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ISOLATION, SCREENING, AND IDENTIFICATION OF BIOCATALYST FROM BACTERIA AND FUNGI IN OIL POLLUTED SOIL

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Abstract: **The continuous activities of companies that deal with substances such as hydrocarbons and their derivatives in the environment are the primary source of pollution in our ecosystem, and they must be eliminated and managed. Biocatalysts secreted by *Saccharomyces cerevisiae, Bacillus cereus, and Pseudomonas aeruginosa* were identified and characterized in this study. The oil-polluted environment is an ideal habitat for isolating lipase-producing microorganisms. At the University of Port Harcourt, Aluu, and Rupkokwu were chosen as case studies because of their distinctive oily environments for isolating lipase-producing microorganisms. The goal of this investigation was to isolate, screen, identify, and characterize lipase-producing fungi and bacteria from oil-contaminated soil. While screening, inoculation on the spot for primary screening, and Lipase synthesis in submerged fermentation were used. To test the effect of temperature on lipase activity, the reaction was carried out at temperatures ranging from 10 to 90 °C. *Pseudomonas aeruginosa* produced the most lipase, followed by *Bacillus cereus*, with a minor amount of lipase in the shape of a zone around the *Saccharomyces cerevisiae* colony. Pseud*omonas aeruginosa* exhibited the greatest enzyme activity of 17.19 U/L after 72 hours of inoculation into the culture medium, and the biocatalyst producing temperature conditions were maximized at 37°C. The enzyme activities of the three isolates were 0.5 U/L after 96 hours of inoculation. The lipase was extremely stable in terms of temperature and organic solvent. This one-of-a-kind feature makes it appealing and practical for industrial applications.**

Keywords: ***Bacillus cereus*, Biocatalyst, Biomaterial, Environment, Enzymes, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*.**

I. INTRODUCTION

Biocatalysts are natural materials that include enzymes derived from biological sources, dead or living microorganisms, derivatives, and entire cells. Chemical reactions can be sped up by using biocatalysts [1]. Enzymes accelerate reactions between reactants and products by being particular and exact in their activities. They also use very little or no energy to do so. Biocatalysts with distinct properties are being developed to replace costly chemical catalysts with toxic side effects being environmentally unfriendly. Biocatalysts are enzymes. Enzymes are proteins; they are denatured by heat and extracted or precipitated with chemical solvents like ethanol and by inorganic salts such as ammonium sulphate [2]. Coenzymes are also proteins that join with low molecular mass organics. Proteins are large, complex molecules that play a number of important tasks in the human body. They are responsible for the structure, function, and regulation of the body's tissues and organs and work in cells. They have the ability to perform a wide range of tasks. Apart from these functions, proteins can also act as biocatalysts and biomaterials. Some of the examples of protein function are Transport, Storage, Enzyme, Messenger Antibody Enzyme, Messenger and Structural Component. In several industrial processes, enzymes are gradually replacing the usage of harsh chemicals. They provide an economical and energy-efficient tool for

future industrial applications [3]. Biocatalysts have recently gotten a lot of attention because of their numerous industrial applications in domains like clinical analysis, organic synthesis, medicines, detergents, food manufacturing, remediation, oil and gas, and fermentation.

Rapid and reliable methods for the isolation, screening, and identification of relevant biocatalysts have become more important as approaches for rational or evolutionary design of novel or modified enzymes have advanced. The basic requirements for the construction of a biocatalyst are selectivity, technology integration, security of supply, and volume efficiency, as reported by [4]. Extracellular and intracellular enzymes are two types of enzymes found in microorganisms. If complete microbial cells can be immobilized directly, extraction and purifying steps can be skipped, resulting in minimal loss of intracellular enzyme function. When cells are immobilized on a solid support, they act as a solid catalyst. For the immobilization of entire microbial cells, three approaches are available: carrier binding, cross-linking, and entrapment. The protein content of SCP (Single Cell Protein) is extremely high. Dried cells of *Pseudomonas* sp. full-grown on normal petroleum-based liquid paraffin contain 69% protein; algae generally possess about 40% protein [5]. The protein content of SCP is entirely dependent on the carbon source employed and the microorganisms that are cultivated in the media.

Proteins have very narrow stability ranges, outside of which they denaturize. Furthermore, degradative enzymes such as proteases and lipases can alter the structure of proteins, resulting in significant changes in their activity and antigenic characteristics. The physicochemical parameters of a biocatalyst are determined during biocatalyst characterization. It entails a series of laboratory tests aimed at determining the impact of temperature variations on enzyme activity, the optimum temperature for its operations, appropriate pH, minimum concentration, enzyme kinetics (rate), thermostability, primary structure, and family members. Environmental pollution through hydrocarbons, chemicals, solvents, and heavy metals are severe issues that the current world is facing. They are detrimental to living organisms, including human beings, and also contribute to economic losses in developing countries [6]. Not all species can adapt to a variety of environments, but they do so in specific environments based on their biotic and abiotic characteristics. Microorganisms, on the other hand, are found everywhere; they have inhabited a wide range of environments for thousands of years, even some of which are deemed "extreme." However, oil pollutes the soil due to a lack of suitable disposal treatment facilities and methods. The physiochemical characteristics of this soil, particularly its oil content, play an essential role in developing its microbial diversity of interest.

Lipases are the biocatalyst of interest in this study. Lipases are hydrolytic biocatalysts in nature that catalyze fats and oils into fatty acids and glycerol by breaking them down. Lipases (E.C. 3.1.1.3) are the subject of dynamic studies on account of their multi-usage. Novel microbial lipases are used in a variety of disciplines, including medicine, biotechnology, oil and gas, detergents, and bioremediation. Nonetheless, there is still a lot of interest in creating new microbial lipases [7, 8, 9]. Expectedly, economical and environmentally friendly biocatalysts are preferred, which supports the suggestion of using microorganism-derived biocatalysts. These biocatalysts are known to be non-toxic, have a lower extraction cost, and are easily recovered during the refining process. In addition, lipase-producing microorganisms (fungi and bacteria) have been found in a variety of environments, including industrial waste, compost heaps, oil-contaminated soil, oilseeds, degraded food, vegetable oil processing plants, and dairy products [10]. Numerous fungal and bacterial enzymes are used in diverse food industrial processes, and *Bacillus cereus, Pseudomonas aeruginosa, and Saccharomyces cerevisiae* are effective lipase producers [11]. Enzymatic systems among microorganisms, such as those found in fungi and bacteria, play an important role in the biodegradation of undesirable substances such as oils. Although bacteria and fungi have long been known in oil-contaminated environments, their role has been uncertain for a number of reasons [12, 13]. Commercially, microbial enzymes are highly received due to many characteristics, such as genetic modifiability, production quantity, and inexpensive production costs. Several approaches for detecting lipase production have been described. Lipases generated by filamentous fungus and bacteria are extracellular, making extraction and purification relatively simple. As established by [14], lipase can catalyze a range of substrates. Many micro-organisms produce lipases, such as *Bacillus subtitles, Bacillus licheniformus, Streptomyoces sp., Bacillus cereus, Pseudomonas aeruginosa, Saccharomyces cerevisiae, Asperillus Niger.* Free lipases are very robust and stable at pH values of 3.5-9.5 and denature at a temperature between 50^{o}C and 60^{o}C.

Microbial fermentation and enzyme-catalyzed in-vitro processes are used to develop biosurfactants. Chemical composition, molecular weight, and source are used to classify them [15]. According to [16], two bacteria were isolated, and the qualities of the biosurfactant produced by these two bacteria were investigated, and it was discovered that when

added to a waxy crude mixture, the pour point was considerably decreased. [17] used certain biobased surfactants to evaluate their capabilities for emulsification and to investigate their antimicrobial effectiveness using the following methods: Surfactant and fatty acyl glutamic acid are investigated by lipase with fatty acids as a substrate. The percentage of conversion was found to be dependent on the concentration of the reaction substrate. Biobased surfactants are environmentally favorable, but the price and supply of crude oil are also important considerations. Pseudomonas lipase reduced the viscosity of crude and the interfacial tension of oil, water, and sand by using waste oil and crude. The fermentation method increased oil recovery, emulsifying activity, and liquid paraffin stability.

II. METHODOLOGY

2.1 Soil sample collection

*O*il-polluted samples were collected near the University of Port Harcourt, Aluu, and Rupkokwu for this investigation. All of the samples were collected and transported to the laboratory in sterilized polythene bags with a sterilized spatula. All of the samples were then mixed to create a composite sample for microbe isolation and biocatalyst development.

2.2 *Equipment and Materials list*

Please see Table 1.

2.3 Lipase-producing fungi and bacteria isolation

In a 250 mL Erlenmeyer flask containing 100 mL sterile physiological saline, one gram of samples (from the composite) were suspended. This was followed by 30 minutes of continual and active stirring at 120 rpm to break up dirt clumps before letting it to rest. The supernatant was decanted and used to make a 10fold serial dilution. 0.1 ml of 10^{-2} and 10^{-3} dilutions were cultured on Potato Dextrose Agar (PDA) at room temperature and Nutrient Agar (NA) incubated at 37^{o}C.

Preparation of mineral medium for the isolation of micro-organisms

Steps:

[1] For fungi (*Aspergillus niger and Saccharomyces cerevisiae*), dissolve 3.9g of Potato Dextrose Agar (PDA) in 100ml Erlenmeyer flasks of distilled water.

[2] Add the following: CaCl2 =0.25, NaH2PO4 =12, KH2PO4 =2, MgSO4 =0.3, $(NH4)_2SO_{4=}$1%.

[3] For *Bacillus cereus and Pseudomonas aeruginosa*, dissolve 2.8g of Nutrient Agar (NA) in 100ml of distilled water in Erlenmeyer flasks.

[4] The contents were autoclaved at 121°C for 15 minutes to sterilize them.

[5] The sterilized medium is poured and sprayed onto plates after cooling.

[6] In sterile test tubes, dissolve 1g of soil sample in 10ml saline water, then inoculate with spores at various dilutions (10^5/mL) and label accordingly.

[7] Seal the plate with plastic wrap and incubate at 30^{o}C for 7 days for fungi and yeast, and 3 days for *Bacillus cereus, Pseudomonas aeruginosa* at room temperature.

Plate to Plate Sub-Culture

[1] Only some bacteria are allowed to grow on selective medium.

[2] In Erlenmeyer flasks for *Bacillus and Pseudomonas*, dilute 2.8g of Nutrient Agar (NA) in 100ml of distilled water.

[3] .Add the following: CaCl2 =0.25, NaH2PO4 =12, KH2PO4 =2, MgSO4 =0.3, $(NH4)_2SO_{4=}$1%

[4] The contents were autoclaved at 121°C for 15 minutes to sterilize them.

[5] After cooling, the sterilized medium pours and sprays over the plates,

[6] Inoculate the organism and allow it to grow.

[7] Seal the plate with plastic wrap and incubate at 37^{o}C for 3 days.

Plate to Plate Sub-Culture

[1] Selective media are used to grow only certain types of fungi.

[2] For fungi, dissolve 3.9g of Potato Dextrose Agar (PNA) in 100 ml of distilled water into Erlenmeyer flasks (*Saccharomyces cerevisiae*, *Aspergillus Niger*).

[3] Add the following: NaH_2PO_4 =12, KH_2PO_4= 2, $MgSO_4$=0.3 $CaCl_2$ =0.25.$(NH_4)_2SO_{4=}$ 1 %

[4] The contents were autoclaved at 121°C for 15 minutes to sterilize them.

[5] After cooling, the sterilized medium pours and sprays over the plates,

[6] Seal the plate and incubate at 30°C for 7 days.

2.4 Innoculation on the Spot for Primary Screening

On Agar plates, lipase-producing microbes were screened using tributyrin as a substrate. Tributyrin Agar of the following composition was used for this purpose: 15.0 gm Agar Agar; 10.0 ml Tributyrin (Glycerol Tributyrate); 990 ml Distilled water; pH: 7.5 Please read Figure 1 to 4 for further information.

We took the following steps:

[1] Only specific media are employed for the development of microorganisms inoculated at a particular spot.

[2] Diluted 2.8g of Nutrient Agar (NA) in 100ml of distilled water in Erlenmeyer flasks for *Bacillus and Pseudomonas***.**

[3] Dissolved 3.9g of Potato Dextrose Agar (PNA) in 100ml Erlenmeyer flasks of distilled water for fungi (*Saccharomyces cerevisiae*, *Aspergillus Niger).*

[4] Added the following: 10ml of substrate(tributyrin),NaH_2PO_4 =12, KH_2PO_4= 2, $MgSO_4$·=0.3 $CaCl_2$ =0.25.$(NH_4)_2SO_{4=}$ 1 %

[7] The contents were autoclaved at 121°C for 15 minutes to sterilize them.

[8] After cooling, the sterilized medium pours and sprays over the plates,

[9] Inoculate the organism at a particular spot and allow it to grow.

[10] Seal the plate with plastic wrap and incubate at 37°C for nutrient agar, PDA at room temperature for 24 hours, 48 hours, 72 hours, and 96 hours and measure the zone of inhibition.

Lipase production is indicated by the formation of a clear zone around the colonies.

2.5 Lipase production in submerged fermentation (secondary screening)

Inoculate 20 mL of inoculum into 500-mL Erlenmeyer flasks containing 180 mL of medium and incubate at 30 °C for bacteria at 120 rpm for 24 hours, 48 hours, 72 hours, and 96 hours, and at 37 °C for fungi at 120 rpm for 24 hours, 48 hours, 72 hours, and 96 hours. Mycelium was harvested by filtration under vacuum and centrifuged for 20 minutes at 4000 rpm. Extracellular enzymes were recovered from the cleared supernatant. Centrifugation at 4000 rpm for 20 minutes separated yeast and bacterium culture samples

2.6 Bacteria and fungi identification from screening

Five vigorous fungal and bacterial cultures were used for further investigation, and they were identified by microscopic analysis using the Lactophenol cotton blue staining method, which involved adding a drop of 70% ethanol to a clean microscopic glass slide, then using a sterile mounter to add a fungal specimen to the drop of alcohol.To ensure that the fungus sample combines effectively with the alcohol, use a needle mounter to tease it from the alcohol. Before the ethanol dries up, add one or two drops of Lactophenol Cotton Blue Solution with a dropper or pipette. Then, without producing any air bubbles, carefully cover the stain with a clean, sterile coverslip. Microscopically examine the stain at a magnification of 40X to look for fungal spores and other fungal structures. Fungi were kept on PDA (Potato Dextrose Agar) medium, while bacteria were kept on NA medium (Nutrient Agar) after performing a gram stain and then observing the colony appearance and individual features. Laboratory instructions were used to identify the specified fungal and bacterial cultures.

2.7 The Influence of Temperature

The reaction was carried out at various temperatures ranging from 10 to 90 °C to determine the effect of temperature on lipase activity. Before beginning the experiment, the crude enzyme, and substrate were incubated at various reaction temperatures, and the enzyme assay was performed as described earlier to determine the optimal incubation temperature.

III. RESULTS AND DISCUSSION

Isolation, screening, and identification of lipase-producing *Saccharomyces cerevisiae, Bacillus cereus, and Pseudomonas aeruginosa* from oil-polluted soil in Rupkokwu, Aluu, and near the University of Port Harcourt were performed in the current study. The soil samples were produced using normal methods from an oil-contaminated soil, and a composite sample was prepared for isolation. Two hundred and twenty plates of the organism were isolated using the standard approach, but only six were discovered to produce lipase (Figure 1 to 4). The isolated cultures were kept on Potato Dextrose Agar (PDA) for *Saccharomyces cerevisiae* (Figure 4) and *Aspergillus niger* (Figure 2), and Nutrient Agar (NA) for *Bacillus cereus* and *Pseudomonas aeruginosa* (Figure 1 and 3).

The clean zone around the colony was seen using Tributyrin Agar Medium for the primary screening. as shown in Figure 5. Maximum lipase production was observed in *Pseudomonas aeruginosa*, followed by *Bacillus cereus* . *Aspergillus niger* did not produce lipase (Figure 2), while a small amount of lipase in the form of a zone (6 mm) was observed around the *Saccharomyces cerevisiae* colony. As shown in the Figure 5, after the fourth day of incubation, *Pseudomonas aeruginosa* (50mm) produced the most lipase, while A. niger (0mm) produced none.

In the current study, maximum lipase synthesis was reported after 3 days of incubation in *Pseudomonas aeruginosa and Bacillus cereus*. Because of its pH stability, thermal stability, substrate activity in organic solvents, and selectivity. lipase from microbes has been exploited as a useful resource [18].

Previous studies have shown that soil contaminated with oils has a diverse microbial diversity. These microorganisms are being studied for their ability to produce lipase. Lipases are found in a wide range of species, including plants, animals, and microbes.

Lipase production varies per species for characteristics such as optimum temperature, optimum pH, and enzyme specificity, according to [19]. Hydrolysis, aminolysis inter-esterification, alcoholysis, esterification, and acidolysis are among the activities that this group of enzymes performs and catalyzes [20].

The mean lipolytic activity of *Pseudomonas aeruginosa* (plate4.3.) and *Bacillus cereus* were approximately 5 and 4.5 times higher than that of *Saccharomyces cerevisiae*, respectively. It could be assumed that the bacterial strain could efficiently oil both for enzyme synthesis and biomass production, whereas the test *Apsergillus niger* demonstrated neither low levels of lipase activity nor high biomass production. Lipase production was absent in some *Pseudomonas aeruginosa, Bacillus cereus,* and *Saccharomyces cerevisia* strains. Please refer to Figure 1 to 4. Lipase production by the test microorganisms revealed significant differences in lipolytic activity.

3.1 *The consequences of temperature on the generation of lipase*

Temperature is a critical parameter that must be controlled, and it varies from organism to organism. From 10°C to 90°C, the optimal temperature for lipase production and bacterial growth was investigated. After 24 hours of culture, 37°C was found to be the optimum temperature for both lipase production and bacterial growth in spectrophotometer readings for lipase assay (Figure 6 to 8). The absorbance at 580nm was measured and converted to enzyme units using Lambert's rule of enzyme activity calculation.

The temperature was found to influence extracellular enzyme secretion, possibly by changing the physical properties of the cell membrane, resulting in lower growth of activity. It is well known that at higher temperatures, protein conformation changes or is degraded, resulting in a decrease in lipase activity, as shown in figure 4.5.The three lipase-producing microorganisms produced lipase at the optimal temperature of 37°C.

The effects of temperature on crude lipase stability were investigated in this study, as shown in Figures 5 to 8. Incubation at temperatures ranging from 40°C to 90°C was found to reduce lipase production, with no lipase activity detected at 90°C.

After 72 hours of inoculation into the culture medium, *Pseudomonas aeruginosa* had the highest enzyme activity of 17.19 U/L and the biocatalyst producing temperature conditions were optimized at 37°C. After 96 hours of inoculation, the enzyme activities of the three isolates were 0.5 U/L.

IV. FIGURES AND TABLES

Table 1: List of Equipment and Materials

S/NO	Equipment/Material
1	Cultures: *Bacillus cereus,Pseudomonas Aeruginosa,Saccharomyces cerevisiae, Aspergillus Niger*
2	Potato dextrose agar PDA, Nutrient agar NA, Nutrient Broth, Sterilized plate for isolation of micro-organism (plate 1)
3	NaH_2PO_4 =12, KH_2PO_4= 2, $MgSO_4$·=0.3, $CaCl_2$ =0.25,.$(NH_4)_2SO_{4=}$ 1 %.
4	Glucose, Olive oil, Filter paper, Ethanol(absolute), Cotton wool, Nutrient broth, Foil paper Electronic Centrifuge , Autoclave pot
5	Gas, Generator, and Fuel

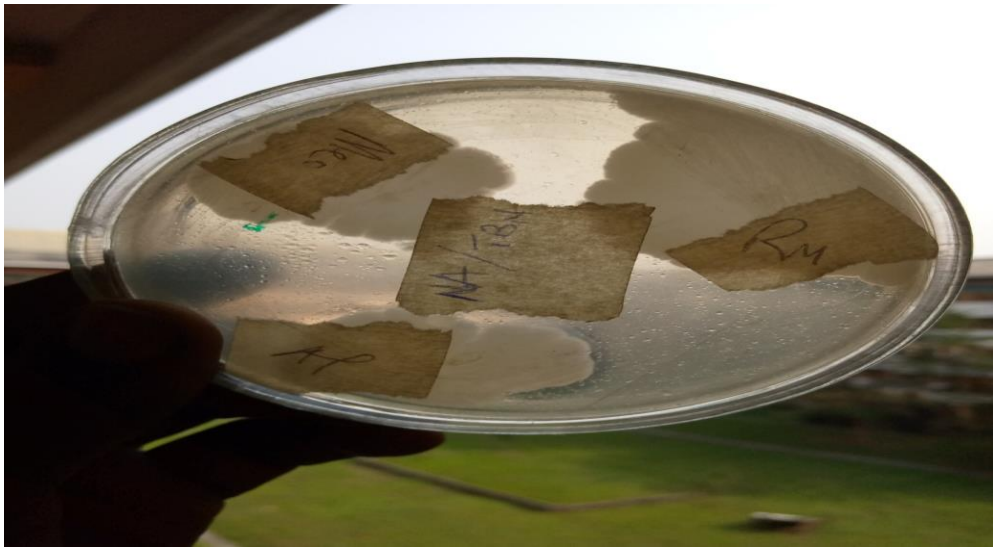

Fig.1: Growth of microorganism *Bacillus cereus* NA+ Substrate with clear zone

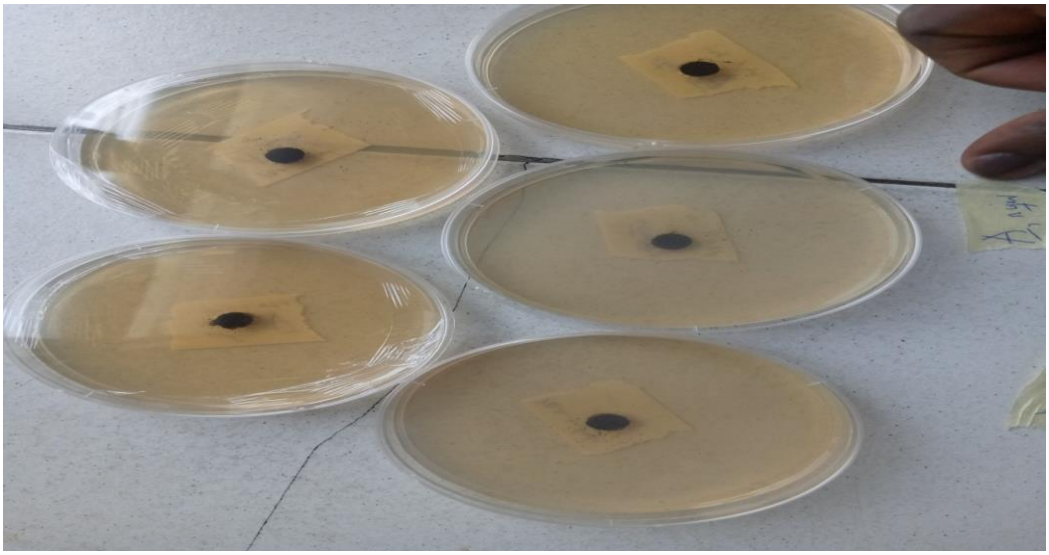

Fig.2: *Apsergillus niger* Sub culture in plate with no zone of inhibition

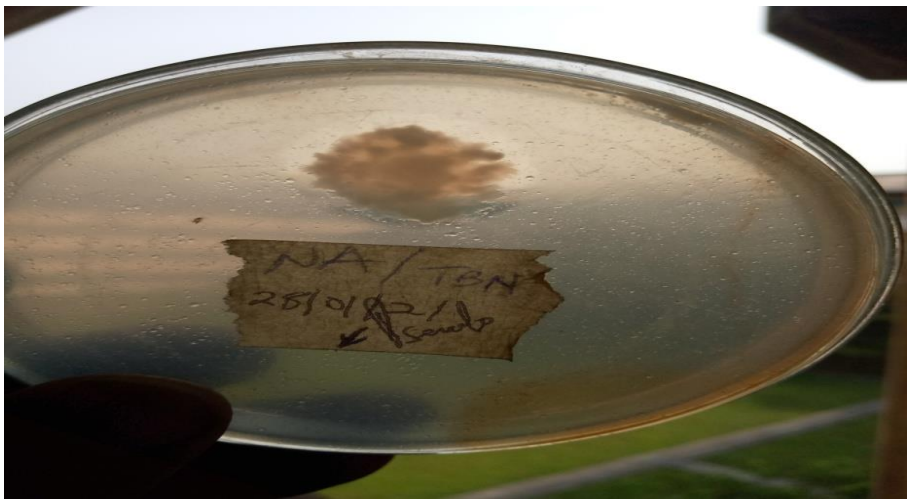

Fig.3: *Pseudomonas aeruginosa* + NA+ Substrate with clear zone

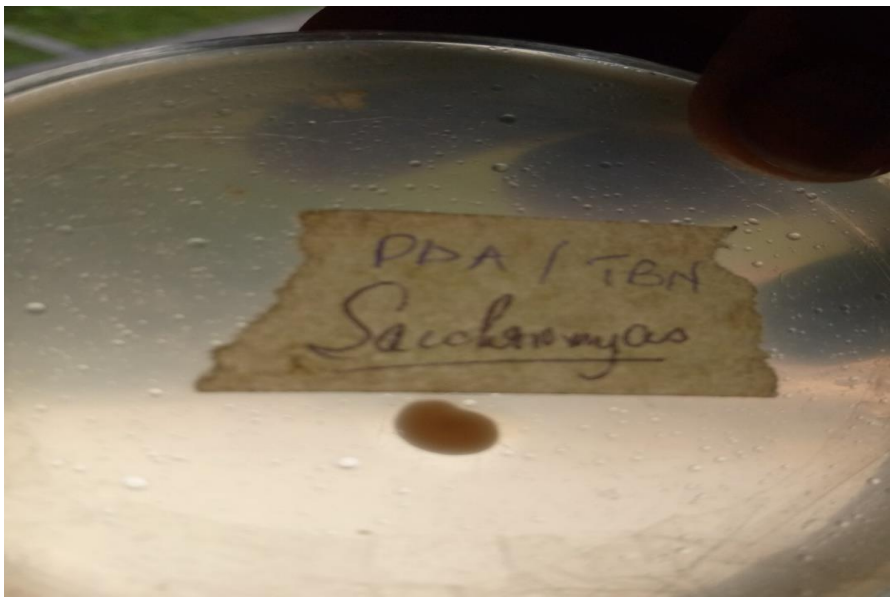

Fig 4: PDA plate+ *Saccharomyces cerevisiae*+ Substrate after 24hours showing zone of inhibition

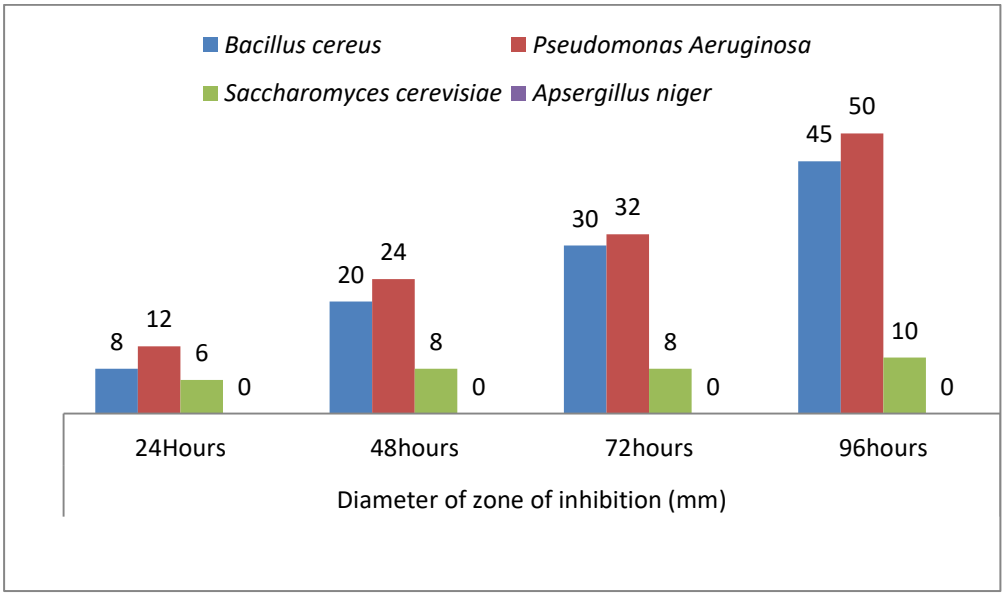

Fig.5: A comparative graph of zone of clearance against different microorganisms

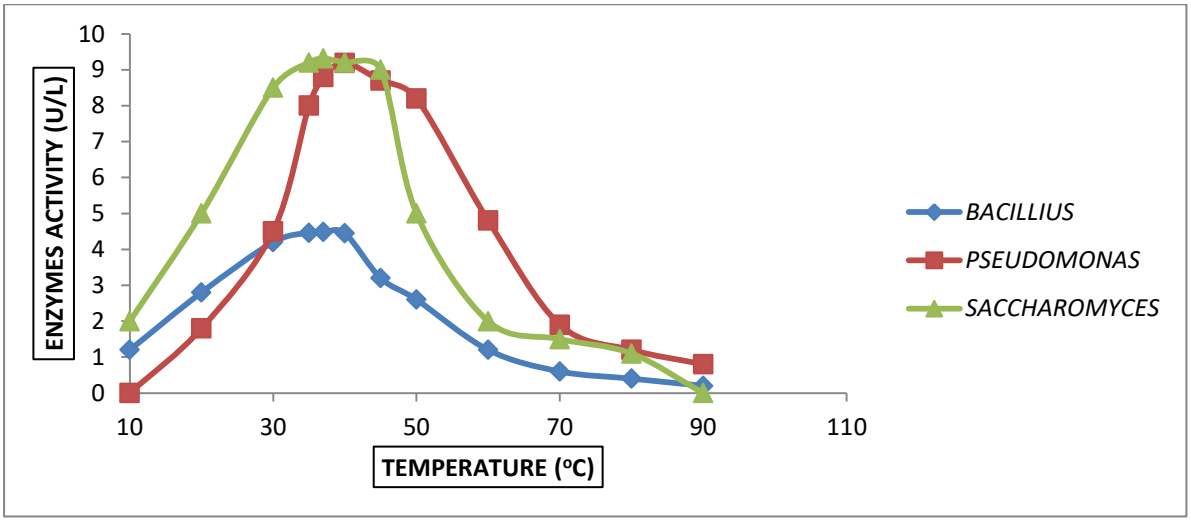

Fig 6: Enzyme Activity Vs Temperature °C lipase assay after 24 hours and trend

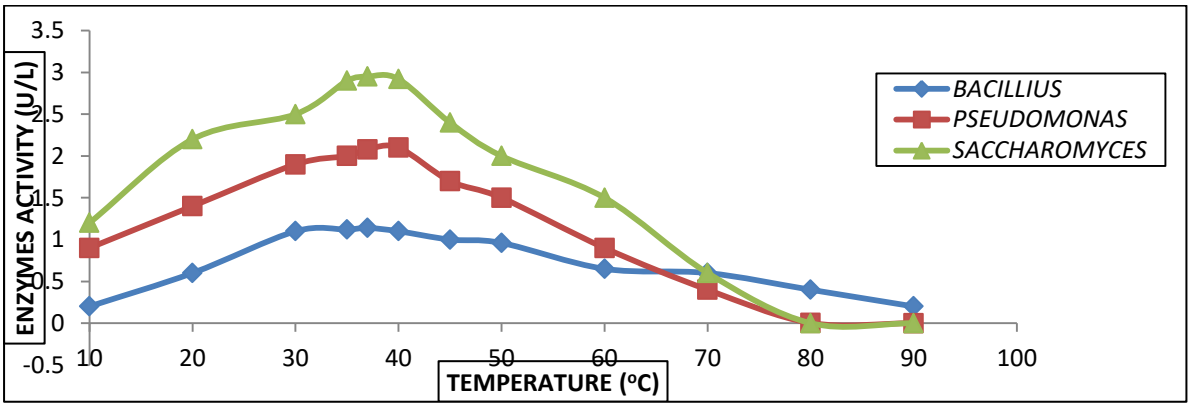

Fig 7: Enzyme Activity Vs Temperature °C assays after 48 hours of inoculation in broth

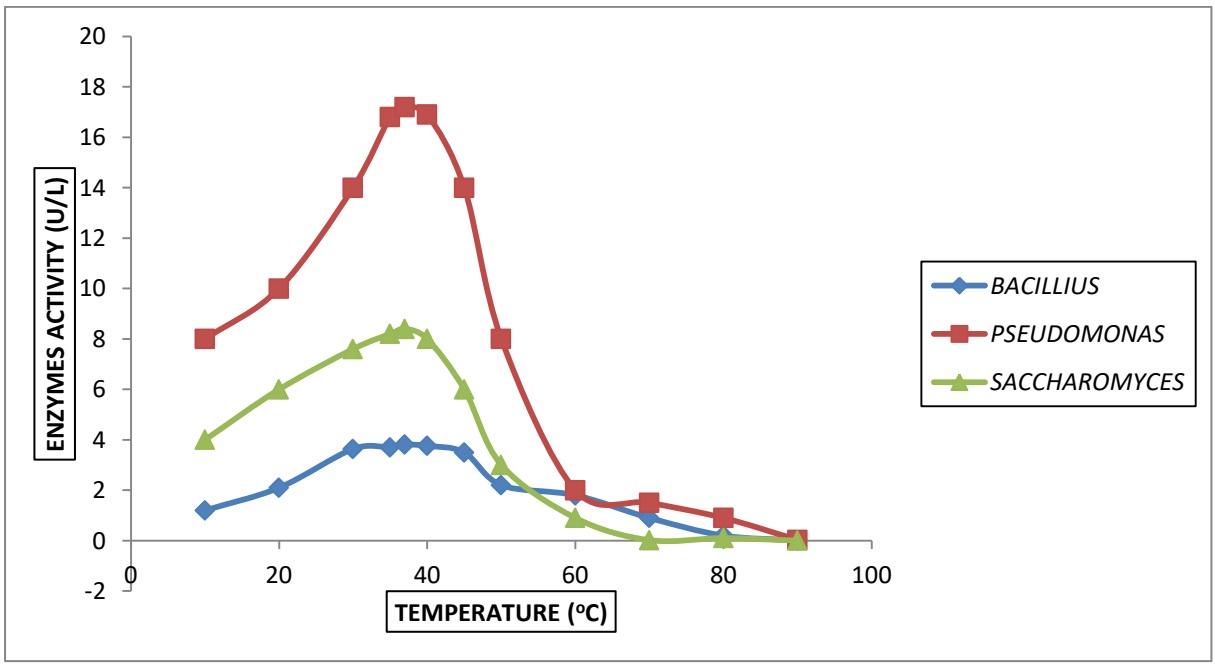

Fig 8: Enzyme Activity Vs Temperature °C after 72 hours of inoculation in broth

V. CONCLUSION

Due to urbanization and industrialization, the accumulation of environmental pollution in the ecosystem has increased dramatically over the past decades. Lipases are universal biocatalysts with important biological and industrial potential. So, research findings on isolation, screening, identification, assay, and production of the enzyme from microbial sources are critical from the standpoints of research, the environment, and commerce. The processing of biocatalysts has a bright future. *Pseudomonas aeruginosa* was the best producer of extracellular lipase after three days of incubation and its synthesis was induced by the presence of a lipid source with high enzyme activity (17.19U/L) at a low cost. More research is needed to enhance the fermentation process in order to achieve increased lipase production with this strain, and more microorganisms and other sources of lipase production should be investigated, despite the fact that these bacteria are usually acknowledged as safe for food, oil and gas sectors, bioremediation, brewing, and pharmaceutical applications.

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