ISSN 2278-2540 | DOI: 10.51583/IJLTEMAS | Volume XV, Issue V, May 2026

Comparative Studies on Lipase Production and Optimization using Bacillus Subtilis and Pseudomonas Aeruginosa

Dr. C. Vinothini[1], Dr. A. Chanthru[2]

[1] Assistant Professor, Department of Biotechnology, E.G.S. Pillay Arts and Science College, Nagapatinam.

[2]Head of the Department of Microbiology, E. G. S. Pillay Arts and Science College, Nagapatinam

DOI: https://doi.org/10.51583/IJLTEMAS.2026.150500209

Received: 14 May 2026; Accepted: 19 May 2026; Published: 15 June 2026

ABSTRACT

The presents study deals with screening of lipase producers from the lipase rich environment like petrol bank, Auto mobile shops from soil sample. The micro organisms have the ability to produce the lot amount of lipase. The sample was collected from (petrol bank, Auto mobile shops). Tributyrin agar medium was prepared 14 strains were isolated from that 2 strains able to produce lipase. The bacterial strain confirmed by biochemical method. The isolated bacterial strain are Bacillus sp. and Pseudomonas sp. The bacterial strains need temperature around 35^0 c, PH 7, olive oil as a carbon source and Incubation for 48hr. If the temperature is higher the enzyme productivity is also high. Thin layer chromatography of lipids revealed that bacillus sp has higher lipase activity than pseudomonas sp.

The present study revealed that extracellular lipase production by
Bacillus sp. and Pseudomonas sp. isolated from soil samples was found to be accelerated at optimized culture conditions such as medium pit, temperature and carbon source. From the result, it could be concluded that the medium pH of 7.0 and temperature 35^0c were optimum for maximizing lipase production by Bacillus sp. and Pseudomonas sp. Inferred that the optimum contain based on this study lipase activity is higher in Bacillus sp. and Pseudomonas sp. than compare other 12 strains.

Keywords: Lipase,Enzyme productivity,Optimization,Pseudomonas sp

INTRODUCTION

Enzymes are biomolecules that catalyze (i.e. Increase that rate of) chemical reactions. Almost all enzymes are proteins in nature. A very important property of an enzyme in its specificity and selectivity.The biological relevance and variability of lipids described above has led to the development of a great variety by lipid – degrading enzymes throughout all kingdoms of life. Among them, lipase are hydrolytic enzymes that catalyses the cleavage of ester bonds in triglycerides and producing glycerol and free fatty acids, are considered to be some of the most important biocatalysts due to their widespread biological functions and due to their biotechnological potential(Bornscheuer, 2002).

Lipases are widely distributed among bacteria, fungi, plants and animal , although they are more frequently found in microorganisms .Their physiological functions are not yet clear for many of them, although they seem to be involved, in general, in the bioconversions of lipids (mainly TAGs) between different organism or into the same organism (Pandey et al., 1999).If bacteria source bacteria is the most important source for lipase. They are many lipase productions bacteria are Bacillus, Bacillus subtitis, Bacillus pumilus, Bacillus termoleavorans, Bacillus stearothermophilus, Bacillus sphaericus, aerogenesa, Staphylococcus epidermidis, Preuclomonase sp. etc.Lipolytic activity of difficult to determine due to the fact that lipases are water soluble enzymes acting on water insoluble substrate concentration at the interface or the use of different detergents must be taken into account to interpret the activity and the enzyme kinetics obtained.

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Chromatographic techniques can be used to detect or quantity FAs released from TAGs and other lipids. These techniques are very sensitive, although, they are time – consuming. The most common ones are: silicic acid columns, thin – layer chromatography, gas – liquid chromatography of FA methyl esters, and high – performance liquid chromatography (HPLC) .The latest is usually performed by detecting b – naphtol or P – nitro phenol released as a result of lipolytic activity on their corresponding FAs ester derivatives. Chromatographic assays are also used for detecting the reaction products of lipases in reactions of synthesis or acyl exchange, as well as for assaying the enantioselectivity of these enzymes.(Jaeges et al., 1999).Bacterial lipases are gaining an increasing interest due to their potential as biotechnological catalysts, as well as by their role as virulence factors in some pathogenic bacteria(Rosenau& Jaeger, 2000).

Understanding the mechanisms of gene expression, folding, and secretion of bacteria lipases, as well as their enzymatic properties is essential for improving the biotechnological application of these enzymes, and for the treatment of lipase – related diseases (Rosenau & Jaeger, 2000).

Lipases are used in situ, and sometimes together with other enzymes, during the elaboration of bread, cheese, and other foods to improve their shelf – life and their rheological properties, or to produce armas or emulgents. Moreover, they are used ex situ to produce flavors, and to modify the structure or composition of AGS by inter – or transesterification, in order to obtain AGs with an increased nutritional value, or suitable for parenteral feeding (Gunstone,1999; Reetz, 2002).Bacteria (and fungal) lipases the most versatile and widely used class of enzymes in biotechnological applications and organic chemistry.

This is reflected by more than 1000 original articles and reviews on lipase and lipase applications that appear each year, and by a the fact that the use of lipase in biotechnology was a business of more than1.5 billion U.S. dollar in year 2000(Jaeger & Eggert, 2002; Gupta et al., 2004).

MATERIALS AND METHODS

Sample Collection and Isolation of Bacteria

The soil was collected from auto mobile shop in Thanjavur, collected in a sterile container and it was brought to the laboratory for further processing. The collected sample was serially diluted upon 10^7dilution using sterile saline as a blank and the dilutes samples were plated into the sterile nutrient agar using spread plate method.

Screening for Lipase Producing Organism

The isolated pure strain were screened for the production of extracellular lipase production using screening medium contain Tributyrine Substrate. The pure culture were streaked at the center of the sterile screening plates were incubated at 375°C for 24 hours only positive andbetter zone formed strain was taken for further study.

Identification of Organisms

The positive strain that produces maximum lipase enzyme was selected and identification by biochemical.

Lipase Production Medium

Composition of Production Medium

Component	Quantity (g/L)
Peptone	10
Yeast extract	5
NaCl	5

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Olive oil	10 mL
$CaCl_2$	0.1
Distilled water	1000 mL

pH adjusted to 7.0 before sterilization.

Screening for Lipase Production

Tributyrin Agar Method

Bacterial isolates were streaked on tributyrin agar plates and incubated at 37°C for 24–48 hours.

Observation

Clear zones around colonies indicated lipase production.

Enzyme Production

The enzyme production was carried out by shake flask fermentation using production medium which comprising of GW cose as a carbon source and amended with peptone as a protein acids substrate with pH_7. Five hundred ml of sterile production broth was prepared in one liter conical flask an 5% inculum was transferred aseptically into the production medium. The inoculated medium was incubated at 37°C for 48hours. The medium was agitated at 200rpm for better aeration and growth of the organism.

Parameter Optimization Studies

Temperature

Sterile production media was prepared and inoculated with 5% inoculums in different test tube each flask was incubated at different temperature such as 35, 40, 45, watch 24hrs. The OD value was observed.

pH

Production media was prepared and inoculums with 5% inoculums in different test tube and pH and medium was adjusted to different pH such as 6, 7, and 8. Using 0.1 N Noah. The flask was incubated at 32°C for 24hrs. The OD was measured.

Carbon Source

Growth and lipase production were determined at 12h interval up to 42h using different carbon source. The growth and lipase production by the organism were highest in olive oil and least in coconut oil (Kamini et al., and Bhushan and Hoon Dail) have also reported olive oil as the carbon source.

Lipase Assay (Confirmation Test)

Titrimetric Lipase Assay

Principle

Lipase hydrolyzes الزيت/olive oil into fatty acids. Released fatty acids are titrated with NaOH.

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Procedure

- Prepare substrate emulsion using olive oil and gum acacia.
- Add crude enzyme extract.
- Incubate at 37°C for 30 min.
- Stop reaction using acetone-ethanol mixture.
- Titrate liberated fatty acids with 0.05 N NaOH using phenolphthalein.

Formula

Lipase Activity (U/mL)=V×N×1000T×Vs\text{Lipase Activity (U/mL)} = \frac{V \times N \times 1000}{T \times V_s}Lipase Activity (U/mL)=T×VsV×N×1000

Where:

- V = Volume of NaOH used
- N = Normality of NaOH
- T = Incubation time
- VsV_sVs = Volume of enzyme sample

Thin Layer Chromotographic

Preliminary characterization of the lipase was done by TLC method and was separated on a silica gel plating using CHCl:H_2O (70:10:0.5$^-$ V/V/V)as developing solvent system with different colour developing reagents. Ninydrin reagent (0.5g inhydrin in 100 ml anhydrous acetone) was used to detect lipopeptide, asyellow spots and lipase are generally identified as yellow spots.(yin.h.,j.Qiang 2008) .

RESULT AND DISCUSSION

Naturally occurring microorganisms' are having ability to produce the various enzymes. Now a day's most of the enzyme is industrially important and human welfare. Lipase is one of the important enzymes, which can be produced from microorganisms.

In this study, the bacterial strain was isolated from auto mobile shops and petrol bank soil from Thanjavur. The samples were inoculated on tributyrin agar plate by serial dilution using sterile distilled water. 14 bacterial strains were isolated from the sample. Then screening medium was prepared from 14bactria strain are produce lipase (Plate: 1). The samples contained 2.06 x 10^6 CFU/ml (plate: 1).

Then the dominant organism were isolated and individually streaked on tributyrin (Hi media 0>1), agar plates and the formation at halo zone around the colony on tributyrin agar was considered as the positive colony for lipase production

Biochemical characters:

Enrichment culture technique enabled the isolation of strain with lipolytic activity in tributyrin media plates. The lipolytic microbes were further and reactions and then identified as Gram positive, rod shaped motile organism. (Table: 1). Finally the morphological and biochemical test indicated that the suspected organism were Bacillus sp. and the lipolytic microbes were further and reactions and then identified as Gram negative, rod shaped, motile organism. The morphological and biochemical test indicated that the suspected organism were Pseudomonas sp.

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The isolated organism were B_1(Auto mobile shop) identified Bacillus sp. based on biochemical characters.

The isolated organism was B_2(petrol bauk) identified as Pseudomonas sp. based on biochemical characters.

Effect of temperature:

Fig: 3 Effects of different incubation temperature on the production of lipase by bacillus sp. The optimum temperature for lipase production by bacillus sp was observed to be 35°C.The effects of different incubation temperature on the production of lipase by Pseudomonas sp. The optimum temperature for lipase production by Pseudomonas sp. was observed to be 35°C.

Effect of pH:

Fig:4 In our study the lipase production by Bacillus sp. produced the maximum lipase at pH 7and lipase was minimum at pH4.

In our study the lipase production by Pseudomonas sp. production the maximum lipase at pH 7and lipase was minimum at pH4.

Effect of carbon source:

Fig:5 Growth and lipase production were determined at 12h interval up to 42h using different carbon source. The growth and lipase production by the organism were higher in olive oil and lower in coconut oil. It have also reported olive as the best carbon source.

Analysis of lipase:

Lipase isolated by acid precipitation method at pH was identified by TLC method. Bacillus sp. and Pseudomonas sp. was identified by yellow colour spot. The volue of Rhamo lipid are 0.78,0.64,oil and petrol. Bacillus sp. have higher lipase activity. The Pseudomonas sp. has low lipase activity.

The dominant organism were isolated by streaking on tributyrin agar plates and the formation of halo zone around the colony on tributryrin agar was considered as the positive colony. Enrichment culture technique enabled the isolation of strain with lipolytic activity in tributrin media plates. The lipolytic microbes were further screened and characterized.

The optimum temperature for lipase production by Bacillus coagulans was observed to be 35°C, However the organism showed on growth below 20°C and 40°C. Similar the same result were also reported by [A. Thomas et al., 2003].

Similar result were obtained, maximum growth was achieved after 44h of incubation at 35°C. The lipase production was detected in the late logarithmic phase (after 20h), and increased until optimum production was achieved, after 48h of incubation. The production of degradative enzymes tends to occur during the late logarithmic phase of growth, when all density is high [Rahman et al., 2005].

Bacillus coagulans produced by the maximum lipase at pH_4, Jose and Kurup have also reported pH_7, for the optimal lipase production in Bacillus pumilus.

The pH of the culture dropped from the initial neutral pH to a pH of 5.3 during the first 12h of incubation. This might be attributed to the production of acids during bacterial growth. However, the pH of the culture medium increased gradually to a pH of 8.5 after 24h. The pH then gradually dropped, and remained unchanged at pH 7.8 after 48h. The rise in pH after 12h of incubation may be due to the utilization of organic acids or the production of alkaline compounds during this period.

The growth and lipase production by the organism were high in olive oil and least in coconut oil (Kamine et al., and Bhushan and Hoon dial) have also reported olive oil and the best carbon source.

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Interpredation

As reported by (Nakashima et al., 1988) the presence of olive oil as growth medium greatly enhanced the lipase activity of bacillus strain (B_1) in the present study. As reported by (Tsuzuki et al., 1999). The presence of olive oil as growth medium greatly enhanced the lipase activity of Pseudomonas sp. strain (B_2) in the present study.

Thin layer chromatography of lipids revealed that Bacillus sp. has higher lipase activity when compared to pseudomonas sp.

Lipase production increased with incubation time up to 48 hours in both organisms.

Maximum enzyme activity was observed at 48 hours:

Bacillus subtilis = 65 U/mL

Pseudomonas aeruginosa = 100 U/mL

After 48 hours, lipase production gradually decreased.

The reduction in enzyme activity at longer incubation periods may be due to:

nutrient depletion,

accumulation of toxic metabolites,

changes in pH,

enzyme degradation The paper conclutes

Pseudomonas aeruginosa produced higher lipase activity than Bacillus subtilis at all incubation periods, indicating its greater efficiency as a lipase-producing organism

Table :1

Biochemical Characterization of Lipase production from **Bacillus** and **Pseudomonas**

Biochemical Characterization of Lipase production from **Bacillus** and **Pseudomonas**

Biochemical Tests for Identification

Biochemical Test Table

Test	**Bacillus subtilis**	**Pseudomonas aeruginosa**
Gram staining	Positive rods	Negative rods
Catalase test	Positive	Positive
Oxidase test	Negative/Variable	Positive

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Citrate utilization	Positive	Positive
Indole test	Negative	Negative
Methyl Red	Negative	Negative
Voges-Proskauer	Positive	Negative
Urease test	Negative	Positive
Motility test	Motile	Motile
Starch hydrolysis	Positive	Negative
Gelatin hydrolysis	Positive.	Positive EFFECT OF PH

B1-Bacillus

B2 = **Pseudomonas**

TABLE-2-Effect of pH

Sample1&2	**PH VALUE**	**OD VALUE**
Bacillus subtilis	**5** **6** **7** **8**	**0.4,** **0.6.** **0.8** **0.6**
Pseudomonas aeruginosa	**5** **6** **7** **8**	**0.2** **0.4** **0.6** **0.4**

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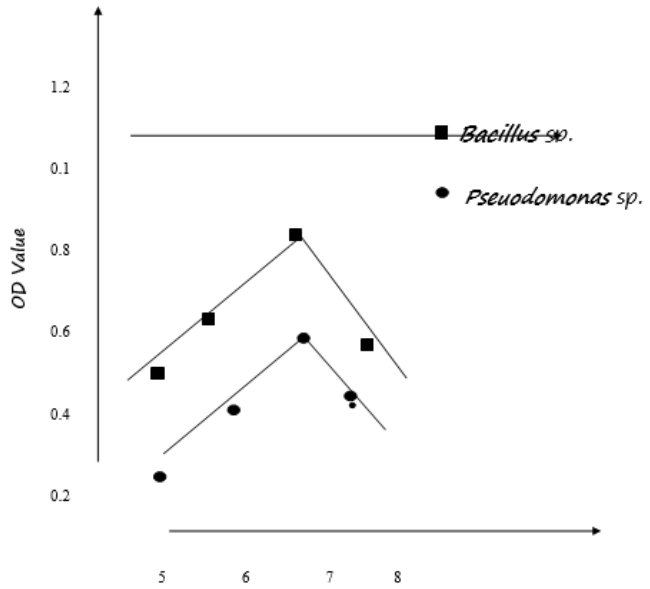
Fig :4

S.No	Soil Sample	Indole	Melthyl	VP	Citrate	TSI	Catalase	Starch	Casein	Gelatin	Nitrate	Acid	Glucose	Arabiose	Maltose	Sucrore	Manittol	Lactose



pH Value

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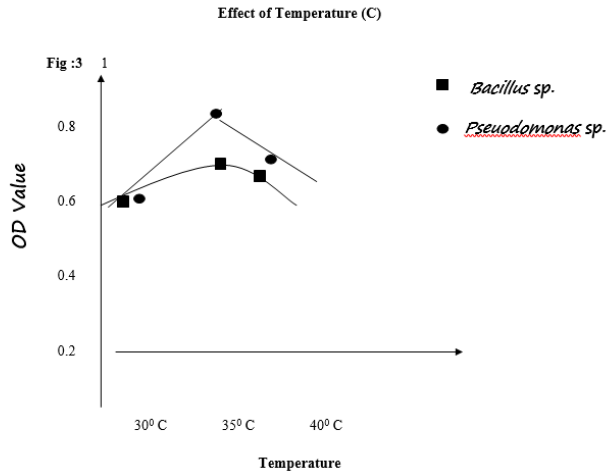
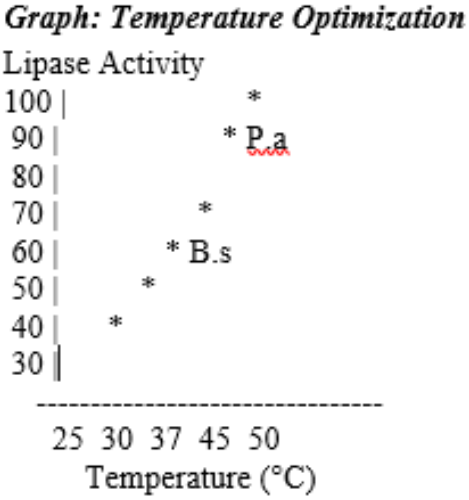


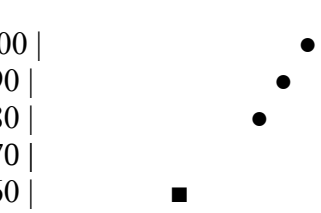
FIG.4



Tab: 5Effect of Incubation Time

Time (hrs)	B. subtilis	P. aeruginosa
24	30	50
48	65	100
72	55	85
96	40	60

Graph: Effect of Incubation Time on Lipase Production



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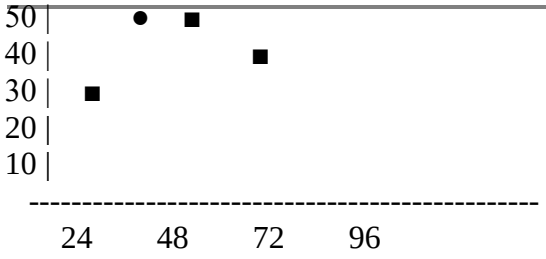
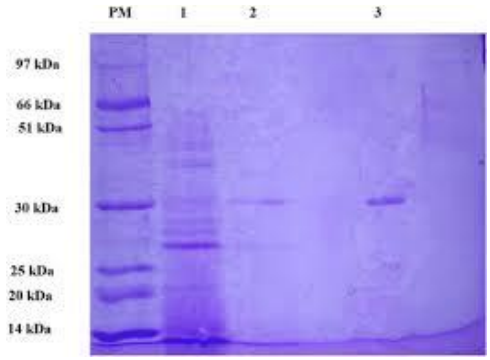


Lipase Enzyme Production in Thin Layer Chromatography

Photo-1



The study demonstrated that both bacterial species produced extracellular lipase, but Pseudomonas aeruginosa showed significantly higher enzyme activity under optimized conditions. Maximum production occurred at:

- pH 7
- Temperature 37°C
- Incubation time 48 hours

The higher lipase yield by Pseudomonas aeruginosa may be due to its strong extracellular enzyme secretion system.

Both Bacillus subtilis and Pseudomonas aeruginosa are efficient lipase producers. However, Pseudomonas aeruginosa exhibited superior lipase activity and better optimization response. These organisms can be exploited for industrial lipase production.

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ISSN 2278-2540 | DOI: 10.51583/IJLTEMAS | Volume XV, Issue V, May 2026

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