

PRODUCTION, PARTIAL PURIFICATION AND IMMOBILIZATION OF TRIACYLGLYCEROL LIPASE FROM INDIGENOUS LIPOLYTIC MICROORGANISMS

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Abstract— Triacylglycerol lipase was produced from indigenous *Aspergillus niger* and *Bacillus* sp. using submerged fermentation. The production process was optimized and lipase was then purified and immobilized. The optimization process improved enzyme production by many folds. Maximum lipase activity was obtained; with microorganisms cultured in medium containing 1% olive oil and 1% soybean meal as substrate, 72hrs incubation at 30°C, pH 6.0 and medium supplementation with 2% glucose, 1% peptone for *A. niger*, 72hrs incubation at 37°C, pH 8.0 and medium supplementation with 1% glucose, 1.5% peptone for *Bacillus* sp. The enzymes were purified using ammonium sulphate precipitation and ion exchange chromatography to achieve 13-14 fold purity with a yield ranging from 53-57%. Lewatite and Amberlite were found to supports of choice for the immobilization of bacterial and fungal lipases, respectively. The immobilized lipase is a promising tool for industrial applications in terms of its retention of enzymatic activity and reusability.

Keywords— Lipolytic; *Aspergillus*; *Bacillus*; Fermentation; Enzyme immobilization.

I. INTRODUCTION

Lipases (E.C 3.1.1.3, triacyl glycerol acylhydrolase) are hydrolytic enzymes that catalyze the hydrolysis of esters (Scheib *et al.*, 1999). On hydrolysis, they convert triglycerides to di, mono-glycerides, fatty acid and glycerols. Lipases are versatile biocatalysts as in non-aqueous environment they catalyze various reactions like esterification, transesterification and interesterification (Abbas *et al.*, 2002). They exhibit unique physicochemical property of catalyzing reactions at lipid-water interface and belong to the group of globular proteins having molecular weight of 20-60 KDa (Geraldine *et al.*, 2008). Serine, histidine and aspartic acids are present at the active site. These residues coordinate to catalyze reactions. Lipases are specific to the substrates. On the basis of this specificity, they are classified into three groups which are fatty acid specific, region specific and non-specific (Imandi *et al.*, 2010).

Lipases are abundantly present in different species of microbes, plants and animals. The energy reserved tissues of the plants have their significant amount (Sharma *et al.*, 2001) such as Maize, castor and rape seeds. Animals can also serve as sources of lipases as they have considerable amounts of lipases in their pancreas. However, pancreatic lipases cannot be used commercially as

the extract contains high concentration of trypsin, animal viruses, different hormones and other undesirable components which can bring unfavorable changes in the desired product (Vakhlu and Kour, 2006). Therefore, on industrial scale, microbes are preferred for production of lipases. Among microbes, filamentous fungi are the main producers of extracellular lipases. Different species of yeasts, fungi and bacteria are capable of producing lipases. They include *Aspergillus*, *Mucor*, *Penicillium*, *Pseudomonas* and *Bacillus*, etc. (Bisht *et al.*, 2012; Huang *et al.*, 2012)

The lipolytic microbes are present in various habitats e.g. soil contaminated with oil, factories which are involved in the processing of vegetable oils and industrial waste etc. Microbial lipases are produced by solid state and submerged fermentation. However, they are produced mainly by submerged fermentation as high enzyme productivity is achieved in this type of fermentation. The production process is affected by three main factors i.e. type of fermentation, design of the bioreactor and composition of media (Rodriguez *et al.*, 2006). Moreover, it is also affected by parameters of fermentation. Various environmental factors also play an important role in the production of lipases which include temperature, pH, agitation and aeration (Burkert *et al.*, 2005).

Lipase is one of the most important and versatile industrial enzyme. It is responsible for the natural generation of new flavors in different dairy products (Liu and Vaughan, 2009). Pretreatment of soybeans with lipases result in the significant reduction in cooking time thus, preventing the nutrient content of soybeans from deleterious effect of excessive cooking (Khetarpaul *et al.*, 2004). During the processing of paper, lipases are used in combination with cellulases and ligninase for the treatment of pulp, as a result the quality and yield of paper improves enormously (Sharma *et al.*, 2002). Lipases also find applications in the production of agrochemicals and drugs (Lotti and Alberghina, 2007). They may also be utilized to enhance degradation of fatty waste and for making polyurethane (Padmapriya *et al.*, 2011). In the manufacturing of different types of noodles and pasta, the quality of wheat flour can be varied when treated with lipases (Ozan and Fadiloglu, 2010). Lipases may be used for biodegradation of plastics (Imandi *et al.*, 2010).

In the present study microorganisms isolated from soil were screened for lipase production. Different culture conditions such as chemical composition of culture

medium, effect of incubation time, incubation temperature, pH, and different substrates were optimized for lipase biosynthesis. The produced enzyme from lipolytic microorganisms was then purified and immobilized on resins. Further investigations may be carried out for large scale lipase production from microbes and their efficient industrial applications.

II. MATERIALS AND METHODS

A. Chemicals

All the chemicals were of analytical grade (Merck, Sigma, USA). Olive oil, canola oil, coconut oil, sunflower oil, pure and crude cotton seed oil, black seed oil (kalonji oil), goose berry oil (aamla oil) and soap berry oil (reetha oil) were purchased from local market.

B. Isolation and screening of lipolytic microorganisms

The fungal and bacterial strains i.e. *Aspergillus niger* and *Bacillus* sp. used in present study were isolated from soil. The microorganisms were cultured on potato dextrose agar and nutrient agar slants and incubated at 30°C and 37°C respectively for 48 hrs. The slants were maintained and stored at 4°C. The bacterial strain was identified on the basis of different morphological and biochemical tests (Holt *et al.*, 1994). The medium proposed by Cipinyle *et al.* (2009) was used to detect the production of lipase from lipolytic microorganisms. The composition of the medium was (g/l) tween 80, 5.0; peptone 10.0; NaCl, 5.0; CaCl₂.2H₂O, 0.1 and agar 18.0, pH 7.0. Ten different culture media were screened, five each for *Aspergillus niger* and *Bacillus* sp. to compare and select the best medium for maximum cell mass and enzyme production (Table I and II).

C. Lipase production

The crude lipase production was performed in 250 ml of Erlenmeyer flasks carrying 50 ml of fermentation media. The fermentation medium used for lipase production by *Aspergillus niger* was composed of (g/l) soy bean meal, 10.0; olive oil, 20.0; glucose, 10.0; K₂HPO₄, 2.0; NaNO₃, 0.5 and MgSO₄.7H₂O, 0.5, pH 7.0 (Iftikhar *et al.*, 2010). The medium was inoculated and incubated at 30°C for 72 hrs at 200 rpm. On the other hand, the fermentation medium used for lipase production by *Bacillus* sp was composed of (g/l) yeast extract, 50.0; K₂HPO₄, 1.0; MgSO₄.7H₂O, 5.0; KCl, 5.0; FeSO₄.7H₂O, 1.0; olive oil, 10.0 and NaNO₃, 3.0, pH 7.2 (Chakraborty and Paulraj, 2009). The medium was inoculated and incubated at 37°C for 72 hrs at 200 rpm. After 72 hrs of incubation, the fermented broth was transferred to centrifugation tubes and was centrifuged for 15 min at 6000 rpm. The supernatant was used for assay of lipase.

D. Lipase activity determination

Titrimetric method was used for activity assay of lipase by titrating liberated fatty acids with alkali (Aly *et al.*, 2012). All the culture media were used for this assay in successive treatments to select the best medium for lipase production. The mixture of assay consisted of 1.0 ml of supernatant along with 10 ml of 10% olive oil homogenized in 10 g of gum acacia, 5 ml of phosphate buffer (pH 7.0), and 2.0 ml of 0.6% of CaCl₂. The reaction mixture

Table I. Composition of fermentation media for lipase production by *Aspergillus niger*

1.	M1	(g/l) Peptone, 1.0; Glucose, 2.0; Yeast extract, 20.0; K ₂ HPO ₄ , 1.0; (NH ₄) ₂ SO ₄ , 5.0; Olive oil, 20.0 (Loo <i>et al.</i> , 2006) (pH 7.2).
2.	M2	(g/l) Peptone, 10.0; Yeast extract, 2.5; MgSO ₄ .7H ₂ O, 0.2; KH ₂ PO ₄ , 0.5; NaNO ₃ , 0.5; Olive oil, 10.0 (Patricia <i>et al.</i> , 2005) (pH 6.0).
3.	M3	(g/l) Peptone, 10.0; Yeast extract, 1.0; MgSO ₄ .7H ₂ O, 0.25; KH ₂ PO ₄ , 1.0; NaNO ₃ , 0.25; Olive oil, 5.0 (Mahadik <i>et al.</i> , 2004) (pH 5.5).
4.	M4	(g/l) Poly peptone, 25.0; Yeast extract, 5.0; MgSO ₄ .7H ₂ O, 0.2; KH ₂ PO ₄ , 7.0; Na ₂ HPO ₄ , 1.2; Olive oil, 2.5 (Hiol <i>et al.</i> , 1999) (pH 6.0).
5.	M5	(g/l) Yeast extract, 10.0; Glucose, 0.5; CaCl ₂ , 0.1; KH ₂ PO ₄ , 1.0; (NH ₄) ₂ SO ₄ , 1.5; Olive oil, 10.0 (Kamariah <i>et al.</i> , 1996) (pH 5.0).

Table II. Composition of fermentation media for lipase production by *Bacillus* sp.

1.	M1	(g/l) Peptone, 4.0; Glucose, 5.0; Yeast extract, 3.0; K ₂ HPO ₄ , 2.0; Olive oil, 10.0; NH ₄ Cl, 8.0; FeSO ₄ .7H ₂ O, 0.06; MgSO ₄ .7H ₂ O, 0.4; NaCl, 1; KCl, 0.1 (Qamsari <i>et al.</i> , 2011) (pH 7.0).
2.	M2	(g/l) Peptone, 2.0; MgSO ₄ .7H ₂ O, 0.4; Olive oil, 10.0; NaCl, 2.5; CaCl ₂ .2H ₂ O, 0.4; (NH ₄) ₂ HPO ₄ , 1.0; Tween 80, 1-2 drops (Hasan Beikdashti <i>et al.</i> , 2012) (pH 7.0).
3.	M3	(g/l) Na ₂ HPO ₄ , 12.8; Glucose, 2.0; NaCl, 0.5; Olive oil, 10.0; NH ₄ Cl, 1.0; KH ₂ PO ₄ , 3.0; MgSO ₄ .7H ₂ O, 0.1 (Heravi <i>et al.</i> , 2008) (pH 7.0).
4.	M4	(g/l) Peptone, 5.0; Yeast extract, 5.0; Olive oil, 10.0; NaCl, 0.5; CaCl ₂ .2H ₂ O, 0.05 (Kumar and Gomes, 2005) (pH 8.5).
5.	M5	(g/l) Peptone, 30.0; Yeast extract, 10.0; Olive oil, 10.0; NaCl, 5.0 (Sugihara <i>et al.</i> , 1991) (pH 7.0).

was incubated at 30°C for 1 h in rotary shaker with 100 rpm. The enzymatic reaction was inhibited by the addition of 20 ml of acetone, ethanol mixture (1:1). The fatty acids which were released during incubation were titrated with 0.1 N NaOH.

One unit of lipase activity is defined as “The amount of enzyme which releases one micro mole (μmol) of fatty acid per minute under specified assay conditions.”

Lipase units will be determined as follows:

$$\text{Lipase activity} = \frac{\Delta V \times N}{V_{(\text{sample})}} \times \frac{1000}{60}$$

where $\Delta V = V_2 - V_1$, V_1 = Volume of NaOH used against control flask, V_2 = Volume of NaOH used against experimental flask, N = Normality of NaOH and $V_{(\text{sample})}$ = Volume of enzyme extract.

E. Determination of dry cell mass

The cell pellets of *Aspergillus niger* were obtained after centrifugation at 6000 rpm. The supernatant was discarded and the pellets were washed with distilled water. After washing, they were filtered by using Whatman filter paper No. 43 and then dried for 2 hrs at 110°C. In case

of *Bacillus* sp, the cell pellets were gained after centrifugation. These pellets were washed with distilled water and then dried at 60°C for 2 hrs.

F. Optimization of fermentation conditions

For optimizing fermentation culture conditions for lipase production, effect of different parameters were studied. The experiment conducted for lipase production was regulated to study these parameters followed by subsequent enzyme activity determination. Varying incubation time (24-120 hrs), incubation temperature (25-45°C) and pH of the medium (5.0-8.0 for *Aspergillus niger* and 6.0-10.0 for *Bacillus* sp.) were investigated. Effect of different carbon sources i.e. glucose, sucrose, fructose, starch, maltose, lactose whereas nitrogen sources i.e. peptone, yeast extract, meat extract, urea, corn steep liquor and a variety of natural oils as substrates were also investigated during the study.

G. Enzyme purification

The purification of lipase was achieved by combination of salt precipitation and ion exchange chromatography. The crude fermentation broth was centrifuged at 3000 rpm for 10 min and the supernatant obtained was used for enzyme purification. Ammonium sulphate precipitation at saturation levels from 20 to 80% was achieved by the addition of varying amounts of the salt to the broth supernatant in combination with stirring and centrifugation (3000 rpm for 10 min) at 4°C. All the pellets obtained at different salt saturation levels were re-suspended in phosphate buffer (pH 7.3) and dialyzed against the same buffer using a dialysis tubing – visking size 9 (MWCO 12-14000 Da) and assayed for enzyme activity and protein content (Bradford *et al.*, 1976). The pellets showing lipase activity were pooled and subjected to further purification through ion exchange chromatography.

The pooled sample was loaded onto DEAE Cellulose Fast Flow column equilibrated with 100mM potassium phosphate buffer (pH 6.5). The column was connected to a Biologic LPC system (BioRad). The bound proteins were eluted with a NaCl salt gradient (0–0.8 M) in the 100mM potassium phosphate buffer (pH 6.5). The flow rate was adjusted as 1.25 ml/min. A single peaks showing lipase activity was collected in a fraction collector and was further concentrated through freeze drying.

H. Immobilization of purified lipase

Three ionic resins namely Amberlite XAD 761, Lewatite VPOC, and Duolite A568 were used for the immobilization of purified lipase. The resins were first equilibrated with phosphate buffer (100 mM pH 7.0) at room temperature for 16 hrs after which, the mixture was vacuum filtered through a membrane filter (0.2µm). The resins collected on the filter were then incubated with the lipase dissolved in phosphate buffer at 4°C for 2 hrs. The vacuum filtered immobilizates were used for further studies. Immobilized enzyme was re-used for upto 10 times to determine the re-usability of the immobilizate. The immobilized enzyme was washed after every assay with phosphate buffer and was re-used in a fresh assay. The Bradford method (Bradford *et al.*, 1976) was used for protein

quantification. The enzyme activity of the first assay was

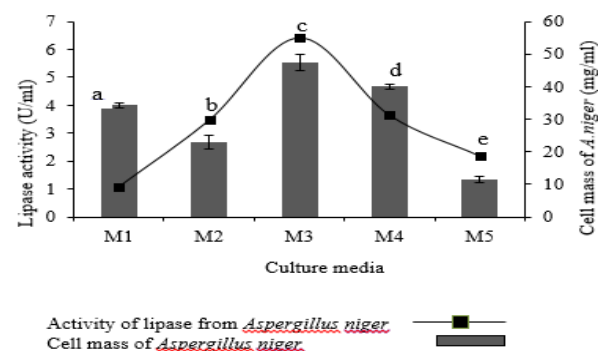


Figure 1. Effect of different culture media on lipase production from *Aspergillus niger**. Y- error bars indicate the standard deviation of means among three parallel replicates. The alphabets differ significantly at $p \leq 0.05$ level.

*Incubation temperature 30°C, incubation time 72 hrs, pH 5.5

considered as 100% relative activity.

I. Statistical Analysis

All the experiments were carried out in triplicates and the results are presented in the form of mean values. The results were subjected to statistical analysis using software CoStat 3.03 CoHort Software, Berkeley, CA 94701. Duncan's multiple range test was applied under one way ANOVA. Significance has been presented in the form of probability ($p < 0.05$) values.

III. RESULTS AND DISCUSSION

A. Screening of lipolytic microbes

The isolation of lipase producing bacterial and fungal strains from different habitats was carried out on Tween 80 agar plates using serial dilutions. The microbial colonies which exhibited white colored zone around their colonies were isolated. 20 bacterial and 20 fungal strains were selected. The selected strains were grown in the fermentation media and screened for lipase production through submerged fermentation. The fungal and bacterial strain, IIB-H and IIB-F showed highest activities of lipase i.e. 5.46 ± 0.06 U/ml and 4.0 ± 0.027 U/ml, respectively (IIB: Institute of Industrial Biotechnology, H and F: sequence of isolation).

The selected bacterial and fungal strains were identified on the basis of different morphological and biochemical characteristics (Holt *et al.*, 1994). They were further identified by 16S rRNA sequencing in another study. The selected bacterial strain was identified as *Bacillus pumilus* while the fungal strain was identified as *Aspergillus niger*.

B. Screening of culture media

For lipase production, ten different culture media were screened. In case of fungi, the culture medium M3 (g/l: Peptone, 10.0; Yeast extract, 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25; KH_2PO_4 , 1.0; NaNO_3 , 0.25; Olive oil, 5.0, pH 5.5) exhibited maximum units of lipase (5.3 ± 0.12 U/ml). Moreover, highest cell mass from *Aspergillus niger* (55.1 mg/ml) was also obtained in the presence of M3 culture medium. The remaining culture media showed less lipase

production (Fig. 1). The decreasing order of lipase yield in different media was $M4 > M1 > M2 > M5$.

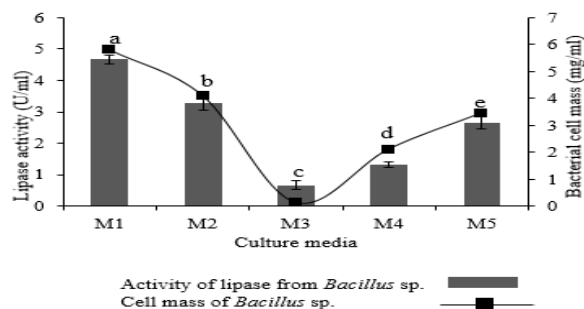


Figure 2. Effect of culture media on lipase production from *Bacillus pumilus**. Y-error bars indicate the standard deviation of means among three parallel replicates. The alphabets differ significantly at $p \leq 0.05$ level.

*Incubation temperature 37°C, incubation time 72 hrs, pH 7.0

Similarly, in case of *Bacillus* sp, the culture medium M1 (g/l Peptone, 4.0; Glucose, 5.0; Yeast extract, 3.0; K_2HPO_4 , 2.0; Olive oil, 10.0; NH_4Cl , 8.0; $FeSO_4 \cdot 7H_2O$, 0.06; $MgSO_4 \cdot 7H_2O$, 0.4; NaCl, 1; KCl, 0.1, pH 7.0) gave the highest production of lipase (4.6 ± 0.02 U/ml) and maximum cell mass of *Bacillus* sp (5.8 mg/ml). As compared to culture medium M1, the rest of the media showed decreased production of lipase (Fig. 2). The decreasing order of lipase yield in different media was $M2 > M5 > M4 > M3$.

Due to high production of lipase from *Bacillus pumilus* and *Aspergillus niger*, the culture media M1 and M3 were selected for further analysis. The maximum production of lipase may occur due to the presence of peptone and glucose in the culture media which are readily metabolizable nitrogen and carbon sources, respectively. Moreover, these media (M1 and M3) contain variety of nutrients that favor microbial growth and enzyme production. KCl and NaCl are the mineral salts that are capable of enhancing the growth of microorganisms. K_2HPO_4 and KH_2PO_4 act as the most effective source of potassium and phosphorus. The presence of $NaNO_3$ increases the fungal growth in the media (Chahinian *et al.*, 2000). The enhanced fungal growth may be due to the specific requirement of sodium or nitrate ions or partial replacement of other limiting component in medium (Jones and Jennings, 1965). On the other hand, other culture media did not support significant amount of lipase production. As compared to simple culture media, the production of lipase is increased in a complex culture media. These media either lack these nutrients or the nutrients are not present in significant amount that increased the production of lipase.

C. Effect of incubation time on lipase production

The effect of varying incubation times (24-120 hrs) on the production of lipase from both the *Aspergillus niger* and *Bacillus* sp. was studied. *Aspergillus niger* gave maximum activity of lipase (6.2 ± 0.31 U/ml) after 72 hrs of incubation. Similarly, *Bacillus* sp. also showed highest activity of lipase (5.1 ± 0.09 U/ml) after 72 hrs of incuba-

tion. Initially, the lipase production was low and gradually began to increase and reached maximum at 72 hrs. However, further increase in the incubation time lowered

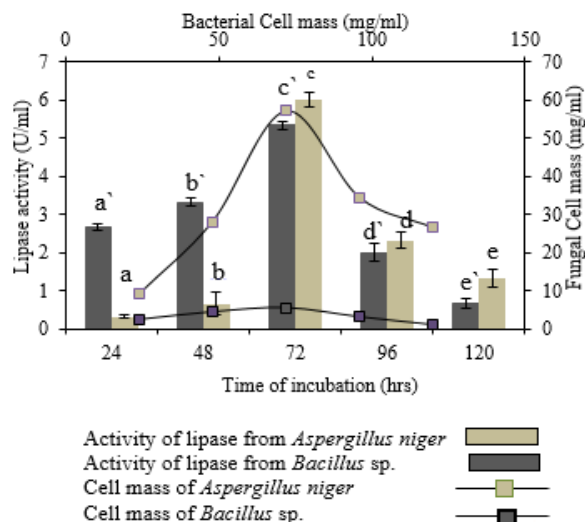


Figure 3. Effect of incubation time on lipase production. Y-error bars indicate the standard deviation of means among three parallel replicates. The alphabets differ significantly at $p \leq 0.05$ level.

the production of lipase (Fig. 3). Maximum cell mass of 57.2 mg/ml was obtained from *Aspergillus niger* and *Bacillus* sp gave 5.6 mg/ml of cell mass after 72 hrs of incubation.

The production of lipase was decreased due to the exhaustion of essential nutrients present in the fermentation medium and accumulation of different by products, thus resulting in decreased yield of desired product. Nadia *et al.* (2010) obtained maximum activity of lipase after 72 hrs of incubation from *Mucor racemosus*. Mukesh *et al.* (2012) obtained highest lipase activity from *Bacillus* sp. after 72 hrs of incubation. Similarly, Tembhurkar *et al.* (2012) also reported that maximum lipase production was achieved from *Pseudomonas* sp. after 72 hrs of incubation. Thus, 72 hrs of incubation was optimized for lipase production from *Aspergillus niger* and *Bacillus pumilus*.

D. Screening of different substrates

The effect of variety of natural oils such as olive oil, canola oil, coconut oil, sunflower oil, pure and crude cotton seed oil, black seed oil (kalonji oil), goose berry oil (aamla oil) and soap berry oil (reetha oil) on the lipase production was analyzed. Both *Aspergillus niger* and *Bacillus* sp. gave maximum activity of lipase in the presence of olive oil (7.3 ± 0.09 U/ml and 5.3 ± 0.03 U/ml, respectively). However, *Aspergillus niger* also produced significant amount of lipase (6 ± 0.21 U/ml) in the presence of crude cottonseed oil whereas moderate production of lipase (4.6 ± 0.19 U/ml) by *Bacillus* sp. was achieved in the presence of coconut oil. Other substrates did not support the high yield of lipase (Fig. 4). Highest cell mass of 62.6 mg/ml from *Aspergillus niger* and 3.8 mg/ml from *Bacillus* was obtained in the presence of olive oil.

The effect of different concentrations of olive oil on lipase production was also evaluated. Maximum activity of lipase (7.3 ± 0.07 U/ml) was obtained from *Aspergillus niger* in the presence of 1% olive oil. *Bacillus* sp. also

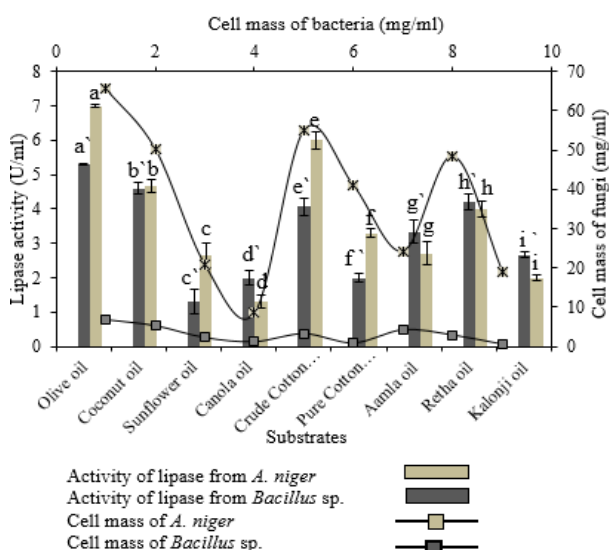


Figure 4. Effect of different substrates (oils) on lipase production. Y-error bars indicate the standard deviation of means among three parallel replicates. The alphabets differ significantly at $p \leq 0.05$ level.

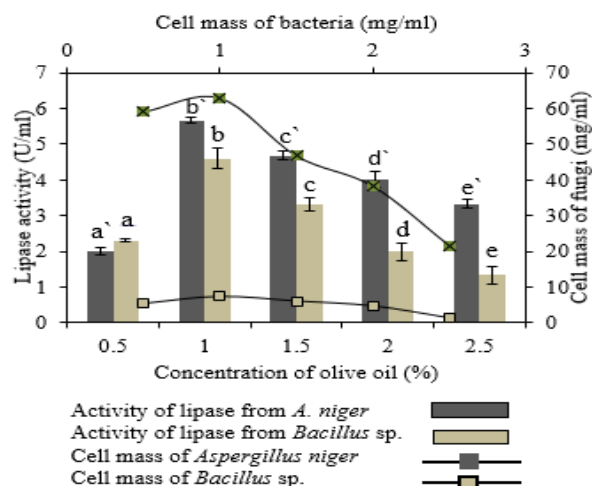


Figure 5. Effect of different concentrations of olive oil on lipase production. Y-error bars indicate the standard deviation of means among three parallel replicates. The alphabets differ significantly at $p \leq 0.05$ level.

gave the highest activity of lipase (5.3 ± 0.21 U/ml) in the presence of 1% of olive oil. Further increase in the amount of olive oil caused a considerable decline in the activity of lipase (Fig. 5). The culture media containing 1% of olive oil as substrate influenced the growth of both *A. niger* and *Bacillus* sp. i.e. 65.9 mg/ml of cell mass from *Aspergillus niger* and 4.6 mg/ml from *Bacillus* sp.

Olive oil has a significant effect on lipase production. In the presence of olive oil, the synthesis of lipase is approximately increased to 13 folds. It has an ability to evoke the secretion of lipase from the microbial cells.

However, it does not have a prominent impact on the microbial growth. Prasad and Manjunath (2012) gained maximum activity of lipase from different species of *Bacillus*, *Pseudomonas* and *Staphylococcus* while utilizing olive oil as substrate.

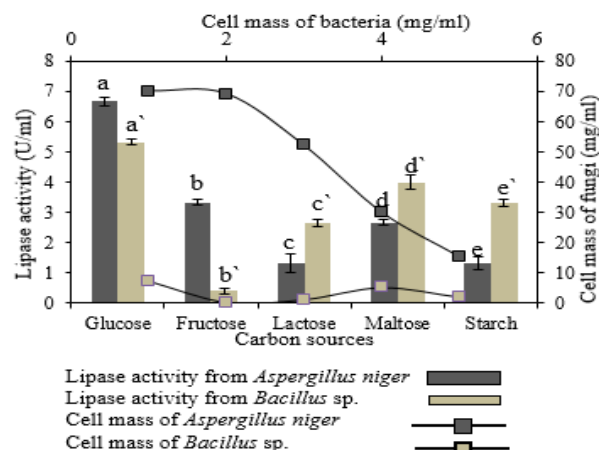


Figure 6. Effect of different carbon sources on lipase production. Y-error bars indicate the standard deviation of means among three parallel replicates. The alphabets differ significantly at $p \leq 0.05$ level.

The lipase production decreases with an increase in the concentration of olive oil. Excess amount of olive oil in the culture medium may have an inhibitory effect on the production of lipase. As, high concentration of olive oil causes less transfer of oxygen in the culture medium, thus the microbial growth and their metabolism is affected (Gaspar *et al.*, 1999). Nadia *et al.* (2010) also obtained maximum lipase activity from *Mucor racemosus* in the presence of 1% olive oil. Similarly, Sirisha *et al.* (2010) gained highest lipase activity from *Staphylococcus* in the presence of 1% olive oil and Tembhurkar *et al.* (2012) reported that *Pseudomonas* sp. also gave maximum lipase activity in the presence of 1% olive oil.

E. Effect of different carbon sources

The effect of various carbon sources i.e. glucose, sucrose, fructose, starch, maltose and lactose on the production of lipase from both *Aspergillus niger* and *Bacillus* sp was investigated. Highest yield of lipase from *Aspergillus niger* and *Bacillus* sp was achieved in the presence of glucose (6.67 ± 0.13 U/ml and 5.3 ± 0.09 U/ml, respectively). Rest of the carbon sources did not play a significant role in lipase synthesis (Fig. 6). Maximum cell mass of 70.1 mg/ml from *Aspergillus niger* and 7.3 mg/ml of cell mass from *Bacillus* sp was obtained in the presence of glucose.

The influence of varying concentrations of glucose on the production of lipase was also studied. *Aspergillus niger* gave maximum activity of lipase (7.3 ± 0.09 U/ml) and highest cell mass (78.2 mg/ml) in the presence of 2% glucose whereas *Bacillus* sp gave maximum activity of lipase (5 ± 0.26 U/ml) and cell mass (6.5 mg/ml), in the presence of 1% glucose. Further increase or decrease in glucose concentration caused a decrease in lipase production (Fig. 7).

Microbes exhibit high rate of activity when they are

grown in a medium containing glucose. Glucose is a highly oxidizable carbon source and is readily available due to its solubility in water. As compared to other carbon sources, it has a marked stimulating impact on lipase synthesis moreover; it also increases the biomass content of the microbes (Fadiloglu and Erkmen, 2002). Fadiloglu

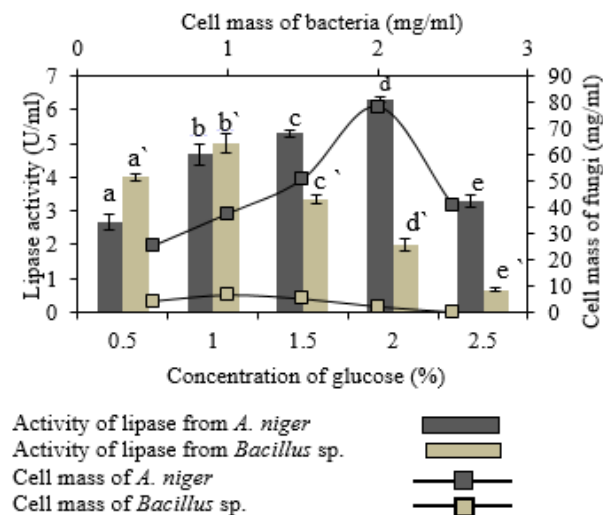


Figure 7. Effect of different glucose concentrations on lipase production. Y-error bars indicate the standard deviation of means among three parallel replicates. The alphabets differ significantly at $p \leq 0.05$ level.

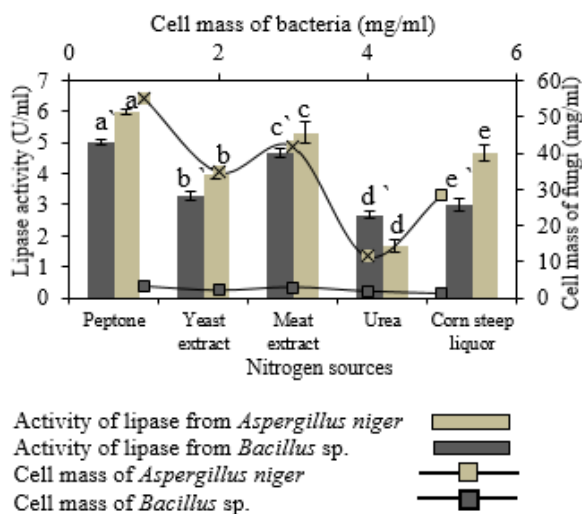


Figure 8. Effect of different nitrogen sources on lipase production. Y-error bars indicate the standard deviation of means among three parallel replicates. The alphabets differ significantly at $p \leq 0.05$ level.

and Erkmen (2002) obtained maximum production of lipase from *Candida rugosa* in the presence of glucose. Heravi *et al.* (2008) obtained maximum production of lipase in the presence of glucose from *Bacillus* sp.

The amount of glucose in the culture medium plays a significant role in the production of lipase. The optimum concentration of glucose stimulates the high production of lipase (Hosseinpour *et al.*, 2011). In the presence of low glucose concentration, initially growth of the cells takes place and after attaining appropriate growth, the rapid synthesis of lipase starts as glucose stimulates the

microbial cells to release lipase. Very high concentration of glucose supports the growth of microbes but it has an inhibitory effect on lipase synthesis (Fadiloglu and Erkmen, 2002). Shahidan *et al.* (2011) obtained maximum activity of lipase from *Pichia pastoris* in the presence of 2% glucose while Qamsari *et al.* (2011) obtained maximum lipase production from *Pseudomonas aeruginosa* in the presence of 1% glucose.

The culture medium supplemented with peptone has a significant effect on the production of lipase. Due to its presence, high microbial growth rate and increased lipase production is attained. As compared to other organic nitrogen sources, peptone contains certain amino acids and co-factors that are required during the synthesis of lipase (Freire *et al.*, 1997). For the utilization of peptone, microbes secrete protease in order to achieve the degradation of the particular nitrogen source. In the presence of peptone, microbes synthesized less amount of protease thus, more production and less degradation of lipase was achieved (Salleh *et al.*, 2006). Fadiloglu and Erkmen (2002) obtained maximum activity of lipase from *Candida rugosa* in the presence of peptone. Burkert *et al.* (2005) achieved highest lipase activity in the presence of peptone from *Geotrichum candidum* while Wolski *et al.* (2009) also reported that maximum activity of lipase was gained from *Penicillium* sp. when peptone was used as nitrogen source. Mukesh *et al.* (2012) achieved maximum activity of lipase from *Bacillus* sp in the presence of peptone. Prasad and Manjunath (2012) reported that in the presence of peptone, different species of *Bacillus*, *Serratia* and *Staphylococcus* gave highest lipase activity. Thus, peptone was optimized for the maximum production of lipase from *Aspergillus niger* and *Bacillus* sp.

The rate of proteolysis of the lipase is greatly enhanced in the presence of high concentration of peptone due to more production of protease enzyme. Nadia *et al.* (2010) obtained maximum production of lipase by *Mucor racemosus* in the presence of 1% peptone while Olusesan *et al.* (2011) achieved highest lipase activity from *Bacillus subtilis* in the presence of 1.5% peptone.

F. Effect of initial pH of culture medium on lipase production

The effect of pH of the medium (5.0-8.0 for *Aspergillus niger* and 6.0-10.0 for *Bacillus* sp.) on the production of lipase was investigated. In case of *Aspergillus niger*, maximum activity of lipase (6.6 ± 0.31 U/ml) was obtained when the pH of the culture medium was adjusted at 6.0. Similarly, *Bacillus* sp. expressed highest lipase activity (4.67 ± 0.15 U/ml) when the pH of the culture medium was adjusted at 8.0. Gradual decrease of lipase activity was observed when the pH was either higher or lower than 6.0 and 8.0 for *Aspergillus niger* and *Bacillus pumilus*, respectively (Fig. 9). When the pH of medium was maintained at 6.0, maximum cell mass of 91.2 mg/ml was obtained from *Aspergillus niger* whereas at pH 8.0, highest cell mass of 5.8 mg/ml from *Bacillus* sp was observed.

The decrease in lipase production occurs as acidic pH causes an inhibitory effect on the enzyme activity. A

change in pH affects the essential amino acids which are present in the active site of the enzyme. Kasana *et al.* (2008) obtained maximum activity of lipase at pH 8.0 with *Acinetobacter* whereas, Tembhurkar *et al.* (2004) obtained highest lipase activity from *Pseudomonas* sp. at pH 8.0. Moreover, Mukesh *et al.* (2012) also obtained highest lipase activity at pH 8.0 from *Bacillus* sp.

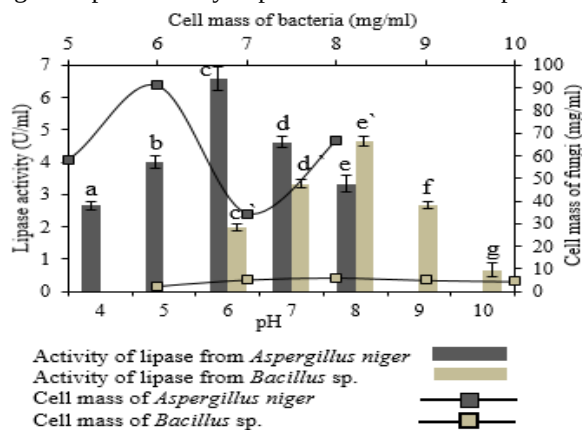


Figure 9. Effect of initial pH of culture medium on lipase production. Y-error bars indicate the standard deviation of means among three parallel replicates. The alphabets differ significantly at $p \leq 0.05$ level.

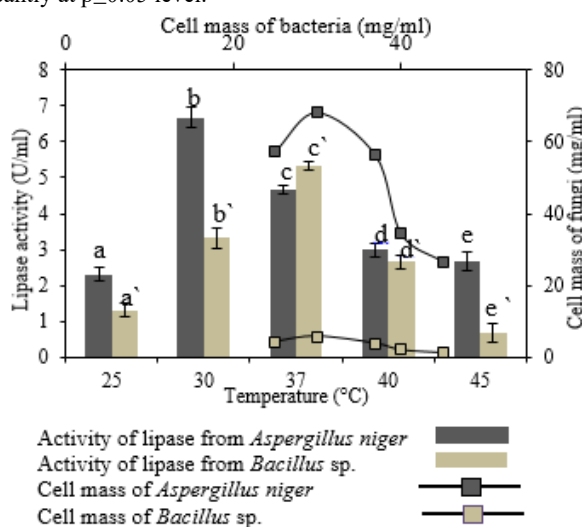


Figure 10. Effect of incubation temperature on lipase production. Y-error bars indicate the standard deviation of means among three parallel replicates. The alphabets differ significantly at $p \leq 0.05$ level.

Generally, fungi have an ability of altering the pH of the medium in which they grow. This change occurs due to the uptake of various anions and cations existing in the culture medium. Fungi are acidophilic in nature and vary the pH of the medium within acidic range of pH scale (Nithiyaa *et al.*, 2012). Ali *et al.* (2010) obtained maximum activity of lipase at pH 6.0 from *Candida lipolytica*. Ulker *et al.* (2011) also obtained maximum lipase activity at pH 6.0 from *Trichoderma harzianum* which is in accordance with our studies.

G. Effect of incubation temperature on lipase production

The effect of incubation temperature (25-45°C) on the production of lipase from both the *Aspergillus niger* and *Bacillus* sp. was investigated. Maximum activity of lipase (6.67 ± 0.29 U/ml) was achieved at 30°C from *Aspergillus niger* whereas *Bacillus* sp. gave highest lipase activity (5.3 ± 0.13 U/ml) at 37°C. Decrease in lipase activity was observed with the increase in incubation temperature (Fig. 10). At the optimized temperature of 30°C, maximum cell mass of 68.2 mg/ml from *Aspergillus niger* was obtained. In case of *Bacillus* sp. 5.8 mg/ml of cell mass was obtained at 37°C.

At high temperature, significant decrease in the yield of lipase was observed because high temperature has an inhibitory effect on the lipase production and microbial growth. With an increased temperature, the rate of reaction is enhanced due to increased kinetic energy of the molecules. As the temperature exceeds the optimum level, the breakdown of hydrophobic and hydrogen bonds takes place, thus disrupting the structure of the enzyme. Similarly, at low temperature, within the cell and in the culture medium, the transportation of nutrients is hindered, thus less yield of product is gained (Rajoka and Malik, 1997). Hosseinpour *et al.* (2011) reported that maximum activity of lipase was obtained from *Aspergillus niger* at 30°C. In case of bacteria, Sirisha *et al.* (2010) obtained maximum lipase activity at 37°C from *Pseudomonas aeruginosa* and *Staphylococcus*, respectively.

H. Purification and immobilization of lipase

Ammonium sulphate precipitation at saturation levels from 20-80% showed that the maximum fungal enzyme activity was found in the 60% fraction (5 U/ml) while maximum bacterial lipase activity was found in the 80% fraction (4.8 U/ml) with specific activities of 2.78 U/mg and 3.7 U/mg, respectively (Table III). The ammonium sulphate precipitated fractions showing lipase activity were further purified using DEAE Cellulose ion exchange chromatography. The fungal lipase after chromatographic purification showed 3.9 U/ml while the bacterial lipase showed 3.0 U/ml of enzyme activity with specific activities of 10.8 U/mg and 10.34 U/mg, respectively. The final 13 and 14 fold purity with a yield of 57 and 53% was achieved after ion exchange chromatography (Table III).

Three different supports *i.e.*, Amberlite XAD 761, Lewatite VPOC, and Duolite A568 were used for the immobilization of purified fungal and bacterial lipases and the activities of the immobilized enzymes were determined (Table IV). The enzymes have different tendencies to bind to these supports through ionic interactions depending on their physical and chemical properties.

The bacterial enzyme immobilized on Amberlite XAD 761 showed maximum activity while the fungal lipase immobilized on Lewatite VPOC showed maximum enzyme activity. Therefore, Amberlite XAD 761 and Lewatite VPOC were found to be best supports for immobilization of fungal and bacterial lipases, respectively. Wang *et al.* (2011) have shown that 96.99% of immobilized lipase activity on NKA-9 resin can be achieved using 0.5% genipin and 86.18% on S-8 with a 0.25% genipin.

Table III. Lipase Purification Summary.

Purification Step	Protein Concentration (mg)	Lipase Activity (U)	Specific Activity (U/mg)	Purification Fold	Recovery (%)
Crude Extract					
<i>Aspergillus niger</i>	82	68	0.83	1	100
<i>Bacillus sp.</i>	76	56	0.74	1	100
(NH₄)₂SO₄ Precipitation					
<i>Aspergillus niger</i>	18	50	2.78	3.35	73
<i>Bacillus sp.</i>	13	48	3.7	3.7	85
DEAE Cellulose Fast Flow					
<i>Aspergillus niger</i>	3.6	39	10.08	13	57
<i>Bacillus sp.</i>	2.9	30	10.34	14	53

Table IV. Lipase Immobilization Summary.

Support	Lipase activity (U/mg support)	Immobilization Efficiency (%)	Re-usability (% relative activity after 10 uses)
Lewatite			
<i>Aspergillus niger</i>	13	62	60
	20	86	75
<i>Bacillus sp.</i>			
Doullite			
<i>Aspergillus niger</i>	10	45	50
	14	60	54
<i>Bacillus sp.</i>			
Amberlite			
<i>Aspergillus niger</i>	18	80	68
	16	64	62
<i>Bacillus sp.</i>			

The re-usability studies revealed that the fungal lipase immobilized on Amberlite XAD 761 retained 68% residual enzyme activity after 10 times use while the bacterial lipase immobilized on Lewatite VPOC retained 75% residual enzyme activity after 10 times use of the enzyme. In a study by Wan *et al.* (2018) immobilized lipase acting as biocatalyst for olive oil emulsion hydrolysis retained activity of 81.6% after 10 cycles of reuse at a temperature of 30 °C and a pH of 7. So it was inferred that the above mentioned supports were not only immobilized the enzyme efficiently but also retained it for a longer time with a very low leakage.

IV. CONCLUSIONS

It is concluded that among the indigenous isolated strains of *Aspergillus niger* and *Bacillus sp.* were best lipase producers. The enzyme production can be maximized several folds by optimization of the fermentation process. A number of fermentation and culture parameters were optimized. It was maximized by incubation for 72hrs, using olive oil as a substrate, glucose as a carbon source and peptone as a nitrogen source. A thirteen to fourteen fold purity of produced lipase was achieved by using DEAE Cellulose ion exchange chromatography. The purified lipase was efficiently immobilized on Amberlite XAD 761 and Lewatite VPOC retaining 68% and 75% residual enzyme activity respectively. The immobilized lipase may be reused and can be very efficient for industrial applications.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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