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Screening and Optimization of Filamentous Fungi for Extracellular Lipase Production from Various Soil Samples Taken In and Around Shimla (HP), India

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Abstract

In the present study samples were collected from different sources such as oil contaminated soil (railway station), moist soil, barren land, garden soils and humus, near dairy and other soil samples in and around Shimla. FromTotal of 48 isolates were isolated from different samples, out of which four were selected on the basis of zone of hydrolysis on tributyrin-agar plates. Among these four isolates, Isolate -3 (Aspergillus niger) gave maximum production of lipase. Further the optimization of production and reaction conditions were carried out. Enzyme gave maximum activity in M3 medium with an inoculum size of 3.6×10^5 spores, at incubation time of 72 h. The enzyme gave considerable amount of activity with maltose and peptone as carbon and nitrogen source respectively at a concentration of 2.0% each at pH 8.0, temperature of 40°C and 150 rpm. During the optimization of reaction conditions, the most favorable reaction conditions was found to be 40°C temperature for 15 minutes of incubation and it was observed that the enzyme showed maximum enzyme activity *i.e.* 4.3 U/mL with Tris HCl buffer (40 mM) of pH 8.0. A 2.17 fold increase in activity of lipase from fungal Isolate -3 (Aspergillus niger) was observed after optimization of culture and reaction conditions.

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Keywords

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Tributyrin Agar,
Potato dextrose agar.

Introduction

Enzymes are considered as nature's catalysts. Lipase (triacylglycerol acylhydrolases) (EC 3.1.1.3) catalyses hydrolysis of long chain acyl glycerol at an oil water interface. These are enzymes belonging to the group of hydrolases that present as main biological function to catalyze the hydrolysis of insoluble triacylglycerols to generate free fatty acids, mono and diacylglycerols and glycerol (Lutz, 2004, Kempka *et al.*, 2008). Ester synthesis is carried out in aqueous media in the presence of various lipases (Lacointe *et al.*, 1996). Lipases are widely distributed in microorganisms, plants, and animal tissues (Pahoja and Sethar, 2002; Pratuangdejkul and Dharmsthiti, 2000). Among them, microorganisms are the most important source of lipases for their diverse properties (Hasan *et al.* 2006). Filamentous fungi are known to be good lipase producers; examples are *Aspergillus niger* (Mahadik *et al.*, 2002), *Fusarium solani* (Maia *et al.*, 2001), *Rhizopus oligosporus* (Ul-Haq *et al.*, 2002) and members of the genera *Geotrichum*, *Mucor* and *Penicillium* (Sharma *et al.*, 2001). The production of enzymes by microorganisms as well as the enzyme yield depends on the nutritional factors

especially carbon and nitrogen sources and physicochemical parameters such as initial pH, incubation temperature and incubation time (Mukesh *et al.*, 2012a, 2012b). Lipases have many uses, including acceleration of cheese curing and others in the food industry (Sharma *et al*., 2001; Hasan *et al*., 2006), the separation of racemic mixtures (Rao *et al*., 1993), detergent production (Hasan *et al*., 2006) effluent treatment (Castro *et al*., 2004; Mendes and Castro 2005; Rosa *et al*., 2006), and the production of cosmetics and pharmaceuticals (Elibol and Ozer, 2000). Interest in lipase enzyme has been greatly developed in the last few years due to their potential applications. They are extensively used in fat hydrolysis as well as in synthesis of triacylglycerol. Lipases are also used in various reactions due to their high specificity, prevention of product and substrate deterioration and decreased energy consumption. The advantage of the enzymatic hydrolysis over the chemical process is less energy requirements and higher quality of the obtained products (Ionita *et al.*, 2001).

Materials and Methods

This study was conducted from January 2014 to June 2014 at Department of Biotechnology, Himachal Pradesh University, Shimla (HP). Total 48 soil samples were collected with the help of soil auger, from different sources such as oil contaminated soil (railway station), moist soil, barren land, garden soils and humus, near dairy and other soil samples in and around Shimla. All the different types of soils were sieved to avoid roots, stones, dry leaves, and other unwanted materials and were air dried. Ten gram of soil samples were collected using a sterile spatula in a sterile Petri plate. For wet and humid soil the trays were exposed to the sunlight till the time the soil got adequately dried. The soil samples were brought to the laboratory and processed immediately. Ten gram of dried processed soil was mixed with 100 ml of sterile distilled water in a conical flask of 250 ml and was shaken well. This homogeneous soil suspension is referred to as stock suspension.

Chemicals and reagents - Potato dextrose agar (PDA), Tributyrin, K_2HPO_4, KH_2PO_4, KCl, $MgSO_4.7H_2O$, NH_4Cl, Yeast Extract, *p*-nitrophenylpalmitate (*p*-NPP), *p*-nitrophenol (*p*-NP), Tween 80, Tris buffer, Peptone, Glucose, NaCl, Beef Extract, Olive oil, Isopropanol, Agar, KCl, Phenol Red, Lactophenol, Potato dextrose broth, NaH_2PO_4 etc. All the chemicals were of analytic grade and were used as received.

Isolation of lipolytic fungi - 1mL each of sample was serially diluted 10^{-1} to 10^{-10} times in sterile saline. 100 µL of each of the dilution was spread over PDA at pH 7.0 and was incubated at 37°C for 48-72 hours. The isolated distinct colonies were carefully picked up depending upon different shape, size, colour and pure line cultures were established by repeatedly streaking single distinct colonies on fresh PDA plates. Pure culture/colonies were maintained on PDA plates for further work. Tween 80 and tributyrin agar plates were used for the isolation of lipolytic fungi.

Screening of lipase producing fungi - PDA plates containing Tween 80 and tributyrin were used for screening of lipase producing fungal isolates. The lipolytic activity was indicated by the appearance of a visible precipitate, resulting from the deposition of crystals of the calcium salt formed by the fatty acid liberated by the enzyme, or as a clearing of such a precipitate around a colony due to complete degradation of the salt of the fatty acid (Kim *et al.*, 2001).

Inoculum preparation - Spores were collected from three to four days old cultures grown on PDA plates using sterilized inoculation loop in 2 mL of sterile buffer saline containing 0.01% Tween 20. Spore concentration was determined by counting spores with a haemocytometer.

Production of lipase - The production medium containing Peptone 1.5%, NaCl 0.5%, $CaCl_2$ 0.1% and Tween 80 1% (Sneath *et al.*,1986) was autoclaved at 15 $lb/inch^2$ pressure at 121°C for 15 min and cooled at room temperature. The sterile production medium (50 mL in 250 mL Erlenmeyer flask) was inoculated with 2.4×10^5 spores and incubated at 37°C for 3 days. The fermentation broth was collected to assay lipase activity. The culture broth was analyzed for lipase activity.

Assay of lipase- Lipase activity was assayed by the method given by Winkler and Stuckmann in 1979 by measuring the micromoles of *p*-nitrophenol released from *p*-nitrophenylpalmitate.

Procedure - 2.9 mL of Tris-HCl buffer (0.1 M, pH 7) was incubated in water bath at 37°C for 10 minutes. Then 60 µl of the substrate (*p*-NPP, 20 mM) was added to the pre-warmed buffer and incubated again at 37°C for 10 minutes. To the pre-warmed solution, 40 µl of enzyme was added and incubated again at 37°C for 10 minutes. The reaction was stopped by chilling at -20°C. The amount of *p*-Nitrophenol released was measured at

410 nm (LabIndia UV/VIS Spectrophotometer Lambda 12) after bringing the tubes to room temperature. A standard curve of *p*-NP was plotted at the selected concentration (0.5-50 µg/mL).The readings were taken in triplicates and the strain showing the highest activity was selected for further study.

Lipase activity - One unit of lipase activity was defined as amount of enzyme required to release one micromole of*p*-NP from the substrate (*p*-NPP) per minute of the enzyme preparation under standard assay conditions.

Optimization of production parameters for extracellular lipase from selected fungal isolate -

Various production parameters such as production medium, inoculum size, incubation time, carbon source and its concentration, nitrogen source and its concentration, pH, temperature and agitation rate were optimized to get maximum production of the Lipase enzyme.

Screening of media for extracellular lipase production- The selected isolate was grown in five different production media (M1-M5) reported by previous workers (Table 1).

Optimization of inoculum size - Inoculum size was optimized by inoculating the production medium with varying spore count *i.e.* 1.2×10^5, 2.4×10^5, 3.6×10^5, 4.8×10^5, 6.0×10^5spores and incubating it for 48 h. Filtered broth was assayed for enzyme activity.

Effect of incubation time on lipase production To study the optimal incubation time for the production of enzyme, the production medium was incubated with optimized inoculum size in the shaker for the time intervals of 24 h, 48 h, 72 h, 96 h, 120 h and 144 h. The culture broth was assayed for enzyme activity.

Effect of carbon source on the production of lipase - Various carbon sources such as Sucrose, Glucose, Maltose and Galactose were used in the production medium at a concentration of 1% (w/v) to check the effect of carbon source on lipase production. The medium without carbon source served as the control. The culture supernatants were assayed for enzyme activity.

Effect of carbon source concentration on lipase production - To optimize the concentration of selected carbon source for maximal enzyme production, different concentrations of optimized carbon source [0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% (w/v)] were used in the production medium and the culture supernatant was assayed for enzyme activity.

Effect of nitrogen source on lipase production - The effect of various nitrogen sources on the production of enzyme was studied by adding 1% w/v of Yeast extract, Peptone, Ammonium chloride, Sodium nitrate, Glycine, Beef extract, Casein, Tyrosine and Arginine to the production medium and incubating the medium at culture conditions optimized previously. The medium without nitrogen source served as the control.

Effect of concentration of nitrogen source on the production of lipase -To optimize the concentration of selected nitrogen source for maximal enzyme production, different concentrations [0.5, 1.0, 1.5, 2.0 and 2.5% (w/v)] were used in the production medium and the culture supernatant was assayed for enzyme activity.

Effect of pH of the medium on the production of lipase - For the optimization of production pH the production medium of varying pH viz. 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 was inoculated with the culture and incubated in the rotary shaker for 72 h at 37°C and lipase activity was determined.

Effect of incubation temperature on lipase production - The most favourable production temperature was studied by incubating the productionmedium at different temperatures *i.e.* 25°C, 30°C, 35°C, 40°C, 45°C and 50ºC. The enzyme activity was checked by using the standard assay method described previously.

Effect of agitation rate on lipase production - For the optimization of agitation rate for the maximum enzyme production the enzyme was agitated at different rpm (rotations per minute) *i.e.* 50 rpm, 100 rpm, 150 rpm, 200 rpm and 250 rpm.

Optimization of reaction parameters for maximum activity of extracellular lipase from selected fungal isolate -

Various reaction parameters such as Effect of reaction time,reaction temperature, different buffer systems,molarity of buffer andbuffer pH on lipase activity were optimized to get maximum production of the Lipase enzyme.

Effect of reaction time on lipase activity - To study the effect of reaction time, enzyme was incubated in reaction medium for different time intervals (5, 10, 15, 20 and 25 min) with 20 mM substrate (*p*-NPP) and Tris buffer (0.1M) of pH 7.0. The reaction was performed using 40 µl of crude enzyme at 37°C incubation temperature.

Effect of reaction temperature on lipase activity - To study the effect of reaction temperature, enzyme activity was assayed at various reaction temperatures (25, 30, 35, 40, 45 and 50ºC) with 20 mM substrate *p*-NPP and Tris buffer (0.05M) of pH 7.0. The reaction was performed using 40 µl of crude enzyme for optimized reaction time.

Effect of different buffer systems on lipase activity - Following buffers were screened for enzyme activity

- 50 mM Sodium citrate buffer (pH 5.0)
- 50 mM Potassium phosphate buffer (pH 7.0)
- 50 mM Sodium phosphate buffer (pH 7.0)
- 50 mMTris-HCl buffer (pH 8.0)

Effect of molarity of buffer on lipase activity - Selected buffer with different molarity *i.e*. 10, 20, 30, 40 and 50 mM was used to perform enzyme reaction.

Effect of buffer pH on lipase activity - Selected buffer with different pH range (6.0, 7.0, 8.0, 9.0 and 10.0) was used and the activity of lipase evaluated for each.

Results and Discussion

48 fungal cultures were isolated from different soil samples. Out of them four culture isolate showed zone of hydrolysis on tributyrin-agar plates and were shifted to PDA plates. Identification of fungus was done on the basic of colony morphology on PDA plate and Lactophenol cotton blue mount (Jagdish Chander, 2009).

Four isolates were screened quantitatively through shake flask fermentation (Table 2). It was found that production of lipases ranged from 0.54 U/mL to 1.98 U/mL. Out of the four strains tested the strain designated as isolate -3 (*Aspergillus niger*) from soil sample from railway station, Shimla gave the maximum production of extracellular lipase (1.98 U/mL).

Optimization of culture conditions for the production of extracellular lipase

The optimization of culture conditions was done for the maximum production of extracellular lipases.

Media optimization - Among the 5 media tested, the maximum production of extracellular lipase (1.98 U/mL) was observed in Medium 3 (M 3) (Table 3). Lowest lipase activity of 0.54 U/mL was observed in Medium 4 (M 4). The reason for higher yield in M 3 might be due to the fact that glucose, NH_4Cl and K_2HPO_4 are easily metabolizable carbon, nitrogen, and potassium source respectively (Elibol and Ozer 2000).

Optimization of inoculum size - The inoculum of size 3.6×10^5 spores showed the maximum enzyme production with an enzyme activity of 2.14 U/mL (Figure 1 and Table 5). It might be due to adequate amount of mycelium produced, which synthesize optimum level of enzyme. As the dose of inoculum increased there was gradual decrease of lipase activity. It might be due to the reason that it consumed majority of the substrate for growth and metabolic processes, hence enzyme synthesis decreased. In a study by (Mathew *et al*., 2008) inoculum of size 4.5×10^7 spores/mL gave the maximum enzyme production in *Penicillium*sp. Another study showed 3.6×10^5 spores/mL as the optimum inoculum size for lipase production from *Penicilliumcyclopium* (Vanot*et al*., 2002).

Effect of incubation time on lipase production - Optimal incubation time for maximum enzyme production was found to be 72 h (2.32 U/mL) (Figure 2). There was considerable enzyme activity till upto 120 h of incubation even though it was lower than 72 h growth. After this it started decreasing, this might have been due to the exhaustion of nutrients, which may lead to the accumulation of toxic products and decreased production of enzyme. It is evident from the results that time course of enzyme production plays a very critical role in enzyme synthesis (Sztajar and Maliszewska, 1988). In another study, 4 days of incubation time was optimized for lipase from *Rhizopus oryzae* (Hiol*et al*., 2000).

Effect of carbon source on production of lipase production - Maximum production of extracellular lipase was obtained from the medium which was supplemented with maltose (1%) as carbon source giving enzyme activity of 2.39 U/mL (Figure 3). *Aspergillus niger* and *Aspergillus terreus* showed maximum lipase production when corn oil (0.4%) was used as carbon source (Abdel-Fattah and Hammad, 2002). *Candida rugosa* lipase gave maximum activity when glucose was used as carbon source in a study carried out by (Fadiloglu and Erkmen, 2002).

Effect of carbon source concentration on lipase production - The various concentrations of maltose were used in the production medium and 2.0 % (w/v) was optimized to give enzyme activity of 2.95 U/mL (Figure 4). There was a consistent decrease in lipase activity when the concentration of maltose increased or reduced from 2%, therefore 2% of maltose was taken as optimum for further studies. In a previous study, 1.25% of Glucose concentration was found to be the optimum for the production of lipase from *Geotrichum candidum* (Gopinath *et al*., 2003). In another study, 1% of soluble starch as carbon source gave maximum production of lipase in *Saccharomyces cerevesiae* (Sherazi *et al*., 1998).

Effect of nitrogen source on lipase production - Among the various nitrogen sources used, organic non-defined nitrogen sources were found to enhance the enzyme production (Figure 5). This might be because of other nutrients and growth enhancers present in them. The enzyme production reached to (3.03 U/mL) maximum when peptone (1% w/v) was used as the nitrogen source. In a previous study, yeast extract was shown as the best nitrogen source for the production of lipase from *Rhizopus delemar* (Espinosa *et al*., 1990). In a study carried out on *Aspergillus* sp. peptone as nitrogen source gave maximum lipase production (Cihangir and Sarikaya 2004).

Table 1. Composition of the media used

Media	Components	Concentration (%)	Reference
M 1	Glucose Peptone $MgSO_4$ K_2HPO_4 Peppermint oil	0.1 0.5 0.01 0.1 1.0	(Kekde and Chavan 2011)
M 2	KH_2PO_4 $MgSO_47H_2O$ KCl Yeast extract Peptone Glucose Olive oil	0.2 0.05 0.05 0.025 0.025 1.25 1.2	(Sharma *et al.*,2012)
M 3	Glucose Olive oil NH_4Cl Yeast extract K_2HPO_4 $MgCl_2$ $CaCl_2$	0.1 3.0 0.5 0.36 0.1 0.01 0.2	(Gupta *et al*., 2004)
M 4	NaH_2PO_4 KH_2PO_4 $MgSO_47H_2O$ $CaCl_2$ Ammonium sulphate Olive oil	0.12 0.2 0.03 0.025 1 2	(Colen*et al., 2006)*
M 5	Peptone NaCl $CaCl_2$ Tween 80	1.5 0.5 0.1 1	(Sneath*et al.*,1986)

Table.2 Extracellular lipase activity shown by different isolates

Isolates	Lipase activity (U/mL)
Isolate-1 (*Penicillium* species)	1.32
Isolate -2 (*Aspergillus fumigatus*)	1.36
Isolate -3 (*Aspergillus niger*)	**1.98**
Isolate -4 (*Rhizopus* species)	0.54

Table.3 Effect of different media on production of extracellular lipase

Name of medium	Enzyme activity (U/mL)
M 1	0.35
M 2	1.36
M 3	**2.08**
M 4	0.54
M 5	1.98

Table.4 Effect of agitation rate on lipase production of Isolate -3 (*Aspergillus niger*)

Agitation rate (rpm)	Lipase activity (U/mL)
50	0.56
100	2.87
150	**3.96**
200	3.19
250	1.58

Fig.1 Effect of inoculum size on the production of lipase of Isolate -3 (*Aspergillus niger*)

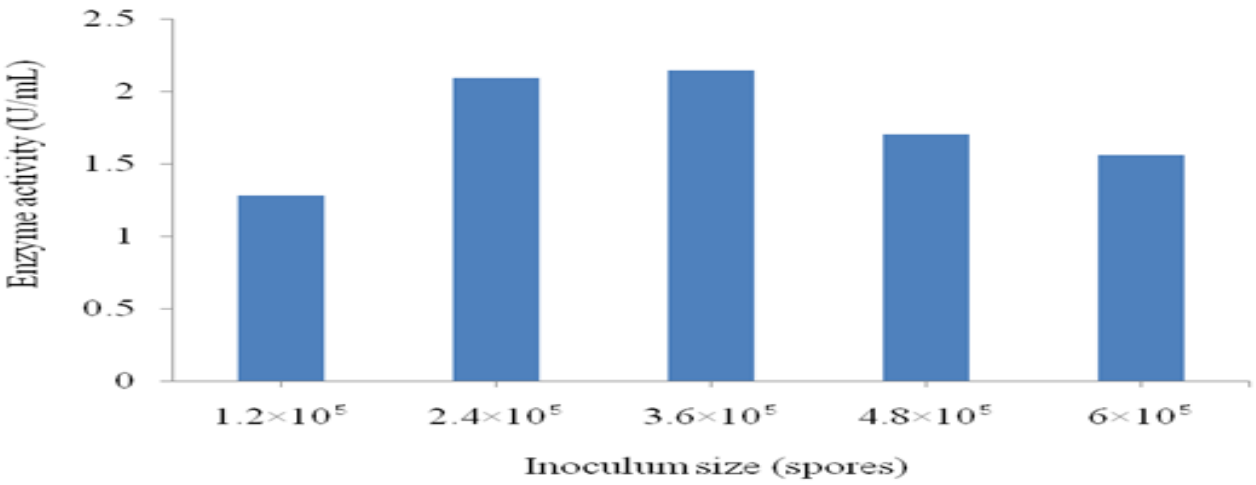


Fig.2 Effect of incubation time on lipase production of Isolate -3 (*Aspergillus niger*)

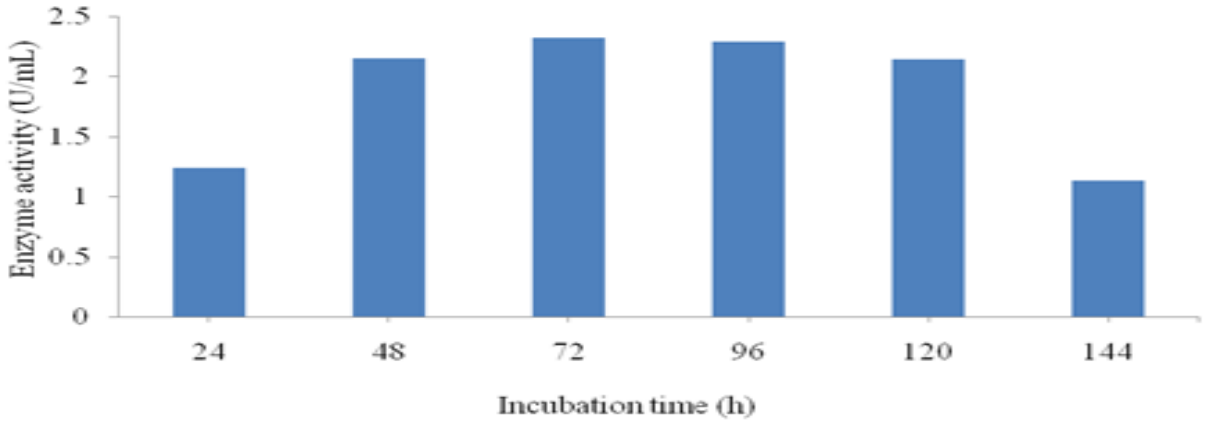


Fig.3 Effect of different carbon sources on lipase production of Isolate -3 (*Aspergillus niger*)

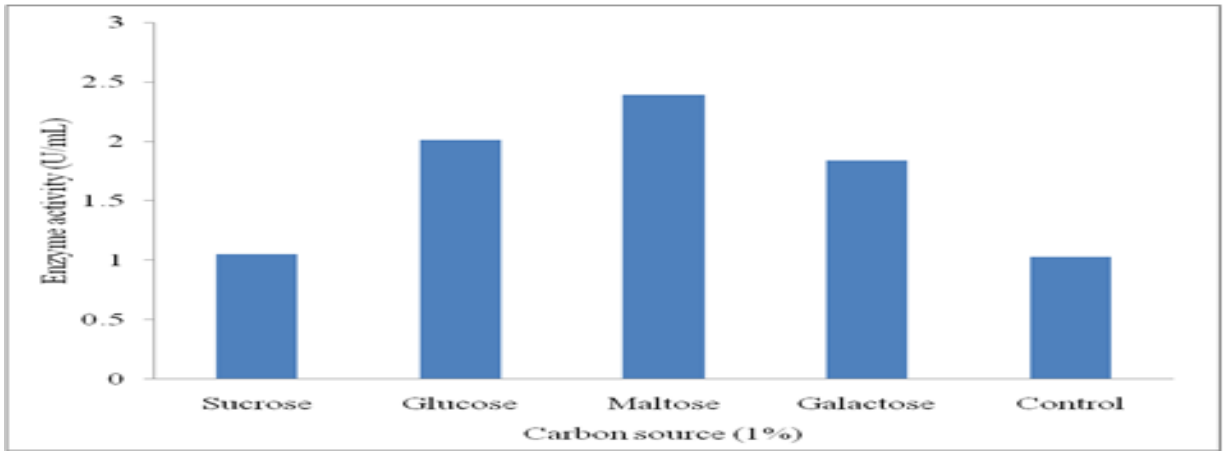


Fig.4 Effect of different concentrations of maltose on lipase production of Isolate -3 (*Aspergillus niger*)

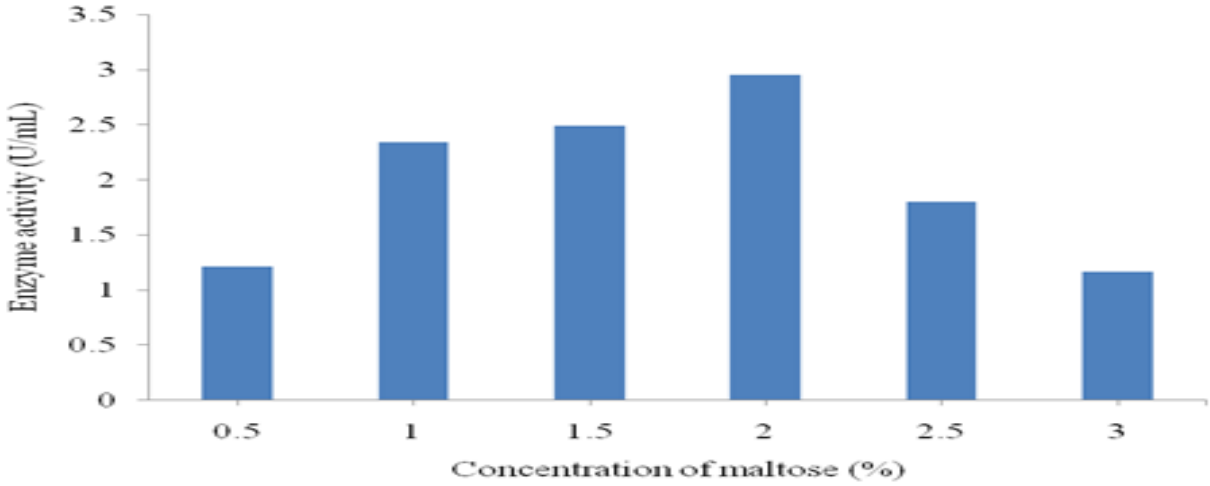


Fig.5 Effect of different nitrogen sources on the lipase production of Isolate -3 (*Aspergillus niger*)

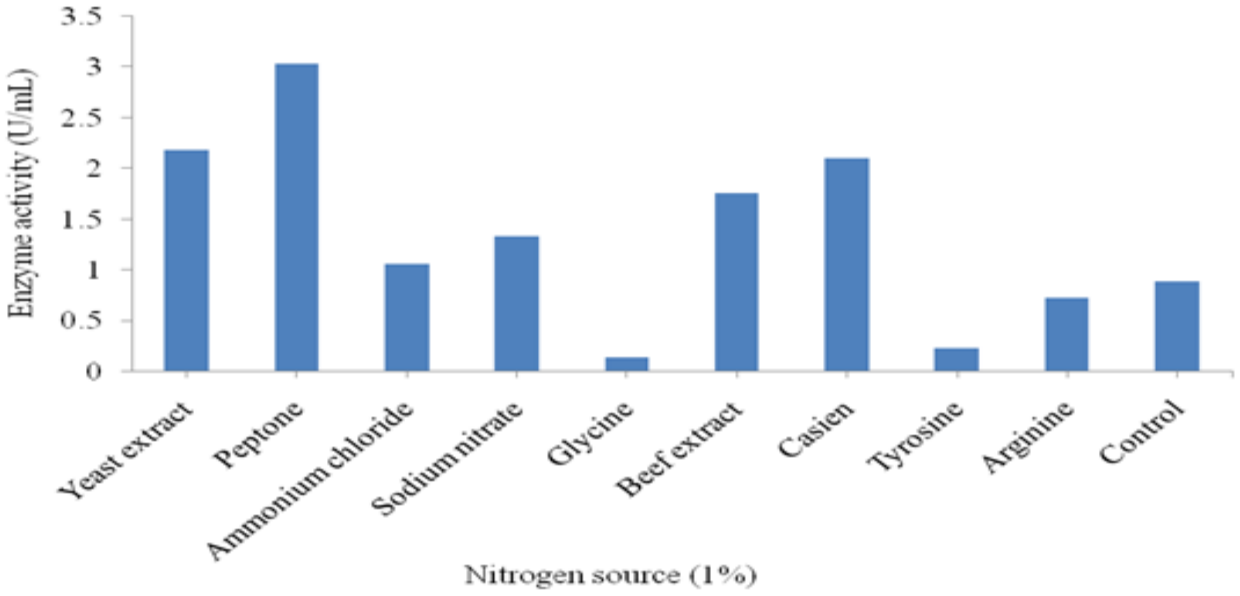


Fig.6 Effect of concentration of peptone on lipase production of Isolate -3 (*Aspergillus niger*)

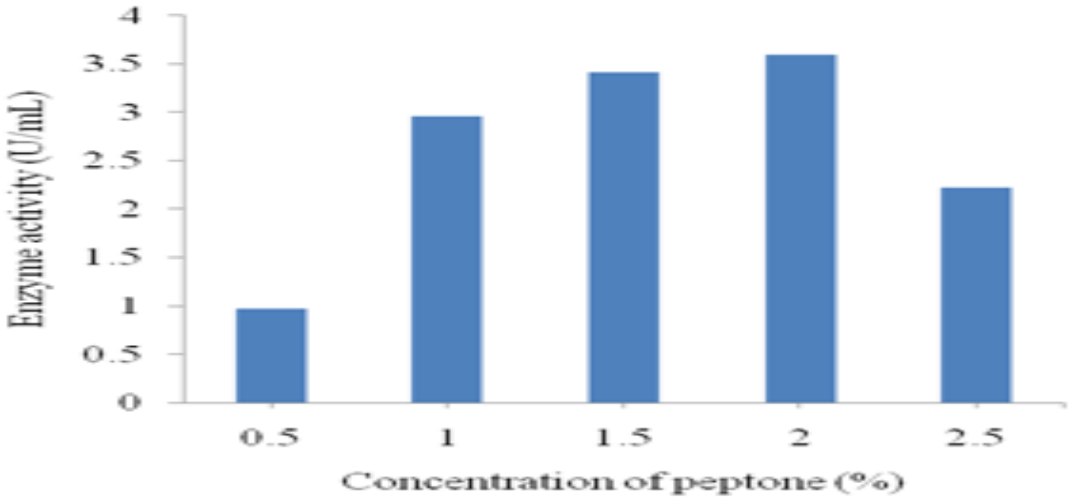


Fig 7. Effect of different pH on the lipase production of Isolate -3 (*Aspergillus niger*)

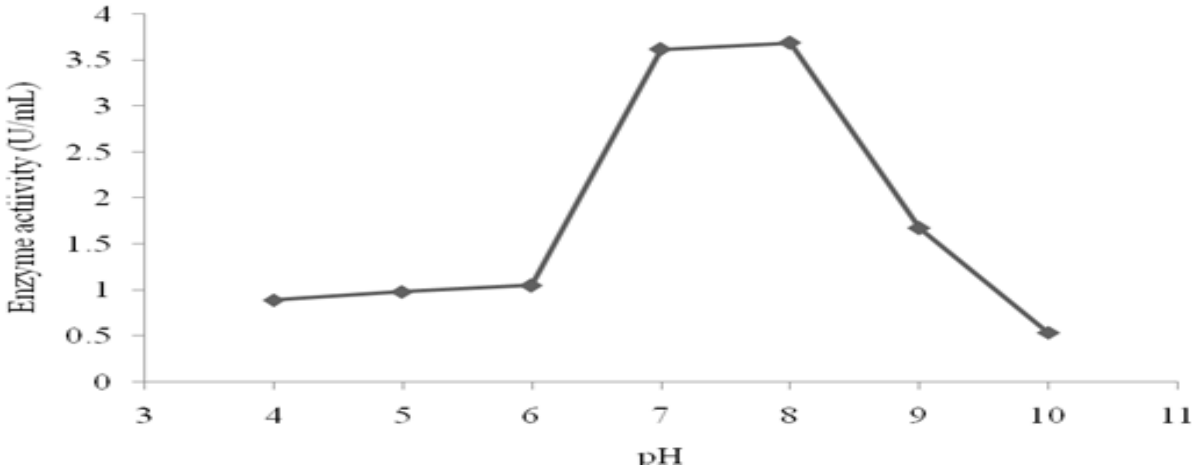


Fig.8 Effect of incubation temperature on the lipase production of Isolate -3 (*Aspergillus niger*).

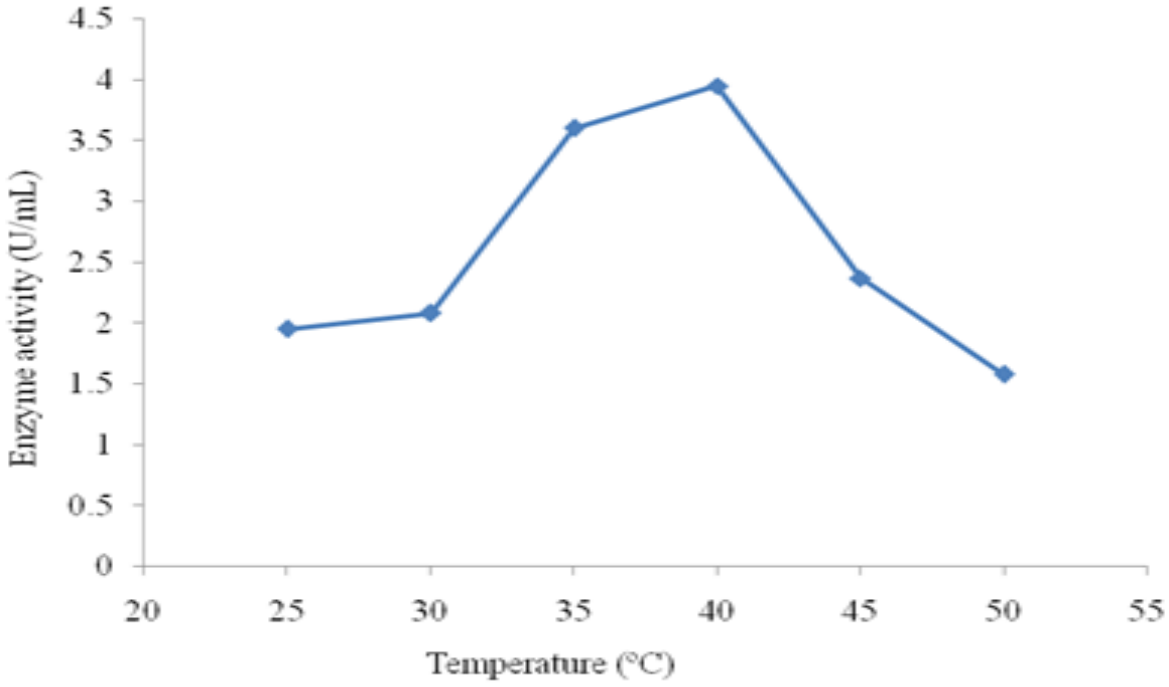


Fig.9 Effect of incubation time on lipase activity

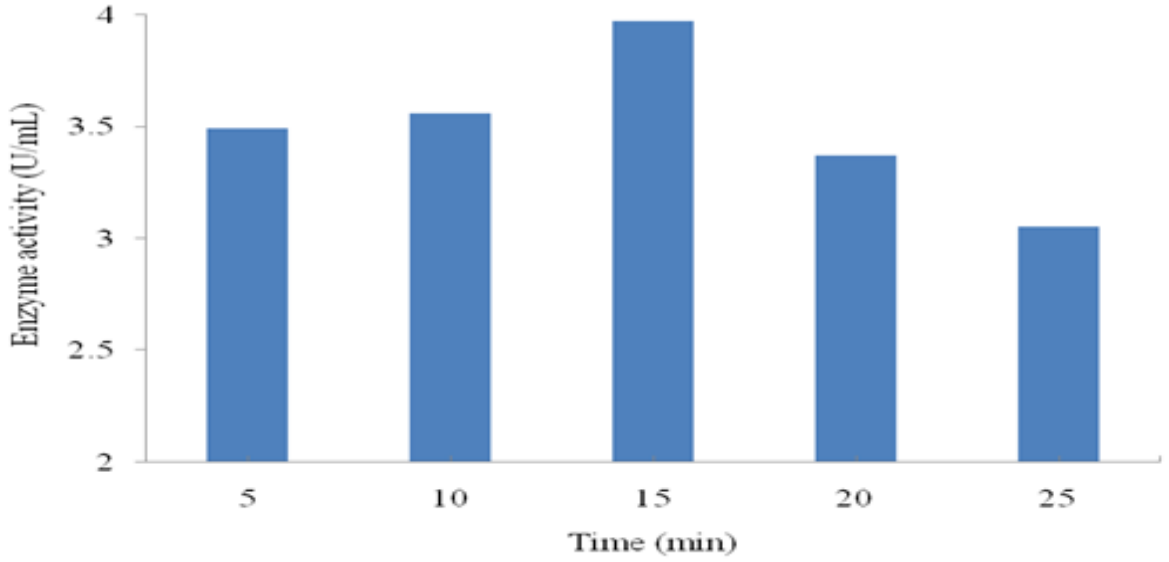
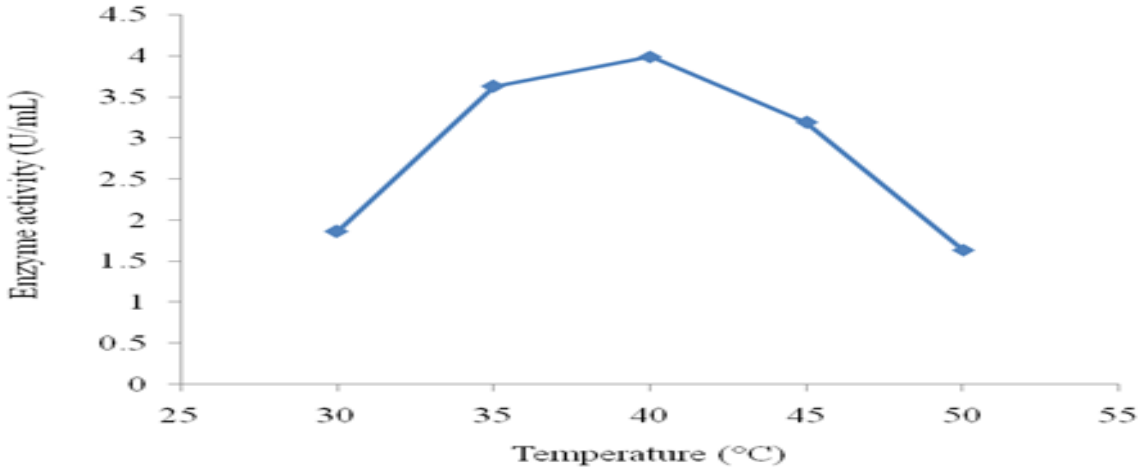


Fig.10 Effect of temperature on lipase activity



Effect of the concentration of nitrogen source on the production of lipase- Of the various concentrations of peptone used in the production medium, 2.0% w/v showed the maximum enzyme production with an enzyme activity of 3.60 U/mL (Figure 6). Further increase in concentration led to decrease in enzyme activity. Therefore 2.0% of peptone was taken as the optimum concentration for further studies. Similarly a research carried out by (Wang *et al.,* 2008) showed maximum lipase production when 2% of peptone was used as nitrogen source in *Micrococcus* sp. In another study, 2% of soyabean powder was optimized as nitrogen source for maximum lipase production from *Saccharomyces cerevisiae* (Shirazi *et al*., 1998).

Effect of pH of the medium on production of lipase - The production of enzyme is very sensitive to the pH of the production medium therefore, optimization of pH is a necessary step for the maximum production of lipases. The maximum production of lipase (3.69 U/mL) was obtained at pH of 8.0 (Figure 7). This might be because organism required a slightly basic pH for its metabolic processes as well as for the production of lipases (Kiran *et al*., 2008). Increase or decrease in pH had negative impact on the production of lipase. In another study, *Fusariumsolani* gave maximum lipase production at pH of 8.6 (Maia *et al*., 1999).

Effect of incubation temperature on lipase production- Temperature is known to influence the metabolic rate of the organism involved in the process, which in turn determines the amount of end product. Most favourable temperature for maximum extracellular lipase production (3.95 U/mL) was found to be 40°C (Figure 8). Increase or decrease in temperature from 40°C caused a decrease in enzyme production, indicating the mesophilic nature of the isolated strain. High production temperature reduces the contamination risk during fermentation (Madhaiyan and Sundaram 2004). However, a moderate optimal temperature for a longer incubation period is beneficial so as to reduce the cost of production. Maximum lipase production was observed at 25°C from *Pseudomonas* sp. (Kulkarni and Gadre, 2013).

Effect of agitation rate on lipase production - The maximum lipase production was obtained at 150 rpm and was therefore taken as the optimum agitation rate for further studies (Table 4). Higher stirring speeds than 250 rpm resulted in mechanical and/or oxidative stress, excessive foaming, disruption and physiological disturbance of the cells, while lower stirring speeds seemed to limit oxygen levels along with the lacking of homogeneous suspension of the fermentation medium and breaking of the clumps of the cells. (Elibol and Ozer2000) also reported that main reason for the negative effect of agitation speed might due to the perturbation of protein structure during the biosynthesis of lipase. Agitation rate of 120 rpm was found to give maximum lipase production from *Fusarium solani* (Maia *et al*., 1999) and *Penicilliumcyclopium* (Vanot *et al*., 2002).

Effect of reaction time on lipase activity - Maximum activity of extracellular lipase (3.97 U/mL) was observed after 15 minutes of incubation (Figure 9). The longer an enzyme is incubated with its substrate, the greater the amount of product that will be formed. However, the rate of formation of product is not a simple linear function of the time of incubation**.**Incubation time of 10 minutes gave maximum activity of lipase from *Penicillium*sp. in a study carried out earlier (Margesin *et al*., 2002).

Effect of reaction temperature on lipase activity - Maximum enzyme activity (3.99 U/mL) was observed at 40°C of incubation temperature (Figure 10). When the temperature was increased or decreased from 40°C there was a consistent decrease in lipase activity. The optimum temperature for lipase activity was 35°C for *Penicillium candidum* (Ruitz *et al*., 2001) and 37°C for *Aspergillus niger* (Kamini *et al*., 1998).

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