

COMPARATIVE STUDY OF PRODUCTION OF EXTRACELLULAR LIPASE FROM FUNGI AND BACTERIA WITH APPLICATION TO DEGRADE FAT AND OIL

*Submitted in partial fulfilment of the requirements for the award of Master of Science
in Medical Biotechnology and Clinical Research*

By

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**INSTITUTE OF SCIENCE AND TECHNOLOGY
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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Ms. SUJITHA I (42101015)** who carried out Project entitled "**COMPARATIVE STUDY OF PRODUCTION OF EXTRACELLULAR LIPASE FROM FUNGI AND BACTERIA WITH APPLICATION TO DEGRADE FAT AND OIL**" under the supervision from September 2023 to April 2024.


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DECLARATION

I, **Ms. SUJITHA I (42101015)** hereby declare that the Project Report entitled **"COMPARATIVE STUDY OF PRODUCTION OF EXTRACELLULAR LIPASE FROM FUNGI AND BACTERIA WITH APPLICATION TO DEGRADE FAT AND OIL"** done by me under the guidance of **Dr. A. DAYANANDAN, Professor (Research)** at the Centre for Molecular and Nanomedical Sciences, Sathyabama Institute of Science and Technology, Chennai, is submitted in partial fulfilment of the requirements for the award of Master of Science in Medical Biotechnology and Clinical Research.

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ABSTRACT

Lipase enzyme plays a major role in several industrial processes. The effective production of lipase enzyme from microorganism in a cost-effective manner is in great demand in the current scenario. This study has aimed in comparing the production of lipase from bacterial and fungal source. The enzyme showed the high total activity of bacterial source of 2141.8 U and total fungal activity was 441.8 U. Further optimization studies for bacteria showed the respective results: pH (6.5-7) 16.83 U/ml, temperature (35°C – 40°C) 17.93 U/ml, incubation time (36 hrs) 18.590 U/ml, effective substrate olive oil (1%) 19.019 U/ml, nitrogen source (Peptone 1.5% (w/v)) 20.651 U/ml and following for fungal source, pH (5) 2.416U/ml, temperature (25°C-27°) 3.185 U/ml, (96 hours) 3.932U/ml, olive oil(1%) 4.281 U/ml and yeast extract 4.359 U/ml enhanced the parameters to be considered for the high production of lipase enzyme. The purification process carried out in this study was ammonium sulphate precipitation, dialysis and column chromatography using Sephadex G-100 as a stationary phase. The characterization studies of partially purified lipase enzymes with parameters enhanced the stability of bacterial and fungal lipase. Metal ions (Ca^{2+}) and detergent (Tween 80) showed effective stability whereas the Ca^{2+} and detergent (Tween 20) showed stability for the fungal lipase. Comparatively, the bacterial lipase showed better activity and stability. The bacteria was able to degrade 12.69% crude oil to compared to 2.44% biodegradation in control at the end of 28 days. Thus, this study of extracellular lipase would be effective in the application of oil degradation.

Keywords: Extracellular enzyme, Lipase, Optimization, Purification, Characterization, Oil degradation.

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ABBREVIATION

SDS-PAGE

kDa	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
NA	Kilo Dalton
NB	Nutrient agar
PDA	Nutrient broth
PDB	Potato dextrose agar
U	Potato dextrose broth
ml	Unit
g	Milli liter
(w/v)	grams
Na ⁺	Weight/volume
Ba ²⁺	sodium
Ca ²⁺	Barium cation
Mn ²⁺	Calcium cation
nm	Manganous cation
	Nanometer

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CHAPTER-1

INTRODUCTION

Lipases are extremely different in character and are ubiquitous in plants, animals and microorganisms. The microbial lipases have reaped much attention industrially than those derived from plants and animals due to their functional ability at extreme temperature, pH, and stability in organic solvents, chemo-selectivity, regio-selectivity, and enantio-selectivity (Iqbal and Rehman, 2015). Microbial lipases are the most important group of biocatalysts used for a variety of different biotechnological applications. Microbial lipases have been used for production of desirable Flavors in cheese and other foods, and for the interesterification of fats and oils to produce modified acylglycerols, which cannot be obtained by conventional chemical interesterification (Al Mohaini *et al.*, 2022).

1.1 HISTORY OF LIPASE

Claude Bernard originally identified lipase in pancreatic juice in 1856. Plant seeds were the first source of evidence for lipases. Traditionally, lipase for commercial use was sourced from animal pancreatic extracts. There are several lipase manufacturers in the natural world. But when lipases' industrial potential increased and the supply of lipases from animal sources was insufficient to meet demand, researchers began looking at microbial sources of the enzyme. The field of fungus lipases was first studied. Lipases are acyl hydrolases of triacylglycerol, which hydrolyses it into glycerol and fatty acids. Numerous plants, animals, and microbes make them, and they are widely distributed in the natural world (Hasanuzzaman *et al.*, 2004).

The remarkable chemo-, regio-, and enantioselectivity of microbial lipases, along with their resistance to temperature, pH, and organic solvent extremes, have drawn significant industrial interest. Strong efforts have been focused on the creation of enzymes with specific features or better performance for industrial applications as a result of the multiple industrial applications of lipases, which have sparked interest in the isolation of new lipases from unique sources (Warangkar and Khobragade, 2009). The remarkable biotechnological potential of microbial lipases can be

attributed to their stability in organic solvents, lack of cofactor requirement, broad substrate selectivity, and strong enantioselectivity. Lipases' great adaptability makes them useful in a variety of industries, including food, dairy, detergent, pharmaceutical, biodiesel manufacturing, leather, textile, paper, and (Musa *et al.*, 2012).

Because of its qualities such as temperature sensitivity, pH dependence, activity in organic solvents, biodegradability, high specificity, and high catalytic efficiency, lipases have been more widely available since the 1980s and are being employed as industrial biocatalysts. Using all mono-, di-, and triglycerides as well as free fatty acids in transesterification, low product inhibition, high activity/yield in non-aqueous media, low reaction time, resistance to changed temperature, pH, and alcohol, and the ability to reuse immobilized enzyme are the most desired properties of lipase. The energy needed to control reactions at unusual temperatures and pressures is further decreased since lipases may conduct reactions in mild pH and temperature circumstances (Kumar *et al.*, 2012).

Due to their broad spectrum of substrate compatibility, which includes both natural and artificial materials, lipases can catalyse a variety of processes (Prasad and Manjunath, 2011). Numerous habitats, including industrial wastes, compost heaps, oilseeds, degraded food, vegetable oil processing industries, and dairy products, have been found to include lipase-producing microorganisms (fungi and bacteria). Filamentous fungus produce lipase in a culture medium, but this process is reliant on the surrounding conditions, which include the presence of an inducer (usually oil) and the right physiological factors including pH, NaCl concentration, temperature, and dissolved oxygen (Mehta *et al.*, 2018). To produce extracellular enzymes with the highest yield possible, ideal growing conditions are even more crucial. Furthermore, it has been shown that a single lipase exhibiting a variety of unique biochemical characteristics can also be obtained (L. P. Lee *et al.*, 2015).

1.2 EXTRACELLULAR LIPASE

Extracellular lipase released into the liquid medium and internal enzymes are the two general forms of fungal lipase, depending on the kind of lipase found in fungus. In contrast to extracellular lipase, which is found outside of cells and is always secreted into the liquid medium before being eliminated from it, intracellular lipase is found inside of cells and is attached to the cell wall or membrane. Furthermore, it

is more practical to clearly explain the product using whole-cell technology. Whole-cell fungal lipase is a more cost-effective option for the practical use of wastewater treatment and biodiesel generation in countries. Consequently, the identification of novel fungal strains possessing elevated intracellular lipolytic activity and selection characteristics will be advantageous for the utilization of whole-cell lipase in biotechnology (Mukherjee *et al.*, 2010).

For the purpose of determining lipase extracellular activity, various screening procedures have been presented; however, assays utilizing agar plates are strongly advised due to their ease of use and reduced cost. However, agar plates are frequently not clear enough to measure the intracellular lipase activity of different strains, and the procedure causes losses during the isolation and selection stages; as a result, additional techniques must be used in addition to agar plates (Wadia and Jain, 2020). In solid-state fermentation systems, filamentous fungus have favourable traits for growth and lipase synthesis. Filamentous fungus grow well on solid substrates because the conditions are comparable to those of their native habitat, which leads to increased enzyme production, less energy and water consumption, and simple aeration. This is a low-cost substitute that might be employed by the agroindustry to produce enzymes or even cleaned up contemporary oil residues (Yasar *et al.*, 2020).

1.3 SOURCES OF LIPASE

An excellent source of these enzymes is moderately halophilic bacteria, which may thrive in medium with 3–15% NaCl. For future industrial uses, it would be beneficial to screen and isolate extreme and moderate halophiles from hypersaline conditions that can manufacture these enzymes (N. Pandey *et al.*, 2016). The *Bacillus cereus* C71, *B. thermoleovorans* ID1, *B. coagulans* BTS3, *Geobacillus* sp. TW1, *Bacillus* sp. strain L2, *B. sphaericus* 205y, *B. bogoriensis* sp. nov., *B. salarius* sp. nov., and *B. sphaericus* JS1 are among the *Bacillus* species that have been found to possess the highest efficiency in lipase production. Numerous saline habitats, including salterns, estuaries, salt lakes, salty foods, sea ice, and deep-sea hydrothermal vents, have been isolated from *Bacillus* species, which exhibit great taxonomic diversity (Kulkarni Pranav, Harkut Nidhi, 2019).

1.4 BACTERIAL LIPASE

Bacteria produce lipases, which are referred to as extracellular enzymes because of this. For *Bacillus prodigious*, *B. pyocyaneus*, and *B. fluorescens*, their existence has been noted as early as 1901, which represent some of today's best studied lipase-producing bacteria now named *Serratia marcescens*.

Pseudomonas lipases, for example, are one of the specific types of bacterial lipases that have received the most attention (Shon *et al.*, 2002). The unique biochemical features that *pseudomonas* lipases exhibit set them apart from lipases made by other microbes, making them extremely fascinating. Activity at alkaline pH and correspondence to heat resistance. Moreover, lipase and bacteria that break down oil may be essential for the bioremediation of lipid waste from homes or restaurants.

In an investigation, a new *Pseudomonas* bacterium that degrades oil was discovered. It generates inducible lipase, which effectively breaks down every type of fat and oil that has been examined, even certain lipid wastes (Bhumibhamon and Phattayakorn, 2003). *Pseudomonas* species, which are common in both soil and water, are highly significant in both science and technology. They belong to a class of organisms with diverse metabolisms that may utilize a broad variety of both simple and complex organic chemicals. The biodegradation of hazardous chemical substances, whether created by nature or by humans, is known to involve them. Moreover, lipase is among the several extracellular enzymes that the bacterium genus *Pseudomonas* produces in large quantities (Sarkar and Chatterji, 2018).

1.5 FUNGAL LIPASES

Well-known lipase producers are *Aspergillus* Mold species. Both external and intracellular lipases are generated by *Aspergillus Niger*. Response surface approach was used to optimize *Penicillium sp. lipase*. *Rhizomucor miehei*, *T. lanuginose*, *Candida rugosa*, *Candida antarctica*, *Mucor*, and *Geotrichum* are among the fungi that make lipases (Geoffry and Achur, 2018). *Pseudomonas aeruginosa*, and *P. fluorescens*, respectively (Jaeger *et al.*, 1994). The produce lipase extracellularly, fungi have been regarded as the finest suppliers of lipase. The following are some of the major genera of filamentous fungi: *Fusarium*, *Acremonium*, *Alternaria*, *Eurotrium*, *Ophiostoma*, *Ashbya*, *Geotrichum*, *Aspergillus* *Penicillium*, *Rhizopus*, and *Rhizomucor* (Bora and Bora, 2012).

1.6 LIPASE ASSAY

Titrimetry is the most straightforward way of lipase test, and it was also employed in our investigations. Other methods of lipase assay include Interfacial tensiometry, Spectroscopy, Chromatography, Immunochemistry, and Conductimetry. Because of their higher potential for large-scale production, lipase enzymes are highly valued from an industrial standpoint (Matsuoka *et al.*, 2009).

1.7 FERMENTATION PROCESS

Recent studies have shown that submerged fermentation is a very efficient method for producing huge quantities of industrially significant enzymes such cellulases, pectinases, and lipases. The most suitable manufacturing method for poor nations is submerged fermentation, which is a promising instrument in the biotechnology industry for the synthesis of microbial enzymes. More benefits of submerged fermentation over traditional solid-state fermentation include better oxygen circulation, lower energy and cost requirements, fewer operational issues, less impact on downstream processing, higher productivity, compactness of the fermentation vessel, lower capital and recurring expenditure, and a resemblance to several microorganisms' natural habitat (Colla *et al.*, 2015).

To create biodiesel, lipases can catalyse the transesterification reaction of lipids. Blood cholesterol is measured using lipases that have been immobilized on electrodes and glucose oxidase, which together act as a lipid biosensor. Additionally, they are crucial to the handling of industrial wastes. In activated sludge, lipases are utilized in the aerobic degradation process, as fats create a thin layer that prevents aeration. Lipases can handle the fat-laden wastewater from the dairy, poultry, and food sectors. In this work, bacteria that produce lipolytic enzymes are isolated and identified from oil cakes, a waste product of the oil industry. In order to use them as inoculums for treating waste polluted with oils, microbes were screened according to their capacity to degrade various types of oils. The research is anticipated to expand upon current understanding of lipase-producing microbes and pave the way for future directions (F. Islam, 2019).

They can also be employed directly in solid fermentation settings as whole-cell lipase. One advantage of this strategy is that entire cells and solid substrates can serve as immobilizing agents for enzyme support, negating the need for an enzyme

purification step. Cotton provides protein, lipids, and Fiber. The degree of lipase production in oil-rich seeds may be related to the pathogenic fungi's Vigor during the biodeterioration process. A gelatinous, dark brown chemical component that is separated from the oil refinery is called soap stock. High concentrations of free fatty acids and greater values for 5–10% of the crude oil mass will be found in soap stock; SSF *Aspergillus Niger* uses these byproducts to produce fungal lipase. Although alkali refining soap stock is a source of fatty acids, it also poses handling, storage, and disposal issues. About 6% of the volume of crude soybean oil that is caustic refined is produced at this rate (Musa *et al.*, 2012).

1.8 REMEDIATION OF LIPID WASTE

Lipid wastes have the potential to cause serious environmental problems, such as oil films on water surfaces that block the transfer of oxygen from the air into the water, killing a variety of aquatic plants and animals C. It has long been practiced in aquatic aerobic and anaerobic settings to bioaugmentation lipid-rich wastes. Additionally, the use of lipolytic enzymes in wastewater treatment has been documented in numerous papers. Microorganisms are a reliable source for lipase synthesis on a big scale. The bioremediation of lipid waste (Cruz Ramírez *et al.*, 2012)(Matsuoka *et al.*, 2009)before their release into the environment has become a growing concern in recent years due to stringent waste disposal regulations .

1.9 LIPASE IN WASTEWATER TREATMENT

The aerobic or anaerobic bioremediation of lipid-rich wastes has been studied. Actinomycetes, fungi, bacteria, and cultures of plant and animal cells are used in the industrial extraction of lipases. Microbial lipases possess versatility in metabolism, making them advantageous in numerous industrial processes. Due to strict environmental rules and the clean and friendly applications of enzymes, enzymatic treatment techniques have attracted increased attention Wastewater from a catering unit with a high fatty content may benefit from the use of *Pseudomonas aeruginosa* LP602, a lipase-producing strain that was isolated from restaurant wastewater¹⁰. In order to lower the biological oxygen demand (BOD) and lipid content from five distinct lipid-rich wastewater sources, the current study set out to assess the effectiveness of individual bacteria and mixed cultures (Ghasemi, Amini, *et al.*, 2011).

1.10 WASTEWATER TREATMENT

Numerous bacteria that can break down hydrocarbons aerobically have been found (Sarkar and Chatterji, 2018). Generally speaking, the Department of Biotechnology, Faculty of utilizing fabrics, can treat wastewater contaminated with lipids by performing basic physical procedures like skimming and adsorbing. On the other side, the emulsion can also be stabilized chemically, and then the process can either be thermal or precipitate. These chemical processes do cost money, though. Because lipase-producing microbes convert lipid wastes into carbon dioxide, water, and biomass, they are of interest to biological wastewater treatment. The current investigation sought to choose strains and improve parameters for efficient fat decomposition in kitchen wastewater (Narasaiah TV and Lavanya Latha, 2014).

1.11 LIPASE IN ADVERSE APPLICATION

1.11.1 Oil degradation

Recombinant lipase has been created to be used in products like laundry detergents, baked goods, and even as biocatalysts in alternative energy schemes to turn vegetable oil into fuel, etc. Because lipase-producing bacteria convert lipid wastes into carbon dioxide, water, and biomass, they may be useful for the biological treatment of wastewater (Celson *et al.*, 2016). Because of their widespread occurrence and enduring nature, crude oil hydrocarbons are regarded as one of the organic contaminants that should be taken very seriously. The environment and human health are harmed by complex molecules found in crude oil, such as alkanes and monocyclic and polycyclic aromatic hydrocarbons. In recent decades, there has been a lot of research on the biodegradation of crude oil. Many microbes and their enzymes have been identified and isolated as a result of these studies. After carbohydrase's and proteases, lipases are the third biggest group of marketed enzymes due to their stability in organic solvents and their ability to catalyse reactions in a wide range of substrates without the need for costly cofactors (Musa *et al.*, 2012).

Additionally, microbial lipases have shorter generation times, and it is easier to produce more of the enzyme by genetically modifying bacterial cells. The purpose of this study was to screen for lipase-producing bacteria in oil-contaminated soil using samples from the night market. These bacteria may serve as a "extraction pool" for extracellular enzymes and as a possible source of microbial-based

breakdown. Additionally, the analysis included lipase and protease differential secretion. Enzymes belonging to the lipase and protease class carry out both synthetic and degradative functions necessary for the microorganism's physiological needs. The comparison can reveal information about the lipase/protease differential secretion in the soil contaminated by oil. To speed up the production of huge amounts of enzymes, it is also possible to alter the strains that excrete these enzymes by growing them in media. The oil pollutants in the night market's gathering area, which are extremely difficult to clean, can also be helped by these enzymes (Veerapagu *et al.*, 2013).

Triacylglycerol (TAG) and the oils and fats that come from food items and are found in the wastewater of restaurants and food companies are the same. Lipase must first hydrolyse TAG into glycerol and fatty acids in order for microorganisms to degrade it. Following this, the fatty acids are absorbed by microorganisms and transformed into acetyl-CoA by the β -oxidation process. TAG hydrolysis happens extracellularly, and lipase is typically released by the cells. The fatty acids produced as a result cause the aqueous field to become slightly acidic. Indeed, we determined from an initial examination of wastewater from multiple grease-traps that its pH was often between 5.5 and 6.5. So, for the in-situ breakdown of oils and fats in grease-traps to be efficient, microbes and secreted lipase need to be active at this pH range. Nevertheless, we discovered that while many of the microorganisms included in commercialized items can grow at pH 7.0, they do not grow at pH 6.0 (Souza *et al.*, 2018) .

CHAPTER-2

LITERATURE REVIEW

The highest lipase production was achieved by the lipase-producing bacteria BLP2 *Pseudomonas Guzzardi* at pH 7, 37°C, and 48 hours of incubation. When the organisms were grown in media supplemented with 1% protease peptone by *Pseudomonas Guzzardi* (168.7 U/ml), there was an increase in enzymatic production. *Pseudomonas Guzzardi* is the perfect strain to produce extracellular lipase at the industrial level, as the current study's findings show. (Matsuoka *et al.*, 2009). The Buriganga river water had the lowest bacterial count (8.3×10^2 cfu/ml) and the highest (1.56×10^7 cfu/gm) in the soil of dairy farms. The Buriganga river water had the lowest percentage of lipase-producing bacteria (23.44%) (Niyonzima and More, 2013) and the highest percentage (94.51) in the soil used to make edible oil. Thirty of the isolates exhibited superior lipase activity. For molecular identification, 10 possible lipase producers were selected. Nine of the taxa were identified according to standard methods, however *Acetobacter liquid facies*, which was previously thought to be a different species, was instead determined to be *Stenotrophomonas maltophilia*. Wastewater that contains a lot of lipids may be treated with the enzyme that the isolated bacteria produced (Culture *et al.*, 2016).

For isolate L13, a greater lipolytic activity of 17.43 U/ml was observed. The most effective bacterial producer of an alkaline lipase was found using conventional techniques, such as morphological, cultural, and biochemical traits. It was determined that *Bacillus flexus* was the isolate L13. *Bacillus flexus* strain XJU1 was designated for the isolate, which showed 99% sequence match to *Bacillus flexus* based on nucleotide homology and phylogenetic analysis. Because the isolate is generating greater quantities of enzyme in alkaline conditions, the screened *Bacillus flexus* from this study can be used to produce an alkaline lipase that is useful in the detergent and leather sectors (Niyonzima and More, 2013).

It is widely recognized that the excretion of lipase, which makes the lipid substrates available to cells, enables microbes to flourish and change vegetable oils. Screening for lipase production from used olive and sunflower frying oils was the goal of the current investigation. Out of the 47 examined strains of bacteria and yeast, lipolytic

activity was produced by the genera *Pseudomonas*, *Bacillus*, *Candida*, *Rhodococcus*, and *Staphylococcus* when they grew on the waste oils. The two highest producers were *Pseudomonas aeruginosa* ATCC 111 (1703.8 U/L) and *Pseudomonas* sp.3AT (2748 U/L) (Haba *et al.*, 2000).

Sixteen pure bacterial strains with higher lipase activity at an acidic pH (6.0) than an alkaline pH (pH 9.0) were isolated from food waste samples using the suggested screening protocols. A unique assay method was also created by deactivating lipase activity by microwave treatment before back titrating, which improved the accuracy of lipase activity detection in acidic environments. By taking this extra step, interferences from residual lipase activity during traditional direct back-titration procedures in the measurement of lipase activity at acidic pH may be reduced. Using the four procedures outlined in this work, approach C (SSC) produced the best acidic-lipase-producing isolate, which was identified as *Aeromonas* sp. C14. It showed optimal lipase activity of 0.7 U/ml at an acidic pH of 6.0 (Chang, 2007). The isolate that produced the largest amount of lipase was *Bacillus vallismortis* BCCS 007, bacterium, with 3.41 ± 0.14 U/mL lipase activity. This is the first report on the isolation and molecular identification of Maharla Lake lipase-producing bacteria (Ghasemi, Rasoul-Amini, *et al.*, 2011).

The Biolog system and biochemical testing were used to identify the microorganisms. The *Bacillus* genus was determined to contain strain ST 7, which displayed the maximum lipolytic activity of 4.58 U/ml. *Ralstonia paucula* was discovered as strain ST 6, which has an activity of 3.51 U/ml. According to the results, the lipolytic activities of strains ST 1, ST 4, ST 8, ST 9, and ST 10 were 2.39, 1.84, 2.38, 1.80, and 2.62 U/ml. While strains ST 8 and ST 9 were identified as *Bacillus* spp., strains ST 1, ST 4, and ST 10 were found to be *Ralstonia paucula*. *Bacillus thermos glucosidasius*, *Bacillus stearothermophilus*, or *Bacillus coagulans* were the tentative identities assigned to strains ST 7 and ST 9, whereas *Bacillus subtilis* was the tentative identity assigned to strain ST 8 (Hamid *et al.*, 2003).

16S rDNA sequencing was used in this work to screen for and identify soil bacteria that produce lipase. With a 17.8-fold purification factor and 583 U/mg specific activity, a novel hyper-thermophilic lipase known as *Bacillus subtilis* LP2 isolate was largely purified by ammonium sulphate precipitation. The substrate was found to be

between 80 KM and pH 7 at 80°C, exhibiting maximum activity. The computed V_{max} values were 18.3 mM and 680 U/mg, with a catalytic efficiency (kcat/KM) of 307 s⁻¹ M⁻¹. Because of its effective catalysis at higher temperatures, these results suggest that *Bacillus subtilis* LP2 lipase can be a useful contender for commercial applications such as organic synthesis and the fats and oils industry (Yasar *et al.*, 2020).

In the pH-Stat method at 55 °C, four of the identified bacterial strains exhibit more activity. Using universal primers, the 16s rRNA gene was amplified by PCR to identify these bacteria. The activity rose by 50% as a result of fermentation. The growing medium—which was created to promote the generation of lipase—boosted activity by a factor of 4.55. ZR-5's crude supernatant was lyophilized, and its activity was assessed following fermentation and cell separation. This strain's 12 kU/g total activity indicates that it may find application in industry. To calculate the specific activity of an enzyme and purify it, more research is needed. Another strategy to think about is immobilization (Rabbani *et al.*, 2013).

Maximum lipase production (11 IU/L) and maximum esterase production (33 IU/L) were found in Production medium 2 after 24 and 36 hours, respectively. Maximum esterase production (extracellular, intracellular, and membrane bound) was also found in Production medium 2 after 24 hours. By esterifying stearic acid with ethanol, the DVL2 lipase/esterase was shown to produce ethyl stearate, as demonstrated by thin-layer chromatography. Furthermore, when DVL2 lipase was used to resolve 1-acetyl phenyl ethanol, a chiral auxiliary, good results were obtained (Kumar *et al.*, 2012). The maximum lipolytic activity, which was examined further. The physiology and biochemistry of this strain resembled those of the Burkholderia genus. While resolution of the phylogeny based on 16S rRNA sequences was insufficient to distinguish Burkholderia species, phylogenetic analysis based on the ferric uptake regulator (fur) gene sequences placed the strain in the clade of Burkholderia cepacia, Genomovar I. The ideal medium for inducing or sustaining lipase production was determined to be 0.5% glucose, 2% peanut oil, 2% peptone, 0.1% (NH₄)₂SO₄, 0.1% K₂HPO₄·3H₂O, and 0.05% MgSO₄·7H₂O at pH 8.0. The fermentation conditions were tuned for this purpose. 37°C was the ideal

temperature for fermentation, and 72 hours was the ideal duration. Lipolytic activity reached 10.5 U mL⁻¹ under these ideal circumstances (Ma *et al.*, 2010).

In order to help researchers select the best strains for lipase production, this article has emphasized and addressed the connection between the production of lipases utilizing different microbial strains, including bacteria, fungus, and yeast. Next, the topic of lipase production under various fermentation systems and lipase producer screening was covered. Later, the impact of several variables has been evaluated and reported with the goal of boosting its production from microbial strains. These variables include temperature, pH, and sources of carbon and nitrogen. Ultimately, lipase enzyme purification methods were compiled, and the most recent research on lipase extraction from microbiological stains was examined and condensed (Bharathi and Rajalakshmi, 2019).

At 48 hours of incubation, the highest level of lipase activity was detected in medium containing rice husk and groundnut peel, enhanced by ammonium nitrate and urea, the optimal sources of carbon and nitrogen. Through biochemical examination, partial purification, lipase activity evaluation at various pH levels, and molecular weight analysis, the isolated bacteria were identified as *Pseudomonas*. Using the ammonium sulphate precipitation spectrophotometric lipase assay, the lipase was partially purified to saturation for enzyme characterisation. *Pseudomonas* lipases are widely utilized in the food, pharmaceutical, pulp, paper, and leather sectors, among other industries, because of their broad substrate specificity, stability, and selectivity (Jamilu *et al.*, 2022).

At 37°C and pH 6.0 ± 0.5, there was a high degree of lipase synthesis during submerged fermentation. Comparing the medium with other carbon and nitrogen sources, the addition of 5% yeast extract and 3% sucrose increased the synthesis of enzymes. The produced lipases underwent dialysis after being partially purified using a precipitation process of 40–60% (w/v) ammonium sulphate. SDS-PAGE was used to assess the molecular weights of the partly purified lipases, which came out to be 32–47 kDa (Bharathi *et al.*, 2019). Trying to isolate possible lipase-producing bacteria from oil mill soil is the main goal of this study. Clear zones and/or opaques surrounding the colonies suggested lipolytic activity, which was initially screened for on agar plates using specific substrates like tributyrin and tween 80.

In all, 28 lipolytic strains were identified from soil samples taken from Myanmar's Mandalay and Myittha Township sesame oil mills. Olive oil was the only particular substrate used in the evaluation of their qualitative lipase activity. The top four strains for lipase production can be identified among them (Wai *et al.*, 2016).

These fungi were determined to be from the *Aspergillus* and *Penicillium* genera using the micro cultivation technique. By using the random amplified polymorphic DNA approach, it was possible to genetically differentiate amongst the most promising strains and identify two fungi that were both good lipase producers and different genetically. One that was isolated from soybean bran was capable to hydrolysing triglycerides with various lengths of fatty acids with efficiency. Only triglycerides containing long-chain fatty acids could be hydrolysed by another isolated from dairy products. With this method, two different groups-the strains with the highest hydrolytic activity and the strains with the lowest or nonproductive hydrolytic activity-could be confirmed (Griebeler *et al.*, 2011).

Using microscopic examination and colony morphology, *Aspergillus Niger*, *Penicillium sp.*, *Alternaria sp.*, *Trichoderma sp.*, and *Aspergillus flavus* were detected. *Aspergillus flavus* and *Alternaria sp.* were determined to be the best extracellular lipase producers in respect to the other test strains. Furthermore, as these microbes can be beneficial for the bioremediation of oil-contaminated sites, studies are required to optimize the fermentation process and produce more lipase from these fungal isolates (Wadia and Jain, 2017).

The primary method for directly screening these lipases is the plate assay technique, which uses a variety of preferred and particular substrates. An indirect method involves cultivating the strains in liquid medium according to protocol, then analysing the activity of the filtrates. The primary method for producing fungal lipase is through submerged and solid-state fermentation processes, which vary greatly in their operating conditions and are mostly dependent on physico-chemical parameters that are important for achieving the best lipase synthesis. Additionally, employing a variety of support materials, the immobilized fungal cells have been used to produce lipase. The screening, synthesis, and use of lipases derived from well-known fungus species are the main topics of this review, which takes all of these factors into consideration (Geoffry and Achur, 2018).

The most productive strain, *Colletotrichum gloeosporioides*, generated 27,700 U/l of lipase under ideal circumstances, and the crude lipase preparation could hydrolyse a variety of substrates, such as tributyrin, lard, and natural oils (Colen *et al.*, 2006). It was possible to get 122 endophytic fungal isolates. After demonstrating strong lipase activity in the plate screening, two isolated fungi were selected for submerged fermentation cultivations with carbon sources such as glucose and cottonseed oil. After 48 hours of fermentation in the culture medium with cottonseed oil as a carbon source, one strain reached a maximum lipase activity of 5.9 U/mL. *Preussia Africana* was the strain's genetic identity. Following lipase purification (purification factor of 18.5), SDS-PAGE analysis revealed the presence of a single protein band with an apparent molecular mass of 64 kDa (Souza *et al.*, 2018).

Aspergillus Niger 13F produced 4.2 U/mL/min after 4 days of incubation at 30°C, while the more potent *Fusarium solani* 7F produced 5.95 U/mL/min of crude lipase. *Penicillium Griseofulvin* 5F, *Aspergillus flavus* 6F, *Fusarium solani* 7F, *Aspergillus Niger* 12F, *Aspergillus Niger* 13F, and *Aspergillus Tereus* 17F were identified as potent fungi culture based on morphological, cultural, and molecular characteristics (18S rRNA gene sequence and phylogenetic analysis) (Patel, 2020). From the samples, 37 bacterial and 17 fungal strains from the genera *Trichoderma* (16 spp.) and *Aspergillus* (one sp.) were isolated. 37.8% was the greatest frequency of incidence for *Acinetobacter* sp. After 24, 48, and 72 hours of incubation, the bacteria's lipolytic activity ranged within 3.0 mm-15.5 mm, 3.0 mm-15.5 mm, and 3.0 mm-16 mm, respectively, when tested on solid agar. At various incubation times, *Acinetobacter* sp. (OG3) exhibited the maximum activity. During the incubation phase, eight isolates exhibited no activity. *Acinetobacter* sp. had the highest growth and *Bifidobacterium* sp. had the lowest during submerged fermentation, while the lipolytic bacteria's range of growth was 0.093-1.003. *Acinetobacter* sp. produced the greatest amount of lipase, which varied between 1.25 U/ml and 8.65 U/ml. *T. FISO1* had the highest growth at 3 and 7 days of incubation, while *T. virens* FSU/AW3 had the highest growth at 14 days of incubation. The growth of the chosen fungal isolate during submerged fermentation ranged within 0.00-0.200a, 0.00-0.25a, and 0.00-0.3190a. The fungi produced lipase at different rates. It ranged from 0.70n to 12.35a U/ml, 1.8k to 19.15a U/ml, and 2.50n to 19.8a U/ml. *Hypocrea patella* FAD1, *T. stromaticum* FSUAW1, and *T. virens* FSU/AW3 produced the most lipase at days

3, 7, and 14, respectively. As per the study, the isolated strains of *Trichoderma* and bacteria have the potential to manufacture lipase (Musa *et al.*, 2012).

The cup-plate method was used to measure the amount of lipase in the broth, and the titrimetric method was used to confirm the enzyme's activity. *Aspergillus* and *Trichoderma* species exhibited the highest lipase-producing activity (8.08 U/mL), while *Mucor* species displayed the lowest enzymatic activity (4.77 U/mL). It was discovered that, in comparison to other fungal members, *Aspergillus* species were the most commonly encountered. Faster solutions to environmental issues can be found by continuously and quickly searching for novel microorganisms and their lipolytic enzyme activity. This is because there is an increasing need for lipases in the industrial sector. Because of this, this component of our current study is valuable (Narasaiah TV and Lavanya Latha, 2014).

Alternaria alternata (A), *Penicillium aurantiogriseum* (B), *Penicillium pinophilus* (C), *Aspergillus fumigatus* (D), *Penicillium purpurogenum* (E), and *Penicillium citrinum* (F) were the detected fungus. To determine which production medium would be best for producing the most lipase, these fungi were point-inoculated on four different production media, namely M1, M2, M3, and M4. Among the production mediums tested, M4 exhibited the highest lipase activity from *Penicillium aurantiogriseum* (isolate B). M2 media had the lowest lipase activity, which was followed by M1 and M3 media. After 96 hours of incubation, isolate B produced lipase at a pH of 6.8 at 30°C, which was the most favourable temperature. Furthermore, the organism's enzymatic production rose when it was cultivated in medium supplemented with glucose as a source of carbon and vegetable oil and corn steep liquid as sources of fat and nitrogen, respectively. From 0.065 IU ml⁻¹ min⁻¹ under unstandardized settings to 16.718 IU ml⁻¹ min⁻¹ under standardized conditions, lipase activity (1 µM of p-nitro phenol ml⁻¹ min⁻¹) increased, according to the study's results. If the environment is slightly acidic, *P. aurantiogriseum* may be a possible source of extracellular lipase synthesis (H. Pandey *et al.*, 2018).

In the plates, the levels of the enzyme's lipase, protease, and laccase were measured. The aim was to identify the fungi that had the most potential for use in biotechnology. Generally speaking, fungi developed at 30 or 37 degrees Celsius. For certain isolates, enzymatic activity was found at this higher temperature. The

relationship between growth rate and enzyme activity is shown in the results: There were 60 fungi with laccase activity, 49 with lipase activity, and no protease activity. Enzymatic activity was typically greater than growth rate, suggesting that the isolated fungus have significant biotechnological potential. The results of the statistical analysis indicate that isolates 31 (*Trametes*) and 8.1 (unidentified) hold greater potential for further investigation as laccase-producing fungus. Conversely, isolates 147.2, 154, and 144.2 (*Fomes*), and *Trametes* (Cruz Ramírez *et al.*, 2012).

Using olive oil as a substrate and glucose or Tween 80 as a supplement, a quantitative measurement of lipase activity was carried out using the titration method. From oil-mill effluent, we have isolated a few lipolytic strains. *Penicillium sp.*, *Aspergillus fumigatus*, and *Aspergillus Tereus* were the three that were shown to be outstanding lipase producers. The medium added with glucose showed increased biomass and lipolytic activity. At a concentration of 1%, tween 80 is regarded as the best inducer as well. Under these circumstances, these isolates produced the highest amount of lipase in less than twenty-four hours ($3.91 \text{ IU mL}^{-1} \pm 0.12$ for *Penicillium sp.*) (Rihani *et al.*, 2018).

The titration approach yielded the enzyme activity. On the sixth culture day, using organic nitrogen rich medium (referred to as PM II) at pH 7, the highest lipase activity of 2.4 U/ml was obtained. In a 5-liter fermenter, lipase production was increased to a pilot scale while adhering to growth parameters of pH 7, temperature of 28°C, stirrer rate of 120 rpm, airflow rate of 30 L/hr, O₂ saturation percentage of 50%, and pressure of 0.05 MPa. For additional tests, the crude enzyme was isolated. Both the productivity and the quality of the enzyme produced can be enhanced via parameter optimization (Rai *et al.*, 2014). The rotary shaker was used to produce lipase for five days at 28±2°C using a minimal salt agar medium that contained olive oil and waste engine oil as the only source of carbon. On phenol red agar medium containing 1% olive oil, 25 of the 30 fungal isolates displayed an oil degradation zone. The most effective fungi that break down lipids were then subjected to a quantitative quantification process utilizing a spectrophotometric approach [420 nm] with oleic acid serving as a benchmark. Based on their morphology, *Penicillium sp.* was shown to be a proficient lipase generating fungal species with lipase activity of 91.80

± 1.2 U/ml. Lipase now under consideration is used in the generation of biofuel (Kulkarni Pranav, Harkut Nidhi, 2019).

Rhizopus stolonifer (with a diameter zone of clearance of 10 ± 0.05 mm) was found to have the second-highest lipase-producing ability, behind *Aspergillus Niger*. *Aspergillus flavus* and *Mucor* sp exhibited respective diameters of 6 ± 0.03 mm and 6 ± 0.01 mm. There was no clearance zone for *Acremonium* sp. or *Penicillium* sp. These findings indicate the existence of lipase-producing fungi in the soil environment of the Kwara State metropolis of Ilorin. These fungi can be used locally to produce the enzyme on a large scale, which has commercial value in the manufacture of leather, detergent, textiles, and as an ingredient in some specialty diets and medications (Abdulumuni *et al.*, 2022).

After 48 hours of batch fermentation, the highest amount of lipases produced by *Trichophyton* sp. was discovered. Similar to this, it was discovered that the microorganism produced its most enzymes at a temperature of 30°C during incubation and at a diluent pH of 7.0. Over the duration of the current investigations, the highest amount of lipases produced was 65.20 ± 1.13 U/g (Iftikhar *et al.*, 2011). Following a 72-hour incubation period at 45°C and pH 10.0, *Aspergillus fumigatus*, which was isolated from oil-contaminated soil, exhibited a good degree of lipase activity while using galactose (1%) as the carbon source and peptone (0.1%) as the nitrogen source. When p-nitrophenyl benzoate was utilized as the substrate, it was discovered that the isolated enzyme gave its best reaction temperature at 40°C and pH 9.0. Metal ions were observed to inhibit the activity of lipase.

One variable at a time produced an increase in lipase synthesis of 6.68 times. According to the results of this investigation, *A. fumigatus* lipase has the potential to be used in the bioremediation, detergent, leather, and pharmaceutical industries (Mehta *et al.*, 2017). The plant that produced the most lipase was found to be *Penicillium wortmanii*. Over the course of seven days, cultures using 5% (w/v) olive oil as the carbon source produced the highest amount of lipase (12.5 U/ml). Crude lipase had optimal pH and temperature values of 7.0 and 45 °C, respectively. The enzyme remained stable at temperatures of 40 and 45 °C, and after an hour at 50 °C, it maintained roughly 55% of its activity (Costa and Peralta, 1999).

After 96 and 120 hours of incubation, the lipase activity was 5.13 ± 0.30 U/ml and 5.22 ± 0.59 U/ml, respectively, in medium containing 1% glucose + 1% olive oil at pH 7.0 and 30°C. At pH 7.0, 30°C, and with either methanol or ethanol in the reaction tube, the optimal lipase activity was discovered. At 20–30°C, lipase was more stable and continued to function at 85%. The identification of isolate Ms.11 as a possible source of lipase capable of catalysing transesterification processes was concluded (Triyaswati and Ilmi, 2020).

Aspergillus versicolor demonstrated elevated amylolytic and gelatinolytic activity, while *Penicillium citrinum* alone shown elevated amylolytic activity. *Aspergillus Niger*, *Absidia corymbifera*, *Fusarium solani*, *Cunninghamella echinulata*, *Mucor racemosus*, *Paecilomyces variotii*, and *Synephalastrum racemosum* were shown to have the highest levels of cellulolytic activity. Maximum lipase activity was shown by the fungal species *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus*, *Cunicula verticillata*, *Curculum pallescens*, *F. oxysporum*, *Geotrichum candidum*, *M. racemosus*, *Pe. citrinum*, *Pe. frequentans*, *Rhizopus stolonifer*, and *Trichoderma viride*. This study validates the presence of isolated fungus in the ambient environment on a variety of substrates, and the findings may offer fundamental information for future research on fungal extracellular enzymes (Gopinath et al., 2005)

CHAPTER-3

AIM AND SCOPE

3.1 AIM

To produce extracellular lipase from fungi and bacteria to degrade fat and oil wastes.

3.2 OBJECTIVE

- To collect the soil sample and isolate the bacterial and fungal culture.
- To screen the production of extracellular lipase from isolated cultures.
- To optimize and maximize the production of lipase.
- To purify the produced enzyme.
- To evaluate the activity and stability of the produced enzyme.
- To compare the efficiency of the enzyme production between bacteria and fungi
- To study the effect of the enzyme on oil degradation.

CHAPTER-4

MATERIALS AND METHODS

4.1 COLLECTION OF SOIL SAMPLES

Oil contaminated soil sample which was contaminated with oil from 60 years was collected from a Mechanical shop in Chennai, Tamil Nadu, India.

4.2 ISOLATION OF LIPASE PRODUCING BACTERIA FROM FUNGI AND BACTERIA

Serial dilution were prepared for 10^{-1} to 10^{-9} dilutions from the soil sample without clumps. The 0.1ml of 10^{-4} and 10^{-5} dilutions was cultivated on nutrient agar (NA) plates and incubated at 37°C for 24hrs to 32hrs for bacteria and the grown bacterial colonies were streaked individually and the pure culture was streaked in nutrient agar slants at 4°C for further analysis.

Serial dilution were prepared for 10^{-1} to 10^{-9} dilutions from the soil sample without clumps. The 0.1ml of 10^{-2} and 10^{-3} dilutions was cultivated on potato dextrose agar (PDA) plates and incubated at 25°C for 72hrs for fungi and the grown fungal colonies were streaked individually and the pure culture was streaked in potato dextrose agar slants at 4°C for further analysis.

4.3 LIPASE SCREENING FOR BACTERIA AND FUNGI

For bacteria, to nutrient agar which was autoclaved at 121°C , tributyrin was added and the isolates have been grown on the tributyrin agar base plates and incubated at 37°C for 36 hours. A zone of clearance was observed to identify the lipase production.

For fungi, to potato dextrose agar (PDA) which was autoclaved at 121°C tributyrin was added and isolates have been grown on the tributyrin agar base plates and incubated at 25°C for 7 days. A zone of clearance was observed to identify the lipase production.

4.4 GRAM STAINING FOR LIPASE PRODUCING BACTERIA

The purified isolated bacterial strain was smeared on the glass slide by heat fixing and then it was primary stained by crystal violet for 1 minute and washed with water

and added Gram's iodine for 1 minute then washed with water and it was treated by decolorizer with ethanol, acetone or by mixing both was washed with water and finally counterstained with safranin for 30 seconds and washed with water. After air drying, the slide was observed under the microscope to identify whether the bacteria was Gram positive or Gram negative.

4.5 LACTOPHENOL COTTON BLUE STAINING FOR LIPASE PRODUCING FUNGI

For staining of fungi, in a clean and dry glass slide one or two drops of the lactophenol cotton blue reagent was added.

With a sterile needle a mycelial mat was transferred onto the fluid and pressed gently so that the stain was mixed easily.

4.6 MEDIA COMPOSITION

Nutrient agar composition for 1000ml was Beef extract 3g, Peptone 5g, Sodium Chloride 5g, Agar 15g, Distilled water 1000ml.

Potato dextrose agar composition for 1000ml was Dextrose 20g, Potatoes (sliced washed) 4g (from 200g infused potato), Agar 20g, Distilled water 1000ml.

Nutrient Broth composition for 1000ml was Beef extract 3g, Peptone 5g, Sodium Chloride 5g, Distilled water 1000ml.

Potato dextrose broth composition for 1000ml was Dextrose 20g, potatoes (sliced washed) 4g (from 200g infused potato), Distilled water 1000ml.

4.7 OPTIMIZATION FOR LIPASE PRODUCING BACTERIA AND FUNGI

For optimization, there are pH, Temperature, incubation time, carbon source, nitrogen source, agitation speed to identify the better lipase production from the bacteria and fungi in their favorable conditions.

4.7.1 pH

The optimum pH to produce enzyme from bacteria in nutrient broth was finalized by adjusting the pH from 2 to 11 in olive oil where the other parameter range are unaltered. After incubation for 24hrs at 37°C, the enzyme activity was calculated to finalize the optimized pH.

The optimum pH to produce enzyme from fungi in potato dextrose broth was finalized by adjusting the pH from 5 to 9 in olive oil where the other parameter range are unaltered. After incubation for 72hrs at 25°C, the enzyme activity was calculated to finalize the optimized pH.

4.7.2 Temperature

The optimum temperature was selected for lipase production the temperature was adjusted from 25 to 65°C in olive oil nutrient broth for bacteria.

The optimum temperature was selected for lipase production the temperature was adjusted from 25 to 65°C in olive oil potato dextrose broth for fungi.

4.7.3 Incubation time

The bacterial culture was inoculated in olive oil nutrient broth and incubated at the temperature of 37°C for time intervals of 24hrs to 72hrs and checked the enzyme activity by centrifuging at 4000rpm for 10mins for bacteria.

The fungal culture was inoculated in olive oil potato dextrose broth and incubated at the temperature of 25°C for the time intervals of 72hrs to 168hrs and checked the enzyme activity by filtering by filter paper.

4.7.4 Carbon source

Olive oil was now replaced with coconut oil, corn oil and gingelly oil with the concentration of 1% (w/v) in the nutrient broth for bacteria.

Olive oil was now replaced with coconut oil, corn oil and gingelly oil with the concentration of 1% (w/v) in the potato dextrose broth for fungi.

4.7.5 Nitrogen source

Different nitrogen sources have been taken like peptone, yeast extract, tryptone, urea, Ammonium Nitrate with the concentration of 1% (w/v) into the nutrient broth for bacteria.

Different nitrogen source have been taken like peptone, yeast extract, tryptone, urea, Ammonium Nitrate with the concentration of 1%(w/v) into potato dextrose broth for fungi.

4.8 DETERMINATION OF LIPASE ACTIVITY BY SPECTROPHOTOMETER METHOD

For enzyme activity, the substrate solution was prepared as follows. 40mg of p-nitrophenyl palmitate was dissolved in 12ml of isopropanol was named as the solution A. For solution B, 0.1g of gum Arabic, 0.4ml of triton x-100, 0.9ml of distilled water are being added. The mixture of 1ml of solution A and of 19ml solution B are mixed and was named as substrate solution.

Now for enzyme assay, 1ml of substrate solution and 0.5ml of sodium phosphate buffer was mixed. 0.1ml of crude enzyme was added. The mixture was kept in dry bath at 37°C for 30 minutes.

To stop the reaction 0.2ml of isopropanol was added and absorbance was measured using spectrophotometer at wavelength of 410nm to calibrate the release of p-nitrophenol.

4.9 PURIFICATION OF LIPASE

4.9.1 Ammonium sulphate precipitation

To the crude enzyme ammonium sulphate (70 %) was added at the temperature of 4°C for 24hrs. The precipitation was seen, and it was centrifuged at 1000rpm for 5 minutes and the supernatant was discarded, and the pellet was mixed with the sodium phosphate buffer for further use.

4.9.2 Dialysis

The precipitation mixed buffer was added to the membrane and it was kept inside a flask filled with sodium phosphate buffer at 4°C for 24hrs. The dialysis membrane was prepared by boiling the membrane in 2 % sodium bicarbonate and 1mM EDTA for 10 mins. The membrane was then rinsed in distilled water. The membrane was stored in ethanol at 4°C.

4.9.3 Column chromatography

Into the 400mg Sephadex, the sodium phosphate buffer was added and kept at 4°C for 4hrs. Then the Sephadex with the sodium phosphate buffer was added slowly in the column chromatography to this the dialyzed lipase was added. Then the enzyme

was eluted with the same sodium phosphate buffer and collected 1ml into 20 tubes as elution fractions. The enzyme activity was carried out for all the fractions.

4.10 SDS-PAGE

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out to measure the molecular weight of the purified lipase from GFC. The concentrations of the separating and stacking gel were 12.5% and 4.5%, respectively. The lipase sample and sample buffer were mixed in a volume ratio of 3:1 and were kept in a boiling water bath for 5 min. The supernatant was injected into the gel lane after the mixture was centrifuged for 5 minutes at a speed of 1000×g. Electrophoresis was performed at a constant voltage of 80V for approximately 1–2h. The gel was stained with a Coomassie Brilliant Blue R250 of 0.25%(w/v), 50%(v/v) methanol and 10%(v/v) acetic acid. The gel was destained using the buffer with 25%(v/v) methanol and 7%(v/v) acetic acid.

4.11 CHARACTERIZATION OF LIPASE

The purified enzyme was characterized by metal ions and detergent to determine the stability and relative activity of the enzyme.

4.11.1 Metal ions

0.5ml of crude enzyme, 0.5ml olive oil and 0.5ml metal ions which are Magnesium chloride, manganese, potassium chloride, zinc sulphate, ferrous sulphate are added to each tube and kept in dry bath at 38°C for 45 minutes and the enzyme activity and the relative activity was calculated.

4.11.2 Detergent

0.1 ml of SDS, Tween (80), Triton x-100 are taken into each tube along with 0.5ml of crude enzyme, 0.5ml of olive oil. The mixture was kept in dry bath at 38°C for 45 minutes. The enzyme activity and the relative activity was calculated.

4.12 APPLICATION OF LIPASE AND DEGRADATION OF CRUDE OIL

The biodegradation studies were carried out by dispensing 5 mL of cell free culture supernatant into 15 mL of sterile mineral salt medium that contain 2 mL of crude oil in a flask. The second flask (control) contained only the mineral salt medium and 2 mL of crude oil without the cell free culture supernatant (enzyme) and the two flasks

were incubated for 28 days at optimum pH and temperature for 7 days interval, two flasks were removed. The total amount biodegradation of the crude oil in the mixture was measured by draining the mixture with diethyl ether contained in a separating funnel with continuous shaking, the separating funnel was placed on the retort stand and this was allowed for the organic phase to separate from the aqueous phase, mineral salts medium was removed by opening the tap while the beaker of a known weight was used to collect the solvent oil mixture. This was placed in the water bath to allow the complete evaporation of the solvent. Therefore, the final weight of the beaker containing the residual oil was documented. The percentage of degradation of crude oil was calculated.

$$\% \text{ Biodegradation} = \frac{\text{Weight of oil (Control)} - \text{Weight of oil (degraded)}}{\text{Weight of oil (Control)}} \times 100$$

CHAPTER-5

RESULTS AND DISCUSSION

5.1 SCREENING OF LIPASE FROM ISOLATED CULTURE

In tributyrin agar, screening around the colony a clear zone has been observed. Due to presence of lipase activity the clear zone was confirmed where the bacteria was producing the extracellular lipase after 24hrs. In (Fig 5.1 a) sample of bacteria, the best clear zone of 2mm was observed.

In tributyrin agar, screening around the colony a clear zone has been observed. Due to the presence of lipase activity the clear zone was confirmed where the fungi was producing the extracellular lipase after 72hrs. A clear zone of 0.5mm was observed (Fig 5.1 d).



Fig: 5.1: Screening of lipase producing bacteria (a) has a clear zone (b) has no clear zone (c) has a clear zone (d) has a clear zone (e) has a clear zone (f) was control

A clear zone was observed which indicated the production of lipase as shown in (Fig 5.1 a, c, d, e). The (Fig 5.1 b) did not show a clear zone which was not producing lipase. (Fig 5.1 f) was control. The better zone was observed in a plate (a).

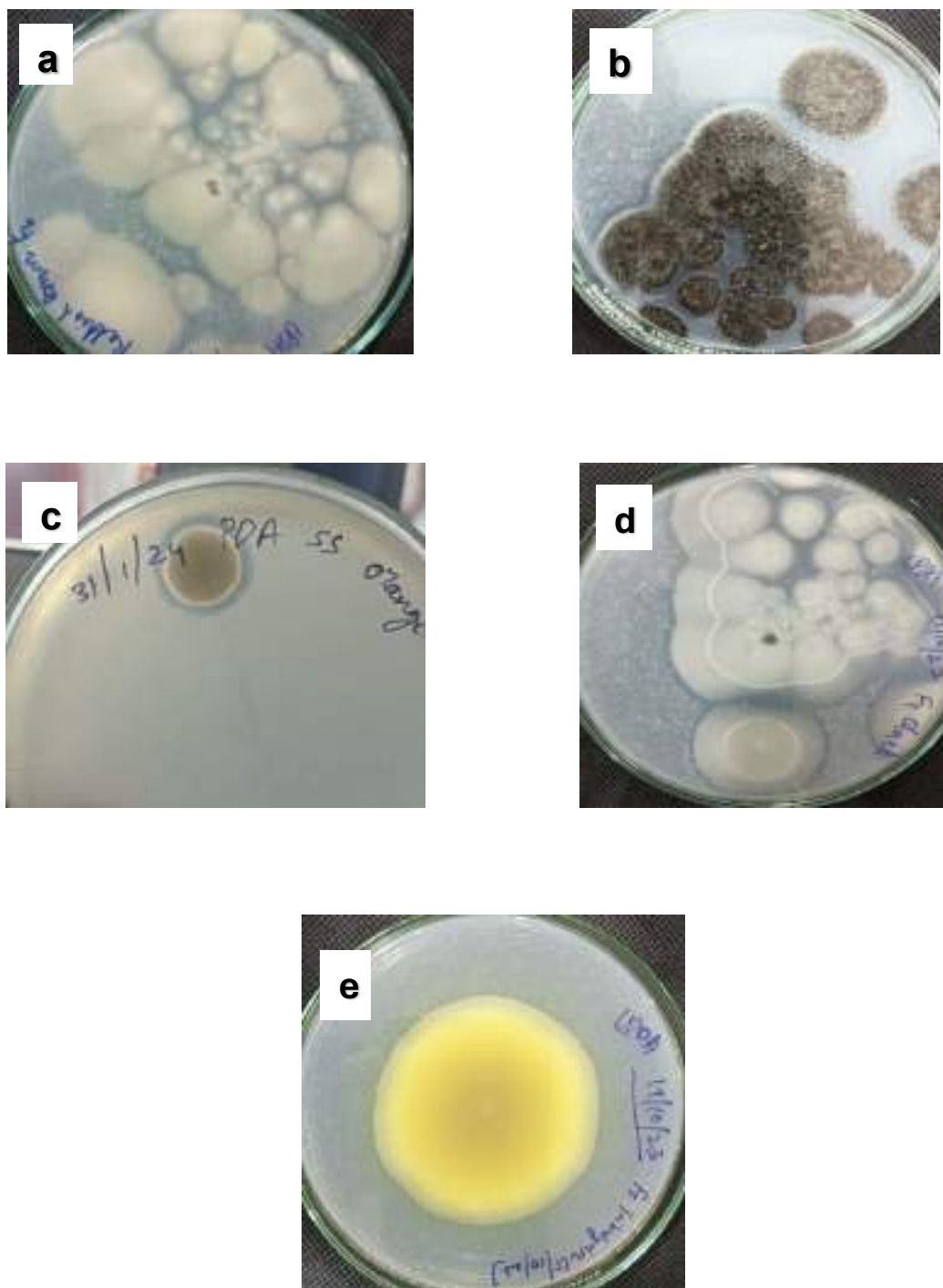


Fig: 5.2: Screening of lipase producing fungi (a) has a clear zone (b) has a clear zone (c) has a clear zone (d) has a clear zone (e) has no clear zone.

A clear zone was observed which indicated the production of lipase as shown in (Fig 5.2 a, b, c, d). The (Fig 5.2 e) did not show a clear zone which was not producing lipase.

5.2 GRAM STAINING FOR LIPASE PRODUCING BACTERIA

Lipase producing bacteria was found to be gram negative and the morphology was short rod.

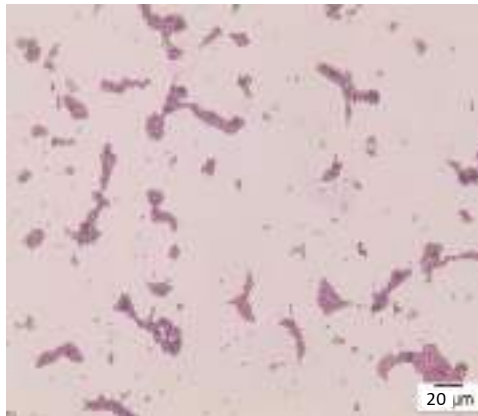


Fig: 5.3: Gram negative short rod (Scale bar =20μm).

5.3 LACTOPHENOL COTTON BLUE STAINING FOR LIPASE PRODUCING FUNGI

Lactophenol cotton blue staining showed the morphology of the fungal sample, including septate hyphae and swollen vesicles that produced phialides, which in turn produced conidia chains. The conidia are green and echinulate, with a diameter of 2.5-3 μm, and are produced in chains from greenish phialides that are 6-8 by 2-3 μm in size.



Fig: 5.4: Lactophenol cotton blue staining (Scale bar = 20μm).

5.4 OPTIMIZATION FOR THE LIPASE ENZYME PRODUCTION

5.4.1 pH

5.4.1.1 Effect of pH on bacteria produced lipase

The effective pH range was optimized for highest lipase production. The maximum lipase activity, 16.833 U/ml was shown in (Fig 5.5), was found in the pH range of 6.5 to 7, while the lipase activity declined at pH values below and above the range. The lipase activity was negatively impacted by the media's acidic content. In the meantime, the lipase's activity was reduced by the culture medium's high basicity. The enzyme's strong basic nature led to denature and stability loss, which progressively reduced activity. Therefore, a pH of 6.5-7 is practically ideal for optimal action.

At 10 °C and pH 7–8, the greatest growth was seen; growth was less pronounced in the alkaline range (pH 9 and 10). The bacteria flourished at several NaCl concentrations (0–30%), with 15% yielding the best growth rates (Abdella and others, 2023). The lipolytic activity of lipases that were produced through submerged fermentation were at their highest at pH 7.2 and temperature +0.417, or +0.55 and +0.417, respectively. The levels of +0.076 and zero (0) to pH and temperature, respectively, which correspond to pH 6.0 and 35°C, were shown to yield the highest lipolytic activity of lipases produced via solid-state fermentation (Colla *et al.*, 2015).

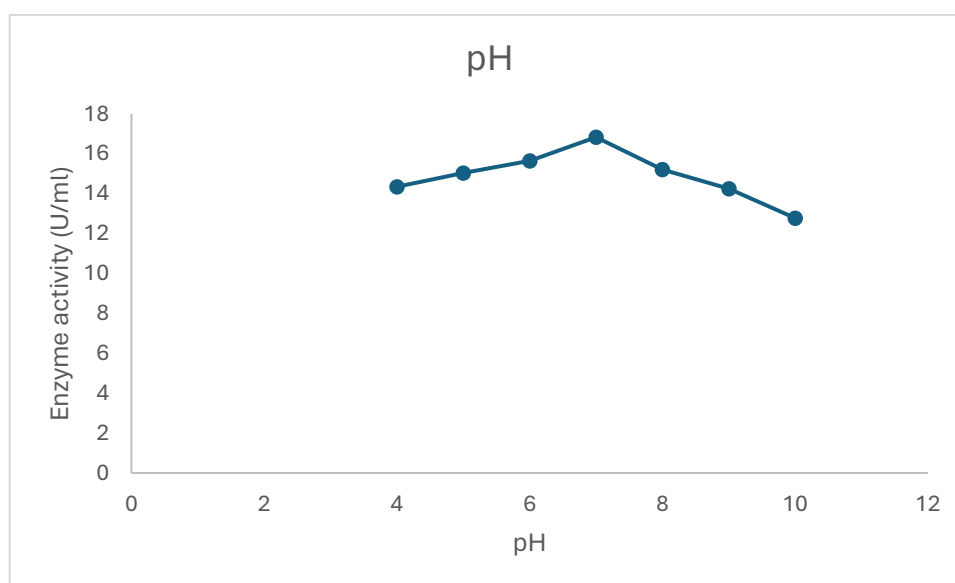


Fig: 5.5: Effect of pH on bacteria produced lipase.

5.4.1.2 Effect of pH on fungi produced lipase

Different outcomes have been seen regarding the fungal isolate's ability to produce lipase when the pH is gradually raised from 4 to 9. The maximum lipase activity, 2.416 U/ml as shown in (Fig 5.6), was found in the pH range of 5, while the lipase activity declined at pH values below and above the range. The lipase activity was negatively impacted by the media's acidic content. In the meantime, the lipase's activity was reduced by the culture medium's high basicity. The enzyme's strong basic nature led to denature and stability loss, which progressively reduced activity. Therefore, a pH of 5 is practically ideal for optimal action.

In tests 1 and 2, high lipase production (27.46 and 30.76 U. mL^{-1}) was observed at pH 5.0. Compared to the initial lipase production process (13.12 U.mL^{-1}), the enzymatic activity (30.76 U.mL^{-1}) was 2.3 times higher (Ribeiro Lima *et al.*, 2019). At 40°C and 60% moisture content, a previously adjusted tri-substrate combination with a ratio of 2:1:1 (RH:CSC:RGH) was used for the SSF process. The tri-substrate mixture's pH is adjusted during the pre-treatment by 0.5 units, ranging from 5.5 to 8. By evaluating lipase activity, the pH's influence was examined. At pH 6, a maximum lipase activity of 25.12 U/gds was found. As the pH increased further, lipase activity decreased; at pH 7.5, lipase activity was 7.14 U/gds (Nema *et al.*, 2019).

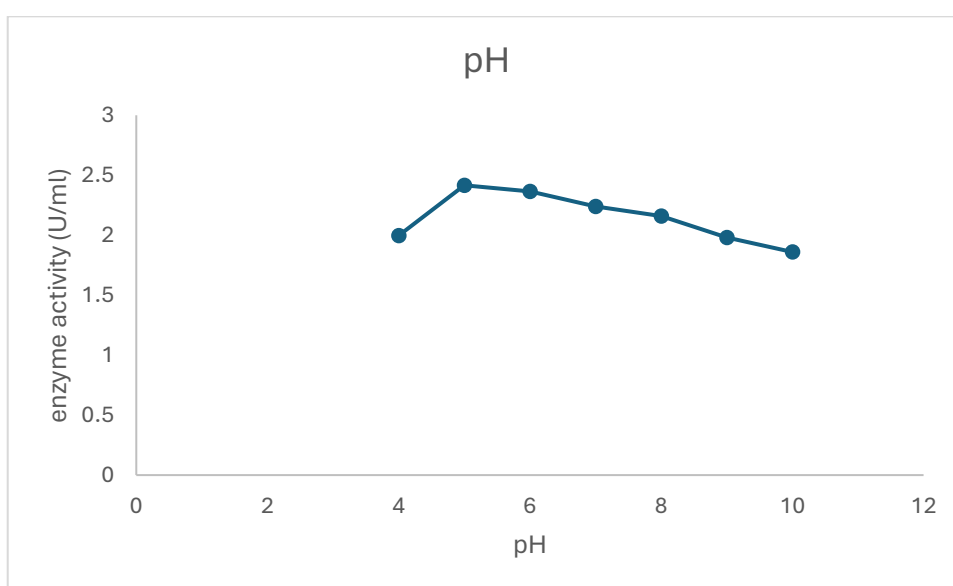


Fig: 5.6: Effect of pH on fungi produced lipase.

5.4.2 Temperature

5.4.2.1 Effect of temperature on bacteria produced lipase

The lipase enrichment medium were incubated within an ideal pH range and temperature range of 20°C to 95°C with the bacterial culture. Following incubation, the activity of lipase was measured. From the range of 35°C to 40°C, an increase in activity of 17.934 U/ml as shown in (Fig 5.7). Subsequent temperature increases caused the enzyme activity to steadily decline. The primary cause of this would be the enzyme's denaturation. One possible explanation for the poor activity at low temperatures could be a lack of accessible temperature. Thus, the steady synthesis and activity of the enzyme depend heavily on temperature. Between 35 and 40 degrees Celsius was the ideal temperature for bacteria improved productivity.

The ideal temperature for the synthesis of lipase 50°C was discovered for lipase. The stability and production of proteins are increased as a result of temperature-regulated enzyme synthesis at the mRNA transcription level and potentially at the translation stages of proteins. The temperature may, however, affect their secretion, perhaps by altering the physical characteristics of the cell membrane. This is another explanation for the higher production temperature. The *Bacillus* sp. LBN2 production trend (Bora and Bora, 2012). After 48 hours of incubation at 37°C, the highest specific activity (23.6 U/mg) was seen. The specific activities were 21.2 and 22.3 U/mg, respectively, at the lower temperatures of 20 and 30°C. The specific activities were 23.1 and 20 U/mg, respectively, at temperatures greater than 37°C, or 40 and 50°C (Hasan *et al.*, 2006).

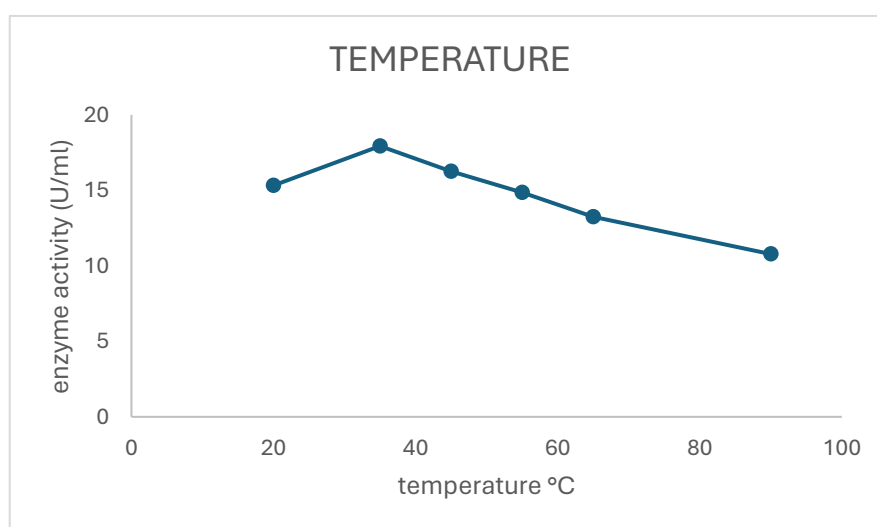


Fig: 5.7: Effect of temperature on bacteria produced lipase.

5.4.2.2 Effect of temperature on fungi produced lipase

The lipase enrichment medium were incubated within an ideal pH range and temperature range of 20°C to 95°C with the fungal culture. Following incubation, the activity of lipase was measured. From the range of 25°C to 27°C, an increase in activity of 3.185 U/ml was seen (Fig 5.8). Subsequent temperature increases caused the enzyme activity to steadily decline. The primary cause of this would be the enzyme's denaturation. One possible explanation for the poor activity at low temperatures could be a lack of accessible temperature. Thus, the steady synthesis and activity of the enzyme depend heavily on temperature. Between 25 and 27 degrees Celsius was the ideal temperature for fungi improved productivity.

Maximum lipase production occurs at a temperature below optimality (15°C) due to *Penicilium* isolation. Numerous reports on the synthesis of lipase from various mesophilic fungus species have been published in the literature. Lipase production in *Penicillium expansum* SM3 has been found to be best at 25, while lipase production in *P. citrinum*, a different species, has been reported to be optimal at 37°C (Pimental *et al.* 1997). The maximal lipase production of *Aspergillus nidulans* WG312 has been observed to occur at 30°C (Mayordomo *et al.* 2000).

It has also been researched in several fungal species belonging to genera like *Alternaria*, *Penicillium*, and *Trichoderma* how lipase is produced at low temperatures (around or below 20°C). It has been observed that on the fifth day of incubation at 20 °C, *Penicillium chrysogenum* produces lipase 68 U/ml. Three of the *Penicillium* isolates used in this study, GBPI_P8, GBPI_P188, and GBPI_P222, can be further investigated because of their capacity to create lipase at a low temperature of 15 °C, while GBPI_P98 can be used because it has the capacity to produce lipase at a temperature of 25 °C (N. Pandey *et al.*, 2016).

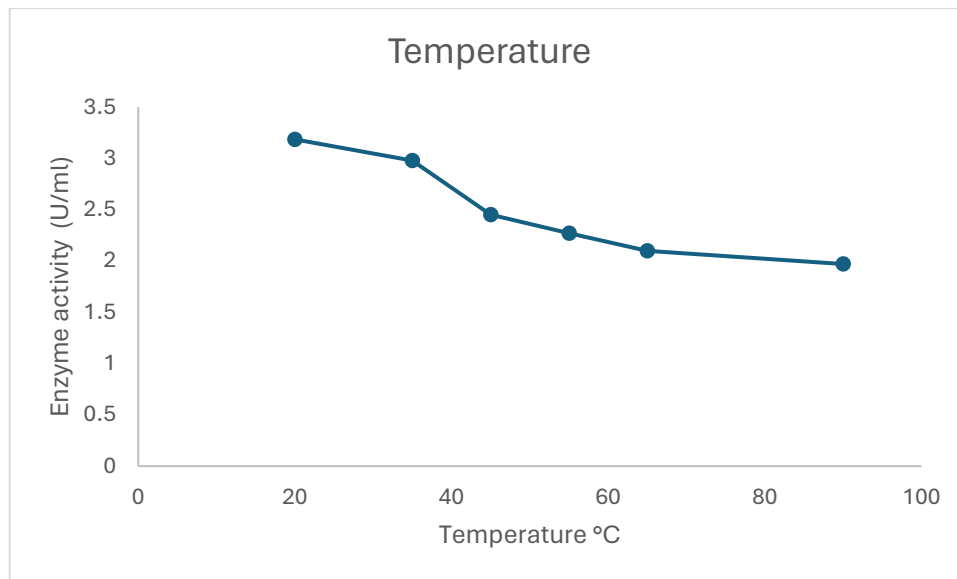


Fig: 5.8: Effect of temperature on fungi produced lipase.

5.4.3 Incubation time

5.4.3.1 Effect of incubation time on bacteria produced lipase

Effective lipase activity with 18.590 U/ml as shown in (Fig 5.9), was seen after 36 hours of incubation. As the amount of time increased, the enzyme activity steadily dropped, which could be because the bacterial strain was in its stationary phase. The ideal incubation period, then, would be 36 hours in order to produce lipase with optimal activity and stability. Compared to the 36-hour incubation period, the earlier incubation time did not exhibit significant activity. Lipase synthesis was carried out using nutrient broth supplemented with 1% olive oil. Using a spectrophotometric approach, cultures were grown at pH 9, 30 °C for 12 hours, and the supernatant was obtained every 3 hours to measure lipase activity. This allowed researchers to ascertain the impact of incubation time on lipase activity and identify organisms with higher lipase activity. When compared to water, which had a value of 162.85 U/L at 9 h, soil had the highest activity of lipase, which was measured at 364.82 U/L at 12 h (Ilesanmi *et al.*, 2020).

After 48 hours of development, *P. fluorescens* reached stationary phase, and after 120 hours, peak lipase activity was seen. After 96 hours and 120 hours of

incubation, respectively, *Serratia marcescens* and *Pseudomonas fluorescens*, which were separated from a bromeliad sample, showed maximum lipolytic activity of 3.85 ± 0.10 U/mL and 3.53 ± 0.24 U/mL (DA SILVA LUZ *et al.*, 2021).

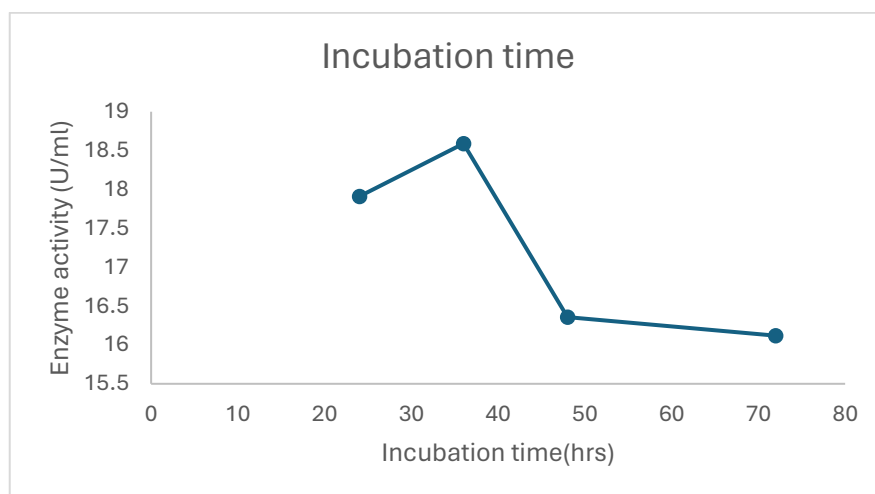


Fig 5.9: Effect of incubation time on bacteria produced lipase.

5.4.3.2 Effect of incubation time on fungi produced lipase

Effective lipase activity with 3.932 U/ml as shown in (Fig 5.10), was seen after 96 hours of incubation. As the amount of time increased, the enzyme activity steadily dropped, which could be because the fungal strain was in its stationary phase. The ideal incubation period, then, would be 96 hours in order to produce lipase with optimal activity and stability. Compared to the 96-hour incubation period, the earlier incubation time did not exhibit significant activity.

After being incubated for 96 and 120 hours, respectively, 5.13 ± 0.30 U/ml and 5.22 ± 0.59 U/ml, that high lipase activity was created. After 96 hours, the lipase production reached its peak at 0.31 ± 0.02 U/mg biomass, and it remained steady until 120 hours, at 0.30 ± 0.04 U/mg biomass. Following a 120-hour incubation period, the output of lipase starts to decrease until 168 hours later (0.05 ± 0.001 U/mg biomass). This is caused by a fall in lipase activity and an increase in biomass (Triyaswati and Ilmi, 2020).

It is confirmed that the fungus attained its peak growth at 15.2 g.L⁻¹ within 36 hours, nearly consuming all of the sucrose at the final concentration of 0.35 g.L⁻¹. Nevertheless, the dry biomass concentration (13.0 g.L⁻¹) somewhat decreased after 48 hours. It took 72 hours to yield the greatest specific activity (57.17 U.mg⁻¹). The

low protein concentration (0,23 g.L⁻¹) in the enzymatic extract is connected to it. The study's lipid source, soybean oil, had a comparable specific activity (56.47 U.mg⁻¹) (Ribeiro Lima *et al.*, 2019).

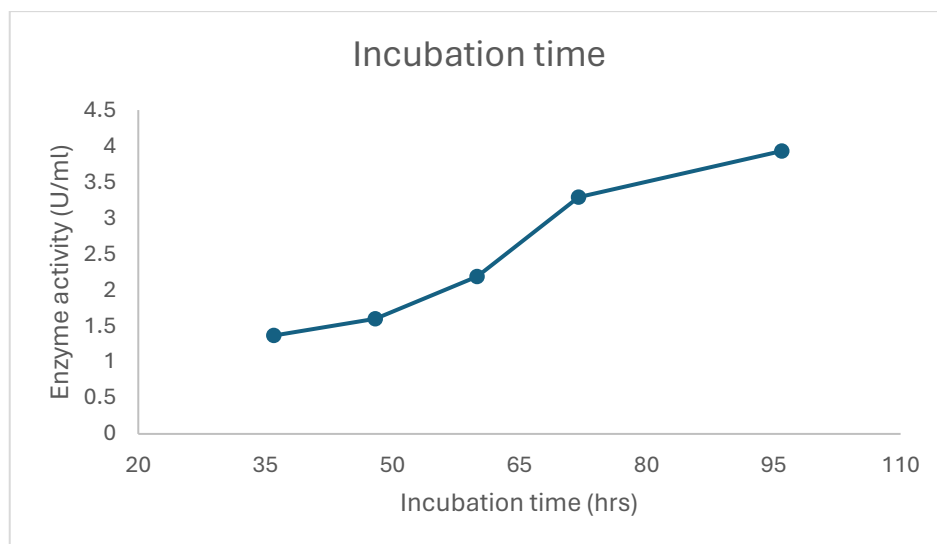


Fig 5.10: Effect of incubation time on fungi produced lipase.

5.4.4 Carbon source

5.4.4.1 Effect of carbon source on bacteria produced bacteria

Different substrates at 1% concentrations were used in place of the lipase production medium. The other settings that were previously optimized were not changed. After 36 hours of incubation, the activity of the lipase enzyme was calibrated. When olive oil was utilized as the substrate, the lipase activity was relatively high (19.019 U/ml) as shown in the (fig 5.11). Concurrently, coconut oil shown increased activity while corn and gingelly oils did not exhibit significant effectiveness. Olive oil can therefore be utilized as a useful substrate for greater production ranges.

Cultures were grown at 30 °C in each production medium for 12 hours in order to assess lipase activity using a spectrophotometric method. This was done to find out the impact of different carbon sources (Tween 20, Tween 80, glucose, and fructose) on lipase production in organisms with higher lipase activity from previous experiment. The lipase activity in the olive oil-containing medium was 364.81 U/L, while the activity in Tween 20, Tween 80, glucose, and fructose was 202.39, 313.69, 61.8, and 106.57 U/L, respectively (Ilesanmi *et al.*, 2020).

The influence of a different oil used as a carbon source in the fermentation medium was demonstrated by the lipase activity, which was measured titrimetrically by hydrolysing olive oil. According to the findings, the fermentation medium containing olive oil had the highest lipase activity (11.00 ± 2.00 U/mL), with sunflower oil and cooking oil waste coming in second and third. (Al Mohaini *et al.*, 2022).

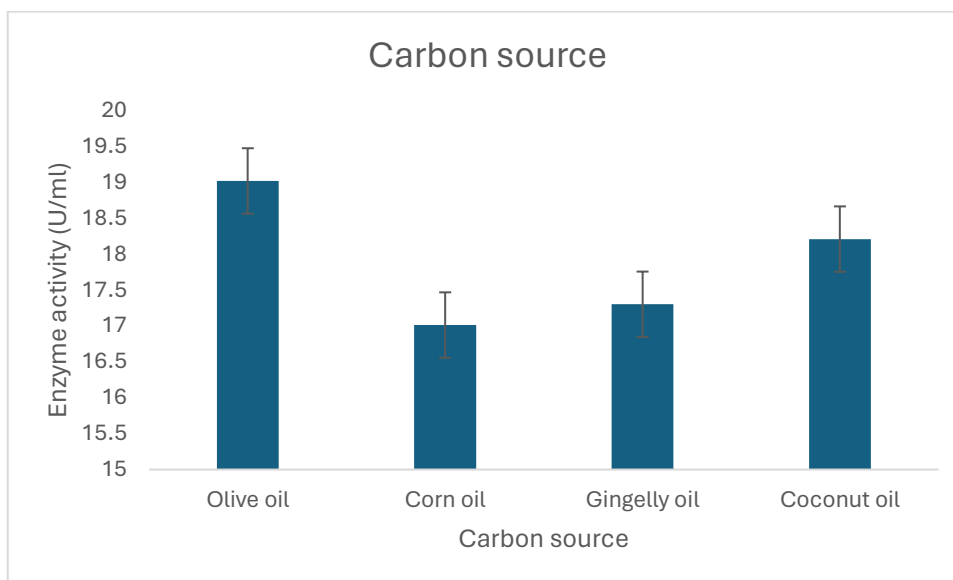


Fig: 5.11: Effect of carbon source on bacteria produced lipase

5.4.4.2 Effect of carbon source on fungi produced lipase

Different substrates at 1% concentrations were used in place of the lipase production medium. The other settings that were previously optimized were not changed. After 36 hours of incubation, the activity of the lipase enzyme was calibrated. When olive oil was utilized as the substrate, the lipase activity was relatively high (4.281U/ml). Concurrently, coconut oil shown increased activity while corn and gingelly oils did not exhibit significant effectiveness (Fig 5.12). Olive oil can therefore be utilized as a useful substrate for greater production ranges.

At three concentrations, the lipase activity at 1.5% glucose syrup was greater than that of other sources, with an activity of 1600 ± 69.1 u/mg in a 24-hour period. Significant variations were seen in the lipase activity at several low-cost carbon source concentrations ($p < 0.05$). Molasses (1.5%) with 1478 ± 34.3 u/mg lipolytic activity in 24 hours was utilized for additional analysis because it is the least expensive of these sources. The addition of 1.5% glucose demonstrated the maximum activity (1573 ± 21.6 u/mg) among high-cost sources when compared to

other sources and concentrations ($p>0.05$). Three different amounts of malt extract were shown to have the lowest lipase activity after 24, 48, and 72 hours (Câmara Ribas *et al.*, 2019).

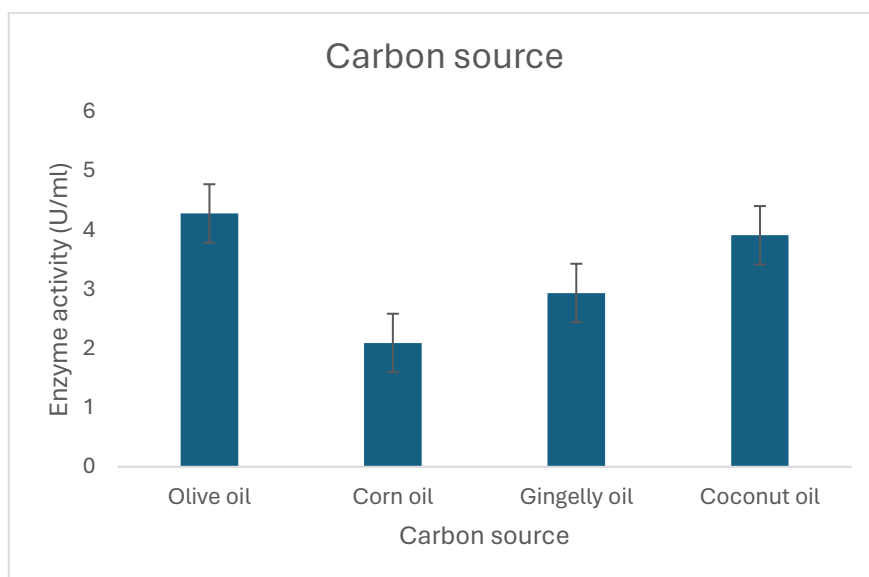


Fig: 5.12: Effect of carbon source on fungi produced lipase

5.4.5 Nitrogen source

5.4.5.1 Effect of nitrogen source on bacteria produced lipase

Using peptone, yeast extract, tryptone, urea, and ammonium nitrate, the impact of the nitrogen supply on the lipase activity was investigated. The other optimal parameters—pH, temperature, and carbon source—remained constant. Comparing lipase activity from different sources, it was discovered that the peptone-incubated lipase had the maximum activity, at 20.651 U/ml as shown in the (Fig 5.13). Comparing the results from other sources to peptone, they were not as depleting. Urea, meantime, exhibited somewhat successful activity.

For lipase synthesis (68.75 U) and cell growth (21.12 g/L), yeast extract was the most effective source of nitrogen. This nitrogen source also showed specific activity (10.57 U/mg) comparable to the control. When *C. viswanathii* was given peptone (YX/S01.625 g/g; YL/S04.590U/g), tryptone (YX/S01.075 g/g; YL/S03.199 U/g), and yeast extract (YX/S01.543 g/g; YL/S04.683 U/g), it demonstrated strong cell growth and lipase production for the other parameters that were examined. Since they give the cells extra nutrients including vitamins, cofactors, and amino acids, these

organic nitrogen sources are typically utilized (Barth and Gaillardin 1997; Tan *et al.* 2004; Darvishi *et al.* 2009).

Lipase was produced in a medium enriched with 1% olive oil and various nitrogen sources. The cultured medium were adjusted to pH 11, temperature 30°C, and timed for 12 hours in order to assess the impact of several nitrogen sources (Yeast Extract, Peptone, Casein, Ammonium Nitrate, and Potassium Nitrate) on lipase synthesis. A spectrophotometric technique was used to evaluate the lipase activity of the supernatant. Yeast extract, peptone, casein, and ammonium nitrate all showed lipase activities of 490.72, 445, 225.6, and 482 U/L, respectively, although potassium nitrate, the medium's nitrogen source, showed no activity at all (Ilesanmi *et al.*, 2020).

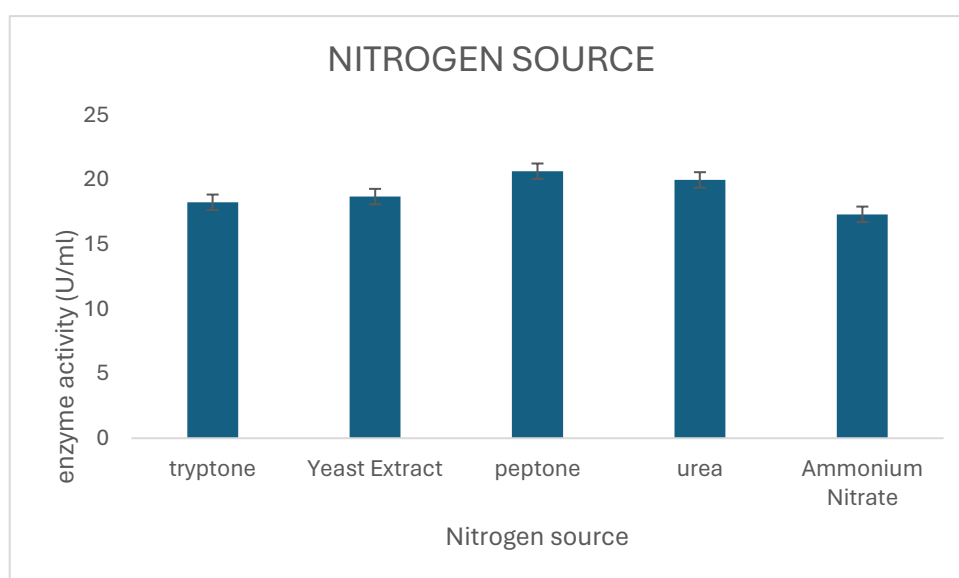


Fig: 5.13: Effect of nitrogen source on bacteria produced lipase.

5.4.5.2 Effect of nitrogen source on fungi produced lipase

Using peptone, yeast extract, tryptone, urea, and ammonium nitrate, the impact of the nitrogen supply on the lipase activity was investigated. The other optimal parameters-pH, temperature, and carbon source-remained constant. Comparing lipase activity from different sources, it was discovered that the yeast extract-incubated lipase had the maximum activity, at 4.359 U/ml. Yeast extract as nitrogen showed better enzyme activity compared to other source (Fig 5.14). peptone, meantime, exhibited somewhat successful activity.

Initial pH has a significant influence on the nitrogen source's effect. For lipase synthesis at pH 6.0, tryptone was the most effective nitrogen source, eliciting an activity of 47.02 u L^{-1} in cell-harboring supernatant and 36.98 u L^{-1} in cell-free supernatant. Lipase activity in the cell containing supernatant was slightly reduced (43.01 u L^{-1}) and lipase secretion was absent at pH 9.0 (Câmara Ribas *et al.*, 2019). Using meat extract as an organic nitrogen source resulted in the highest lipase activity. Compared to the reference (658.09 U/L), the enzyme activity in this instance (1226.03 U/L) increased by about 1.9 times. Dobrev and colleagues (2015).

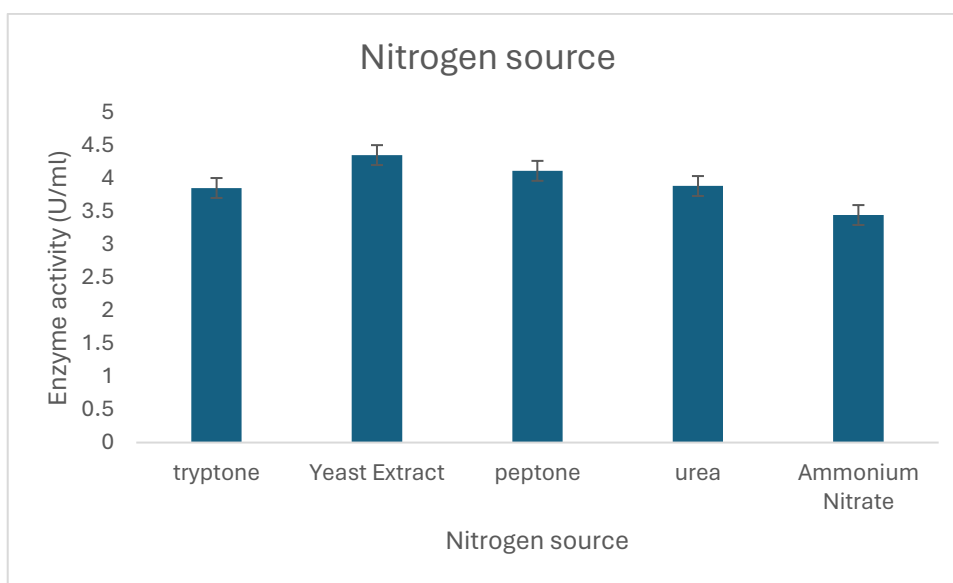


Fig: 5.14: Effect of nitrogen source on fungi produced lipase

5.5 LIPASE ACTIVITY USING SPECTROPHOTOMETRIC METHOD FOR CRUDE AND PURIFIED ENZYME

5.5.1 Enzyme activity of bacterial lipase enzyme

The enzymatic activity of the crude, precipitated ammonium sulfate, dialysis, and chromatography samples were all calibrated. The substrate used to measure the enzyme's activity was p-nitrophenyl palmitate. The substrate that was utilized in the investigation was quite successful in bringing the generated lipase's enzyme activity into uniformity.

After the growth in conditions including supplements for efficient growth, the crude supernatant was measured for activity. While the total activity was 2141.8 Units, the unit activity was determined to be 156.41 U/ml. Following column chromatography,

the unit activity of the partly purified lipase enzyme was determined to be 27.6119 U/ml. About 138.0595 Units was the total activity. While column chromatography using Sephadex G-100 improves the lipase enzyme's activity and stability, precipitation with ammonium sulphate and dialysis of the same did not significantly improve purification.

A study carried out by (Priyanka *et al.*, 2019) showed highest activity of lipase as 0.91 IU/mL Whereas the purified lipase of the present study showed effective activity of 97.61 U/ml.

Table 5.1: Purification of bacterial lipase enzyme by enzyme activity

Bacterial Sample	Enzyme volume ml	Absorbance	Unit activity u/ml	Total activity u
Supernatant	100	1.121	21.418	2141.8
Precipitate	5	0.855	11.716	58.58
Dialysis	5	0.757	10.09	50.45
Sephadex G-100	5	0.727	27.6119	138.0595

5.5.2 Enzyme activity of fungal lipase enzyme

The enzymatic activity of the crude, precipitated ammonium sulfate, dialysis, and chromatography samples were all calibrated. The substrate used to measure the enzyme's activity was p-nitrophenyl palmitate. The substrate that was utilized in the investigation was quite successful in bringing the generated lipase's enzyme activity into uniformity.

After the growth in conditions including supplements for efficient growth, the crude supernatant was measured for activity. While the total activity was 441.82 units, the unit activity was determined to be 4.418 U/ml. Following dialysis, the unit activity of the partly purified lipase enzyme was determined to be 1.09 U/ml. About 5.45 Units

was the total activity. Precipitation with ammonium sulphate and dialysis of the same did not significantly improve purification.

However, neither the fungus *Aspergillus sp.* F18 nor the fungus *Talaromyces sp.* F15, which were isolated from the *M. guianensis* root, demonstrated high enzymatic activity (0.37 U/ml) or 0.34 U/ml) (Ribeiro Lima *et al.*, 2019).

Table 5.2: Purification of fungal lipase enzyme by enzyme activity

Fungal Sample	Enzyme volume ml	Unit activity u/ml	Total activity u
Supernatant	100	4.418	441.8
Precipitate	5	3.716	18.58
Dialysis	5	1.09	5.45

5.6 COLUMN CHROMATOGRAPHY

The lipase enzyme's elution fraction and absorbance. The elution fractions 12, 13, and 14 had the highest absorbance, according to the observation. After the three fractions were combined, 138.05 U of total activity was discovered. Following the further elution, a gradual decline in activity was noted. The lipase enzyme was successfully purified with high activity and stability utilizing column chromatography using Sephadex G-100 as the stationary phase, as opposed to the dialyzed enzyme. A report on thermostable lipases from the gram-positive thermophilic bacterium *Bacillus thermoleovorans* ID-1, showed lowest total activity after the purification with column chromatography whereas the present study has effective readings (D. W. Lee *et al.*, 2001).



Fig: 5.15: Ammonium sulphate precipitation



Fig: 5.16: Dialysis



Fig: 5.17: Column chromatography

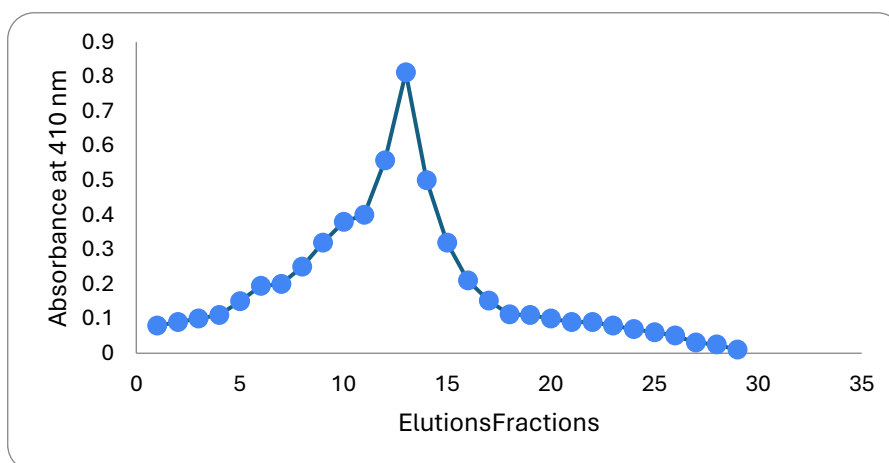


Fig: 5.18: Absorbance at 410 nm of fractions of column chromatography of the dialyzed lipase.

5.7 SDS PAGE

Following chromatography, the molecular weight of the partially purified sample was determined to be between 150 and 160 kDa. The purified lipase enzyme used in this investigation had a molecular weight of about 155 kDa, but the crude sample showed a wide range of bands. The MW of the enzyme was also determined by SDS- PAGE using the same marker proteins and found to be almost same as that found by gel filtration 87 KDa (M. a Islam *et al.*, 2009). Determination of molecular mass of the partially purified enzyme was carried out by SDS-PAGE. Result of this application, the enzyme revealed one band as 110.5 kDa (YasemIn, 2017).

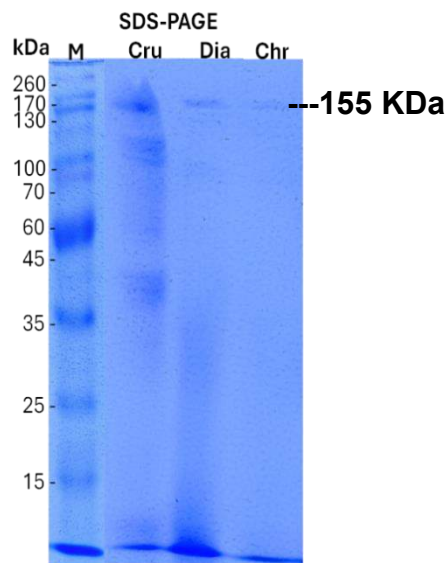


Fig: 5.19: SDS-PAGE (Lane-1) Marker, (Lane-2) Crude enzyme, (Lane-3) Dialyzed enzyme, (Lane-4) Column chromatography purified enzyme.

5.8 CHARACTERIZATION OF LIPASE

5.8.1 Effect of metal ions and detergent

5.8.1.1 Bacterial lipase

Several metal ions have been shown to cause the fatty acids at the oil–water interface to form their corresponding metal salts, freeing up lipase to act on oil molecules. At both the 1 mM and 10 mM concentrations, the lipase activity and stability were significantly enhanced by the presence of Ca^{2+} as shown in (fig 5.20). Ca^{2+} has been shown to be essential for the conformational stability of the enzyme. Compared to other metals, Ca^{2+} significantly increases the activation of pseudomonas sp (Joseph, Ramteke, and Thomas 2008). By creating a salt bridge and altering the lipase's conformational shape, the metal ion gives the enzyme more stability.

Surfactants can activate lipases in molecular bioimprinting and are essential for the multifunctionality and variety of features of enzymes. According to Holmberg (2017), the presence of non-ionic surfactants usually has a stronger positive impact on lipase than that of anionic or cationic surfactants. Tween 80 should have more stability than other detergents as shown in (Fig 5.21) . Tween 20 had an impact on

the stability of the purified lipase, in contrast to the findings published by (Priyanka, Kinsella, *et al.*, 2019). Next to SDS, Triton X 100 demonstrated superior stability.

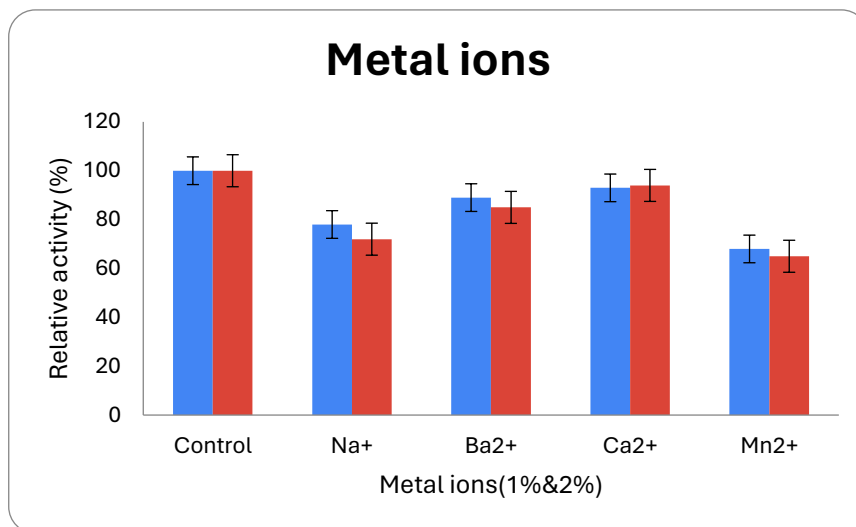


Fig. 5.20: Effect of metal ions (bacterial lipase).

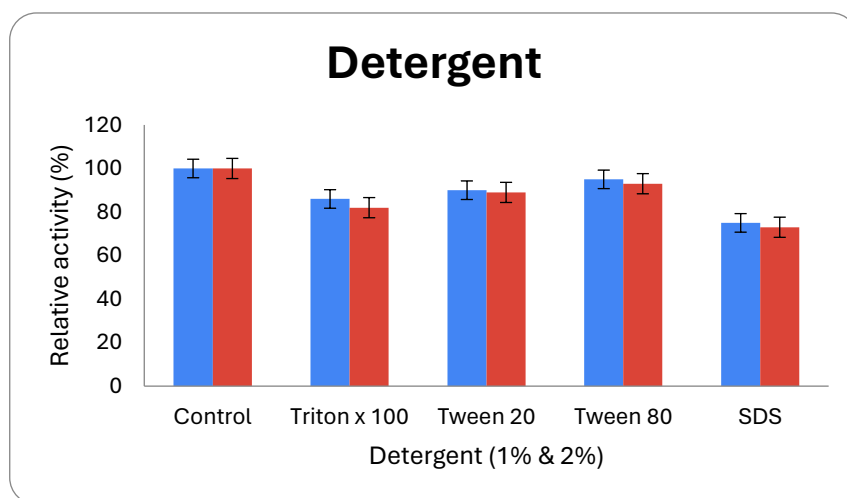


Fig. 5.21: Effect of detergent (bacterial lipase).

5.8.1.2 Fungal lipase

Several metal ions have been shown to cause the fatty acids at the oil–water interface to form their corresponding metal salts, freeing up lipase to act on oil molecules. At both the 1 mM and 10 mM concentrations, the lipase activity and stability were significantly not much effected by the presence of Ca²⁺. Other metals found to affect the relative stability of the partially purified enzyme.

The effect of various detergent on the enzyme activity and stability was analysed. At both the 1 mM and 10 mM concentrations, the lipase activity and stability were significantly barely affected by the presence of Tween 20. Other detergents found to affect the relative stability of the partially purified enzyme.

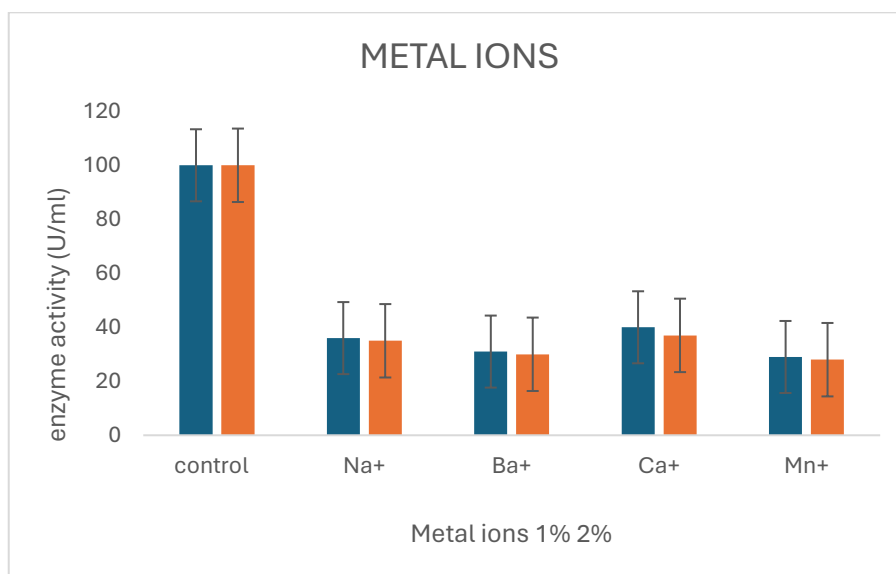


Fig: 5.22: Effect of metal ions (fungal lipase)

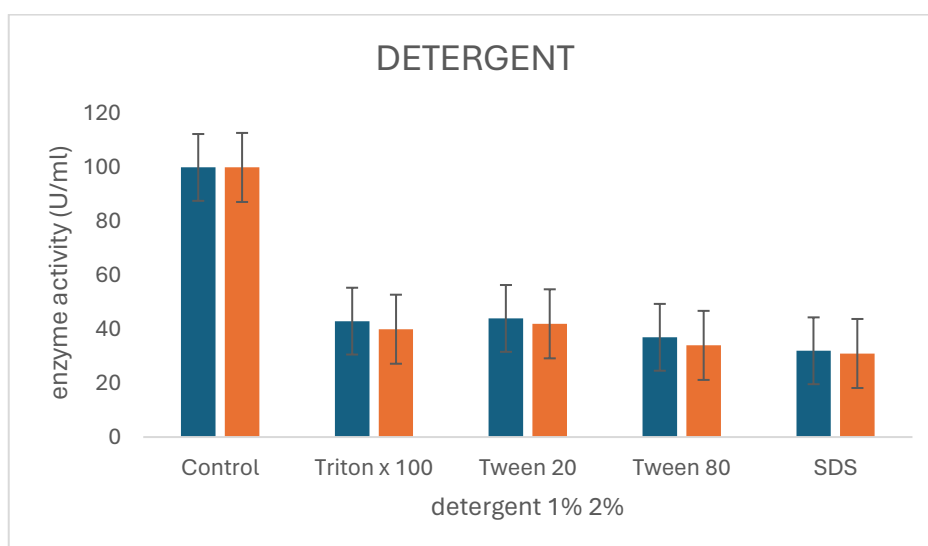


Fig: 5.23: Effect of detergent (fungal lipase)

5.9 APPLICATION

The bacteria was able to degrade 12.69% crude oil to compared to 2.44% biodegradation in control at the end of 28 days.

The study shows that *Pseudomonas aeruginosa* shows higher percentage of degradation compared to *B. subtilis* suggesting that *P. aeruginosa* is a more efficient degrader of crude oil when compared to *B. subtilis* was reported 65% degradation of crude oil sample treated with *Pseudomonas sp.* and 40% degradation with crude oil sample treated with *Bacillus sp* (Abubakar *et al.*, 2024)

CHAPTER-6

SUMMARY AND CONCLUSION

The investigation shown that altered media composition and purification techniques might produce lipase effectively. Using olive oil as a substrate would be very beneficial for producing lipase efficiently. After partial purification, the total activity was approximately 138.0595 U/mg without any additional activity loss. High activity optimization was used to produce lipase and enzyme characterisation research confirmed stability and increased activity. Comparatively, bacterial isolate had better enzyme activity and stability compared to the fungal isolate. The enzyme produced by these organisms at a particular optimal activity, showed potential in biodegradation of hydrocarbon contaminated area. The percentage crude oil degradation revealed further that the lipase produced by culture could be used for the biodegradation of crude oil. The bacteria was able to degrade 12.69% crude oil to compared to 2.44% biodegradation in control at the end of 28 days. Comparatively, the bacterial lipase showed better activity and stability. Thus, this study of extracellular lipase would be effective in the application of oil degradation.

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