EFFECT OF CARBON AND NITROGEN SOURCES ON FUNGAL LIPASE PRODUCTION USING SOLID STATE FERMENTATION

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CERTIFICATION

This is to certify that this research project titled **"EFFECT OF CARBON AND NITROGEN SOURCES ON FUNGAL LIPASE PRODUCTION USING SOLID STATE FERMENTATION"** was carried out by ADEBOYE, Tolulope Mary, with matriculation number 16010101003. This project meets the requirements governing the award of Bachelor of Science (B.Sc) Degree in Microbiology department of biological sciences of Mountain Top University, Ogun State, Nigeria and is approved for its contribution to knowledge and literary presentation.

ADEBOYE M. TOLULOPE Date

MR G.E. ADEBAMI

(Project Supervisor) Date

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.

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ADEBOYE M. TOLULOPE Date

DEDICATION

I dedicate this work to God almighty for his divine strength, wisdom and for his guidance.

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ABSTRACT

The increasing interest in the development of lipase is attributed to the possible biotechnological applications of these enzymes. Lipases are hydrolytic enzymes that have occupied a major role as commercial biocatalysts in hydrolyzing triglycerides to free fatty acids and glycerol. Microorganisms isolated from restaurant wastewater polluted soil were screened for fungal lipase production on solid agar. Morphological and biochemical characteristics of the best isolate were investigated. The effect of carbon sources including monosaccharide (glucose), disaccharide (sucrose), polysaccharide (starch) and agro wastes (plantain peel, banana peel, starch, wheat bran and rice bran) and nitrogen sources were investigated. A total of twelve (12) fungi were isolated. There was significant difference ($p \geq 0.005$) in growth and lipase activity on solid medium. The diameter of growth ranged from 23.0^{k} – 53.0^{a} mm while lipase activities ranged from 2.0^{g} – 10.5^{a} mm. Isolate TPD1 identified as *Trichoderma harzianum* gave the best mycelium growth and activity. Lipase production using different carbon sources ranged from 88.96^{e} – 156.42^{a} U/mL. Glucose (156.42^{a} U/mL) supported the highest lipase production followed in order by sucrose (132.21^{b} U/mL), mannitol (129.44^{c} U/mL) and plantain peel (104.65^{d} U/mL) while the least production was recorded in rice bran (85.56^{g} U/mL). Lipase production using organic and inorganic nitrogen sources ranged from 88.96^{e} – 128.01^{a} U/mL peptone supported the highest production (128.01^{a} U/mL) while Urea provided the least support (88.96^{e} U/mL). The study has shown that *Trichoderma harzianum* TPD1 isolated from wastewater polluted soil is a potential lipase producer and can be harness for industrial production.

Key words: lipase production, carbon source, lipase, nitrogen source, solid state fermentation (SSF), *Trichoderma harzianum*.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND OF THE STUDY

The interest in microbial lipase production has increased in the last decade (Mehta *et al*., 2017), because of its large potential in manufacturing applications as food additives, fine chemicals, waste water treatment, cosmetics, pharmaceutical, leather and medicine, alongside the quest for sustainable production to meet up with the increasing demand for lipase (Kumar and Ray, 2014).

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are enzymes that catalyze triacylglycerol hydrolysis into glycerol and free fatty acids (Sharma and Kanwar, 2014). Lipases are industrial biocatalysts involved in a variety of novel reactions occurring in both aqueous and non-aqueous media (Kumar *et al*., 2016). Moreover, they are well known for their exceptional ability to perform a large range of chemo-, regio-and enantio-selective transformations (Kumar *et al*., 2016). Besides their enantio-selective, chemo-selective, or regio-selective catalysis, since lipases are versatile for synthesis and hydrolysis, their effect on enzyme biotechnology has become more prominent (Helal *et al*., 2017).

In nature, they are pervasive and are obtained from several plants, animals and microorganisms (Thakur, 2012). Due to their capacity to stay active under extremes of temperature, pH and organic solvents, and as a result of their chemo-, regional and enantioselectivity, microbial lipases have attracted special industrial interest (Chandra *et al*., 2020). Apart from the hydrolysis of triglycerides, lipases can catalyze a variety of chemical reactions which include esterification, trans-esterification, acidolysis and aminolysis (Joseph *et al*., 2008).

Due to the specificity of their substrates and resilience under different chemical and physical conditions, fungal lipases have been used extensively (Mehta *et al*., 2017). Fungi capable of producing lipases are present in a variety of environments, including oil-contaminated soils, vegetable oil waste, dairy industries, seeds and degraded food industries (Ko *et al*., 2005). Fungal enzymes are inherently extracellular and can be easily extracted, this greatly decreases costs and makes this source superior to bacteria (Mehta *et al*., 2017). Fungal species which produce lipases are *Candida rugosa, Candida Antarctica, T. lanuginosus, Rhizomucor miehei, Pseudomonas, Mucor* and *Geotrichum* (Chandra *et al*., 2020).

Solid state fermentation (SSF) in the absence or near absence of free water is known as the fermentation process on moist solid substrates (Santis-Navarro *et al*., 2011). Enzymes of industrial interest traditionally have been produced by submerged fermentation (SmF) (Dobrev *et al*., 2018). SSF is preferred over SmF as it is cost effective, eco-friendly and it delivers high yield of enzyme (Doriya *et al*., 2016). As a solid substrate, the SSF method utilizes agricultural and industrial waste. In addition, through low moisture content, the amount of contamination in solid state fermentation is greatly reduced (Doriya *et al*., 2016).

1.2 STATEMENT OF PROBLEM

This research was made with the twofold purpose of producing fungal lipase using solid state fermentation and to show by means of this procedure, the factors affecting lipase production.

1.3 JUSTIFICATION

Due to the growing increase in industrialization and the need for sustainable enzyme production, an extensive study is carried out to propose enhanced solutions to lipase production. Lipases in non-aqueous media can catalyze esterification, interesterification, and transesterification reactions. This versatility makes lipases the enzymes of choice for the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries with potential applications. Fungal species produce extracellular lipases. In nature, fungal enzymes are extracellular and can be easily removed, which greatly decreases costs and makes this source favored over bacteria. Lipase production using solid state fermentation is cost effective and delivers high yield of the enzyme.

1.5 AIM AND OBJECTIVES OF THE STUDY

The aim of this research is to produce extracellular lipase from fungal species isolated from soil samples using solid state fermentation.

i. To isolate fungi from fatty acid and diesel contaminated soil samples.

ii. To screen the isolates for lipase production and selection of the best lipase producer.

iii. To identify the selected isolates using morphological and biochemical characterizations.

iv. To produce lipase using solid state fermentation and determination of the effect of carbon and nitrogen sources.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 FUNGI

Fungi are a diverse and widespread group of species belonging to the kingdom Fungi (Carris *et al*., 2012) which was first considered as the fifth kingdom by Whittaker (Whittaker, 1959). Despite difficulties defining the limits of the group, mycologists have defined fungi as eukaryotic, nucleated, spore-bearing, achlorophyllous organisms which generally reproduce sexually and asexually and whose usually filamentous, branched somatic structures are typically surrounded by cell walls containing cellulose or chitin or both (Alexopoulos *et al*., 1962; Leera *et al*., 2019).

They contain no photosynthetic pigments, however, and are chemo-organotrophs (Carris *et al*., 2012). Most of them develop aerobically and their energy is obtained by oxidation of organic compounds (Buckley, 2008). Fungi are the principal decomposers in ecological systems (Kathleen *et al*., 2015). Mycology is regarded as the study of fungi (Alexopoulos *et al*., 1962).

They are abundant worldwide (Alsohaili and Bani-Hasan, 2018). Fungi are the main components of nearly every ecosystem on Earth, including marine habitats that range from high alpine lakes to the deep ocean (Grossart *et al.*, 2019). Kingdom Fungi is among the most complex classes of species on Earth, and is an important part of the environment that regulates soil carbon cycling, plant growth, and pathology (Tedersoo *et al.*, 2014). Fungi play an important role in organic matter decomposition and in the cycling and exchange of nutrients (Yuvaraj *et al*., 2020).

At least 100,000 named species of fungi are recognized (Brandt and Warnock, 2015). However the number of undiscovered species has been estimated to range from 1 million to more than 10 million, and it has been estimated that between 1,000 and 1,500 new species are described per year (Buckley, 2008; Guarro *et al*., 1999). Of the identified species of fungi, less than 500 are generally associated with human or animal diseases, and no more than 50 are capable of causing infection in otherwise healthy individuals (Brandt and Warnock, 2015).

In industry, fungi are relevant as well (Azizan *et al*., 2016). Yeasts, a form of fungus, are being used in the baking and fermentation of sugars into carbon dioxide and ethanol in the wine and

brewing industries (Walker and Stewart, 2016). Mushrooms, another fungal community, are a source of food (Valverde *et al*., 2015). Other fungi are important for cheese and antibiotic production (Bagyaraj and Arpana, 2006).

Fungi can be harmful, resulting in major economic losses (Lucca, 2007). Many plant diseases (wheat rust, corn smut, potato blight, root rot and stem rot) are caused by pathogenic fungi (Almeida *et al*., 2019), some human diseases (ringworm, athlete's foot and histoplasmosis) and some animal diseases (dog and cat mange).Wood, clothes, food deterioration and decay are caused by the fungus that grow in wet, damp climates (Bagyaraj and Arpana, 2006).

2.1.1 GENERAL CHARACTERISTICS OF FUNGI

Fungi constitute a distinct category of eukaryotic species, which vary from other groups, such as plants and animals, in a variety of aspects (Brandt and Warnock, 2019)**.** Fungal cells are encased within a rigid cell wall, often consisting of chitin, glucan, chitosan, mannan, and glycoproteins in a number of combinations (Leera *et al*., 2019). These features contrast with animals, which have no cell walls and the plants which have cellulose as the major cell wall component (Brandt and Warnock, 2019). As with other eukaryotic organisms, fungal cells have a true nucleus with a surrounding membrane, and cell division is followed by meiosis or mitosis (Bueno and Silva, 2014).

Fungi can be multicellular or unicellular (Naranjo-Ortiz and Gabaldon, 2019). In multicellular organisms, the basic structural unit is a chain of multinucleate, tubular, filament-like cells (termed a hypha). The vegetative stage of most multicellular fungi consists of a mass of branching hyphae, called a mycelium or a thallus (Brandt and Warnock, 2019). As a result of apical growth with mitotic cell division, each individual hypha has a rigid cell wall and increases in length (Bueno and Silva, 2014). In the more primitive fungi, the hyphae remain aseptate (without cross walls). In more advanced groups, however, hyphae are divided into sections or cells by the formation of more or less frequent cross-walls, known as septa. Such hyphae are referred to as being septate. Fungi that exist in the form of microscopic multicellular mycelium are commonly called molds (Brandt and Warnock, 2019; Bueno and Silva, 2014).

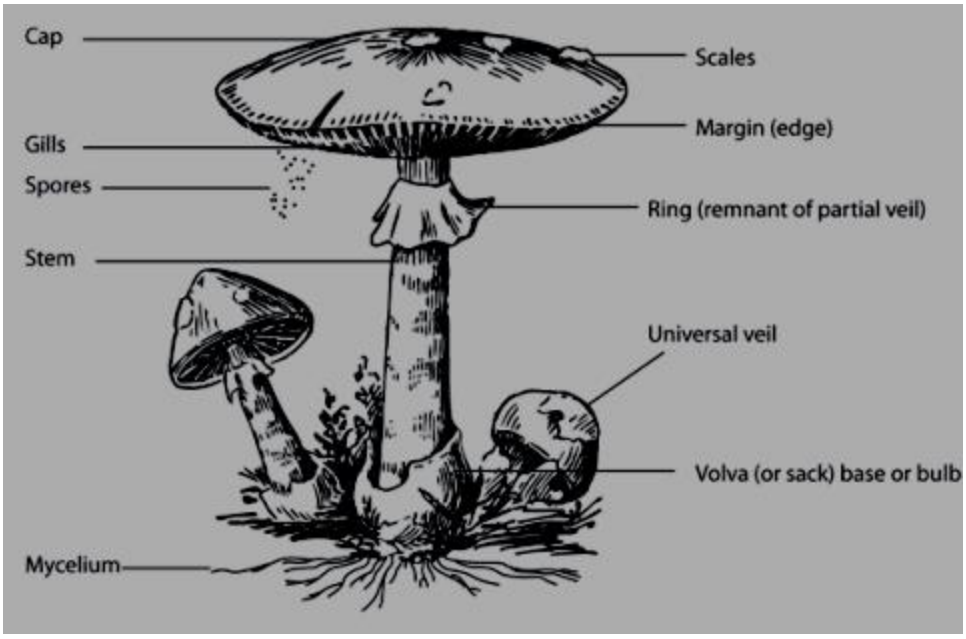

Figure 2.1: Structural diagram of a mushroom (Kamalakannan *et al*., 2020)

2.1.2 FUNGAL CELL STRUCTURE

All the morphological forms (yeast and hyphae) of fungi are surrounded by a rigid cell wall (Samantha, 2015).The cell wall's main component is chitinous fibrils enclosed in a matrix of polysaccharides, proteins (acid phosphatase, α-amylase, and protease), lipids and inorganic salts (calcium, magnesium, phosphorus) (Gow *et al*., 2016; Samantha, 2015). Chitin is a (β1, 4) linked polymer of N acetyl-D-glucosamine (GlcNAc).It is synthesized by chitin synthetase present in the chitosome (cell organelle) (Samantha, 2015). The degree of cross-linking is defined by the plasticity (extensibility) of the wall, while the pore size (permeability) is a property of the wall matrix. The scaffold forms the wall's inner layer, and the matrix is primarily contained in the outer layer (Nobel *et al*., 2001).

Table 2.1 The chemical composition of cell walls of selected groups of fungi (dry weight of total cell wall fraction, in per cent) (Webster and Weber, 2007).

Group	Example	Chitin	Cellulose	Glucans	Protein	Lipid
Oomycota	*Phytophthora*	0	25	65	4	2
Chytridiomycota	*Allomyces*	58	0	16	10	-
Zygomycota	*Mucor*	9	0	44	6	8
Ascomycota	*Saccharomyces*	1	0	60	13	8
	Fusarium	39	0	29	7	6
Basidiomycota	*Schizophylum*	5	0	81	2	-
	Coprinus	33	0	50	10	-

2.1.3 FUNGAL NUTRITION AND GROWTH

Fungi are heterotrophic, that is they lack chlorophyll, and thus need preformed organic carbon compounds for their nutrition (Brandt and Warnock, 2019). Fungal organisms are primarily composed of water (69-90%), carbohydrates, proteins and lipids (Table 2.2). Fungi live and derive their nutrition from a food source or medium by secreting enzymes into the external substrate and absorbing the released nutrients via their cell wall (Brandt and Warnock, 2019).

Table 2.2: Proximal compositions of fungi (Bueno and Silva, 2014)

Class of compound	Dry weight (%)
Carbohydrates	16-85
Lipids	0.2-87
Proteins	14-44
RNA	1-10
DNA	0.15-0.3
Ash	1-29

Fungi are chemoorganoheterotrophic organisms (Leera *et al*., 2019). Chemical compounds are used as an energy source and organic compounds as an electron and carbon source are used (Yuvaraj *et al*., 2020). They obtain their nutrition by absorption (osmotrophic) either from the environment (saprophyte) or the host (parasite). Most of the saprophytic moulds grow aerobically in artificial culture medium at 20–30^{o}C (Kuhn and Ghonnoum, 2003).

The pathogenic yeasts and yeast phase of dimorphic fungi prefer to grow at 37^{o}C (Boyce and Andrianopoulos, 2015). High humidity, acidic pH (3.8–5.6), high sugar concentration (4–5%), carbon, phosphorus, sulphur and traces of potassium, magnesium, iron and calcium are required for optimum fungal growth (Adinarayana *et al*., 2003).The peptone in the media and keratin in the skin act as a nitrogen source (Yuvaraj *et al*., 2020). Nitrogen is required to synthesize amino acids for building proteins, purines and pyrimidines for nucleic acids, glucosamine for chitin and various vitamins (Samanta, 2015).

Most fungi use nitrogen as nitrate that is reduced to nitrite and ammonia as well. None of them can directly fix nitrogen (Carris *et al*., 2012). The growth rate of fungi is slower than bacteria and the medium is easily contaminated with bacteria. Antibiotics (e.g. chloramphenicol) and antifungal (e.g. cycloheximide) are added in the media to prevent the bacterial and saprophytic fungi contamination (Carris *et al*., 2012). The cycloheximide is inhibitory against the growth of

certain pathogenic fungi and yeasts such as *Aspergillus fumigatus*, *Candida albicans* and *Cryptococcus* (Vandeputte *et al*., 2012).

2.1.4 FUNGAL REPRODUCTION

The fungi reproduce by means of asexual and sexual or para-sexual reproduction (Carris *et al*., 2012). Asexual reproduction is sometimes called somatic or vegetative and it does not involve union of nuclei, sex cells or sex organs. Sexual reproduction is characterized by the fusion of two nuclei (Lee *et al*., 2010). Most fungi reproduce by spores and have a body called thallus composed of microscopic tubular cells called hyphae (Carris *et al.,* 2012). Basically, these spores range between 40 and 800 μm in diameter, each of which may have hundreds or thousands of nuclei (Carris *et al*., 2012). Spore is a unit of survival or dispersion capable of germinating to produce a new hypha consisting of one or more cells. These spores may be produced singly or in clusters. Unlike plant seeds, fungal spores do not have an embryo, but they contain the food reserves required for germination (Bareke, 2018). As part of their life cycles, many fungi develop more than one form of spore. These spores can be formed by an asexual process involving mitosis (mitospores), or through a sexual process called meiosis (meiospores) (Carris *et al*., 2012).

2.1.5 SHARED FEATURES OF FUNGI WITH OTHER ORGANISMS

Along with other eukaryotes with chromosomes that include DNA with non-coding regions called introns, fungal cells contain membrane-bound nuclei and coding regions called exons (Ivaschenko *et al*., 2009). They are comprised of soluble carbohydrates and storage compounds, including sugar alcohols, disaccharides and polysaccharides (Deacon, 2005). Fungal cells also have mitochondria and an intrinsically complex internal membrane structure like the endoplasmic reticulum and the Golgi apparatus (Mullock and Luzio, 2013).

Fungi, like animals, are heterotrophs; instead of fixing carbon dioxide from the atmosphere, they use complex organic compounds as a carbon source, as do some bacteria and most plants (Ren and Yuan, 2015). Moreover, fungi can not absorb nitrogen from the atmosphere (Brandt and Warnock, 2019). Like animals, they need to get it from their food. However, unlike most animals that eat food and then digest it internally in specialized organs, fungi conduct these steps in the opposite order; digestion precedes ingestion (Brandt and Warnock, 2019). Exoenzymes are first transferred out of the hyphae, where nutrients are stored in the environment (Brandt and

Warnock, 2019). As in animal cells, polysaccharides are stored by glycogen, a branched polysaccharide rather than amylopectin, a less densely branched polysaccharide, and amylose, a linear polysaccharide, as present in plants.

2.1.6 DIVERSITY IN FUNGI

Fungi are present in a wide variety of habitats and flourish, including harsh conditions such as deserts and areas with high amounts of salt or ionizing radiation, as well as deep sea sediments (Grossart *et al*., 2019). They perform some of life's most significant fundamental functions and have some of the greatest biotechnological ability, but only 7% of the total estimated fungal species on Earth are identified (Tang *et al*., 2006).

Taxonomists have identified about 120,000 species of fungi, but the global biodiversity of the fungal kingdom is not completely known. An estimate from 2017 indicates that there could be between 2.2 and 3.8 million species (Hawksworth and Lucking, 2017). In mycology, various approaches and definitions have generally differentiated organisms. The classification of fungal taxonomy has traditionally been based on the morphological characteristics, for example spores or fruit structures.

It is possible to distinguish between the biochemical and physiological characteristics of organisms, such as their ability to metabolize certain biochemicals and their response to chemical analysis. The definition of biological species discriminates among species on the basis of their ability to mate (Tang *et al*., 2006). The use of molecular methods to study diversity, such as DNA sequencing and phylogenetic analysis, has vastly improved precision and added robustness to genetic diversity estimates within different taxonomic groups (Li *et al*., 2018).

2.2 FUNGAL CLASSIFICATION

According to Ainsworth (1966), the kingdom is divided into two divisions namely (Gupta *et al*., 2012; Katoch and Kapoor, 2014):

Division Myxomycota,

Division Eumycota

2.2.1 Division Myxomycota (False fungi)

They lack cell walls and are rare species (Clark and Haskins, 2015). Possess either a plasmodium, a mass of naked, multinucleate protoplasm, which feeds by ingesting particulate matter and shows amoeboid movement, or pseudo plasmodium, an aggregation of separate amoeboid cells (Helmenstine, 2018). Both are of a slimy consistency, hence they are also called 'Slime moulds'. It is divided into four classes; Acrasiomycetes, Hydromyxomycetes, Myxomycetes, Plasmodiophoromycetes.

2.2.2 Division Eumycota (True fungi)

It is divided into five subdivisions (Helmenstine, 2018) Ascomycotina, Basidiomycotina, Deuteromycotina, Mastigomycotina and Zygomycotina

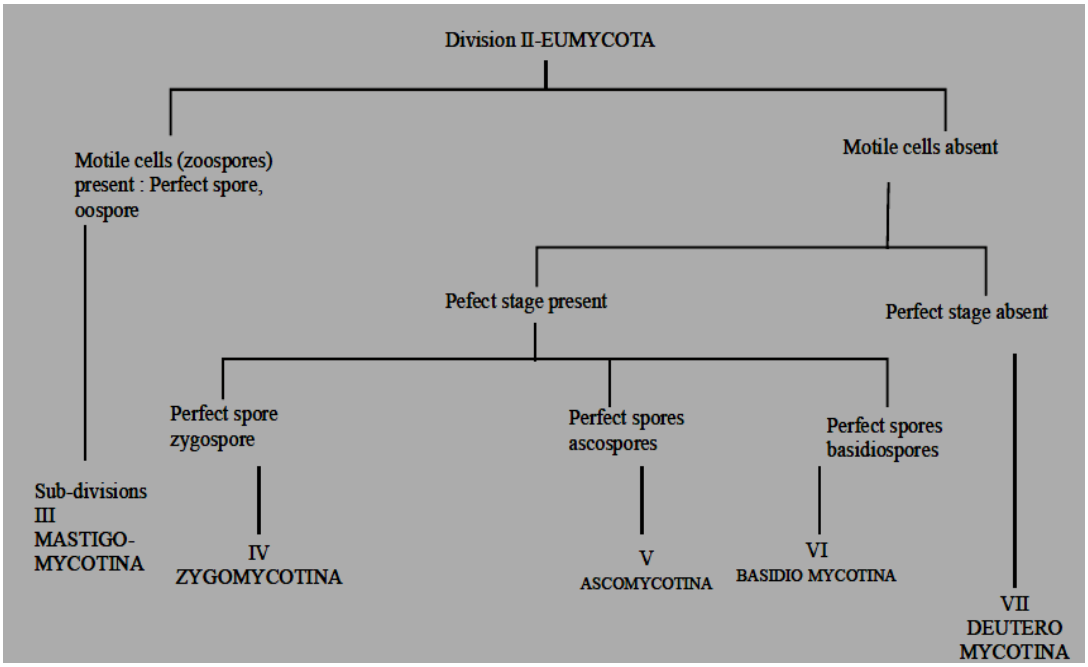

Figure 2.2: Classification of Eumycota (Brandt and Warnock 2019).

2.3 ECONOMIC IMPORTANCE OF FUNGI

Fungi are vital for their ecological roles (Blackwell *et al.*, 2012). Furthermore, a variety of fungi are utilized in food processing and flavoring (baker's and brewer's yeasts, *Penicillium* in cheese-making) and also to produce antibiotics and organic acids. Secondary metabolites are also known to be produced by fungi, such as enzymes used in situ lipid metabolism and multifaceted industrial applications (Mehta *et al.*, 2017).

2.3.1 Role of Fungi in Agriculture

Fungi are tremendous decomposer of organic waste material and they degrade cellulose, lignins, gums and other organic complex substances. They are also responsible for plant hormone development (Table 2.3). Organic matter degradation and soil aggregation are the main functions of filamentous soil fungi. In addition to this property, bound species of *Alternaria*, *Cladosporium*, *genus Aspergillus*, *Dematium*, *Gliocladium and Humicola*, produce substances such as organic compounds in the soil and may therefore also be used for soil organic matter maintenance. (Yuvaraj and Ramasamy, 2020). Plant growth regulators and chemical fertilizers have been used to increase crop production (Islam, 2008; Metting, 1993).

Table 2.3: Agricultural application of fungi (Yuvaraj and Ramasamy, 2020)

Fungi Species	**Product**	**Application**
Fusarium moniliforme	Gibberellins	Plant growth hormone
Fusarium graminearum	Zearalenone	Growth promoter in cattle
Phytophtora palmivora	DeVine	Control of milkweed vine
Colletotrichum sp	Collego	Control of northern joint vetch
Chondrostereum purpureum	Chontral	Control of hardwoods
Phanerochaete gigantea	Rotstop	Control of butt rot of conifers

Diseases are the main harmful effects the fungi have on commercial agriculture. They trigger a number of diseases in both crops and animals. Damping off disease that affect seedlings of almost all types of crops are the most common and essential fungal crop diseases e.g. tomatoes, beans, spinach, and tobacco, downy mildews of grapes, ergot disease of rye which results in the formation of poisonous sclerotia in the rye kernel, brown rot of stone fruits (Yuvaraj and Ramasamy, 2020). This affects fruits like cherries, peaches, apricots and plums, sugar cane red rot disease.

2.3.2 Role of Fungi in Medicine

Fungi play important roles in many aspect of human life including medicine, food and farming etc (Beg *et al*., 2004). Medicinal fungi are those fungi that use biotechnology to produce or can be induced to generate medically significant metabolites (Mehta *et al*., 2017). The spectrum of medicinally active compounds known includes antibiotics, anti-cancer drugs, cholesterol inhibitors, psychotropic drugs, immunosuppressants and even fungicides (Kidd *et al*., 2016).

Several fungal secondary metabolites are of great commercial importance (Jadon *et al*., 2020), these groups of organisms are well known to naturally produce antibiotics to kill or inhibit the growth of bacteria (Dumancas *et al*., 2014). Fungi's role in antibiotic development was first established by Sir Alexander Fleming in 1929 (Alexopoulos *et al*., 1996). He extracted penicillin from *Penicillium notatum*. It was the first antibiotic to be widely used. Penicillin is an organic substance lethal to microorganisms (Dumancas *et al*., 2014). It kills bacteria especially gram positive bacteria.

Streptomycin is obtained from *Streptomyces griseus* (Westhoff *et al*., 2020). It is of great value in medicine. It destroys many organisms which are not killed by penicillin particularly the gram-negative organisms. A numbers of antibiotics have also been extracted from Aspergillus cultures (Al-Fakih and Almaqtri, 2019).

Mushrooms, such as *Antrodia camphorata*, *Ganoderma* spp., *Hericium erinaceus*, *Lignosus rhinocerotis* and *Pleurotus giganteus*, have a long history of use in enhancing the peripheral nervous system (Phan *et al*., 2015). Nerve growth factors are important for the survival, maintenance, and regeneration of specific neuronal populations in the adult brain (Petruska and Mendell, 2009).

There are countless roles fungi have played and are still playing till date in the medical field (Abdel-Razek *et al*., 2020). In medicine, fungi are used to fight various diseases such as cancer (Patel and Goyal, 2012), bacterial infections, diabetes etc. Research has shown that a species of Ganoderma (*Ganoderma applanatum*) contains compounds with anti-tumor and anti-fibrotic properties (Hyde *et al*., 2019).

For all the benefits of fungi, there is also a drawback to its role in medicine, Aspergillosis is a disease caused by *Aspergillus flavus* (Bazaz and Denning 2019). It occurs in people with lung

diseases and weak immune system (Bazaz and Denning 2019). Mucormycosis occurs in people with weak immune system and it is caused by fungi in the order mucorales (Ibrahim *et al*., 2012). Some fungal species have also been attributed to skin infections such as ringworm (White *et al*., 2014). Neoformans infection this infection is caused by *Cryptococcus neoformans* which infects the brain and causes meningitis (King, 2018). Fungi cause so many other infections like Athlete foot, fungal nail infection, fungal eye infection etc (White *et al*., 2014).

2.3.3 Role of Fungi in Petroleum Industries

The oil industry, also known as the petroleum industry, includes the processes of extraction, processing, refining, transport (often by pipelines and oil tankers) and distribution of petroleum products worldwide (Demain *et al*., 2004). Bioremediation is the environmental application of biotechnology (Leera *et al*., 2019) and is defined as the use of biological agents or organisms to breakdown pollutants in the environment by the process of biodegradation (Elshafie, 2007). Major groups capable of using petroleum hydrocarbons are found to be distinct strains of soil fungi, including *Graphium*, *Fusarium, Penicillium*, *Paecilomyces*, *Acremoniun*, *Mortierella, Giiocladium, Trichoderma* and *Sphaeropsidales* (Leera *et al*., 2019). Various fungal species are utilized in the petroleum industry (Table 2.4).

Table 2.4: Petroleum hydrocarbon degradation by different species of filamentous fungi (Leera *et al*., 2019).

Fungi	**Compound**
Trichoderma harzianum	Naphthalene
Aspergillus spp.	Crude oil
Cunninghamella elegans	Phenanthrene
Aspergillus niger	n-hexadecane
Cunninghamella elegans	Pyrene
Aspergillus ochraceus	Benzo[a] pyrene
Penicillium spp	Crude oil

2.3.4 Role of Fungi in Industry

The industrial applications of fungi are many and varied (Hyde *et al*., 2019). Many fungi are beneficial to humans and have been economically and industrially exploited (Leera *et al*., 2019). In reality, fungi form the base of several major industries. There are a variety of industrial processes that take good account of the biochemical activities of certain fungi (Azizan *et al*., 2016). In the agriculture - based markets, the functions of fungi are as follows:

2.3.4.1 Alcoholic Fermentation

Fungi are typically used for the processing of ethanol, organic acids, antibiotics and enzymes such as fungal lipase, cellulase, gluconase and glycosidase in the fermentation industry (Hyde *et al*., 2019; Leera *et al*., 2019). *S. cerevisae* and *Monilia* sp. are used in ethanol production. The use of yeast (*Saccharomyces cerevisae)* to make alcohol and carbon dioxide uses the fermentation process to break down sugars (Maicas, 2020). Up to 50% of the sugar can be converted to alcohol, but rarely surpasses 15% because fungi are sensitive to high concentration of alcohol. There are three main products of this process: beer, wine and sake (Leera *et al*., 2019).

2.3.4.2 Organic Acids

An organic acid is an organic, acidic compound (Kulshrestha *et al*., 2012). Fungi, in particular *aspergilla* are well known for their potential to overproduce a variety of organic acids (Liaud *et al*., 2014). Oxalic acid, citric acid, gluconic acid, gallic acid, fumaric acid (Table 2.5) etc., are essential organic acids produced commercially as a result of the biochemical activities of moulds (Hyde *et al*., 2019).

Table 2.5: Various organic acids and their corresponding producing microorganisms. (Hyde *et al*., 2019).

Organic acid produced	**Fungi**
Gluconic acid	*Penicillium* and *Aspergillus*
Gallic acid	*Aspergillus gallomyces*
Oxalic acid	*Aspergillus niger*
Ethanol	*Saccharomyces cerevisae*
Citric acid	*Aspergillus niger*

2.3.5 Role of Fungi in Enzyme Production

Most commercially important enzyme-producing fungi are recognized as belonging to the genera *Rhizopus* sp., *Aspergillus* sp., *Penicillium* sp., *Geotrichum* sp., *Mucor* sp., and *Rhizomucor* sp (Rodrigues *et al*., 2016). Based on their excellent capacity for extracellular protein production, filamentous fungi are perhaps the most preferred source of industrial enzymes (Jun *et al*., 2011).

Within the cell, exoenzymes are synthesized and then secreted outside the cell (Kamalanathan *et al*., 2020), where their function is to break down complex macromolecules into smaller units for growth and assimilation to be taken up by the cell. For example: *Trichoderma reesei* is known to secrete large amounts of cellulases (Keshavarz and Khalesi, 2015). Invertase is extracted from *Saccharomyces cerevisae* (Qureshi *et al*., 2017). It hydrolyses sucrose into glucose and fructose. Due to their possible use in food processing, pharmaceuticals, cosmetics, detergents and the leather industry, fungal lipases have gained attention (Adinarayana, *et al*., 2003).

For many applications, fungal enzymes have drawn attention because fungi can grow on low-cost materials and secrete large quantities of enzymes into the culture medium, which eases down-stream processing (Anitha and Palanivelu, 2013). Several fungal enzymes are available commercially including amylases, cellulases, lipases, phytases, proteases, and xylanases (Lakshmi *et al*., 2014; Singh *et al*., 2016). There is a general interest in the positive environmental effects of manufacturing processes and the use of enzymatic reactions instead of organic solvents or chemical reactions is highly regarded (Chapman *et al*., 2018) Some examples of significant fungal enzymes and enzyme sources that are used in many applications are given in Table 2.6.

Table 2.6: Examples of enzyme sources and applications of fungi (Hyde *et al*., 2019).

Enzymes	Applications	Fungal Sources	References
Amylases	Starch hydrolysis in starch manufacturing industries	*Penicillium fellutanum* *Aspergillus fumigatus* *Aspergillus niger* *Rhizopus stolonifer*	Sahoo *et al*., 2014 Ko *et al*., 2005 Ko *et al*., 2005 Saleem and Ebrahim, 2014
Cellulases	Animal feed industry	*Aspergillus niger* *Lentinula edodes*	Ko *et al*., 2005 Pachauri *et al*., 2017
Keratinases	Biomass conversion into biofuels	*Aspergillus oryzae*	Anitha and Panivelu, 2013
Laccase	Biopulping biobleaching deinking in pulp and paper industry.	*Agaricus subrufescens*	Ergun and Urek, 2017
Lipase	Degradation of fat in wastewater treatment; animal feed industry; pulp paper industry; detergent industry; food processing; textile industry; pharmaceutical industry	*Aspergillus niger* *Fusarium solani* *Penicillium* sp *Trichoderma* sp *Mucor* sp *Pseudomona aeruginosa*	Falony *et al*., 2006 Facchini *et al*., 2015 Ulker *et al*., 2011 Vallado *et al*., 2011 Falony *et al*., 2006

2.4 LIPASE

Lipases (triacylglycerol acyl hydrolases, (E.C. 3.1.1.3) are a group of enzymes that catalyze triacylglycerol hydrolysis to glycerol and fatty acids (Kumar and Ray, 2014). They are ubiquitous in nature and are created by various plants, animals and microorganisms (Thakur, 2012). Due to their recent and multiple applications in oleochemistry, organic synthesis, detergent formulation and nutrition, lipases have become one of the prominent products among biocatalysts (Gunasekaran and Das, 2005; Saxena *et al*., 2003). At the water lipid interface, lipases are special in fat hydrolysis into fatty acids and glycerol and reversing the reaction in non-aqueous media (Saxena *et al*., 2003). They catalyze both triglyceride hydrolysis and the synthesis of glycerol and long chain fatty acid esters. They also act as they biocatalysts for alcoholysis, acidolysis, esterification and aminolysis (Gunasekaran and Das, 2005).

2.4.1 PROPERTIES OF LIPASE

Since the 1980s, the amount of lipases available has increased (Arife *et al*., 2016). Due to their properties including bio-degradability, they have been used as commercial biocatalysts (Chapman *et al*., 2018), high specificity (Amoah *et al*., 2016) high catalytic efficiency (Amoah *et al*., 2016b), temperature (Liu *et al*., 2015), pH dependency, activity in organic solvents (Singh *et al*., 2016), and non-toxic nature. The ability to utilize mono-, di-, and tri-glycerides as well as free fatty acids in transesterification, is the most desirable feature of lipase (Sharma *et al*., 2016b).

Moreover, under mild pH and temperature conditions, lipases can react, subsequently reducing the energy used for direct responses at extraordinary temperatures and pressures (Mehta *et al*., 2017) In general, lipases are stable in organic solvents with few activation or inhibition exceptions (Patil *et al*., 2011). Lipases produced from *Bacillus* sp. have been found to be extremely stable in hydrophobic organic solvents and are slightly more effective if short chain alkanes, benzene, and toluene are present at the rate of 10–50% (v/v) (Kumar *et al*., 2016).

2.4.2 SORCES OF LIPASE

Lipases are produced by plants, animals and several microorganisms such as bacteria, actinomycetes, fungi and yeast (Raveendran *et al*., 2018).

2.4.2.1 PLANT LIPASE

Lipases produced from seed plants have recently been the subject of great attention as biocatalysts (Barros *et al*., 2010). In some cases, owing to some very fascinating characteristics such as specificity, low cost, availability and ease of purification, these enzymes have advantages over animal and microbial lipases, representing a perfect substitute for future commercial exploitation as industrial enzymes (Polizeli *et al*., 2008; Barros *et al*., 2010). Plant lipases, such as oilseeds, are often present in energy reserve tissues (Seth *et al*., 2014). They function as biocatalysts that are advantageous due to their high substrate specificity, low manufacturing costs and easy pharmacological acceptance because of their eukaryotic origin (Mazou *et al*., 2016). Plant lipases thus offer greater potential in the organic synthesis, food, detergent and pharmacology industries for industrial applications (Seth *et al*., 2014).

2.4.2.2 ANIMAL LIPASE

In animals, pig and human pancreas derived lipases are better known and more studied than any other lipase (Riberio *et al*., 2011). They are involved in many stages of lipid metabolism in these organisms, including fat digestion, adsorption, reconstitution, and metabolism of lipoproteins (Riberio *et al*., 2011).

2.4.2.3 MICROBIAL LIPASE

Lipases with microbial origin are the most widely used enzyme class in biotechnological and biochemical applications (Thakur, 2012). Lipases from different microorganisms have been isolated, including bacteria (Gram-positive and Gram-negative), fungi, yeast and actinomycetes (Gunasekaran and Das, 2005). Microbial sources are superior to plants and animals for enzyme production, and this can be due to the simplicity of which they can be cultured and genetically engineered (Hasan *et al*., 2006).

Microbial lipases have received a great deal of industrial attention compared to plant and animal lipases (Chandra *et al*., 2020) due to their ability to work at high temperatures, pH and stability in organic solvents, chemo-selectivity, regio-selectivity and enantio-selectivity (Sharma and Kanwar, 2014). In addition, they have increased production yields, ease of genetic modification, rapid growth in low-cost media, which makes production more suitable (Thakur, 2012).

Carvalho *et al*. (2008) isolated a bacterium strain from petroleum-contaminated soil and codified as Biopetro-4. After investigation of several inducers on lipase activity, the maximum value obtained was 1,675uml-1 after 120hr of fermentation. Maximum lipase activity of 1 084 U gds−1 was achieved using the *Pseudomonas aeruginosa* PseA solvent resistant strain by Mahanta *et al*. (2008). Alkan *et al*. (2007) developed extracellular lipase by *Bacillus coagulans* and achieved a maximum lipase activity of 149 U gds−1 after 24 h of fermentation. Fernandes *et al*. (2007) achieved a maximum lipase activity of 108 U gds−1 after 72 h of fermentation by *B. cepacia*.

2.5 FUNGAL LIPASE

Fungi have been considered as best lipase sources (Falony *et al*., 2006; Kumar and Ray, 2014) because of extracellular lipase production (Narishman and Bhimba, 2015; Ramos-Sanchez *et al*., 2015) As modern technology favors the use of batch fermentation and low cost extraction techniques, fungal lipases have advantages over bacterial lipases (Mehta *et al*., 2017). The development of fungal lipase varies depending on the strain, the composition of the growing medium, cultivation conditions, pH, temperature, and the type of source of carbon and nitrogen (Cihangir and Sarikaya, 2004). The isolation and selection of new strains is motivated by the industrial demand for new lipase sources with different catalytic properties. Lipase-producing microorganisms have been identified in different habitats, such as industrial waste, processing plants for vegetable oil, agricultural plants and oil-contaminated soil and oil seeds, among others (Thakur, 2012).

Adinarayana *et al*. (2003) studied the lipase production by *Aspergillus* species and obtained maximum enzyme yield of 1934U/g. In another work, Costa *et al*. (2017) isolated a strain of *Aspergillus niger* from agro-industrial wastes and obtained an expressive activity of 19.844U/g using solid state fermentation. Vishnupriya *et al.* (2010) studied the lipase production by *Streptomyces grisesus* and obtained maximum enzyme activity of 51.9U/ml. Colen *et al*. (2006) isolated 59 lipase-producing fungal strains using enrichment culture techniques from Brazilian savanna soil. For the isolation and growth of fungi in primary screening assays, an agar plate medium containing bile salts and olive oil emulsion was used. Twenty one strains were chosen based on the ratio of the lipolytic halo radius and the colony radius. Eleven strains were considered and, among them, the strain identified as *Colletotrichum gloesporioides* was the most productive (Colen *et al*., 2006).

Amin and Bhatti (2014) analyzed the impact of physicochemical parameters on *Penicillium fellutanum* lipase production using canola seed oil cake as a substrate. Maximum lipase activity of 521 units/gram dry substrate (U/gds) was achieved after 48 h of reaction time in a medium containing 10 g canola seed oil cake as a substrate complemented by 2% olive oil as an inducer and 50% moisture content at an initial pH of 4.0 using 2 mL inoculum at 30°C.

2.6 LIPASE PRODUCTION

Lipases are the most versatile industrial enzymes and are known to cause a variety of bioconversion reactions (Gunasekeran and Das, 2005). The enzyme production market has since expanded significantly (Prakasan *et al*., 2016). A growing number of enzymes can be produced affordably due to an improved understanding of production biochemistry, fermentation processes, and recovery methods. Advances in enzyme usage techniques have also increased significantly (Mehta *et al*., 2017). In addition, the number of enzymes used in commerce continues to multiply because of the many different transformations that enzymes can catalyze (Thakur *et al*., 2014). Production of lipase is influenced by the type and concentration of sources of carbon and nitrogen, culture pH, growth temperature and concentration of dissolved oxygen (Elibol and Ozer, 2001). Microbial lipases have been produced by both solid state and submerged cultures (Colla *et al*., 2015).

2.6.1 SOLID STATE FERMENTATION

Solid state fermentation is the process that takes place in a solid medium in the absence of water (Singhania *et al.*, 2010), but the substrate requires moisture to support the growth and metabolic activity of microorganisms (Thomas *et al.,* 2013). The most appropriate technique for growing microorganisms is solid state fermentation (Bhargav *et al*., 2008). Solid state fermentation is currently used as an economical alternative technique to submerged fermentation in a variety of applications such as antibiotics and enzyme production (Thomas *et al*., 2013), whereby raw materials and processing are cheap with less processing risk (Guerra *et al.,* 2003).

This technique involves the use of agricultural substrates with various nitrogen sources, carbohydrates, nutrients such as starch, cellulose, pectin and fibers (Kumar and Ray, 2014) because it has the potential to produce desired microbial products more efficiently. The different substrates traditionally fermented by solid state fermentation include agricultural waste (Table 2.7) such as rice husk, wheat bran, beans, sugar cane and lemon peel (Kumar and Ray, 2014).

This technique involves inoculation and growth of microbes on solid substrate maintaining low moisture content (Manan and Web, 2017)

Table 2.7: Comparison of lipase production in various fungal microorganisms (Kumar and Ray, 2014).

Microbes	**Solid Substrate**	**Inducers**	**Fermentation conditions**			**Lipase Activity (U/mL)**	**References**
			Temp. (oC)	**pH**	**Time (hr)**		
Fusarium oxysporum	Wheat bran	Cetyl trimethylammonium bromide	40	8.5	96	111.48	Angelo *et al*., 2014
Aspergillus flavus	Wheat bran and castor oil cake	n.r	30	7.0	96	121.35*	Tosacano *et al*., 2013
Aspergillus niger	Rice bran	n.r	30.3	6.87		121.53*	Hosseinpour *et al*., 2012
Candida rugosa	Coconut oil cake	Urea, Peptone and Maltose	n.r	n.r	96	87.76*	Benjamin and Pandey, 2004
Aspergillus niger	Shea butter cake	Tween 20	30	7.0	168	3.35*	Salihu *et al*., 2013
Penicillium restrictum	Babassu oil cake and Olive oil	n.r	37	7.0	24	5.8	Castilho *et al*., 2000
Aspergillus terreus	Mustard oil cake	n.r	30	6.0	96	1566.6	Sethi *et al*., 2013

Code: * U/g; n.r. – not reported

The selection of an effective solid substrate for the fermentation process is essentially a critical factor and requires the screening of various agro-industrial materials to increase microbial growth and enzyme production (Krishna, 2008). The type of solid substrate selected for this process is mainly related with cost and availability of other solid substrates (Kumar and Kanwar,

2012). There are however, some important factors affecting fungal lipase production in solid state fermentation. These factors are substrate size, inoculum size, moisture content, temperature, inducers, pH, nitrogen source, surfactants and spore formation (Kumar and Kanwar 2012).

2.7 FACTORS AFFECTING LIPASE PRODUCTION

Microbial lipases are mainly extracellular and medium composition and physicochemical factors such as temperature, pH and dissolved oxygen greatly influence their production (Salihu and Alam, 2012).These enzymes are typically produced as triacylglycerols, fatty acids, hydrolysable esters, tweens, bile salts, and glycerol in the presence of lipid substrates such as oils or any inducers (Gupta *et al*., 2004).

2.7.1 EFFECT OF NUTRITIONAL FACTORS

2.7.1.1 Carbon sources

In microorganisms, carbon sources are important substrates for energy generation (Salihu and Alam, 2012). In all types of microbial sources, carbon sources play an important role in lipase induction. *Aspergillus terreus* showed a strong yield in the production of lipase with mustard seed oil as carbon source (Sethi *et al*., 2012). Increased production of lipase in fungal strains is achieved using an amalgam of olive oil cake and sugar cane bagasse as carbon source (Fatima *et al*., 2020). Olive oil cakes have reported an increase in lipase production in comparison to other carbon sources (Zarevucka, 2012). The use of Tween 80 has helped to improve the recovery of *Acinetobacter* sp (Fatima *et al*., 2020). Non-lipidic carbon sources, such as glucose, fructose, sucrose, etc., have also documented increased production of lipase in addition to lipid carbon sources. The effect on the production of lipase by *Aspergillus sydowii* of various non-lipid sources of carbon (galactose, glucose, xylose, fructose, lactose, maltose, sucrose and mannitol) and lipid sources of carbon (coconut oil, palm oil, cucumber oil, olive oil, mustard oil, sunflower oil and neem oil) was investigated by Bindiya and Ramana (2012).

2.7.1.2 Nitrogen source

Nitrogen plays a major role in the synthesis of lipase. In various microbial organisms, various organic and inorganic sources of nitrogen have played an important role in increasing the production of lipase (Fatima *et al*., 2020). *Rhizopus sp*. has been grown by applying urea to the lipase cultivation medium, which is more efficient in lipolytic activity (Rodriguez *et al*., 2006).

Aspergillus sp. was also used for the production of lipase in a mixture of peptone and other nitrogen extract (Colonia *et al*., 2019). Lipase activity was also influenced by mixture of organic nitrogen sources as described by Ulker *et al*. (2011) where maximum lipase activity by *Trichoderma harzianum* was achieved in a medium containing glucose and peptone as source of carbon and nitrogen, respectively, while minimum activity was obtained with glucose and yeast extract medium. However, Bindiya and Ramana (2012) used various nitrogen sources (1% w/v) to investigate their influence on lipase production by *Aspergillus sydowii*. The nitrogen sources used in the study were $NaNO_3$, KNO_3, NH_4NO_3, NH_4Cl, $(NH_4)_2SO_4$, urea, beef extract, malt extract, yeast extract, tryptone and peptone. Highest activity (49 U/mL) was observed using NH_4Cl.

2.7.2 EFFECT OF PHYSICAL FACTORS

2.7.2.1 Temperature

Temperature influences secretion of extracellular enzymes by changing the physical properties of the cell membrane (Valeria *et al*., 2003). Mahmoud *et al*. (2015) investigated impact of temperatures on lipase production by incubating the cultures of *Aspergillus terreus* at various temperatures viz. 10 ºC, 20 ºC, 30 ºC and 45 ºC. Highest activity of lipase was obtained at 45 ºC (15 U/mL), which was followed by 30 ºC (12 U/mL), 20 ºC (9.5 U/mL) and 10 ºC (3.0 U/mL). Similarly, Mukhtar *et al*. (2015) studied the impact of various incubation temperatures ranging from 25 to 55 ºC on the productivity of lipase by *Aspergillus niger*. Highest production was achieved at 30 ºC, followed by 35 ºC, 40 ºC, 45 ºC, 25 ºC, 50 ºC and 55 ºC. Sumathy *et al*. (2012) reported that cultures of *Aspergillus niger* were incubated at 25 ºC, 30 ºC and 37 ºC, followed by estimation of activity of lipase after 48 h, 72 h, 96 h and 120 h. Highest activity of lipase was achieved in the culture incubated at 30 ºC after 96 h of incubation. Bindiya and Ramana (2012) investigated lipase production by *Aspergillus sydowii* at various incubation temperatures. Highest activity (64 U/mL) was obtained at 32 °C, followed by 30 °C, 28 °C, 26 °C, 24 °C, 22 °C, 20 °C and 34 °C, respectively.

2.7.2.2 pH

Potent lipolytic fungi such as *Aspergillus* sp., *Geotrichum* sp., *Rhizopus* sp., *Rhizomucor* sp., *Mucor* sp. and *Penicillium* sp. are able to grow and secret extracellular lipases within the pH range of 6.0 to 8.0 (Sharma *et al*., 2016a). Bindiya and Ramana (2012) investigated lipase

activity by *Aspergillus sydowii* at different pH of fermentation broth. pH 8.0 was the best for high yield of lipase, followed by pH 7.5, 8.5, 7.0, 9.0, 6.5 and 6.0. Similarly, Mahmoud *et al*. (2015) investigated influence of various pH on production of lipase by growing *Aspergillus terreus* at various pH ranging from 2.0-12.0. An optimum pH of 8.0 was observed to yield highest lipase activity (15 U/mL). *Aspergillus terreus* demonstrated the lipase activity of 12 U ml-1 at pH 12.0, while the activity was not found at pH 2.0, which indicates that alkaline conditions promotes fungal lipase growth and production.

2.8 APPLICATION OF FUNGAL LIPASE

The upsurge in the market for lipase in industrial applications is due to their inherent versatility (Geoffry and Rajeshwara, 2018). Fungal lipases are a significant group of biotechnologically essential enzymes due to the versatility of their properties and ease of mass production (Singh and Mukhopadhyay, 2012). Fungal lipases have versatile enzymatic and substrate specificity properties, making them very desirable for industrial applications (Ray, 2015). Many researchers have investigated the industrial applications of fungal lipases (Treichel *et al.,* 2010).

2.8.1 Lipase in Pharmaceutical Industry

Enzymes have many advantages over chemical synthesis in the pharmaceutical industry, justifying the rising demand for lipase (Singh and Mukhopadhyay, 2012). These benefits include mild conditions that inhibit isomerization, epimerization, racemisation, and rearrangement reactions; enantio and regio selectivity; and immobilized lipase reuse. The capacity of lipases to overcome racemic mixtures through the synthesis of a single enantiomer is currently being utilized by the pharmaceutical industry for drug development.

Mushrooms with medicinal impact are used for pharmaceutical products (Mohamed *et al*., 2011). Few essential mushrooms with pharmacological properties are *Agaricus brasiliensis*, *Ganoderma lucidum*, *Lentinula edodes*, *Coriolus versicolor*, *Pleurotus ostreatus*, *Grifola frondosa*, *Termitomyces*, etc. (Ganeshpurkar *et al*., 2010). Important biologically active substances such as immunosuppressive, antimicrobial, antiviral, nematic and hypocholesterolemic agents are contained in fungi (Hyde *et al*., 2019).

2.8.2 Lipase in Food Processing Industries

The lipase used in food processing, industry is for the modification and breakdown of biomaterials (Singh and Mukhopadhyay, 2012)**.** Fats and oils are essential food constituents and their alteration is one of the key areas needing new economic and green technologies in the food processing industry (Gupta, 2003). On an industrial scale, a large number of fat-clearing lipases are made. For milk products and the processing of other foods, such as meat, vegetables, fruit, smoked carp, milk products, baked foods and beer products, most of the commercial lipases produced are used to improve flavor (Mehta *et al*., 2017). Lipases from *A. niger*, *Rhizopus oryzae*, *Candida cylindracea* have been used in bakery products (Singh and Mukhopadhyay, 2012).

2.8.3 Lipase in Textile Industry

The use of fungal lipase in textile industry is becoming increasingly important (Mehta *et al*., 2017). Lipases are used in the textile industry to help remove size lubricants in order to provide a fabric with greater absorbency to improve the dyeing speed (Gupta *et al*., 2017). Polyester has some primary advantages in the textile industry, such as softness, high strength, washability, stain, stretch, machine abrasion, and wrinkle resistance. For use in the development of yarns, garments, rugs, and textiles, synthetic fibers have been enzymatically modified. It concerns the alteration of the properties of a polyester fiber in such a way that these polyesters are not prone to post-modification treatment (Singh and Mukhopadhyay, 2012).

2.8.4 Lipase in Waste Treatment

In activated sludge and other aerobic waste processes, lipases are used where thin layers of fat are continuously extracted from aerated tank's surfaces to allow the transport of oxygen. This skimmed fat-rich liquid is digested with lipase (Mehta *et al*., 2017), such as that from *C. rugosa*. Lipases are also assisting the usual performance of anaerobic digesters (Su *et al*., 2016). In many industrial activities, successful solids breakdown and the clearing and prevention of fat blocking or filming in waste systems are critical (Islam and Datta, 2015). Jeganathan *et al*. (2009) evaluated the hydrolysis of wastewater with high oil and grease (O&G) concentration from a pet food industry using immobilized *C. rugosa* lipase (CRL) as a pretreatment step for anaerobic treatment through batch and continuous-flow experiments.

2.8.5 Lipase in Detergent Industry

Fungal lipases are commonly used in commercial laundry detergents and domestic detergents as additives, and this will minimize the environmental load of detergent products by saving resources by allowing lower washing temperatures to be used (Saisubramanian *et al.*, 2006). In the approximately 13 billion tons of detergents produced annually an estimated 1,000 tons of lipase are added. Due to its thermostability, high activity at alkaline pH and stability against anionic surfactants, *Humicola lanuginosa* lipase is ideal as a detergent additive. Lipases used as detergents also include those from *Candida*. Other detergent uses include dishwashing, bleaching composition, decomposition of lipid pollutants in solvents for dry cleaning, liquid leather cleaner, cleaning of contact lenses, washing, degreasing and reconditioning of water using lipases along with oxido-reductases (Mehta *et al.*, 2017).

2.8.6 Lipase in Paper Industry

Lipolytic enzymes are used to extract pitch, the wood lipid fraction that interferes with paper pulp production. They also help to eliminate lipid stains and prevent the development of sticky materials through paper recycling (Dube *et al*., 2008; Hasan *et al*., 2006). In Japan, Nippon Paper Industries developed a pitch control system that used *Candida rugosa* derived fungal lipase to hydrolyze up to 90% of the triglycerides (Sharma *et al*., 2001). In1990, Hata and coworkers at Jujo Paper Company claimed that by lowering the triglyceride content of ground wood pulp, lipases may minimize pitch issues. A lipase obtained from *Candida cylindrica*, when added to the ground wood stock chest, reduced pitch problems and talc consumption considerably. *Candia antartica* lipase A (CALA) was used in pitch control in the paper industry (Maria *et al*., 2005).

2.8.7 Lipase in Biodiesel Production

Biodiesel is a group of esters formed in the presence of catalysts by a transesterification reaction between fatty acids and alcohol (Mehta *et al*., 2017). The processing of biodiesel from waste and non-edible vegetable oil substantially decreases the cost of biodiesel production, thus preventing disputes between food security and energy protection, and is seen as an essential step in reducing emissions and recycling waste oil (Gashaw *et al*., 2015; Narwal and Gupta, 2013).

The production of biodiesel has risen sharply in the last decade from approximately 950 liters in 2000 to nearly 17,000 million liters in 2010 with the European Union as the world's major producer, accounting for 53% of global biodiesel production (Eryilmaz *et al*., 2016). In 2000,

biodiesel represented around 5% of the world's biofuel production and in 2011 biodiesel share accounted for around 20% of total biofuel production (Eryilmaz *et al*., 2016). This rise appears to continue and, as estimated by the United Nations, biodiesel production is expected to exceed 41,000 million liters in 2022. Higher thermostability and short-chain alcohol-tolerant capabilities of lipase make it very convenient for use in biodiesel production (Bacovsky *et al*., 2007; Kato *et al*., 2007). Most yeast lipases and fungal lipases involved in biodiesel production are produced from *A. niger*, *C. antarctica*, *C. rugosa*, *R. miehei*, *R. oryzae*, and *Thermomyces lanuginose* (Fan *et al*., 2012).

Production of biodiesel has been reported by using immobilized *Candida Antarctica* lipase-catalyzed methanolysis of soybean oil (Ho *et al*., 2007). The most promising biocatalyst for the enzyme-catalyzed biodiesel synthesis was immobilized lipase from *Candida rugosa* on Sepabeads EC-OD (Winayanuwattikun *et al*., 2011). In a recent study, biodiesel production from Chinese tallow kernel oil has been catalyzed by *Candida rugosa* lipase (CRL) in ionic liquid (Su *et al*., 2016).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials and Equipment

The following materials and equipment were used for the experiment: Petri dishes, cover slips, glass slides, conical flask, inoculating needle, cotton wool, aluminum foil, dropper, inoculating loop, test tubes, pipette, burette, McCartney bottle, spatula, Erlenmeyer flask, beaker, syringe, Bunsen burner, water bath, incubator and autoclave.

3.2 Culture Media and Reagents

The media used during the experiment were: Potato Dextrose Agar (PDA), Tributyrin Agar, Nutrient broth. The reagents used during the experiment include: Lactophenol blue, ethanol, thymolphthalein indicator, physiological saline, Tween solution, Saline solution (2 g/L KH2PO4, 1 g/L MgSO4, FeSO4·7H2O (0.63), MnSO4 (0.01), ZnSO4 (0.62)), Sodium Hydroxide (NaOH), Hydrochloric Acid (HCL).

3.3 Isolation of Fungal Species

3.3.1 Sampling

The soil samples were obtained from selected restaurants and diesel contaminated sites. The soil samples were collected from a depth of 5-10cm using a sterile spatula and stored in sterile glass vials (Alhamdani *et al*., 2016). The bottles were labeled properly and transported to the laboratory for examination and subsequent analysis (Sagar *et al*., 2013). The samples were stored at 27^{o}C when not in use (Nwuche *et al*., 2011).

3.3.2 Serial Dilution

Serial dilution was performed for isolation of lipolytic fungi (Rajeshkumar *et al*., 2013). One gram of the respective soil sample was weighed and placed in a sterile test tube with 9 mL of distilled water. Five-fold serial dilutions were prepared aseptically. The sterile test tubes were taken and labeled per dilutions ranging from 10^{-1} to 10^{-5}. 1 g of the respective soil sample was weighed and added to the first dilution blank of 9.0 mL of distilled water. 1.0 mL of the first dilution blank (10^{-1}) was added to the second dilution blank (10^{-2}). The tubes were shaken and serial dilution was done till the last tube dilutions (10^{-5}). 0.1 mL of the diluents was inoculated

using the pour plate method in a disposable petri-dish with Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA).

3.4 Preparation of Culture media

3.4.1 Potato Dextrose Agar

Potato Dextrose Agar (PDA) was used for the cultivation of fungi. PDA is a general-purpose medium for the cultivation of yeast and mold that can be supplemented with antibiotics to inhibit bacteria growth (Sadar, 2019). Maintaining aseptic methods and conditions, 39 g of commercially prepared Potato Dextrose Agar was measured and dissolved in 1 liter of sterile distilled water in a conical flask and mixed thoroughly. Chloramphenicol antibiotic was added to prevent bacterial growth. The mixture was boiled to dissolve the culture medium completely. The Agar was then sterilized by autoclaving at 121^{o}C for 15 minutes. The medium was allowed to cool to 45^{o}C before pouring into the sterile petri dishes.

3.4.2 Sabouraud Dextrose Agar

Sabouraud Dextrose Agar (SDA) was used for the isolation, cultivation and maintenance of non-pathogenic and pathogenic species of fungi and yeasts. Maintaining aseptic methods and conditions, 65g of commercially prepared Sabouraud Dextrose Agar was measured and dissolved in 1 liter of sterile distilled water in a conical flask and mixed thoroughly, chloramphenicol antibiotic was added to prevent bacterial growth. The mixture was boiled to dissolve the culture medium completely. The Agar was then sterilized by autoclaving at 121^{o}C for 15 minutes. The medium was allowed to cool to 45^{o}C before pouring into the sterile petri dishes.

3.4.3 Pour Plate method

Pour plate method was used to culture the fungal colonies on PDA (Potato Dextrose Agar) and SDA (Sabouraud Dextrose Agar). 0.1 mL from the serially diluted samples (10^{-1}, 10^{-3} & 10^{-5}) was poured into the sterile petri plates, the petri plates were shaken for uniform distribution of the sample. After few minutes, the molten Potato Dextrose Agar and Sabouraud Dextrose Agar were poured gently into the respective petri plates. After solidification, the plates were incubated at room temperature for 3-5 days. The sterility of the media was confirmed by incubating an un-inoculated agar plate labeled as the control. (Wadia *et al*., 2017). Distinct fungal colonies were selected from the heterotrophic colony and were transferred using a sterilized inoculating loop

into a new culture medium to get a pure culture. Chloramphenicol antibiotics were added to the culture medium (PDA and SDA) to prevent bacterial contamination. The plates were then incubated and stored in a plastic bag at 4^{o}C.

3.5 Screening of Lipolytic Fungal Species

Screening of lipase producing fungi was done using tributyrin as a substrate on agar plates. For this purpose, Tributyrin agar (HiMedia) was used with the following composition: Peptone: 5.0 gm; Yeast extract: 3.0 gm; Agar Agar: 15.0 gm; Tributyrin (Glycerol Tributyrate): 10.0 mL; Distilled water: 990 mL; pH: 7.5. All the isolated fungal culture was inoculated on the TBA plates and incubated at 27^{o}C up to 7days. The formation of opaque zones around the colonies is an indication of lipase production by the organisms (Wadia *et al*., 2017) The diameter(d) of the colonies and the diameter (D) of total clear halos were also determined. The strains that yielded higher halos (D-d) were selected as potential fungi for lipase production (Griebeler *et al*., 2009). The lipase producing fungal strain was transferred to agar slants for storage at 4^{o}C.

3.5.1 Preparation of Agar Slant

Grown fungal colonies were purified on Tributyrin (HiMedia) slants. The Agar slants were made by preparing agar in a sterilized Erlenmeyer flask, distributing it into test tubes with the aid of a sterilized syringe, thereafter an indicator (Victoria blue) was added to the medium, the tubes were sterilized and laid at angle of 45^{o} to make a slanted surface as they solidify (Caprette, 2017). The fungal colonies were inoculated on the agar slants by streaking method.

3.6 Identification of Screened Fungi

After screening, the potent fungal cultures were inoculated on Potato dextrose Agar and incubated at room temperature for identification and further studies.

3.6.1 Morphological identification of the selected isolate on solid agar

The morphological characteristic of the selected isolate grown on PDA was studied for growth rate, growth pattern, colony texture, and pigmentation (Promputtha *et al*., 2005).

3.6.2 Biochemical Identification of Isolates using Lactophenol Blue Stain

A portion of the growth of the colony was teased out using the borer and an inoculating needle and mounted on the slide with a drop of lactophenol blue on it and covered with a cover slip. The preparation was examined under a light microscope with an attached camera (Motic Mc Camera

2.0-megapixel digital colored camera) connected to a computer for the microscopic photography of the fungi. The essence of this was to observe the exact arrangement of the conidiophores and the way the spores are produced. The identities of these fungi were certified using cultural, morphological as well as comparing them with confirmed representatives of different species in relevant texts. (Alexopoulos *et al*., 1996; Ellis *et al*., 2007).

3.7 Lipase production using Solid State Fermentation

The medium for solid-state fermentation was prepared with 10 g/L wheat bran as carbon source. The medium was added to 71% (v/w) of saline solution containing KH2PO4 (0.2% w/v) and $MgSO_4$ (0.1% w/v) and 2% of yeast extract as nitrogen source inside 300 mL Erlenmeyer's flasks containing 50 g of the medium. 2% olive oil was added as lipase inducer (Colla *et al*., 2014). 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 120 rpm (Bertolin *et al*., 2001). The fermented brans were kept at −20°C until use.

3.7.1 Lipase Extraction

Lipase extraction from the fermented bran obtained in solid-state fermentation by the fungal strain was carried out by adding 10 mL of phosphate buffer (pH 7.0) at 1 g of fermented medium, followed by agitation of 160 rpm for 30 min. at 35°C. The extract was cotton-filtered and used as enzyme extract in subsequent reactions (Colla, 2009).

3.7.2 Lipase Assay

Lipase activity was assayed by using p-nitrophenyl palmitate (p-NPL) as substrate. Reaction mixture contained 2 µL of 0.03 g/L p-NPL, 6 µL of 0.05 M phosphate buffer and 2 µL of enzyme extract (lipase) incubated at 30 °C for 10 min. Reaction was terminated by adding 30µL of absolute ethanol. Absorbance was measured using spectrophotometer at 410 nm wavelength. (Ilesanmi *et al*., 2020) One unit of enzyme was defined as the amount of enzyme that releases 1µmol of *p*-nitrophenol from the substrate.

3.8 Effect of carbon source on lipase production

The effect of carbon source such as wheat bran, banana peel, rice bran, plantain peel, glucose, sucrose, mannitol and starch on lipase production under solid-state fermentation was

investigated. The production medium was similar to the one described above containing 10 g/L carbon source, KH2PO4 (0.2% w/v), $MgSO_4$ (0.1% w/v) and 2% of yeast extract as nitrogen source inside 300 mL Erlenmeyer's flasks containing 50 g of the medium. 2% olive oil was added as lipase inducer (Colla *et al*., 2014). 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 120 rpm. The fermented brans were kept at −20◦C until use.

3.9 Effect of nitrogen source on lipase production

The effect of nitrogen source such as yeast extract, peptone, beef extract, ammonium sulphate, sodium nitrate, potassium nitrate and urea on lipase production was under solid-state fermentation was investigated. The production medium was similar to the one described above containing 10 g/L wheat bran, KH2PO4 (0.2% w/v), $MgSO_4$ (0.1% w/v) and 2% of nitrogen source inside 300 mL Erlenmeyer's flasks containing 50 g of the medium. 2% olive oil was added as lipase inducer (Colla *et al*., 2014). 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 120 rpm. The fermented brans were kept at −20◦C until use.

3.9.1 Statistical Analysis

The values for each parameter were calculated and presented as means of duplicates. Data was analysed 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 (Aforiji *et al*., 2019).

CHAPTER FOUR

4.1 RESULT

Fungi isolated from diesel and restaurant wastewater contaminated soil were screened for lipase production. A total of 12 morphologically different fungi were isolated. The identities of the isolates were established using their morphological and biochemical characteristics.

Table 4.1 shows the result of the screening of the isolates for lipase production on solid agar. At the end of incubation periods, the diameter of growth of the isolates ranged from 23.0^{k} – 53.0^{a} mm. Isolate TPD1 had the highest diameter of growth while isolate TPS4 had the lowest. Moreover, there was a significant difference ($p \geq 0.05$) in lipase activity of the isolates. Lipase production measured based on the zone of clearance of the isolates ranged from 2.0^{g} - 10.5^{a} mm. Isolate TPD1 had the highest lipase activity while isolate TPS6 had the lowest activity. Four (4) isolates including TPD3, TPS1, TPS2 and TPS9 did not show any lipase activity throughout the incubation period. Isolate TPD1 exhibited excellent lipase activity and was selected for further study.

Figure 4.1 shows the morphological identification of the selected isolate (TPD1). The isolate is a rapidly growing mould which matures within 3 to 5 days. Growth begins as fluffy white tufts which then compact and appear woollier. Green tufts later developed within the colony due to the production of conidia which appeared as concentric rings. The reverse is typically a light yellow to pale orange.

Figure 4.2 illustrates the microscopic image of the selected isolate, following staining with lactophenol blue dye. The microscopic appearance showed septate and hyaline hyphae. Conidiophores appeared rather short, branching at wide angles while Phialides are flask or ampule shaped (inflated at the base), which again extend from the conidiophore at wide angles. Conidia are round to ellipsoidal and can be smooth or rough walled depending on the species. Based on the results of morphological and biochemical characterizations, the probable identity of the isolate was concluded to be *Trichoderma harzianum*.

The influence of various carbon sources on lipase production by isolate TPD1 was investigated. There was significant difference in lipase production ($p \leq 0.05$) in the presence of different carbon sources including monosaccharide (glucose), disaccharide (sucrose), polysaccharide (starch) and

agro wastes (plantain peel, banana peel, starch, wheat bran and rice bran). Lipase activity ranged from 85.56^{g} – 156.42^{a} U/mL as seen in Figure 4.3. Glucose (156.42^{a} U/mL) supported the highest lipase production follow in order by sucrose (132.21^{b} U/mL), mannitol (129.44^{c} U/mL) and plantain peel (104.65^{d} U/mL) while the least production was recorded in rice bran (85.56^{g} U/mL).

Figure 4.4 illustrates the impact of different inorganic and organic nitrogen sources on the activity of extracellular lipases. There was significant difference in lipase production in the presence of different organic and inorganic nitrogen sources. Lipase production ranged from 88.96^{e} – 128.01^{a} U/mL. The highest level of lipase activity (128.01^{a} U/mL) was recorded in the presence of peptone followed in order by yeast extract (126.56^{b} U/mL), sodium nitrate (109.84^{c} U/mL), ammonium sulphate and potassium nitrate (106.58^{e} U/mL) while Urea provided the least support (88.96^{e} U/mL).

Table 4.1: Screening of fungal isolates for lipase activity

Isolates	Diameter of growth (mm)	Lipase activity (mm)
TPD1	53.0[a]	10.5[a]
TPD2	30.0[j]	9.0[b]
TPD3	50.0[c]	0.0
TPD4	31.0[i]	4.5[f]
TPS1	32.0[h]	0.0
TPS2	51.0[b]	0.0
TPS4	23.0[k]	5.0[e]
TPS5	41.0[g]	8.0[c]
TPS6	41.0[g]	2.0[g]
TPS7	48.0[d]	10.5[a]
TPS8	46.0[e]	7.0[d]
TPS9	43.0[f]	0.0

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

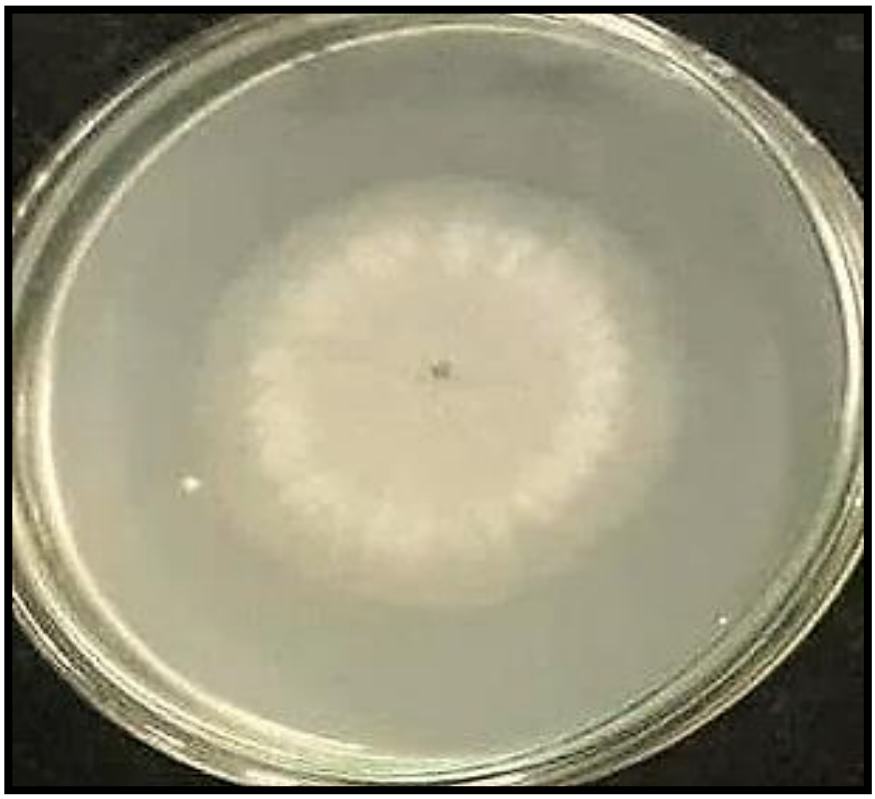

Figure 4.1: Morphological appearance of the isolate on PDA.

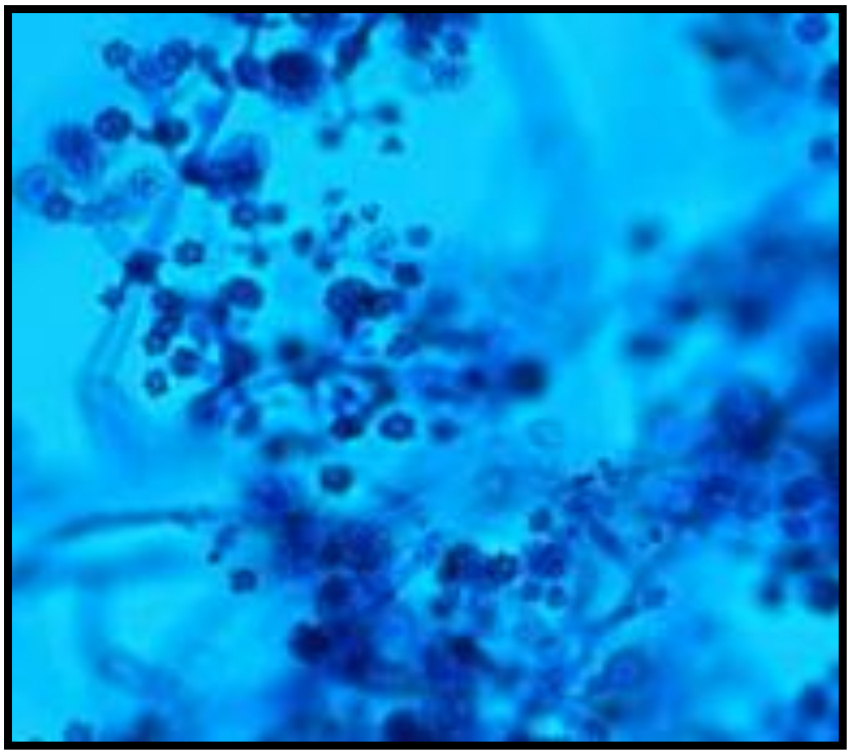

Figure 4.2: Microscopic image of the isolated *Trichoderma* sp.

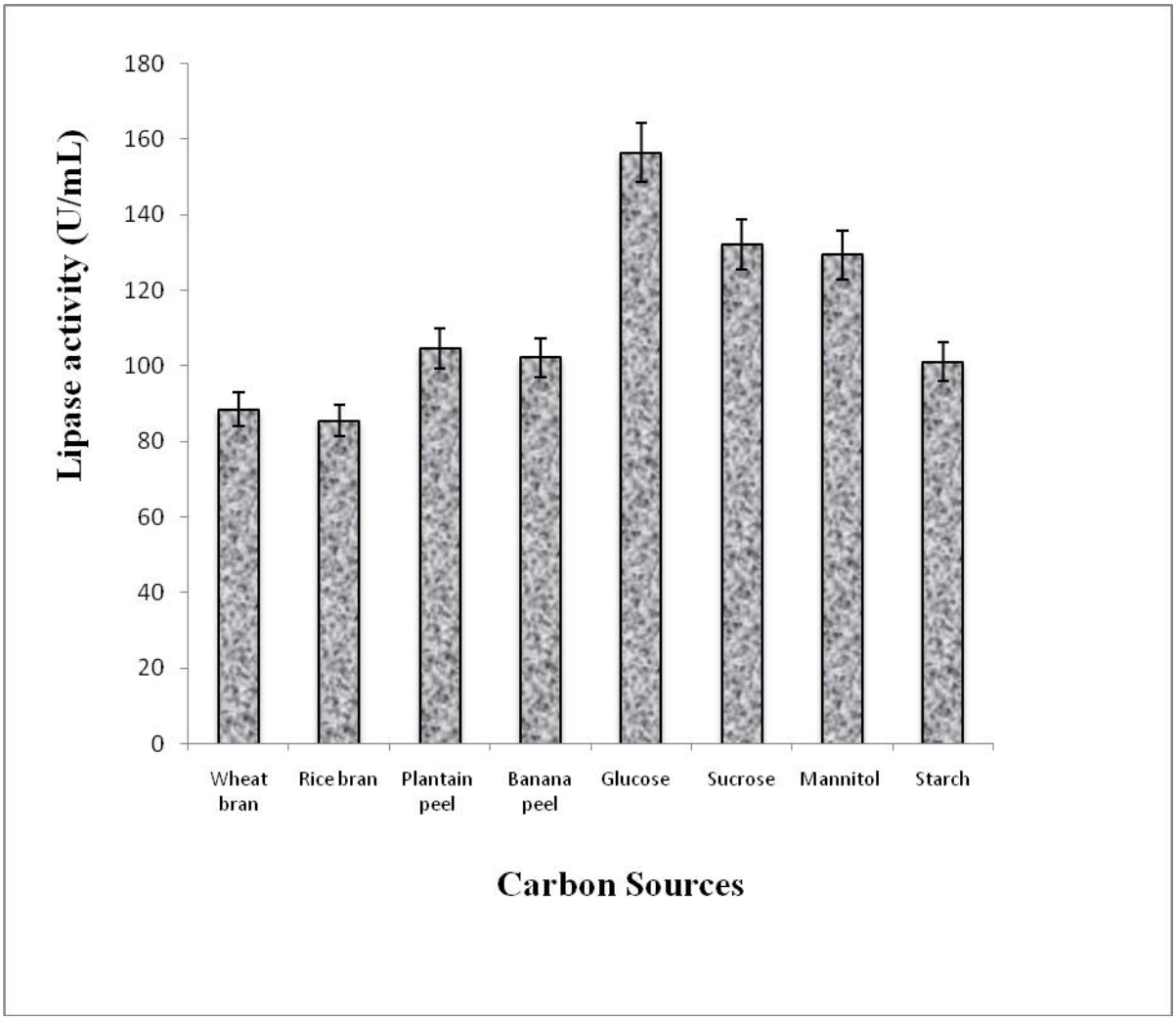

Figure 4.3: Effect of carbon source on lipase activity

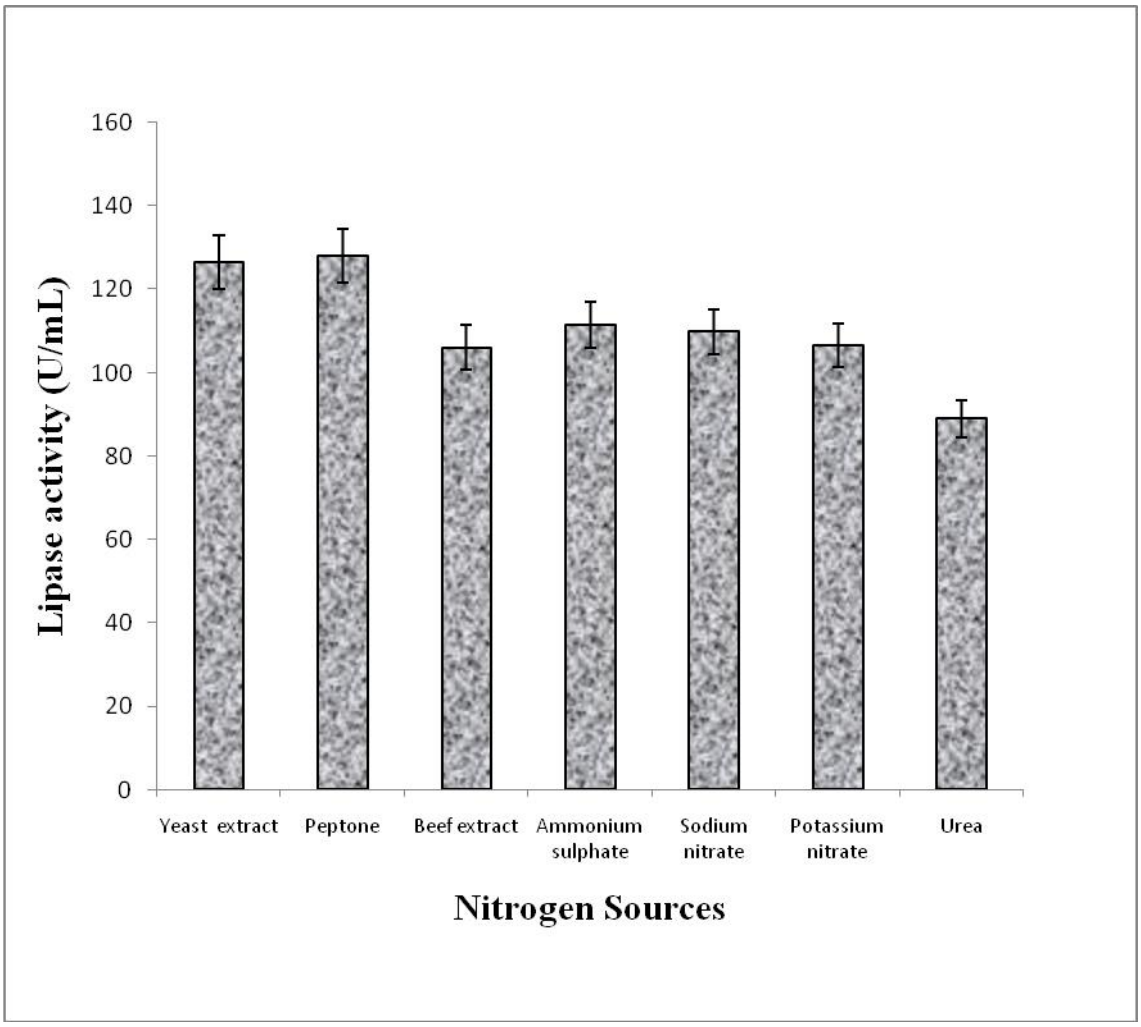

Figure 4.4: Effect of nitrogen source on lipase activity

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

5.1 DISCUSSION

The presence of lipase-producing fungi from environmental samples has been previously reported (Ramos-Sánchez *et al*., 2015; Rihani and Soumati, 2019). Akyil and Cihangir (2018) similarly screened environmental samples for lipolytic fungi. Moreover, Musa and Adebayo-Tayo (2012) referenced 17 distinct fungal sources from which common lipases are derived. Similar results were reported by Yalçın *et al*. (2014) who screened 120 filamentous fungi isolated from active samples of petroleum sludge, soil and wastes collected from petroleum refineries and soils polluted by petroleum for lipase production. Twelve (12) of these fungi were reported to be a good source of lipase.

Fungi are good source of extracellular lipase. The morphological and biochemical characterization observed in this study for the selected isolate (TPD1) was similar to the report of Nwuche and Ogbonna (2011) on *Trichoderma* sp. The genus Trichoderma has been previously reported to be a good producer of lipase (Akyil and Cihangir, 2018; Wang *et al*., 2018, Rantasalo *et al*., 2019). Similar to this study, Rihani and Soumati (2019) reported *T. harzianum* isolated from oil contaminated soil as a good source of lipase for industrial production. Ulker *et al*. (2011), reported the isolation of *Trichoderma harzianum* which showed an excellent lipolytic activity, from soil samples collected from different regions in Turkey.

Several factors have been known to affect lipase expression. The key contributors to lipase production are media components such as: carbohydrates and nitrogen, oils, fatty acids and sugar esters (Salihu *et al*., 2011). Factors like carbon or nitrogen sources and their engrossments have always been of enormous attraction to the industrialists and scientific community for the cut price media formulation (Singh *et al*., 2016).

Different carbon sources have different effect on the enzyme production. From this study, the highest lipase activity (156.42 U/mL) was observed in the presence of glucose as carbon source. Similar results were obtained by Rehman *et al*. (2019), who utilized different carbon sources for lipase production by *Pleurotus ostreatus*. They observed maximum lipase activity (2654 U/gds) when glucose was supplemented as a carbon source. In contrast, Rihani and Soumati (2019)

reported 1.58 U/mL as the maximum lipase production by *Trichoderma harzianum*, when the medium was supplemented with olive oil in the absence of glucose and Tween 80. Similar results were obtained by Falony *et al*. (2006) and Ramos-Sánchez *et al*. (2015) who reported that sugar substrates only favor the growth of microorganisms but not the synthesis of lipase, whereas oleic acid, and olive oil enhance its synthesis.

Maximum lipase activity (128.01 U/mL) was observed with peptone as nitrogen source. Similar results were reported by Ulker *et al*. (2011), who observed maximum lipase activity (1.25 g/L) using glucose and peptone as carbon and nitrogen sources respectively. Likewise, Akeed and Al-halaby (2018) reported that peptone was found to enhance the production of lipase by about 94.8% compared to the cultivation without the addition of any nitrogen source. However, Kebabci and Cihangir (2012) concluded that the addition of ammonium compounds led to the best increase in lipase production. Likewise, Lopes *et al*. (2016) reported maximum lipase activity (486 U/g) when the fermentation medium was supplemented with ammonium sulphate.

5.2 CONCLUSION

In conclusion, samples of diesel and restaurant wastewater contaminated soil were collected, cultured and screened for lipolytic fungi. Out of 12 isolates screened, isolate TPD1 showed the highest growth and lipase activities on solid agar. The probable identity of the isolate as *T. harzianum* was established based on its morphological and biochemical characteristics. Both glucose and peptone supported the highest lipase productions among the carbon and nitrogen sources investigated. Isolate TPD1 is a good source of lipase production and can be harness for further study with the aim for industrial production.