SCREENING AND PRODUCTION OF LIPASE FROM LIPOLYTIC FUNGI UNDER SUBMERGED FERMENTATION

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A RESEARCH PROJECT SUBMITTED TO THE DEPARTMENT OF BIOLOGICAL SCIENCES, COLLEGE OF BASIC AND APPLIED SCIENCES, MOUNTAIN TOP UNIVERSITY, MAKOGI, IBAFO, OGUN STATE, NIGERIA.

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CERTIFICATION

This is to certify that this research project titled **"SCREENING AND PRODUCTION OF LIPASE FROM LIPOLYTIC FUNGI UNDER SUBMERGED FERMENTATION"** was carried out by ADESINA, Victor Oluwaseyi, with matriculation number 16010101009. This project meets the requirements governing the award of Bachelor of Science (B.Sc.) Degree in Microbiology, Department of Biological Sciences of the Mountain Top University, Ogun State, Nigeria and is approved for its contribution to knowledge and literary presentation.

Mr. G.E. ADEBAMI	DATE
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DECLARATION

I hereby declare that this project report written under the supervision of Mr. Adebami G.E is a product of my own research work. Information derived from various sources has been duly acknowledged in the text and a list of references provided. This research project report has not been previously presented anywhere for the award of any degree or certificate.

ADESINA, Victor O. Date

DEDICATION

This project is dedicated to Almighty God that has helped me throughout my stay in Mountain Top University and for sparing my life till this moment.

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My sincere and utmost appreciation goes first to my Lord and Saviour, the creator of the whole universe for His unending mercy and grace towards me for the past four years in Mountain Top University, also till the time of writing this project successfully. All thanks to His Name.

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ABSTRACT

Lipases are triacylglycerol acyl hydrolases with the enzyme number EC 3.1.1.3. They catalyze the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids. in this study, fungi were isolated from diesel and restaurant wastewater polluted soil and screened for lipase production on solid agar. The morphological and biochemical characteristics of the best isolate were determined. The effects of carbon and nitrogen sources on lipase production were also investigated. A total of 11 fungi were isolated. The diameter of growth ranged from 24.6^{i}- 47.0^{a} mm and lipase production ranged from 0.0^{h} mm- 10.5^{a} mm. Isolate VPD4 had the best lipase activity and was identified as *Fusarium oxysporum*. Carbon sources had significant difference ($p \geq 0.05$) in lipase production which ranged from 88.09^{h} U/mL- 188.65^{a} U/mL and nitrogen sources had significant difference ($p \geq 0.05$) in lipase production which ranged from 79.92^{g} U/mL - 182.22^{a} U/mL) respectively. Glucose (188.65^{a} U/mL) and Peptone (182.22^{a} U/mL) supported the highest lipase production. This study has shown that *Fusarium oxysporum* isolated from a diesel polluted soil is a good lipase producer and can be further investigated for industrial purpose.

Keywords: fungi, lipases, nitrogen source, carbon source, *Fusarium oxysporum*.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Lipases are triacylglycerol acyl hydrolases with the enzyme number E.C. 3.1.1.3 which are enzymes that involve in the catalysis of the hydrolysis of triacylglycerol to glycerol and fatty acids (Treichel *et al.,* 2010). They are found everywhere and are gotten from various animals, plants and microorganisms (Thakur, 2012). Microbial lipases produced by microorganisms have attained industries' attention due to their activeness under temperature, pH, organic solvents, chemo- regio and enantioselectivity (Bodh *et al.*, 2017). In addition, lipases can also catalyze different chemical reactions such as esterification, trans-esterification, acidolysis and aminolysis (Joseph *et al.,* 2008).

In order to obtain enantio and regio- selective substrates, lipases are often used in the catalysis of the hydrolysis of wide non- natural substrate (Thakur, 2012). The usefulness of these enzymes in the industries has brought about interest in isolating new lipases from various sources, and strong efforts have been concentrated on the enzymes for better performances in industrial applications. The potentials of microbial lipases biotechnologically include its stability in organic solvents, possession of wide substrate specificity and high enantio-selectivity (Treichel *et al.,* 2010). In the last decade, lipases have gained more recognition over proteases and amylases, specifically in the area of organic synthesis. The enantio-selective and regio- selective nature of lipases have been demonstrated for the resolution of chiral drugs, fat modification, synthesis of cocoa butter substituents, biofuels, synthesis of personal care products and flavour enhancer (Sundar and Kumaresapillai, 2013).

Among the various microorganisms, fungi are known to be among the lipase producers (Thakur, 2012). Due to their substrate stability when subjected under different physical and chemical conditions, fungal lipases have gained a lot of industries' attention (Akshita *et al.,* 2017). Fungal enzymes generally are extracellular therefore making it easy to be extracted from fermentation mixture which significantly reduced the cost of enzyme extraction and makes these extracted enzymes preferable over bacterial enzymes (Bodh *et al.,* 2017). Lipase

was discovered first in the year 1856 by Claude Bernard in the pancreatic juice (Chandra, 2020) and was demonstrated first in the seeds of plants which was first reported by Novo Nordisk in 1994 who discovered the first recombinant lipase 'Lipolase' which was gotten from the fungus *Thermomyces lanuginosus* and expressed in *Aspergillus oryzae* (Thakur, 2012).

1.2 STATEMENT OF PROBLEM

This research was made to produce lipase extracellularly from fungi and to show the production cost as an obstacle to the production of fungal lipase.

1.3 JUSTIFICATION OF THE STUDY

Lipases are majorly produced extracellularly by fungi and they are known to be one of the leading enzymes with proven abilities for their contribution to the industries. Basically, lipases from fungi have gained attention in the industries because they can remain active under temperature, pH, and organic solvents. Hence, lipase production using submerged fermentation has gained high positivity and great yield of products.

1.4 AIM OF THE STUDY

The aim of this research is to produce extracellular lipase from fungi species isolated from soil samples.

1.5 OBJECTIVES OF THE STUDY

The objectives of the study include:

i. Isolation and culturing of lipase producing fungi from fatty acid contaminated soi samples located at selected restaurant shops in Ogun State.
ii. Screening of the isolates for lipase production and selection of the best lipase producer
iii. Identification of the selected isolates using morphological and biochemical characterizations
iv. Production of lipase under submerged fermentation

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 FUNGI

A fungus is a eukaryote that externally digests food and directly absorbs the nutrients through its cell wall. A fungus inhabits different environments such as soil, leaves, root, fruits, water and food (Maheswari and Komalavalli, 2013, Rebecca *et al.,* 2012). Some environmental factors such as pH, temperature, moisture and type of nutrients affect the growth of fungi (Gaddeyya *et al*., 2012). The Kingdom Fungi is divided into Division Myxomycota (false fungi) and Division Eumycota (true fungi).The study of fungi is called Mycology. Fungi are abundant everywhere. Examples of fungal species include *Aspergillus, Fusarium, Penicillium, Thermomyces, Humicola, Acremonium, Eurotrium* etc. Fungi present in the soil function in the maintenance of soil fertility and this is influenced by the properties of the soil and the activities of humans (Bao *et al*., 2012). They also decompose organic matter and play important roles in nutrient cycling.

Fungi participate in nutrition that brings about the improvement of the health and development of the plant (Mulani and Turukmane, 2014), which makes them a high-priority concern because plants are sources of food for consumers and of great economic importance to farmers (Prabakaran *et al.,* 2011). Udoh *et al*. (2015) isolated some fungal species from fruits and vegetables that were responsible for post-harvest spoilage of these fruits and vegetables.

Fungi participate in varieties of activities and these activities can either be beneficial or non-beneficial. Some of these fungi are decomposers, harmful to other microorganisms as parasites or pathogens, some of them are beneficial in the process of symbiotic association with animals, plants and algae (Christopher *et al.,* 2012). Fungi are capable to grow on vertebrates and invertebrate animals. There are various fungal infections or mycoses (disease caused by fungi). The most common fungal infection is caused by dermatophytes i.e. a fungal species that colonizes dead tissues including skin, finger nails and toe nails. Dermatophytes cause superficial infections including ring worms that are difficult to treat but rarely serious (Christopher *et al.,* 2012). *Candida* species is an example of fungi that causes yeast infections

right in the mucosal tissues of individuals and they can also cause candidiasis in babies and immunocompromised individuals. *Aspergillus funmigatus* is another example of fungi species that produces small, airborne spores that are frequently inhaled and the fungi starts to grow by invading the lungs thus causing a disease known as aspergillosis (disease of the lungs) (Stiles *et al.,* 2012).

2.1.1 Morphological Characteristics of Fungi

Fungi constitute an important category of eukaryotes which varies from other groups, such as plants and animals. Fungal cell are enveloped within a rigid cell wall, consisting of chitin, glucans, mannans and glycoproteins (Leera *et al.,* 2019). These features differ from animal cell which has no cell wall and the plant cell which contains cellulose and various cell wall components (Brandt and Warnock 2015). In similarities to other eukaryotic organisms, fungal cells have true nucleus with a surrounding membrane and cell division is followed by mitosis or meiosis. Fungi can be single celled (unicellular) or multiple celled (multicellular). In multicellular organisms, the basic structural unit is a chain of multinucleate, tubular, filament-like cells. In most multicellular fungi, the vegetative state contains a mass of branching hyphae, called mycelium. A rigid cell wall is contained in each individual hypha in which as a result of apical growth increases in length as a result of apical growth. The hyphae remain without cross walls in primitive fungi. Fungi are heterotrophic, that is they lack chlorophyll, and thus need performed organic carbon compounds for their nutrition. Fungi primarily are composed of water (69- 90%), carbohydrates, proteins and lipids. Fungi live embedded in a food source or medium and by the derivation of nutrition by secreting enzymes into the external substrate and the nutrients released from the cell wall is being absorbed (Brandt and Warnock, 2015).

2.1.2 REPRODUCTION OF FUNGI

Fungi reproduce by two different methods of reproduction. These are asexual reproduction and sexual reproduction. Most fungi have a body called thallus containing microscopic tubular cells called hyphae and they reproduce by spores (Christopher *et al.,* 2013). Basically, these spores range from 40- 800 µm in diameter, each of which can contain hundreds or thousands of nuclei. A survival or dispersal unit, consisting of one or a few cells, that is capable of germinating to produce a new hypha is called a spore. These spores may be formed

singly or in clusters. Fungal spores generally lack an embryo, but contain food reserves needed for germination. As part of their life cycles, many fungi produce more than one type of spore. These spores may be produced through asexual reproduction including mitosis (mitospores), or through a sexual process called meiosis (meiospores) (Christopher *et al.*, 2012).

2.1.3 ECONMIC IMPORTANCE OF FUNGI

Fungi are vital for their ecosystem functions (Blackwell, 2011). Fungi play important roles in agriculture, food, medicine and in the industry (Beg *et al.*, 2004). Directly or indirectly, fungi are beneficial to human being. The filamentous fungi present in the soil play important role such as organic matter degradation and soil aggregation. Aside these properties, species of *Alternaria,* genus *Aspergillus, Cladosporium, Dematium, Gliocladium and Humicola* manufacture substances such as soil organic compounds which could be necessary for soil organic matter maintenance (Yuvaraj and Ramasamy, 2020). They are useful in the preparation of medicine. Examples of such fungal species are *Penicillium notatum, Claviceps purpurea, Aspergillus proliferous* etc. Fungi also help to produce antibiotics. The role of fungi in producing antibiotics was first established by Sir Alexander Fleming in 1929 (Alexopoulos *et al.*, 1996). He extracted penicillin from *Penicillium notatum.* Penicillin is an organic substance lethal to microorganisms. It kills bacteria especially gram positive bacteria. In medicine, fungi are being used to fight different diseases such as cancer, bacterial infections, diabetes etc. Research has shown that a species of Ganoderma contains compounds contains anti-tumor and anti-fibrotic properties (Hyde, 2019). Different strains of soil fungi are found to be capable of utilizing petroleum hydrocarbons. Examples are *Fusarium, Penicillium, Acremonium, Mortierella* and *Trichoderma*. Fungi are important in enzymes production which is useful in various industries (Leera *et al.,* 2019).

2.2 LIPASE

Lipases are triacylglycerol acyl hydrolases with the enzyme number E.C 3.1.1.3 that catalyze the hydrolysis of triacylglycerol to glycerol and fatty acids (Treichel *et al.,* 2010). Lipase producers are found everywhere in nature, including different plants, animals and microorganisms. The biological function of lipases is the catalysis of the hydrolysis of

triacylglycerols to produce di-acylglycerols, mono-acylglycerols, glycerol and fatty acids (Patil *et al*., 2011).

The reasons for the potentiality of microbial lipases are ability to remain stable in organic solvent, no cofactors needed, possession of a broad substrate specificity and exhibition of a high enantio-selectivity (Akshita *et al.,* 2017). At various stages such as fat digestion, adsorption and metabolism of lipoprotein, lipases are also involved in the metabolism of lipid in eukaryotes (Balashev *et al.,* 2001). Lipases accounts nearly 10% of the enzyme market and they are recognized as one of the prominent enzymes (Salihu *et al.,* 2013).

2.2.1 General Properties of Lipase

Lipases are stable enzymes that act on lipid and also on variety of natural and artificial reactants because can catalyze diversified reactions. Because they can perform specific biotransformation has made them to be more useful in the food, cosmetics, detergents and pharmaceutical industries (Gupta *et al.*, 2017). According to Menoncin *et al*. (2010), it was stated that since the 1980s, the number of available lipases has increased which resulted to the huge achievements of expressing enzymes in microorganisms, as well as increasing demands for these enzymes with specific properties such as stability, pH, specificity and temperature. According to Sharma and Shukla (2011), 75% of industrial enzymes including lipase are hydrolytic in action and this has stated the general fact that they remain enzymatically active in organic solvent, including several studies to analyze the activities of microbial lipases under different environmental conditions.

2.2.2 Microbial Sources of Lipase

Microorganisms are recognized as the best source of extracellular lipases especially fungi (Facchini *et al.*, 2016). Microorganisms with the abilities to produce lipases can be seen in different habitats, such as, dairy product industries, vegetable oil waste and deteriorated foods (Sharma *et al*., 2001). This indicates the fact that nature also contributes a tremendously to the identification of new lipase sources and this has improved the search for microorganisms from natural sources with improved lipolytic properties. Microorganisms have so many advantages including the ability to catalyze different reactions, production of high products, enhanced stability and reduced production costs (Tan *et al.*, 2003). This is why microbial lipases (i.e. lipases gotten from microorganisms) are significant due to low production cost, stability and availability (Patil *et al.*, 2011). They are more useful than lipases gotten from animals or plants because of the catalytic activities that are present. Among the microorganisms producing lipase, fungi are known to be one of the best lipase producing microorganisms. Fungi are widely recognized as the best lipase sources and are used preferably for industrial applications; especially in food industry (Facchini *et al.*, 2016). Fungal lipases are extracellular, thus making it easy to extract reducing the extraction cost (Urgyn *et al.*, 2017). Some of the fungi species that produces lipase are known to belong to the genus of *Rhizopus sp., Aspergillus sp., Penicilium sp., Mucor sp.,* and *Rhizomucor sp* (Tan *et al.*, 2003).

Fungal lipases are diversified in their enzymatic properties which makes them useful for industrial application. They are part of an important group of biotechnologically important enzymes because of their properties and easy mass production (Ray, 2015). Differences in fungal lipase production is according to the fungal strain, growth medium composition, cultivation conditions, pH, temperature and carbon and nitrogen sources (Cihangir and Sarikaya, 2004). The production of fungal lipase occurs during the late logarithmic phase or stationary phase; thus the cultivation period may vary in accordance with the microorganism's growth rate (Akshita *et al.,* 2017). The fungal strains are preferred for lipase production since it is secreted extracellularly and extraction of the lipase is easy (Sumathy *et al.,* 2012). According to Gupta *et al*.(2017), there are three detective factors of lipase-positive fungi. They are fungal growth, fungal lipase production and a sensitive method to detect lipase activities.

Growth conditions affect the synthesis of lipase by microorganisms which include the presence of activators and inhibitors, incubation temperature, pH, inoculum amount and oxygen (Thakur, 2012). Carbon source is known as the main factor that influences lipase production. These enzymes production is on the basis of the presence of a lipid, such as olive oil or any other inducer, such as triacylglycerol, fatty acids and tweens (Gupta *et al.,* 2017). They play important functions in different stages of lipid metabolism in eukaryotes which include fat digestion, absorption, reconstitution, and metabolism of lipoprotein.

The table below shows different microorganisms, time, lipase activities, type of fermentation and raw material.

Table 2.1 Different fungi, time, lipase activities, type of fermentation and raw materials. Journal of Biotech research (Akshita *et al.,* 2017).

Microorganisms	**Time (h)**	**Lipase activity (U/mL)**	**Type of fermentation**	**Raw material**
Penicillium aurantiogriseum	48	25	SmF	Soya bean oil
Rhizopus rhizopodiformis	24	43	SSF	Olive oil cake-Bagasse

Rhizopus pusillus	25	10.8	SSF	Olive oil cake-Bagasse
Penicillium restrictum	24	30	SSF	Babassu oil cake
Rhizopus oligosporus TUV-31	48	76.6	SSF	Egg yolk
Candida cylindracea	179.5	23.7	SmF	Oleic acid

Code: SSF - Solid State Fermentation; SmF - Submerged Fermentation

2.3 SUBMERGED FERMENTATION

Submerged fermentation is the process of microorganisms' cultivation in liquid media containing soluble carbon source and nutrients. This process involves the growing of microorganisms selected in closed vessels which contain rich nutrients and a high oxygen concentration. Submerged fermentation has been used in the large- scale culture of microorganisms in fermenters, and the recovery of useful products contained within the microbial cells. This process makes use of some common substrates such as molasses, liquid media, fruit and vegetable juices and sewage. Submerged fermentation takes place in batch, continuous or fed- batch culture systems. A batch culture system is a system in which nothing is removed or added to an environment after a medium of appropriate composition is inoculated with living cell. Fed- batch culture is a modified form of batch culture in which transition from exponential growth of the microorganism to the stationary phase is prevented by adding fresh medium from time to time.

2.3.1 Applications of Submerged Fermentation

According to Ito *et al*. (2001), it was stated that microbial lipases are mostly produced through submerged fermentation. Coradi *et al*. (2013) mentioned that due to the recovery of extracellular enzymes and the determination of biomass is easy by facilitating through centrifugation, lipases have been produced by submerged fermentation. Submerged fermentation is important in secondary metabolites extractions that are needed in liquid forms. It is traditionally used to produce enzymes that are microbially derived because filamentous fungi are able to flourish in submerged fermentation medium. At industrial levels, a cost

effective synthesis involving low cost culture media under submerged fermentation would be optimal as lipases are in great demand (Grazia and Trono, 2015).

2.3.2 Advantages of Submerged Fermentation

Submerged fermentation has different choices of microorganisms under abundant supply of moisture that are capable of growth. Submerged fermentation has the well- established technological basis for scaling the process to industrial production capacity and fungal lipase production is greatly influenced by the initial pH, moisture content, and temperature, amount of substrate, inoculum size and oxygen concentration that is dissolved (Amin and Bhatti, 2014). Submerged fermentation has a large impact on productivity, leading to higher yields and improved product characteristics compared to solid state fermentation. The reproducibility of submerged cultivations is not often challenged by medium heterogeneity. Submerged fermentation technology has the advantages of short period, low cost and high yield. Products purification is easier and in liquid culture, fermentation control is simple achievement of reduction in fermentation times can be possible. Also, it can benefit the production of secondary metabolites and decrease in production costs (Arun *et al.,* 2017).

2.3.3 Disadvantages of Submerged Fermentation

According to Ramos- Sanchez *et al*. (2015), filamentous fungi cannot show grow more efficiently in submerged fermentation. There is no potential to produce desired microbial products more efficiently in submerged fermentation leading to low volumetric productivity (Kumar, 2014). Fungal strains used in submerged fermentation produces undesirable results leading to relatively lower concentration of the products (Kumar, 2014). Lipase activity obtained in submerged fermentation is lower (Azeredo *et al.,* 2007). The complexity of fermentation machineries in submerged fermentation leads to high energy demand and high capital (Leda *et al.,* 2000).

2.4 Factors affecting Lipase Production

Variety of conditions has been described which stimulate fungi lipase production. The important stages in a biological process are optimization to improve a system and to increase

the efficiency of the process without cost increase. There are certain environmental factors that play significant role during enzyme production and metabolic activities.

2.4.1 Carbon Source

Carbon sources play important roles in lipase production in all types of microbial sources. The increase in lipase production in fungal strains is accomplished by addition of various carbon sources such as glucose, Tween 80, starch, galactose, fructose, maltose, lactose etc. According to Zarevucka (2012), *Aspergillus terreus* observed a good yield in lipase production with mustard seed oil as the carbon source. Also, in contrast to other carbon sources, an increase in lipase development was recorded with olive oil cakes (Zarevucka, 2012).

2.4.2 Nitrogen Source

Nitrogen plays significant role in lipase production. Diverse organic and inorganic sources of nitrogen have played basic roles in the higher production of lipases from various microbial species (Priyanka *et al.,* 2019). According to Bõhm and Boos (2004), it was stated that organic nitrogen sources produces better results than the inorganic nitrogen sources. This means that inorganic nitrogen sources are consumed quickly and this can cause the low or no efficiency of the lipase (Bõhm and Boos, 2004) while organic nitrogen sources can supply amino acids and many cell growth factors, which is needed for cell metabolism and protein synthesis (Helal *et al.,* 2017).

2.4.3 Temperature

Different incubation temperature influences fungal lipase production. Bharathi *et al.,* (2019) stated the fact that optimum temperature is important for lipase secretion as higher yield of lipase production was observed at 37^{o}C. De Souza *et al*. (2019) observed that low temperature reduces the output of lipase production while higher temperature maximizes the output of lipase production.

2.4.4 pH

Different pH levels ranging from 3 to 8 are used to test for the effectiveness of fungal lipase production. If there is a sudden drop in lipase activity at pH 8, the initial basic medium could enhance intracellular pH of the cell and destabilize the enzyme synthetic network (Stockar *et*

al., 2006). Turati *et al.*, (2019) observed that fungal lipase production was higher at acidic pH where an increased lipase production was observed at pH 4 of the reaction medium.

2.5 Applications of Lipase

Fungal lipases intheir enzymatic properties are diversified in which they are useful in various applications in the industries (Andualema and Gessesse, 2012, Ray, 2015). They are important group of biotechnologically enzymes due to their potent properties and easy mass production. The industrial applications of fungal lipases have been reviewed by many researchers (Treichel *et al.*, 2010).

The different applications of lipase include:

2.5.1 Lipases in Food Processing Industry

Fats and oils are important constituents of foods and their modification is an area in food processing industry that demands novel economic and green technologies. Most of the lipases produced commercially are used for flavor development in dairy products and other foods processing, such as vegetable, fruits, meat, baked foods, milk products, and beer. Lipases from *Aspergillus niger, Rhizopus oryzae, Candida cylindracea* have been used in bakery products (Akshita *et al.*, 2017). Applications of lipases in food industries are given in the table below:

Table 2.2: Applications of lipases in the food industry (Journal of Biotech research, Akshita *et al.*, 2017)

Food industry	Action	Product of application
Dairy foods	Hydrolysis of milk, fat, cheese ripening, modification of butter fat	Development of flavoring agents in milk, cheese and butter.
Bakery foods	Flavor improvement	Shelf- life extension, volume improvement.
Beverages	Improved aroma	Alcoholic beverages, e.g. wine

Food dressings	Quality improvement	Mayonnaise, dressings and whipping
Health foods	Trans- esterification	Health foods
Meat and fish	Flavor development	Meat and fish product, fat removal
	Tran- esterification	Cocoa butter, margarine,
Fats and oils	Hydrolysis	fatty acids, glycerol, mono and diglycerides.

2.5.2 Lipase as biosensor

In clinical diagnosis and in food industry, the quantitative determination of triacylglycerol is of great importance. The lipid sensing device as a biosensor is rather cheaper and less time consuming as compared to the chemical methods to determine triacylglycerol. Basically, lipase is being used to generate glycerol from the triacylglycerol in the sample and to measure the glycerol released by an enzymatic method. Lipase biosensor is also used for lipid determination in clinical diagnosis (Gupta *et al.,* 2017).

2.5.3 Lipases in ester synthesis

Lipases have been useful for the synthesis of esters. The esters produced from short chain fatty acids have applications as flavoring agents in food industry. As reported earlier, esterification of sulcatol and fatty acids in toluene was catalyzed by *Candida rugosa* lipase (Bodh *et al.,* 2017). The esterification reaction of lauryl alcohol and palmitic acid with *C. Antarctica* lipase as the catalyst is being known to give a yield of more than 90% of lauryl palmitate under optimized conditions (Gupta *et al.,* 2017).

2.5.4 Lipases in bioremediation

Bioremediation for waste disposal is a new avenue in lipase biotechnology. Lipases have been extensively used in waste water treatment. Species of fungi can be useful in the environment to remove oil spillage, which brings about restoration and help in oil processing in the industries. Some examples of fungi that have been described as tolerant to a variety of pollutants and indicated as potential bioremediation agents in soil are the genera of

Trichoderma, Fusarium, *Penicillium, Aspergillus*, *Cladosporium*, *Mortierella*, *Beauveria,* and *Engyodontium* (Gupta *et al.*, 2017).

2.5.5 Lipases in Textile Industry

The use of lipase gotten from fungi is becoming important in the textile industries. As such, they are used in lubricants removal in order to provide the fabric better absorbency and enhance levelness in dyeing (Gupta *et al.,* 2017). Together with alpha amylase, lipases are being used for the desizing of cotton fabrics at the commercial scale. *Aspergillus oryzae* lipase is an example of a modifying agent that modified PET (Polyethylene terephthalate), which improves the hydrophobicity and anti- static ability of the fabric (Akshita *et al.,* 2017).

2.5.6 Lipases in detergent industry

Fungal lipases are being used as additives in detergents for laundry and detergents used at home, which reduce the environmental load of detergent products, as it saves energy by enabling a lower wash temperature (Saisubramanian *et al.,* 2006). Lipase gotten from *Humicola lanuginosa* is useful as a detergent due to its thermostability, high activity at alkaline pH and stability towards anionic surfactants. Lipases used as detergents also include those from *Candida*. The other applications of detergents are in dish washing, in a bleaching composition, lipid contaminants decomposition in dry solvents, liquid leather cleaner, contact lens cleaning, washing, degreasing and water reconditioning by using lipases along with oxido- reductases (Bodh *et al.*, 2017).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Equipment

500 ml cylindrical flask, filter paper, oil- contaminated soil sample, test tubes, cotton wool, petri dishes, glass slides, cover slips, conical flask, 1 L Erlenmeyer's flasks, inoculating loop, Bunsen burner, Incubator, autoclave, weighing balance, oven, water bath, microscope.

3.2 Culture media and Reagents

Potato Dextrose Agar (PDA) Glucose, 30 $g \cdot L^{-1}$; $NaNO_3$, 2.0 $g \cdot L^{-1}$; K_2HPO_4, 1.0 $g \cdot L^{-1}$; $MgSO_4.7H_2O$, 0.5 $g \cdot L^{-1}$; KCl, 0.5 $g \cdot L^{-1}$; HCl 1.5 mol/L or NaOH 1 mol/L;$FeSO_4.7H_2O$, 0.001 $g \cdot L^{-1}$; Yeast extract, 1.0 $g \cdot L^{-1}$ Agar- agar, 20 g L^{-1},10% wheat bran, Peptone (0.3 $g.L^{-1}$), TWEEN 80 Solution (0.1% v/v), soy bean oil (20g/L), Saline solution (2 g/L of KH_2PO_4, 1 g/L of $MgSO_4$, and 10 mL/L of trace solution containing $FeSO_4 \cdot 7H_2O$ (0.63 mg/L), $MnSO_4$ (0.01 mg/L), $ZnSO_4$ (0.62 mg/L).

3.3 Sample Collection

Soil samples were collected from selected restaurants and diesel- contaminated sites from a depth of 5- 10cm using a sterile spatula and they were stored in a sterile McCartney bottle. The bottles were then labeled properly and moved to the laboratory for examination and for subsequent analysis. Furthermore, all the samples were stored at 27^oC when not in use.

3.4 Preparation of culture media

Maintaining aseptic conditions and methods, 39g of commercially prepared Potato Dextrose Agar was measured and dissolved in a sterile distilled water of 1 litre in conical flask and was mixed thoroughly. Also, the mixture was boiled to dissolve the culture medium completely. After boiling, the agar was then sterilized by autoclaving at 121°C for 15 minutes and allowed to cool to 45°C before pouring the medium into the sterile petri dishes.

3.5 Isolation of Fungal Species

The isolation of microorganisms was performed by serial dilution. 1g of the soil samples were serially diluted in distilled water of 9mL inside the test tubes and 1ml was taken from each test tubes and added into another 9ml for 10^{-1} and another 1mL was taken from 10^{-1} and added

to another 9mL of distilled water for 10^{-2} and then another 1mL was taken from the 10^{-2} and added to another test tube of distilled water of 9mL to get 10^{-3} and then another 1mL was taken from 10^{-3} and added to another test tube of distilled water of 9mL to get 10^{-4} and then another 1ml was pipetted from 10^{-4} and added to another test tube of distilled water of 9mL to get 10^{-5}.

After the serial dilution method, the pour plate technique followed. For the Potato Dextrose Agar plate, pour plate technique were used for plating of samples using 3 empty sterilized petri dishes. After the serial dilution method, 0.1mL was pipetted from the test tubes labeled 10^{-1}, 10^{-3} and 10^{-5} and was carefully dropped at the centre of the 3 empty petri dishes and the agar was poured (observing aseptic methods and conditions) after it had been cooled. The Agar plates were incubated at 27^{o}C for 5 days under aerobic condition.

After the incubation of the agar for 3- 5 days, the subculture method follows maintaining aseptic methods and conditions. The distinct fungal colonies of interest were selected from the heterotrophic colony and were transferred using a sterilized inoculating loop into a new culture medium to get a pure culture. Also, streptomycin was added to the culture medium (PDA) to prevent bacterial growth. The plates were then incubated and stored in a plastic bag at 4^{o}C.

3.6 Screening of Fungal isolates for lipase production

The screening of lipase producing fungi was done using the tributhyrin agar; as a substrate on agar plates. Tributhyrin agar (HI Media) was used for the following compositions: Peptone: 5.0gm; Yeast extract: 3.0gm; Agar- Agar: 15.0gm; Tributhyrin (Glycerol Tributyrate): 10.0mL; Distilled water: 990 mL; pH: 7.5. The clear zones formed around the colony indicate the production of lipase. All the isolated fungal cultures were then inoculated on the Tributhyrin Agar plates and at a temperature of 27^{o}C, the plates were incubated up to 7 days. The formation of the opaque zones around the colonies is an indication of lipase production by the organisms.

3.7 Identification of Selected Fungi

After screening, the potent fungi species were inoculated on Potato Dextrose Agar and incubated at room temperature for identification and further studies.

3.7.1 Morphological Identification

The cultures were examined and identified when they sporulate. The growth of fungi on solid agar was based on their growth pattern, colony, texture, pigmentation and growth rate of the colonies on the PDA in order to identify the fungi accurately.

3.7.2 Biochemical identification

Lactophenol blue staining is used for staining fungi. It comprises of phenol crystals, cotton blue, lactic acid, glycerol and distilled water. A drop of Lactophenol cotton blue reagent was placed on a clean and dry glass slide. Using a sterilized inoculating loop, a little out of the fungal culture was picked and made into a thin preparation leading to the making of a smear on the glass slide after which a cover slip was then placed on the glass slide. The glass slide was placed on the microscope and examined under a light microscope with an attached camera connected to a computer for the microscopic photography of the fungi. The essence of these is to observe the exact arrangement of the conidiophores and the way the spores are being produced.

3.8 Lipase Production using Submerged Fermentation Process

The submerged fermentation processes was prepared in a 300mL Erlenmeyer flask with 100 mL of the medium containing 15 g/L glucose, 2 g/L KH_2PO_4, 1 g/L $MgSO_4$, and 10 mL/L of trace solution containing (mg/L) $FeSO_4 \cdot 7H_2O$ (0.63), $MnSO_4$ (0.01), $ZnSO_4$ (0.62), 20 g/L of yeast extract as nitrogen source and 20 g/L of soybean oil as inducer. The pH was adjusted to 7.0 using HCL 1.5 mol/L or NaOH 1 mol/L. Afterwards, the medium was autoclaved at 103 kPa for 20 mins. After inoculation, the cultures were incubated for 4 days at 30°C with agitation of 160 min^{-1} (Bertolin *et al*., 2001). The effects of other carbon sources were also investigated by replacing wheat bran with banana peel, rice bran, plantain peel, glucose, sucrose, mannitol and starch.

3.9 Lipase Assay

Lipase assay was determined using the colorimetric method. This entails two solutions which were prepared for the lipase assay. The first solution used contained 90 mg of *p*-nitrophenyl palmitate that was dissolved in 30 mL propane-2-ol. The second solution used contained 2g Triton X-100 and 0.5 g of gum arabic dissolved in 450 mL (Tris- HCl 50 mM) buffer at a pH

of 8.0. The assay solution was prepared by adding 1 ml of solution 1 to 9 ml of solution 2 to get an emulsion that remains stable for 2 hours. The assay mixture contained 900 µl of the emulsion and 100 µl of the appropriately diluted enzyme solution. The liberated *p*-nitrophenol was measured at 410 nm using spectrophotometer. One unit of the enzyme was defined as the amount of enzyme that releases 1 µmol of p-nitrophenol from the substrate (Saeed *et al.*, 2009).

3.10 Effect of Carbon Source on Lipase Production

The effects of carbon sources such as glucose, galactose, fructose, maltose, lactose, sucrose, mannitol and starch on lipase production were investigated. The submerged fermentation processes was prepared in a 300mL Erlenmeyer flask with 100 mL of the medium containing 15 g/L carbon source, 2 g/L KH_2PO_4, 1 g/L $MgSO_4$, and 10 mL/L of trace solution containing (mg/L) $FeSO_4 \cdot 7H_2O$ (0.63), $MnSO_4$ (0.01), $ZnSO_4$ (0.62), 20 g/L of yeast extract as nitrogen source and 20 g/L of soybean oil as inducer. The pH was adjusted to 7.0 using HCl 1.5 mol/L or NaOH 1 mol/L. Afterwards, the medium was autoclaved at 103 kPa for 20 mins. After inoculation, the cultures were incubated for 4 days at 30°C with agitation of 160 rpm (Bertolin *et al*., 2001).

3.11 Effect of Nitrogen Source on Lipase Production

The effect of nitrogen source such peptone, beef extract, ammonium sulphate, sodium nitrate, potassium nitrate and urea on lipase production under submerged fermentation were investigated. The production medium contained 15 g/L glucose, 2 g/L KH_2PO_4, 1 g/L $MgSO_4$, and 10 mL/L of trace solution containing (mg/L) $FeSO_4 \cdot 7H_2O$ (0.63), $MnSO_4$ (0.01), $ZnSO_4$ (0.62), 20 g/L nitrogen source and 20 g/L of soybean oil as inducer. The pH was adjusted to 7.0 using HCl 1.5 mol/L or NaOH 1 mol/L. Afterwards, the medium was autoclaved at 103 kPa for 20 mins. After inoculation, the cultures were incubated for 4 days at 30°C with agitation of 160 rpm (Bertolin *et al*., 2001).

3.12 Statistical Analysis

The values for each parameter were calculated and presented as means of duplicates. Data was analyzed using Analysis of Variance (ANOVA) with Duncan Multiple Range Test for

significance at $P \leq 0.05$. Standard deviation was not shown. Data were also presented in tables (Aforijiku *et al.*, 2019).

CHAPTER FOUR

4.0 RESULT

A total of eleven (11) different fungal species were isolated and screened for mycelium growth and lipase production. These isolates were identified using their morphological characteristics.

Table 4.1 shows the results for the screening of the isolates for mycelium growth and production of lipase on solid agar. At the end of the incubation period, there was significance difference ($p \geq 0.05$) in diameter of growth and lipase activity. The mycelium growth range from 24.6^{i} mm – 47.0^{a} mm. Isolate VMS4 had the highest diameter of growth (47.0 mm) while isolate VPD6 had the lowest (24.6 mm). Lipase production measured based on the diameter of zone of clearance of the isolates ranged from 5.0^{g} U/mL – 10.5^{a} U/mL. Isolate VPD4 has the highest lipase activity (10.5 U/mL) while isolates VPD3 had the lowest. Both isolates VMS3 and VMS5 could not produce lipase at the incubation conditions investigated. Based on the screening results, isolate VPD4 was selected for further investigation.

Figure 4.1 shows the morphological identification of the fungal isolate that was selected (VPD4). The isolate grew rather rapidly to produce of-white cotton-like colony. The reverse was rather a pale brown to yellow colouration.

Figure 4.2 illustrates the microscopic image of the fungal isolate that was selected following staining with Lactophenol blue stain. The microscopic appearance showed the hyphae are hyaline (clear/non-pigmented) and are septate (show divisions or walls within the hyphae). Conidiophores are short and usually non-septate. The conidiophores have an inflated appearance as their sides are not parallel but slightly bulge out in the middle. As these conidiophores extend from the aerial mycelium, they are produced. Microconidia are usually non-septate, ellipsoidal and are straight or slightly curved in shape as they are abundantly produced from the tip of these phialides. Microconidia are produced singly and not in chains. These microconidia may accumulate around the tip of the phialide if not dispersed. On the basis of the results of the morphological and biochemical characterizations, the probable identity of the selected isolate was *Fusarium oxysporum*.

Figure 4.3 summarizes the effects of carbon sources on lipase production by isolate VPD4. There was a significance difference ($p \geq 0.05$) in production of lipase in the presence of various carbon sources such as monosaccharide (glucose, and galactose), disaccharide (maltose, lactose, and sucrose), polysaccharide (starch), alcohol sugar (mannitol). The highest lipase activity was recorded when glucose (188.65^{a} U/mL) was added to the substrate followed by fructose (172.45^{b} U/mL), galactose (165.56^{c} U/mL), and maltose (163.23 U/mL), while the least lipase activity was recorded when starch (88.09 U/mL) was used as carbon source.

Figure 4.4 shows the effects of different organic and nitrogen sources on the activity of extracellular lipases. There was a significance difference ($p \geq 0.05$) in the production of lipase using different organic and inorganic nitrogen sources. Lipase production ranged from 79.92^{g} U/mL – 182.22^{a} U/mL. The highest lipase activity (182.22^{a} U/mL) was recorded in peptone followed in order by yeast extract (175.43^{b} U/mL), Ammonium Sulphur (173.08^{c} U/mL), and beef extract (168.61^{d} U/mL), while urea (79.92 u/mL) provided the lowest activity.

Table 4.1: Screening of Isolates for Lipase activity

Isolates	Diameter of growth (mm)	Lipase activity diameter (mm)
VPD1	37.0^{f}	9.0^{b}
VPD2	39.0^{e}	7.0^{d}
VPD3	37.0^{f}	5.0^{g}
VPD4	44.0^{b}	10.5^{a}
VPD5	41.0^{d}	8.0^{c}
VPD6	24.6^{i}	5.5^{f}
VMS1	32.0^{g}	6.0^{e}
VMS2	32.0^{g}	7.0^{d}
VMS3	42.0^{c}	0.0
VMS4	47.0^{a}	6.0^{e}
VMS5	29.0^{h}	0.0

Mean followed by different superscript within a column are significantly different (P≥0.05).

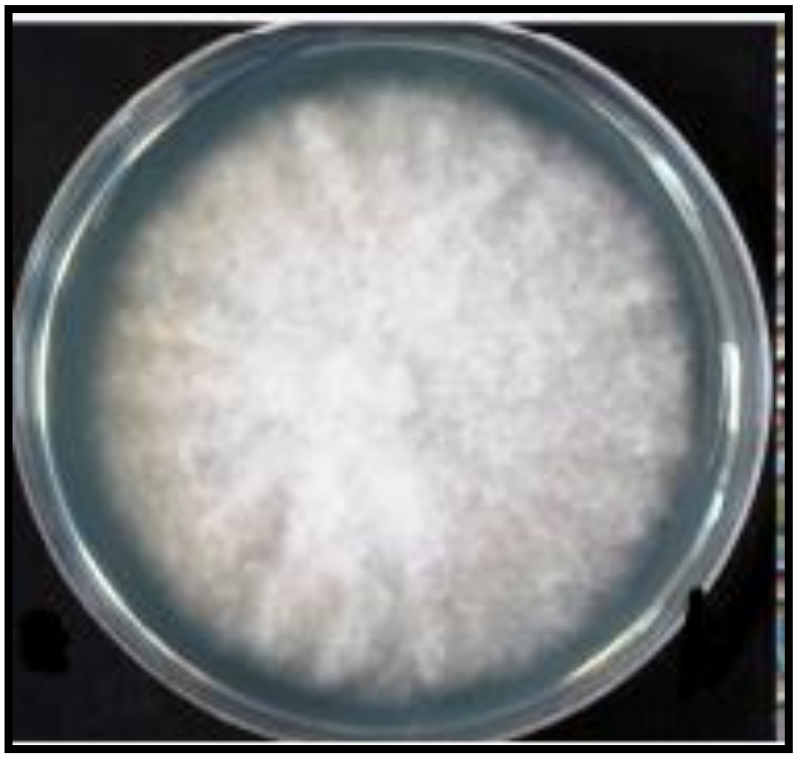

Figure 4.1: Morphological appearance of the isolate on PDA

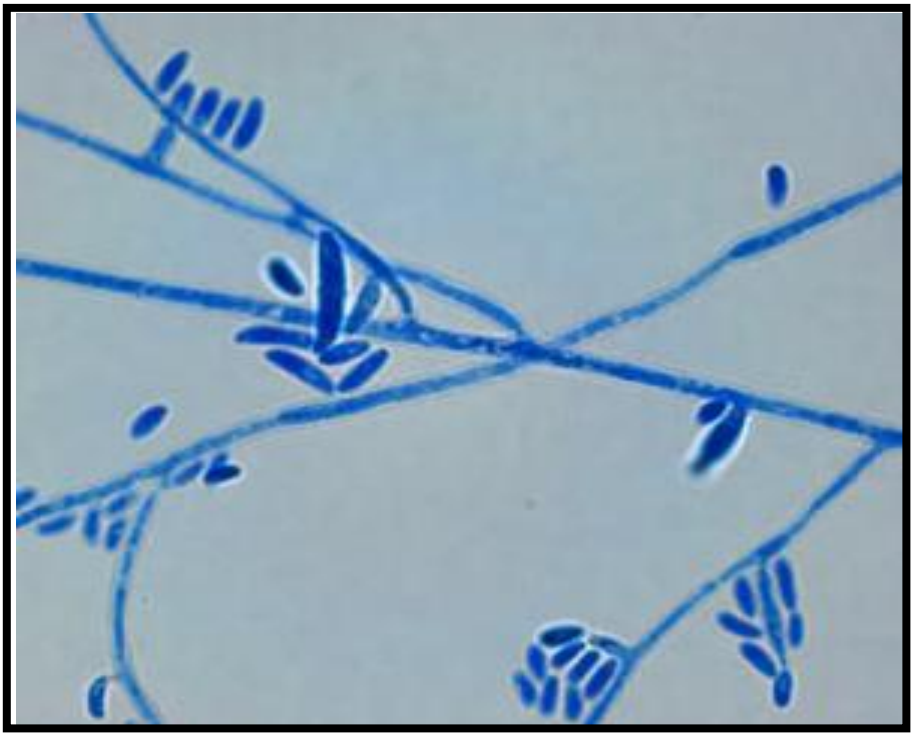

Figure 4.2: Microscopic image of the isolate *Fusarium oxysporum*

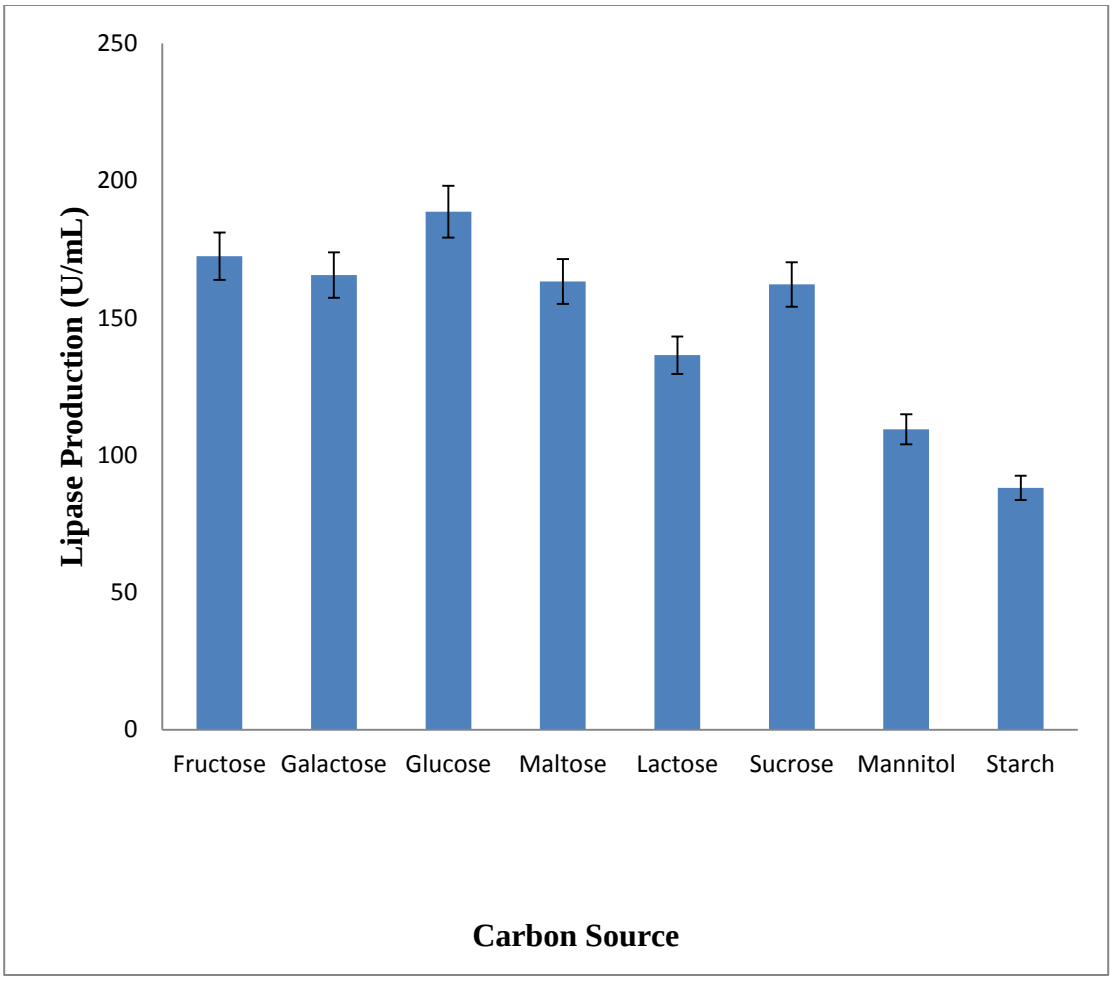

Figure 4.3: Effect of carbon source on lipase activity

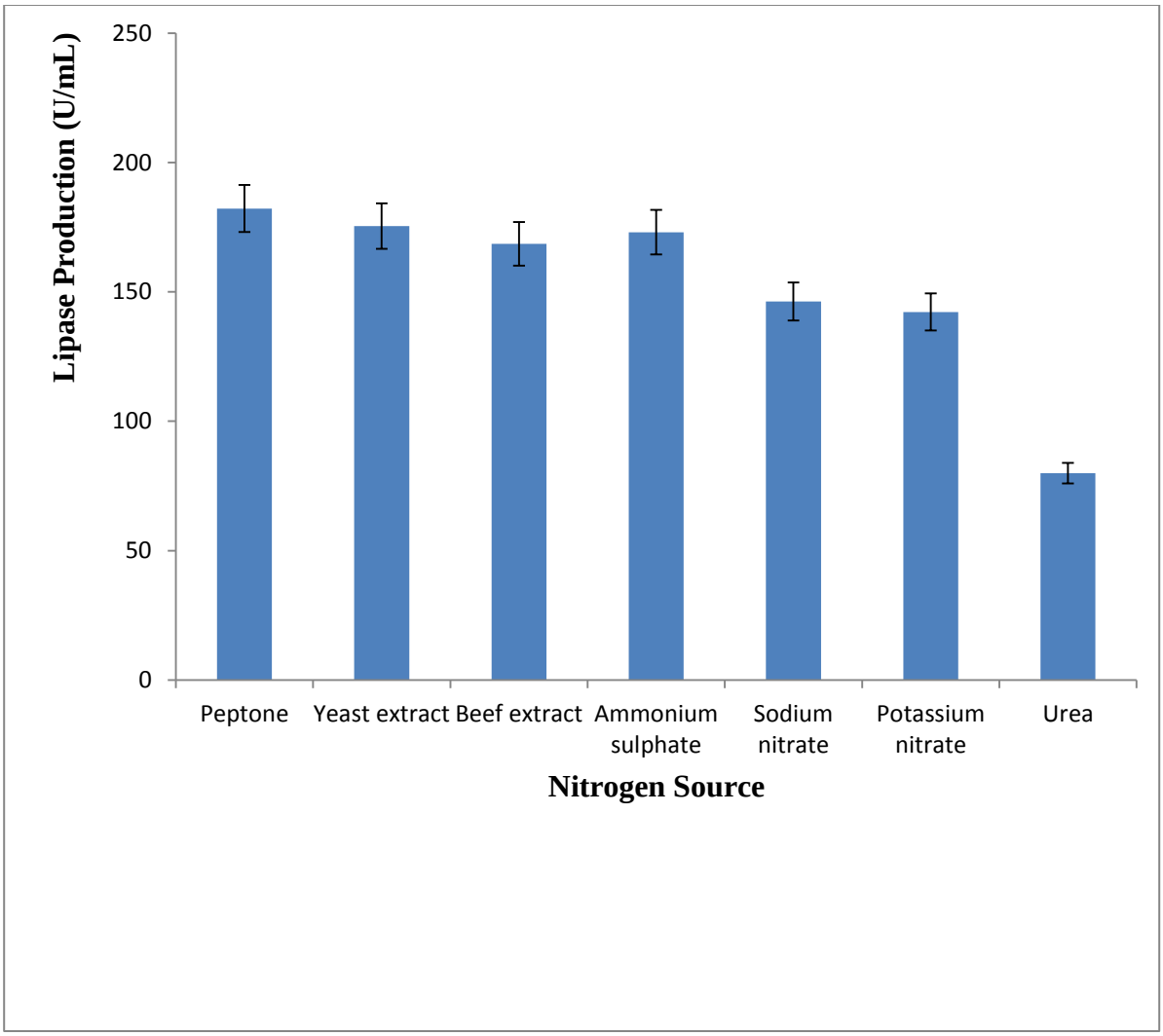

Figure 4.4: Effects of Nitrogen Source on lipase activity

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

5.1 DISCUSSION

The presence of lipase-producing fungi from environmental samples has been previously reported. Musa and Adebayo- Tayo (2012) reported similar findings by screening 17 distinct fungal sources from which lipases were derived. Also, Yalçin *et al.* (2014) screened a total of 120 fungal species gotten from various environments such as wastewater, diesel contaminated site to be able to produce lipase and exhibit lipase activities.

The morphological and the biochemical characteristics of the selected isolate observed in this study shows that the isolate is *Fusarium oxysporum*. The morphological and biochemical characteristics were similar to the report by Janaina *et al.* (2006). Also, Hala *et al.,* (2010) isolated *Fusarium oxysporum* to be a good source of lipase. Similar to the study, Fernando and Jose- Vicente (2001) also isolated *Fusarium oxysporum* to give the best yield and a good source of lipase.

Several nutritional factors such as nitrogen and carbon sources have known to affect lipase production. As such, their optimizations have always been of great attraction to industries due to the reduction in production cost (Kumar and Mausumi, 2012). In this study, among the different carbon sources investigated, glucose supported the highest production of lipase (188.65 U/mL) while starch resulted in the lowest production (88.09 U/mL). It therefore shows that glucose favoured the fungal growth while starch does not. Similarly, Arun *et al.* (2016) reported the same result whereby lipase production by *Aspergillus niger* shows a high value when glucose was added but got the highest production of lipase in the mixture of olive oil and glucose. In contrast, Falony *et al*. (2006) reported that lipase activity (0.53 U/mL) by *Aspergillus niger* was high in the presence of olive oil but later changed to 0.99 U/mL when the medium was further supplemented with glucose. Also, Sarkar and Laha (2013) reported similar results with the mixture of glucose and olive oil showing the increase in lipase production by *Aspergillus niger.*

Different nitrogen sources were used to determine their effects on lipase production. Among the nitrogen sources used, it was recorded that peptone was the best nitrogen source resulting in the highest lipase activity (182.22 U/mL) while urea gave the lowest support for lipase production (79.92 U/mL). All the tested nitrogen sources increased the production except urea. Urea was harmful to the cells at the tested concentration. Urea probably denatured the cellular proteins, therefore fungal growth and productivity of lipase was decreased (Arun *et al.*, 2016). Similarly, Cihangir and Sarikaya (2004) reported the same findings that the optimum lipase activity (14.83 U/mL) by a novel isolate of *Aspergillus* sp was achieved when the medium was supplied with peptone. In this report, peptone was the optimum source of nitrogen. Also, Aulakh and Prakash (2010) reported the same result whereby peptone was the most suitable nitrogen source for the lipase production (21.80 U/mL) by *Aspergillus* sp. In contrast, Salihu *et al*. (2013) reported that lipase production was increased by *Aspergillus niger* when the medium was supplemented with $(NH_4)_2SO_4$. In contrast to this study, Amira *et al.* (2020) utilized different nitrogen sources where it was observed that yeast extract supported the maximum lipase activity (741.54 U/mL) while potassium nitrate exhibited the minimum lipase activity (449.0 U/mL) when added to the substrate.

5.2 CONCLUSION

In conclusion, soil samples from diesel and restaurant wastewater polluted site was collected, cultured and screened for lipolytic fungal production. Out of 11 isolates screened, isolate VPD4 showed the highest growth and lipase activity on solid agar. The morphological and biochemical characteristics of the isolate showed the identity of the fungi as *Fusarium oxysporum*. Glucose (188.65 U/mL) and peptone (182.22 U/mL) respectively supported the highest lipase production among different nitrogen and carbon sources investigated. These findings suggest that adequately optimized lipase production from *Fusarium oxysporum* could be a potential source of enzyme for industrial use.