International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2013): 6.14 | Impact Factor (2013): 4.438

# Studies of the Estimation of Lipase Production Capability of Some Fungal Species and their Application in Oil Spillage Degradation

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**Abstract:** ***During this study three Aspergillus species (A.niger, A. paraciticus and A. aculeatus) were screened for lipase production shown with varying lipolytic activities. The most active one was A. niger followed by A. paraciticus. On the other hand, the least lipase productive organism was A. aculeatus. The fermentation was carried out in shake flask culture employing a liquid media than results was compared with control, and found that all three fungal strains were shown lipase production. Effect of various factors (carbon, nitrogen, time period, pH, temperature, activator and inhibitor) on production of fungal lipase, result were shown that carbon source Tween-20, nitrogenous source -yeast extract , temperature 35°C and 55°C, pH 9 and 10 supported the production of lipase activity in fermentation media, whereas glycerol and peptone showed moderate effects. Mostly $Co^{2+}$ $Mg^{2+}$, $Zn^{2+}$, $Mn^{2+}$ & $Fe^{2+}$ act as enhancer of fungal lipase activity, only in few fungal species they inhibit lipase activity where as $Hg^{++}$ and SDS slightly enhances lipase activity. During optimized conditions lipase activity highly increased in all three fungal species. Also studied the role of these three lipolytic fungal species activity on various oil spillages (Soyabean, Mustard and Coconut Oil) degradation and result shown that maximum lipolytic activity were found in A. niger and A. paraciticus in soyabean oil and A. aculeatus in mustard oil. This study revealed that all fungal strains have capability to degrade soybean, mustard and coconut oil. It also shown that they have ability to degrade other oil spillages or hydrocarbons.***

**Keywords**: Lipase, *Aspergillus* species, fermentation, temperature, screening, oil spillages.

## 1. Introduction

Microorganisms and their enzymes are used in a wide range of biotechnological applications [1],[2],[3],[4],[5]. Enzyme catalysed reactions are highly efficient and selective, are less polluting, and usually require mild conditions and less energy, which leads to the lowering the cost of production, because of these reasons the improvement of manufacturing processes with microbial enzymes is one of the most important area of research because [6],[7]. Increasing interest in isolating new enzymes and new enzyme-producing strains for their use in various fields due to increased awareness of environment and cost issues for major Research and Development. Among these enzymes, lipases, carboxylesterases, cellulases, xylanases, pectinases, amylases and proteases are some of the most important enzymes [6],[8],[9]. Currently microbial lipases are very diverse in their enzymatic properties and substrate specificities, which make them attractive for industrial applications [10],[11],[12]. Microbial lipase catalyzed the hydrolysis of triacyl glycerol to glycerol and free fatty acids in contrast to esterases [13], [14]. Novel biotechnological applications have been successfully recognized using lipases for the synthesis of biopolymers and biodiesel, the production of new pharmaceuticals, agrochemicals, and flavour compounds [15],[16]. So, in the present study an attempt has been made to isolate potential fungal strain(s) which are capable of producing active lipase which can fit in the conditional processes. The lipase thus obtained was further analyzed in optimization conditions for their better production.

## 2. Materials and Methods

### 2.1 Fungal Strains

Fungal Strains of *Aspergillus niger, A. paraciticus* and *A. aculeatus* were obtained from "School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur" and the cultures were maintained on Potato Dextrose Agar (PDA) and Basal media.

### 2.2 Rapid screening of fungi for the production of lipase

Tributyrin agar medium was used for assay of enzyme productivity according to the tributyrin clearing zone technique [17]. Fungal colonies that have lipolytic activity showed clear zone.

### 2.3 Lipase production

Inoculums were prepared by inoculated fungal strains spores on experimented media [18] to obtain standard concentration of approximately 1.2x $10^7$ spores /ml. Prepared inoculums were grown in a modified form of fermentation medium according to this composition (g/L) 30-peptone, 2.0-$KH_2PO_4$, 0.5- $NaN0_3$, 0.5- KCl, 0.5-$MgSO_4.7H_20$, 1.0-$CaCl_2$, silicone MS Antifoam A -40ppm and 20% (w/v) emulsified triglycerides [19]. Fermentation was done in an orbital incubator shaker (REMI, 9001:2000) at 25°C, pH- 4, and 50rpm for 3 days [18]. Total protein extraction were done by filtration of mycelia and dried with chilled acetone ether (1:1) mixture, then crushed with 0.1 M sodium phosphate buffer (pH-7). Sample was centrifuged at 12500 rpm for 10 minutes and obtained supernatant and broth were used to

determined intra and extracellular lipase activity [18]. Protein purification was done by 80% ammonium sulphate precipitation method than estimated by Folin Lowry method at 660nm absorbance [20].

### 2.4 Lipase Assay

Lipase activity was assayed quantitatively using 2ml phosphate buffer taking 2 ml phosphate buffer pH 7.0, 1 ml 10% (w/v) emulsion of olive oil in gumacacia, 0.5 ml of 0.1M $CaCl_2$ and 1 ml enzyme solution and incubating the mixture at 37°C for one hour. The reaction was stopped by adding 5 ml mixture of ethanol: acetone (1:1) and the whole mixture was titrated against 0.05 M NaOH using phenolphthalein as indicator. The blank used and the activity unites were [21].

"A unit of lipase activity was defined as the amount of enzyme that would liberate one micromole of free fatty acids per minute from the substrate under the assay conditions described."

### 2.5 Effect of Physico-Chemical Parameters on Lipase Production

Lipase production was optimized by altering various physic-chemical and culture conditions observing the effect at 25°C in Orbital Incubator Shaker (REMI, 9001:2000) at 50 rpm for appropriated incubation periods for 1-3 days.

**2.5.1 Effect of Incubation periods:** The effect of incubation period on lipase production for *Aspergillus* sps. were studied measuring the enzyme activity, growth at different incubation period (2-5) days at 25°C, 50 rpm and pH 7.

**2.5.2 Effect of different Incubation pH and temperatures:** The effect of pH on lipase activity was determined by incubating 0.1ml of protein precipitate in 0.4 ml of appropriate buffers. To this, 0.5 ml of olive oil (1% w/v) was dissolved and the reaction mixture was incubated at 37°C for 5 minutes. The effect of temperature was determined by incubating 0.5 ml of proper diluted enzyme and 0.5 ml of olive oil (1% w/v in 0.2M sodium acetate buffer pH 4) for 5 minute at different temperatures (ranges from 25°C, 35°C, 45°C and 55°C).

**2.5.3 Effect of Different media components:** The media can be classified into 3 categories that are carbon, nitrogen and effectors. Effect of various supplements on lipase production medium was studied by adding different carbon sources (glycerol and T-20), Nitrogen sources (peptone and yeast extract) as equivalent amount present in basal medium. **(in mm)**While the effect of activators and inhibitors like ($NaCl_2$, $CaCl_2$, $CoCl_2$, $ZnSO_4$, $MgCl_2$, $MnSO_4$, $CuSO_4$, $FeCl_2$, $HgCl_2$ and SDS) was determined by incubating 0.5 ml of olive oil (1% w/v in 0.2M sodium acetate buffer pH 4) at 37°C for 5 minutes.

**2.6 Fungal Lipase Production in Optimized Conditions:** At optimized source of carbon, nitrogen, activator, inhibitor, pH, and temperature of the fermentation medium fermentation were done for all three fungal species and isolated protein used for checking lipase activity assay.

### 2.7 Application of Fungal Lipase in Oil Spillages Degradation

Various vegetable oils like (Soybean/Mustard/Coconut oil) were used as source of carbon , nitrogen (Yeast Extract), activator, inhibitor, pH, and temperature of the fermentation medium was adjusted according to optimized conditions in an Orbital Incubator Shaker at 50 rpm for appropriated incubation periods. Composition of media was selected from (Kouker and Jaeger) with slightly modification (removing of agar and Rhodamine B from media). The hydrolysis of various oils was confirmed by lipase activity [22].

## 3. Results and Discussions

**3.1 Screening of fungal strains and optimization of the enzyme production:** In this study, nine fungal strains *A. niger*, *A. parciticus*, and *A. aculeatus* were screened for lipase production. Table (1) shown that all the tested strains, produced lipase with varying lipolytic activities. The most active lipolytic activities were observed in *A. niger*, (33.0mm) and after that *A. parciticus* (6.0mm) was shown average lipolytic activity. On the other hand, the least lipase productive organism was *A. aculeatus* (1.0mm). In previous studied of ecological screening for lipolytic molds *Aspergillus* was found to be the excellent producers of lipase [23].

**Table 1:** Screening of liapase producing fungal species Diameter of Tributyrin clreaing zone by lipolysis activity.

| Lipase enzyme of Fungal sps. | Average of Growth of Fungal Sps. in Trybutyrin Assay Media (in mm) | Average of Growth of Fungal Sps. in Trybutyrin Assay Media+ Hydrolysis | Diameter of Tributyrin clreaing zone (in mm) |
|---|---|---|---|
| *A. aculeatus* | 1.0 | 2.0 | 1.0 |
| *A.* | 4.0 | 10.0 | 6.0 |
| *A. niger* | 10.0 | 15.0 | 5.0 |

**3.2 Estimation of Total protein Produced by Fermentation of three fungal species:** In this study, three fungal strains *A. niger*, *A. parciticus*, and *A. aculeatus* were compared with standard protein estimation method of Lowry to estimate the production of total protein during fermentation. Table (2) shown that all the tested strains, produced protein in varies concentration.

**Table 2:** Estimation of various fungal protein concentrations by folin Lowery method

| Fungal sps. | Protein Concentration (in mg/ml) |
|---|---|
| *A. aculeatus* | 2.6 |
| *A. paraciticus* | 2.7 |
| *A. niger* | 2.9 |

**3.3 Production Fungal Lipase in fermentation medium:** In this study, lipase activity assay were performed to determine the lipase production and these results was compared with control, and found that all three fungal strains were shown lipase activity in (Table 3). Lipase production of fungal strains observed reading were (in U/ml) *A. niger* (2.7), *A. parciticus* (2.4), and A. *aculeatus* (2.5).

**Table 3:** Production of fungal lipases in fermentation medium through various fungal species

| Various Fungal sps. | Enzymatic Activity in (U/ml) |
|---|---|
| Controll | 1.8 |
| *A. aculeatus* | 2.4 |
| *A. paraciticus* | 2.5 |
| *A. niger* | 2.7 |

### 3.4 Effect of Physio- Chemical Parameters

#### 3.4.1 Effect of different Carbon sources (Glycerol & T-20)

Due to glycerol and Tween-20 effects on lipase production of fungal strains observed reading were (in U/ml) *A. niger* (2.7 & 5.6), *A. parciticus* (2.4 & 5.3) and *A. aculeatus* (2.5 & 4.5) (Table 4). Result was shown that both carbon supplement media support the production of lipase activity, but the highest lipase activity was observed on Tween-20 source of carbon, whereas glycerol showed moderate effects. Previously studies was reported optimum activity in *Aspergillus niger* KG-17, *Aspergillus flavus* KG-18 were also showing very nice production profile with an average when T-20 as a carbon source [24]. Previously results revealed that use of glycerol as main carbon source rather than glucose or lipid supported mycelial growth and lipase production [25].

**Table 4:** Effect of different Carbon sources (Glycerol & T-20) on various Intra-cellulal fungal Lipase productions

| Lipase enzyme of Fungal sps. | Enzymatic Activity in Carbon source (Glycerol) in (U/ml) | Enzymatic Activity in Carbon source (T-20) in (U/ml) |
|---|---|---|
| *A. aculeatus* | 2.4 | 5.3 |
| *A. paraciticus* | 2.5 | 4.5 |
| *A. niger* | 2.7 | 5.6 |

#### 3.4.2 Effect of different Nitrogenous sources (Peptone & Yeast extract)

Effect of various nitrogen sources such as Peptone & Yeast extract (equimolar amounts) were shown in (Table 5). Result were found that addition of yeast extract the maximum lipase production, whereas peptone support the on the biosynthesis of lipase. Due to peptone & yeast extract effects on lipase production of fungal strains observed reading were (in U/ml) *A. niger* (2.9 & 4.9), *A. parciticus* (2.6 & 6.8) and *A. aculeatus* (2.7 & 5.4). Maximum productivity of the enzyme in *Aspergillus flavus & Candida rugosa* with yeast extract [26]. Previously studied revealed the same results as present studies [27].

**Table 5:** Effect of different Nitrogenous sources (Peptone & Yeast extract) on various Intra-cellular fungal Lipase productions

| Lipase enzyme of Fungal sps. | Enzymatic Activity in $N_2$ source (Peptone) in (U/ml) | Enzymatic Activity in $N_2$ source (Yeast Extract) in (U/ml) |
|---|---|---|
| *A. aculeatus* | 2.6 | 6.8 |
| *A. paraciticus* | 2.7 | 5.4 |
| *A. niger* | 2.9 | 4.9 |

#### 3.4.3 Effect of different Incubation periods (2, 3, 4, & 5 days)

Results shown that *A.* niger (14.2), *A. parciticus* (35.5), *A. aculeatus* (15.0) were produced maximum lipase activity (in U/ml) at 2nd of incubation (Table 6), but with the increasing the day of incubation lipase production was reduced and there was no activity found in these three fungal species. Previous studies reported that lipase yield was maximum in 2-3 days of incubation in *Aspergillus* sp. [28].

**Table 6:** Effect of different Incubation periods (2, 3, 4, & 5 days) on various Intra-cellular fungal Lipase productions

| Lipase enzyme of Fungal sps. | After 2-Day's Incubation Enzymatic Activity in (U/ml) | After 3-Day's Incubation Enzymatic Activity in (U/ml) | After 4-Day's Incubation Enzymatic Activity in (U/ml) | After 5-Day's Incubation Enzymatic Activity in (U/ml) |
|---|---|---|---|---|
| Controll | 3.9 | 3.9 | 3.9 | 3.9 |
| *A. aculeatus* | 15.0 | 4.4 | 5.2 | 2.5 |
| *A. paraciticus* | 35.5 | 5.3 | 5.8 | 2.4 |
| *A. niger* | 14.2 | 5.6 | 5.2 | 2.7 |

#### 3.4.4 Effect of different Temperatures range from (25°C, 35°C, 45°C, & 55°C)

The optimum temperature for lipase activity (in U/ml) of *A. aculeatus* were 35°C (7.1) than lipase activity which was decreased with the increasing temperature. Whereas *A. niger* (6.5) and *A. parciticus* (5.3) shown maximum lipase activity at 55°C (Table 7). Previous study reveal that *A. aculeatus*, lipase activity a temperature range from 20 to 50°C was tested and showed that 35°C was the optimum incubation temperature, While lipases of *A. niger* are stable at 50°C [29, 19].

**Table 7:** Effect of different Temperatures range from (25°C, 35°C, 45°C, & 55°C) on various Intra-cellular fungal Lipase productions.

| Lipase enzyme of Fungal sps. | At 25°C Enzymatic Activity in (U/ml) | At 35°C Enzymatic Activity in (U/ml) | At 45°C Enzymatic Activity in (U/ml) | At 55°C Enzymatic Activity in (U/ml) |
|---|---|---|---|---|
| Controll | 4.3 | 3.1 | 4.7 | 3.0 |
| *A. aculeatus* | 7.2 | 7.1 | 4.5 | 5.0 |
| *A. paraciticus* | 5.0 | 5.1 | 4.1 | 5.3 |
| *A. niger* | 4.9 | 3.5 | 5.6 | 6.5 |

#### 3.4.5 Effect of different Incubation pH (4-10)

Table (8) results shown that there were no lipase activity found in pH range from (4-6) & pH-8 accept in *A. niger* (5.1). Optimum pH for maximum lipase activity was found pH-9 for *A. parciticus* (3.0) and *A. aculeatus* (3.5) pH- 10 for *A. niger* (11.2). In previous study indicate that the initial pH of the growth medium is also important for lipase production.

**Table 8:** Effect of different pH range from (4 to 10) on various Intra-cellular fungal Lipase productions

| Lipase enzyme of Fungal sps. | Controll | *A. aculeatus* | *A. paraciticus* | *A. niger* |
|---|---|---|---|---|
| pH-4 | 67.0 | 52.7 | 64.7 | 54.6 |
| pH-5 | 65.5 | 47.4 | 62.7 | 62.7 |
| pH-6 | 16.1 | 11.1 | 11.9 | 11.8 |
| pH-7 | 3.1 | 6.2 | 4.8 | 5.6 |
| pH-8 | 3.7 | 2.5 | 2.8 | 5.1 |
| pH-9 | 1.0 | 4.5 | 4.0 | 4.5 |
| pH-10 | 3.5 | 5.9 | 5.9 | 11.2 |

**3.4.6 Effect of activators and inhibitors in Lipase Activity**: The effect of activators and inhibitors with respect to control shown that *A. aculeatus* lipase activity were activated by $Co^{2+}$ (10) and *A. niger by* $Co^{2+}$ (6.6), $Mn^{2+}$ (8.1) & $Cu^{2+}$ (6.3) also. Due to $Ca^{2+}$ their was no enhancements in lipase activity accept in *A. niger*. Mostly $Co^{2+}$ $Mg^{2+}$, $Zn^{2+}$, $Mn^{2+}$ & $Fe^{2+}$ act as inhacer of fungal lipase activity, only in few fungal species they inhibit lipase activity where as $Hg^{++}$ and SDS slightly enhances lipase activity. Previous study indicated that lipolytic activity was enhanced by $Mg^{2+}$, $Zn^{2+}$, $Co^{2+}$ and $Mn^{2+}$, but was inhibited by $Cu^{2+}$, $Ba^{2+}$ and $Hg^{2+}$, while $Ca^{2+}$ had no effect, while SDS had negligible effects on enzymatic activity [24].

**Table 9(a):** Effect of 100µl of different Activators & Inhibitors (in concentration of 5mg in 10ml Distilled Water) on various Intra-cellular fungal Lipase productions

| Lipase enzyme of various Fungal sps. | For $Fe^{2+}$ Enzymatic Activity in (U/ml) | For $Zn^{+}$ Enzymatic Activity in (U/ml) | For $Hg^{2+}$ Enzymatic Activity in (U/ml) | For $Co^{2+}$ Enzymatic Activity in (U/ml) | For SDS Enzymatic Activity in (U/ml) |
|---|---|---|---|---|---|
| Controll | 4.6 | 3.8 | 4.1 | 0.4 | 4.2 |
| *A. aculeatus* | 6.8 | 4.0 | 5.7 | 10. | 7.0 |
| *A. paraciticus* | 5.0 | 6.5 | 6.8 | 4.0 | 6.0 |
| *A. niger* | 5.3 | 6.7 | 5.8 | 6.6 | 4.0 |

**Table 9(b):** Effect of 100µl of different Activators & Inhibitors (in concentration of 5mg in 10ml Distilled Water) on various Intra-cellular fungal Lipase productions

| Lipase enzyme of various Fungal sps. | For $Mg^{2+}$ Enzymatic Activity in (U/ml) | For $Cu^{2+}$ Enzymatic Activity in (U/ml) | For $Mn^{2+}$ Enzymatic Activity in (U/ml) | For $Ca^{2+}$ Enzymatic Activity in (U/ml) | For $Na^{2+}$ Enzymatic Activity in (U/ml) |
|---|---|---|---|---|---|
| Controll | 4.7 | 0.2 | 1.9 | 5.9 | 3.4 |
| *A. aculeatus* | 4.3 | 4.7 | 7.1 | 5.9 | 5.0 |
| *A. paraciticus* | 7.6 | 7.5 | 8.6 | 4.3 | 5.8 |
| *A. niger* | 7.0 | 6.3 | 8.1 | 7.9 | 7.7 |

**3.5 Production of various fungal Lipases in Optimized Conditions:** Lipase production of fungal strains in optimized condition observed reading were (in U/ml) *A. niger* (13.9), *A. parciticus* (13.8) and *A. aculeatus* (9.9) (Table 10). Result shown that in optimized condition lipase production were high.

**Table 10:** Production of various Intra-cellular fungal Lipases in Optimized Conditions

| Lipase enzyme of Fungal sps. | Enzymatic Activity in (U/ml) |
|---|---|
| Controll | 2.0 |
| *A. aculeatus* | 9.9 |
| *A. paraciticus* | 13.8 |
| *A. niger* | 13.5 |

**3.6 Application of Fungal Lipase in Oil Spillages Degradation:** Maximum lipolytic activities (U/ml) were found in *A. niger (*5.50) and *A. paraciticus* (6.00) in soyabean oil, *A. aculeatus* (8.90) in mustard oil (Table 11). This study revealed that all fungal strains have capability to degrade soybean, mustard and coconut oil, it also shown that they have ability to degrade other oil spillages [32].

**Table 11:** Role of Fungal lipase in various Oil Spillage degradation

| Lipase enzyme of various Fungal sps. | Lipase Enzyme Activity in (U/ml) in Soyabean Oil | Lipase Enzyme Activity in (U/ml) in Mustard Oil | Lipase Enzyme Activity in (U/ml) in Coconut Oil |
|---|---|---|---|
| Controll | 2.90 | 2.90 | 2.90 |
| *A. niger* | 5.50 | 5.10 | 6.00 |
| *A. aculeatus* | 3.60 | 8.90 | 6.31 |
| *A. paraciticus* | 6.00 | 5.90 | 6.50 |

## 4. Conclusions

Lipases are ubiquitous enzymes of considerable physiological significance and industrial potential. Lipases catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids, in contrast to esterases. Microbial lipases are currently receiving much attention with the rapid development of enzyme technology due to their multifold properties. During present study all three fungal species *A. niger*, *A. paraciticus* and *A. aculeatus* have ability to produced lipase enzyme in tributyrin agar medium and in modified form of fermentation medium both extra and intracellulary. The fermentation conditions such as carbon sources, nitrogenous sources, incubation time, pH, temperature, activator and inhibitors were optimized for all three fungal species. In optimized conditions intracellular lipase activity were increased highly and maximum activity found in A. niger. Application of all fungal lipolytic activity was used for detection in degradation of soybean, mustard and coconut oils. Study was concluded that they all fungal species have capapbility to degrade soybean, mustard and coconut oil hydrocarbons means they also have ability to degrade other oil spillages. For future prospect lipase quantity and quality can be more enhance by either produce genetically manipulate microorganisms or by protein engineering.

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