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Optimization Of Lipase Synthesis By Pseudomonas Aeruginosa Strain Av311222

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ABSTRACT
Lipases are commonly found in nature, breaking the fat-generating glycerol and free fatty acid (FFA) functioning as biocatalyst. Lipases in general are synthesized by animals and microbes. However, industrially the microbes are exploited for the synthesis of lipase. Which depends on many factors and the difference among strain of the microbe is also reported. This study analyse different conditions for extracellular lipase production by a bacterial strain isolated from lipid-rich fish market soil. The investigated isolate was identified as P. aeruginosa strain AV311222 by analysing 16S rRNA. The objective of the present study is to determine the activity of lipase under various physiochemical conditions, i.e. temperature, incubation time, inoculum size, substrate, carbon source, organic/inorganic nitrogen sources, surfactant and metal ions. It is noticed that the maximum lipase production was at the medium temperature range of 30 ℃42 ℃, with the pH 6.0, 1% of inoculum size and incubated up to 24 hours. The maximum lipase activity was recorded at the optimized factors containing lactose (1%), meat extract (1%), ammonium dihydrogen phosphate (1%), calcium chloride (1%), Tween 80 (1%) and 1% of palm oil respectively. The findings revealed that the combined approaches enabled rapid and cost effective lipase production.
Keywords: *Lipase, Optimization, P. aeruginosa, Strain AV311222, 16SrRNA.*

INTRODUCTION

Lipases (triglycerol acyl hydrolases, EC 3.1.1.3), catalyse the breakdown of carboxylic ester bonds with triglycerides, producing diacylglycerol, monoacyl glycerol, free fatty acids (FFA), and glycerol as end byproducts (Wagget and Pfaendtner, 2024). Lipases are employed in various industries not limited to flavour enhancement, treatment of fatty effluent, production of biosurfactants and biodiesel production (Kumar *et al.*, 2023). They are also widely employed in leather industries and in detergents.

Lipase in general is synthesized by animals, plants, and microorganisms. Extracting lipase from animals is almost banned due to the ethics involved. From plants, the quantity or nature of lipases are not desirable. However, microbes have the potency in synthesizing extracellular enzyme and can also be modified according to the industrial needs. Microbial lipases have been extensively studied due to their interesting characteristics, such as appropriate stability, high substrate presence, and greater activity under environmental conditions. Among the microbes, bacteria are most preferable due to their growth rate and synthesis of more lipase when compared to their biomass. Further, Bacteria are among the best- adapted species in utilizing lipid, carbon and nitrogen source residues based on their ability to grow on the surface of various substrates in submerged conditions.

Among bacteria, *Pseudomonas* and *Bacillus* species play a major role in the manufacture of industrial microbial lipases, where production depends upon the inducers and the requirement of a lipid carbon source (Eskandari *et al.*, 2024). Especially *P. aeruginosa* contribute to the maximal lipase production in the environment and produces industrially important lipases in batch and submerged fermentation through the cultures, significantly exhibiting major cost-effective contributions (Bier, 1995). Thus, continuous approach

on finding a novel strain in synthesis of lipase at economical cost is utmost important. The aim of the study is to find the cost-effective, significant bacterial strain and optimizing various factor in maximum synthesis of lipase.

MATERIALS AND METHOD

Sampling site

Soil Samples were collected from a fish market in Bhestan, Surat, Gujarat in India. The samples were collected and held in a sterile, sealed, polythene bag and brought to the laboratory. Sample was processed immediately for the isolation of bacterial species.

Screening of Lipase synthesizing Bacteria

The bacteria from the soil sample were isolated using serial dilution method. A gram of soil sample was dispersed in 10 ml of sterile distilled water to make the dilution as 1:10 dilution and further dilutions were made in 1ml of diluted sample dispersed in 9 ml of sterilised water. Two ml of supernatant was added to the 20ml of nutrient broth (peptone 0.5%, HM Peptone 0.15%, yeast extract 0.15%, sodium chloride 0.5% pH 7.4 ± 0.2) amended with 1% olive oil and incubated in a shaker for two days at 30°C. Then, the culture was serially diluted, and 200 µl was added to a prescreened medium of 1% tributyrin agar plates comprising special peptone 5g/L, yeast extract 3 g/L, 1% tributyrin, and agar 15 g/L, pH 7.5 ± 0.2 at 37°C for 24 hours. Strains producing halo zones were inoculated into 100 ml of nutrient broth, which was incubated at 37°C for 24 hours, in a shaker at 100 rpm. Bacteria were cultured in basic production media containing olive oil (1%), peptone (5g/1l), and yeast extract (3g/l) for 24 hours at 37°C (Ebrahimpour *et al.,* 2008). The supernatant was separated from the differential strains by centrifugation (10000 xg, 10 min, and 4°C), and their lipase activity was evaluated using a titration-based lipase test.

Determination of Lipase activity

Lipase activity was assessed using the pH titration method, whereas free fatty acids produced by hydrolysing olive oil were tested using the pH-stat method. Under standard assay conditions, one unit was defined as the number of enzymes capable of releasing 1 µM of fatty acid per minute (Edgar, 2004).

Molecular identificationand phylogenetic analysis

Genomic DNA was extracted from microbial sample using the Biobee spin EXpure Microbial DNA isolation kit. The 16S rRNA gene was amplified through PCR (Polymerase Chain Reaction), and the amplicon was sequenced. Following isolation, quantitative and qualitative studies were done with 50 ng of DNA for DNA amplification, and the following two degenerative primers were constructed (Table 1).

Cycling conditions include denaturation at 95°C for 2 minutes, followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 2 minutes. PCR products were purified with the Montage PCR cleaning-up kit (Millipore). Sequencing reaction performed using the ABI PRISM® BigDyeDM terminator cycle sequencing kit with AmoliTaq® DNA polymerase (FS enzyme) (Applied Biosystem).

The 16S rRNA gene sequence was analyzed through NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Program MUSCLE 3.7 was used for multiple alignments of sequences. The obtained aligned sequences were treated with G blocks 0.91b, which removed poorly aligned locations and divergent regions. Finally, PhyML 3.0 aLRT was used for phylogeny analysis, and HKY85 as a substitution model.

Optimization of lipase production

Various carbon source (Dextrose, Glucose, Lactose, Maltose, Starch and Sucrose - 1% w/v), organic nitrogen source (Beef extract, Meet extract, Peptone, Urea and Yeast extract – 1% w/v), inorganic nitrogen source (Ammonium dihydrogen phosphate, Calcium nitrate, Potassium nitrate, Sodium nitrate - 1% w/v), surfactants (Glycerol, Triton X-100, Tween 20, Sodium dodecyl sulphate and Tween 80 - 1% w/v), metal ions (Calcium chloride, Magnesium chloride, Potassium chloride, Sodium chloride and Zinc Chloride - 1% w/v) and varioussubstrates (Castor oil, Coconut oil, Cotton seed oil, Ground nut oil, Palm oil an Sesame oil – 1%

w/v) with varying incubation time (12, 24,36,48, and 60 Hour), temperature (30,40,50, and 60°C), pH (4,5,6,7,8, and 9) and inoculum size (1, 2,3,4, and 5%) were tested by one factor-at-a time strategy.

RESULTS AND DISCUSSION

Selection of Microorganisms

The bacterial strains isolated from fish market soil producing the clear zone in 1% tributyrin agar plates were selected for further studies. Among the isolated strains, the strain demonstrating maximum zone of clearance was isolated. The bacterial strain was initially characterised based on its morphological and culture properties, exhibiting smooth, viscous, convex, opaque, and white colonies on tributyrin agar (Fig. 1a) and the strain was gram-negative rod (Fig. 1b). The strain demonstrating zone formation of 10 mm on tributyrin medium at 37°C after 24 hours of incubation is presented in Figure (Fig. 1c). The 16S rRNA gene sequencing analysis confirmed that *P. aeruginosa strain* AV311222 had 100% homology with*P. aeruginosa* strain CL8 on the evolutionary distance and the phylogenetic tree study (Fig. 2).

Accession numbers

The sequence was submitted to GenBank, and accession numbers was received as PQ000241.

Optimisation of different factors for maximum lipase production

During the study, lipase activity of *P. aeruginosa* strain AV311222 was detected in culture supernatant at the basal medium containing olive oil (1%), peptone (5g/L), and yeast extract (3g/L) for 24 hours at 37°C. The basal media was prepared by the supplement with a 1% concentration level of carbon source, nitrogen source, inorganic nitrogen source, metal ions, surfactants, oils, pH, temperature, incubation time, inoculum size, and inorganic salts. The level of optimum variables were analysed in every step.

Impact of temperature on lipase production

The effect of temperature on lipase production was determined at a temperature range of 30-70°C, and high lipase production was recorded at 30°C as 7.51 U/ml (Fig. 3a). The study revealed that there is an increase in lipase production from the temperature between 37°C to 42°C and beyond the activity was found to decrease. A similar kind of study (Nguyen *et al.*, 2024) on *Bacillus subtlis* showed that a significant temperature of 30°C was found to be the optimum and a substantial decrease in lipase production was observed. *Serratia marcescens* EGHK-19 strain was found to produce high lipase activity, at 30°C after incubation of 24 hours (Issa *et al.*, 2024). At rising temperature, the enzymatic protein is found to denature thus reducing the activity of Lipase.

Impact of pH on lipase production

The pH of the growth medium is one of the most critical environmental parameters influencing microbial cell development and biochemical metabolism. The study revealed that an optimum pH of 6 was determined for lipase synthesis, and a maximum activity of 7.41 U/ml was obtained from *P. aeruginosa strain AV311222* (Fig. 3b). Optimum pH of 7 was recorded for the bacteria, *Bacillus* sp. (Ertugrul *et al.*, 2007).

Impact of incubation time on lipase production

The influence of incubation time on lipase production was analysed using basal media at the duration interval of 24 hours. The maximal lipase activity of 7.46 U/ml (Fig. 3c) was recorded after 24 hours of incubation. After 24 hours, the growth declined from the stationary phase because of the lack of nutrient and oxygen whichlimited the growth. Few studies reported 24-hour lipase production by using mustard oil cake using *Klebsiella aerogenes* and *Enterobacter hormachei* (Haryati et al,. 2024).

Impact of inoculum size on lipase production

The inoculum size is the most important factor for the production of lipase by the microorganism.[18] In thisexperiment, different inoculum sizes (1,2,3,4, and 5%) from 24-hour cultures were employed to evaluate the influence of inoculum size on lipase production. The maximal lipase activity of 4.29 U/ml was received at the 1% w/v of inoculum size (Fig. 3d). It is noticed that, high concentration of inoculum exhibited reduced lipase activity. This is attributed to utilization of low nutrient when compared to that of inoculum size. This

is in agreement with the previous study that 1% of inoculum size has significantly increased lipase activity by *Bacillus pumilis* (Savalia and Dungrechiya, 2022) and *Geobacillus* sp. Strain (Ebrahimpour *et al.,* 2008).

Impact of carbon source on lipase production

A carbon source plays a unique function in the metabolism of *Pseudomonas spp*. However, these carbon sources (1%) were examined for enzyme activity at 24 hours of incubation with maximum lipase production. In order to investigate the effects of carbon sources, viz., glucose, lactose, maltose, sucrose, dextrose monohydrate, and starch were used. As shown in Fig. 4a, high lipase activity of 4.08 U/ml was obtained when lactose was used as a carbon source and that significantly recorded high lipase activity than any other carbon sources studied. Current result is in agreement with the previous work on the 1% lactose carbon source which obtained the maximum lipase activity (Tembhurkar *et al.,* 2012).

Impact of organic and inorganic nitrogen sources on lipase production

Nitrogen sources would suggestively affect the pH of the medium; hence, several organic and inorganic nitrogen sources (1%) were explored for the optimization of lipase production (Esakkiraj *et al.,* 2010). Optimization of lipase production was observed with different organic nitrogen sources among which meat extract as a nitrogen source exhibited maximum lipase activity. The meat extract served as the best organic nitrogen source for the synthesis of lipase which gives a higher lipase activity of 7.5 U/ml (Fig. 4b). Similar report of usage of meat extract (0.5%) for *Staphylococcus epidermidis CMST*-Pi 1 exhibited the maximum lipase activity. Among the inorganic nitrogen sources, ammonium dihydrogen phosphate has recorded high lipase activity of 10.99 U/ml than other inorganic nitrogen sources (Fig. 4c).

Impact of Substrate on Lipase production

The substrate specificity of lipases is very significant for their analytical and commercial purposes. Thus, bacterial lipases are generally produced in the presence of oil or any other lipid substrate, like fatty esters, fatty acids, or glycerol, in the presence of any complex carbon and nitrogen source. In order to investigate the substrates on lipase production, various edible oilswere tested, such as coconut oil, castor oil, groundnut oil, sesame oil, palm oil, and cottonseed oil. The data presented in Fig. 4dsignifies that lipase activity of 5.90 U/ml was recorded for the palm oil used at 1% and the cottonseed oil is used exhibits a low level of lipase activity at 3.65 U/ml. In an agreement with similar study it was found that palm oil increased lipase activity to 13.83 U/ml significantly under optimal conditions ((Elgharbawy *et al.,* 2014). The usage of industrial waste of palm oil as a substrate resulted in the synthesis of maximum level of lipase of 15.41 U/mlwas achieved by *Aspergillus niger* (Silveira *et al.,*2016)

Impact of Surfactant on Lipase Production

The effect of surfactants on lipase optimisation was determined by analysing the lipase production with different surfactants (1%) like Tween 20, sodium dodecyl sulphonate (SDS), glycerol, triton, x-100, and tween 80. Fermentation media containing Tween 80 showed maximum lipase production (7 U/ml) after 24-hour incubation (Fig. 4e). While other surfactants, viz., sodium dodecyl sulphate (SDS), Tween 20, Triton x-100, and glycerol, showed a lower activity of 3.5 U/ml, 3.12 U/ml, 1.51 U/ml, and 0.79 U/ml respectively. The Maximum level of lipase production was observed when the substrate formed an emulsion, thereby presenting an interfacial area to the lipase (Saktaweewong *et al.,* 2011). A similar study used Tween 80 as a carbon source to influence lipase productivity via *Schizochytricum* sp (Byreddy *et al.,* 2017).

Impact of metal on lipase production

Metal ion activity has been hypothesised to be owing to changes in the solubility and behaviour of ionised fatty acids at interfaces, as well as changes in the catalytic properties of lipase (Barik *et al.,* 2022). The sensitivity of lipase to the effects of different metal ions on lipase production is shown in Fig. 4f.Some metal ions affect lipase activity, even though Zn^{2+} had the strongest inhibitory activity (0.433 U/ml), followed by calcium chloride (7.89 U/ml), potassium chloride (3.84 U/ml), magnesium chloride (6.16 U/ml) and sodium chloride (3.25 U/ml). It is evident from this study that, Zn^{2+} is extremely inhibitory in its effect on lipase activity (Zouaoui, B. and Bouziane, 2012).

CONCLUSION

The present study determined that extracellular lipase production by *P. aeruginosa* strain AV311222 isolated from the scattered soil of fish market from Gujarat. The synthesis of lipase wasoptimized by culture conditions, viz., temperature, pH, incubation time, inoculum size, carbon source, substrate, organic/inorganic nitrogen source and various metal ion compositions. The study revealed the usage of 30℃-42℃, pH 6.0, and 1% of inoculum size for the period of 24 hours produces more lipase for the strain, i.e. *P. aeruginosa strain* AV311222. Increased lipase activity can be further achieved by using various factors containing lactose (1%), Meat extract (1%), Ammonium dihydrogen phosphate (1%), calcium chloride (1%), Tween 80 (1%) and palm oil (1%). The study provides, cost-effective method for maximum lipase synthesis and inspires next level Response surface methodology (RSM) to obtain the high quality of lipase.

Declaration of competing interest

The authors declare that they have no known competing interests.

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Table 1: Primer design for analysis of 16S rRNA sequencing.

Primer	Sequence
Forward (27F)	5' AGAGTTTGATCTGGCTCAG 3'
Reverse (1492R)	5' TACGGTACCTTGTTACGACTT 3'

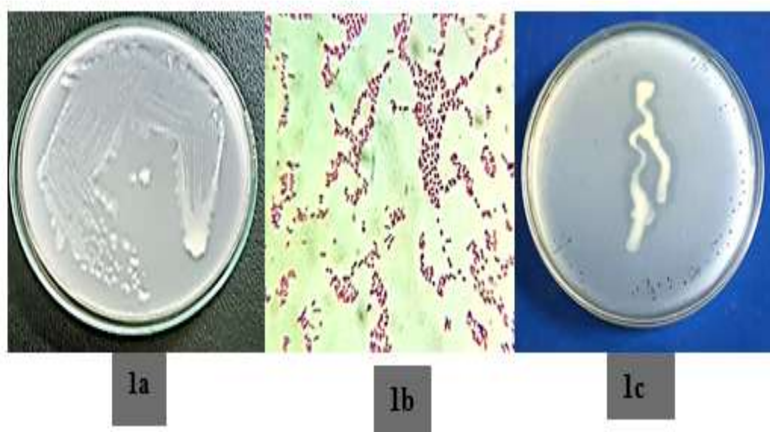


Fig. 1. a. Quadrant strike for morphological identifications; b. Gram stain for identified as gram-negative bacillus (40 X); c. 1% Tributyrin agar plate showing the lipase production,

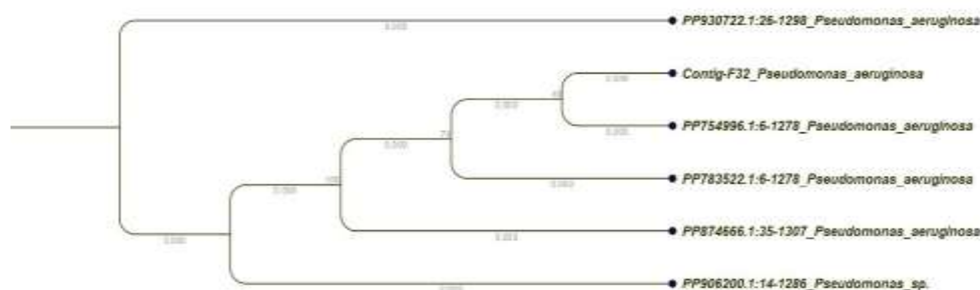


Fig. 2. Phylogenetic analysis of *P. aeruginosa* strain AV311222, 16S rRNA gene sequence with other P. aeruginosa/strains.

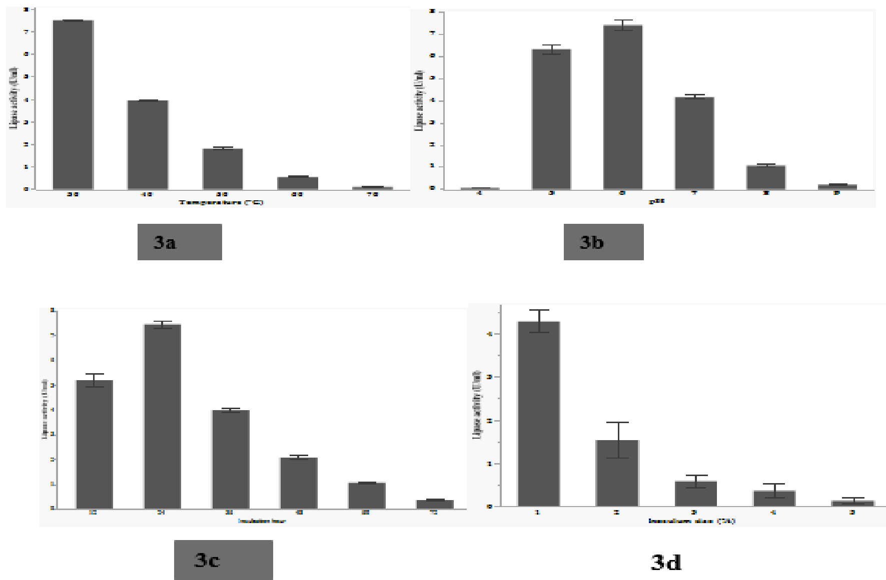
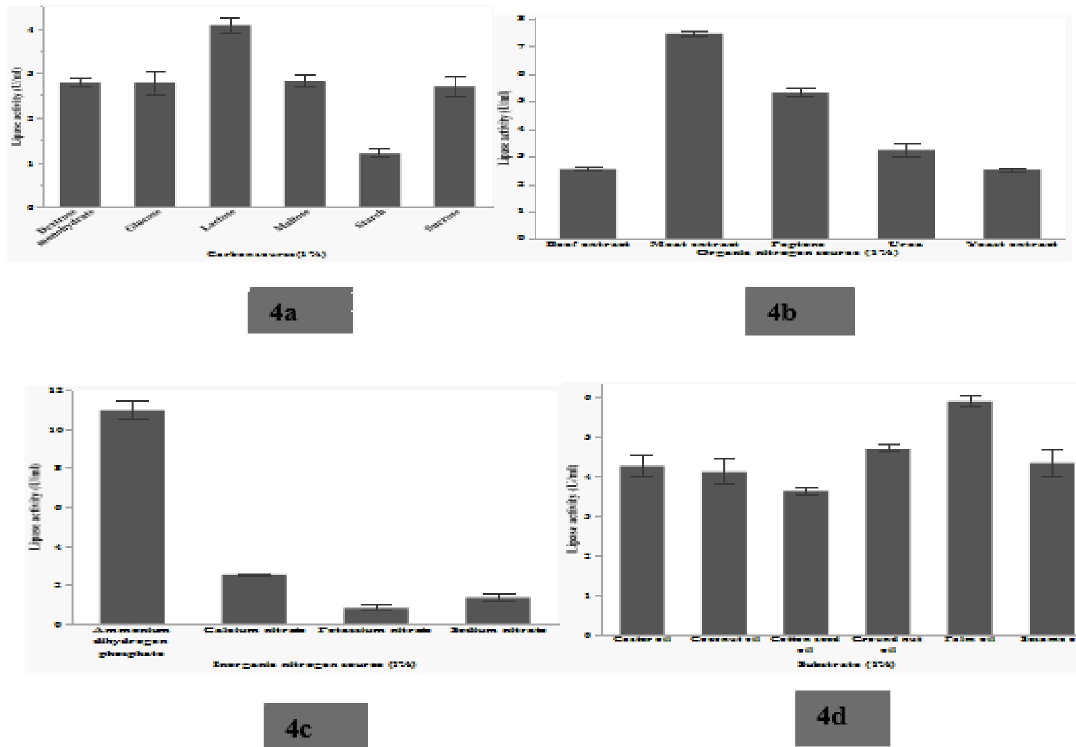


Fig. 3. Lipase activities represented by mean ± Standard Deviation (n=3), Effect of differential factors to the basal medium on lipase production by *P. aeruginosa* strain AV311222a. Effect of temperature on activity of lipase production b. Effect of pH on activity of lipase production; c. Effect of Incubation time on activity of lipase production; d. Effect of Inoculum size on activity of lipase Production.



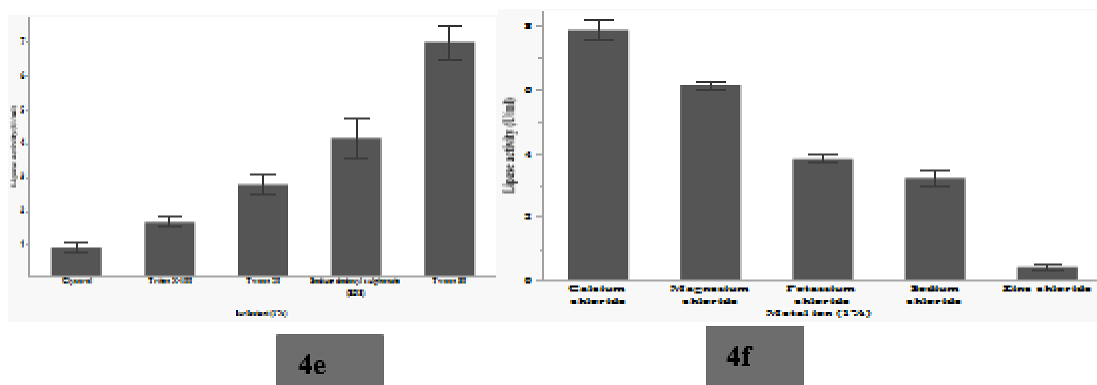


Fig. 4: Lipase activities represented by mean ± Standard Deviation (n=3), Effect of differential factors as additives (1% w/v) to the basal medium on lipase production by *P. aeruginosa* strain AV311222(Temperature 37℃; pH: 7.0, agitation: 100 rpm) a. Effect of carbon source (1%) on activity of lipase production ; b. Effect of organic nitrogen source (1%) on activity of lipase production; c. Effect of inorganic nitrogen source (1%) on activity of lipase production; d. Effect of substrate (1%) on activity of lipase production; e. Effect of surfactant (1%) on activity of lipase production ; f. Effect of metal ion (1%) on activity of lipase production.