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Effect of Environmental and Nutritional Parameters on the Extracellular Lipase Production by *Aspergillus niger*

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Abstract. The present investigation was carried out to evaluate the effect of different growth conditions on lipase production by *Aspegillus niger*. The extracellular lipase producing fungus was isolated from spent bleaching earths. Optimizations of physical and chemical parameters were done for maximum lipase production using this isolate. Growth of the organism and lipase production were measured usig varying pH (4 – 9), incubation temperature (20 – 30 °C), incubation time (8 – 80 hrs.), carbon sources, nitrogen sources, and shaking speed. Enhanced lipase production was observed at 24 °C, pH 7 and after 72hrs of incubation. Olive oil 5 % was observed as the most effective carbon source and Yeast extract 1.0 % as the most effective nitrogen source for lipase production. The optimum shaking value to get maximum lipase activity by *Aspergillus niger* was 200 rpm.

Introduction

Lipases are serine hydrolases of considerable industrial applications and physiological importance that can catalyze numerous reactions such as esterification, alcoholysis, hydrolysis, aminolysis and interesterification [1-3]. Lipases have a great prospect for merchant applications owing to their broad substrate specificity and selectivity. Lately almost lipases produced commercially are presently obtained from yeasts and fungi. Fungal lipases have received awareness because of their extensive use in cosmetics, pharmaceuticals, food processing, leather and detergents industry [4, 5].

The lipase enzyme one of the esterases group, which hydrolyse esters of glycerol with idealy long-chain fatty acids. They take action at the boundary generated by a hydrophobic lipid substrate in a hydrophilic aqueous medium. Lipases (Triacylglycerol acylhydrolases, E.C. 3.1.1.3) are omnipresent enzymes of considerable industrial importance and physiological significance [6, 7]. A considerable property of lipase(s) is the intense increase in lipase efficiency observed when the substrate starts to form an emulsion, by that means presenting to the enzyme an interfacial area [6, 8]**.** This property defined as interfacial activation of lipase.

Special types of Lipases act as a catalyst biological enzyme and can be used in place of alkali or acid as a catalyst. Biodiesel production using these types of enzymes could offer several potential application and features [9, 10]. Lipases have a number of singular features such as regiospecificity, stereo specificity, substrate selectivity and capability to catalyze a heterogeneous reaction at the interface of water insoluble and water soluble systems. Lipases often have other activities such as; cholesterol esterase, amidase, phospholipase, isophospholipase , cutinase and other esterase type of activities [11-13]**.** Lipases are widely applied in the synthesis of fine chemicals and pharmaceuticals, fat and oil industry, food industry, manufacture of paper, cosmetics production, and waste water treatment [14-16]**.**

Microorganisms produced by Lipase have been found in various occupant such as industrial wastes, oilseeds, soil contaminated with oil, vegetable oil processing factories, dairies product and decaying food [17], coal crest and compost blend [18]. Lipase-producing microorganisms include actinomyces, fungi, yeasts, and bacteria. A simple and reliable method for detecting lipase activity in microorganisms has been described by different researchers. Tween 80 (surfactant) in a solid substrate has been used extensively to identify a lipolytic activity by those researchers [19-21]. Lipase preparation by the organisms can be specified by the formation of dark zones around the colonies. Modifications of this assay could be done by use of different surfactants in combination with foot oil and Cu^{2+} salts [22, 23]. Also, examination (screening) of lipase producers on agar plates is considerably done by using tributyrin as a substrate and fine zones around the colonies suggest production of lipase. This examination systems making use of chromogenic substrates have also been described [24].

In various industries bacterial lipases have essential enzymes applications, because of its characteristic features such as; non-toxic, friendly for environment and production of no harmful residues. Recently, they are widely uses in chemical, surfactant and detergent, agricultural, pharmaceutical industry, perfume and cosmetic [25, 26]. Especially, they are applied for biodiesel productions such as lipase enzyme from Acinetobacter venetians RAG-1 using transesterification process. They are greatly influenced by physic-chemical and nutritional factors such as; pH, temperature, nitrogen & carbon sources and presence of lipids, dissolved oxygen, agitation, and inorganic salts [27-29]. Lipidic carbon sources appear to be generally main for obtaining a high lipase yield; however a few authors have obtained good yields in the absence of fats and oils [30-32]. In this paper, the effect of carbon, nitrogen sources, pH, growth temperature, shaking speed and incubation period on lipase production by isolated lipase-producing fungal isolate is described.

1. Materials and Methods

1.1. Chemicals

Chemicals and media for isolation and lipase assay like *p*- Nitrophenylpalmitate and tributyrin were of high grades and purchased from Sigma and Hi-Media Laboratories, India.

1.2. Sample Collection

For the present study, the extracellular lipase producing fungus was isolated from spent bleaching earths in sterile bottles and plastic bags by dilution plate method [33].

1.3. 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. 1 a, b. shows a pure culture of Aspergillus niger on potato dextrose agar media plate. Fig. 1c,d,e. shows Microscopic view of the *Aspergillus niger* at different magnification.

1.4. Lipase production medium

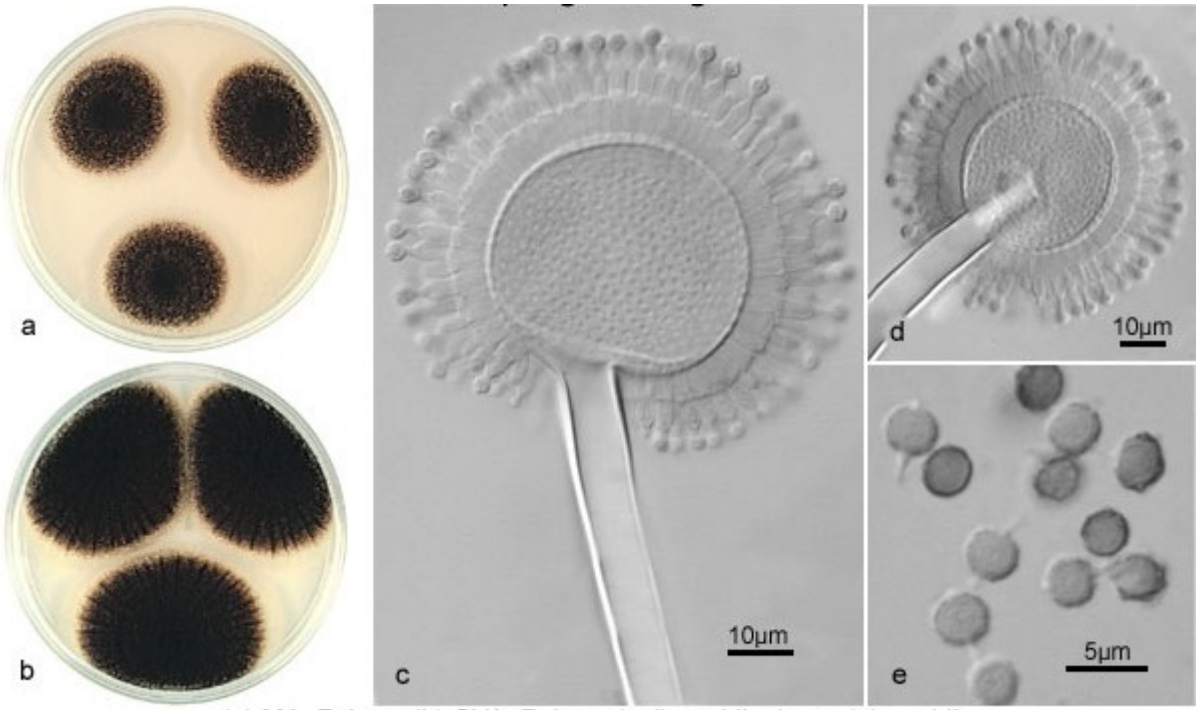
The liquid medium for optimization contained olive oil 5%, peptone 5gm/l, yeast extract 5gm/l, glucose 5gm/l, NaCl 3gm/l and $MgSO_4.7H_2O$ 0.5gm/l which was used as standard. Different physical and chemical parameters were altered while keeping the others constant in the media during the study for enhanced lipase production.

1.5. Factors affecting lipase production

To optimize the lipase activity produced by *Aspergillus niger*, the effect of different carbon sources, growth temperatures, nitrogen sources, pH, incubation periods, shaking speeds and gamma irradiation were tested .

1.5.1. Incubation Period

The time course of lipase production was studied in the enzyme production medium in shake flasks incubated for 60 hrs. An inoculum of 5.0% was added to 50 ml of medium, in 500-ml Erlenmeyer flasks and incubated at 150 rpm on a rotary shaker, at 26°C, for 80 hrs. Samples were removed periodically at 8 hrs. interval and lipase activity in the culture supernatant was determined.



(a) MA, 7 days; (b) CYA, 7 days; (c,d) conidiophore; (e) conidia.

Figure 1. Full Identification of *Aspergilluis niger* ADM110.

1.5.2. pH and incubation temperature

The effect of pH and temperature of the fermentation medium for lipase production was performed by varying pH of the medium (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0), whereas the other parameters were unaltered. For selection of optimum temperature for the production of lipases, the temperatures varying from 20 to 30°C and keeping the other parameters fixed, while the cultures were incubated at 20, 22, 24, 26, 28 and 30 °C.

12.5.3. Shaking speed

Effect of agitation speed on lipase production was performed by incubating the enzyme production medium with inoculated culture in an orbital shaking incubator at 26°C at varying agitation speed 0, 50, 100, 150, 200 and 250 rpm for 80 hrs.

12.5.4. Lipidic carbon sources and glucose

To evaluate the effect of carbon source on the production of lipase by *A. niger*, cultures were done with the addition of 1.0 % (w/v) of palm, corn, cottonseed, sunflower and olive oils, each separately. For comparison, a culture was also grown with 1.0 % (w/v) glucose as the sole carbon source.

1.5.5. Nitrogen sources

Diverse nitrogen sources (both organic and inorganic) were tested individually by supplementing each of the selected N- sources used at 1.0% (w/v) in the production broth. The enzyme activity was affected by the variety of nitrogen sources such as: Yeast extract, meet extract and peptone for organic sources, while ammoniums sulfate, ammonium chloride and urea as inorganic sources.

1.6. Lipase Assay

The crude enzyme was obtained by centrifugation at 10,000 rpm, 4ºC for 10 min. Lipase activity was determined by spectrophotometric method using *p*-NPP (*ρ*-nitrophenyl palmitate) at pH 8.0 [34, 35]**.** The coefficient of extinction of *p*-nitrophenol (*p*-NPP), 1.5 x 10^4 L/mol/cm, was determined by measuring absorbance at 410 nm after incubation for 15 min with the enzyme. One unit was defined as the amount of enzyme liberating 1µmol of *ρ*-nitrophenol per min at 37ºC.

2. Results and Discussions

2.1. 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 production very significantly and maximum lipase activity (25 U/ml) was observed after 72 h of incubation time. After long incubation time, lipase production 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 72hrs. The lipase activity which had been recorded was 25 U/ml, as indicated in Fig. 2.

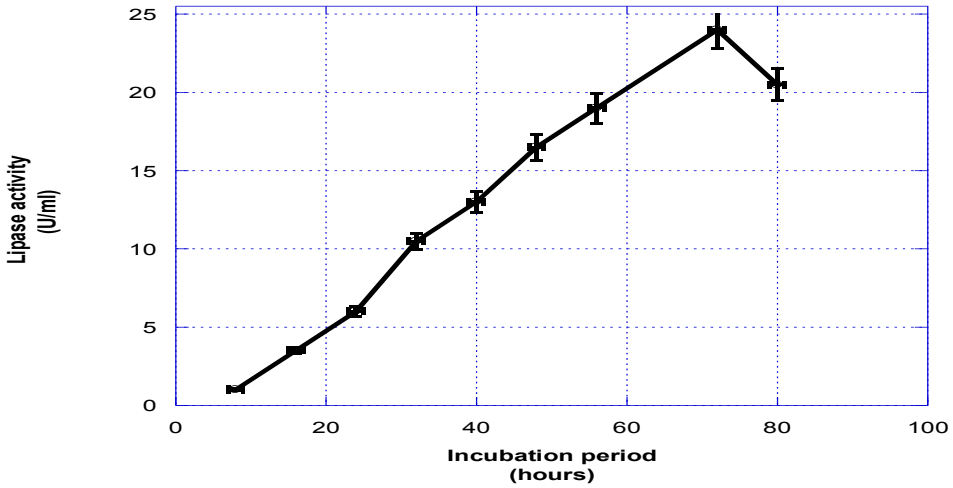


Figure 2. Relation between incubation period and lipase activity by *A. niger*.

2.2. Effect of lipid carbon sources and glucose

Vegetable oils such as soybean, corn, olive, cottonseed, sunflower and palm oils, amongst others, are sited as inducers of lipase production, comprising, at times, the sole source of carbon in the medium [36, 37].

Fats and vegetable oils inducers (Lipidic carbon sources) appear to be generally important for obtaining a high lipase yield. Vegetable oils were used for inducers on lipase production of lipase-producing microorganism. The effect of lipidic carbon sources on lipase production was investigated at 26◦C and 150 rpm for 80hrs. Considering 80 hrs of fermentation time, the maximum lipolytic activity was 24 U/ml in the presence of olive oil, as showed in Table (1). The maximum lipolytic activity was 12 U/ml in the presence of olive oil by *P. aurantiogriseum* [38]. *A. niger* produced lipase in a lipid free medium but required an inducer for improved production [39]. Stoytcheva *et al*., (2010) recorded that the maximum lipolytic activity was 12 U/ml in the presence of olive oil by *P. aurantiogriseum*. *A. niger* produced lipase in a lipid free medium but required an inducer for improved production [40].

Table 1. Relation between different lipidic carbon source and lipase activity by *A. niger.*

Lipidic Carbon sources	Lipase activity (U/ml)
Palm oil	12.0
Corn oil	6.6
Olive oil	**24.0**
Sunflower oil	10.5
Cotton seed oil	17.0
glucose	3.5

2.2.1. Effect of the carbon source concentration:

The effect of the carbon source concentration on lipase production 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 production of lipase by (ADM110), as shown in Table (2).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 production of lipase by (ADM110).

Table 2. Relation between different olive oil concentrations and lipase activity by *A. niger.*

Olive oil concentration (%)	Lipase activity (U/ml)
0.5	**25**
1.0	23
1.5	18
2.0	10

2.3. Effect of nitrogen sources

The effect of nitrogen sources on growth and lipase production was investigated at 26°C and 150 rpm using olive oil as carbon source. In this work, Yeast extract, meet extract and peptone were used as the organic nitrogen source and ammonium sulfate, ammonium chloride and urea was used as inorganic source. The obtained result indicated that yeast extract was effective nitrogen source for both of growth and lipase production by *A. niger.* 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 were obtained when ammonium sulfate, peptone, meet extract, urea and ammonium chloride were used, respectively, as indicated in Table 3. Thanagrit Boonchaidung *et al*., 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[1, 41].

Table 3. Relation between different nitrogen source and lipase activity by *A. niger.*

Nitrogen source (1.0%)	Lipase activity (U/ml)
Meet extract	20.0
peptone	22.0
Ammonium sulphate	24.0
Ammonium chloride	12.5
Yeast extract	**25.0**
Urea	19.5

2.3.1. Effect of the nitrogen source concentration:

To study 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, comparatively high nitrogen concentrations are typically required in order to support the production of lipases over the production of other enzymes [42]. 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 Table (4). Some researchers recorded that the maximum lipolytic activity in a medium that contained yeast extract (0.5%) as the nitrogen source by a Brazilian strain of Pe. Citrinum [43]. Decreases in yeast extract concentration decrease the attainable lipase activity. Replacement of yeast extract with ammonium sulfate diminished lipase production [44].

Table 4. Relation between different concentrations of yeast extract and lipase activity by *A. niger.*

Yeast extract (%)	Lipase activity (U/ml)
1.0	15.0
2.5	22.0
5.0	**26.0**
10.0	21.0

2.4. Effect of the Incubation temperature

Generally, temperature is a critical factor that has to be controlled and it varies from organism to organism. Temperature influences secretion of extra cellular enzymes by changing the physical properties of the cell membrane [45]. As indicated in Fig. 3. The optimum temperature for lipase production is 24 °C and differences as little as 1 °C can considerably decrease the yield**.** Production of lipases by fungi of the genus *Penicillium* are mostly incubated between 25 and 30 °C, most often at 28 °C.

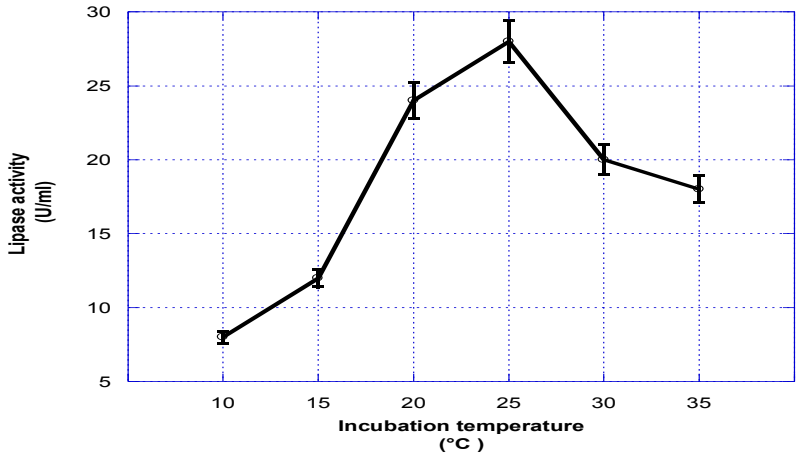


Figure 3. Relation between different Incubation temperature and lipase activity by *A. niger.*

2.5. Effect of the pH values

The pH was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 using 0.1 N NaOH or 0.1 N HCl under the following conditions; 72 hrs incubation period, 25 ^{0}C, 1.0 % olive oil, 5.0% yeast extract, 200 rpm and 1.0 ml inoculum size. The results show that by increasing the pH range, the activity of the tested isolates for lipase production increased till it reaches its maximal value at pH 7.0. As the pH increased over 7.0, lipase production decreased. Many investigators results agreed with this, in their research the optimal pH value for lipase production by microorganisms increased till certain value of pH and then decreased again. The maximal lipase yield by *Penicillium roqueforti* obtained at pH 5.5 [46]. While, *Saccharomyces lipolytica* produce maximal yield of lipase at pH 9.5 [47]. In this study the maximum enzyme activity was found to occur at pH 7, as reported in Fig. 4.

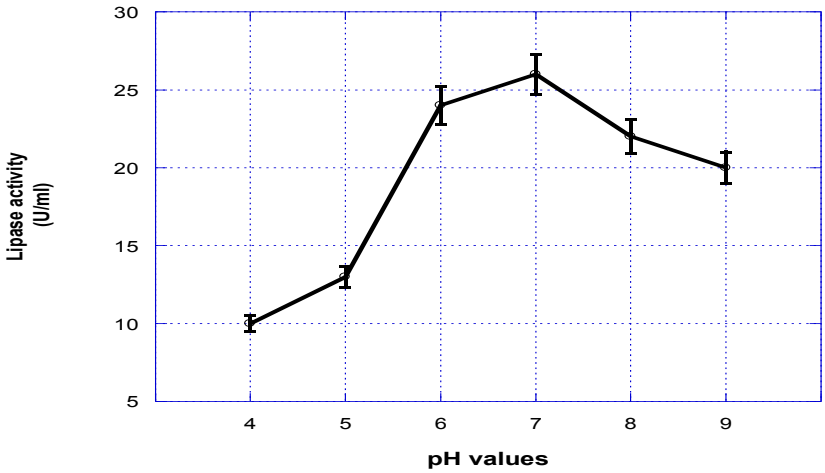


Figure 4. Relation between different pH values and lipase activity by *A. niger.*

2.6. Effect of Agitation speed

Shaking speed has great effect on lipase production by aerobic microorganisms as increasing shaking rate increases the availability of dissolved oxygen. In addition shaking may also make condition of higher availability of the carbon source to microorganisms [48]. However, at higher agitation speed, there was a reduction in growth as well as lipase production [32]. The optimum shaking value to get maximum lipase activity by *A. niger* was 200 rpm under the same conditions mentioned above. The lipase activity obtained was 26 U/ml as represented in Fig. 5. *Rhizopus sp* showed highest lipolytic activity 19 U/ml at 200 rpm [18]. *R. oligosporus required* static incubation for maximum lipase production [49].

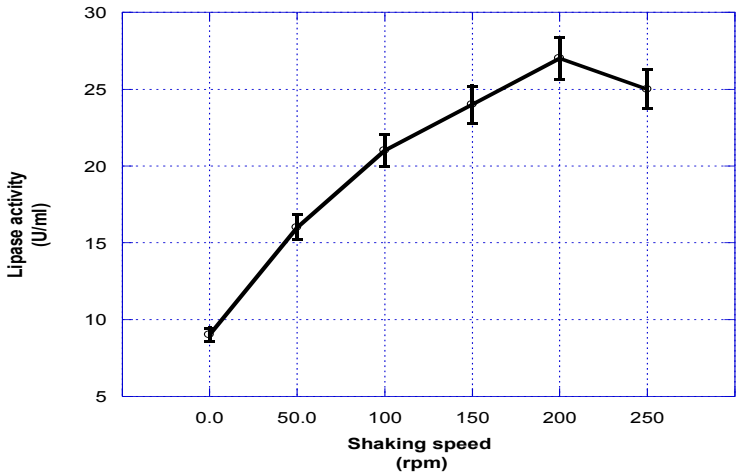


Figure 5. Relation between different shaking speed and lipase activity by *A. niger.*

2.7. Effect of Inoculum size:

Different size of inoculum ranging from 1ml to 6 ml with an interval of 1.0 ml was tested by (ADM110). Maximum extracellular lipase production (25 U/ml) was obtained when 1.0 ml of spore inoculum was used. The relation between spore count and absorbance was listed in Table 5. Lipase activity obtained by (ADM110) was listed in Table 6 and represented graphically in Fig. 6. Further increase in inoculum size leads to a gradual decrease in lipase activity. Ushio *et al*., (1996) also optimized 1.0ml of inoculum for maximum lipase production[50]. Imandi *et al*., (2013) reported a 2ml of inoculums [51, 52].

Table 5. Relation between optical density and cell count.

Absorbance (nm)	Cell count
1.0	4.6×10^7
2.0	8.7×10^7
3.0	3.4×10^8
4.0	7.9×10^8
5.0	2.1×10^9
6.0	5.4×10^9

Table 6. Relation between different Inoculum size (ml) and lipase activity by *A. niger*.

Inoculum size (ml)	Lipase activity (U/ml)
1.0	25.0
2.0	23.0
3.0	19.0
4.0	16.0
5.0	11.0
6.0	8.0

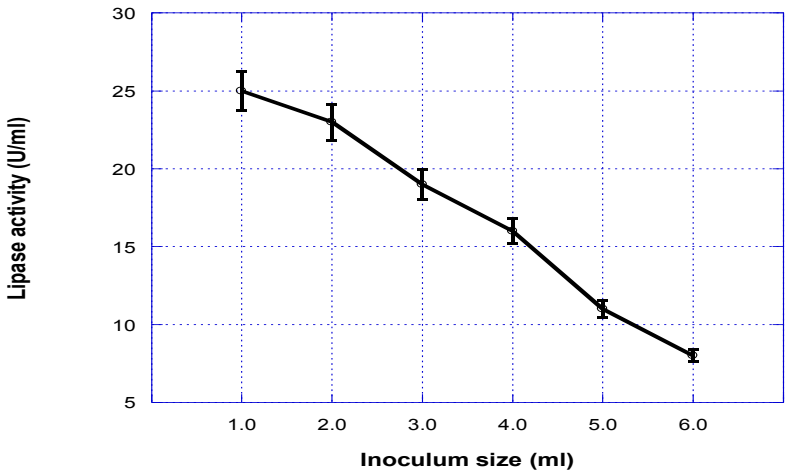


Figure 6. Relation between different Inoculum size (ml) and lipase activity by *A. niger*.

2.8. Effect of Gamma Irradiation on lipase activity

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 represented in Fig.7. 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 [53]. 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[24]. Many researchers reported that R. oligosporus showed maximum extracellular lipases biosynthesis (13.75±1.5 U ml-1) at dosage of 140 k Rad [54, 55].

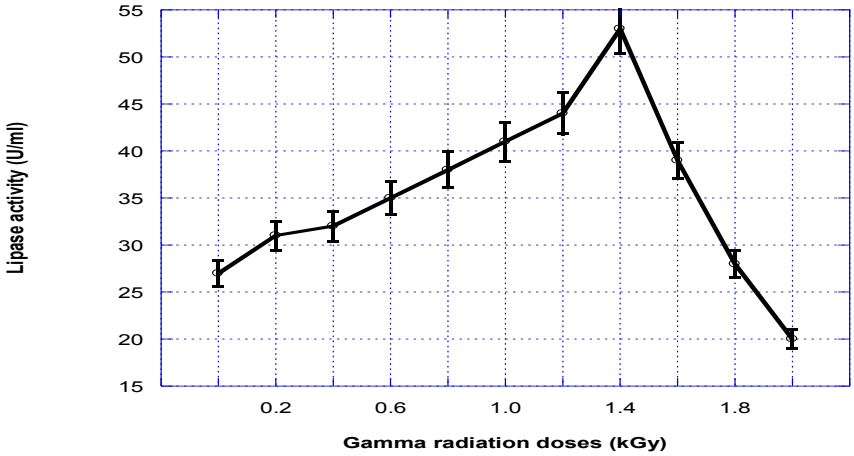


Figure 7. Relation between different γ -radiation doses (Gy) and lipase activity by *A. niger.*

Conclusions

The results of the present study provide useful information for the optimization of culture conditions such as fermentation time, pH, temperature, agitation speed, carbon sources and nitrogen sources to provide the best lipase production by *A. niger.* These results show clearly that lipase producing fungus is widespread in oil contaminated soil. The optimized growth conditions developed in this study can be used for a large scale in industrial purposes. A gradual decrease in the enzyme activity after exposure to the different doses of 1.6 and 1.8 kGy was observed. Some strategies and planning can be applied like the use of agro-industrial residues in order to decrease the enzyme production costs, i.e. sugarcane molasses as inducer or carbon sources for lipase production to estimate the feasibility of lipase application in biodiesel production. Lipase-catalyzed by transesterification process for biodiesel production should be investigated for further study.

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