International Journal of Science and Research (IJSR)
ISSN: 2319-7064
Index Copernicus Value (2016): 79.57 | Impact Factor (2017): 7.296

Isolation and Optimization of Growth Conditions for Lipolytic Bacteria from Soil

Hemkala

Abstract: ***Isolation and screening of bacteria from these natural environment can be supposed to be useful for obtaining bacterial species with potential of producing lipase enzyme. Therefore , in the present study isolation of lipase producing bacteria was aimed from soil source contaminated with oil which acts as a protein source. The soil sample for this purpose was collected from petrol pump of sector- 42 ,Chandigarh, India . The bacterial species was isolated by culturing on Tributyrin agar media , The colonies which appeared on the plate were further streaked on Tributyrin-agar plates to get pure culture. All the bacterial isolates were further processed for lipolytic assay followed by optimization of different bio-chemical parameters for maximal enzyme activity. The various objectives of the study are :- 1) Isolation and screening of lipolytic bacteria from oily soil. 2) Optimization of growth conditions for lipase production from selected strain. 3) Evaluation of carbon and nitrogen sources for maximal enzyme activity. 4) Effect of agitation speed on lipase enzyme. Characterization of lipase produced from the selected strain.***

1. Introduction

The large part of the earth's biomass is represented by lipids. They are the most important source of energy, play structural roles in membranes and are involved in signaling events. To be able to carry out these functions, lipids require lipolytic enzymes during their metabolism. Lipolytic enzymes catalyze the turnover of these water-insoluble compounds [Gilham and Lehner 2005]. They also breakdown lipids and make them mobile within the cells of individual organisms [Beisson *et al.* 2000]. Lipolytic enzymes are grouped into 3 main categories, which are esterases, phospholipases and lipases [Arpigny and Jaeger 1999]. Different lipases have been identified in and isolated from bacteria, fungi, plants, and animals. Lipases (triacylglycerol hydrolases, EC 3.1.1.3), are lipolytic enzymes that catalyze the hydrolysis of ester bonds of triacylglycerols at the oil-water interface (Arpigny & Jaeger, 1999). Their remarkable stability in water-restricted environments has made them key enzymes in biotechnological applications (Klibanov,2001. Lipase catalyzed reactions are stereoselective and regioselective in nature . Therefore, there is an increasing interest for lipases and lipase producing strains, as lipase-catalyzed reactions show high selectivity under mild conditions, with no requirement for added cofactors . Lipolytic enzymes have numerous industrial applications . The number of lipases has been increasing as a result of the developments in cloning and expression of enzymes from microorganisms, but still the demand for the biocatalysts with novel and specific properties such as specificity, stability, pH, and temperature is increasing. This has led to enhanced interest in isolation of new lipolytic micro-organisms from diverse habitat.

Research on lipases is focused particularly on structural, characterization, general characterization of performance and industrial applications (Bornscheuer, 2000). Development of lipase based technologies for the synthesis of novel compounds is rapidly expanding the use of these enzymes (Liese *et al*., 2000). In addition to hydrolysis of triglycerides, lipases can catalyze a variety of chemical reactions; which include esterification, transesterification, acidolysis, alcoholysis, aminolysis. The high versatility of lipases, recognized as the most important and versatile group of biocatalysts in biotechnology (Hassan *et al*., 2006) allows their applications in different industries like food, detergent, pharmaceutical, leather, textile, cosmetic, paper and oleo-chemicals (Kademi and Leblanc, 2004). Lipases can catalyze a variety of chemical reactions; which include esterification, transesterification, acidolysis, alcoholysis, aminolysis. Which allows their applications in different industries like food, detergent, pharmaceutical, leather, textile, cosmetic, paper and oleo-chemicals (Kademi and Leblanc, 2004). Lipase producing strains have been isolated from industrial wastes, oil and fats processing factories, soil (Sharma *et al*., 2001; Colen *et al*., 2006).

Enzymes are widely applied in the field of scientific research, cosmetic production, medical diagnostics and chemical analysis, therapeutic applications and industrial catalysis in the special syntheses (Sharma *et al*, 2001). Lipases have unique characteristics that they carry out reactions at the interface between aqueous and non-aqueous media. This is due to their ability to utilize relatively a wide spectrum of substrates, high stability towards extremes of temperature, pH and organic solvents. Lipases occur widely in nature but only microbial lipases are commercially significant (S.E *et al*, 2007). Lipases produced from microbial source, in general is more economical and stable. Bacterial lipases are extracellular and are greatly influenced by nutritional and physio-chemical factors such as temp, pH, nitrogen and carbon source, inorganic salts , agitation speed and dissolved oxygen concenteration. The naturally occurring environment provide good protein source for micro-organisms . Isolation and screening of bacteria from these natural environment can be supposed to be useful for obtaining bacterial species with potential of producing lipase enzyme. Therefore , in the present study isolation of lipase producing bacteria was aimed from soil source contaminated with oil which acts as a protein source.

2. Material and Methods

The study was conducted at Microbial Biotechnology Laboratory of Post Graduate Govt. College for Girls, Sector-42, Chandigarh. The entire work was done, stepwise, as follows:

1) Sample collection;
2) Isolation of lipolytic bacteria;
3) Screening of isolates for lipase activity;

4) Culturing and characterization of the isolates;
5) Lipase activity assay; and
6) Optimization of media parameters such as effect of nitrogen , carbon sources and agitation speed on lipase activity.

2.1 Chemicals

All the chemicals used in the study were of analytical or microbiological grade, obtained from Merck (India) Ltd., Nice, Fisher Company and HiMedia. The different types of oil like coconut oil , mustard oil, sunflower oil, olive oil and ghee used in the media for the growth of lipolytic organism were purchased from local market.

2.2 Sample Collection

The soil sample was collected from crude oil polluted soil of fuel filling station of Sector 42, Chandigarh .It was used for the isolation of lipolytic bacteria using qualitative and quantitative screening, and for optimization of various media parameters . The sample was collected with the help of spatula in sterile polythene zipper bags and was stored at 4°C for further use.

2.3. Isolation of Lipase Producing Bacteria

Requirements:
Tributyrin agar base containing 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, 1% (v/v) tributyrin and 2% (w/v) agar (pH 7.0).

Procedure
- The soil sample was weighed (approx. 1g), serially diluted upto 10^{-8} and spread-plated onto tributyrin agar base.(Sarada *et al*.,1998).
- The Plates were incubated at 37 °C for 48 hours.
- Pure cultures of the isolates were maintained on minimal media agar slants (0.3% yeast extract, 0.5% sodium chloride, 0.5% peptone and 2% agar ; pH 7.0) and were subcultured every 15 days.

2.4 Screening of the Isolates for Lipase Activity

Lipolytic organisms were screened by qualitative plate assay.

Procedure
- Isolates were grown on tributyrin agar base plates .
- These plates incubated at 37° C for 48 hours.

Observation
Zone of clearance was observed due to hydrolysis of tributyrin (Jaeger *et al*.,1994, Ertugrul *et al*.,2007 , Kim *et al*.,2001).

2.5 Characterization of the Isolates

The isolate showing maximum one of clearance, was given the name MZ and was selected for further analysis. Morphological and biochemical characteristics of the isolate were studied according to Bergey's Manual of Determinate Bacteriology(Holt *et al* .,1996).Morphological tests were based on Gram staining while the biochemical tests performed were catalase test and starch hydrolysis test. In addition to this, the IMViC tests were performed for bio chemical characterization. The various tests done were as follows.

2.5.1 Biochemical Tests

Sr.No	Substrate , Tests
01	Indole
02	Methyl red
03	Voges Proskauer's
04	Citrate Utilization
05	Glucose
06	Adonitol
07	Arabinose
08	Lactose
09	Sorbitol
10	Mannitol
11	Rhamnose
12	Sucrose

2.5.2. Gram Staining
A procedure invented by the Danish scientist and physician **Hans Christian Joachim Gram** in 1884, Gram staining is differential staining procedure that differentiates bacteria into two groups viz., Gram positives and Gram negatives, based on the differences in their cell wall composition. Gram positives are stained purple while Gram negatives are stained pink.

Requirements:
- Crystal violet;
- Gram's iodine;
- Alcohol; and
- Safranin

Procedure:
- Application of crystal violet (primary stain) to heat fixed smear of bacterial culture for 20 seconds;
- Addition of Gram's iodine for 1 minute;
- Decolourization with alcohol for 10- 20 seconds; and
- Counterstaining with safranin for 20 seconds.

NOTE: Wash the slide with water after each step.

2.5.3. Catalase Test
Some bacteria contain proteins that reduce oxygen (O_2), resulting in the production of hydrogen peroxide (H_2O_2) and, in some cases, an extremely toxic superoxide (O_2).Accumulation of these substances will result in death of the organism as they are powerful oxidizing agents and destroy cellular constituents very rapidly unless they are enzymatically degraded.

Many bacteria possess enzymes that help protection against toxic oxygen products . Facultative anaerobes and obligate aerobes usually contain the enzymes superoxide dismutase, to catalyze the destruction of superoxide , and either catalase or peroxidase , which catalyze the destruction of hydrogen peroxide as follows:

$$2O^{-}_2 + 2H^{+} \xrightarrow{\text{Superoxide dismutase}} O_2 + H_2O_2$$

$$2H_2O_2 \xrightarrow{\text{Catalase / peroxidase}} 2H_2O + O_2$$

Catalase activity is very useful in differentiating between various groups of bacteria. For example, the morphologically similar *Enterococcus* (catalse negative) and staphylococcus (catalase positive) and *Staphylococcus* (catalse positive) can be differentiated using this test.

Procedure:
- Agar slants were inoculated with the organism and incubated at 37° C for 24 hours.
- 1ml of hydrogen peroxide was allowed to flow over the slants.

Observation:
Formation of air bubbles indicated a positive result.

2.5.4. Starch Hydrolysis Test
The main purpose of this test is to check whether a microbe can utilize starch, a complex carbohydrates, as a source of carbon and energy for growth. Starch utilization is accomplished by an enzyme called α- amylase. The test for starch hydrolysis is determined using starch agar medium. After inoculation and overnight incubation, the plates are flooded with iodine solution. Iodine forms blue-black complex with starch in the medium. Clear halos surrounding the microbial colonies are indicative of their ability to hydrolyze starch in the medium due to presence of the enzyme α-amylase.
- Using sterile techniques, single streak inoculation was done on starch agar plates and incubated at 37 °C for 24 hours.
- Then the plates were flooded with iodine solution.

Observation:
Formation of clear zone around the microbial colony indicates positive result.

2.6 Lipase Assay

The selected bacterial colony i.e., MZ, was assayed for extracellular lipase activity using titrimetric method with olive oil as substrate.

Procedure:
- Olive oil (10% v/v) was emulsified with gum arabic (5% w/v) in 100 mM potassium phosphate buffer (pH 7.0).
- 100 µl of enzyme was added to the emulsion and incubated for 15 minutes at 37 °C.
- The reaction was stopped and fatty acids were extracted by the addition of 1.0 ml of acetone: ethanol solution (1:1).
- The amount of fatty acids liberated was estimated by titrating with 0.05 M NaOH until pH 10.5 using phenolphthalein indictor.(Jensen, *et al., 1983).*The activity of enzyme was expressed in terms of enzymes units.

Calculation of Lipase Activity

$$\text{Lipase Activity (Units/ml)} = \frac{\text{Volume of alkali consumed} \times \text{Normality of NaOH}}{\text{Time of incubtion} \times \text{Volume of enzyme soltuion}}$$

One unit of lipase activity is defined as the amount of enzyme required to hydrolyze µmol of fatty acids from triglycerides.

2.7 Optimization of Media Parameters for Profound Enzyme Activity

Medium components significantly affect the enzymatic activity of micro organisms (Zhang *et al.*,2009). For this reason, it is essential to assess the nutritional requirements (carbon and nitrogen sources) towards enhanced enzymatic activity. Keeping this in view, the effect of various carbon and nitrogen sources on lipase activity was studied as follows.

2.7.1 Effect of Different Oils as Carbon Source on Lipase Activity
To evaluate different carbon source for maximum lipase activity by the isolate MZ Olive oil present in growth media was replaced with different oils like ghee, coconut oil, groundnut oil, sunflower oil and mustard oil at a final concentration of 1% (v/v) by keeping
the temp. at 37^{o}C and pH 7 .0.

Procedure:
- Tributyrin broth in five different flasks was inoculated with pure culture from the isolate MZ.
- The flasks were then incubated with different carbon sources like ghee, coconut oil, groundnut oil, sunflower oil and mustard oil.
- Broth was harvested after 48 hours and centrifuged at 10,000 g for 30 minutes at 4 °C .
- The supernatant was then assayed for lipase activity using titrimetric method.

2.7.2 Effect of Different Nitrogen Sources on Lipase Activity
Various organic nitrogen sources like yeast extract, tryptone and peptone and inorganic sources like NH_4Cl and $NaNO_3$ respectively were added to the broth at a final concentration of 1% (w/v) by keeping the temp. at 37^{o}C and pH 7.0.

Procedure:
- Tributyrin broth in five different flasks was inoculated with pure culture from the isolate MZ.
- The flasks were then incubated with different nitrogen sources like yeast extract, tryptone, peptone , NH_4Cl and $NaNO_3$,.
- Broth was harvested after 48 hours and centrifuged at 10,000 g for 30 minutes at 4 °C.
- The supernatant was then assayed for lipase activity using titrimetric method.

2.7.3 Effect of Agitation Speed on Lipase Activity
To determine the optimal agitation speed for peak enzyme activity, the isolate MZ was cultured in an orbital shaker was kept at 37 °C ,pH 7.0 at varying agitation speeds from 120 to 200 rpm along with stationary culture.

Procedure:
- Tributyrin broth in five different flasks was inoculated with pure culture from the isolate MZ.
- The flasks were then incubated at varying agitation speed like 120,140,160,180 and 200.
- Broth was harvested after 48 hours and centrifuged at 10,000 g for 30 minutes at 4 °C.

- The supernatant was then assayed for lipase activity using titrimetric method.

2.8 Statistical Analysis

It was done for all the above said parameters in triplicates.

Sr. No.	Sample Collection	Area	Month of Collection	Temperature	pH	colour
01	Fuel filling station	Sector-42 Chandigarh	February	22°C±	6.5	Brownish black

3. Results and Discussion

Isolation, Screening and Identification of Lipolytic Bacteria.

3.1 Sample collection

The soil sample was collected from crude oil polluted soil of fuel filling station of Sector 42, Chandigarh. It was processed for isolation of lipolytic bacterial strain using qualitative and quantitative screening and for optimization of various media parameters. The sample was collected with the help of spatula in sterile polythene zipper bags and was stored at 4°C for further use. The various characteristics of collected soil sample are given below:

Figure 1: Oil contaminated Soil sample used for the isolation of lipolytic bacteria.

3.2 Isolation and screening of Lipase Producing Bacteria

Soil samples were serially diluted and plated on to tributyrin agar by spread plate method (Sarada *et al.*,1998).Plates were incubated at 37°C for two days. Colonies with zone of clearance were picked and maintained on minimal media agar slants (yeast extract, NaCl, Peptone and 2% agar, pH 7.0) and were then subcultured every 15 days.

Figure 2: a) Zone of clearance(2.4mm) around colonies plated on tributyrin agar medium.

b) Colonies were obtained on streaked tributyrin agar medium.

3.3 Morphological and biochemical characterization of the isolate MZ

Enrichment culture techniques enabled the isolation of strains from oil contaminated soil with lipolytic activity in tributyrin agar plate . The isolate MZ showing the maximum zone of clearance was selected for further analysis. The morphological and bio chemical characterization of lipase producing bacteria was done in accordance with the Bergey's Manual of systematic bacteriology (Holt *et al.*,1996) .

The lipolytic microbe were further screened and characterized by its features and reactions and then identified as gram positive cocci.(Table 1). Finally , morphological and bio chemical tests indicated that the suspected organism is likely to be belonging to genus *Staphylococcus*.

3.3.1 Gram staining

A procedure invented by the Danish scientist and physician **Hans Christian Joachim Gram** in 1884, Gram staining is differential staining procedure that differentiates bacteria into two groups viz., Gram positives and Gram negatives, based on the differences in while Gram negatives are stained pink their cell wall composition. Gram positives are stained purple

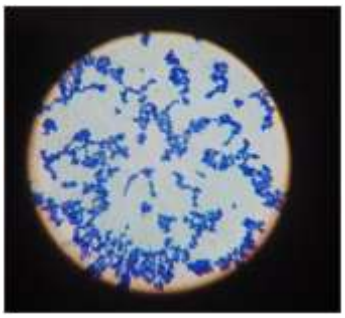

Figure 3: Purple colour indicates the presence of gram positive bacteria

3.3.2 Starch hydrolysis test

The main purpose of this test is to check whether a microbe can utilize starch, a complex carbohydrates, as a source of carbon and energy for growth . Starch utilization is accomplished by an enzyme called α- amylase. The test for starch hydrolysis is determined using starch agar medium. After inoculation and overnight incubation, the plates are flooded with iodine solution. Iodine forms blue-black complex with starch in the medium. Clear halos surrounding the microbial colonies are indicative of their ability to

hydrolyze starch in the medium due to presence of the enzyme α-amylase.

Figure 4: The isolate MZ showing starch hydrolysis.

3.3.3 Catalase test

Catalase is a enzyme that breaks H_2O_2 into H_2O and O_2.

$$2H_2O_2 \xrightarrow{\text{Catalase / peroxidase}} 2H_2O + O_2$$

Catalase activity is very useful in differentiating between various groups of bacteria. For example, the morphologically similar *Enterococcus* (catalse negative) and staphylococcus (catalase positive) and *Staphylococcus* (catalse positive) can be differentiated using this test. The evolution of oxyzen is indicated by the appearance of bubbles which is indicative of positive catalase test.

Figure 5: Air bubbles indicated a positive result for Catalase test

3.3.4 Biochemical Characterization

The various IMViC tests performed are given in the below mentioned Table 1.

Table 1: Morphological and Biochemical characterization of the isolate MZ

Sr. No	Substrates,Tests	Results
01	Gram staining	Positive
02	Morphology	Cocci (in cluster)
03	Indole	Negative
04	Methyl red	Negative
05	Voges Proskauer's	Negative
06	Citrate Utilization	Negative
07	Glucose	Positive
08	Adonitol	Negative
09	Arabinose	Negative
10	Lactose	Positive
11	Sorbitol	Negative
12	Mannitol	Negative
13	Rhamnose	Negative
14	Sucrose	Positive

3.4 Optimization of media for profound Enzyme Activity

Bacterial lipases are influenced by the composition of the growth medium, cultivation condition and many physio-chemical (pH and Temp.) and nutritional factors (carbon, nitrogen and lipid sources) (Jaeger *et al* 1994) . For this reason, it is essential to assess the nutritional requirements (carbon and nitrogen sources) towards enhanced enzymatic activity. The influence of the components of growth medium on microbial lipase activity varies from one organism to another. Keeping this in view , the below mentioned parameters were optimized one by one. The lipase activity was analysed by titrimetric assay for different parameters.

3.4.1 Effect of Nitrogen Sources on Lipase Activity

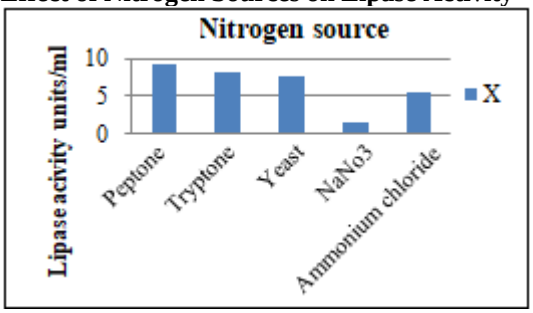

Figure 6: Effect of Nitrogen source on lipase activity.

Both organic and inorganic nitrogen sources play an important role in enzyme synthesis. Inorganic nitrogen sources can be exhausted from the culture media quickly while organic nitrogen sources can supply many cell growth factors and amino acids which are needed for cell metabolism and enzyme synthesis. In our study the enzyme activity was found to be maximum for peptone (9.2± 0.25 U/ml) followed by tryptone (8.2±0.20U/ml) and yeast extract (7.5±0.15 units/ml) (fig.6). Sirisha *et al* 2010 has also reported peptone as the best nitrogen source for lipase production. To explain superiority of peptone as an organic nitrogen source over other complex nitrogen sources, Freire *et al* 1997 suggested that peptone contains certain co-factors and amino acids that match the physiological requirements for lipase bio synthesis. The inorganic nitrogen sources i.e. sodium nitrate and ammonium chloride also showed their influence on enzyme activity. Ammonium chloride was found to be an excellent nitrogen source giving lipase activity of 5.47±0.23 U/ml as compared to sodium nitrate 1.47±0.25. Generally micro organisms exhibit high yield of lipase when organic nitrogen sources are used, which have been used for lipase production by various Bacillus species and Pseudomonas species (Sharma *et al* 2002, Kathiravan *et al*

3.4.2 Effect of diferent oils used as carbon source on Lipase Activity

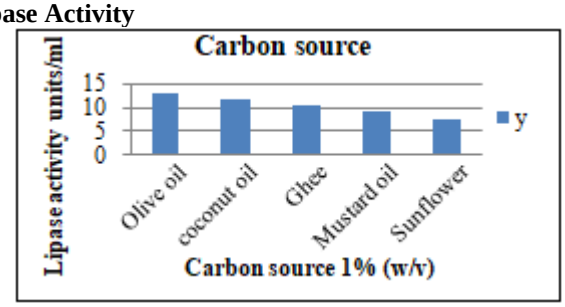

Figure 7: Effect of carbon source

The major factor for the expression of lipase activity has always been reported as the carbon source ,the different oils used as carbon source enhanced lipase activity from 7.4 to 12.8 U/ml which indicated a decent increment in lipase activity (fig:7).The lipase activity was found to be maximum

for olive oil (12.84±0.28 U/ml) followed by coconut oil (11.72±0.12 U/ml) and ghee (10.51±0.30 U/ml) while optimizing the process for carbon source . The results show that the enzyme was more efficient to act on oils with long chain mono unsaturated fatty acids. The lipase production by the isolate MZ vary widely under different growth condition and indicates that the medium supplemented with triglycerides enhances the enzyme production.The results are in good accordance with earlier reports were triglycerides supplementation in the medium has been shown to be indispensable for lipase production (Del Rio *et al.*, 1990; Marek and Dednarski 1996; Cihangir and Sarikaya, 2004; Mohan *et al.*, 2008; Sirisha *et al.* ;2010; Papdampriya *et al*.,2011). Gupta *et al* .,2004 and Acikel *et al* .,2011 have also reported variations in induction of lipase production depending on the type of lipid sources used.

3.4.3 Effect of Agitation speed on Lipase Activity

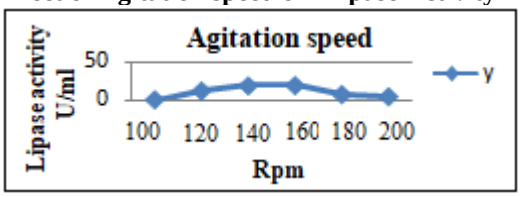

Figure 8: Effect of agitation speed on lipase activity mean

The isolate showed maximum lipase activity after 48 hours of incubation at 37°C, pH 7 with 160 rpm agitation speed (fig.8). From the results, it is clear that agitation is required for the bacteria to produce lipase since there was no lipase production at stationary conditions . Agitations at 120 rpm to 200 rpm enhanced the lipase activity. The optimum agitation speed for the production of lipase was 160 rpm (19.56±0.21 U/ml). The high agitation speed indicates the elevated aeration requirement by the bacteria. Also shaking may be required to create a condition for better bio availability of nutrients confirming the earlier reports (Nahas 1998;Rao *et al* ., 1993;Cihangir and Sarikaya 2004).The effect of agitation speed on lipase production varies depending upon the bacterial strain. Optimum lipase production for most Bacillus species was found at lower agitation rates(Gupta *et al.*, 2004). In contrast production of lipase by Pseudomonas putida 3SK was maximum at 500rpm(Lee and Rhee,1994). Shaking affects cell growth and lipase production by modulating the oxygen transfer rate and at lower agitation rate, there has been reports of occurrence of oxygen limitation (Iftikar *et al*., 2008, Gupta *et al.*, 2004). On the contrary , high levels of aeration decreased lipase production in S. carnosus (Genovefa *et al*.,1994). Sirisha *et al.*, 2010 observed maximum enzymatic activity at agitation speed of 160 rpm which coincides with our study.

4. Conclusion

The bacterial lipases are one of the enzymes having huge market demand. The isolated staphylococcus species isolate has showed the production of extracellular lipase. Optimization studies on media parameters for maximum lipase activity was done on isolated lipolytic bacteria . The various results were summarized as follows;

1) To study the effect of nitrogen source on lipase activity various organic and inorganic nitrogen sources were used . The enzyme activity was found to be maximum for peptone followed by tryptone and yeast extract.
2) The effect of carbon source on lipase activity was studied using different oils and the activity was found to be maximum for olive oil followed by coconut oil and ghee.
3) The optimum agitation speed for the production of lipase was found to be 160 rpm after 48 hours of incubation at 37°C ,pH 7.0.
4) The extracellular lipase enzyme can be further purified and the optimized growh conditions developed in this study can be used used in different industrial applications.

Abbreviations

ACE	Angiotenin-Converting enzyme
°C	Degree celsuis
G	Gram
MZ	Maximum zone
M	Molar
Approx.	Approximately
H_2O_2	Hydrogen peroxide
O_2	Oxygen
$2H_2O$	Water
NH_4Cl	Ammonium chloride
$NaNO_3$	Sodium nitrate
PUFA	Polyunsaturated fatty acid
%	Percentage
Temp.	Temperature
V/V	Volume/Volume
W/V	Weight/Volume
Mm	Millimetre
Rpm	Revolutions per minute
U	Units

References

[1] A koh,C.C,S.W,Chan Lee and J.E Shaw.,2007.Ezymatic approach to biodiesel production .J.argic Food Chem 55.8995-9005.
[2] Alberghina L, Schmid RD, Verger R, editors. Lipases: structure, mechanism and genetic engineeringWeinheim: VCH, 1991.
[3] Antczak, M.S., A. Kubiak, T. Antczak and S. Bielecki, 2009. Enzymatic biodiesel synthesis-key factors affecting efficiency of the process. Renew. Energy, 34: 1185-1194.
[4] Arpigny, J., & Jaeger, K. (1999). Bacterial lipolytic enzymes: classification and properties. *Biochemical Journal*, 343 W. , 177-183.
[5] Azim A, Sharma SK, Olsen CE, Parmar VS. Lipase catalysed synthesis of optically enriched a-haloamides. Bioorg Med Chem 2001;9:1345 – 8.
[6] Balashev K, Jensen TR, Kjaer K, Bjornholm T. Novel methods for studying lipids and lipases and their mutual interaction at interfaces: Part I. Atomic force microscopy. Biochimie 2001;83:387 – 97.
[7] Balaji, V. and P. Ebenezer, 2008. Optimization of extracellular lipase production in Colletotrichum gloeosporioides by solid state fermentation. Ind. J. Sci. Technol., 1: 1-8.
[8] Berglund P, Hutt K. Biocatalytic synthesis of enantiopure compounds using lipases. In: Patel RN, editor. Stereoselective biocatalysis. New York: Marcel Dekker, 2000

[9] Bjorkling, F., Godtfredsen, S.E., Kirk, O., 1991. The future impact of industrial lipases. Trends Biotechnol. 9, 360 – 363.
[10] Bornscheuer UT, editor. Enzymes in lipid modificationWeinheim: Wiley-VCH, 2000.;