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Production and optimization of lipase enzyme by *Pseudomonas* sps.

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ABSTRACT

Lipase (Triacylglycerol acylhydrolase, E.C. 3.1.1.3) is one of the well known industrial useful enzymes as it can catalyze different reactions including hydrolysis of triglycerides, transesterification and chiral synthesis of esters under natural conditions. In the present study, the lipase enzyme producing bacterial strain was isolated from soil contaminated with groundnut oil and identified as *Pseudomonas* sps based on its morphological, physiological and biochemical characteristics. The optimal parameters for lipase enzyme production were carried out by using different carbon and nitrogen sources in the medium maintained at pH-7 through submerged fermentation. Various chemical and physical conditions carried out in the present study, Olive oil, Glucose and Ammonium sulphate were served as best carbon and nitrogen sources for lipase production by *Pseudomonas* sps grown at 30°C during 24h incubation period respectively.

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KEYWORDS

Pseudomonas sps;
Lipase enzyme;
Production;
Optimization;
Submerged fermentation.

INTRODUCTION

Lipases or Triacylglycerol acylhydrolase (E.C.3.1.1.3) are hydrolytic enzymes that catalyze reversibly the cleavage of ester bonds of triglycerides^[1]. Lipases are unique serine esterases whose catalytic activity is greatly enhanced at the lipid-water interface, a phenomenon known as "Interfacial activation". Microbial lipases have been isolated, purified and biochemically characterized from a variety of microbial sources and it has been common feature in many types of yeast such as *Geotrichum candidum*, *C.antarctica*, *C.rugosa*, and moulds such as *A.niger*, *A.oryzae*, *Penicillium cyclopium*, *Rhizopus miehei* and bacteria like *Chromobacterium viscosum*^[2].

C.rugosa lipases have been termed of great significance for their diverse biotechnological potential^[3]. Existence of *C.rugosa* lipase isoforms has been reported by several authors^[3-5]. They used a strain of *C.rugosa* for extracellular lipase production in submerged fermentation (SmF) and solid state fermentation (SSF)^[6-9]. Different studies have been carried out on the selection of lipase production^[10-12], but the information available on the fermentation process is scanty. It was reported that strains of bacteria and yeast screened, the genera *Pseudomonas*, *Bacillus*, *Candida*, *Rhodococcus*, and *Staphylococcus* grow on the waste oils and produced lipolytic activity. The highest producers of lipase were *Pseudomonas* sps and *P.aeruginosa*^[13].

Based on the versatile reaction properties of lipases are widely used in industries such as food, chemical, pharmaceutical, detergent and organic synthesis industries^[14-16] and used as biocatalyst in the synthesis of life saving drugs^[4,17,18]. These include synthesis of Nikkomycin-B, non-steroidal anti inflammatory drugs such as Naproxen, Ibuprofen, Suprofen and Ketoprofen, antiviral agent Lamivudine etc. They have also been used for the synthesis of antitumour drugs, alkaloids, antibiotics etc.^[4]. Due to the high industrial applications of lipase enzyme an attempt was made in the present study to isolate prominent bacterial strain and optimization of lipase production through submerged fermentation by the bacterial strain.

MATERIALS AND METHODS

Collection of sample

Soil sample was collected from the site contaminated with oil spilled from ground nut oil mill (oil refinery) situated in Kurnool District, Andhra Pradesh, India. The collected soil was air dried and preserved in sterile polythene bag for further studies.

Isolation and screening of lipolytic bacteria

One gram of soil was suspended in 10 mL of sterile distilled water to form 10%(w/v) suspension and the same amount of this suspension was inoculated in to the flask containing 50 mL of enrichment medium following ingredients in g/L (K_2HPO_4 -2.5, $(NH_4)_2SO_4$ -1.3, $MgSO_4 \cdot 7H_2O$ -0.5, Yeast Extract -0.5, filter sterilized urea 6.5 mL (200g/L stock), and Oliveoil-20mL and the medium pH was adjusted to pH 7.0 After autoclaving the flasks were incubated at 30°C for 7 days at 100rpm on rotary shaker. The bacterial culture was isolated and maintained on nutrient agar medium.

The isolated bacterial culture was screened on minimal medium (MM). According to this 1 % (v/v) refined olive oil' tween-80 and tween-20 are added to the minimal medium. The medium contains following ingredients, for 1 liter of medium, 200mL of 5X minimal salt solution, 800mL of water and 15g of agar. After autoclaving, 2mL of 1M $MgSO_4$ (sterile filtrated), 0.1mL 1M $CaCl_2$ (sterile filtrated) and 20mL of 20% glucose (sterile filtrated) (carbon source) solution added to media. 5X minimal salt solution contains following in-

gredients (g/L) 64- $Na_2HPO_4 \cdot 7H_2O$, 15- KH_2PO_4 , 2.5- NaCl and 5- NH_4Cl (nitrogen source) autoclaved at 121°C, 1 bar for 15 minutes.

The lipolytic activity of *Pseudomonas* strain was screened on Minimal Medium agar containing 1% high refined olive oil (v/v) and fluorescent dye Rhodamine-B (0.005%) (Dissolve in distilled water and sterile filtrated). Orange-colored fluorescent halos around lipase producing colonies could be seen when these agar plates were exposed to UV light at 350nm.

Extracellular protein estimation

The total extracellular protein content was measured by the method of Lowry^[19] using Bovine serum albumin as standard.

Assay of lipase enzyme

Extra cellular lipase activity was measured in culture supernatants after centrifugation (10000 rpm for 10 min). 20 mL of oil was added to 80 mL of 2% Poly Vinyl alcohol solution and sonicated. The reaction mixture composed of 5 ml olive oil emulsion, 4 ml glycine-NaOH buffer (0.1 M, pH 9.0), and 1 ml of enzyme sample was incubated at 30°C in shaking water bath at 180 rpm for 1 h. At the end of the incubation, the emulsion was broken by addition of 20 ml acetone: ethanol mixture (1:1) and the liberated fatty acids were titrated with 0.05 N NaOH^[20].

Effect of physical parameters on lipase production

The effect of physical parameters (pH, temperature and incubation period) on lipase production was carried out on production medium containing the chemical ingredients (g/l) (Peptone 10, olive oil 10, yeast extract- 5, NaCl -1, NaH_2PO_4 -6.08, Na_2HPO_4 - 8.63, at pH 7.4. One ml of sterile $MgSO_4 \cdot 7H_2O$ stock solution (500 g/l) was added after autoclaving). 0.1% culture was inoculated into the autoclaved medium. The flasks were incubated on rotary shaker at 100 rpm, at 30°C for 2 days. The culture were collected at different time intervals and centrifuged at 1000rpm for 10min at 4°C. Lipase activity was estimated by titrimetry method.

Effect of carbon and nitrogen sources for lipase production

Hundred microliteres of bacterial suspension was

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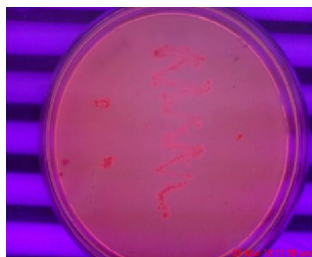


Figure 1 : Lipase positive colonies on agar plate containing Rhodamine B dye and olive oil

transferred to 250ml of Erlenmeyer flasks containing 100ml of production medium consisting of following ingredients (g/L). Peptone -5; Yeast Extract-2.5; K_2HPO_4 -1, $MgSO_4 \cdot 7H_2O$ -0.2, Castor oil- 5ml and marinating medium pH 7.. The flasks were incubated on rotary shaker at 100 rpm, at 30°C. Samples were removed at two different time intervals i.e., 24 and 48hrs. The samples were centrifuged at 10,000rpm for 10 min at 40°C and collected the supernatant for lipase enzyme measurement. For the determination various carbon and nitrogen sources for production of lipase, castor oil was replaced by the respective carbon source, at equal carbon content equivalent to 5g/L. Replacing peptone with various nitrogen sources, on equal nitrogen content basis i.e., 4g/L, assessed the effects of nitrogen sources.

RESULTS

Isolation and screening of lipolytic bacteria

The lipolytic bacteria was isolated from the soil and identified as *Pseudomonas* spp, based on the morphological and biochemical properties listed in the TABLE 1.

The production of lipase activity by *Pseudomonas* strain was screened on minimal agar medium containing olive oil and rhodamine-B plates incubate at 30°C. Orange colour was observed under UV illumination gave a positive result for lipase activity by *Pseudomonas* spp (Figure 1). Rhodamine- B gave a more convenient result than Tween- 20 and Tween- 80 used in the present study.

Estimation of total extracellular protein

The amount of total extracellular protein liberated by *Pseudomonas* spp was estimated and listed in the (Figure 2). The amount of protein was estimated as

76.64 μ g/mL/min at 24h of incubation period, after that the amount of protein gradually declined to 25.32 μ g/mL/min.

Effect of time incubation on lipase production

Two types of oils used in the present study olive oil induced the lipase production than the coconut oil. With increasing the incubation period lipase activity improved upto 24 hours there onwards declined in both oils the maximum lipase activity observed at 24 hrs incubation. For instance The lipase activity of *Pseudomonas* spp was maximum (8 μ ml/min) in medium containing olive oil and minimum in coconut oil (6.9 μ ml/min) (Figure 3).

Effect of temperatures on lipase production

The effect of temperature for production of lipase enzyme by *Pseudomonas* spp was studied and shown in figure 4. Among the different temperatures maintained in the present study, maximum lipase enzyme activity was found to be 8 μ ml/min at 30°C by *Pseudomonas* spp (Figure 4).

Effect of pH on lipase production

The enzyme was most active between the pH range of 6- 9. Maximum lipase activity found to be 9 μ ml/min at pH 7 (Figure 5). Constant lipase activity was estimated at 8 and 9 pH levels as 6 μ ml/min.

Effect of carbon and nitrogen sources on lipase production

The lipase production with various carbon sources from the bacterial strain *Pseudomonas* spp grown in the medium showed that the enzyme production was maximal, when olive oil was used as carbon source. *Pseudomonas* spp utilize several vegetable oils as well as hexoses and pentoses but it showed poor growth with disaccharides and polysaccharides. *Pseudomonas* spp secreted lipase enzyme activity of 8 U/mL with olive oil as a sole carbon source (Figure 7). Among the sugars used as carbon source in the present study glucose served as best carbon source for lipase production by *Pseudomonas* spp (Figure 6).

The effect of nitrogen sources on lipase production by *Pseudomonas* spp was tested. Ammonium sulphate was the best among all the nitrogen sources tested in the present study. For instance the lipase activity of bac-

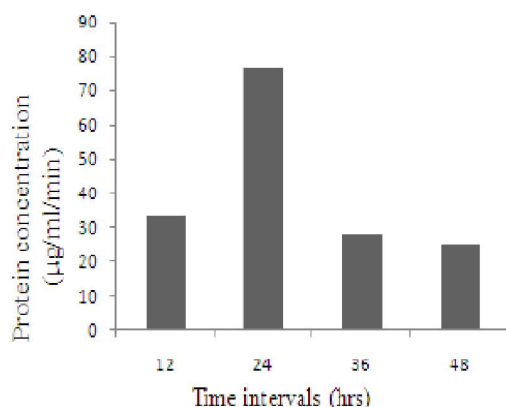


Figure 2 : Total protein content at different time intervals

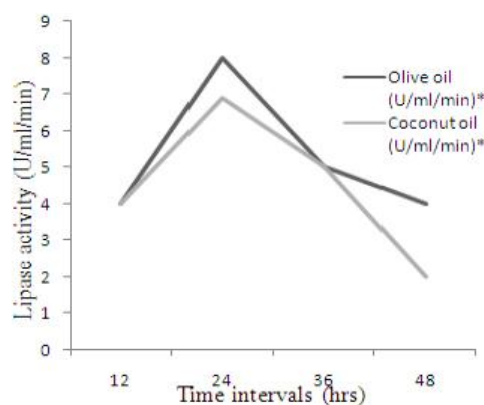


Figure 3 : Lipase production at different time intervals

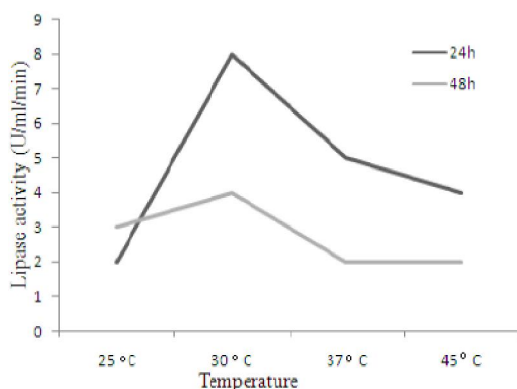


Figure 4 : Effect of different temperatures on lipase production

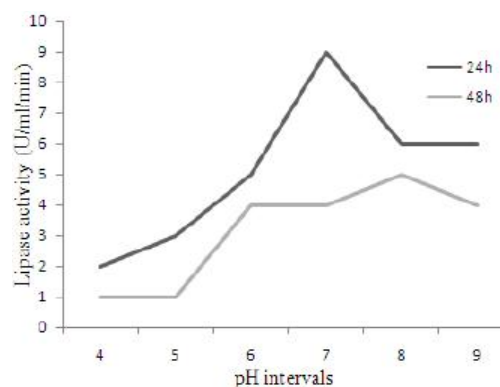


Figure 5 : Effect of medium pH on lipase production

terial culture grown in the medium contains Ammonium sulphate as nitrogen source was 8 µ/mL (Figure 8). Generally, the lipase production was very low with tryptone; whereas other organic and inorganic nitrogen sources produced more than 4-7 µ/mL of enzyme.

DISCUSSION

Agar plate assay is the most frequently used procedures for screening of lipase enzyme existant on Petri dishes. In this assay colored halos appear around the particular lipase producing colonies. Generally three substrates which were mostly used for plate assay are Tributylglycerol (tributyrylglycerol), Tween-80 and Tween -20. If Tweens are used as substrates for lipase production, no need of dyes for visualization^[21]. They give the white clear zones around the colonies when hydrolysis is pointing out either esterase or lipase activity^[22]. The fluorescence dye Rhodamine- B agar plate assay is describe as a true lipase assay which indicate the zone of lipolysis as an orange fluorescence under UV light at 350nm. Rhodamine- B in the presence of

olive oil forms a fluorescence complex with free fatty acids^[21].

Similarly Janegllberta^[23] reported that *Pseudomonas aeruginosa* strain EF2 grown in continuous culture gave the maximum protein content at 24 hours of incubation. Gobbetti^[24] also reported that the proteinaeous compound from the culture supernatant of *P. fluorescens* ATCC 948 were grown in nutrient broth medium and they estimated total protein content as 1652 mg/min.

Neelima Kulkarni^[20] reported that the lipase activity of *Pseudomonas fluorescens* was estimated at 32h incubation time. The maximum growth and lipase production was obtained after 72 h by *Bacillus coagulans*^[5]. Akram Kashmiri^[25] were reported the maximum extracellular lipase activity of 7.3 U/mL by *Trichoderma viride* at 48h incubation time.

The lipase enzyme exhibited optimum activity at 40°C by *Pseudomonas fluorescens* MTCC 2421^[26]. Sztajer^[7] stated that the temperature optimum for oil hydrolysis between 50 and 55°C, for a lipase from *Pseudomonas fluorescens*. The temperature optima

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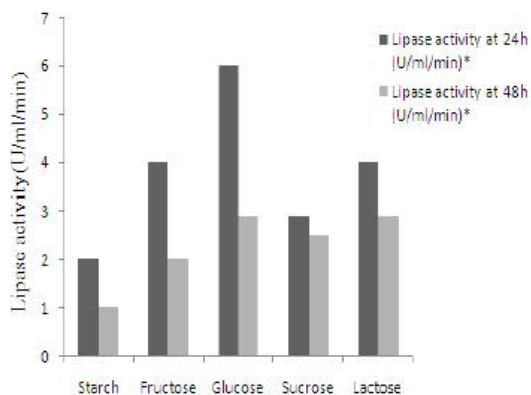


Figure 6 : Effect of carbon source on lipase production

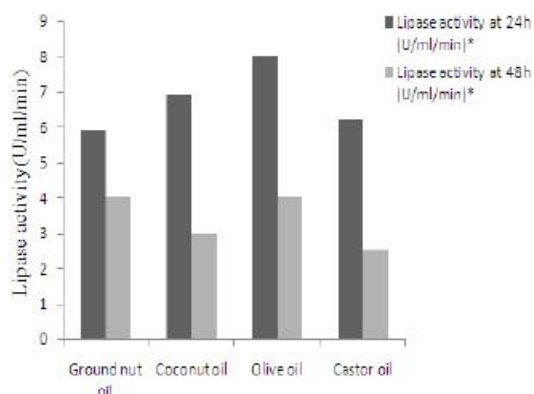


Figure 7 : Effect of oil source on lipase production

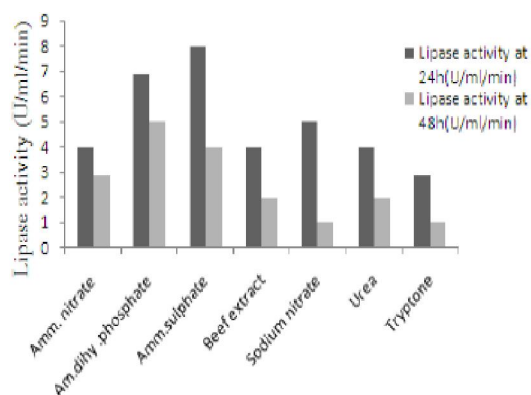


Figure 8 : Effect of nitrogen source on lipase production

around 55-65°C have been reported for lipases from *Pseudomonas* spp^[11,28-30]. The higher lipase activity of 5U/mL by *Bacillus* sps at 40°C were reported on slaughterhouse soil^[31]. *Pseudomonas* sp. strain KB700A were reported maximum activity at 35°C^[32] which was a low temperature for lipase production by compared with previously reported. In the present study, when compared with the reported authors, *Pseudomonas* sps was produced a maximum lipase at 30°C tem-

TABLE 1 : Morphological and biochemical characterization of bacterial isolate

S.No	Property	Isolate
1	Pigment production	Green
2	Colony size	2mm
3	Fluorescence under U.V.light	Blue
4	Gram's staining	-ve
5	Spore staining	-ve
6	Motility	+ve
7	Indole production	-ve
8	Methyl red production	-ve
9	V-P reaction	-ve
10	Citrate hydrolysis	+ve
11	Casein hydrolysis	+ve
12	Gelatin hydrolysis	+ve
13	Urea hydrolysis	-ve
14	Catalase test	-ve
15	Oxidation	+ve
16	Fermentation	-ve
17	TSI agar test	+ve

Note: +ve = Positive test; -ve = Negative test

perature. From this it shows that some what different than the other microorganisms for producing lipase enzyme.

Most bacterial lipases were reported to have pH optimum on alkaline condition. Among Gram positive bacteria, a *Bacillus subtilis* lipase^[20] was shown to have a very alkaline pH optimum between 10 and 11.5. *Pseudomonas* lipases were reported to have optimum pH in acidic^[29] as well as alkaline environment^[28,30,33]. In the present study also lipase had shown optimum activity on alkaline condition. In contrast *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Propionibacterium acnes*^[34] lipases were again exception to this, because they were more stable in the acidic range. The lipase from *Pseudomonas fluorescens*^[35] retained more than 80% of its activity following treatment at a pH range of 7-11.5 for 1h. Kojima et al.^[36] was reported the lipase from *Pseudomonas fluorescens* AK102 to be stable over pH range 4-11. Similarly lipase from *Pseudomonas* sp. was reported to be stable over pH range 5-9 with more than 70% activity retention for 2 h^[30].

Sztajer and Maliszewska^[35] have reported that starch was the best carbon source for lipase produc-

tion by *Pseudomonas fluorescens*. In *Pseudomonas fluorescens* 2D, the lipase production was inhibited by glucose^[37]. The maximum lipase activity 1.46 IU/mL was obtained during the submerged fermentation in a medium containing glucose at 2 % and olive oil at 2 % by *Aspergillus niger*^[38]. The present bacterial culture was able to synthesize lipase in presence of glucose and also enhanced the lipase production in the presence of olive oil. In this study, isolation was done from groundnut oil refinery but it shows sensitivity to olive oil.

Makhzoum^[37] reported that arginine, threonine and lysine as well as some ammonium salts of mineral acids supported good growth and lipase production by *Pseudomonas fluorescens*. Tryptone and casamino acids in combination with ammonium were reported to be the best nitrogen sources for lipase production by *Acinetobacter calcoaceticus* strains^[39]. Sibel Fadilo Glu and Osman Erkmen^[40] reported high yields of enzyme activity (5.58 μmL^{-1}) were obtained with yeast extract and protease-peptone in the medium with olive oil by *Candida rugosa*.

CONCLUSION

In the present study lipase producing bacteria was isolated from groundnut oil contaminated soil. The physical conditions for lipase production were optimized at temperate 30°C, pH 7 and 24hrs incubation period. Olive oil, glucose and ammonium sulphate were shown best carbon and nitrogen sources for the lipase production by *Pseudomonas* spp.

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