the biodegradation process by cleaning contaminated environments (Cooney *et al.,* 1985). When liquid petroleum hydrocarbon release in an environment the amount of carbon increase and phosphorus or nitrogen quality reduce that effect oil degradation. Phosphorus and nitrogen in fresh water and seawater cause nutrient-deficient area and then plant require a huge amount of nutrients. It is necessary to increase amount of nutrient for degradation oil pollutant. While large concentration of nutrients inhabits process of biodegradation (Atlas, 1985).

Several scientists worked on nutrient levels and reported NPK (nitrogen, potassium, and phosphorus) high level as an inhibiting factor for degradation specially in the presence of aromatic hydrocarbon. Nutrient type and quality affect degradation mostly biodegradation of hydrocarbon (Singh, 2020). Aerobic microorganisms during degradation use different types of the nutrient include sulphur, manganese, nitrogen, small amount of phosphorus and iron but phosphorus and nitrogen are important nutrients for natural degradation of hydrocarbon

1.5.2.3. Oxygen

Oxygen is most important element for the biodegradation of hydrocarbon. Petroleum degradation's first step starts in presence of oxygen while it requires for overall degradation process as an important element. In aerobic conditions use the large amount of oxygen and their amount reduce in soil microbes uses molecular oxygen for respiration during the overall

degradation pathway, so oxygen is sufficient for the degradation of hydrocarbon (Zhou and Crawford, 1995).

Mostly 3-4 ml oxygen used in the degradation of 1ml hydrocarbon into water and carbon dioxide. The biodegradation process used large quantity of oxygen due to high amount of carbon and hydrogen in petroleum but low amount of carbon dioxide (Fragkou, 2021). Water in lake, ocean and harbors have large concentration of oxygen on surface due to air, water, wave action and wind but oxygen concentration decrease in depth (Xue *et al*., 2015).

In deep water resource, degradation process is anaerobic due to low concentration of oxygen. If petroleum covered with sediments, scatter and go down to deep require longer time for degradation. The presence of oil polls on surface of water stops the reclamation of oxygen that require for degradation process, due to this different mechanical process used to remove oil and make boundary for oxygen penetration (Ward and Overton, 2020).

Oil movement depends on presence of oxygen, type of soil, concentration of moisture and microbes degrading ability that contribute to replacement and re-aeration. All these conditions are important for biodegradation of petroleum hydrocarbon. However, oxygen concentration will be high dependent on microorganism used in degradation (Varjani and Upasani, 2017).

1.6. The Role of Microorganisms on the Biodegradation of Petroleum Hydrocarbon

Rising amount of microbiological research has been devoted to bioremediation of petroleum and petroleum products contaminated sites using microorganisms. Over 200 bacteria, 103 fungi and 14 algal genera, encompassing over 500 species, have been identified as potential degraders of hydrocarbon contaminants (Head *et al*, 2006; Yakimov *et al*., 2007). Notable among these are the bacterial species *Arthrobacter, Flavobacterium, Pseudomonas sp*. (most predominant), *Sphingomonas* (a novel Pseudomonas sp.) and *Acinetobacter* (Uzoamaka *et al*, 2009). Others include *Micrococcus, Alcaligenes, Bacillus, Marcaxella and Comomanas*. Bacteria of the subphyla a-, B- and o-proteobacteria are well established for their hydrocarbon degrading capabilities (Mahjoubi *et al*., 2013).

Microbial methods that are used to monitor the biodegradation of the petroleum hydrocarbon should contain molecular indicators that are chemical, biochemical, and microbiological. The purpose is to measure the activity rates of the microorganisms and to reach

the level of the pollution reduction that is accepted. Biodegradation by *Mycobacterium, Arthrobacter, Pseudomonas, Aspergillus, Rhodococcus, Chlorella, Penicillium, Candida,* and *Cyanobacteria* could be classified as some of the main components for the removal and remediation of the hydrocarbons from the environment (Pandey *et al.*, 2016).

1.7 Enzymes

Enzymes are biological catalysts that facilitate the conversion of substrates into products by providing favorable conditions that lower the activation energy of the reaction (Belguith *et al.*, 2009). An enzyme may be a protein or a glycoprotein and consists of at least one polypeptide moiety. The regions of the enzyme that are directly involved in the catalytic process are called the active sites (Guo and Xu, 2005).

1.7.1 Lipase enzymes

Lipases are glycerol ester hydrolases that have the ability to act on acylglycerols to liberate fatty acids and glycerol (Gupta *et al.*, 2004). They are water-soluble enzymes that act in catalyzing the hydrolysis of ester bonds in water-insoluble lipid substrates (Guo and Xu, 2005). Lipases are ubiquitously produced by the plants (Bhardwaj *et al*., 2001; Belguith *et al.,* 2009); animals (Carriere *et al.,* 1994); and microorganisms (Olempska-Beer *et al*., 2006).

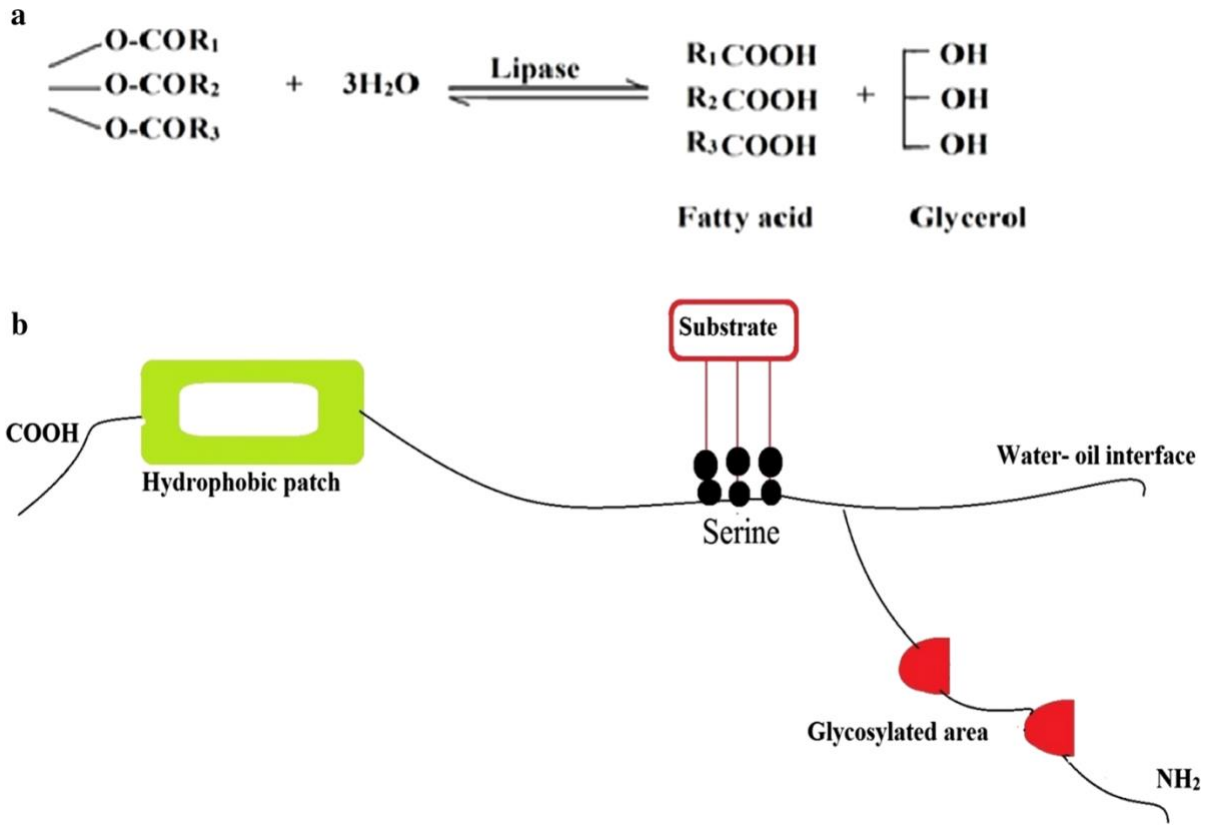
Microbial lipases are the preferred potent source due to several industrial potentials (Hasan *et al*., 2006; Riaz *et al*., 2010; Veerapagu *et al,*. 2013). They are present in many bacteria, fungi, plants, and animals (Rabbani *et al*., 2013; Sugahara *et al*. 2014). A considerable number of lipase producing microorganisms such as *Pseudomonas fragi, Staphylococcus aureus*, and *Burkholderia glumae* have been described (Ghanem *et al.* 2000; Gupta *et al*. 2004; Sangeetha *et al*. 2014).

Lipase degrades lipids derived from a large variety of microorganisms, animals and plants. Research works have shown that lipase is closely related with the organic pollutants present in the soil (Sirisha *et al*., 2010). Lipase activity was responsible for the drastic reduction total hydrocarbon from contaminated soil. Research undertaken in this area is likely to progress the knowledge in the bioremediation of oils spill (Margesin *et a*l., 1999; Riffaldi *et al*., 2006).

Lipases have been extracted from bacteria, plant, actinomycetes, and animal cell (Bornscheuer 2002). 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).

Generally, bacterial lipases are glycoproteins but some extracellular bacterial lipases are lipoproteins (Abdul-Hammid *et al.*, 2013). Previous studies had reported the isolation of extracellular lipase from various type of bacterial species, including Bacillus sp. and Pseudomonas sp. (Bouaziz *et al.*,2011; Sagar *et al.*,2013). 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 (Sagar *et al.*, 2013).



(**a**) Hydrolysis of triglyceride converts into glycerol and fatty acid.

(**b)** Representation of a molecule of lipase with its features

Figure 1. The hydrolysis of triglyceride and representation of a molecule of lipase with its features. (Chandra *et al.*, 2020)

Lipase activity was found to be the most useful indicator parameter for testing hydrocarbon degradation in soil (Margesin *et a*l., 1999; Riffaldi *et al*., 2006). Most common bacterial lipases are reportedly obtained from *Bacillus subtilis, Bacillus pumilus, Bacillus licheniformis, Bacillus coagulans, Bacillus stearothermophilus,* and *Bacillus alcalophilus* (Bhosale *et al*, 2012). In addition, *Pseudomonas aeruginosa, Burkholderia multivorans, Burkholderia cepacia,* and *Staphylococcus caseolyticus* are also reported as bacterial lipase producers (Ertugrul *et al*., 2007). Most commercially important lipase-producing fungi belonged to the genera *Rhizopus* sp., *Aspergillus* sp., *Penicillium* sp.,*Geotrichum* sp., *Mucor* sp. and *Rhizomucor* sp.

1.7.2 Properties and characteristics of lipases

The molecular weight of lipases is in the range of 19–60 kDa and reported to be monomeric protein (Carpen *et al.,* 2019). The position of the fatty acid in the glycerol backbone, chain length of the fatty acid, and its degree of unsaturation are the factors of which the physical properties of lipases count on the sensory and nutritive values of given triglyceride also affected by these features (Tong *et al.,* 2016).

Several lipases catalyze a number of useful reactions such as esterifcation due to their activeness in organic solvents (Damasco *et al.,* 2013). Lipases displayed pH dependent activities, generally at neutral pH 7.0 or up to pH 4.0 and 8.0 lipases are stable, *Chromobacterium viscosum*, *A. niger* and *Rhizophus sp.,* produced extracellular lipases are active at acidic pH, and *P. nitroaeducens* produced alkaline lipase and active at pH 11.0. Under certain experimental conditions lipases have capability to reversing the reactions which leads to esterifcation and interesterifcation in the absence of water (Damasco *et al.,* 2013).

For the expression of lipase activities the cofactors are not necessary but calcium is the divalent cation stimulates the activity. Co, Ni^{2+}, Hg^{2+} and Sn^{2+} inhibited the lipase activities drastically and Zn^{2+}, Mg^{2+}, EDTA and SDS inhibited slightly (Melani *et al.,* 2020). The half-life values determined temperature stability profiles of lipases and lower temperature shows more stability.

According to the region-specificity lipases divided into two groups and revealed with acyl glycerol substrate. Without display of region-specificity only fatty acids are discharged from all three positions of glycerols in the first group of lipases (Priyanka *et al.,* 2019). The fatty acids region-specifcally discharged from the 1, 3 positions of acylglycerols in the second group of lipase

(Gaschler and Stockwell 2017). Triacylglycerol hydrolysed by lipases and constructed 2-monoacylglycerol and free fatty acids 1, 2-(2, 3)-diacylglycerols. In *A. arrhizus, R. delemar, C. cylindracea a*nd *P. aeruginosa* the partial stereo-specifcity have been detected in the hydrolysis of triacylglycerols. These enzymes may be used to extract optically pure esters and alcohols due to these properties (Silveria *et al.,* 2017).

At low water activity using the organic media offers an exceptional prospect over variation of the solvent. So, varying the properties of the solvents an enzyme's specificity may be transformed (Gaschler and Stockwell 2017). Any solvent may utilize a substantial influence on the catalytic properties of an enzyme due to the possession of soft structures and delicate. The biosynthesis lipase may be influenced by various physical and chemical factors, such as agitation rate, temperature and initial pH.

The agitation rate is considered an important factor in the production of lipase (Liu *et al*., 2011). An increase in the agitation rate improves the oxygen transfer rate and mixing efficiency, allowing an increase of cell growth and lipase production. However, high agitation rates may cause shear stress, which leads to negative effects on cell growth and enzyme activity. Liu and co-workers (2011) evaluated different agitation speeds (50 to 400 rpm) on lipase production by *Burkholderia sp.* and observed agitation speed showed significant effects on the overall lipase productivity and cell growth and the maximum overall lipase productivity occurred at 100 rpm.

In other study, Cihangir and Sarikaya (2004) investigated the effect of agitation on the lipase activity produced by *Aspergillus sp*. The cultures conducted with agitation (150 rpm) showed enzymatic activity of 16.50 U/mL, while under static conditions, it reached 6.32 U/mL. The authors reported that the agitation speed also influenced the biomass production. Lipase production by *Aspergillus oryzae* was also influenced by the agitation rate and the best results were found on using 300 rpm in 10-liter fermentor.

Aeration also plays an important role on lipase production by different microorganisms. Lipase production by *Pseudomonas fragi, Pseudomonas aeruginosa* and *Rhizopus oligosporus* was reduced by vigorous aeration (Ghosh *et al.,* 1996). The temperature of incubation is also a critical factor in the production of lipase and may vary in different microorganisms (Ramani *et al.,* 2010). Optimization of the physical parameters for lipase production by using *Arthrobacter sp.* and the study of the effects of different pH values (8.0, 9.0 and 10.0) and temperatures was done (Sharma *et al*., 2009).

1.7.3. Bacterial lipases

Lipase was initially detected in 1901, *B. prodigiosus* and *B. fluorescens*, presently *Serratia marcescens* and *P. fluorescens* are observed today as the best lipase producing bacteria subsequently (Mobarak-Qamsari *et al.,* 2011; Veerapagu *et al.,* 2014). Bacterial lipases square measure a major cluster of enzymes that supply immense potential for various applications, and there's intensive enthusiasm for recognizing and making microorganism lipases. It can be isolated from different plant and animal sources but lipase isolated from microbial sources are considered over other sources (Geoffry and Achur 2018).

In most bacteria, the production of enzyme is affected by the certain polysaccharides. Lipoproteins and glycoproteins are bacterial lipases. (Muthazhagan and Thangaraj 2014). Some bacterial lipases are thermo-stable while most of the bacterial lipases are reported as constitutive and nonspecific in their substrate specificity (Tembhurkar *et al.,* 2012)

1.7.4. Fungal lipases

Fungal lipases have been studied since the 1950's, due to their affluence in pH and thermal stability, activity in organic solvents, substrate specificity, and downstream processing (Iftikhar *et al.,* 2010). Major filamentous genera of fungi that produce lipase enzyme are *Rhizopus, Aspergillus, Penicillium, Mucor, Ashbya, Geotrichum, Beauveria, Humicola, Rhizomucor, Fusarium, Acremonium, Alternaria, Eurotrium* and *Ophiostoma* (Costa *et al.,* 2012).

Other species such as *Candida rugosa, Candida antarctica, T. lanuginosus, Rhizomucor miehei, Pseudomonas, Mucor* and *Geotrichum*. *Colletotrichum gloesporioides* produced 27,700 U/l of lipase are the most productive strain identified from the Brazilian savanna soil through the use of enrichment culture techniques (de Almeida *et al.,* 2013).

1.8. *Bacillus subtilis*

` *Bacillus subtilis is a* rod-shaped and an aerobic bacterium which possess Gram-positive , and catalase-positive properties (Su *et al.,* 2020). *Bacillus subtilis* cells are typically about 4–10 micrometers (μm) long and 0.25–1.0 μm in diameter, with a cell volume of about 4.6 fL at stationary phase (Yu, 2014). As with other members of the genus *Bacillus*, it can produce an endospore, to survive extreme environmental conditions (temperature and desiccation) (Madigan and Martinko, 2005).

Bacillus subtilis has the ability to move quickly in liquids due to the possession of heavy flagella. It secretes numerous enzymes to degrade a variety of substrates, enabling the bacterium to survive in a continuously changing environment (Su *et al.,* 2020). *Bacillus subtilis* has proven highly amenable to genetic manipulation, and has become widely adopted as a model organism for laboratory studies, especially of sporulation, which is a simplified example of cellular differentiation. In terms of popularity as a laboratory model organism, *B. subtilis* is often considered as the Gram-positive equivalent of *Escherichia coli*, an extensively studied Gram-negative bacterium (Ruiz *et al.,* 2022*).*

1.9.Phenol

The heavy production and use of phenol in industrial activities make it a major eco contaminant in most wastewater facilities, such as oil refineries, pharmaceuticals and plastic industries (Whiteley and Bailey, 2000; Arutchelvan *et al*., 2005). Many aquatic life forms, including microorganisms, plants and fishes, face a risk of having cancer (developing cancerous growths) and being mutated in the environments that contain phenol contaminants.

Finding an efficient method to remove phenol effectively has attracted more concern for the environmental remediation and the health welfare of human beings, plants and animals. It has been reported in literature that a few microorganisms can utilize phenol as the sole source of carbon and energy (Paller *et al*., 1995; Barbosa *et al*., 1996), numerous bacterial species have been isolated and characterized as phenol-degrading microbes (Geng *et al*., 2006). These bacterial species include Pseudomonas putida (Müller *et al*., 1996), *Rhodococcus erythropolis* (Prieto *et al.,* 2002), *Bacillus subtilis* (Tam *et al.,* 2006), *Bacillus brevis*, *Serratia marcescens* (Yao *et al*., 2006), *Arthrobacter citreus* (Karigar *et al*., 2006), *Alcaligenes faecalis*, *Sphingomonas* (Liu *et al*., 2009) and *Acinetobacter* (Luckarift *et al*., 2011)

1.10. Benzene

The chemical formula for benzene is C_6H_6, i.e it has 6 hydrogen- H atoms and six-carbon atoms and has an average mass of about 78.112. The structure has a six-carbon ring which is represented by a hexagon and it includes 3-double bonds. The carbon atoms are represented by a corner that is bonded to other atoms (Lide, 2005). Benzene was discovered in the year 1825 by Michael Faraday in illuminating gas. In the year 1834, a German chemist naming Eilhardt Mitscherlich heated the benzoic acid with lime, which produced benzene

Later In the year 1845, a German chemist named A.W. von Hofmann isolated this benzene from coal tar. Benzene is widely utilized chemicals that are involved in various manufacturing products that we use daily such as plastics, detergents, and rubber. Benzene is harmful especially to the tissues that form blood cells. How benzene affects human health depends on how much and how long a person is exposed to it.

Benzene has been recognized as a toxic substance that causes acute and chronic health problems. The inhalation of benzene vapors causes irritation to the eyes and upper respiratory tract. Skin contact with solvents containing benzene causes dry, itching, cracked and fissured skin. Long term inhalation of high levels of benzene results in headaches, dizziness, nausea, convulsions, coma and eventually death. Scientific evidence has established that employee exposure to low levels of benzene can cause leukemia and other diseases of the blood-forming organs, such as multiple myeloma, a cancer of the plasma cells as well as anemia and low blood count which can result in tiredness, lung infections and bruising of the skin.

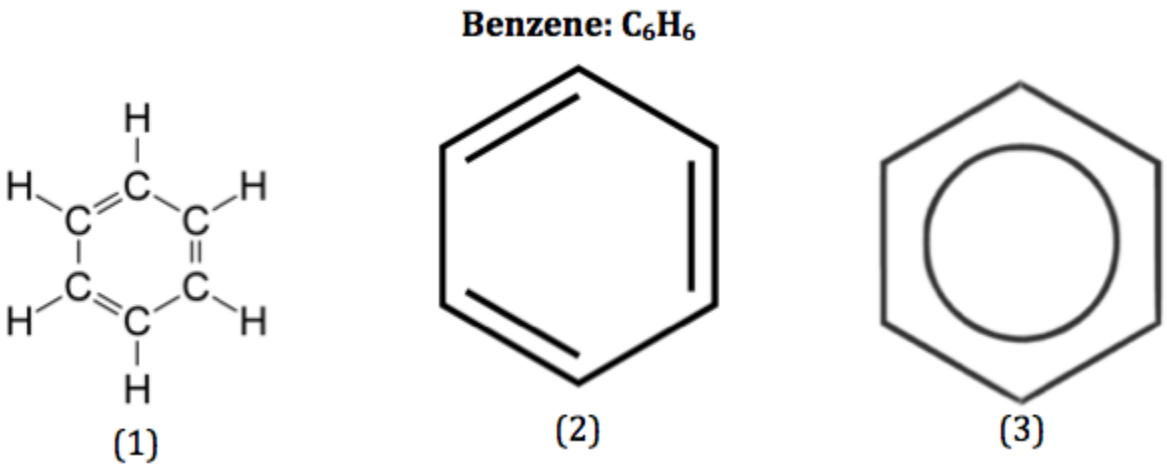


Figure 2. Structure of benzene (Otar and Kinjo, 2020).

1.11. Hexane

Hexane is a component in gasoline and is used as an extractant. The structure of hexane is shown in figure 3. Hexane degradation by bacteria is comparatively not fast due to its low solubility (Lee *et al*., 2010).

Figure 3. Structure of hexane (National Center for Biotechnology Information, 2023)

1.12. Justification

Despite the numerous studies conducted on bioremediation around the world, this study will in addition to previous studies on biodegradation of hydrocarbons reveal the importance of using enzyme produced by indigenous bacteria to remediate and degrade oil contaminated environment.

1.13. Aims and objectives

The aim of this study is to isolate and identify the best lipase producing bacteria from hydrocarbon polluted soil and optimization of the lipase production with capability to biodegrade different hydrocarbons. The objectives of the study include;

i. Isolate bacteria from petroleum contaminated soils

ii. Pre-screen the isolate for their ability to grow using the hydrocarbon (phenol) as their sole source of carbon.

iii. Identify the isolates with positive result in (ii) using biochemical techniques

iv. Screen isolates for their capability to produce lipase enzyme.

v. Use the best isolate to optimize lipase production conditions with

vi. screen the best isolate for ability to degrade phenol, hexane and benzene

vii. Identify best isolate using molecular method.

CHAPTER TWO

2.0. MATERIALS AND METHODS

2.1 Collection of samples

The soil samples were collected into sterile polythene bags from an automobile workshop at Asadam, Ilorin and transported to laboratory for analysis.

2.1.2 Hydrocarbon sample collection

Phenol was collected from the department of Microbiology stock, University of Ilorin. It was of analytical grade. Hexane and benzene were collected from the chemistry department of The Nigerian Stored Products Research Institute, (NSPRI) Ilorin.

2.2 Sterilization of Equipment

All materials used were adequately sterilized prior to use and after use to avoid contamination. Glassware were properly washed with detergent and sterilized in the oven at 170°C for 60 minutes The laboratory bench was swabbed with cotton wool soaked in 70% ethanol. The disposable pipette tips used were sterilized prior to use. Autoclavable materials used were washed and autoclaved for 15 minutes at 121°C. Inoculating loops and straight wire loops were sterilized by heating to redness before and after use. All inoculation and isolation were carried out near an open flame to avoid contamination (Fawole and Oso, 2007).

2.3 Physiochemical parameter of soil sample

2.3.1 Determination of Soil Moisture Content

Five grams of the soil sample was weighed into pre-weighed crucible. The weighed sample was dried in the oven at 80°C for 24 hours and allowed to cool. The loss in the weight of the moisture content was expressed as percentage weight of the sample (Fawole and Oso, 2007).

The moisture content was calculated using the formula below;

$$\text{Moisture content (\%)} = \frac{(W_2 - W_3)}{(W_2 - W_1)} \times 100$$

Where W_1 = weight of crucible only

W_2 = weight of crucible + fresh soil sample

W_3 = weight of crucible + oven dried soil

2.3.2 Determination of Soil pH

Twenty grams of soil was weighed and introduced into a 100 ml beaker. Exactly 20 ml of distilled water was later introduced into the beaker and the suspension was left for twenty minutes, with occasional stirring with a glass rod to enable it reach equilibrium. At the end of twenty minutes, a pH meter was used to determine the pH of the suspension (Jones *et al*., 2000).

2.3.3 Determination of Soil Temperature

The temperature of the soil was determined by inserting a thermometer into the soil. The temperature was read and recorded when a steady temperature was obtained (Jones *et al*., 2000).

2.3.4 Determination of Soil Organic Matter Content

Two crucibles were heated to constant weight in an oven at 105^{o}C for 24 hours and one gram of oven dried soil was put in it. The crucible with the soil were weighed and put in a desiccator to disallow moisture absorption. The sample with the crucible was removed and immediately placed on wire gauze on Bunsen flame supported by tripod stand. It was then heated to give a glowing red coloured soil for 30 minutes with occasional stirring with a mounted needle after which the crucible was placed in a desiccator to cool and then weighed. The heating procedure was repeated after which a constant weight was obtained. The loss in weight represents the organic matter content and this was calculated as percentage of initial weight of the soil sample using the following formula (Fawole and Oso, 2007).

$$\text{Soil organic matter (\%)} = \frac{(W_1 - W_2)}{W} \times 100$$

W = initial weight of oven dried soil.

W_1= weight of crucible + oven dried soil

W_2 = weight of crucible + soil after furnacing

2.4. Preparation of Media

The media used for the isolation and characterization of bacteria from the sample, biodegradation tests and others are Nutrient agar, Mineral salts medium, MR-VP broth, Simmons' citrate agar, Sugar fermentation agar, etc. The preparations of the media were done according to the manufacturers instructions as follows:

2.4.1 Preparation of Nutrient Agar

Nutrient agar was prepared by weighing 28g of nutrient agar powder and dispensing same into 1000 ml of distilled water in a sterile conical flask. The solution was homogenized by heating on a Bunsen burner and mixed intermittently with a spatula. The conical flask containing the agar was plugged with cotton wool and wrapped with an aluminium foil. It was then sterilized in the autoclave for 15minutes at 121°C (Fawole and Oso, 2007).

2.4.2 Preparation of Simmons' Citrate Agar

Exactly 28g of Simmons' citrate agar was suspended into 1000ml of distilled water and the mixture was thoroughly heated to boiling in order to homogenize the mixture. The mixture was then dispensed into test tubes and sterilized at 121°C for 15minutes. The medium was allowed to set in slant (Fawole and Oso, 2007).

2.4.3 Preparation of Media for Sugar Fermentation

Exactly 65 g of the sugar (glucose, lactose, sucrose) were suspended in 1000 ml of sterile distilled water. The mixture was heated to boiling to dissolve the medium completely. The mixture was dispensed into test tubes. They were then sterilized by autoclaving at 121°C for 15minutes (Fawole and Oso, 2007).

2.5. Enumeration of Bacteria

One gram (1g) of the soil sample was added to 9 ml of sterile distilled water in a test tube to make a dilution factor of 10^{-1} and serially diluted to 10^{-2} using sterile pipettes. This was continued until 10^{-5} dilution. 1 ml of the 10^{-5} and 10^{-3} dilution were aseptically pipetted and inoculated in a sterile Petri dish that contained the already autoclaved nutrient agar. The plates were incubated in an inverted position at 37 °C for 24 hrs.

Developed colonies were counted using a colony counter and the number of bacteria per ml of the sample was recorded and expressed as mean cfu m-1. Isolated colonies were purified by

picking and streaking repeatedly on nutrient agar plates to obtain pure cultures. Pure bacterial cultures were kept in slants and stored at 4°C for further use.

2.6. Isolation of phenol utilizing bacteria

The isolated bacteria were inoculated on an enrichment medium using a method by Hasanuzzaman *et al* (2004) that contains mineral salt medium (MSM) supplemented with 1% phenol as the sole carbon source. The components of MSM used are KH2PO4 (0.5g), Na2HPO4 (1.4g), MgSO4.7H2O (0.2g), KNO3 (0.3g), and (NH4)2SO4 (1g) and distilled water (1L). Ten grams of hydrocarbon contaminated soil was added to 90ml of sterile distilled water and then agitated for 2 minutes.

1 ml from the mixture was serially diluted using 10-fold dilution up to 10^{-5}. 1 ml the 10^{-3} and 10^{-5} tubes were plated using pour plate method on sterile mineral salts agar plates containing 2% purified agar and supplemented with 0.5% hydrocarbon and then incubated at 37ºC for 7days. The organisms growing on the agar plates are purified and isolated after which they are preserved on mineral salts agar slants containing the hydrocarbon cuts at 4ºC in the refrigerator (Amanchukwu *et. al*., 1989).

2.7. Biochemical tests

2.7.1. Indole test

The indole test screens for the ability of an organism to degrade the amino acid tryptophan and produce indole. It is used as part of the IMViC procedures, a battery of tests designed to distinguish among members of the family Enterobacteriaceae. The indole test is done by inoculating the test organism into tryptophan soya broth which contain tryptophan which is prepared by following the manufacturer's (Biomark) guide. After 48hrs of incubation KOVAC's reagent is added into the broth and if indole positive a cherry red color ring is indicative.

2.7.2. Methyl Red test (MR- Test)

This test is among the tests that are used to differentiate among the Gram-Negative bacilli in the family Enterobacteriaceae. The test measures the final acidity of a culture in buffered medium containing glucose and peptose. The MR VP medium is prepared following the manufacturer's (Biomark) guide at 121°C for 15minutes. After incubation, the pH indicator Methyl Red is added to the broth. Methyl Red is red at pH below 4.4 (this would be a positive result) and yellow at pH above 6.0. An orange color indicates an intermediate pH and would be considered a negative result.

2.7.3. Voges-Proskauer (VP) test

This is a test used to detect acetoin in a bacterial broth culture. The test is performed by preparing the Voges-Proskauer broth and autoclaving it at 121°C for 15minutes following the instruction of the manufacturer (Biomark). It is then inoculated with bacteria and incubated for 24 hours after which alpha naphthol and potassium hydroxide is added to the broth culture. A cherry red color indicates a positive result, while a yellow- brown color indicates a negative result (Cappuccino *et al* 1996).

2.7.4. Citrate utilization test

The citrate test is performed by inoculating microorganism into organic synthetic media, "Simons Citrate broth" which is prepared following the instruction of the manufacturer by dissolving 24.2g of powder in 1L, homogenise by heat and autoclaving at 121°C for 15minutes.

Bromothymol blue is used as an indicator. When the citric acid is metabolized, carbon dioxide is generated which combines with sodium and water to form sodium carbonate which is an alkaline product which is responsible for change in color from green to blue and this constitute positive test (Cappuccino *et al* 1996).

2.7.5 Catalase production test

In this test, the organism is subjected to 3% H_2O_2 solution and catalase enzyme acts on it just as it could H_2O_2 is produced inside the cell. Catalase lies close to the cell membrane. A positive result is detected by the formation of air bubbles. The test is done to check whether the test

organism produce catalase or not. A drop of the reagent was dropped on a clean glass slide and the bacterial colonies was put on it. Effervescence formation was observed for positive organisms (Cappuccino *et al* 1996).

2.7.6. Urease test

This test distinguishes members of genus Proteus from other lactose non fermenting enteric microbes. Urease test is performed by growing test organisms on urea broth or agar medium containing pH 6.8 indicator phenol red. During incubation microorganism processing urease will produce ammonia that raises the pH of the medium to pH 8.1 or greater.

The test is done if the given organism produces enzyme urease or not. Urea agar (basal medium) was prepared and dispensed into tubes and sterilize. Glucose and phenol red is added to the basal medium and steamed for 1 hour and filtered sterilized urea solution was added and all the contents was mixed well and dispense into sterile test tubes. The test organisms are inoculated and incubated at 37oc for 24-48 hours. Color change is observed.

2.7.7. Fermentation of carbohydrate

This test is performed to check the ability of bacteria to ferment sugar. The fermentation medium used which is a nutrient broth was prepared by dissolving 13g of nutrient broth in 1L of distilled water in a conical flask and 0.5g of the sugars viz.: lactose, glucose, fructose, sucrose. sorbitol were added to form a sugar nutrient broth mixture. Phenol red was added as indicator.

10ml of each broth was then dispensed into McCartney bottles and Durham tubes were placed in the bottles in the inverted position ensuring that there is no air/gas space in it. The media were then sterilized by autoclaving at 121°C at 15 Psi for 15 minutes following the manufacturer's (Rapid Labs UK) instruction. This was allowed to cool, then the organisms were inoculated in them and incubated at 37°C for 24hours.Positive tubes had color change and gas production in the Durham's tube (Cappuccino *et al* 1996).

2.7.8. Starch hydrolysis

This test is performed to test the utilization of starch by bacteria by producing the enzyme amylase. Sterile starch agar medium is poured on to the sterile Petri dishes and allowed to solidify. The test organism is streaked on the plate and incubated for 48 hours at 37°C. The plates are flooded with gram's iodine and excess iodine is drained off. Plates are examined for the zone of clearance around the growth for each organism.

2.8 Screening of Lipase-Producing Bacteria

Pure isolated bacterial cultures were screened for lipase activity using tributyrin agar medium (Ahmed *et al.,* 2010). The tributyrin agar medium pH 7.5 was prepared with 5 g/L peptone, 3 g/L yeast extract, 15 g/L agar, 10 ml/L tributyrin in distilled water. The medium was sterilized for 15 min by autoclaving with 15 lb pressure at 121 °C and cooled. The aliquot was transferred to petri dishes and allowed to solidify. A loopful of each pure culture was streaked each onto tributyrin agar plates separately and incubated at 37°C for 24 h (Bharathi *et al.,* 2017). After incubation, the clear zone of hydrolysis around the colonies indicated the presence of lipase activity. Positive cultures that showed maximum zone of hydrolysis were selected for further studies.

2.8.1. Secondary screening

Tween80 agar medium pH7.0 was prepared with 10 g/L peptone, 20 g/L agar, 5 g/L NaCl, 0.1 g/L CaCl2·2H2O, in distilled water, sterilized for 15 min by autoclaving with 15 lb pressure at 121 °C and cooled to 45°C. 10 ml/L of Tween 80 was added to the cool media. Aliquots were transferred to Petri dishes and allowed to solidify. A loopful of each pure culture was streaked onto Tween80 agar plates separately and incubated the inoculated plates at 37°C for 48 h. After incubation, a white precipitate around the colony indicates lipase activity.

2.9. Optimization of culture conditions for maximum lipase production

2.9.1. Determination of optimum incubation time

The organism was grown in nutrient broth containing appropriate 1% oil (olive oil); pH of the medium to was adjusted to 9.0. Cultures were incubated at 30 °C and samples were taken at 3 h interval over a period of 12 h for lipase assay.

2.9.2. Determination of a suitable carbon source

Different kinds of carbon sources (phenol, Tween80, benzene and hexane) were used as they might induce lipase production. Bacterial culture was inoculated into nutrient broth containing each kind of carbon source. Cultures were incubated at 37 °C for 12 h taking samples for lipase assay every 3 h.

2.9.3. Optimum pH

The organism was grown in nutrient broth containing appropriate 1% oil (olive oil); media were adjusted to different pH (3, 5, 7, 9 and 11) and incubated at 30 °C for 12 h.

2.9.4. Determination of suitable nitrogen source

Different kinds of nitrogen sources (Yeast extract, Peptone, Casein, Ammonium Nitrate and Potassium Nitrate) were used in production media as they might affect lipase production. Bacterial culture was inoculated into nutrient broth containing each kind of nitrogen source. Cultures were incubated at 30 °C for 12 h taking samples for lipase assay every 3 h.

2.9.5. Effect of Temperature on Lipase Production

The effect of temperature on lipase activity was determined using the above method. The organism was cultivated in the growth medium at different temperatures, which ranged from 30°C to 90°C for 24 hours. The lipase activity and growth in the culture supernatant were determined.

2.9. Effect of Metal ion on Lipase Production

Lipase activity was also determined in the presence of metal ions by incubating the culture supernatants separately in different flasks with 1mM solution of each salt, i.e., $ZnCl_2$, $MgCl_2$, $CuSO_4$ and $CaCl_2$ at 37°C for 1 h.

2.10. Molecular characterization of the isolate

Molecular analysis based on 16SRNA was done at Inqaba biotec, Ibadan, Nigeria for further identification of the isolate. The processes involved the use of DNA extraction, polymerase chain reaction (PCR) and nucleotide sequencing characterize the isolates.

2.10.1. Extraction of Genomic DNA

The isolates were inoculated into a sterile nutrient broth after which they were incubated for 24hours at 37°C. The extraction of the isolates' DNA was done using kit extraction method. The kit used was the QIAamp DNA Mini Kit (250) cat no. 51306. The procedure involves inoculating a loopful of the organism into a sterile nutrient broth after which it was incubated at 37°C for 24 hours. About 2000microlitre of the broth containing organism was measured into an Eppendorf tube and the tube was placed in an Eppendorf centrifuge and centrifuged for about 10 minutes at 14000rpm. Buffer AE was placed into 70°C water bath. After discarding the supernatant, 180 micro litre of ATL was added to the pellet (to lyse the cell wall) and 20 micro litre of Proteinase K was also added (to remove the protein content). The sample was vortexed and then incubated at 56°C for about 2-3 hours until completely lysed using a shaking heat block set at 500rpm. The tube was briefly centrifuge again to collect condensation. A 200ul of buffer aluminum was added and mixed by vortexing for 15 seconds. The tube was then incubated at 70°C for 10 minutes. The tube was briefly centrifuged again to collect condensation. A 230ul of ethanol was added and mixed by vortexing for 30 seconds, the sample was carefully applied to QIAmp spin column, centrifuged at 600rpm for 1 minute and the spin column was placed in a clean 2ml collection tube and the filtrate was discarded. The sample was centrifuged at full speed for 3minutes and column was placed in a labelled 1.5ml tube containing 200 microlitre of pre-heated (70°C). Buffer AE was afterwards added, the tube was incubated at 70°C for 5 minutes and centrifuged at 600rpm for 1 minute. The filtrate solution (200microlitre) was the placed back into the spin column and then 200microlitre of the pre-heated 70°C buffer AE was added. A tube was incubated at 70°C for 5 minutes again, centrifuged at 600rpm for 1 minute. The spin column was discarded and ran through agarose and Nano Drop to check the quality.

2.10.2. Nano-drop Procedure

3microlitre of elution buffer was dropped on the lower pedester of the spectrophotometer to initiate the process. This was to blank the system. 3microlitre of the isolated DNA was placed on the lower pedester of the spectrophotometer after blanking and measure to obtain the result. The concentration of a good DNA is usually above 100ng/ug and purity should be between 1.8 and 2.0. To further ascertain the quality of the DNA agarose testing was performed on the DNA extracted. The procedure used for this testing was the gel electrophoresis.

2.10.3. Sample preparation

A 3 microlitre of loading dye was added to equal volume of the DNA centrifuged at 1500 rpm for 1 minute. The solidified gel was dropped in the electrophoresis tank; the comb is removed thereby leaving wells on the gel. The DNA was dropped into the well made on the agarose gel and the electrophoretic machine was switched on and allowed to run for some times. The gel was brought out of the tank after about 30-45 minutes and inserted into the ultra violet transilluminator which projects the DNA picture on the screen of the monitor.

2.10.4. Polymerase Chain reaction

PCR is used to amplify a specific region of a DNA strand (the DNA targeted). Most PCR method typically amplify DNA fragments of between 0.1 and 10 base kilo pairs (kbp), although some techniques allow for amplication of fragments up to 40kbp in size (Cheng *et al.*, 1994). The PCR was carried out in a reaction volume of 10-200microlitre in small reaction tubes (0.2-0.5ml volumes) in a thermal cycler. The thermal cyclers heats and cools the reaction tubes to achieve the temperatures require at each step of the reaction. The thermal cycler uses the Peltiere effect which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current. Thin-walled reaction tubes were used so as to prevent thermal conductivity to cause rapid thermal equilibration. Also, the thermal cycle poses a heated lid to prevent condensation at the top of the reaction tube. The reagents used and their concentrations were: primer 1 (for the forward reaction), 0.5microlitres; primer 2 (for the backward reaction), 0.5microlitres; a 1.0microlitre of 10x buffer, a 0.5microlitre of 2.5dNTPs (Deoxynucleotide triphosphates), a 0.06 microlitres of taq polymerase, 2.84 microlitres of autoclaved distilled water and 3.0 microlitres of the isolated DNA. These were all mixed in a reaction tube, vortexed and

inserted into the PCR machine for the reaction. The primers used for the two reactions were the 16S primers (Chen *et al*., 2013).

2.10.5. The Reaction

This involves three (3) major processes which are denaturation, annealing and extension. The reaction was subjected to heating at a temperature range of 94-96°C for a period of 1-9 minutes so as to initialize the reaction. This yields single-stranded DNA molecules. The DNA was denatured by heating the reaction to 94-98°C for 20-30 seconds. The hydrogen bonds between complementary bases were broken to produce single-stranded DNA molecules. The reaction temperature was afterwards lowered to 50-65°C for 20-40 seconds at allow for annealing of the primers to the single-stranded DNA template. The polymerase then bond to the primer-template hybrid and began DNA formation. The reaction temperature was increased to 72°C (because the polymerase used was Taq polymerase). At this step, the DNA polymerase then synthesizes a new DNA complementary to the DNA polymerase synthesizes a new DNA complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. Final elongation was performed by setting the temperature at 70-74°C for 5-15 minutes. This was to ensure that any remaining single-stranded DNA is fully extended. Finally, the reaction was held at 10°C for an indefinite time for short-term storage of the reaction.

2.10.6. Testing for quality of the reaction

To ascertain the quality of the polymerase chain reaction, the product was subjected to testing, using the procedure stated below. Three tablets of agarose was dissolved in 100ml of 0.5 TBE (Trizma base, Boric acid and EDTA) buffer in a conical flask. The solution was autoclaved in a microwave for 3 minutes and cooled under a gently flowing tap water. 5 microlitres of Ethydium bromide was added to the solution, shaken, poured into gel tray and allowed to solidify. 3 ml of loading dye was added to 3microlitre of the PCR product centrifuged at 1500rpm for 1 minute. The solidified gel was dropped in the electrophoretic tank; the comb was removed thereby leaving wells on the gel. 2microlitre of ladder was dropped into the first well and PCR products were dropped into the horizontal wells made on the agarose gel and the electrophoretic machine was

turned on and allowed to run for some minutes (30-45 minutes). The ladder used is the 1kb ladder. The gel was brought out of the tank and inserted into the ultra violet trans-illuminator which projects the picture of the chain reaction on the screen of the monitor for assessment.

2.10.7. Polymerase chain reaction product purification

Two volumes (20ul) of absolute ethanol was added to the PCR product and incubated at room temperature for 15 minutes. It was spin down at 10,000rpm for 15 minutes. The supernatant was then decanted. It was again spun down at10,000 rpm for 15 minutes. 2 volumes (40ul) of 70% ethanol were added and the supernatant was decanted. It was then air dried after which 10ul of ultra pure water was added. After running it through the 1.5 agarose for about 30minutes, the gel was removed and placed in the UV trans- illuminator in order to re-ascertain the quality of the product. After this, the purified product was taken for sequencing.

2.10.8. Sequencing

A half-reaction of 20ul final volume, 4ul of ready reaction premix and 2ul of Big Dye Sequencing Buffer was used. The ready reaction premix was at a concentration of 2.5X and volume of 4ul. Big Dye Sequencing buffer was at a concentration of 5X and volume of 2ul, the primer had no concentration but volume was 3.2pmol, template also had no concentration but quantity between 1-50ng, water used had volume up to 20ul. The final volume of the concentration was 1X and 20ul volume. The buffers were optimized to avoid deterioration of sequence quality. The tubes were placed in a thermal cycler and set to correct volume. An initial denaturation was performed at Rapid thermal ramp to 96°C for one minute. The following were repeated for 25cycles; rapid thermal ramp to 96°C for 10 seconds. Rapid thermal ramp to 60°C for 4 minutes, finally, rapid thermal ramp to 4°C and it was ready to purify. The content of the tubes were spin down in a micro centrifuge.

2.10.9. Purifying Extension Products

A 96-well reaction plate was removed from the thermal cycler and briefly spun. 5ul of 125mM EDTA was added to each wells making sure the EDTA reaches the bottom of the wells. 60ul of 100% ethanol was then added to each well. The plate was sealed with aluminum tape and mixed by inverting 4 times. It was then incubated at room temperature for 15 minutes, A Beckam Allegra

6ACentrifuge with a GH-3 8A rotor was set at 4°C and the plate was spun at 1650rpm for 45 minutes. The plate was then incubated and spun again up to 185rpm and was then removed from the centrifuge. A 60ul of 70% ethanol was added to each well. With the centrifuge set to 4°C, the plate was inverted again and spun up to 185rpm for 1 minute and was then removed from the centrifuge. The sample was then re-suspended in injection buffer and covered with aluminum foil and stored at 4°C. It is important to make sure the wells are dried and Speed-Vac can be used to dry the plate for 15 minutes and also make sure that the samples are protected from the light while drying. The product from the purification process was loaded on the 3130xl genetic analyser from Applied Biosystems to give the sequences of the DNA.

2.10.10. Nucleotide Blasting

Translated nucleotide sequences coding for each isolates were then analyzed for similarities by using BLASTN tool (www.ncbi.nlm.nih.gov:80/BLASTN/). This gave the name of the bacteria up to strain level (Wael *et al*., 2013).

2.11. Statistical analysis

The data obtained were subjected to descriptive statistics using the Mean and standard deviation of the mean. One-way analysis of variance (ANOVA) test under Completely Randomnized Design (CRD) was used in interpreting the results. *P* value of less than 0.05 was considered to indicate statistical significance. SPSS statistical analysis software was used. Excel plots were used for the line graphs.

CHAPTER THREE

3.0. RESULTS

3.1. Physiochemical parameters of soil samples

The moisture content of soil samples ranged from 5.20 to 8.80. The pH reading obtained showed the soil samples were slightly acidic with values ranging from 5.4 to 6.3. The temperature range was 21°C to 33^{0}C and the organic matter content ranged from 4.30 to 9.00. This is presented in Table 1.

3.2. Enumeration and isolation of bacteria isolates

Developed bacteria colonies were observed and counted using a colony counter and the number of bacteria per ml of the soil samples which ranged from 9 CFU/ml to 28 CFU/ml observed and shown in table 2.

3.3. Isolation, morphological and biochemical characterization of phenol utilizing bacteria isolates

4 out of the 6 bacterial isolates showed maximum zone of clearance on mineral salt medium having phenol as its carbon source. The 4 isolates were identified using biochemical characterization and morphological characterization which are shown in tables 3 and 4. The biochemical characterization of the four isolates revealed them to belong to species of *Bacillus*, *Pseudomonas* and L*actobacillus*.

Table 1. Physiochemical parameters of the soil samples

Samples	Moisture Content (%)	pH	Temperature (°C)	Organic Matter (%)
A	$7.27^{a} \pm 0.65$	$5.87^{ab} \pm 0.18$	$23^{a} \pm 1.15$	$7.10^{a} \pm 1.11$
B	$5.67^{a} \pm 0.37$	$5.83^{ab} \pm 0.26$	$26^{ab} \pm 0.57$	$6.26^{a} \pm 0.67$
C	$7.30^{a} \pm 0.29$	$6.10^{ab} \pm 0.06$	$30^{b} \pm 3.46$	$6.33^{a} \pm 0.81$
D	$7.07^{a} \pm 0.93$	$6.27^{b} \pm 0.33$	$27.3^{ab} \pm 1.45$	$7.33^{a} \pm 1.53$
E	$6.10^{a} \pm 0.44$	$5.53^{a} \pm 0.07$	$27^{ab} \pm 1.73$	$5.88^{a} \pm 0.68$
F	$6.97^{a} \pm 1.02$	$6.10^{ab} \pm 0.06$	$30^{b} \pm 0.87$	$6.01^{a} \pm 0.37$

Key: Table shows Mean ± Standard Error (SE) of triplicate readings/values. Means with unshared superscripts are significantly different ($p<0.05$)

Table 2. Enumeration of bacteria from contaminated soil samples

Soil Samples	Dilution (10^3) (CFU/ml)	Dilution (10^5) (CFU/ml)
A	25	13
B	19	11
C	14	9
D	28	10
E	17	11
F	23	10

Table 3. Biochemical characteristics of the bacteria isolates

Biochemical Tests	isolate A	isolate B	isolate C	isolate D
Gram reaction	+	-	+	+
Motility	+	+	-	+
Endospore	+	-	-	+
Catalase	+	+	-	+
Oxidase	-	+	-	-
Indole	-	-	-	-
Coagulase	-	-	-	-
Citrate utilization	+	+	-	+
Methyl red	-	-	-	-
Vogues Proskeaur	+	-	-	+
Gelatinase	+	+	-	+
Urease	-	-	-	-
Glucose	+	-	+	+
Lactose	-	-	+	-
Sucrose	+	-	+	+
Starch	+	-	+	+
Gas production	-	-	-	-
Oxygen relationship	OA	O	FA	OA
probable organisms	*Bacillus* spp	*Pseudomonas* spp	*Lactobacillus* spp	*Bacillus* spp

Key: + denotes positive test, - denotes negative test, FA= facultative anaerobe, OA= obligate aerobe

Table 4. Morphological characteristics of bacteria isolates that utilized phenol

Morphology characteristics	***Bacillus* spp**	***Pseudomonas* spp**	***Lactobacillus* spp**	***Bacillus* spp**
Shape	Rods	Spherical	Rods	Rods
Cell arrangement	Pairs	Irregular clusters	Pairs	Pairs
Colony form	Circular	Flat	Circular	Circular
Colour	Yellowish	Green	Creamy	Yellowish

3.4. Screening and Identification of Lipolytic Bacteria

Among the 4 bacterial isolates, isolate D (*Bacillus* sp) had the highest zone of clearance recorded as $4.23^{c} \pm 0.17$mm in Mean ± Standard Error (SE) of triplicate readings. Isolate B (*Pseudomonas* sp) had the least zone of clearance with $2.30^{a} \pm 0.15$ mm as shown in table 5. Isolate D was selected for further studies on the basis of the screening test.

3.5. Optimization of culture conditions for maximum lipase production

3.5.1. Determination of optimum incubation time

The incubation time effect was tested on lipase activity at different time intervals (3-12 hours) and the obtained results are shown in figure 4. At 3 hours, the relative enzyme activity was 100U/l, 6 hours was 150 U/l, 9 hours was 250U/l and 12 hours was 350U/l.

3.5.2. Determination of a suitable carbon source

Relative growth and lipase activity was observed when cultivated in medium supplemented with phenol, tween 80, benzene and hexane as shown in figure 5. There was no relative growth in the presence of benzene, the highest relative growth rate, 90% was in the presence of tween 80, 40% was recorded for phenol and 80% for hexane. The highest lipase activity, 320 U/l was in the presence of hexane, 300 U/l for tween 80, 150 U/l the lowest lipase activity was for phenol. No lipase activity was observed in the presence of benzene.

3.5.3. Determination of optimum pH

The highest enzyme activity was recorded at pH 7 as 340 U/l and the lowest enzyme activity was recorded at pH 5 as 130 U/l as shown in figure 6.

3.5.4. Determination of suitable nitrogen source

Yeast extract and ammonium nitrate both had lipase activity were the nitrogen sources with the highest enzyme activity at 450 U/l while no enzyme activity was observed in the presence of potassium nitrate as shown in figure 7. The lipase activity for peptone was 420 U/l and casein had 180 U/l.

3.5.5. Determination of optimum temperature

The best temperature for the production of lipase enzyme is at 50^0C while the lowest enzyme activity was at 30^0C as shown in Figure 7. Temperatures 30^0C, 40^0C, 50^0C, 60^0C, 70^0C, 80^0C and 90^0C had 145 %, 275% , 350%, 340%, 300%, 240% and 200% relative enzyme activity respectively.

3.5.6. Effect of *Bacillus subtilis on* petroleum compounds

The ability of *Bacillus subtilis* to degrade some petroleum compounds, Benzene, phenol and hexane was studied and the result is shown in table 6. Phenol and hexane were degraded while benzene did not undergo degradation.

3.6. Effect of Metal ions on Lipase Production

Enhanced lipase enzyme activity was observed in the presence of Zinc ions as 97%, Magnessium ions as 95%, with the highest lipase activity 110% in the presence of Calcium ions while the lowest enzyme activity, 80% was in the presence of Copper ions as shown in Figure 9.

3.7. Molecular identification of the *Bacillus subtilis*

The molecular identification of the isolate with high lipase producing potential based on the

different screening methods was done and the bacterial isolate was identified as *Bacillus subtilus.* The GenBank accession and the percentage ID is shown in table 7.

Table 5. Lipolytic zones of the bacterial isolates

Isolate	lipolytic zone (mm)
A	$3.27^{b} \pm 0.17$
B	$2.30^{a} \pm 0.15$
C	$2.70^{a} \pm 0.15$
D	$4.23^{c} \pm 0.17$

Key: Values are Mean ± Standard Error (SE) of triplicate readings/values. Means with different superscripts are significantly different ($p<0.05$)

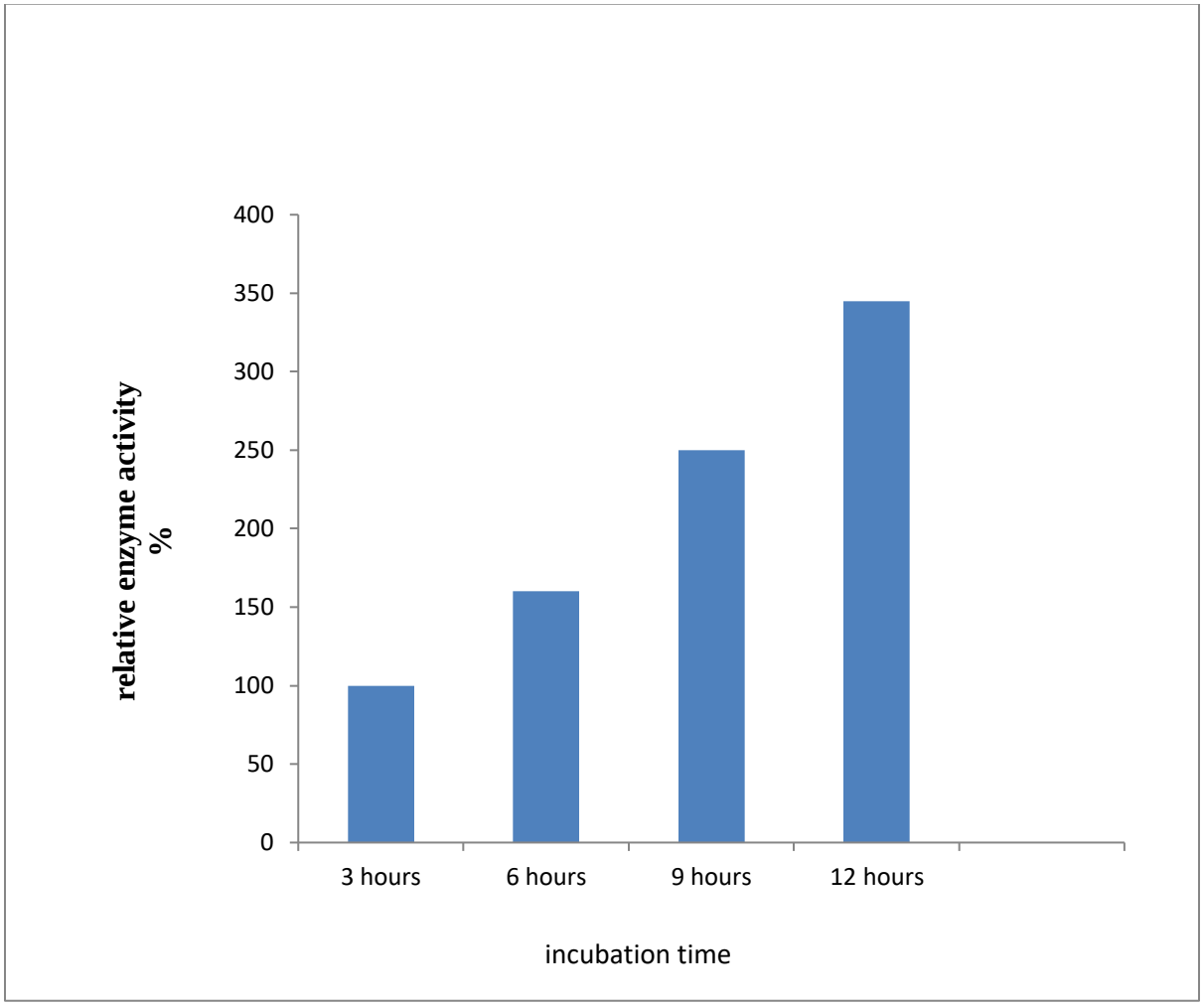


Figure 4. Effect of incubation time on lipase production.

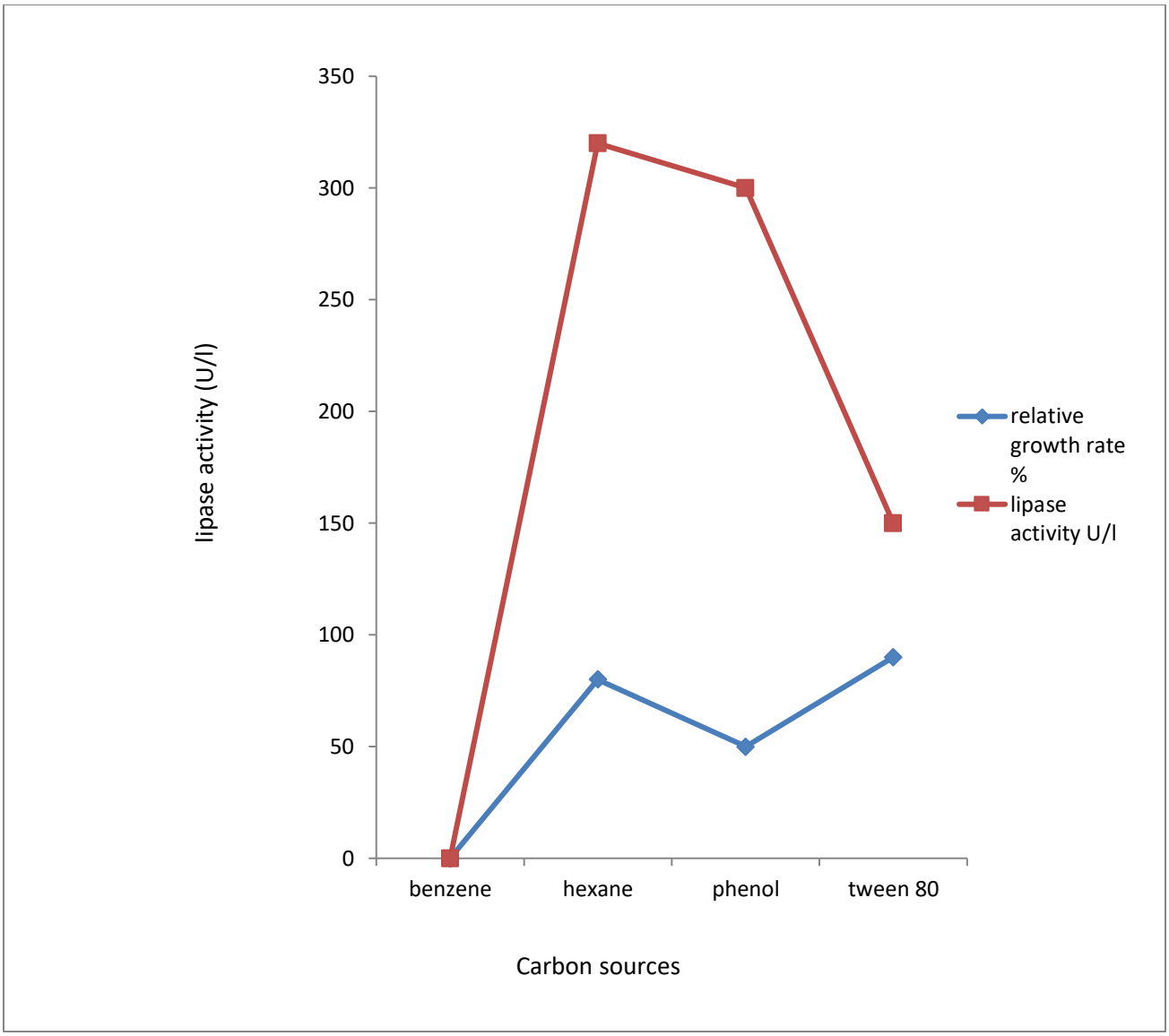


Figure 5. Effect of carbon source on lipase production.

Table 6. Effects of *Bacillus subtilis* on petroleum compounds

PC	Benzene	Phenol	Hexane
B.subtilis	-	+	+

Key: PC=petroleum compounds, += degraded, -= not degraded

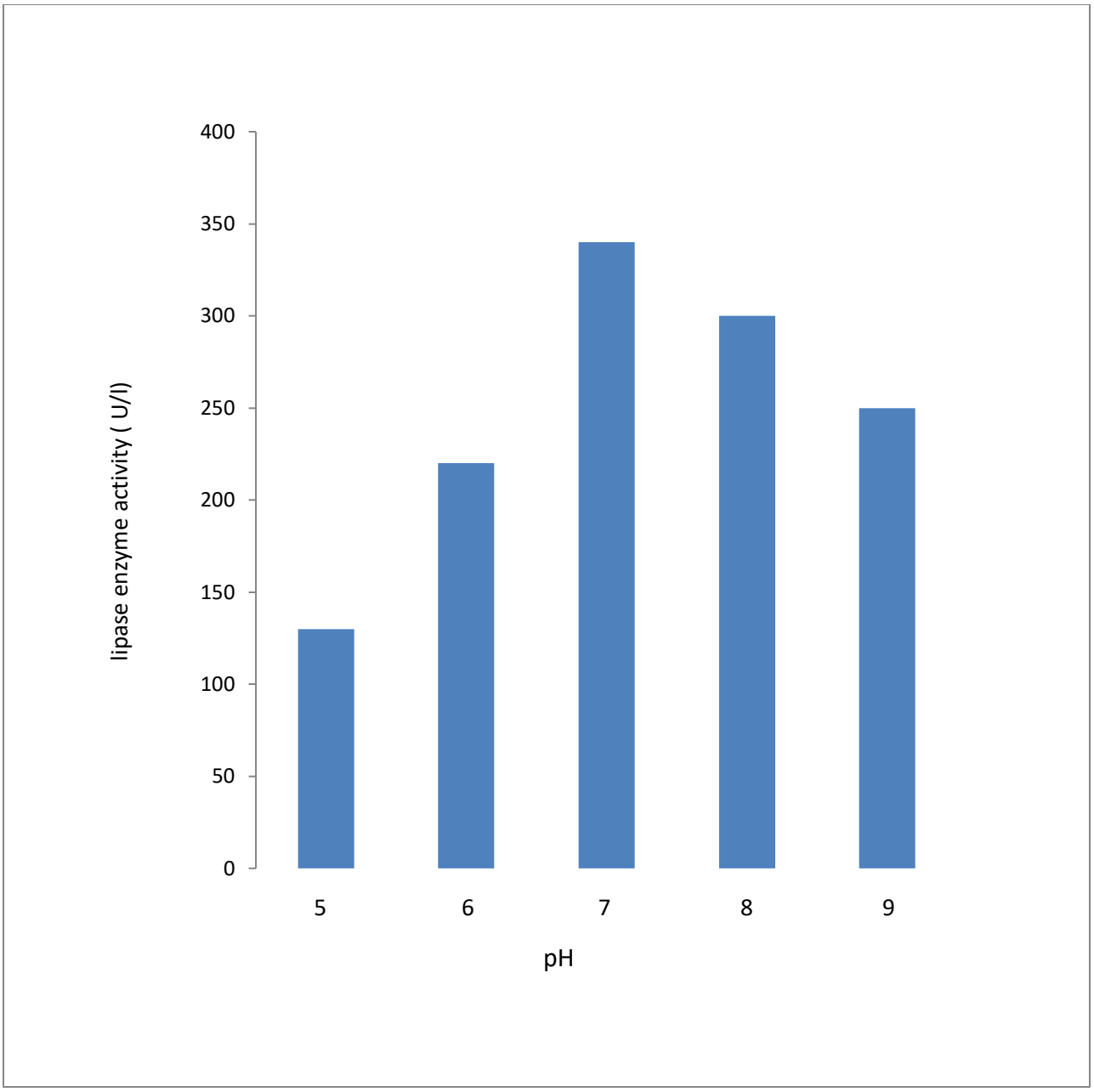


Figure 6. Effect of pH on lipase production.

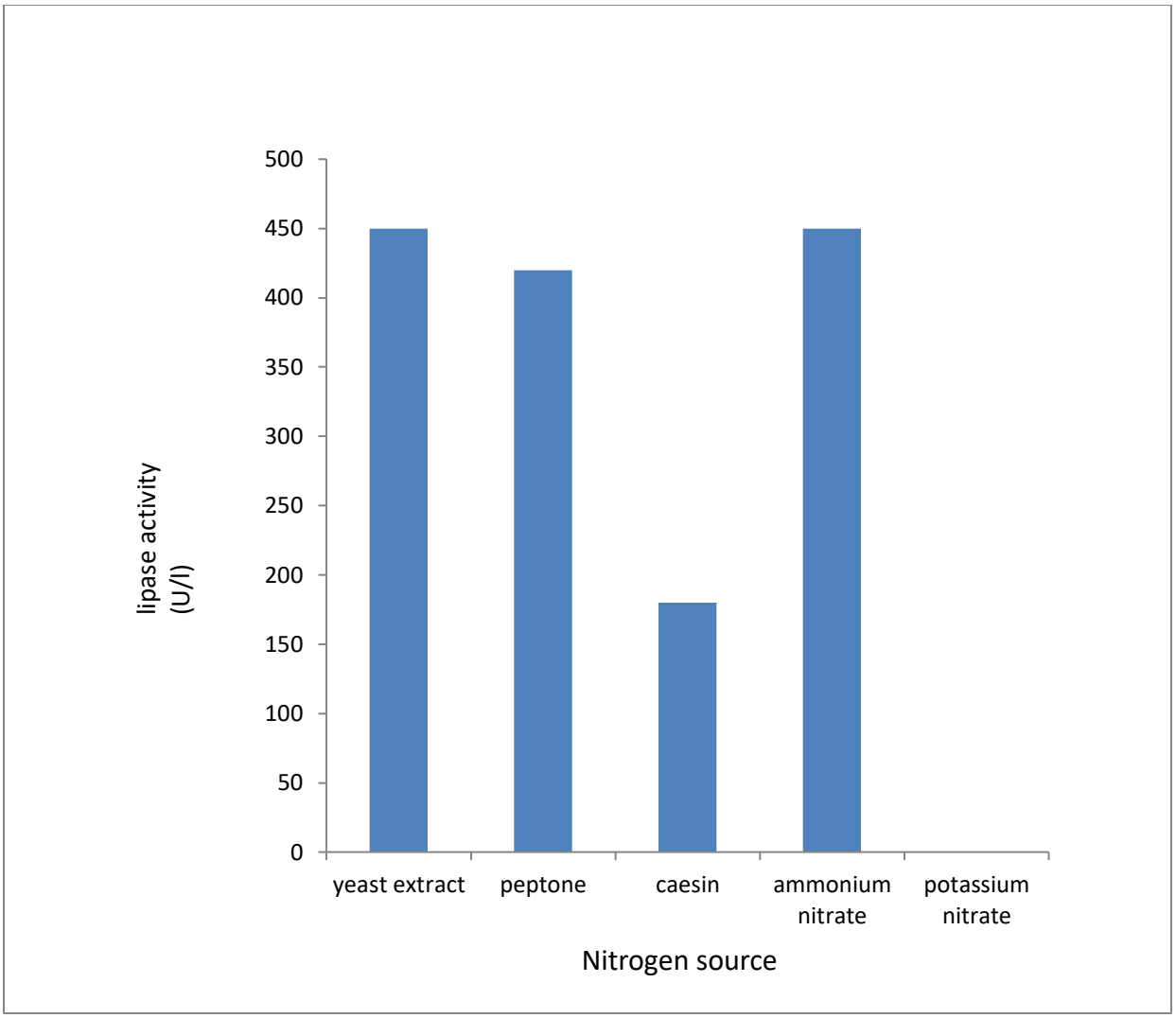


Figure 7. Effect of nitrogen source on lipase production

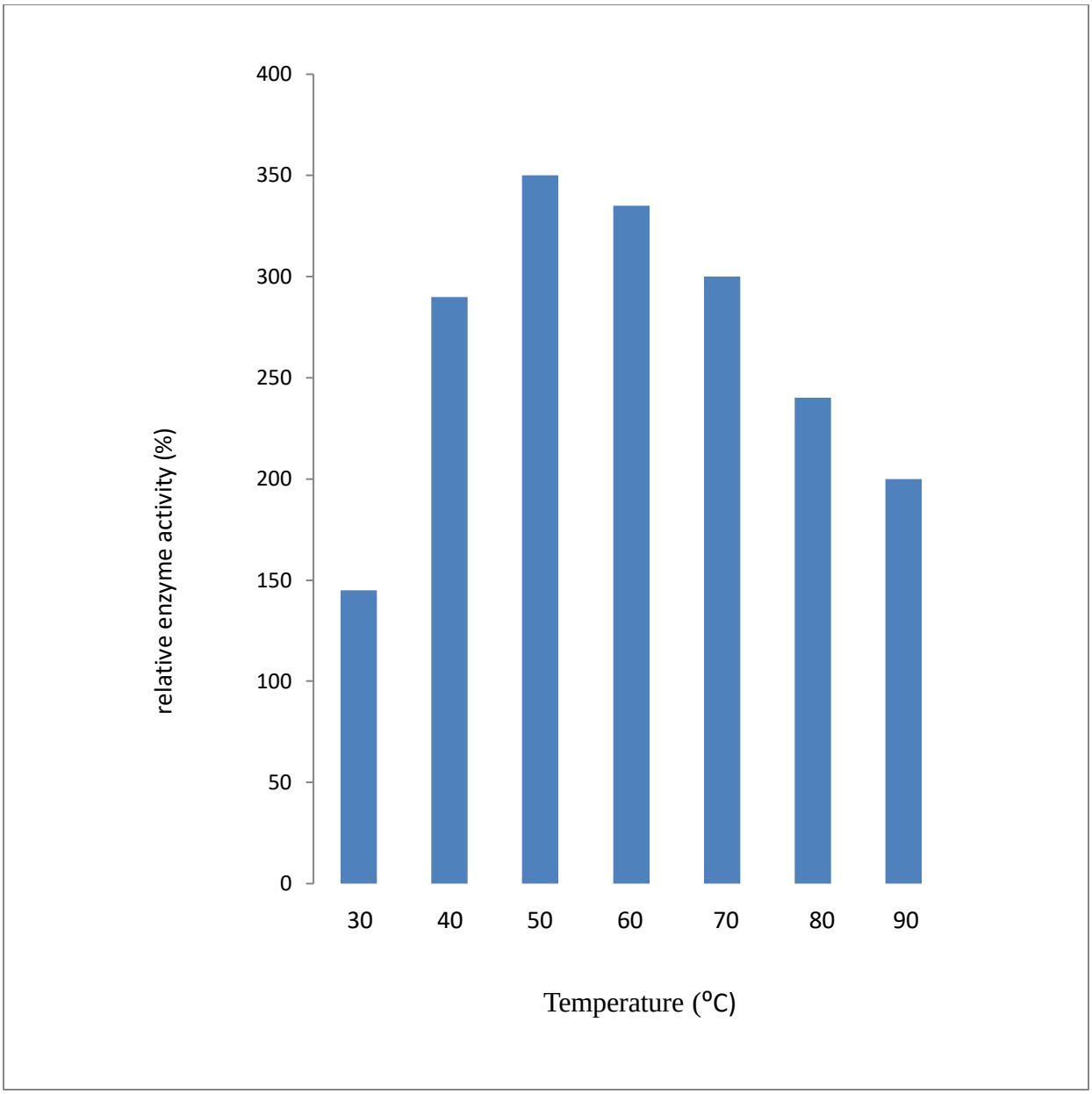


Figure 8. Effect of temperature on lipase production

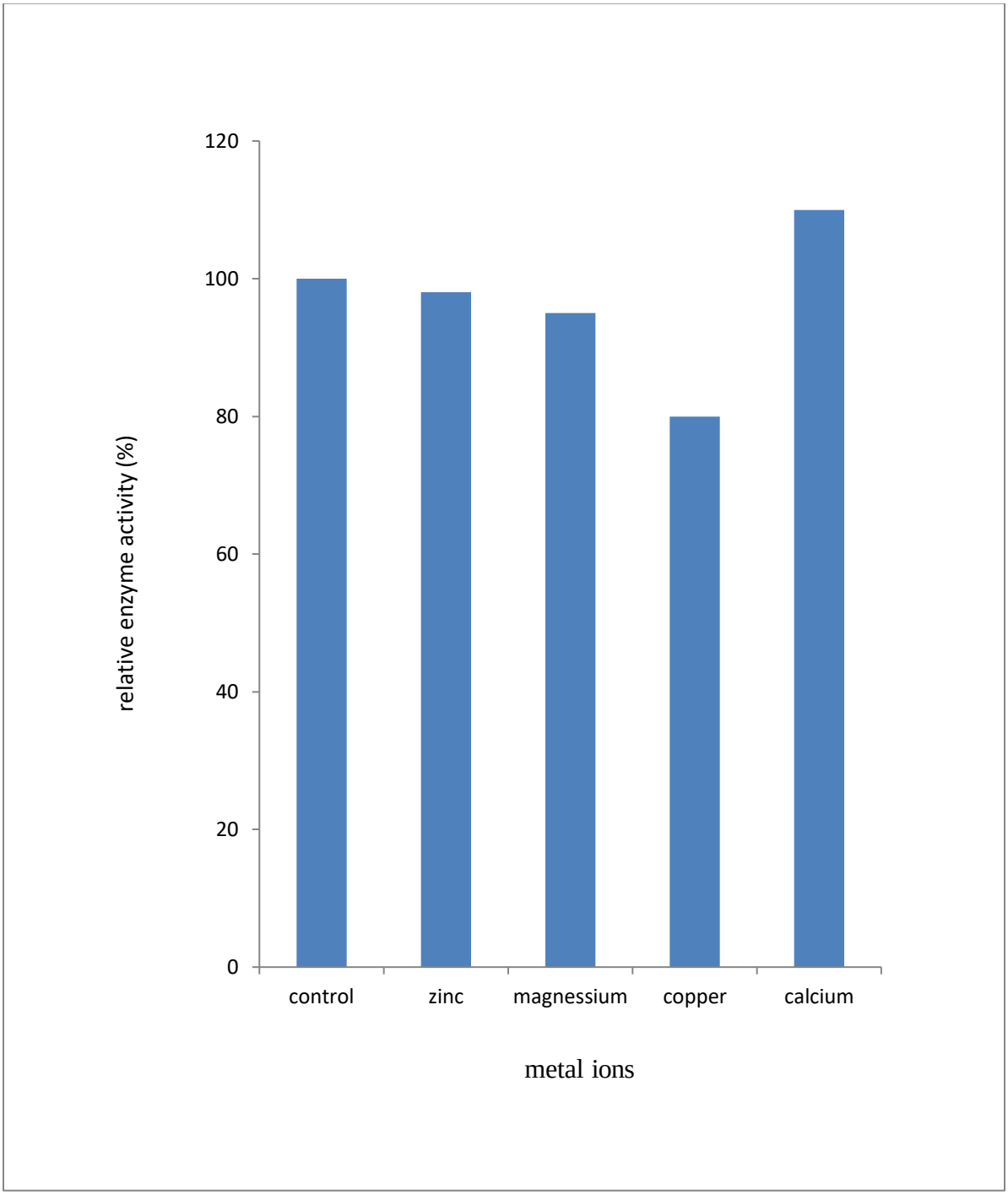


Figure 9. Effect of metal ions on lipase production.

Table 7. Molecular identification of isolate D

Name of sample	D
Percentage ID	100%
Predicted organism	*Bacillus subtilis*
GenBank accession	CP104097.1

CHAPTER FOUR

4.0. DISSCUSSION

4.1. Discussion

The moisture content of the soil samples reported in table 1 is different from that which Devatha *et al.* (2019) reported while working on the investigation of physical and chemical characteristics on soil due to crude oil contamination and its remediation. They reported 7.6 to 11.3 as moisture content values while the soil moisture in this study ranged from 5.20 to 8.80. This may be because the soil moisture content depends on the climatic conditions and as it varies from season to season, constant values cannot be established. The soil samples in this study were slightly acidic ranging from 5.4 to 6.9 which is relatively similar to the works of Ibiyemi *et al*. (2022) and Devatha *et al.* (2019) that reported 5.58 to 5.45 and 6.54 to 7.68 as pH values while they worked on the petroleum hydrocarbon-contaminated soil bacteria. Most bacteria are neutrophiles and grow best at near neutral pH 5.9 to 8.0 as reported by Jin and Kirk (2018). This explains why high values of bacterial count were recorded from the soil samples in this study.

The temperature range of the soil samples ranged from 21-33^{0}C organic content value reported in this study was close to that which was reported by Adetitun (2017) who reported the temperature of the soil sample as 35.80^{0}C. The organic content of the soil samples reported in this study ranged from 4.95 to 9.20 are in contrast to the report by Orgu and Olannye (2021) with values 5.44 to 6.87 in oil contaminated soil. The influence of soil organic matter was said to be the most significant factor dominating interactions during bioremediation, especially when the pollutants in soil are hydrophobic, the organic fraction of soil, and the extent of the sorption is related to the percentage of soil organic matter.

The result of the bacteria load from the contaminated soil samples revealed high level of bacteria load ($9x10^5$CFU/ml-$13x10^5$CFU/ml) as shown in table 2. Similar bacteria load ($10.0x10^5$CFU/ml) was reported by Musa (2019) in the study carried out on isolation and identification of diesel oil-degrading bacteria in used engine oil contaminated soil. High bacterial count could be due to high percentage of organic matter in the soil, normal soil microflora, the

ability of the isolated bacteria to utilize petroleum compounds as a carbon source and due to relatively high moisture content. The ability to isolate certain oil degrading bacteria from an environment is commonly taken as evidence that they are the active degraders of the constituents in that environment.

Only 4 isolates showed zone of clearance in this study, and were identified using biochemical methods amd morphological methods which revealed the isolates to be species of *Bacillus, Pseudomonas* and *Lactobacillus*. Prominent among the bacteria found in the oil contaminated soil in this study is *Bacillus* spp and this could be attributed to their ability to produce spores which enable them to survive in a different environment including hydrocarbon polluted soils as what Ghazali *et al.* (2004) reported. This agrees with earlier reports by Kumar *et al*. (2012) which stated *that Bacillus* sp., *Pseudomonas* sp., *Micrоccus* sp. *and Aeromonas* sp, are among bacteria found in oil-contaminated areas and Ibiyemi *et al.* (2022) that also also reported the frequent occurrence of *Bacillus* sp., *Pseudomonas aeruginosa., Staphylococcus* sp., *Escherichia coli., Actinobacter with Bacilus.* spp as the highest.

The bacterial isolates in this study were further screened for their ability to grow using phenol as the sole carbon source. The 4 bacterial isolates were able to utilize phenol this agrees with Cesarini *et al*. (2014) that the ability to isolate certain oil degrading microorganisms from the oil contaminated soil is usually taken as evidence that those organisms are the active degraders. . Positive producer bacterial strains for lipase activity on tween agar medium were detected by the appearance of lipolytic zones around the tested bacterial colonies and the isolated bacteria were examined for their potentiality to produce lipase on tween agar base medium. In the screening medium, *Bacillus subtilis* had the highest zone of clearance at 12mm while *Lactobacillus* sp had the least 5mm as shown in table 5. This is in contrast to findings by Lee *et al*. (2015) that reported zone of clearance *Bacillus* sp between 20mm to 30mm. This may be because the zone of clearance is a direct indicator of the amount of enzymes secreted by bacteria. Anbu *et al.* (2011) studied the lipase activity by different microbial strains using tween as substrate and they reported that this substrate is more convenient and easy to use for lipase detection.

The least relative enzyme activity was observed at 3 hours while the highest was at 12 hours which agrees with Ilesanmi *et al.* 2020 who reported that the highest lipase activity 364.82U/l was at 12 hours and the least 150U/l at 3 hours. The incubation time for enzyme

production is governed by the characteristics of the culture and is based on growth rate. This means that the longer an enzyme is incubated with its substrate, the greater the amount of product that will be formed. The increase in enzyme activity can be caused by the rapid growth of bacteria because the bacteria have adjusted to the nutrients available in the substrate as reported by Murtius *et al.* (2022).

Among the carbon sources used, tween 80 was the best carbon source for *Bacillus subtilis* lipase production in this study. Carbon source is an important substance for energy production in microorganisms. Pokagu *et al. (*2009) revealed that since lipases are inducible enzymes and are always produced in the presence of a lipid source or any other inducer, the major factor for the expression of lipase activity has always being the source of carbon. Tween 80 recorded the highest growth rate (90%) of *Bacillus subtilis* in this study, it was able to stimulate lipase activity (300U/l) in comparison with Martinez and Nudel (2002) that reported that the addition of Tween 80 enhanced lipase production with 16, 142-fold. Hexane stimulated a high growth rate (80%) of *Bacillus subtilis* in comparison with findings by Maia *et al.* (2001) that reported hexane as a good carbon source. Low growth rate of *Bacillus subtilis* (40%) and lipase activity (150 U/l) was observed with phenol as a carbon source.

Bacillus subtilis degraded phenol and hexane however it did not degrade benzene as investigated in this study. Benzene as carbon source showed no positive effect on lipase production and growth rate of *Bacillus subtilis.* This agrees with Adetitiun (2018) who revealed that benzene not degraded by *Bacillus subtilis* in his work. This might be because *Bacillus subtilis* was the only individual bacterium used instead of a hydrocarbon degrading bacterium consortorium (microbial distribution).

The enzyme activity of this study was at its maximum value at optimum pH of 7.0 as shown in figure 6 and as the pH value increased above optimum pH, enzyme activity decreased. This may be because have an optimum pH range each and changing the pH outside of this range will affect relative enzyme activity. This result is in agreement with the report of Iqbal and Rehman (2015) that reported optimum pH as 7.0 for lipase production. This is because increasing or changing the pH outside of this range will slow enzyme activity. Bacterial lipases have a neutral or alkaline optimum pH although, a comprehensive review on all bacteria lipases done by Gupta *et al*. (2004)

stated that maximum activity of lipases at pH values higher than 7 has been observed in many cases. This shows that pH is an important factor of enzyme activity.

Ammonium nitrate (450U/l), yeast extract (450U/l) and peptone (420U/l) were both preferred for lipase production in this study as shown in figure 7 which is similar to the studies by Ilesanmi *et al.* (2020) revealed that there was no enzyme activity for potassium nitrate while yeast extract (490.72 U/l), ammonium nitrate (482 U/l), casein (225.6 U/l) and peptone (445U/l) out of five tested nitrogen sources showed lipase activity. Nitrogen sources play an important part in the biosynthesis of bacterial lipases and are highly important for the growth of the bacteria also. They have the tendency to stimulate lipase production more than carbon sources. Organic nitrogen sources (peptone, yeast extract, e.t.c) increases lipase and cell productivity during lipase production. Both organic and inorganic nitrogen sources supply nitrogen, as Thakur *et al* (2014) stated that amino acids and cell growth factors necessary for the synthesis of enzymes.

Lipase activity in this study showed maximum activity at 50°C which is agreement to the optimum temperature 50°C reported by Iqbal and Rehman (2015). An increase in temperature increased the rate of the lipase activity in this study while the rate of lipase activity reduced as the temperature was being increased. This is because the temperature increases with corresponding increase in the rate of enzyme activity and the optimum activity is reached at the enzyme's optimum temperature. A continued increase results in a sharp decrease in activity as the enzyme's active site changes shape. This explains the reason the lipase activity decreased as the temperature increased above the optimum temperature.

Figure 9 shows that the lipase production in this study was activated by calcium ions while magnessium ions, zinc ions, and copper ions inhibited its activity. This agrees with Iqbal and Rehman (2015) that reported lipase production in the presence of calcium ions and no activity in the presence of copper ions. Sharma *et al.* (2002) also reported stimulation in lipase production from *Bacillus sp*.RSJ1 in the presence of calcium chloride even though most other metal ion salts were inhibitory to lipase production. Divalent cations stimulate, or inhibit enzyme production in microorganisms. The effect of different metal ions on lipase activity determines the inhibitory ability of metal ions during lipase enzyme production. This is because in the presence of calcium ions, the mixed micelle-complex is active and opens a new pathway for lipolysis.

The molecular characterization of the bacterial isolate with the highest lipase production revealed the isolate to be *Bacillus subtilis* with GenBank Accension numbers CP104097.1.

4.2. Conclusion

The ability of *Bacilus subtilis* to degrade phenol, benzene and hexane in this study revealed that hydrocarbon utilizing or degrading bacteria readily isolated from oil contaminated soil samples can be used in the biodegradation of hydrocarbon polluted soil. The lipase produced by *Bacillus subtilis* which was isolated from oil contaminated soil under optimum conditions for growth which were influenced by the type of carbon and nitrogen sources, the culture pH, the growth temperature and presence of metal ions. In conclusion, lipase enzyme production through a microbial process could be a better way to manage and remove petroleum related contaminants from the soil and even the environment.

REFERENCES

Abada, E. A. E. (2008). Production and characterization of a mesophilic lipase isolated from *Bacillus stearothermophilus* AB-1. *Pak. J. Biol. Sc*i, 11:1100-1106.

Abdul-Hammid, A.M, Khedeer, M.K. and Forman, M.S. (2013).Production and purification of lipase from Pseudomonasecepacia and study some effected conditions on production, Diyala *Agricultural Sciences Journal*, 5(2):5-12

Abed, R.M., Al-Sabahi, J., Al-Maqrashi, F., Al-Habsi, A. and Al-Hinai, M. (2014). Characterization of hydrocarbon-degrading bacteria isolated from oil-contaminated sediments in the sultanate of Oman and evaluation of bioaugmentation and biostimulation approaches in microcosm experiments. *Int. Biodeterior. Biodegrad.* 89: 58–66.

Adeline, Ting, S. Y., Carol, Tan, H. C. and Aw, C. S. (2009). Hydrocarbon-degradation by isolate *Pseudomonas lundensis* UTAR FPE2. *Malaysian Journal of Microbiology*, 5(2): 104-108.

Adetitun, D. O. (2017). Biodegradation of hydrocarbons by autochthonous bacteria isolated from kerosene and gasoline experimentally contaminated soil. *Submitted Ph.D* (Doctoral dissertation, Thesis. University of Ilorin).

Adetitun, D. O., Sulaiman, R. D. and Oguntoye, M. B. (2019). Utilization of Decane, Heptane, Petrol and Hexadecane by Fungi Isolated from Engine Oil Contaminated Soil in Ilorin. *Journal of Applied Sciences and Environmental Management*, 23(9): 1671-1676.

Agbogidi, O. M., Okonta, B. C. and Dolor, D. E. (2005). Socio–economic and environmental impact of crude oil exploration and production on agricultural production: a case study of Edjeba and Kokori communities in Delta State of Nigeria. *Global Journal of Environmental Sciences*, *4*(2):171-176.

Amanchukwu, S. C., Obafemi, A. and Okpokwasili, G. C. (1989). Hydrocarbon degradation and utilization by a palm-wine yeast isolate. *FEMS Microbiology letters*, *57*(2): 151-154.

Arutchelvan, V., Kanakasabai, V., Nagarajan, S. and Muralikrishnan, V. (2005). Isolation and identification of novel high strength phenol degrading bacterial strains from phenol-formaldehyde resin manufacturing industrial wastewater. *Journal of Hazardous Materials*, 127(1-3): 238-243.

Atlas, R. M. (1985). Effects of hydrocarbons on microorganisms and petroleum biodegradation in arctic ecosystems. *Petroleum effects in the arctic environment*, 63-100.

Barbosa, A. M., Dekker, R. F. H. and Hardy, G. S. (1996). Veratryl alcohol as an inducer of laccase by an ascomycete, Botryosphaeria sp., when screened on the polymeric dye Poly R-478. *Letters in Applied Microbiology*, *23*(2): 93-96.

Bennet, J.W., Wunch, K.G. and Faison, B.D. (2002). Use of fungi biodegradation. Manual of Environmental Microbiology 2nd ed., Washington, D.C. ASM Press. 960-971.

Belguith, H., Fattouch, S., Jridi, T. and Ben Hamida, J. (2009). Immunopurification and characterization of a rape (Brassica napus L.) seedling lipase. *Afr J Biotechnol*.; 12(21): 3224-3234.

Bhardwaj, K., Raju, A., and Rajasekharan, R. (2001). Identification, purification and characterization of a thermally stable lipase from rice bran. A new member of the phospholipase family. *Plant Physiol*, 127: 1728-1738.

Bhosale, H. J. Kadam, T. A. Sukalkar, S. R. and Adekar, S.D. (2012) Lipase production from Bacillus species using soybean oil cake as substrate. *Int. J. Pharm. Biol. Arch*, 3(5): 213-221.

Bornscheuer, U. T., Bessler, C., Srinivas, R. and Krishna, S. H. (2002). Optimizing lipases and related enzymes for efficient application. *TRENDS in Biotechnology*, *20*(10): 433-437.

Bouaziz, A.H., Horchani, N.B., Salem, Y. and Gargouri, A.S. (2011). Expression, purification of a novel alkaline staphylococcus xylosus lipase acting at high temperature. *Biochemical Engineering Journal*, 54(3):93-102

Boopathy, R. (2000). Factors limiting bioremediation technologies. *Bioresources Technology* 74: 63–67.

Carpen, A., Bonomi, F., Iametti, S., Marengo, M. (2019). Effects of starch addition on the activity and specificity of food-grade lipases. *Biotechnol Appl Biochem*, 66(4):607–16.

Cappuccino, J. G., and Sherman, N. (1996). A laboratory manual in general microbiology.

Cerqueira, V. S., Maria do Carmo, R. P., Camargo, F. A. and Bento, F. M. (2014). Comparison of bioremediation strategies for soil impacted with petrochemical oily sludge. *International Biodeterioration & Biodegradation, 95*: 338-345.

Chaillan, F., Le Flèche, A., Bury, E., Phantavong, Y. H., Grimont, P., Saliot, A. and Oudot, J. (2004). Identification and biodegradation potential of tropical aerobic hydrocarbon-degrading microorganisms. *Research in microbiology*, *155*(7):587-595.

Chandra, P., Enespa, Singh, R. and Arora, P.K. (2020). Microbial lipases and their industrial applications: a comprehensive review. *Microb Cell Fact*, 19:169.

Chandra, S., Sharma, R., Singh, K. and Sharma, A. (2013). Application of bioremediation technology in the environment contaminated with petroleum hydrocarbon. *Annals of Microbiology*, 63: 417–431.

Cheesbrough, M. (2006). District Laboratory Practices in Tropical Countries. Cambridge University Press. USA pp. 132-143

Cihangir, N. and Sarikaya, E. (2004). Investigation of lipase production by a new isolate of Aspergillus sp. *World Journal of Microbiology and Biotechnology*, *20*:193-197.

Cooney, J. J., Silver, S. A. and Beck, E. A. (1985). Factors influencing hydrocarbon degradation in three freshwater lakes. *Microbial ecology*, *11*: 127-137.

Costa, T.M., Hermann, K.L., Garcia-Roman, M., Valle, R.D.C.S.C. and Tavares, L.B.B. (2012). Lipase production by *Aspergillus niger* grown in different agro-industrial wastes by solid-state fermentation. *Brazil J Chem Eng*, 34(2):419–27.

Damaso, M.C.T., Salum, T.F.C., Terzi, S.D. and Couri, S. (2013). Assay methods for lipase activity. In: Vermelho AB, Couri S, editors. Methods to determine enzymatic activity. *Bentham: Rio de Janeiro*, p. 161–94.

Das, N. and Chandran, P. (2010). Microbial Degradation of Petroleum Hydrocarbon Contaminants: An Overview. *Biotechnology Research International,* 1-13.

Das, N. and Chandran, P. (2011) Microbial Degradation of Petroleum Hydrocarbon Contaminants: An Overview. *Biotechnology Research International,* 941:8-10.

Dana, L. D. and Bauder, J. W. (2011). A General Essay on Bioremediation of Contaminated Soil, Montana State University, Bozeman, Mont. 1:23-41.

de Almeida, A.F., Tauk-Tornisielo, S.M. and Carmona, E.C. (2013). Acid lipase from *Candida viswanathii*: production, biochemical properties, and potential application. *Bio Med Res Int*, 18:1–10.

Devatha, C.P., Vishnu Vishal, A. and Purna Chandra Rao, J. (2019). Investigation of physical and chemical characteristics on soil due nto crude oil contamination and its remediation. *Applied Water Science*, 9: 1-10.

Dua, M., Singh, A., Sethunathan, N. and Johri, A.2003. "Biotechnology and bioremediation: successes and limitations," *Applied Microbiology and Biotechnology*, vol. 59, no. 2-3, pp. 143–152.

Ekanem, J. O. and Ogunjobi, A. A. (2017). Hydrocarbon degradation potentials of bacteria isolated from spent lubricating oil contaminated soil. *Journal of Applied Sciences and Environmental Management*, 21 (5): 973-979.

Ertugrul, S., Donmez, G. and Takag, S. (2007). Isolation of lipase producing Bacillus sp. from olive mill wastewater and improving its enzyme activity. *J. Hazard. Mater*, 149: 720-724.

Facundo, J. M., Vanessa, H. and Teresa, M. L. (2001). Biodegradation of Diesel Oil in Soil by a Microbial Consortium. *Water, Air and Soil Pollution,* 128 (3-4). 313-320.

Fawole, M.O. and Oso, B.A. (2007). Laboratory Manual of Microbiology, ed, Ibadan. *Spectrum Books Limited*, 15-35.

Fragkou, E. (2021). In Situ Aerobic Bioremediation of Sediments Polluted with Petroleum Hydrocarbons: A Critical Review. *Journal of Marine Science and Engineering*, 9(9): 1003.

Frazar Chris. (2011). Agency guidelines for petroleum contaminated soil and debris, Vermont State agency of Natural Resources Waste Management Division. *WMD magazine*, 67-69.

Fritsche, W. and Hofrichter, M. (2000). "Aerobic degradation by microorganisms," in Environmental Processes- Soil Decontamination, *J. Klein*, Ed., pp. 146–155, Wiley-VCH, Weinheim, Germany.

Gaschler, M.M. and Stockwell, B.R. (2017). Lipid peroxidation in cell death. *Biochem Biophys Res Commun*, p ;482(3):419–25.

Geetha, S.J., Joshi, S.J. and Kathrotiya, S. (2013). Isolation and characterization of hydrocarbon degrading bacterial isolates from oil contaminated sites. *APCBEE procedia*, 5:237-241.

Geoffry, K. and Achur, R.N. (2018). Optimization of novel halophilic lipase production by *Fusarium solani* strain NFCCL 4084 using palm oil mill effluent. *J Genet Eng Biotechnol*, p;16(2):327–34.

Ghanem, E. H., Al-Sayed, H. A. and Saleh, K. M. (2000). An alkalophilic thermostable lipase produced by a new isolate of Bacillus alcalophilus. *World Journal of Microbiology and Biotechnology*, *16*: 459-464.

Ghazali, F.M., Rahman, R.N.Z.A., Salleh, A.B. and Basri, M. (2004). Biodegradation of hydrocarbons in soil by microbial consortium. *International Biodeterioration and Biodegradation*, 54 (1): 61–67.

Ghosh, P. K., Saxena, R. K., Gupta, R., Yadav, R. P. and Davidson, S. (1996). Microbial lipases: production and applications. *Science Progress (1933-)*: 119-157.

Guo, Z., and Xu, X. (2005). New opportunity for enzymatic modification of fats and oils with industrial potentials. *Org Biomol Chem*, 3(14): 2615- 2619.

Gupta, R., Gupta, N. and Rathi, P. (2004). Bacterial lipases: an overview of production, purification and biochemical properties. *Appl. Microbiol. Biotechnol*, 64(6): 763-781.

Hasan, F., Shah, A.A. and Hameed, A. (2006). Industrial applications of microbial lipases. *Enzyme Microb Technol*, 39: 235-251.

Hasanuzzaman, M., Kathryn, M., Umadhay-Briones, S.M., Morita, N., Nodasaka,, Y. and Yumoto, I. (2004). Isolation, identification, and characterization of a novel, oil degrading bacterium, *Pseudomonas aeruginosa* T1. *Curr Microbiol*, 49: 108-114.

Head, I. M., Jones, D. M. and Röling, W. F. (2006). Marine microorganisms make a meal of oil. *Nature Reviews Microbiology*, *4*(3): 173-182.

Ibiyemi, M.F., Julius, O.O. and Oluwasusi, V.O. (2022). Microbiological and physiochemical analysis of oil contaminated soil from major auto mechanic workshops in Ado-Ekiti metropolis. *World Journal of Advanced Research and Reviews*, 15 (1): 223-238.

Iftikhar, T., Niaz, M. and Zia, M.A. (2010). Production of extracellular lipases by Rhizopus oligosporus in a stirred fermentor. *Braz J Microbiol*, 41(4):1124–32.

Ilesanmi, O.I., Adekunle, A. E., Omolaiye, J. A., Olorode, E. M. and Ogunkanmi, A. L.(2020). Isolation, optimization and molecular characterization of lipase producing bacteria from contaminated soil. *Scientific African,* 8, e00279.

Iqbal, S. A. and Rehman, A. (2015). Characterization of lipase from Bacillus subtilis I-4 and its potential use in oil contaminated wastewater. *Brazilian archives of biology and technology*, *58*: 789-797.

Jin, Q. and Kirk, M. F. (2018). pH as a primary control in environmental microbiology: thermodynamic perspective. *Frontiers in Environmental Science*, *6*:21.

Jinyong, Y. (2014). Integrated lipase production and in situ biodiesel synthesis in a recombinant Pichipastot is yeast: An efficient dual biocatalytic system composed of cell catalysts. *Biotechnology for Biofuels*, 3(2):7-14

Jones, A., Reed, R. and Weyers, J. (2000). Practical skills in Biology. Pearson Educational Limited, Edinburgh Gate, England. P 27-29.

Kumar, D., Kumar, L., Nagar, S., Raina, C., Parshad, R. and Gupta, V. (2012). "Screening, isolation and production of lipase/esterase producing *Bacillus* sp. strain DVL2 and its potential evaluation in esterification and resolution reactions". *Scholars Research Library Archives of Applied Science Research* (4): 1763-1770.

Lee, E. H., Kim, J., Cho, K. S., Ahn, Y. G. and Hwang, G. S. (2010). Degradation of Hexane and other Recalcitrant Hydrocarbons by a Novel Isolate, *Rhodococcus* sp. EH831. *Environmental Science and Pollution Research International*, 17(1): 64-77.

Lee, L. P., Karbul, H. M., Citartan, M., Gopinath, S. C., Lakshmipriya, T. and Tang, T. H. (2015). Lipase-secreting Bacillus species in an oil-contaminated habitat: promising strains to alleviate oil pollution. *BioMed research international*,82:05-75 .

Leung, M. (2004). "Bioremediation: techniques for cleaning up a mess," *Journal of Biotechnology*, vol. 2, pp. 18–22.

Lide, D. R., ed. (2005). *CRC Handbook of Chemistry and Physics* (86th ed.). *Boca Raton (FL):* CRC Press. ISBN 0-8493-0486-5.

Liu, Y. J., Zhang, A. N. and Wang, X. C. (2009). Biodegradation of Phenol by using Free and Immobilised Cells of *Acinetobacter* sp. XA05 and *Sphingomonas* sp. FG03. *Biochemical Engineering Journal*. 44:182-192.

Liu, P.W., Wang, S.Y., Huang, S.G. and Wang, X.C. (2013). Effects of soil organic matter and ageing on remediation of disel-contaminated soil. *Environ Technol,* 33:22-24.

Lloyd, A. C., and Cackette, T. A. (2001). Diesel engines: environmental impact and control. *Journal of the Air & Waste Management Association*, *51*(6): 809-847.

Luckarift, H. R., Sizemore, S. R., Farrington, K. E., Fulmer, P. A., Biffinger, J. C., Nadeau, L. J. and Johnson, G. R. (2011). Biodegradation of medium chain hydrocarbons by *Acinetobacter venetianus* 2AW immobilized to hair-based adsorbent mats. *Biotechnology progress*, *27*(6): 1580-1587.

Mahjoubi, M., Jaouani, A., Guesmi, A., Amor, S. B., Jouini, A., Cherif, H. and Cherif, A. (2013). Hydrocarbonoclastic bacteria isolated from petroleum contaminated sites in Tunisia: isolation, identification and characterization of the biotechnological potential. *New biotechnology*, *30*(6):723-733.

Mandal, A. K., Sarma, P. M., Singh, B., Jeyaseelan, C. P., Channashettar, V. A., Lal, B. and Datta, J. (2012). Bioremediation: an environment friendly sustainable biotechnological solution for remediation of petroleum hydrocarbon contaminated waste. *ARPN Journal of Science and Technology*, *2*(8): 1-2.

Maia, M. M. D., Heasley, A., De Morais, M. C., Melo, E. H. M., Morais Jr, M. A., Ledingham, W. M. and Lima Filho, J. L. (2001). Effect of culture conditions on lipase production by Fusarium solani in batch fermentation. *Bioresource technology*, *76*(1): 23-27.

Marinescu, M., Dumitru, M. and Lacatusu, A. (2009). Biodegradation of Petroleum Hydrocarbons in an Artificial Polluted Soil. *Research Journal of Agriculture Science*. 41:(2).

Margesin, R., Zimmerbauer, A. and Schinner, F. (1999). "Soil lipase activity—A useful indicator of oil biodegradation," *Biotechnology Techniques*, vol. 13, no. 12, pp. 859–863.

Margesin, R. and Schinner, F. (2001). Biodegradation and bioremediation of hydrocarbons in extreme environments. *Applied Microbiology and Biotechnology*, 56 (5–6): 650–663

Martinez, D. A., and Nudel, B. C. (2002). The improvement of lipase secretion and stability by addition of inert compounds into *Acinetobacter calcoaceticus* cultures. *Canadian journal of microbiology*, *48*(12): 1056-1061.

Megharaj, M., Ramakrishnan, B., Venkateswarlu, K., Sethunathan, N. and Naidu, R. (2011). Bioremediation approaches for organic pollutants: a critical perspective. *Environment international*, *37*(8): 1362-1375.

Melani, N.B., Tambourgi, E.B. and Silveira, E. (2020). Lipases: from production to applications. *Sep Purif Rev,* p.;49(2):143–58.

Mishra, S., Jyot, J., Kuhad, R. C. and Lal, B. (2001). Evaluation of inoculum addition to stimulate in situ bioremediation of oily-sludge-contaminated soil. *Applied and environmental microbiology*, *67*(4): 1675-1681.

Mobarak-Qamsari, E., Kasra-Kermanshahi, R. and Moosavi-nejad, Z. (2011). Isolation and identification of a novel, lipase-producing bacterium, *Pseudomnas aeruginosa* KM110. *Ran. J. Micro*., 3 (2): 92-98

Mohan, S.T., Palavasem, A. and Immanuel, G. (2008). Isolation and characterization of lipase-producing Bacillus strains from oil mill waste. *African Journal of Biotechnology*, 7(15): 2728-2735.

Mrozik, A., Piotrowska-Seget, Z. and Labuzek, S. (2003). Bacterial degradation and bioremediation of Polyctclic Aromatic Hydrocarbons. *Polish Journal of Environmental Studies.* 12(1): 15-25.

Müller, R. H., Rühl, D. and Runge, S. A. (1996). Biodegradation of solid lipid nanoparticles as a function of lipase incubation time. *International Journal of Pharmaceutics*, *144*(1): 115-121.

Murtius, W. S., Hari, P. D. and Putri, I. N. (2022). The Effect of Incubation Time to The Activity of Lipase Produced by *Bacillus thuringiensis* on Coconut (Cocos nucifera L.) Dregs. In *IOP Conference Series: Earth and Environmental Science*,1: 20-76.

Musa, S. I. (2019). Isolation and identification of diesel oil-degrading bacteria in used engine oil contaminated soil. *Journal of Applied Sciences and Environmental Management*, 23(3): 431-435.

Muthazhagan, K. and Thangaraj, M. (2014). Production and partial characterization of lipase by *Bacillus* sp. isolated from vellar estuary sediment. *IJSIT*, 3:639–53.

National Center for Biotechnology Information (2023). *PubChem* Compound Summary for CID 8058, n-HEXANE. Retrieved March 22, 2023.

Olempska-Beer, Z.S., Merker, R.I. and Ditto, M.D. (2006). Food processing enzymes from recombinant microorganisms-a review. *Reg Toxicol Pharmacol*, 45: 144-158.

Ogru, K. I. and Olannye, P. G. (2021). Microbial studies of biosurfactant producing bacteria from crude oil contaminated soil. *Journal of Applied Sciences and Environmental Management*, *25*(9):1729-1735.

Ota, K. and Kinjo, R. (2020). Inorganic benzene valence isomers. *Chemistry–An Asian Journal*, *15*(17): 2558-2574.

Paller, G., Hommel, R. K. and Kleber, H. P. (1995). Phenol degradation by *Acinetobacter calcoaceticus* NCIB 8250. *Journal of basic microbiology*, *35*(5): 325-335.

Pandey, P., Pathak, H. and Dave, S. (2016). Microbial ecology of hydrocarbon degradation in the soil: a review. *Research Journal of Environmental Toxicology*, *10*(1): 15.

Popoola. B. M. and Olateru Comfort. T. (2018). Characterisation of a Bacterium Lipase (*Bacillus subtilis*) from Vegetable Oil Polluted Soil. *International Journal of Applied and Natural Sciences (IJANS*), 55-66.

Pramila, S., Fulekar, M. and Bhawana, P. (2012). E-waste-A challenge for tomorrow. *Research Journal of Recent Sciences*, 2277- 2502.

Prasad, M.P. and Manjunath, K. (2011). "Comparative study on biodegradation of lipid-rich wastewater using lipase producing bacterial species," *Indian Journal of Biotechnology*, 1:121–124.

Prieto, M. B., Hidalgo, A., Serra, J. L. and Llama, M. A. J. (2002). Degradation of Phenol by *Rhodococcus erythropolis* UPV-1 Immobilized on Biolite® in a Packed-Bed reactor. *Journal of Biotechnology*. 97:1-11.

Priyanka, P., Tan, Y., Kinsella, G.K., Henehan, G.T. and Ryan, B.J. (2019). Solvent stable microbial lipases: current understanding and biotechnological applications. *Biotechnol Lett*, 41(2):203–20.

Rabbani, M., Bagherinejad, M.R., Sadeghi, H.M., Shariat, Z.S., Etemadifar, Z. and Moazen, F,. (2013). Isolation and characterization of novel thermophilic lipase secreting bacteria. *Braz J Microbiol*, 44: 1113- 1119.

Ramesh, S., Kumar, R., Devi, A. and Balakrishnan, K. (2014). Isolation of a lipase producing bacteria for enzyme synthesis in shake flask cultivation. *Int J Curr Microbiol App Sci*, 3(3): 712-719.

Ramani, K., L. John Kennedy, M. Ramakrishnan, K. and G. Sekaran (2010). "Purification, characterization and application of acidic lipase from *Pseudomonas gessardii* using beef tallow as a substrate for fats and oil hydrolysis." *Process Biochemistry*,10: 1683-1691.

Riaz, M., Shah, A.A., Hameed, A. and Hasan, F. (2010). Characterization of lipase produced by *Bacillus* sp. FH5 in immobilized and free state. *Ann Microbiol*, 60: 169-175.

Riffaldi, R., Levi-Minzi, R., Cardelli, R., Palumbo, S. and Saviozzi, A. (2006). "Soil biological activities in monitoring the bioremediation of diesel oil-contaminated soil," *Water, Air, and Soil Pollution*, 4: 3–15.

Ruiz-Urigüen, M., Shuai, W., Huang, S. and Jaffé, P. R. (2022). Biodegradation of PFOA in microbial electrolysis cells by *Acidimicrobiaceae* sp. strain A6. *Chemosphere*, *292*; 133-506.

Sagar, K., Bashir, Y., Phukan, M.M. and Konwar, B.K. (2013). Isolation of lipolytic bacteria from waste contaminated soil: A study with regard to process optimization for lipase. *International Journal of Scientific Technology Research*, 2(3):12-17.

Sangeetha, R., Arulpandi, I. and Geetha, A. (2014). Molecular characterization of a proteolysis-resistant lipase from *Bacillus pumilus* SG2. *Braz J Microbiol*, 45: 389-393.

Santhini, K., Myla, J., Sajani, S. and Usharani, G. (2009). Screening of *Micrococcus* sp from oil contaminated soil with reference to bioremediation. *Botany Research International*, 2(4): 248-252.

Seo, J. S., Keum, Y. S. and Li, Q. X. (2009). Bacterial degradation of aromatic compounds. *International journal of environmental research and public health*, *6*(1): 278-309.

Sharma, R., Soni, S.K., Vohra, R.M., Jolly, R.S., Gupta, L.K. and Gupta, J.K. (2002). Production of extracellular alkaline lipase from a *Bacillus* sp. RSJ1 and its application in ester hydrolysis. *Ind J Microbiol*; 42: 49-54. 48.

Sharma, D., Sharma, B. and Shukla, A. K. (2011). "Biotechnological approach of microbial lipase: a review," *Biotechnology*, 1:23–40.

Sihag, S., Pathak, H. and Jaroli, D. P. (2014). Factors affecting the rate of biodegradation of polyaromatic hydrocarbons. *International Journal of Pure & Applied Bioscience*, *2*(3): 185-202.

Silveira, E.A., Moreno-Perez, S., Basso, A., Serban, S., Mamede, R.P., Tardioli, P.W. and Guisan, J.M. (2017). Modulation of the regioselectivity of *Thermomyces lanuginosus* lipase via biocatalyst engineering for the Ethanolysis of oil in fully anhydrous medium. *BMC Biotechnol*,17(1):88.

Singh, P.; Patil, Y. and Rale, V. (2019). Biosurfactant production: Emerging trends and promising strategies. *J. Appl. Microbiol*., 126:2–13.

Sirisha, E. M., Lakshmi, N. and Rajasekar, N. (2010). Isolation and optimization of lipase producing bacteria from oil contaminated soils. *Advances in Biological Research*, 4(5):249-252.

Stephen, E. and Eugene, U.M. (2012). Microbiological and physiochemical properties of soil polluted with lubricating oil in Ayingba, Kogi state, Nigeria. *Nigerian Journal of Technology Research,* 7(2): 49-52.

Strong, P. J. and Burgess, J. E. (2008) Treatment methods for wine-related and distillery wastewaters: a review. *Bioremediation Journal* 12: 70-87.

Su, Y., Liu, C., Fang, H. and Zhang, D. (2020). *Bacillus subtilis*: a universal cell factory for industry, agriculture, biomaterials and medicine. *Microb Cell Fact,* 19: 173.

Sugahara, V.H. and Varea, G.S. (2014). Immobilization of *Beauveria bassiana* lipase on silica gel by physical adsorption**.** *Braz Arch Biol Technol.*; 57: 842-850.

Tebyanian, H., Hassanshahian, M. and Kariminik, A. (2013). Hexadecane-degradation by *Teskumurella* and *Stenotrophomonas* strains isolated from hydrocarbon contaminated soils. *Jundishapur Journal of Microbiology*, *6*:7.

Thakur, V., Tewari, R. and Sharma, R. (2014). Evaluation of production parameters for maximum lipase production by *P. Stutzeri* MTCC 5618 and scale-up bioreactor, *Chin. J. Biol.* 1–14.

Tong, X., Busk, P.K. and Lange, L. (2016). Characterization of a new sn-1, 3-regioselective triacylglycerol lipase from *Malbranchea cinnamomea*. *Biotechnol Appl Biochem*, 63(4):471–8.

Ullah., N, Daud, M., Hidayatullah, Shabir, M., Özkan A.T., Ullah, S. and Qasim, M. (2015). Screening identification and characterization of lipase producing soil bacteria from upper dir at Mardan Khyber Pakhtunkhwa, Pakistan. *Int. J. Biosci*, 6(2):49-55.

Uzoamaka G, Taise, F. and Muote-Okafor, F. (2009). Hydrocarbon degradation potentials of indigenous fungal isolates from petroleum contaminated soils. *Physical and Natural Sciences*, 2 (1): 1-6.

Varjani, S. J., Rana, D. P., Bateja, S., Sharma, M. C. and Upasani, V. N. (2014). Screening and identification of biosurfactant (bioemulsifier) producing bacteria from crude oil contaminated sites of Gujarat, India. *Int J Inno Res Sci Eng Technol*, *3:*2.

Varjani, S. J. and Upasani, V. N. (2017). A new look on factors affecting microbial degradation of petroleum hydrocarbon pollutants. *International Biodeterioration & Biodegradation*, *120*:71-83.

Veerapagu, M., Narayanan, A.S., Ponmurugan, K. and Jeya, K.R. (2013). Screening, selection identification production and optimization of bacterial lipase from oil spilled soil. *Asian Journal of Pharmaceutical and Clinical Research*, 6(3):62-67.

Veerapagu, M., Sankara, A., Jeya, K. and Alagendran, S (2014). Isolation and identification of a novel lipase producing bacteria from oil spilled soil. *Int J Innov Res Sci Eng Technol,* 3(5):111–9.

Vidali, M. (2001). "Bioremediation. An overview," *Pure and Applied Chemistry,* 7: 1163–1172.

Ward, C. P. and Overton, E. B. (2020). How the 2010 Deepwater Horizon spill reshaped our understanding of crude oil photochemical weathering at sea: a past, present, and future perspective. *Environmental Science: Processes & Impacts*, *22*(5): 1125-1138.

Whiteley, A.S. and Bailey, M.J. (2000). Bacterial Community Structure and Physiological State Within an Industrial Phenol Bioremediation System. *Applied Environmental Microbiology*. 66: 2400–2407.

Xue, J., Yu, Y., Bai, Y., Wang, L. and Wu, Y. (2015). Marine oil-degrading microorganisms and biodegradation process of petroleum hydrocarbon in marine environments: a review. *Current microbiology*, *71*: 220-228.

Yakimov, M. M., Timmis, K. N. and Golyshin, P. N. (2007). Obligate oil-degrading marine bacteria. *Current opinion in biotechnology*, *18*(3): 257-266.

Zhan, J., Zhang, A., Heroux, P., Li, X., Li, Z., Zhao, J., Guo, Y. and Liu, Y., (2019). Gasoline degradation and nitrogen fixation in soil by pulsed corona discharge plasma. *Sci total environ*, 661:266-275.

Zhou, E. and Crawford, R. L. (1995). Effects of oxygen, nitrogen, and temperature on gasoline biodegradation in soil. *Biodegradation*, *6*(2): 127-140.