Journal of Pharmacology &Drug Development

eISSN: 2958-6801

OPEN ACCESS

Extracellular Lipase Production by *Aspergillus niger* Isolated From Industrial Oil Crops Using Submerged Fermentation

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Received 3/Oct/2025, Accepted 23/Nov/2025, Published 1/Dec/2025

ABSTRACT

The screening, production, and optimization of extracellular lipase from a fungus Aspergillus niger isolated from different sources were examined in this work. The isolated fungi were screened on tributyrin hydrolysis method to detect exogenous lipolytic activity. A promising lipase producing isolate of A. niger-6 was selected and identified as the highest lipase-producing strain. The optimal conditions were determined for: inducer oil, carbon source, nitrogen source, initial pH value, incubation temperature, incubation period, inoculum size, and shaking speed for lipase production using one factor at a time approach. Maximum lipase production was observed in 1% olive oil, 2% glucose, 2g/l ammonium sulphate, pH6, 30°C, 5days, 1x106spores/ml-1, and 150 rpm, respectively.

Keywords: Lipase, Extracellular enzyme, Optimization, Submerged Fermentation, Aspergillus niger

INTRODUCTION

Fungi produce various types of enzymes that enable them to decompose complex compounds found in nature. The production of lipases enzymes by microorganisms is low and has no commercial importance [(1)]. The production of such enzymes has increased since 1988 when "Norsk Hydro Company" produced "Lipolase" enzyme, which is the first commercially produced fat-dissolving enzyme in the world and is used for cleaning purposes. This enzyme works at its maximum efficiency at low temperatures (30°C), and a Japanese washing powder was produced called "Hi-Top Lipolase". This enzyme was produced by "Recombinant DNA Technology", this enzyme is the first of its kind produced by this technique in the world, where a gene was obtained from a selected strain of the fungus *Thermomyces lanuginosus* and transferred to the fungus *Aspergillus oryzae*, which is easy to grow compared to the first fungus to produce a large amount of this enzyme [(2)]. Lipases (EC 3.1.1.3) are triacylglycerol acyl hydrolases, a class of hydrolyzed enzymes that catalyze the hydrolysis of triacylglycerol to glycerol, acyl glycerol and free fatty acids. Lipases have long-chain of triacyl-glycerol. The solubility of this group of enzymes in water is very low since the reaction is catalyzed at the fat-water interface. Due to the high stability of these enzymes in extreme temperatures, pH and organic solvents, they are highly efficient in catalytic reactions in aqueous and non-aqueous media [(3)]. The lipase enzyme is found in animals, plants and microorganisms. Interest in lipase of microbial origin has increased more than those derived from other sources. It has become an industrial interest because of its functional capabilities and desirable properties in harsh conditions, as well as its stability in organic solvents and chemical selectivity [(4)]. Lipase enzymes are the most important biocatalysts in the field of biotechnology, because of their ability to carry out chemical and biological transformations, making it of increasing importance with the possibility of its application in many industries such as pharmaceuticals, cosmetics, foodstuffs, leather, textiles, biodiesel production, paper industries, and detergents [(5)].

The lipase enzyme is produced by the fungus *A. niger* through fermentation processes and various carbon sources such as sugars and fats are used to grow the fungus and induce it to produce lipase enzyme.
It was found from previous studies that the use of sucrose has a positive effect in the production of lipase. Different vegetable oils were used as catalysts for lipase synthesis, in addition to being a source of carbon. It was found that ammonium salts as a nitrogen source have a positive effect on growth of fungi and lipase production by *A. niger* [(6)]. Production of various enzymes via submerged fermentation (SmF) has many benefits, the fermentation medium is a homogeneous liquid medium that contains dissolved nutrients, the fermentation process is easy to monitor, the heat is evenly distributed over the fermentation medium, ease of extraction of enzymes produced by microorganisms in the fermentation medium [(7)].

Lipases produced from fungi are of great importance in biotechnology because of the ease of production, the specificity of the substrate, in addition to the unique properties possessed by the enzymes produced, which make them have multiple uses in industrial applications [(8)]. Submerged fermentation has some disadvantages compared to solid state fermentation, in which the electrical energy consumption is higher, equipment expenditures are higher, the process is very sensitive to breakdowns in aeration, subject to more contamination which leads to yield loss and total breakdown of the batches involved [(9)].

Aim of Study

Given the economic importance of lipase enzyme and the scarcity of studies related to its production from fungi. This research sheds light on the possibility of producing lipase from *Angier* and determine the optimal conditions for its production.

Materials and Methods

Isolation of fungi from oil seeds

The infected oil seeds with molds were collected from Baghdad, Mosul, Kirkuk, Anbar, and Kut. All seeds were collected during August and September. All seeds were washed thoroughly in tap water to remove dust. The infected seeds were placed in 1% sodium hypochlorite solution (v/v) for 10 minutes for surface sterilization and then washed three times with sterile distilled water to remove the effect of the sterilizer. The seeds were placed on clean, sterile filter paper until dried. One gram of sterilized seeds were mixed thoroughly in 100 ml of sterile distilled water in a conical flask and shaken thoroughly. From this initial suspension, serial dilutions were prepared. One ml of the dilution 1/1000 was transferred into plates containing potato dextrose agar (PDA) medium with chloramphenicol to prevent bacterial growth. The plates were incubated at 28°C for 7 days to get the desired growth. This approach is modified from [(10)] .

Identification of the Isolated Fungal Strain

Fungal isolates were identified based on morphological characteristics and microscopic characteristics. The morphological characteristics, such, the colonies surface and reverse, color, texture, and edges, were recorded. While microscopic characteristics were examined by adding a drop of lacto-phenol cotton blue stain on a clean glass slide to which fungal structures (mycelium and spores) were added. The stain was softly mixed with fungal structures and a cover slip was placed over the sample. The prepared slide was examined at 40x under compound light microscope. The slide culture technique was also used to identify micro-morphological characteristics.

Ten strains of *A.niger* were identified according to [(11, 12)] for selecting the strain with the highest lipase production, while the remaining isolated fungal species were neglected. The isolated strains of *A.niger* were activated by transferring of spores into basic salt medium agar slants which contain :
3.0g.L^{-1} $NaNO_3$, 1.0g.L^{-1} K_2HPO_4 , 0.5g.L^{-1} $MgSO_4 \cdot 7H_2O$, 0.5 g.L^{-1} KCl , 0.01g.L^{-1} $FeSO_4 \cdot 7H_2O$, 30.0g.L^{-1} at pH 5.5. Olive oil (1%) was added as inducer. Media was autoclaved at 121°C, 15 psi for 15 min. An agar slant was prepared by permitting the agar in a tube to solidify while the tube is in a slanted position for some time until hardening. The slants were inoculated with isolated fungi and incubated at 28°C for 7 days. Finally, the slants were kept in the refrigerator at 4°C until use [(6)] .

Inoculum preparation

The slants of *A.niger* strains, separately, were suspended in sterile saline 0.85% of NaCl containing 0.01% of Tween 80 in order to separate the spores. Spores were separated by filtration through gauze. A spore suspension with concentration $1x10^5$ spores /mL was used as an inoculum. The number of spores per mL in the suspension was count and determined by haemocytometer.

Selection of lipase-producing fungi

Primary screening

A qualitative assay was carried out using tributyrin hydrolysis method (THM) to detect the ability of the isolated fungi to produce lipase enzyme. The decomposition medium consists of : Yeast extract 3gm, peptone 5gm, agar 20gm, tributyrin 10 ml and distilled water 1Liter. The detection medium was prepared by dissolving all materials (except tributyrin) in an amount of distilled water and the mixture was mixed using a magnetic stirrer, then the volume was adjusted to 1 L with distilled water, pH was set at 5.8, then sterilized the medium with autoclave at a temperature of 121°C, pressure of 1bar, for 20 min. Tributyrin was sterilized separately in a special glass vial. The detection medium was cooled and poured into sterile Petri dishes, then sterile Tributyrin was distributed on the plates evenly and the dishes were left to solidify. After incubating the isolated fungi for 7 days, a piece of 5mm diameter is being transported to each plate which contains the detection medium. The dishes were incubated at 28°C for 5 days. The ability of fungi to produce lipase was revealed in terms of the diameter of the transparent halos formed around the fungal colonies due to the decomposition of tributyrin in the medium by the lipase enzyme that was secreted in the detection medium [13].Three replicates were used for each treatment. The ability of each fungus to produce lipase was calculated by the following relationship :

$$\text{Ability of the fungus to produce lipase} = \frac{\text{transparent halo diameter(mm)}}{\text{fungal colony diameter (mm)}}$$

Secondary screening

For this purpose, the medium of submerged fermentation was used, it consist of (g/L): sucrose 20 , KH_2PO_4 1.0 , NH_4NO_3 2.0 , $CuSO_4.7H_2O$ 0.06 , $MgSO_4.7H_2O$ 2.0 . The pH was adjusted to 5.5, and 1% (v/v) olive oil was added. 50 ml of medium was distributed in 250ml Erlenmeyer flasks capacity to provide better aeration. All flasks were autoclaved for 20 min., 121°C, 15psi. The sterilized medium was inoculated with $1x10^5$ spore /mL , then the cultures were incubated in rotary shaker at 120rpm for 5 days at 28°C [14]. This culture medium was used later for the production of lipase enzyme and the optimization process was performed on it.

Dry biomass weight Estimation

In order to determine the fungal biomass, the mycelium was filtered through filter paper Whatman No.1 Pre-weighed. The filtrate was used for enzyme assays. The filtered mycelium was washed with
distilled water and dried in an electric oven at 40°C for 24h., then it was weighed. The fungal biomass was measured by calculating the difference between the two weights using a sensitive balance [15].

Enzyme assays

Titration method was used for the assessment of lipase activity. The reaction mixture consist of 5 mL of olive oil emulsion substrate, 20mL of potassium phosphate solution with concentration of 0.1M and 1mL of crude enzyme extract. The mixture was incubated in a shaker incubator at a temperature of 30°C for 30min. with shaking at 130 rpm. The reaction was stopped by adding 15mL of the ethanol-acetone mixture with a ratio of 1:1. The released fatty acids were titrated against 0.05N of NaOH until the solution turned to pink. Blank assays were conducted adding the enzyme just before titration. One unit (U) of lipase activity was defined as the amount of enzyme that produces 1 µmol of fatty acid per minute under assay conditions [16].

$$\text{Lipase activity} = \frac{\mathbf{\Delta V \times N \times 1000}}{\text{V sample} \times \text{T}}$$

Where; $\Delta V = V2 - V1$, V1 is the volume of NaOH as control, V2 is the volume of NaOH as experimental, N is the normality of NaOH, V is the enzyme volume , and T is the incubation time.

Protein estimation

Protein concentration of crude enzyme was determined by Bradford method using Bovine Serum Albumin standard based on the reaction of the enzyme with 0.5 mL Bradford reagent at 30°C for 2 min. The absorbance read at λ 595 nm. The soluble protein was determined by converting the absorbance value into standard curve [17].

The specific activity

The specific activity of the enzyme extract was calculated as the ratio of lipase activity to protein content.

$$\text{Specific activity} = \frac{\text{Enzyme activity (U/ml)}}{\text{Protein concentration (mg/ml)}}$$

Determination of reducing sugars

The concentrations of total reducing sugars were quantified by the 3,5-dinitrosalicylic acid (DNS) method [18].

Optimization of Parameters for Lipase Production

The submerged fermentation medium parameters were optimized, include inducer oils, carbon sources, nitrogen sources, initial pHs, incubation temperature, incubation periods, inoculum size, and shaking speed. The plan adopted in the medium optimization was to optimize one parameter at a time and then include it at optimum value in the next step of optimization. The lipase activity, total protein content, and specific activity were determined in culture filtrates. Each experiment was done in triplicate.

Determine the optimal inducer oil

Different oils were used for this purpose, including olive oil, corn oil, sunflower oil, sesame oil, coconut oil, and castor oil. Olive oil in the medium of submerged fermentation was replaced with 1% (v/v) of the tested oils, while keeping the remaining parameters constant.

Determine the optimal carbon source

Different sugars were used for this purpose, including fructose, maltose, lactose, glucose, galactose, and starch. Sucrose in the submerged fermentation medium was replaced separately with concentration each at 20.0 g/L of the tested sugars , while keeping the remaining parameters constant.

Determine the optimal nitrogen source

To determine the optimum nitrogen source for lipase production, organic and inorganic nitrogen sources were used. Ammonium nitrate in the medium of submerged fermentation was replaced separately with casein (Cas.), yeast extract (YE), urea (Ur.), tryptone (Try.), peptone (Pep.), sodium nitrate (Sod.Nit.), ammonium sulfate (Amm.Sul.), ammonium chloride (Amm.Chl.), with concentration each at 2.0 g/L , while keeping the remaining parameters constant.

Determine the optimal initial pH value

To determine the optimum initial pH value of SmF medium for lipase production, the medium was adjusted at different initial pH values ranged from 4 to 8 while keeping the remaining parameters constant.

Determine the optimal incubation temperature

Eight different incubation temperatures ranged from 20 to 50°C were used, while keeping the remaining parameters constant.

Determine the optimal incubation period

To determine the optimum incubation period for lipase production, seven different time period ranged from 2 to 8 days were used, while keeping the remaining parameters constant.

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Determine the optimal inoculum size

To determine the optimum inoculum size, different concentration of *A.niger* spores were used, include $1x10^3$ - $1x10^8$ spores/mL.

Determine the optimal shaking speed

The effect of shaking speed on lipase production by *A. niger*-6 was evaluated by using a flask system. The following parameters were unchanged: 1% olive oil , 2% glucose , 2g/L ammonium sulphte , pH6 , incubation at 30°C for 5 day , inoculum size $1x10^6$ spores /ml. 300 ml of liquid media of lipase production were distributed into 250 ml conical flasks and sterilized by autoclaving. Then all the culture media were inoculated. One of the flasks was incubated in the static method, while the rest of the flasks were incubated separately in a shaking incubator at the following speeds: 0, 50, 100, 150, 200, and 250 rpm.

Statistical Analysis

Each experiment was performed in three replicates. Excel program was used to calculate the mean, range and standard deviation of each experimental result**.**

RESULTS AND DISCUSSION

Selection of the strain with the highest production of lipase

The results in Table 1 showed that all the fungal isolates were able to form a transparent halo around the fungal colonies but with different degrees due to the decomposition of the tributyrin compound by the extracellular lipase enzyme which secreted into culture medium and release butyric acid which dissolved in water.

The isolate *A. niger*-6 showed the highest ability to produce lipase enzyme with ability of 1.68 . This isolate showed the largest dry biomass during its growth on a broth culture medium reached to 11.52g/L. In addition, this isolate produced the greatest amount of enzyme with specific activity up to 9.13 U/mg, while the isolate *A. niger* 9 revealed the weakest ability to produce the enzyme with ability of 1.08. This isolate also showed the lowest dry biomass during growth on a broth medium reached to 8.36g/L. In addition, this isolate gave the lowest amount of enzyme with specific activity of 6.07U/ml.

These differences are attributed to the presence of genetic discrepancies between the isolates, the insufficient incubation period to stimulate the fungus to produce the enzyme, the inability of the fungus to exploit compounds in the culture medium, or the inappropriate pH of the culture medium for the growth of the fungi. Although the genotype of any organism is stable, It is affected by changes in the environment, which are reflected in its genetic characteristics [(19)] .

After conducting qualitative and quantitative tests of the enzymes produced by different *A. niger* isolates, the isolate *A.niger*-6 with the highest enzyme production was selected as the best isolate for lipase production in subsequent experiments.

Table 1: Selection of the strain with the highest lipase production

Isolate No.	Isolate source	The fungus ability to lipase production	Fungal biomass (g/L)	Enzyme activity (U/ml)	Protein Concentration (U/mg)	Specific activity (U/mg)
A.niger-1	Corn seeds	1.33	9.87±0.07	6.00	0.72	8.33
A.niger-2	Coconut fruits	1.18	8.91±0.09	5.92	0.70	7.46
A.niger-3	Peanut seeds	1.26	8.98±0.05	5.89	0.75	7.85
A.niger-4	Castor seeds	1.12	8.77±0.04	5.77	0.91	6.34
A.niger-5	Sunflower seeds	1.29	9.69±0.15	6.3	0.78	8.07
A.niger-6	Olives	1.68	11.52±0.09	7.3	0.80	9.13
A.niger-7	Sesame seeds	1.28	9.58±0.11	6.08	0.79	7.69
A niger -8	Cotton seeds	1.21	9.08±0.09	6.01	0.87	6.91
A. niger- 9	Soybean seeds	1.08	8.36±0.04	5.41	0.89	6.07
A.niger- 10	Flax seeds	1.31	9.62±0.06	6.4	0.86	7.44

Optimization of Parameters for Lipase Production

Lipases are typically microbial enzymes with several applications in fermentation and biochemical processes [20]. In this study, the physio-chemical parameters for getting the highest yield of lipase were optimized. These parameters are assumed to be necessary for microbial production of enzymes. The amount of enzyme extracted was determined using a spectrophotometer. The enzyme activity and the amount of protein was determined and then the specific activity of the enzyme produced in each experiment was calculated.

Determine the optimal inducer oil

Data obtained revealed that olive oil and coconut oil were potent inducers for lipase production by *A. niger*-6, in which the specific activity were 9.92U/mg and 7.52U/mg, respectively. On the other hand, castor oil with specific activity 5.44U/mg was the less inducer for enzyme production (Figure1).

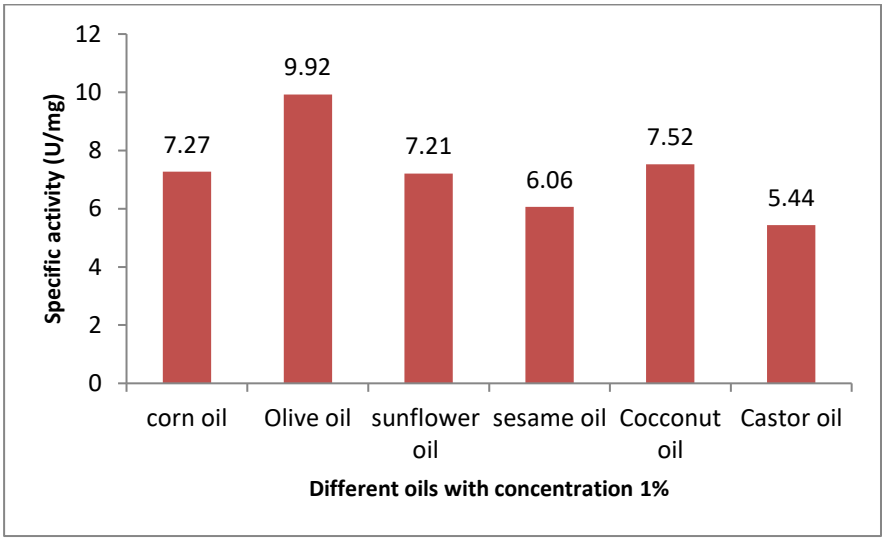

Figure 1: Effect of different inducer oils with concentration 1% for lipase production by *A. niger*-6 , using SmF, initial pH5.5, 1x10^5 spore.mL^{-1}, shaking speed 120rpm, incubation at 28°C for 5 days.

Extracellular lipases of microbial origin are often inducible, and in the presence of stimuli such as tween, glycerol, soapstock, oils, fatty acids, and triacylglycerol the cell produces these enzymes and excreted them into the enclosing environment [21]. Research was founded that amid the natural plants oils, olive oil confirmed to be an potent inducer for lipase production, because of the elevated content of linoleic and oleic acids [22]. The results of this study are consistent with the results of [23] , which founded that olive oil is regarded as the greatest inducer of lipase production by *Rhizopus* sp. ZAC3. Also,[24] was reported that olive oil stimulated the production of extracellular lipase by *Penicillium citrinum*, with 0.1% olive oil significantly increasing the extracellular enzyme activity compared to the basic medium. Furthermore,[25] was founded that olive oil has the best stimulating effect among the oils used to produce the extracellular lipase enzyme from different *Yarrowia* yeast strains, but the stimulating concentration of the oil varies from one strain to another. It is demonstrated that lipase enzyme production is generally increased in the presence of oil components. These oils act as a substrate, carbon sources and inducers for lipase production.

Determine the optimal carbon source

The data obtained indicated that the addition of glucose gave the highest specific activity 10.96 U/mg , followed by fructose with specific activity 9.35U/mg. Sucrose with specific activity 8.84U/mg as carbon source additive was found to be relatively good in stimulation of lipase production. While, starch with specific activity 4.65U/mg was significantly suppressed lipase production.

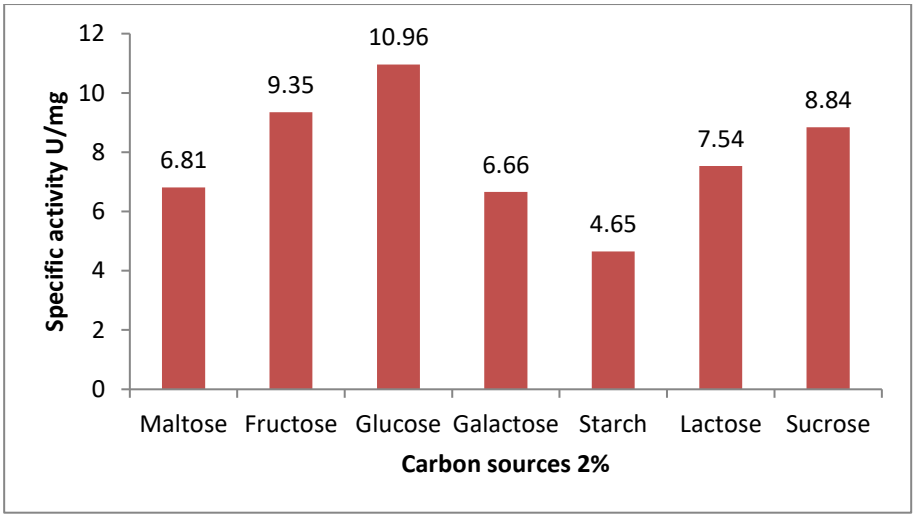

Figure 2: Effect of different carbon sources with concentration 2% on lipase production by *A. niger*-6 using SmF, initial pH5.5, 1% olive oil, 1x10^5 spore.mL^{-1}, shaking speed120rpm, incubation at 28°C, for 5 days.

The results obtained from the Figure 2 showed that the carbon source in the culture medium has highly effect on the lipase synthesis by selected strain *A. niger*-6. The results of this study were consistent with the results of [26]. who found that lipase production by *Antrodia cinnamomea* was increased substantially upon the addition of glucose, when it comprised 8% of the modified ME and YM media. Also, the results are agreement with [14]) who found that maximum lipase production by *A.carbonarius* was obtained when using 2% glucose as carbon source. While the results of this study was a little different with [27], who found that the best carbon source to stimulate the production of lipase by *P.citrinum*, was sucrose.

Determine the optimal nitrogen source

The results showed in Figure 3 that the best inorganic nitrogen source for lipase production by *A.niger*-6, was ammonium sulfate with specific activity 12.74U/mg , while peptone was the best as organic nitrogen source with specific activity 12.36U/mg. Also, it was found that the casein was the least effective nitrogen source for lipase production with specific activity 11.21U/mg.

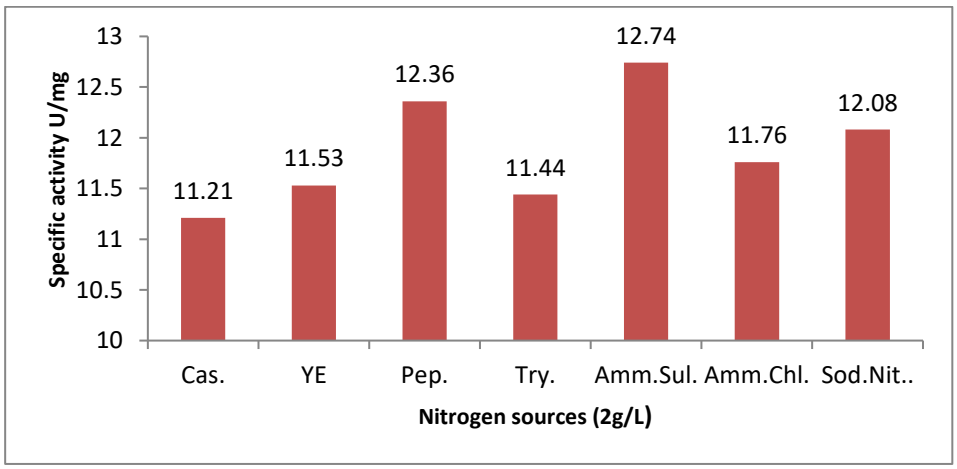

Figure 3: Effect of different nitrogen sources (2g/L) for lipase production by *A. niger* -6 using SmF, initial pH5.5, glucose 2%, 1% olive oil, 1x10^5 spore.mL^{-1}, shaking speed 120rpm, at 28°C, for 5 days

Nitrogen is an important biological element, such as the bases of the amino acids that build up proteins and enzymes. Organic and inorganic nitrogen sources play an important role in the biosynthesis of lipase by microorganisms [28]. Inorganic nitrogen sources can be used immediately, while organic nitrogen sources can provide cells with amino acids and growth factors needed for enzymes synthesis and cell metabolism [29]. This fact is consistent with the results in this study, which

indicated that the best nitrogen source was ammonium sulfate, which is an inorganic source of nitrogen for lipase production by *A.niger*. The results of [30] revealed that ammonium sulfate as nitrogen source is the best option for lipase production by *A.niger* when utilization of palm oil mill effluent. The findings of [31] was proved that ammonium sulfate was stimulated of lipase production in several strains of *Penicillium* fungi [32] indicated that ammonium sulfate improved lipase production by *Penicillium camemberti* AM83. The best yield up to 13 U/mL of lipase production by *Penicillium aurantiogriseum* after incubation for 72 h, was achieved with the medium provide with ammonium sulfate [33]. While both [34] was noted increased the lipase activity that produced by the wild strain of *Penicillium citrinum* KU613360 when peptone was added to the culture medium as nitrogen source. Also, highest yield of lipase production by two strains of *A.niger* named LPF-5 and HN1, was achieved in the medium containing peptone (1%) as nitrogen source [35].

Determination the optimum initial pH value

The effect of initial pH (4-8) on lipase production by *A. niger*-6 was investigated. Data in Figure 4 showed that the increased enzyme production by *A.niger*-6 was observed in initial pH 6 with specific activity up to 14.06 U/mg, followed by initial pH 6.5 (13.35 U/mg). While minimum yield of the lipase was achieved in SmF medium with initial pH4 (7.78 U/mg).

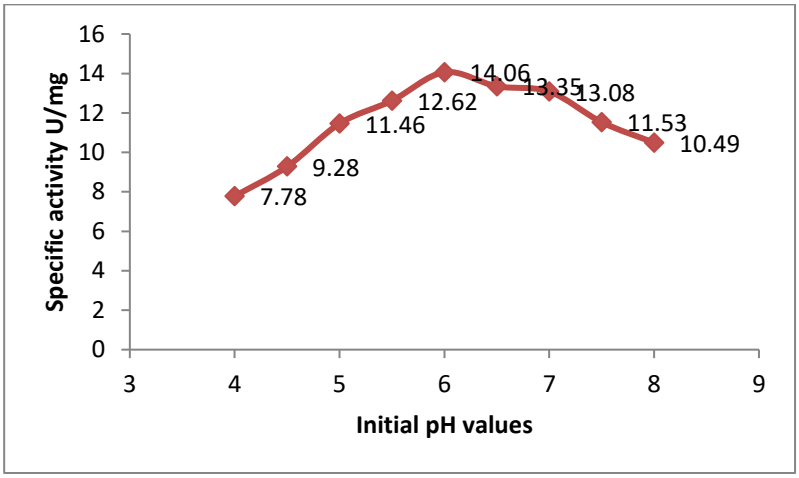

Figure 4: Effect of different initial pH for lipase production by *A. niger* -6 using SmF, 1% olive oil, 1x10^5, glucose 2%, spore/mL, shaking speed 120rpm, at 28°C, for 5 days.

The results of the current study are consistent with [36] which was found that the highest yield of lipase enzyme that produce by *A.oryzae* and *A.flavus* was obtained when initial pH was 6. While, [37] was found the highest yield of lipase production by *A. oryzae* when initial pH was 5.5.

The initial pH can influence microbial growth and ultimately the product formation due to its effect on the solubility of nutrients, ionization of the substrate, and its availability to the microorganisms. Filamentous fungi are able to flourish over a wide Initial pH range in submerged fermentation conditions [38].

The aforementioned outcome can be explained by the enzyme's ionization state, which changes with pH and may have an impact on the enzymatic reaction's pace. Ionic interactions determine the enzyme's and its active site's conformation and/or flexibility. As a result, the side groups ionization state varies depending on the pH, which could disrupt these forces and change or denature the protein structure [39, 6] . Because of this, every enzyme has an ideal pH at which it functions at its best, a pH at which it reaches high levels of enzyme activity.

Determination of the optimum incubation temperature

Extracellular lipase production was obtained at various temperatures ranged from 20 to 50ºC. The results showed that the optimum temperature for lipase production by *A.niger-6* was found to be 30°C with specific activity up to 14.85 U/mg. However, the decrease or increase in the incubation temperature lead to a decrease enzyme activity as it was illustrated in Figure 5.

One of the most significant physical and environmental elements influencing the synthesis of enzymes is temperature. The production of microbial lipase is also significantly impacted by temperature, which can alter the physical characteristics of cell membranes and affect the extracellular enzyme's secretion. When using the shake-flask approach, an ideal temperature is essential for the production of enzymes [40, 41]. investigated the impact of varying incubation temperatures ranging from 25 to 55°C, on *A. niger* lipase enzyme synthesis, at 30°C, the maximum lipase production was recorded. [42] examined the effect of different incubation temperatures 10, 20, 30, and 45°C) on lipase production by *A. terreus*. They

found at 45 °C, the highest lipase activity was achieved, and as the temperature decreased, the enzyme activity was decreased. While, [43] found that the best temperature for producing lipase enzyme by *A.niger* was 40°C using submerged fermentation.

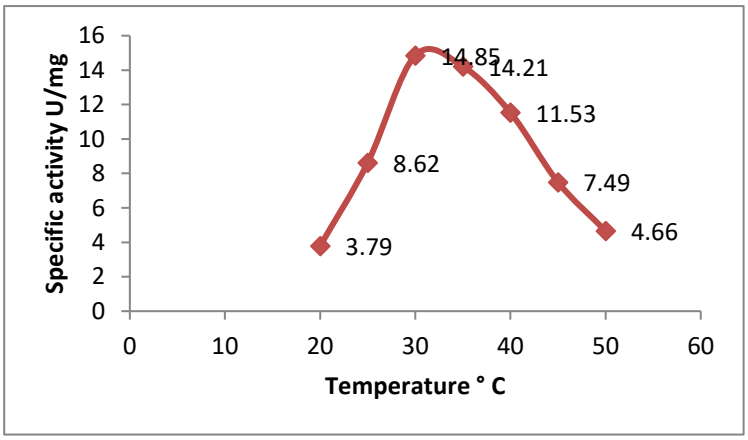

Figure 5: Effect of different temperature for lipase production by *A. niger* -6 using SmF, initial pH6, 1% olive oil, 10^5 spore.mL^{-1}, shaking speed 120 rpm, for 5 days.

Determination of the optimum incubation period

Lipase production by *A.niger*-6 was observed from 2 to 8 days. The results in Figure 6 revealed that maximum specific activity was achieved after 5 days incubation period up to (14.18 U/mg), then, on the sixth day, the enzyme activity begins to gradually decrease.

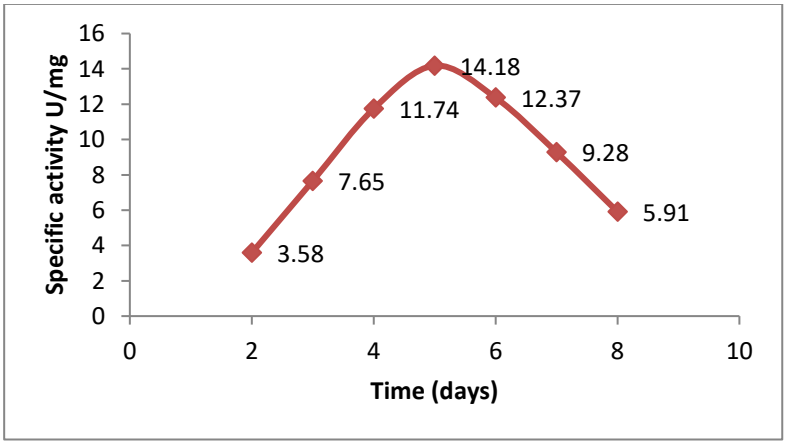

Figure 6: Effect of different incubation period for lipase production by *A. niger* -6 using SmF, initial pH6, 1% olive oil, glucose 2%, 10^5 spore.mL^{-1}, shaking speed 120 rpm, at 30°C.

The obtained results found that the rate of enzyme production significantly increased with the increase in the incubation period and reached its maximum activity in day 5 which were specific activity up to 14.18U/mg. Lipase activity was significantly inhibited by incubation times both above and below the ideal incubation times. The release of proteolytic enzymes, which are known to denaturize lipase, may be the source of the decrease in enzyme activity since day 6 of fermentation. [44] Mention that this may also be ascribed to decrease in nutrient availability in the medium at the end of the cultivation process or catabolic inhibition of enzyme.

The results of this study are consistent with [44] who found that *A. oryzae*, *A. flavus*, and *A. fumigatus*, were given highest lipase activity on day 5. In a study conducted by [45] who found that highest lipase production was achieved on day 5 of incubation period for strains of Aspergilli. While [43] found that the optimum incubation period of *A.niger* for lipase production was day 3 at a temperature of 40°C. In a study conducted by [14] to determine the optimum conditions for the production of crude lipase from *A. carbonarius* IMI 366159, who found that highest lipase production (1.3U/ml) was achieved in day 4 at 30ºC. In a study conducted by [46] who found that maximum lipase production by *A. niger*, occurred at the day 4 of incubation which is differ to our result. In another report, [47] prove that maximum lipse production by *A. niger*

occurred on the day 6 of fermentation, this notice differs with our present results. Another contrast that was noted was acquired by [48] who prove that highest lipase production by *Aspergillus* sp occurred at day 7.

Determination of the optimum inoculum size

As illustrated in Figure 7, when the inoculum size of *A.niger* spores was $1x10^6$ spores /ml, the lipase enzyme's highest specific activity up to 16.48U/mg, and any change in the inoculum size led to a decrease in the enzyme activity.

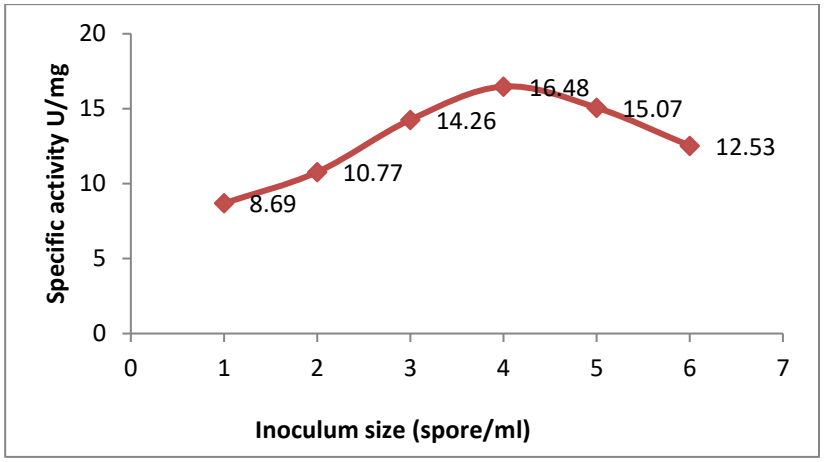

Figure 7: Effect of different inoculum size on lipase production by *A. niger* -6 using SmF, initial pH 6, 1% olive oil, glucose2%, shaking speed 120 rpm, incubation at 30°C for 5 day.

Production of lipase and lipase activity was affected by microorganism, cultivation conditions, substrate [49] , carbon and nitrogen sources [50]. Increasing of inoculum size tends to reduce lipase activity and protein content. There was an optimum condition of inoculum size to generate a lot of lipase activity. In addition to increasing spore biomass, the higher inoculum size also reduces enzyme synthesis and limits the amount of nutrients in the substrate [51]. The results of our study were in agreement with [52] who found that inoculum size $1x10^6$ spoes/ml of *A.niger* that grown on coconut pulp waste, was gave the highest specific activity of the lipase enzyme.

The results of our study differed from those of [43] who found that the best inoculum size of *A.niger* spores for lipase production was $2.5x10^7$ spores/ml by using SmF method. In a study conducted by [53] for production of lipase from *A.niger* NRRL-599 using response surface, they found that specific activity of lipase was 181.8U/mg, and increased with increasing the inoculum size till it attained a maximum at 4% v/v. Increasing the inoculum size than 4% v/v decreases the production of enzyme occurred. This may be due to the fact that there is an increase in cell mass conformation and a decrease in nutrients. In another study conducted by [54] who indicated that the lipase activity gradually increased with increasing the inoculum size up to 8% v/v that gave the maximum activity. Increasing the inoculum size than 8% v/v decreases the lipase production. This may be due to enhancement in cell mass formation and exhaustion of nutrients.

Determine the optimal shaking speed

The results in Figure 8 show the effect of shaking speed on lipase production. The highest specific activity (17.65U/mg) of lipase was obtained at a shaking speed of 150rpm, but any increase or decrease in the shaking speed results in a decrease in the specific activity of the enzyme.

In static culture, it was observed a good growth of the fungus as a mycelium on the surface of the culture medium with many sporangium on the surface of the mycelium. With increasing shaking speed, the mycelium slowly transformed into a circular granule. Thus, it was found that the shaking speed affected the morphological characteristics of the mycelium of *A.niger*-6.

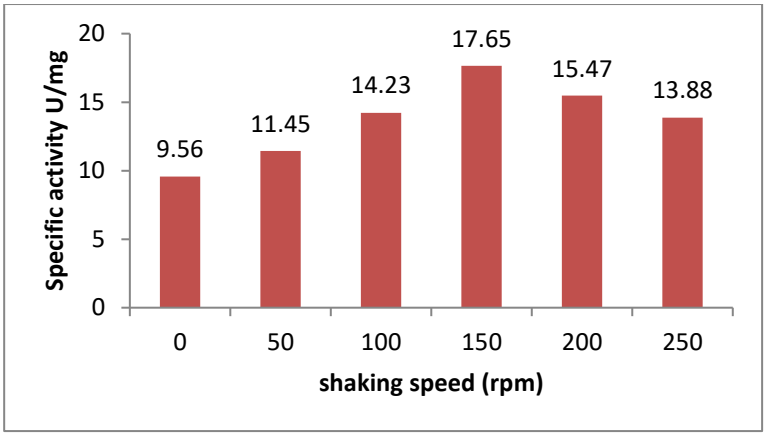

Figure 8: Effect of different shaking speed on lipase production by *A. niger* -6 using SmF, 1% olive oil , 2% glucose , 2g/L ammonium sulphte , initial pH6 , incubation at 30°C for 5 day , inoculum size 1x10^6 spore ml^{-1}

Shaking is a critical parameter in improving the production of enzymes as shaking increases the concentration of dissolved oxygen [55] In addition, shaking increases the accessibility of nutrients for quicker cell division, this enhances nutrient dispersion throughout the entire medium, hence increasing lipase synthesis [56]**.** In this study, 150 rpm produced high specific activity (17.65U/mg). This result was predictable since *A.niger* is an aerobic fungus, therefore it will grow quickly in a situation with high dissolved oxygen [57].

Shaking speed was found to affect lipase production in a flask system. In a study conducted by who found that higher shaking speeds than the optimum speed (150 rpm) decreased pectinase production which due to crash between the suspended fungal cells in the growth media as well as shear stresses. It was observed that the increase in enzyme activity is directly proportional to the increase in shaking speed, up to 150 rpm, after which the enzyme activity begins to decrease as the shaking speed increases. This is completely consistent with our found in this study, the highest specific activity was obtained at a shaking speed of 150rpm. After that, when the shaking speed was increased, the enzyme activity decreased.

Different microorganisms have different oxygen needs. For oxidative reactions, oxygen works as a terminal electron acceptor, providing energy for cellular processes. The amount of mixing in the shake flask system and the accessibility of nutrients were both impacted by the shaking speed [58].

As well as increasing the amount of dissolved oxygen in the culture media, shaking offers sufficient mass, mixing, and heat transition. While higher shaking speeds lead to decrease enzymes production, lower shaking speed usually impacts microbial growth because of scanty oxygen in the culture medium [59]. Greater shaking speeds cause shear pressures to form between the suspended microbial cells in the culture medium, which lowers production because of cell collision-induced cell damage. Also, shear stresses can affect the fungal cell in a number of ways. It can alter fungal growth and yield production, beside create morphological changes by damaging the fungal cell structures both inside and outside [60]. Thus, the optimal shaking speed is needed to be determined so as to gain greatest enzyme production.

CONCLUSION

This study identified *A. niger* as a producer of extracellular lipase and under optimal conditions of the process parameters, a significant increase in lipase yield was achieved. The correlation analysis conducted on all the experiment results revealed the interdependence of the physicochemical parameters and lipase production in the fungus, which provides essential basic information to achieve the large-scale production of lipase by *A. niger*. The ability and optimal conditions to produce extracellular lipase by *A. niger* were determined. The various nutritional and environmental parameters were optimized.

ACKNOWLEDGMENT

This work was funded by the Department of Pharmacognosy, College of Pharmacy, Al-Nahrain University, Baghdad, Iraq.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

FUNDING

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript

ETHICS STATEMENTS

The study didn't need ethical approval from an ethics committee .

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أنتاج انزيم اللاييبيز الخارج خلوي بواسطة العفن*Aspergillus niger* المعزول من المحاصيل الصناعية الزيتية بأستخدام تقنية التخمر المغمور

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الخلاصة

في هذا البحث تم غربلة عشرة سلالات من الفطرAspergillus niger المعزول من مصادر مختلفة لانتاج انزيم اللاييبيز خارج خلوي وتم تحسين عملية الانتاج من خلال استخدام ظروف مختلفة. الفطريات المعزولة تم فحصها لانتاج اللاييبيز بطريقة التحلل المائي للتريبيوترين لمعرفة قدرة ونشاط هذه الفطريات على تحليل الدهون. من خلال النتائج تم اختيار العزلة A.niger-6كأقوى عزلة منتجة للانزيم . تم اختيار افضل زيت محفز على انتاج الانزيم ، أفضل مصدر كاربون ، أفضل مصدر نيتروجين ، أفضل اس هايدروجيني ، درجة حرارة مثلى للحضن ، أفضل فترة للحضن ، أفضل حجم للقاح ، وافضل سرعة الهز، وكانت النتائج على التوالي : 1% زيت الزيتون ، 2% كلوكوز ، 2غم /لتركبريتات الامونيوم ، pH6، 30 درجة مؤية ، 5 أيام حضن ، 106×1 سبور /مل ، 150دورة بالدقيقة.

الكلمات المفتاحية: الليباز، الإنزيم خارج الخلية، التحسين، التخمير المغمور، أسبيرجيلوس نيجر