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ORIGINAL ARTICLE

Enhancement of Lipase Biosynthesis by *Aspergillus niger* using Gamma Radiation

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ABSTRACT

Key words:

Lipase biosynthesis optimization, *Aspergillus niger ADM 110*, Gamma radiation

Objectives: *The present study describes the isolation, identification and screening of fugal isolates for the biosynthesis of extracellular lipase enzyme.* ***Methodology:*** *Thirteen fungal cultures were isolated from cotton seeds, waste cake of cotton seed oil and spent bleaching earth, samples by serial dilution method. All isolates were initially selected qualitatively on tributyrin agar plates and were shifted to the slants of PDA for maintenance and storage at 4°C. Quantitative screening for extracellular lipase biosynthesis by isolated fungi was carried out in shake flasks and the most potent fungal isolate which producing 25 U ml^{-1} of enzyme was selected. The isolate was then identified on the basis of standard morphological measurements and was assigned the code Aspergillus niger ADM 110.* ***Results:*** *Enhanced lipase biosynthesis was observed at 25 °C, pH 7, 1.0 ml (4.60 × 10^{7}) of spore inoculum and after 72 h of incubation. Olive oil 5 % was observed as the most effective carbon source and yeast extract 5.0 % as the most effective nitrogen source for lipase biosynthesis. The optimum shaking value was 200 rpm. Aspergillus niger ADM 110 was subjected to Gamma radiation in order to improve its lipolytic potential.* ***Conclusion:*** *Using the optimized conditions, maximum lipase biosynthesis has been obtained by using 0.14 kGy of gamma radiation with enzyme activity 53 U/ml as compared to the parent strain (unirradiated) with enzyme activity 30 U/ml.*

INTRODUCTION

Lipases (E.C. 3.1.1.3) triacylglycerol (TAG) acylhydrolases, are ubiquitous amoung microbs, plants and animals which catalyze the hydrolysis of ester linkages of triglycerides to form glycerol and fatty acids[1]. Lipases can catalyze numerous reactions such as hydrolysis, interesterification, esterification, alcoholysis and aminolysis [2,3,4]. Lipases have a great potential for commercial applications due to their selectivity and broad substrate specificity. Nowadays, most lipases produced commercially are currently obtained from fungi and yeasts. Fungal lipases have received attention because of their potential use in food processing, pharmaceuticals, cosmetics, detergents and leather industry [5].

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Lipases are produced widely in nature, but fungal lipases have received increased attention due to the high biosynthesis titers obtained with these microorganisms. Among fungi, well-known producers are *Penicillium restrictum*[6], *Candida rugosa*[7], *Colletotrichum gloeosporioides*[8], *Aspergillus niger*[9] and *Sporobolomyces ruberrimus*[10]. However, lipase biosynthesis levels and activity changes according to each microorganism and especially with the conditions employed in the cultivation. Thus, it is important to analyze and evaluate the variables and conditions that may influence lipase biosynthesis and activity.

Lipase biosynthesis is influenced by the type and concentration of carbon and nitrogen sources, the culture pH medium, the growth temperature, and the dissolved oxygen concentration[11]. Lipidic carbon sources seem to be generally essential for obtaining a high lipase yield; however, a few authors have produced good yields in the absence of fats and oils [12].

In this study, the effect of carbon, nitrogen sources, pH, growth temperature, shaking speed and incubation

period on lipase biosynthesis by isolated lipase-producing fungal isolate is described. This work also aimed at study the effect of exposure *Aspergillus niger ADM 110* to different doses of gamma radiation for enhancement lipase biosynthesis.

METHODOLOGY

Chemicals

Media and chemicals for isolation and lipase assay like *p*- Nitrophenylpalmitate and tributyrin were of high grades and purchased from Sigma and Hi-Media Laboratories, India, (all chemicals were analytical grade).

Isolation of microorganisms

Microorganisms growing in an organic matter rich environment were isolated from, cotton seeds, waste cake of cotton seed oil and spent bleaching earth samples and were transported in sterile plastics bags and stored at 4^0C when not used immediately. Ten grams of samples (from each site) were suspended in 250 mL Erlenmeyer flask containing 100mL sterile physiological saline (PS; Oxoid). This was followed by constant and vigorous stirring for 30 minutes at 120 rpm to dislodge soil clumps before allowing settling. The supernatant was decanted and a 10–fold serial dilution made from it. Approximately, 100µL of each dilution was spread inoculated on a mineral salts agar medium formulated by a modification of the methods of ***Kashmiri et al.*** and ***Bapiraju et al.***[13,14]**.** The medium contained (g/l) palm oil 10 (emulsified in Tween 80 at 10ml oil: 1ml Tween); $(NH_4)_2SO_4$, 5; Na_2HPO_4, 6; $KH2PO_4$, 2; $MgSO_4$, 3; $CaCl_2$, 3 and Agar 12. The pH of the medium was adjusted to 5.0 using citrate-phosphate buffer (0.5M). Plates were incubated at 28°C for 5–7 days and checked every day for growth. Developed colonies were purified and transferred to the mineral salts agar slants for storage. In addition, fungal colonies were subcultured on Potato Dextrose Agar (PDA, Oxoid) plates for macro and micro-morphological identification.

Purification and identification of isolated microorganisms:

Each fungus isolated and subcultured on (PDA) slant . The fungi identified according to external morphological characters using **Barnett *et al.*** for the genera and **Pitt *et al.***[15,16] for the species. Pure isolates only were subcultured on slants of (PDA) and kept in refrigerator for further investigation.

Primary screening of the fungal isolates for their lipolytic activities

For the detection of fungal lipolytic activity, tributyrin agar clearing method was used [17]**.** Twenty ml of tributyrin agar medium was inoculated with a loopful of isolate and incubated at 30ºC for five days. The composition of tributyrin agar medium is (g/l): $(NH_4)_2SO_4$, 5; Na_2HPO_4, 6; KH_2PO_4, 2; $MgSO_4.7H_2O$, 3; $CaCl_2.2H_2O$, 3; agar 20 and tributyrin, 10ml with pH 6.0. Lipolytic zone of the isolates was measured and these isolates were subjected to secondary screening by submerged fermentation.

Secondary screening for lipase biosynthesis

For the biosynthesis of lipase, the selected fungal isolates were cultivated in a biosynthesis medium containing olive oil (source of natural triglyceride, triolein) as the sole carbon source under submerged fermentation conditions and assayed for the lipolytic activity of the culture filtrates. The flasks were incubated at 30ºC for 4 days on a rotary shaker (150 rpm). The culture broth was filtered and the clear filtrate was used as the source of crude enzyme.

Lipase biosynthesis medium

The medium for biosynthesis contained (g/l): olive oil, 50; peptone, 5; yeast extract, 5; glucose, 5; NaCl, 3 and $MgSO_4.7H_2O$, 0.5. The enzyme biosynthesis medium was optimized by varying Different environmental parameters such as carbon and nitrogen sources, pH, growth temperatures, shaking speeds and incubation periods. *Aspergillus nigerADM110* was inoculated into enzyme biosynthesis medium. The culture filtrate was assayed in triplicate for enzyme activity.

Assay of lipase activity

The assay was modified from that described by **Pencreac'h *et al.***[18]. The assay mixture contained 90 µl of 8.25 mM p-nitrophenyl palmitate (Sigma-Aldrich) in isopropanol and 810 µl of 50 mM Tris-HCl, pH 8.0, with 0.5% (w/v) Triton X-100 and 0.12% (w/v) arabic gum preheated to 40 °C. To initiate the reaction, 100 µl of lipase solution was added. The change in absorbance at 410 nm was monitored for 5 min at 40 °C using a spectrophotometer (UV- Vis Cary 60, Agilent, Malaysia). The activity was calculated from the difference in absorbance between 2 and 5 min with a standard curve for the hydrolysis product, p-nitrophenol. One enzyme activity unit was defined as the biosynthesis of 1 µmol of p-nitrophenol per min at 40°C.

Optimization for Extracellular lipase biosynthesis

The effect of different carbon sources on lipase biosynthesis was studied using 1.0 % (w/v) palm, corn, cotton seed, sunflower and olive oils, each separately. For comparison, a culture was also grown with 1.0 % (w/v) glucose as the sole carbon source. All the carbon sources were sterilized separately and added. Organic and inorganic nitrogen sources like yeast extract, meet extract, peptone, ammoniums sulfate, ammonium chloride and urea were used at 1.0 % (w/v) and added into the media.

The biosynthesis medium was adjusted to different pH ranging from 4.0 to 9.0 with 1.0 variation whereas the other parameters were unaltered. For selection of optimum temperature for the biosynthesis of lipases, the temperatures varying from 20 to 30°C and keeping the

other parameters fixed, while the cultures were incubated at 10, 15, 20, 25, 30 and 35°C. The time course of lipase biosynthesis was studied in the enzyme biosynthesis medium in shake flasks incubated for 80 hrs. Samples were removed periodically at 8 hrs interval and lipase activity in the culture supernatant was determined. The effect of agitation on lipase biosynthesis was studied at varying agitation speed (0, 50, 100, 150, 200 and 250) rpm. To determine the effect of gamma radiation on lipase biosynthesis, the fungal strain was grown on PDA for 8 days and subjected to gamma radiation at doses (0.2, 0.4, 0.6, 0.8, 1, 1.2,1.4 ,1.6, 1.8 and 2.0 kGy). All irradiation processes were carried out at the military technical college (MTC) Cairo. Irradiation facility was Co-60 Gamma chamber 4000-A India. The source gave average dose rate 2.325 kGy/h at the time of experiments.

RESULTS AND DISCUSSIONS

Isolation of microorganisms from the collected samples:

Microorganisms growing in an organic matter rich environment were isolated from, cotton seeds (CS), waste cake of cotton seed oil (WCS) and spent bleaching earth (SBE) samples. Thirteen fungal isolates were obtained from the samples which were able to grow on PDA medium at 25± 2º C for 5-7 days as indicated in table (1).

The Obtained microorganisms were thirteen isolates; four isolates from (CS), three isolates from (WCS) and six isolates from (SBE).

Primary and secondary screening of fungal isolates for their lipolytic activities:

All the thirteen fungal isolates obtained in pure culture were analyzed to detect their lipolytic activities using tributyrin agar medium. Among the isolates, ten possess lipolytic activities after three successive subculturing on tributyrin agar plates. These ten isolates were secondary screened to obtain the most potent fungal isolate. The results of primary detection of lipase activity of the isolated strains were recorded in table 1.

All the positive isolates were subjected to submerged fermentation conditions and assayed for lipolytic activity quantitatively. Since tributyrin is not a substrate for lipases alone, all the selected positive isolates are confirmed for their lipolytic activity by the hydrolysis of natural triglyceride (triolein) under submerged fermentation conditions and the results are presented in table (1). From the result the isolate (*A. niger ADM110*) showed maximum lipolytic activity of 25.0 U/ml among all the positive isolates.

Table 1: Isolation and screening of fungal isolates for their lipolytic activity.

Source of isolates	fungal isolates	Zone of hydrolysis (mm)	Lipase activity (U/ml)
Cotton Seeds (CS)	*Aspergillus fumigatus ADM101*	**(-ve)**	**0.0**
	Alternaria sp. ADM102	**3.0**	**5.1**
	Aspergillus flavus ADM103	**10.2**	**15.6**
	Rhizopus arrhizus ADM104	**4.5**	**6.9**
Waste Cake Of Cotton seed Oil(WCS)	*Alternaria sp. ADM105*	**12.5**	**19.8**
	Penicillium chrysogenum ADM106	**1.5**	**5.0**
	Cladosporium sp. ADM107	**3.2**	**10.1**
Spent Bleaching Earth (SBE)	*Penicillium italicum ADM 108*	**(-ve)**	**0.0**
	Rhizopus delemar ADM109	**9.6**	**14.8**
	Aspergillus niger ADM110	**16.0**	**25.0**
	Penicillium roqueforti ADM111	**4.0**	**9.9**
	Mucor sp. ADM112	**(-ve)**	**0.0**
	R.arrhizus ADM113	**12.0**	**18.7**

Fungus identification

The fungus used for the present study was first selected on the basis of formation of zone of hydrolysis on tributyrin agar media and identified on the basis of morphological, biochemical and physiological characteristics at the Regional Center of Mycology and Biotechnology, El-Azhar University, Cairo.

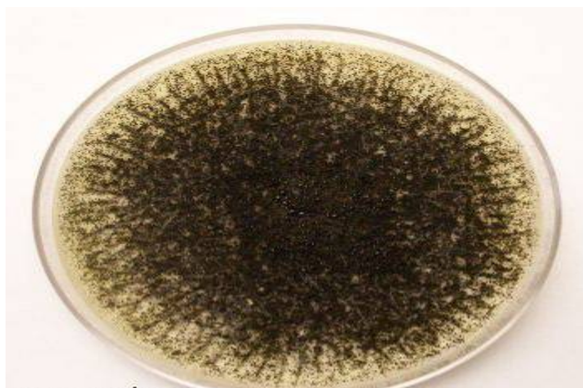

Fig. 1a: Pure culture of *A. niger ADM110*

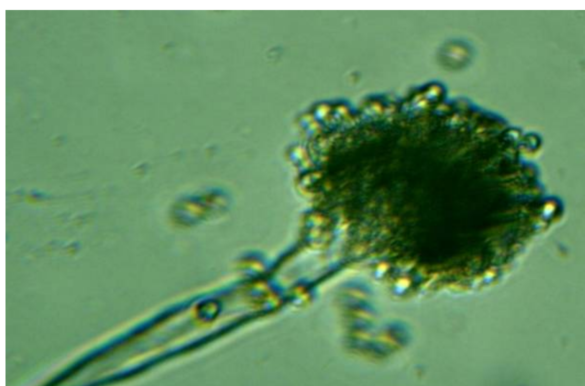

Fig. 1b: Microscopic view of the *A. niger ADM110*

Fig. 1a: shows a pure culture of Aspergillus niger on potato dextrose agar media plate, figure **1b**. Shows Microscopic view of the *A. niger ADM110* at 40X.

Effect of incubation Period

The amount of lipase produced was investigated after every 8 h up to 96 h. The result indicates that incubation time affected lipase biosynthesis very significantly and maximum lipase activity (25 U/ml) was observed after 72 h of incubation time (Fig. 2). After long incubation time, lipase biosynthesis was turned down as its activity was found 18 U/ml after 80 h of incubation whereas mycelial biomass was rapidly stimulated throughout the fermentation period.

The optimum incubation period for lipase activity by the most potent isolate was 72h. The lipase activity which had been recorded was 25 U/ml, as indicated in Figure3.

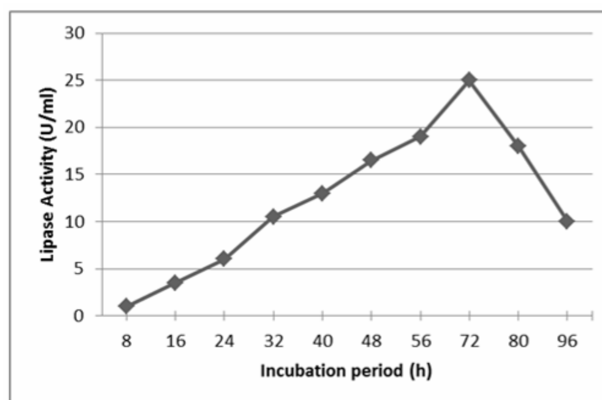

Fig. 2: Relation between incubation period and lipase activity by *A. niger ADM110*.

Effect of lipidic carbon sources and glucose

Lipidic carbon sources or inducers such as fats and vegetable oils seem to be generally essential for obtaining a high lipase yield, vegetable oils was used for inducers on lipase biosynthesis of lipase-producing microorganism. The effect of lipidic carbon sources on lipase biosynthesis was investigated at 26◦C and 150 rpm for 80h. Considering 80 h of fermentation time, the maximum lipolytic activity was 24 U/ml in the presence of olive oil, as showed in Figure 3. V. M.G. LIMA *et al.*[19] reported that the maximum lipolytic activity was 12 U/ml in the presence of olive oil by *P. aurantiogriseum*. Lipase biosynthesis occurred in medium without lipids, but for improved biosynthesis an inducer was needed [20].

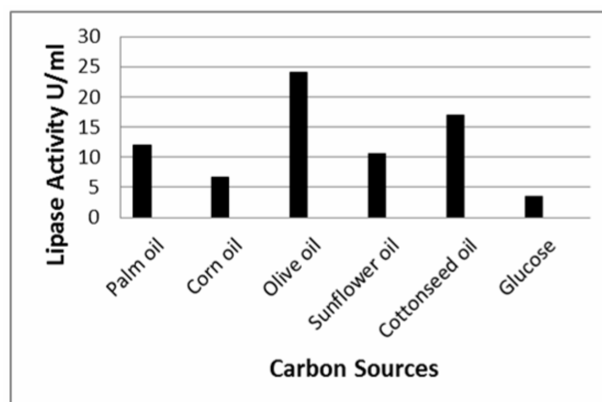

Fig. 3: Relation between different lipidic carbon source and lipase activity by *A. niger ADM 110*

Effect of different concentrations of olive oil:
The effect of the concentration of the carbon source on lipase biosynthesis was studied with the addition of different concentrations (0.5, 1, 1.5 and 2.0 %) of olive oil. The carbon source concentration has a strong influence on the biosynthesis of lipase by *A. niger ADM110*, as shown in fig. 4. with an increase in olive oil concentration there was a decrease in the peak lipolytic activity attained. Fermentations done with 1.5 and 2 % olive oil had much lower peak activities, indicating an inhibitory effect on the biosynthesis of lipase by *A. niger ADM110*.

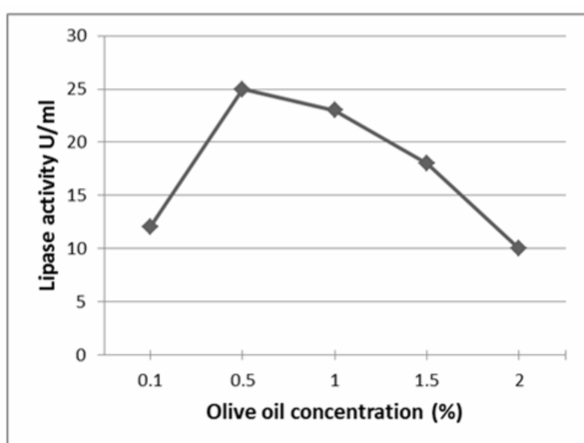

Fig. 4: Relation between different olive oil concentrations and lipase activity by *A. niger ADM 110*.

Effect of nitrogen sources

The effect of nitrogen sources on growth and lipase biosynthesis was investigated at 26◦C and 150 rpm using olive oil (0.5 %) as carbon source. In this work, yeast extract, meet extract and peptone were used as the organic nitrogen sources and ammonium sulfate, ammonium chloride and urea were used as inorganic nitrogen sources. The obtained results in fig. (5) indicated that yeast extract was effective nitrogen source for lipase biosynthesis by *A. niger ADM110*. Maximum lipase activity of 25.0 U/ml was significantly observed by yeast extract when added as a nitrogen source, while lipase activities of 24.0, 22.0, 20.0, 19.5 and 12.5 U /ml were obtained when ammonium sulfate, peptone, meet extract, urea and ammonium chloride were used, respectively, as indicated in Figure 5.

Thanagrit Boonchaidung *et al*. [21] reported that the maximum lipase activity of 1.134 U/mL was significantly observed by yeast extract when added as a nitrogen source by *Candida sp*. KKU-PH2-15.

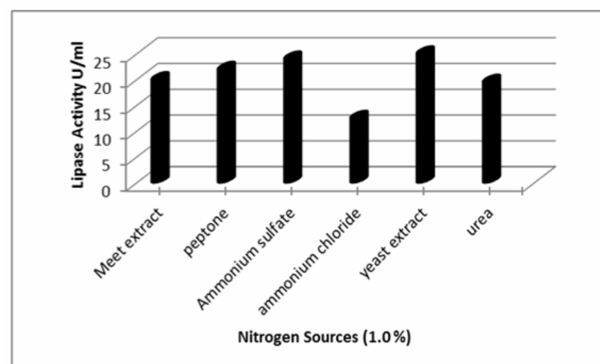

Fig. 5: Relation between different nitrogen source and lipase activity by *A. niger ADM110*.

Effect of different concentration of yeast extract:

To evaluate the effect of different concentrations of the nitrogen source, cultures were done with increasing concentrations of yeast extract while the concentration of olive oil was maintained constant (0.5%), in such a manner as to give nitrogen concentration of 1, 2.5, 5 and 10. %. For fungi, relatively high nitrogen concentrations are typically required in order to favor the biosynthesis of lipases over the biosynthesis of other enzymes [22]. The maximum lipolytic activity obtained with a nitrogen concentration of 5% was 26 U/ml and with a concentration of 2.5% was 22 U/ml as shown in fig. (6). Pimentel *et al.* [23] recorded that the maximum lipolytic activity in a medium that contained yeast extract (0.5%) as the nitrogen source by A Brazilian strain of *P. citrinum was 409 IU/ml*.

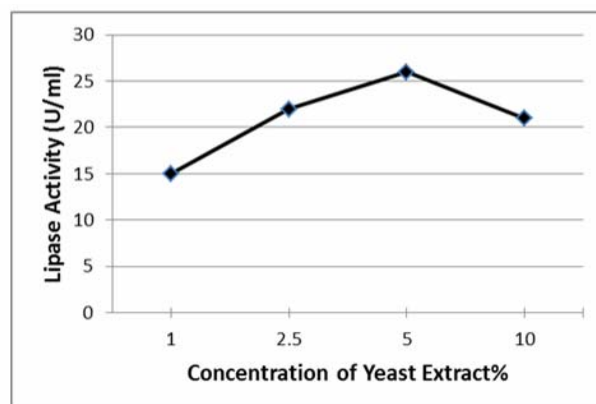

Fig. 6: Relation between different concentrations of yeast extract and lipase activity by *A. niger ADM110*.

Effect of inoculum size:

Different size of inoculum ranging from 1mL to 6ml with an interval of 1.0 (4.63×10^7 spores/ ml) was tested by *A. niger ADM110*, and Maximum extracellular lipase biosynthesis (25 U/mL) was obtained when 1.0 ml of spore inoculum was used. Lipase activity obtained by *A. niger ADM110* was represented graphically in fig. 7. Further increase in inoculum size resulted in a gradual decrease in lipase activity. Ushio *et al.*[24] also

optimized 1.0 ml of inoculum level for maximum lipase biosynthesis. Imandi *et al.*[25] reported a 2 ml of inoculum.

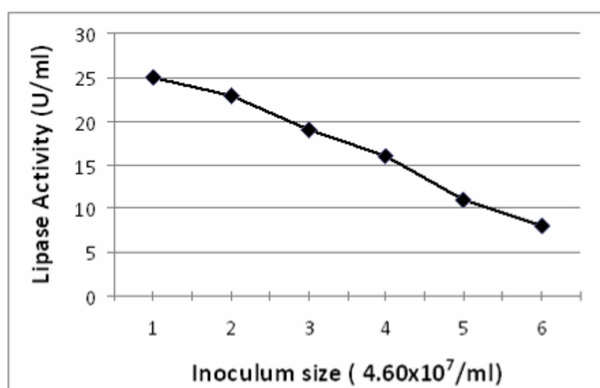

Fig. 7: Relation between different Inoculum size (4.60×10^7/ ml) and lipase activity by. *A. niger ADM110.*

Effect of incubation temperature

Temperature is a critical parameter that has to be controlled and it varies from organism to organism. Temperature influences secretion of extracellular enzymes by changing the physical properties of the cell membrane. As indicated in Fig. 8. The optimum temperature for lipase biosynthesis is 25 °C and above this, can considerably decrease the yield. Cultures for biosynthesis of lipases by fungi of the genus *Penicillium* are generally incubated at between 25 and 30 °C, most often at 28°C [26].

Effect of pH values

Increasing the pH range till it reaches its maximal value at 7.0 increased the activity of the tested isolates for lipase biosynthesis. As the pH increased over 7.0, lipase biosynthesis decreased. These results agree those obtained by many investigators, who claimed that optimal pH value for lipase biosynthesis by microorganisms varied. *Penicillium roqueforti* produced the maximal lipase yield at pH 5.5 [27], while the maximal yield of lipase was produced at pH 9.5 by *Saccharomyces lipolytica* [28]. In this study the maximum enzyme activity was found to occur at pH 7, as reported in Fig. 9.

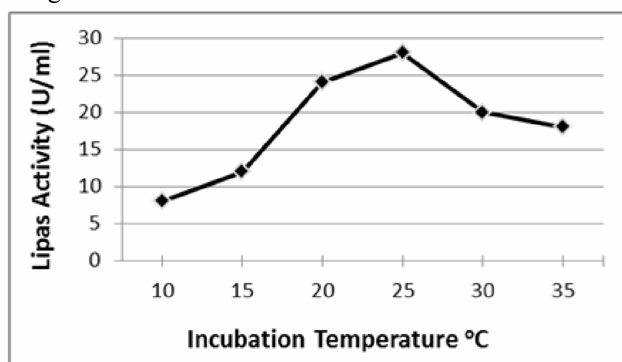

Fig. 8: Relation between different Incubation temperature and lipase activity by *A. niger ADM110*.

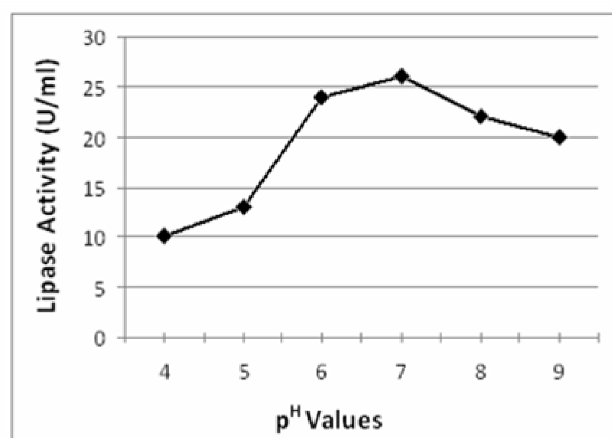

Fig. 9: Relation between different pH values and lipase activity by. *A. niger ADM110*.

Effect of Agitation speed

Shaking speed has great effect on lipase biosynthesis by aerobic microorganisms, as increasing shaking rate increases the availability of dissolved oxygen. In addition shaking may also create condition of higher availability of carbon source to microorganisms [29]. However, at higher agitation rates, there was a reduction in lipase biosynthesis[30,31]. The optimum shaking value to get maximum lipase activity by *A. niger ADM110* was 200 rpm. The lipase activity obtained was 26 U/ml as represented in Fig. 10. *Rhizopus sp* showed highest lipolytic activity 19 U/ml at 200 rpm [32].

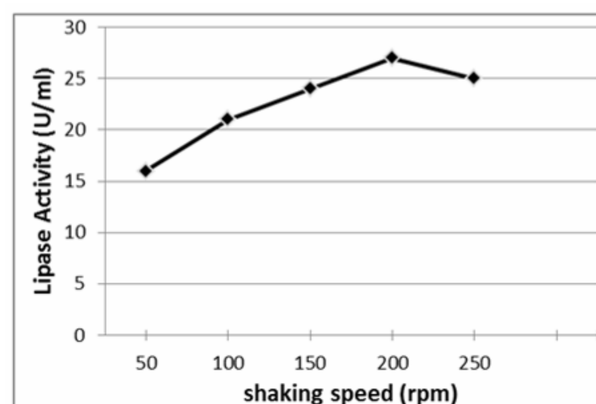

Fig. 10: Relation between different shaking speed and lipase activity byA. niger.

Effect of gamma radiation

A range of doses (0.2 – 2.0 kGy) of gamma radiation at an interval of 0.2 kGy were applied to *A. niger ADM110*. It was found that at dosage of 1.4 kGy, *A. niger ADM110* showed maximum extracellular lipases activity (53 U/ ml) as reported in table 2. Worthy, exposure of microbial cells to ionizing

radiation as gamma radiation leads to chemical then to metabolic or physiological changes. Irradiation makes an additional stress to cells, which changes their organization. Irradiation affects cell proteins, enzymes, nucleic acids, lipids and carbohydrate. Ionizing radiation at high doses may be lethal to microorganisms. On the other hand, low-dose irradiation of microorganisms my produce mutations which may be desired in producing products of importance such as antibiotics, organic acids, amino acids, vitamins, alcohols, pigments and enzymes. This enhancement of enzyme biosynthesis might have been due to either, an increase in the gene copy number or the improvement in gene expression, or both [33,34,35,36]. A gradual decrease in the enzyme activity after exposure to the different doses of 1.6 and 1.8 kGy was observed. The reduction of the enzyme activity also obtained at dose 2.0 kGy. This could be explained by damage or deterioration in the vitals of the microorganism as radiation causes rupturing in the cell membrane .This major injury to the cell allows the extracellular fluids to enter in to the cell. Inversely, it also allows leakage out of ions and nutrients which the cell brought inside. Membrane rupture may result in the death of a cell and decrease in the enzyme synthetic activity due to radiation exposure [36]. TEHREEMA IFTIKHAR *et al.,* (2010) reported that *R. oligosporus* showed maximum extracellular lipases biosynthesis (13.75±1.5 U ml^{-1}) at dosage of 140 k Rad[37].

Table 2. Relation between different γ -radiation doses (kGy) and lipase activity by *A. niger ADM110*.

γ -radiation doses (kGy)	Lipase activity (U/ml)
Unirradiated (control)	**27**
0.2	**31**
0.4	**32**
0.6	**35**
0.8	**38**
1.0	**41**
1.2	**44**
1.4	**53**
1.6	**39**
1.8	**28**
2.0	**20**

CONCLUSIONS

The results of the present study provides useful information about the isolation, purification and screening of fungal strain for their lipolytic activities is optimization of culture conditions such as pH ,temperature, fermentation time, shaking speed, carbon sources and nitrogen sources to provide the best lipase biosynthesis by *A. niger ADM110*. These results show clearly that lipase producing fungus is widespread in oil contaminated samples. The optimized growth conditions developed in this study can be used for a large scale in industrial purposes. In order to reduce the enzyme biosynthesis costs, some strategies can be applied like the use of agro-industrial residues as carbon or inducer sources for lipase biosynthesis to evaluate the feasibility of lipase application in biodiesel biosynthesis. Lipase catalyzed transesterification for biodiesel biosynthesis should be investigated for further study.

An important aspect of this study is the possibility of application of gamma radiation in a manner to increase the enzyme biosynthesis to its maximum value at a certain dose of gamma irradiation above which the enzyme activity gradually decrease.

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