# ISOLATION, SCREENING AND PRODUCTION OF LIPASE FROM BACILLUS SUBTILIS

**BY**

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## CERTIFICATION

This is to certify that this research project titled "**ISOLATION, SCREENING AND PRODUCTION OF LIPASE FROM BACILLUS SUBTILIS"** was carried out by Ayoola, Pamilerin Opeyemi, with matriculation number 17010101026, 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.

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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 have been duly acknowledged in the text and a list of references provided. This research project report has been previously presented anywhere for the award of any degree or certificate.

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**AYOOLA, Pamilerin O.** **Date**

## DEDICATION

I dedicate this Project work to the Almighty God for His ever-continuous mercies, guidance, protection, and favor all through my stay at Mountain Top University, and for the successful completion of this body of work. I also dedicate this work to my parents – Mr. and Mrs. Ayoola – and my guardians – Mr. and Mrs., Akinola for their financial, spiritual, psychological, and moral support.

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## ABSTRACT

The production of commercial enzymes, including lipase from bacteria has always been the industrial choice due to its economic and commercial feasibility. In this study, bacterial isolates from diesel and wastewater polluted soil were screened for lipase production on solid agar. Morphological and biochemical characteristics of the best isolate were investigated. The effect of carbon sources including monosaccharide (glucose, and galactose), disaccharide (maltose, lactose, and sucrose), polysaccharide (starch), alcohol sugar (mannitol) and nitrogen sources were investigated. A total of 14 bacteria were isolated. Lipase activity ranged from $1.5^{f}$ - 5.0; $2.5^{g}$ - $6.0^{a}$ and 3.5g - $9.5^{a}$ at 24, 48 and 72 hrs respectively. Isolate PMD1 identified as *Bacillus subtilis* gave the best lipase activity in all the incubation periods. There was significant difference ($p \geq 0.005$) in the presence of different carbon and nitrogen sources employed for lipase production. Lipase production ranged from $31.09^{h}$ - $88.65^{a}$ (U/mL) and $42.11^{g}$ - $86.35^{a}$ (U/mL) for carbon and nitrogen sources respectively. Glucose ($88.65^{a}$ U/mL) and peptone ($86.35^{a}$ U/mL) supported the highest lipase production. The study has shown that *Bacillus subtilis* isolated from diesel 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), *Bacillus subtilis*.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background of Study

Enzymes are natural impetuses (otherwise called biocatalysts) that accelerate biochemical responses in living creatures. They can likewise be removed from cells and afterward used to catalyse a wide scope of monetarily significant procedures (Robinson, 2015). For instance, they assume significant jobs in the creation of improving specialists and the alteration of anti-microbials. They are utilized in washing powders and different cleaning items, and they assume a key job in scientific gadgets and measures that have clinical, criminological, and natural applications. The word 'enzyme' was first utilized by the German physiologist Wilhelm Kuhne in 1878, when he was portraying the capacity of yeast to deliver liquor from sugars and it is gotten from the Greek words en (which implies inside) and *zume* (which means yeast) (Robinson, 2015). The usage of proteins in mechanical procedures stirs extraordinary enthusiasm because of its accessibility and preferences corresponding to concoction impetuses, greater explicitness, lower vitality utilization, and speed up (Gupta *et al*., 2015). In 2010, the worldwide market for mechanical catalysts was assessed at $3.3 billion and is depended upon to reach $8.0 billion by 2015. Lipase as of now positions third among the most at present promoted proteins after protests and carboxylases (Gupta *et al*., 2015).

Lipases are compounds that catalyse the aggregate or fractional hydrolysis of fats and oils, discharging free unsaturated fats, diacylglycerols, monoacylglycerols, and glycerol (Villeneuve *et al*., 2000). These chemicals vary from esterases (carboxylesterase) that demonstrate just on water-solvent carboxylic ester atoms (Verger, 1997). Under explicit conditions, lipases additionally catalyse blend responses, for example, esterification, transesterification

(interesterification, acidolysis, and alcoholysis), aminolysis (the union of amides), and lactonization (intermolecular esterification) (Gupta *et al*., 2015). Lipases do not need cofactors, work in a wide pH run, is consistent at high temperatures, have high particularity and show regio-, chemo-and enantioselectivity (Villeneuve *et al*., 2000). Inferable from their extraordinary properties, lipases are generally utilized in different mechanical areas, for example, food, pharmaceutics, biofuels, oleochemical, material, agro-concoction, paper assembling, beauty care products, and numerous others. In the food business, lipases can be utilized as flavour modifiers by the union of short-chain unsaturated fats esters and alcohols and to acquire results of expanded dietary benefit by adjusting the triacylglycerol structure (Verma *et al*., 2012). In a bread shop, lipases are potential up-and-comer substitutes for emulsifiers (Colakoglu and Ozkaya, 2012). In the wine business, these catalysts are utilized to deliver trademark wine esters. Lipases can be utilized in numerous procedures, for example, to orchestrate auxiliary lipids, low-calorie lipids, and milk fat and in aging cheddar (Gupta *et al*., 2015).

Lipases are universal in nature and are created by plants, creatures, and microorganisms (Barros *et al*., 2010). They have likewise been accounted for in higher plants, for example, Castor bean (*Ricinus communis*) and rapeseed (*Brassica napus*), as referenced by (Gunasekaran *et al*.2005). Microbial lipases are financially critical due to low creation cost, more prominent steadiness, and more extensive accessibility than different sources (Patil *et al*., 2011). The significance of lipases in the mechanical application has been encouraged by the accompanying variables: (1) acts over a wide scope of pH and temperature (2) High explicitness (3) don't require co-elements and (4) catalyse a wide scope of responses. They have likewise demonstrated viable and guarantee a fundamental job in isolating enantiomers, having enantio, locale, and sound system selectivity of high requests (Gunasekaran *et al*., 2005).

Microbial enzymes are habitually more accommodating than mixes got from plants or animals because of the uncommon variety of synergist practices available, the extraordinary returns possible, effortlessness of genetic control, standard deftly in view of nonattendance of infrequent instabilities and quick improvement of microorganisms on unassuming media (Hasan *et al*., 2006). Microbial synthetic compounds are also consistent than their contrasting plant and animal impetuses and their creation are logically useful and safer. Pretty much 2% of the world\'s microorganisms have been attempted as impetus sources (Patil *et al*., 2011).

Bacterial strains are commonly increasingly utilized as they offer higher exercises contrasted with yeasts and will in general have an unbiased or antacid pH optimum and are regularly thermostable (Patil *et al*., 2011). Hereditary and ecological control to expand the yield of cells to build the catalyst movement of the cells by making the chemical of intrigue constitutive or by inciting it or to create changed proteins might be utilized effectively utilizing microbial cells in view of their short-age times, their generally basic, nourishing prerequisites, and since screening strategies for the ideal attributes are simpler (Patil *et al*., 2011).

The closeness of lipases has been exactly on schedule as in 1901 for *Bacillus prodigious*, *Bacillus pyocyaneus*, and *Bacillus fluorescens* which address the current best-inspected lipase making microorganisms as of now named *Serratia marcescens*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, independently (Hasan *et al*., 2006). Impetuses hydrolyzing fatty substances have been thought well over 300 years and the limit of the lipases to catalyze the hydrolysis and the mix of esters has been seen just about 70 years earlier (Hasan *et al*., 2006).

The utilization of compounds in numerous synergist measures has brought about investigations prompting critical improvement of the chemical properties (Mateo *et al*., 2007). Perhaps the most

significant and broadly utilized methods is protein immobilization in which impetuses are joined to a strong help that is insoluble in the response blend (Zhang *et al*., 2015). The best preferred position of immobilization is that it altogether improves the security of the biomolecules under different response conditions and upgrades the reusability of biomolecules over progressive reactant cycles (Zhang *et al*., 2015). Also, subsequent to restricting the protein particles, the impetuses change from a homogeneous to a heterogeneous structure, which encourages basic division of the biocatalytic framework from the response combination and results in results of higher virtue (Mateo *et al*., 2007). Different immobilization strategies have been created, including adsorption, covalent authoritative, entanglement, embodiment, and cross-connecting (Sheldon *et al*., 2007). These vary in the sort and character of the collaborations framed and, in the structure, and kind of the help materials utilized. Choice of the most suitable immobilization technique and backing material relies emphatically upon the sort and state of the reactant cycle just as the kind of the chemical (Sheldon *et al*., 2007).

Lipases find immense application in various areas such as dairy and food industry, oil industry, medicine, pharmaceutical, cosmetic perfumes detergents, agriculture etc. (Sharma *et al*., 2001). Hence, lipases are today the choice of catalysts for all biochemical processes due to high versatility in their catalytic behaviour (Joseph *et al*., 2012). Microbial lipases have acquired exceptional mechanical consideration because of their solidness, selectivity, and expansive substrate particularity (Sharma *et al*., 2001). Numerous microorganisms are known to be likely makers of extracellular lipases, including microscopic organisms, yeast, and parasites (Joseph *et al*., 2012). They are impervious to solvents and are abused in an expansive range of biotechnological applications.

## 1.2 STATEMENT OF THE PROBLEM

Production of unwanted by-products during industrial processes which requires extensive downstream processes to remove because of using chemical catalysts thereby adding to the cost of production as well as reduction in product quality.

## 1.3 JUSTIFICATION OF THE STUDY

Because of its high precision and economic benefits, without any impact on the environment, enzymes are used as biological catalysts because enzymes can be used instead of harsh conditions and harsh chemicals, as the effect is that energy saving, and pollution control could be beneficial. To minimise the cost of enzyme production, the technique of immobilizing enzyme is being applied. Immobilization of enzyme limits enzyme movement and allows flow processing and easy recovery.

## 1.4 AIM OF THE STUDY

The aim of this research is to produce lipase from bacterial isolate

## 1.5 OBJECTIVES OF THE STUDY

The objectives of the study include:

i. Isolation and culturing of bacteria from oil contaminated soil sample.
ii. Screening and selection of the best lipase producers
iii. Identification of bacterial isolates using morphological and biochemical characterizations.
iv. Production of lipase using submerged fermentation technique

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 LIPASE

Lipases (triacylglycerol acyl hydrolases EC 3.1.1.3) are inevitable proteins of the stunning physiological criticalness and mechanical potential. Lipases catalyze the hydrolysis of triacylglycerols to glycerol and free unsaturated fats Lipases are possibly initiated when adsorbed to an oil-water interface (Martinelle *et al*., 2015). Lipase will part-emulsify esters of glycerin and long-chain unsaturated fats, for example, triolein, and tripalmitin. Lipases are serine hydrolases (Martinelle *et al*., 2015). Lipases show little movement in fluid blueprints dissolvable substrates. In eukaryotes, lipases are secured with different times of lipid ingestion including fat planning, assimilation, reconstitution, and lipoprotein osmosis. In plants, lipases are found in vitality to save tissues (Martinelle *et al*., 2015).

### 2.1.1 Mechanism of Lipase Activity

Lipase actuation at the lipid-water interface of triacylglycerides, within the sight of colipase and bile salts, is known as interfacial initiation (Thomas *et al*., 2005). For the hydrolysis response to happen, colipase grapples lipase to the lipid-water film of the micelle which causes a surface change on lipase (Thomas *et al*., 2005). Colipase's four hydrophobic circles associate with the hydrophobic climate of the triacylglyceride. This starts dynamic site official to the lipid, and a top opening to uncover a progressively hydrophobic condition for the triacylglycerol (Crandall *et al*., 2001 This thus permits the triacylglycerol to interface with key dynamic site deposits like the synergist group of three (Crandall *et al*., 2001).

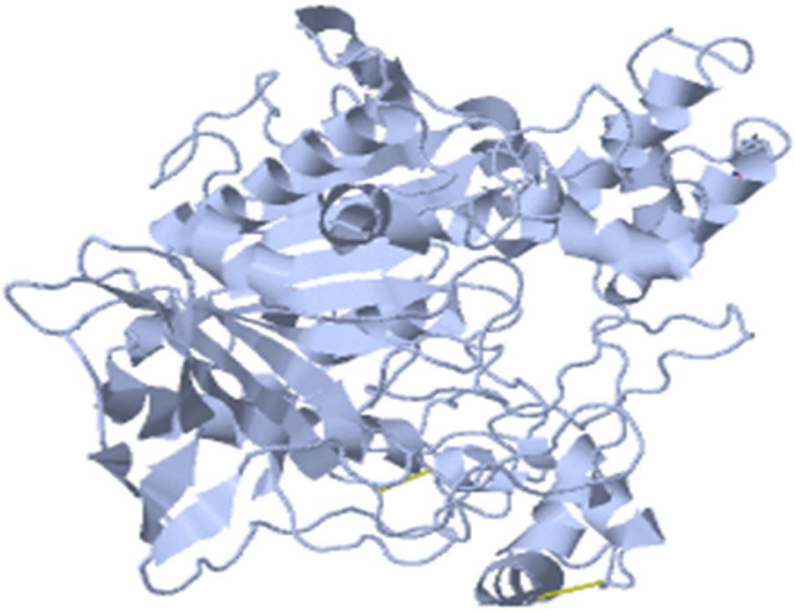

**Fig 2.1: Structure of glycosylated pancreatic lipase**

### 2.1.2 Characteristics of Lipase

Lipase (triacylglycerol acyl hydrolases EC 3.1.1.3) is an inescapable protein with significant physiological noteworthiness and modern potential (Sharma *et al*., 2001). Lipases catalyse the hydrolysis of triglycerides to glycerol and free unsaturated fats (Sharma *et al*., 2001). They are solvent in water and hydrolyse insoluble substrates to increasingly polar lipolytic items (Kanwar, 2006). The principal lipase was distinguished by Claude Bernard in 1856 and from that point on, they have been recognized in microorganisms, plants, and creatures (Kanwar, 2006).

Lipases are one of a kind as far as catalysing fat hydrolysis to unsaturated fats and glycerol, which happen on the water-lipid fringe, and as far as an opposite response in a non-water condition (Erdemir *et al*., 2009). After contact with an insoluble substrate in water, an adjustment in the protein's adaptation happens (Huang *et al*., 1989). This wonder of interfacial action is these days a region of concentrated investigations performed by crystallographers, natural

chemists, sub-atomic scientists, scientific experts, and biochemical designers (Huang *et al*., 1989).

The properties of lipases, much the same as lipases themselves, differ. An attractive element is the warm security of this chemical (Lee, 2009). The wellspring of thermally safe lipases is as a matter of first importance microscopic organisms, and the warm toughness is connected to their structure (Lee, 2009). The warm soundness is affected by such factors as pH and the nearness of substantial metals. All exercises at the degree of protein designing are intended to improve their soundness. A significant improvement of the opposition of numerous lipases might be gotten by their immobilization on substrates and in numerous sorts of bearers (Sharma *et al*., 2001).

## 2.2 SOURCES OF LIPASE

Lipases are created by a few microorganisms principally microbes and organisms just as plants, and creatures (Pahoja and Sethar, 2002). The business essentialness of microbial lipases is fundamentally credited to their huge assortment of synergist exercises, simplicity of hereditary control and high return, combined with remarkable development of the creating microorganisms in economical media and nonattendance of occasional changes (Benjamin and Pandey 1996).

### 2.2.1 Plant Lipase

Lipase movement has been recognized in the different tissues of plants, yet generally, high fixation is found in seeds. Seeds are commonly rich in triacylglycerols, which fill in as a conservative wellspring of vitality for the recently rising plant (Brockerhoff and Jensen, 1974). During germination of the seed, the saved triacylglycerols are vanished since the unsaturated fats cannot be oxidized to give vitality until they are discharged from the triacylglycerol. Lipases are presumably rated controlling during germination and the action of the lipase is high during

germination (Brockerhoff and Jensen, 1974). The most generally considered oilseed grains as for lipase extraction and portrayal is shown in Table 2.1.

**Table 2.1: Examples of plant lipases**

| Common name | Scientific Name | References |
|---|---|---|
| **Bean Lipase** | *Pentaclethra macrophylla* | Enujiugha *et al*., 2004 |
| **3Sunflower Seed Lipase** | *Heliantus annuus* | Sadeghipour and Bhatla, 2003 |
| **Canola Lipase** | *Brassica napus* | Sana *et al*., 2004 |
| **Barbados Nut Lipase** | *Jatropha curcas* | Abigor *et al*., 2002 |
| **Lupin Lipase** | *Lupinus luteus* | Borek *et al*., 2006 |
| **Linseed Lipase** | *Linum usitatissimum* | Sammour, 2005 |
| **Coconut Lipase** | *Cocos nucifera* | Ejedegba *et al*., 2007 |
| **French Peanut Lipase** | *Panchira aquatica Bombacaceae* | Polizelli *et al*., 2008 |
| **Almond Lipase** | *Amygdalus communis* | Yesiloglu and Baskurt, 2008 |
| **Laurel Lipase** | *Laurus nobilis* | Isbilir *et al*., 2008 |
| **Black-Cumin Lipase** | *Nigella sativa* | Dandik and Aksoy, 1996 |
| **Rice Lipase** | *Oryza sativa* | Borgston and Borckman, 1984 |
| **Wheat Lipase** | *Triticum aestivum* | Korneeva *et al*., 2008 |

| | | |
|---|---|---|
| **Corn Lipase** | *Zea mays* | Huang *et al*., 1988 |
| **Oat Lipase** | *Avena fatua* | Mohamed *et al*., 2000 |
| **Barley Lipase** | *Hordeum vulgare* | Kubicka *et al*., 2000 |
| **Sesame Lipase** | *Sesamum indicum* | Wanasundara *et al*., 2001 |
| **Sorghum Lipase** | *Sorghum bicolor* | Uvere and Orji, 2002 |

### 2.2.2 Animal Lipase

Pancreatic lipase can fill in as a model and model for their stomach-related lipases, the cleaning of lipase has ordinarily been done from the dried-out and defatted $CH_3$) 2CO powder of pig pancreas (Pahoja and Sethar, 2002). Dietary fats influence wellbeing and ailment, the osmosis of dietary fats into the body necessitates that they can be processed by lipases (Pahoja and Sethar, 2002). Lipase (pancreatic triglyceride lipase) is basic for the productive processing of dietary fats, the solid enzymatic movement was confined on the outside of mucous cells of the gastric mucosa in creatures at postpartal day 1 after ingestion of milk, The compound found in the gastric juice of the creature is competent to hydrolyze the essential ester obligations of triglycerides with ideal pH 7.0 (Pahoja and Sethar, 2002).

**Table 2.2: Example of mammalian lipases**

| Source | Type |
|---|---|
| **Human being** | Human pancreatic lipase |
| **Horse** | Horse pancreatic lipase |
| **Pig** | Pig pancreatic lipase |
| **Guinea pig** | Guinea pig pancreatic lipase |

### 2.2.3 Microbial Lipase

Lipase creating microorganisms has a place with microscopic organisms, growths, yeasts, and actinomycetes (Sharma *et al*., 2001). The lipases found among these microbial sources are exceptionally different and routinely change from one another in physical, substance, and characteristic properties (Sharma *et al*., 2001). Various lipolytic impetuses are sensible for business utilize such factors as pH broaden, the obstruction of emulsification and surfactants, temperature flexibility, accumulating capacity is huge idea in the decision and improvement of a monetarily supportive thing (Sharma *et al*., 2001).

#### 2.2.3.1 Lipase Producing Bacteria and Actinomycetes

Microscopic organisms' lipases were first seen in the year 1901 in the strains *Serretia marescens* and *Pseudomonas aeruginosa* (Hasan *et al*., 2006). Bacterial strains are in effect continually screened and improved for lipase creation. Different archives on the creation of bacterial lipase especially from *Pseudomonas* and *Bacillus* sp., *P*. *aeruginosa* (Madan *et al*., 2010), *P. fluorescens* (Yang *et al*., 2009), *B. pumilis* (Sangeetha *et al*., 2010a), *B. thermocatenulatus*

(Quyen *et al*., 2003), *B. subtilis* (Ahmed *et al*., 2010), *B. licheniformis* (Sangeetha *et al*., 2010b,), *B. cereus* (Dutta *et al*., 2009) and *B. halodurans* (Ramchuran *et al*., 2006). The extracellular bacterial lipases are of extensive business significance as their mass creation is a lot simpler. A few items dependent on bacterial lipase have been propelled effectively in the market in the previous barely any years.

**Table 2.3: Examples of bacterial lipases (Sharma *et al*., 2001)**

| **Bacterial Group** | **Genus** | **Species** | **Reference** |
|---|---|---|---|
| Gram-positive | *Bacillus* | *B.megaterium, B.cereus, B. stearothermophilus B.subtilis, B. brevis, B. thermocatenulatus,* B. *alcalophilus,* B. *atrophaeus* | Ghanem *et al*., 2000, Godtfredsen, 1990, Kim *et al*., 2002 |
| | *Staphylococcus* | *S. canosus, S. aureus, S. hyicus, S. epidermidis, S. warneri* | Tahoun *et al*., 1985 |
| | *Lactobacillus* | *L. delbruckii* | El-Sawah *et al*., 1995 |
| | *Streptococcus* | *S. lactis* | Sztajer *et al*., 1988 |
| | *Micrococcus* | *M. freudenreichii, M. luteus* | Hou, 2004 |
| | *Propionibacterium* | *P. acne,* Pr. Granulosum | Sztajer *et al*., 1988 |
| | *Burkholderia* | *Burkholderia sp., B. glumae* | Jaeger *et al*.,1998 |
| Gram-negative | *Pseudomonas* | *P.aeruginosa, P.fragi, P. mendocina, P. putida 3SK, P. glumae* | Koritala *et al*., 2007 |

| | | | |
|---|---|---|---|
| | | *P. fluorescens, P. aureofaciens* | |
| | *Chromobacterium* | *C. Viscosum* | Diogo *et al*., 1999 |
| | *Acinetobacter* | *pseudoalcaligenes, A. radioresistens* | Chen *et al*., 1999 |
| | *Aeromonas* | *A. Hydrophila,* | Lotrakul and Dharmsthiti, 1997 |
| Actinomycetes | *Streptomyces* | *S. coelicolor, S. cinnamomeu, S. fradiae* | Sztajer *et al*., 1988 |

### 2.2.3.2 Lipase Producing Fungi and Yeast

Among the various microorganisms recognized as the wellspring of lipases, filamentous organisms are accepted as the fantastic wellspring of extracellular lipase for large-scale manufacturing at the mechanical level (Maia *et al*., 2001). The significant expense of lipase creation is a principle issue in its application in mechanical procedures. Hence, an assortment of endeavours has been made to diminish its creation cost (Maia *et al*., 2001). Growths are for the most part picked as lipase makers since they produce extracellular catalysts that can be essentially isolated from the aging media (Maia *et al*., 2001)

**Table 2.4: (Sharma *et al*., 2001).** Examples of fungi lipases

| Group | Genus | Species | Reference |
|---|---|---|---|
| Fungi | *Rhizopus* | *R. Delemar, R. Oryzae, R. Arrhizus R. Nigricans, R. Nodosus* | Klein *et al*., 1997; |
| | *Aspergillus* | *A. flavus, A. niger, A. japonicus* | Long *et al*., 1998 |

| | | | |
|---|---|---|---|
| | | *A. oryzae, A. fumigatus, A. repens* | |
| | *Penicillium* | *P. Cyclopium, P. Citrinum* | Chahinian *et al*., 2000 |
| | | *P. Roqueforti, P. Fumiculosum.* | |
| | *Mucor* | *M. miehei, M. Javanicus, M. Circinelloides, M. Hiemalis, M. racemosus* | Rantakyla *et al*., 1996; |
| | *Ashbya* | *A. gossypii* | Stahmann *et al*., 1997 |
| | *Geotrichum* | *G. candidum* | Sugihara *et al*., 1994; |
| | *Beauveria* | *B. bassiana* | Hegedus and Khachatourians, 1988 |
| | *Humicola* | *H. lanuginose* | Ghosh *et al*., 1996; |
| | *Rhizomucor* | *R. miehei* | Weber *et al*., 1999; |
| | *Fusarium* | *F. oxysporum, F. heterosporum* | Rapp, 1995 |
| | *Acremonium* | *A. Strictum* | Okeke and Okolo, 1990 |
| | *Alternaria* | *A. brassicicola* | Berto *et al*., 1997 |
| | *Eurotrium* | *E. herbanorium* | Kaminishi *et al*., 1999 |
| | *Ophiostoma* | *O. piliferum* | Brush *et al*., 1999 |
| Yeasts | *Candida* | *C. rugosa, C. tropicalis, C. Antarctica, C. cylindracea, C. parapsilosis* | Wang *et al*., 1995. |

## 2.3 TYPES OF MICROBIAL LIPASES

The lipases delivered by creatures can be generally utilized for various exercises in various structures, for example, extracellular, intracellular, immobilized, and regiospecific. Extracellular lipase insinuates the use of the impetus that has been as of late isolated from the conveying animal and cleaned using different systems, Intracellular lipase suggests the usage of the compound while it is up 'til now contained in the making living things (Robles-Medina *et al*., 2009). Both extracellular and intracellular lipase could be immobilized using a solid assistance (Jegannathan *et al*., 2008).

### 2.3.1 Extracellular Lipase

Microbial lipases are for the most part extracellular which can be created by lowered aging or strong state aging. The maturation procedure is typically trailed by the sanitization process to build the level of virtue in this manner to improve the biocatalyst movement of the compound (Balaji *et al*., 2008). The huge sterilization venture for conveying extracellular lipase is an incredible technique and it depends upon the root and structure of the lipase (Saxena *et al*., 2003). The enormous degree making of extracellular lipases should be moderate, brisk, basic, and viable. Most immobilized lipases, which are financially available, are very extracellular (Robles-Medina *et al*., 2009).

### 2.3.2 Intracellular Lipase

It is the utilization of conservative cells for what it's worth for the intracellular creation of lipases or parasitic cells immobilized inside permeable biomass uphold particles all in all biocatalyst speaks to an appealing cycle for mass creation of biodiesel and polyesters (Iftikhar *et al*., 2008). The usage of lipase found in the cells is alluded to as intracellular lipase (Robles-Medina *et al*.,

2009). A few microorganisms utilized as a wellspring of lipase can unexpectedly immobilize on specific backings.

Numerous microorganisms and higher eukaryotes deliver lipase. Most financially helpful lipases are microbial starting point. Lipase-creating microorganisms have been found in different territories, for example, mechanical squanders, vegetable oil, preparing manufacturing plants, journals, soil polluted with oil, oilseeds, and rotting food (Sztajer *et al*., 2008) just as from manure loads, coal tips, and underground aquifers (Wang *et al*., 1995).

## 2.4 ENZYME SELECTIVITY

### 2.4.1 Substrate Selectivity

A significant trait of compounds is selectivity, which is their capacity to segregate between two unique substrates (Hedfors *et al*., 2010). When the substrate has more than one site that the chemical can follow up on, the protein may have various specificities towards the various destination's dependent on their structure geometry and their reactivity (Svedendahl *et al*., 2009). The selectivity can be evaluated as the proportion between the specificities towards the various destinations.

There are various sorts of substrate selectivity, for example,

i. Chemo selectivity: Catalysts can separate between substrates having diverse concoction gatherings
ii. Regioselectivity: Catalysts can be specific for one of two comparable gatherings on a similar substrate particle.
iii. Stereoselectivity: Catalysts show selectivity between stereoisomers of a chiral substrate atom. This implies one of the isomers responds quicker than the others.

### 2.4.2 Enantioselectivity

Unadulterated enantiomers of chiral mixes have equivalent physical properties yet can cause various reactions in organic frameworks (Kobayashi and Makino, 2009). For instance, one enantiomer of ethambutol is utilized to treat tuberculosis while different causes visual deficiency. The partition procedure of the two enantiomers is called goals. The explanation behind the enantioselectivity of catalysts is that the enzyme- transition state restricting contrasts between the two enantiomers relying upon the complementarity between the dynamic site and the enantiomers (Lutz and Bornscheuer, 2009). The premise of motor goals is the distinction in synergist proficiency that the protein has towards the two enantiomers. This makes chemicals alluring impetuses in the pharmaceutical business for the goals of different chiral mixes. To increment or alter the enantioselectivity, a few compounds have been exposed to building work, both by objective plan and by arbitrary mutagenesis (Hedfors *et al*., 2010).

## 2.5 LIPASE PRODUCTION

The creation of microbial lipases is grown predominantly by lowered maturation (Singh and Mukhopadhyay, 2012), utilizing seat scale or mechanical bioreactors (Asih *et al*., 2004). For the most part, lowered aging could be led by cluster, however, efficiency is expanded by the fed group or constant procedures (Singh and Mukhopadhyay, 2012).

### i. Production of Lipase in Solid-State Fermentation

Solid substrate fermentation is a process in which microorganisms develop on strong substrates with low water substance to create modernly significant catalysts like; lipase (Mahadik *et al*., 2002) just as other a few compounds protease, cellulase, amylase, xylanase (Strong squanders from the creation business of vegetable oils and other agro-mechanical deposits, for example, Niger seed oilcake wheat grain (Mahadik *et al*., 2002), rice wheat (Rao *et al*., 1993) and jatropha

seed cake (Mahanta *et al*., 2008) have been generally utilized for the creation of mechanical proteins, anti-toxin, bio-pesticides, nutrients and other biochemicals (Gutarra *et al*., 2005). These substrates are magnificent help for microbial development just as intriguing wellspring of supplements and requiring low or no supplementation of straightforward carbon in the aging cycle (Mahanta *et al*., 2008) acquired lipase and protease from *Pseudomonas aeruginosa*, from strong state aging by utilizing *Jatropha curcas* seed cake as a substrate (Santis-Navarro *et al*., 2011) revealed the lipase from microbial consortia, from strong state aging by utilizing vegetable oil-refining waste created an extracellular lipase by *Bacillus coagulans* from strong substrate maturation by utilizing melon squander. The mechanical interest of lipase has been expanding a result of its tremendous application in the dairy enterprises, oil preparing ventures, treatment of sleek wastewater and biodiesel creation (Gutarra *et al*., 2005). During the creation of huge measure of compound in enterprises, pre-treatment system of substrate are not possible at bigger scope due to cost of the synthetics, longer pre-treatment methods like establishing, sieving the substrate and explicit hardware with high energy necessities which expands by and large expense of the chemical creation by 30-45 %. In this aging cycle a zero-esteem substrate like agro-modern waste (oil cake) are utilized in non-sterile climate which diminished the information energy, rejection of hardware for sanitization and decrease in the work cost and time for ease catalyst creation. Cellulase, xylanase and L-lactic corrosive were created in open aging (nonsterile condition) to diminish the expense of creation (Kumar *et al*., 2011; Roussos and Raimbault, 1993).

### ii. Production of Lipase in Submerged Fermentation

Submerged fermentation (SmF) method of assembling biomolecules in which catalysts and other responsive mixes are lowered in a fluid, for example, liquor, oil, or a supplement stock (Azeredo

*et al*., 2007). Lowered Aging (SmF) uses free-streaming fluid substrates, for example, molasses and stocks. The cycle is utilized for an assortment of purposes, generally in modern assembling. The substrates are used quickly; henceforth should be continually supplanted/enhanced with supplements. This maturation procedure is most appropriate for microorganisms, for example, microbes that require high dampness content. An extra bit of leeway of this method is that sanitization of items is simpler (Pinheiro *et al*., 2008). SmF is principally utilized in the extraction of optional metabolites that should be utilized in fluid structure. The cycle includes taking a particular microorganism, for example, parasites and setting it in a little shut cup containing the rich supplement stock. A high volume of oxygen is likewise needed for the cycle. The creation of compounds at that point happens when the microorganisms cooperate with the supplements on the stock bringing about them being separated. The bioactive mixes are discharged into the aging stock (Teng Y *et al*., 2008).

### 2.5.1 Factors Affecting Microbial Lipase Production

Microbial lipases are as often as possible extracellular, and their assembling is generously roused through medium organization notwithstanding physicochemical factors, for example, temperature, pH, and broke up oxygen (Gupta *et al*., 2004) .These compounds are ordinarily created inside the presence of lipid substrates which remember oils or any inducers for the state of triacylglycerols, unsaturated fats, hydrolysable esters, Tweens, bile salts, and glycerol (Gupta *et al*., 2004; Sharma *et al*., 2001).

#### 2.5.1.1 Effect of Nutritional Factors

**i. Carbon Sources**

Carbon resources work fundamental substrates for strength fabricating in microorganisms. Lipidic carbon sources work inducers and are by and large fundamental for getting an

unnecessary lipase yield (Benjamin and Pandey 2016) affirmed that *C. rugosa* lipase fabricating transformed into relatively extended with the development in attention to olive oil and greatest creation become completed at 10 % (v/v) olive oil focus. The creation of a thermostable lipase from thermophilic *Bacillus sp*. Strain Wai 28A 45, inside the presence of tripalmitin at 70 °C, gets characterized by methods for (Janssen *et al*. 1994). Media with tripalmitin, tristearin, and trimyristin carbon assets have been tried, and tripalmitin gets situated to be the magnificent inducer of lipase hobby. Catalyst interest becomes not distinguished when glucose turned into the solitary carbon source, affirming that the presence of an inducer is fundamental for *Penicillium aurantiogriseum* to give lipases (Lima *et al*., 2003). In any case, the necessity for sugar as a carbon source like lipids shifts with the microorganism. In any case, regularly, media enhanced with glucose alongside fatty oils animate the lipase creation in *Rhizopus nigricans* as articulated with the guide of (Ghosh *et al*. 1996). Both olive oil and Tween-80 propelled the assembling of extracellular lipase in *Penicillium citrinum* at 0.1 and 0.7 % (v/v), respectively (Maliszewska and Mastalerz, 1992).

**ii. Nitrogen Sources**

Both natural and inorganic nitrogen sources have been generally utilized for lipase creation. In *Aspergillus wentii, Mucor racemosus* and *R. nigricans* lipase yield changed into more grounded by methods for adding peptone in the creation medium on the centralization of 20 g/L (Ghosh *et al*., 1996). Be that as it may, for lipase creation through *Rhodotorula glutinis*, inorganic nitrogen assets, for example, ammonium phosphate appear to support lipase producing (Papaparaskevas *et al*., 1992). Nitrogen assets which incorporate corn steep alcohol and soybean feast animated *P. Citrinum* lipase creation to a lesser amount than peptone; while urea and ammonium sulfate repressed the lipase synthesis (Sztajer and Maliszewska, 1989).

### iii. Inorganic Minerals

Various microorganisms require diverse inorganic minerals for their development and lipase creation. Inorganic salts in type of $MgSO_4$, $(NH_4)2SO_4$, NaCl, $K_2HPO_4$, $BaCl_2$ are needed for greatest lipase creation by *Hendersonula toruloidea* (Odibo *et al*., 1995). If there should arise an occurrence of *Candida sp*. 99-125, culture medium containing (w/v) soybean oil, 4.187%, soybean powder 5.840%, $K_2HPO_4$ 0.284%, $KH_2PO_4$ 0.1%, $(NH_4)_2SO_4$ 0.1%, $MgSO_4$ 0.05% and Range 60 0.1% was discovered to be the ideal for lipase creation and nonappearance of any of these parts influence the life form's development and lipase action (He and Tan, 2006). By and large, Magnesium salt is needed by most microorganisms because of its capacity to play some administrative capacities related with expanded adenosine triphosphate digestion and nucleic acid synthesis (Bankar *et al*., 2009).

#### 2.5.1.2 Effects of Physical Factors

The physical parameters which incorporate pH, temperature, unsettling, air circulation, and inoculum have excellent impact at lipase fabricating. A portion of these boundaries are better overseen in bioreactors, that is mechanical vessels in which microorganisms are developed under oversaw conditions.

### i. Temperature

Most lipase producing microrganisms are mesophilic in nature (creating in moderate temperature by and large somewhere in the range of 25 and 40 °C). Be that as it may, some psychrophilic and thermophilic life forms had been expressed in the writing. Lipase creation via a wild-kind Brazilian pressing factor of *P. Simplicissimum* demonstrated a movement of 90 U/g

after 72 h hatching within the sight of an extensive buildup of the babassu oil industry. The compound got situated to have high exercises at 35 to 60°C (Gutarra *et al*., 2009).

**ii. pH**

pH is a level of sharpness or basicity of a medium, plays a basic capacity in deciding the sort of life forms that can colonize a substrate. 59 lipase-delivering parasitic strains were disengaged from Brazilian savanna soil by utilizing improvement culture methodologies. The greatest productive pressing factor analyzed as *Colletotrichum gloesporioides*, demonstrated lipase exercises somewhere in the range of 27.7 and 27.4 U/ml while refined in shaken fluid medium underneath antacid conditions at pH assortment of 7.4 to 8.4 (Colen *et al*., 2006). Most lipases that can be vigorous at exceptionally acidic pH of between 1.5 to 2.0 are explicitly from mammalian sources, for example, Gastric lipase. Nonetheless, *Aspergillus niger* NCIM 1207 demonstrates a serious level of extracellular lipase while refined at pH 2.5 (Mhetras *et al*., 2009). Likewise, high lipase producing microorganisms such as *Rhizopus* sp., *Aspergillus* sp., *Penicillium* sp., *Geotrichum* sp., *Mucor* sp., and *Rhizomucor* sp. (Treichel *et al*., 2010), *Bacillus* sp., *Pseudomonas* sp., *Burkholderia* sp. (Gupta *et al*., 2004), *C. Cylindracea* and *Yarrowia lipolytica* (Vakhlu and Kour, 2006) grow and bring lipases at pH ranges of 6 to 8.

**iii. Aeration and Agitation**

Aeration and agitation are a couple of the actual components that are significant in upgrading and streamlining the lipase creation. Consequently, an expansion of microorganisms and protein fabricating is experiencing unsettling and air circulation dependent on oxygen conveyance during the lipase creation measure principally in bioreactors (Fickers *et al*., 2006). The impacts of both media and system boundaries (air circulation and fomentation) on lipase producing

utilizing *Rhodotorula mucilaginosa* MTCC 8737 in 1.5-L stirred tank reactor with molasses as sole production medium became studied via (Potumarthi *et al*.2008).

**iv. Inoculum Concentration**

The measure of inoculum present during the maturation way can influence the lipase creation in numerous microbial lines. Accordingly, exorbitant inoculum sizes may not continually achieve better lipase yield, every now and again bring about oxygen, and supplement exhaustion inside the way of life media and along these lines influencing the overall efficiency (Rahman *et al*., 2005).

## 2.6 APPLICATION OF LIPASE

Lipases are broadly utilized inside the preparing of fat and oils, cleansers and degreasing definitions, food handling, the amalgamation of best compound substances and drugs, paper assembling, and creation of beautifying agents, and recommended drugs (Rubin and Denis, 1997a). Lipase might be utilized to help up the corruption of greasy waste (Masse *et al*., 2001) and polyurethane (Takamoto *et al*., 2001).

### 2.6.1 Lipase in The Detergent Industry

In view of their capability to hydrolyse fat, lipases find the main use as added substances in business clothing and family cleansers. Cleanser lipases are exceptionally chosen to satisfy:

low substrate explicitness for example (a capacity to hydrolyse fats of different pieces); capacity to look up to enormously brutal washing conditions (pH 10-11, 3–6 0C); capacity to oppose ominous surfactants and chemicals (straight alkyl and proteases) which can be vital elements of numerous cleanser details. Lipases with the favored homes are acquired through a mix of

nonstop screening (Yeoh *et al*., 1986; Wang *et al*., 1995) and protein engineering (Kazlauskas and Bornscheuer, 1998).

### 2.6.2 Lipase in Food Industry

Fats and oils are significant components of meals. The dietary and tangible cost and the actual homes of fatty oils are significantly impacted by utilizing components, for example, the area of the unsaturated fat inside the glycerol spine, the chain length of the unsaturated fat, and its level of unsaturation. Lipases grant us to adjust the properties of lipids by changing the area of unsaturated fat chains inside the glyceride and supplanting one or extra of the unsaturated fats with new ones (Pabai *et al*., 1995). Cocoa margarine, unreasonable value fat, comprises of palmitic and stearic acids and has a dissolving purpose of around 37 $^{0}$C. Softening of cocoa margarine inside the mouth creates a fit cooling sensation in items including chocolate. Lipase-based age with respect to blended hydrolysis and amalgamation responses is utilized economically to update a portion of the considerably less pertinent fat to cocoa spread substitutes (Undurraga *et al*., 2001). Lipases were utilized for the improvement of flavors in cheddar aging, pastry kitchen product, and refreshments (Kazlauskas and Bornscheuer, 1998). Additionally, lipases are utilized to helpful asset expulsion of fats from meat and fish items (Kazlauskas and Bornscheuer, 1998).

### 2.6.3 Lipase in Pulp and Paper Industry

Pitch or the hydrophobic segments of wood (explicitly fatty oils and waxes), causes exorbitant issues in mash and paper make (Jaeger and Reetz, 1998). Lipases are utilized to get rid of the pitch from the mash delivered for paper making. Nippon Paper Ventures, Japan, has built up a

pitch-control strategy that utilizes the *Candida rugosa* parasitic lipase to hydrolyze as much as 90 % of the lumber fatty oils.

### 2.6.4 Lipase in Organic Synthesis

The utilization of lipases in characteristic synthetic union is transforming into an expanding number of fundamental. Lipases are utilized to catalyze a wide type of chemo-, regio-, and stereoselective varieties (Rubin *et al*., 1997b ; Berglund and Hutt, 2000). Greater part of lipases utilized as impetuses in natural science are of microbial starting point. These compounds compositions at the hydrophilic-lipophilic interface and endure normal solvents inside the reaction mixes.

### 2.6.5 Lipase in Bioconversion in Aqueous Media

Hydrolysis of esters is for the most part finished utilizing lipase in - stage watery media (Vaysse *et al*., 1997; Chatterjee *et al*., 2001). The hydrolysis of p-nitrophenyl palmitate (pNPP) in n-heptane by a lipase training of *P. Cepacia*. Lipase entangled in a hydrophobic sol-gel framework for a choice of adjustments (Jaeger and Reetz, 1998). The lipase-acyl transferase from *C. parapsilosis* has been demonstrated to catalyze greasy hydroxamic corrosive biosynthesis in a biphasic fluid/watery medium. The substrates of the reaction were acyl benefactors (unsaturated fat or unsaturated fat methyl ester) and hydroxylamine. The switch of acyl establishment from a giver ester to hydroxylamine (aminolysis) was catalyzed specially contrasted with the response of loosened unsaturated fats. This element made the *C. Parapsilosis* chemical the impetus of inclination for the immediate bioconversion of oils in a fluid medium (Vaysse *et al*., 1997). (Vaysse *et al*., 1997).

### 2.6.6 Lipase in Bioconversions in Organic Media

Enzymes in common media without a free watery fragment are perceived to show valuable extraordinary properties, and this has immovably introduced nonaqueous protein structures for amalgamation and biotransformation (Klibanov, 1997). Lipases had been broadly researched for various nonaqueous biotransformation (Therisod *et al*., 1987; Klibanov, 1990).

### 2.6.7 Lipase in Resolution of Racemic Acids and Alcohols

Stereoselectivity of lipases has been utilized to clear up different racemic natural corrosive blends in immiscible biphasic frameworks (Klibanov, 1990). Racemic alcohols likewise can be settled into enantiomerically normal deskwork through lipase-catalyzed transesterification. Esterification reaction in non-aqueous media the utilization of lipase-B from *C. Antarctica* gets stereoselective closer to the R-isomer of ketoprofen in an achiral dissolvable along with isobutyl methyl ketone and (S+)- carvone. A refined lipase training from *C. Rugosa* got when contrasted with its rough partner in anhydrous and scarcely hydrated hydrophobic characteristic solvents. The sanitized lipase schooling changed into substantially less dynamic than the rough protein in dry n-heptane, while the presence of little consideration of water drastically initiated the purged chemical anyway not, at this point the unrefined compound inside the esterification of racemic 2-(four-chlorophenoxy) propanoic corrosive with n-butanol (Tsai and Dordick, 1996).

### 2.6.8 Lipase in Regioselective Acylation

Lipases acylate positive steroids, sugars, and sugar subsidiaries with unreasonable regioselectivity. Monoacylated sugars have been delivered in anhydrous pyridine from triethyl carboxylates and assorted monosaccharides (Therisod *et al*., 1987).

### 2.6.9 Lipase in Ester Synthesis

Lipases had been adequately utilized as an impetus for the combination of esters. The esters comprised of short-chain unsaturated fats have applications as seasoning specialists in the food business (Vulfson, 1994). Methyl and ethyl esters of long-chain acids had been utilized to supplement diesel energizes (Vulfson, 1994). Esterification of five positional isomers of acetylenic unsaturated fats (distinctive chain lengths) with n-butanol changed into examined (Falsehood *et al*., 1998), the utilization of eight extraordinary lipases. A most attractive pre equilibrium water revenue cost got important for acquiring a high pace of esterification of (R, S) - ibuprofen. (Arroyo *et al*., 1999).

### 2.6.10 Lipase in Oleochemical Industry

The utilization of lipases in oleochemical handling saves power and limits warm corruption all through alcoholysis, acidolysis, hydrolysis, and glycerolysis (Vulfson, 1994). Despite the fact that lipases are planned via nature for the hydrolytic cleavage of the ester obligations of triacylglycerol, lipases can catalyze the contrary reaction (ester union) in a low water climate. The hydrolysis and esterification can happen simultaneously in a way called interesterification. Depending at the substrates, lipases can catalyze acidolysis, alcoholysis, and transesterification (Balcao *et al*., 1996).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Materials and Equipment

The following materials were used for this experiment: Petri Dishes, Distilled water, test-tubes, pipette, spreader, petri dishes, Erlenmeyer flask, inoculating loop, olive oil, nutrient Agar (axiom medical), tween 80 agar (SIGMA), phenol red agar, tributyrin agar, sterile, carbon source (glucose), nitrogen sources (yeast extract, peptone),. The following equipment was used for the experiment: Incubator (Torrey pines scientific), autoclave (surgifriend medical England), weighing balance (mettler toledo), pH meter.

## 3.2 Samples Collection

Soil samples contaminated with vegetable oil from restaurant and diesel located in Ogun State were collected with the guide of sterile spatula from the profundity of 0.5 to 1.0 cm inside a sterile 100 mL glass bottles and immediately transferred to the laboratory for examination and further analysis.

## 3.3 Isolation and Screening of Lipase Producing Bacteria

Soil samples were diluted serially from $10^{-1}$ up to $10^{-6}$ in sterile distilled water, each dilution was cultured on nutrient agar plates by Spread plate method to obtain isolated colonies after 24 hours of incubation (Patel and Desai 2018).

The Pure bacterial isolates were screened for lipase production by streaking the Bacterial colonies on Tributyrin agar Plate ((0.5% peptone, 0.3% yeast extract, 1% tributyrin and 2% agar, pH 7.0) and incubated at 37˚C for 48 hours. The isolates were observed for zone of clearance and measured accordingly (Patel and Desai 2018).

### 3.4 Identification of the Selected isolate

The selected isolate was identified using morphological and biochemical characteristics.

#### 3.4.1 Morphological Characterization

Morphological characterization of a pure culture of the best isolate on tributyrin agar was done using colonial, cellular and pigment appearances on culture plates (Olutiola *et al*., 2000).

#### 3.4.2 Biochemical Characterization

The biochemical test such as: sugar utilization test, indole production test, methyl red test, Voges-Proskauer test, citrate utilization test, starch hydrolysis test, catalase test, sporulation test, oxidase test, were studied and characterization (Olutiola *et al*., 2000).

**i. Gram Staining Technique**

A smear of the chosen strain was prepared on a clean glass slide and therefore the smear was allowed to air-dry then heat fixed, the warmth fixed smear was flooded with crystal violet and washed under water after 1 minute, it had been flooded with mordant gram's iodine, The smear was decolorized with 95 % ethyl alcohol and washed with water then counter stained with safranin for 45 seconds so the smear was allowed to dry and examined under oil immersion (100x) (Patel *et al*., 2016).

**ii. Sugar Utilization Test**

A loopful culture of chosen potential isolates was innoculated to the sugar stock (10 % watery test sugar arrangement (glucose) in 10 mL basal medium (supplement stock or 1 % peptone water), 1 mL (1 % Andrade's marker and pH 7.5) and was hatched at $37^0$C for overnight. Corrosive creation change the shade of medium to pink and gas creation was closed from a little air pocket in modified Durham's cylinder kept in the test tube (Patel *et al*., 2016).

### iii. Indole Production Test

A loopful culture of chosen selected isolates was innoculated into tryptone broth (1% tryptone water, 0.5 gm NaCl, 100 mL water and pH 7.4) and hatched at $37^0$ C for overnight. 3-4 drops of xylene were included medium after brooding and shaken overwhelmingly. The two layers could separate and 1 mL of Kovac's reagent was added gradually. The development of pink shading ring showed positive test (Patel *et al*., 2016).

### iv. Methyl Red Test

A loopful culture of chosen potential isolates was innoculated into glucose phosphate stock (5 gm glucose, 5 gm $K_2HPO_4$, 5 gm peptone, 1000 mL water and pH 7) and hatched at $37^0$ C for 48-72 hours, 5 drops of methyl red pointer were added inside the medium after brooding. The occasion of red shading showed positive test (Patel *et al*., 2016).

### v. Voges-Proskauer Test

A loopful culture of chosen potential disconnects were immunized into glucose phosphate stock (5gm glucose, 5 gm peptone, 1000 mL water and pH 7) and hatched at $37^0$ C for 48-72 hours, 0.6 mL of a-naphthol and 0.2 mL KOH arrangement was added after brooding and shaken well. The occurence of red tone shows positive test (Patel *et al*., 2016).

### vi. Citrate Utilization Test

The potential isolates were streaked on the outside of Simon's citrate agar incline (0.2 g sodium citrate, 0.02 g $MgSO_4$, 0.5 gm NaCl, 0.1 gm ammonium dihydrogen orthophosphate, 0.005 g bromothymol blue, 100 mL $H_2O$, 4 g agar, pH 6.9) vigorously and in this manner the inclination was brooded at $37^0$ C for 48-72 hours. The occasion of dark blue tone inside 24-48 hours demonstrated positive outcome (Patel *et al*., 2016).

### vii. Starch Hydrolysis Test

All potential isolates were cultured on the on the starch agar plate (3.0 g meat remove, 10.0 g dissolvable starch, 12.0 g agar and 1000 mL refined water) and was brooded at $37^{o}$ C for 48-72 hours, the plates were overwhelmed with Lugol's iodine (2.5 g iodine, 5 g potassium iodide, and 10 mL refined water) after hatching. The vibes of clear dismal zone around development demonstrated positive outcome (Patel *et al*., 2016).

### viii. Catalase Test

The potential isolates were streaked on the outside of supplement agar incline intensely and brooded at $37^{0}$ C for 24 hours, 1 mL of hydrogen peroxide was added over the development on agar incline after hatching. The fast appearance and support creation of gas bubbles showed positive outcome (Patel *et al*., 2016).

### ix. Oxidase test

All potential isolates were streaked on the outside of supplement agar incline (3 g meat separate, 5 g peptone, 15 g agar, 1000 mL refined water and pH 7) intensely and hatched at $37^{o}$C for 24 hours, after brooding, a settlement was picked and a smear was set up on a channel paper saturated with 1 % tetraethyl-phenylenediamine dihydrochloride arrangements. The arrangement of violet tone with 45-60 seconds showed positive outcome (Patel *et al*., 2016).

## 3.5 Lipase Production

The isolate with most elevated zone of clearance on Tributyrin plate was set up in a 300mL Erlenmeyer carafe 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 follow arrangement containing (mg/L) $FeSO_4 \cdot 7H_2O$ (0.63), $MnSO_4$ (0.01), $ZnSO_4$ (0.62), 20 g/L of yeast remove as nitrogen source and 20 g/L of soybean oil as inducer. The pH was acclimated to 7.0 utilizing HCl 1.5 mol/L or NaOH 1 mol/L. Thereafter, the

medium was autoclaved at 103 kPa for 20 mins. After innoculation, the cultures were hatched for 4 days at 30°C with tumult at 120 rpm (Bertolin *et al*., 2001). The lipase activity in the supernatant was determined by using spectrophotometric method (Saeed *et al*., 2005).

### 3.6 Lipase Assay

Lipase assay was resolved utilizing the colorimetric strategy. This involves two arrangements that were ready for the lipase examination. The principal arrangement utilized containing 90 mg of p-nitrophenyl palmitate that was disintegrated in 30 mL propane-2-ol. The subsequent arrangement utilized contained 2g Triton X-100 and 0.5 g of gum Arabic broke down in 450 mL (Tris-HCl 50 mM) cushion at a pH of 8.0. The measure arrangement was set up by adding 1 ml arrangements meant 1 to 9 ml of aarrangements2 to get an emulsion that stays stable for 2 hours. The examine blend contained 900 µl of the emulsion and 100 µl of the fittingly weakened chemical arrangement. The freed p-nitrophenol was estimated at 410 nm utilizing a spectrophotometer. One unit of the catalyst was characterized as the measure of protein that discharges 1 µmol of p-nitrophenol from the substrate (Saeed *et al*., 2005).

### 3.7 Effect of Carbon Source on Lipase Production

The impact of carbon sources, for example, glucose, galactose, fructose, maltose, lactose, sucrose, mannitol, and starch on lipase creation were researched. The lowered aging cycles was set up in a 300mL Erlenmeyer cup with 100 mL of the medium containing 15 g/L carbon sources, 2 g/L $KH_2PO_4$, 1 g/L $MgSO_4$, and 10 mL/L of follow arrangement containing (mg/L) $FeSO_4 \cdot 7H_2O$ (0.63), $MnSO_4$ (0.01), $ZnSO_4$ (0.62), 20 g/L of yeast remove as nitrogen source and 20 g/L of soybean oil as inducer. The pH was changed in accordance with 7.0 utilizing HCl 1.5 mol/L or NaOH 1 mol/L. A short time later, the medium was autoclaved at 103 kPa for 20

mins. After vaccination, the way of life were hatched for 4 days at 30°C with disturbance of 160 rpm (Bertolin *et al*., 2001).

### 3.8 Effect of Nitrogen Source on Lipase Production

The impact of nitrogen source such peptone, beef extract, ammonium sulfate, sodium nitrate, potassium nitrate and urea on lipase creation under lowered maturation were examined. The creation medium contained 15 g/L glucose, 2 g/L $KH_2PO_4$, 1 g/L $MgSO_4$, and 10 mL/L of follow arrangement 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).

### 3.9 Statistical Analysis

Data obtained were analysed using analysis of variance with SPSS version 15.0. The level of tested significance was at $P \geq 0.05$ (Ire and Ike 2014).

# CHAPTER FOUR

## 4.0 RESULTS

Bacteria isolated from diesel and restaurant wastewater contaminated soil were screened for lipase production. 14 morphologically different bacteria 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, lipase activities ranged from $1.5^{f} – 5.0^{a}$, $2.5^{g} – 6.0^{a}$ and $3.5^{f} – 9.5^{a}$ for 24, 48 and 72hrs respectively. At 24 hrs, isolate PMD1 gave the highest lipase activity while isolate PPS3 and PMD6 gave the least activity. At 48 hrs, isolate PMD1 gave the highest lipase activity while isolate PMD6 gave the least activities. At 72 hrs, isolate PMD1 gave the highest lipase activity while isolate PMD6 gave the least activities. In all cases, five (5) isolates including isolates PPS1, PPS2, PPS4, PMD4 and PMD5 did not produce any noticeable activity throughout the incubation period. Isolate PMD1 exhibited excellent lipase activity and was selected for further study.

Table 4.2 shows the morphological identification of the selected bacterial isolate. The isolate is round in shape, creamy colour, entire, convex elevation, dry consistency and moderate growth size.

Figure 4.1 shows the Gram's reaction of the isolate after staining with Gram staining dye. The isolate appeared as purple rod in colour showing that it is Gram positive bacteria.

Table 4.3 shows the biochemical identification of the selected isolate. The result of the biochemical screening of the isolate showed that the isolate is motile and positive for Oxidase, Vogues Proskauer and Catalase test while negative for Coagulase, MR, Indole, Urease, Citrate,

and Starch hydrolysis. Glucose fermentation carried out showed that the isolate can ferment glucose but lack gas production. Based on the results of morphological and biochemical characteristics of the isolates, the probable identity was concluded to be *Bacillus subtilis*.

The influence of various carbon sources on lipase production by isolate PMD1 was investigated. There was significant difference in lipase production in the presence of different carbon sources including monosaccharide (glucose, and galactose); disaccharide (maltose, lactose, and sucrose); polysaccharide (starch); and alcohol sugar (mannitol). Lipase activity ranged from $31.09^{h}$ - $88.65^{a}$ U/mL as seen in Figure 4.2. Glucose ($88.65^{a}$ U/mL) supported the highest lipase production followed in order by fructose ($82.45^{b}$ U/mL), and sucrose ($82.21^{c}$ U/mL) while the least production was recorded in starch ($31.09^{h}$ U/mL).

Figure 4.3 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 $42.11^{g}$ - $86.35^{a}$ U/mL. The highest lipase activity of $86.35^{a}$ U/mL was recorded in the presence of peptone followed in order by Ammonium sulphate ($81.55^{b}$ U/mL), and yeast extract ($80.46^{c}$ U/mL), while urea provided the least support ($42.11^{g}$ U/mL).

**Table 4.1. Screening of isolates for lipase activities.**

| Isolates | Lipase activity diameter (mm) | | |
|---|---|---|---|
| | Incubation time (hr) | | |
| | **24** | **48** | **72** |
| **PPS1** | 0.0 | 0.0 | 0.0 |
| **PPS2** | 0.0 | 0.0 | 0.0 |
| **PPS3** | $1.5^{f}$ | $2.5^{g}$ | $3.5^{g}$ |
| **PPS4** | 0.0 | 0.0 | 0.0 |
| **PPS5** | $3.5^{c}$ | $5.5^{b}$ | $6.5^{d}$ |
| **PPS6** | $3.0^{d}$ | $5.0^{c}$ | $8.0^{b}$ |
| **PPS7** | $4.5^{b}$ | $4.5^{d}$ | $7.5^{c}$ |
| **PPS8** | $3.0^{d}$ | $4.0^{e}$ | $6.0^{e}$ |
| **PMD1** | $5.0^{a}$ | $6.0^{a}$ | $9.5^{a}$ |
| **PMD2** | $2.5^{e}$ | $3.5^{f}$ | $5.5^{f}$ |
| **PMD3** | $3.5^{c}$ | $4.5^{d}$ | $6.5^{d}$ |
| **PMD4** | 0.0 | 0.0 | 0.0 |
| **PMD5** | 0.0 | 0.0 | 0.0 |
| **PMD6** | $1.5^{f}$ | $2.5^{g}$ | $3.5^{g}$ |

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

**Table 4.2 Morphological Identification of the Selected Isolate**

| Morphological Parameters | Characteristics |
|---|---|
| **Shape** | Round |
| **Colour** | Creamy |
| **Elevation** | Convex |
| **Edge** | Entire |
| **Size** | Moderate |
| **Consistency** | Dry |

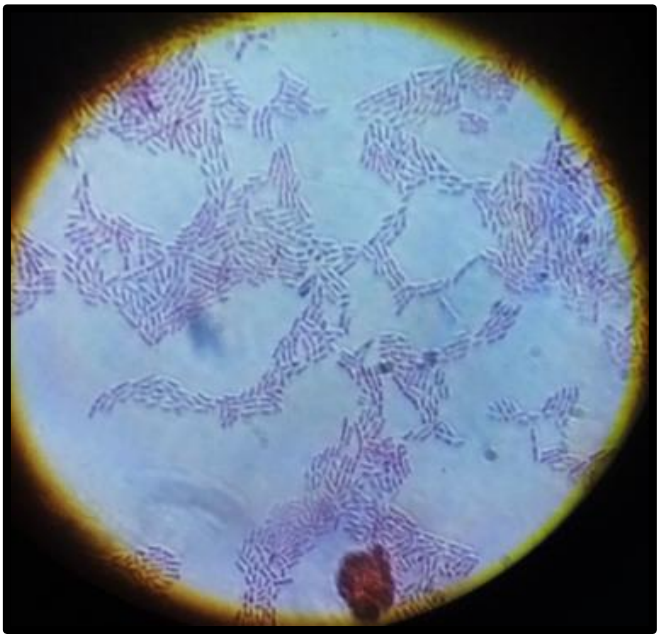

**Fig 4.1 Microscopic characteristics of isolate PMD1**

**Table 4.3 Biochemical Identification of the Selected Isolate**

| Biochemical Parameters | Characteristics |
|---|---|
| **Gram reaction** | Positive with rod shape |
| **Sporulation** | Spore forming |
| **Motility** | + |
| **Catalase** | + |
| **Coagulase** | - |
| **Voges - Proskauer** | + |
| **Methyl Red** | - |
| **Oxidase** | + |
| **Indole** | - |
| **Urease** | - |
| **Citrate** | - |
| **Glucose fermentation** | Acid/no gas |
| **Starch hydrolysis** | - |

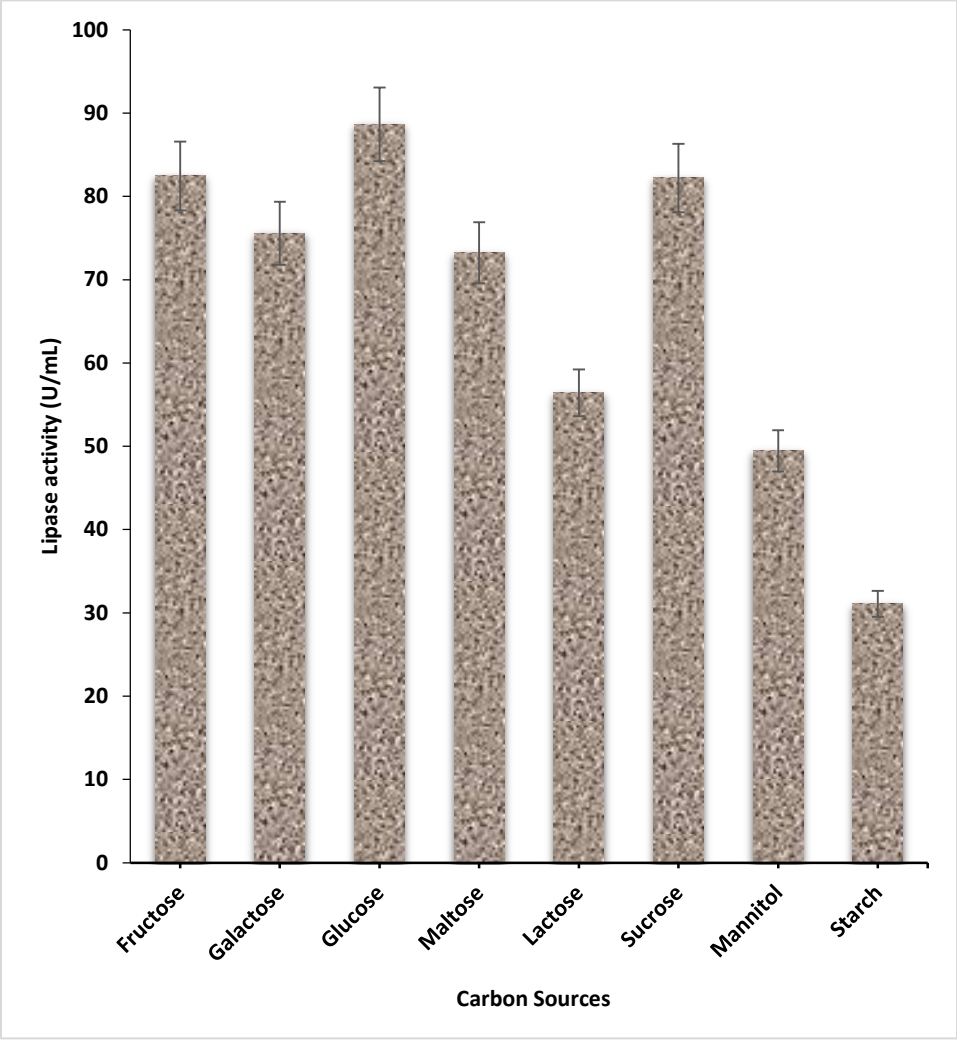


**Figure 4.2: Effect of carbon source on lipase production**

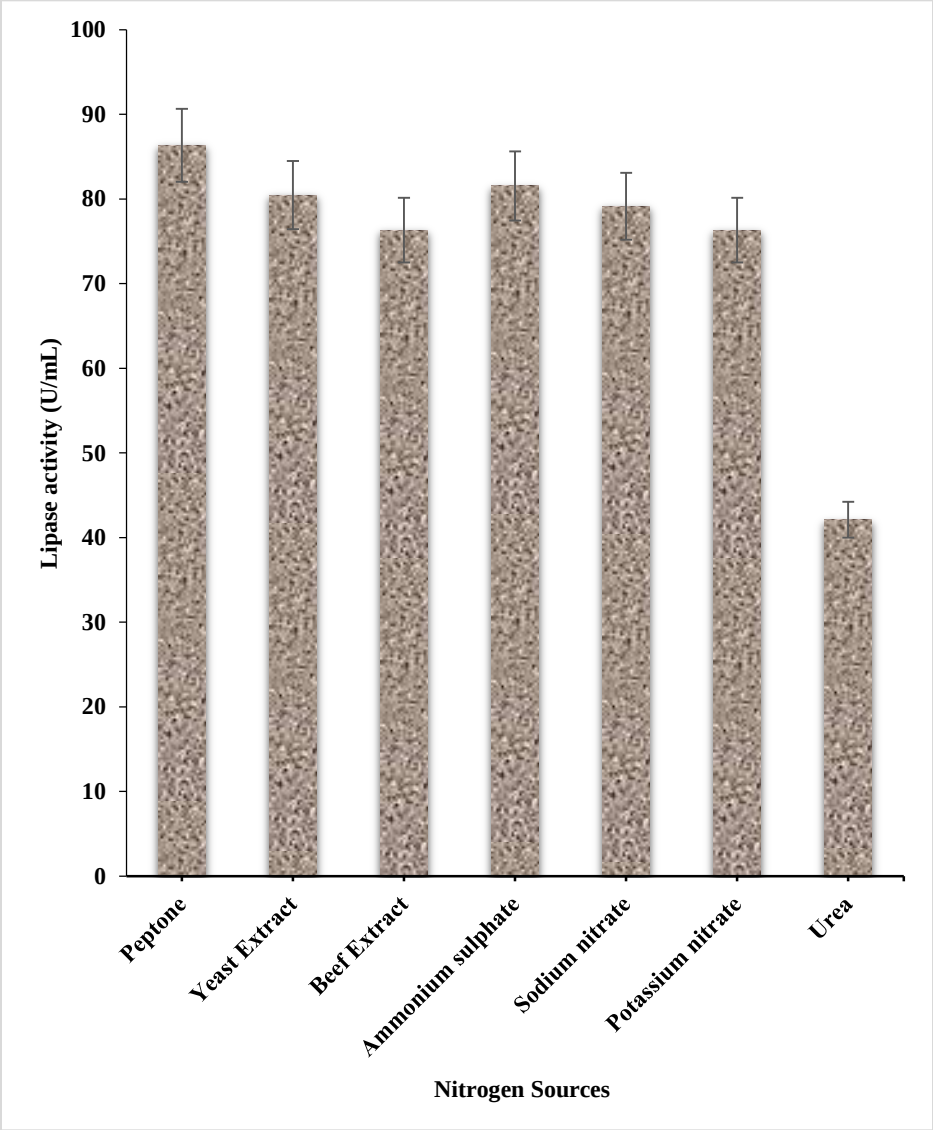


**Figure 4.3: Effect of nitrogen source on lipase production**

# CHAPTER FIVE

## 5.0 DISCUSSION AND CONCLUSION

## 5.1 DISCUSSION

The presence of lipase producing bacteria from environmental samples have been previously reported. Dahiya and Purkayastha (2011) reported the isolation and screening of thirty bacterial strains from oil industry soil samples where the highest lipase activity was 4.1 U/mL. Patel and Desai (2018), in a report, screened 41 bacteria isolates from oil-contaminated soil for lipase activity and it was observed that only 20 showed lipolytic activity.

Bacteria are good sources of extracellular lipase. The morphological and biochemical characterization observed in this study for the selected isolate PMD1 was similar to the report of Kumar *et al* (2011) on *Bacillus* sp. The genus Bacillus has been previously reported to be good producers of lipase. Similar to the study, Patel *et al*. (2016) reported *Bacillus* sp. isolated from waste materials as a good source of lipase for industrial applications. Moreover, Kumar *et al*. (2011) documented the potential of *Bacillus* sp. as a lipase producer. The observed result in this study has been previously reported. Mahza *et al*. (2017) reported that *Bacillus subtilis* showed the highest lipase production from several other bacterial species. Additionally, Suci *et al*. (2017) also reported maximum lipase production from *Bacillus subtilis*.

Nutritional factors have been reported to influence the expression of lipase activity (El-Batal *et al*., 2016) Carbon and nitrogen sources as well as inducers in form of oils, fatty acids and sugar esters are the most contributing factors during lipase production (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 (Asgher *et al*., 2016).

Different carbon sources have different effect on the enzyme production. From this study, highest lipase activity (88.65 U/mL) was observed in the presence of glucose as carbon source. Similar to our study, Nwachukwu *et al*. 2017 reported maximum lipase production by *Serratia sp* using glucose as a carbon source. Contrary to our study, Dahiya and Purkayastha (2011) utilized different carbon sources for lipase production by *Bacillus* sp. and observed maximum lipase activity (4.25 ± 0.020 IU mL) using olive oil as a carbon source.

Maximum lipase activity (86.35 U/mL) was observed with peptone. Similar results were reported by Brevil *et al*. (1997) who revealed peptone as the best source of nitrogen in *Cellotrichum glosoproides* using pongamia oil cake. Patel and Desai, (2018) also reported peptone as the best source of nitrogen for lipase production by several strains of bacteria including *Staphylococcus*. Contrary to our study, Dahiya and Purkayastha reported ammonium nitrate as the best nitrogen source for *Bacillus* sp. isolated from oil industry samples.

## 5.2 CONCLUSION

In conclusion, samples of diesel and restaurant wastewater contaminated soil were collected, cultured, and screened for lipolytic bacteria, Out of 14 isolates screened, isolate PMD1 showed the highest growth and lipase activities on solid agar. The probable identity of the isolate as *B. subtilis* was established based on its morphological and biochemical characteristics. Both glucose (88. 65 U/mL) and peptone (86 .35 U/mL) supported the highest lipase productions among the carbon and nitrogen sources investigated. Isolate PMD1 is a good source of lipase production and can be harness for further study with aim for industrial production.