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Optimization of Lipase Production from Egyptian *Aspergillus niger* Isolates

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Abstract

Microbial lipase plays a crucial role in industrial biotechnology by contributing to a wide range of industrial products worldwide. Seeking potential lipase producers and enhancing their productivity represent a current urge. This study focuses on optimizing the culture conditions required for the extracellular production of lipase by *Aspergillus niger* isolates through submerged fermentation. *Aspergillus niger* was isolated from different herbal seeds and oily sources. Seven fungal isolates could be morphologically identified by the Assiut University Mycological Center (AUMC) and were cultivated on Czapek's Dox Medium. Various pH values, temperatures, oil concentrations, and metal ion (Mn^{2+}, Zn^{2+}, Cu^{2+} and Mg^{2+}) concentrations were investigated to assess their impact on isolates growth, total extracellular protein concentration, and lipase activity. For the isolate AUMC 16212, optimum lipase production was reached at (pH 5 / 37°C / 3% corn oil / 10 mM Mn^{2+} / 10 mM Cu^{2+} / 2 mM Zn^{2+} / 22 mM Mg^{2+}). While that from AUMC 16213 isolate was (pH 5 / 37°C / 3% corn oil/ 8 mM Mn^{2+} / 10 mM Cu^{2+} / 2 mM Zn^{2+} / 22 mM Mg^{2+}) The culture conditions of two isolates of *Aspergillus niger* (AUMC 16212 and 16213) could be optimized for potential lipase production which is of ascending industrial and economical value.

Keywords: Lipase; *Aspergillus niger*; submerged fermentation; enzyme activity; industrial biotechnology

1. Introduction

Lipases are classified as triacylglycerol acyl hydrolases (EC 3.1.1.3) within the serine hydrolase class. They play a vital role in the hydrolysis of fats and oils to produce glycerol and free fatty acids [1]. Notably, they are able to catalyze a wide array of chemical reactions in both aqueous and non-aqueous media and can effectively hydrolyze ester bonds of insoluble substrates. These unique versatility positions microbial lipases as a practical and manageable source of lipase compared to those derived from plants or animals[2-3].

The use of microbial lipases contributes to environmental sustainability, as these enzymes can efficiently biodegrade lipid waste under mild conditions, a significant improvement over traditional lipid degradation processes [4]. Additionally, microbial lipases do not require factors, exhibit a broad substrate range, and function under diverse environmental conditions, including variations in temperature, pressure, and pH, while achieving high conversion rates [5-6].

Fungal lipases are sourced from various fungal species and yeasts [7], with *Aspergillus spp.,* Alternaria spp., Penicillium spp., and Fusarium spp. were notable contributors. Among these, *Aspergillus* strains, particularly *A. niger*, stand out as prolific lipase producers, secreting large quantities of lipase extracellularly compared to other microorganisms [8-9]. Furthermore, *A. niger* is recognized as "generally recognized as safe" by the Food and Drug Administration (FDA) [10].

Fungal lipases occupy a crucial role in industrial biotechnology, finding applications in diverse sectors such as, detergents [11], pharmaceuticals [12], food processing [13], and biodiesel manufacturing [12,14]. The global lipase market is expected to surpass USD 797.7 million by 2025 [15].

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Consequently, there is a continuous demand to identify new lipase producers and develop bioprocesses that enhance productivity, constituting the principal challenges faced by the lipase industry [16].

This study is dedicated to investigating the optimal conditions required for extracellular lipase production from Egyptian *A. niger* isolates using submerged fermentation.

2. Materials and Methods

2.1. Fungal isolation, *Aspergillus niger* identification, and cultivation

Seven fungal isolates were obtained from various origins: five were isolated from different herbal seeds, and two were isolated from oily sources, i.e., bone marrow and corn oil. They were all tested for their lipolytic activity, and the highest two isolates were identified morphologically as *A. niger* strains by the Assiut University Mycological Center (AUMC), and given the following Identification No: AUMC 16212 & AUMC 16213).

The isolates were cultivated on Czapek's Dox agar medium (g/l) [16]: sucrose 30, sodium nitrate 2, potassium dihydrogen phosphate 1, magnesium sulfate 0.5, potassium chloride 0.5, ferrous sulfate 0.01, and agar-agar 15. The plates were incubated for 7 days at 28 °C. Lipase activity was assessed for all isolates **(section 2.4.)**, and the two isolates demonstrating the highest lipolytic activity were selected for further investigations. Pure cultures of these fungi were maintained on Czapek's Dox Agar slants and stored at 4 °C.

2.2. Lipase production by submerged fermentation

Czapek's Dox medium **(section 2.1.)** was prepared. Two disks were cut and inoculated in separate triplicate flasks, each containing 100 ml of Czapek's Dox medium in 250 ml conical flasks. These flasks were then placed in a shaking incubator set at 150 rpm and maintained for 5 days at 28 °C. Following the incubation period, fungal mycelia were separated from the medium using Whatman No.1 filter paper, and the resulting filtrate was subjected to centrifugation at 12,000 rpm for 10 min.

In the experiment involving the addition of corn oil to the medium, a separating funnel was employed after filtration. The clear filtrate in the lower phase was isolated for subsequent investigations.

In both scenarios, an equal volume of ice-cold acetone was added to the filtrate, left for 1 h at -20 °C, and then centrifuged in a cooling centrifuge for 10 min at 12,000 rpm at -4 °C.

The resulting pellet was resuspended in 500 µl of 0.1 M phosphate buffer pH 8, and the sample was preserved at -20 °C for further analysis.

2.3. Optimization of lipase production

The following conditions were investigated for the two isolates:

2.3.1. Effect of pH

The isolates were tested at three different pH levels: pH 5, pH 7, and pH 9. All flasks contain 100 ml Czapek's Dox medium. The adjustments to pH were achieved using NaOH and HCl addition.

2.3.2. Effect of temperature

The fungi were cultivated at three distinct temperatures: 28 °C, 37 °C, and 50 °C. All flasks contain 100 ml medium. The pH of the Czapek's Dox medium was adjusted according to pH which gave the highest lipase enzyme activity from the previous experiment (pH 5)..

2.3.3. Effect of oil concentration in the medium

Various volumes of sterile corn oil were individually added to each flask, including 1 ml, 2 ml, 3 ml and 4 ml. Corn oil was the sole carbon source added to medium (i.e. glucose was not added to Czapek's Dox medium). In each flask, volumes of medium were omitted corresponding to volumes of oil added to keep the total volume 100 ml. All flasks were incubated at pH 5 and 37 °C (pH and temperature that gave the highest lipase enzyme activity from the previous experiments).

2.3.4. Effect of metal ions concentration

Four metal salts ($MnSO_4 \cdot H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$ and $MgSO_4 \cdot 7H_2O$) were selected. Various concentrations were prepared for Mn^{2+} (8 mM, 10 mM, and 12 mM), Cu^{2+} (8 mM, 10 mM, and 12 mM), Zn^{2+} (2 mM, 4 mM and 6 mM) and Mg^{2+} (18 mM, 20 mM, and 22 mM). All flasks were incubated at pH 5 and 37 °C (pH and temperature that gave the highest lipase enzyme activity from the previous experiments).

2.4. Estimation of lipase activity

A commercially available kit, Spectrum, Egypt, (Catalog No.: 281 001), was employed. Briefly, in a 96-well microplate, 30 µl of the crude lipase extract was combined with 100 µl of R1 and incubated for 10 min at 37 °C. Subsequently, 25 µl of R2 was introduced, followed by another 10 min incubation at 37 °C. Absorbance$_1$ (A_1) was recorded, and the reaction was further incubated for 20 min at 37 °C to obtain Absorbance $_2$ (A_2). The final product was measured at 580 nm using BiotekPowerWave HT 340 microplate reader.

2.5. Estimation of total protein concentration

A commercially available kit, Spectrum, Egypt, (Catalog No.: 211 001), was employed. In a 96-well microplate, 10 µl of the crude lipase extract was added to 200 µl R and incubated for 5 min at room temperature. Absorbance was measured at 623 nm using BiotekPowerWave HT 340 microplate reader.

2.6. Estimation of dry weight

The fungal biomass was filtered using Whatman No.1 filter paper and was placed in oven at 50 ^oC overnight and the dry weight was estimated.

3. Results and Discussion

3.1. Taxonomical identification of the fungal isolates

the Assiut University Mycological Center (AUMC) confirmed that the two isolates were *Aspergillus niger* van Tieghem and were given the AUMC Identification No: 16212 and 16213.

3.2. Lipase enzyme activity

The kit employed relies on a colorimetric reaction using a synthetic substrate known as 1,2-o-Dilauryl-rac-glycero-3-glutaric acid-(6-methylresorufin)-ester (DGMRE). When this substrate is cleaved by lipase, it generates the colored product, Methylresorufin. The increasing absorbance of the red Methylresorufin is measured photometrically at 580 nm. The kit sensitivity is 3 U/l and the reagent shows linearity up to 300 U/l.

$$y = 0.005\ x$$

where, y is the absorbance at 580 nm, x is the enzyme activity represented in U/l, and the standard deviation was R^2 = 0.9887.

A standard curve was established using known lipase concentrations against $A_{\lambda=580}$. The isolate (AUMC 16212) displayed the highest enzyme activity at 154.511 U/l, while that of the AUMC 16213 reached 100.6 U/l.

3.3. Optimization of lipase concentration from *A. niger* isolates

Measurement of total protein is based on its binding to the indicator dye bromocresol green (BCG) in pH 4.1 to form a blue-green colored complex. The intensity of the blue-green color is directly proportional to the concentration of proteins in the sample. It is determined by monitoring the increase in absorbance at 623 nm [17].

The following equation was used to measure protein concentration.

$$y = 0.0303\ x$$

where, y is the absorbance at 623 nm, x is the protein concentration represented in mg/ml, and the standard deviation was R^2 = 0.9732.

The kit sensitivity is 10 mg/ml and the reagent shows linearity up to 70 mg/ml.

3.3.1. Effect of pH

Both isolates indicated no direct correlation between lipase enzyme activity and protein concentration. As shown in Fig. (1), pH 5 was the best pH for enzyme activity for isolate AUMC 16212 (28.311 U/l) and isolate AUMC 16213 (28.356 U/l). While our results indicated that the best pH for total protein concentration was pH 9 for both isolates (14.85 mg/ml and 15.18 mg/ml, respectively). The highest dry weight for AUMC 16212 was at pH 7 (0.891 g) and for AUMC 16213 was at pH 5 (1.07 g).

Our results are consistent with [18] who worked on *A. niger* C to produce lipase by submerged fermentation, they found that the activity of lipase is optimum in a more acidic pH (5.0-6.0) medium. Another study stated that the optimum pH for lipase activity from *A. niger* and *A. oryzae*was pH 6 and pH 6.25 respectively [19]. Others found that a pH of 7.5 was the optimum pH for lipase production from *A. niger*when using 1% fructose as the carbon source, 1% yeast extract as the nitrogen source. Moreover, a study on A. flavus demonstrated that pH 7.15 resulted in the highest lipolytic activity [20]. However, other microbial lipase from *Bacillus licheniformis* favors alkaline conditions (pH 8) for optimum lipase activity [21].

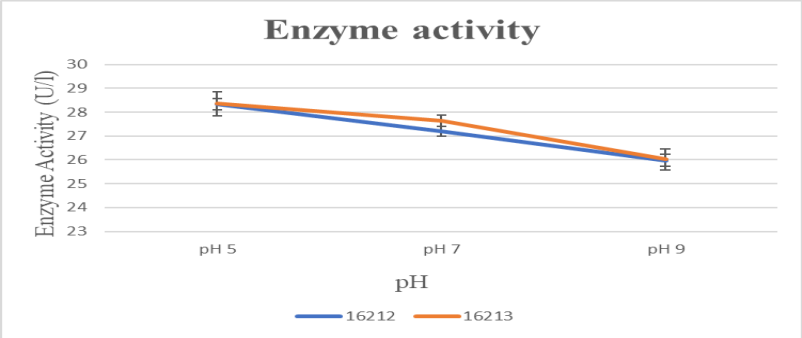

Figure 1: Effect of pH on lipase activity

3.3.2. Effect of temperature

Both isolates AUMC 16212 and AUMC 16213 exhibited their highest enzyme activity at 37 ºC (26.756 U/l and 30.689 U/l, respectively) as shown in Fig. (2). While the highest total protein concentration was at 28 ºC (15.578 mg/ml and 14.477 mg/ml, respetively). The highest dry weight for AUMC 16212 was at 37 ºC (1.119 g) and for AUMC 16213 was at 28 ºC (1.206 g).

Some studies support our results and report that constitutive lipase activity (i.e. not induced by adding oil to medium) from *A. niger* MTCC 2594 [22], *A. carneus*[23] and *A. costaricaensis*[15] showed maximum activity at 37°C. Other than *Aspergillus* species, *Bacillus subtilis* lipase showed similar results [24].

Moreover, lipase enzyme produced from *A. niger* under different incubation conditions or from another lipase producing organisms showed maximal activity at lower and higher temperatures than 37°C. A study reported that the highest lipase production occurred at 40 ºC[25]. Other strains of *A. niger* recorded higher optimum values for lipolytic activity (55°C) [18]. On the other hand, other strains recorded optimum values of lipase activity lower than 37ºC.*A. niger* F7-02 showed the maximum lipase activity at an incubation temperature of 30 ºC[26].

However, at 45 °C most *A. nigerspp.* experienced growth inhibition, leading to no enzyme production due to cellular protein damage [1], except for some isolates including *A. niger* MH078571 and *A. niger*MH079049, which displayed a heat resistance and continued to produce lipase at higher temperature [27][28].

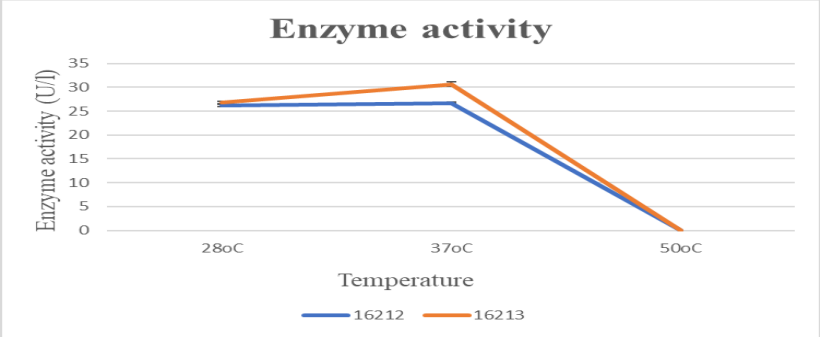

Figure 2: Effect of temperature on lipase activity

3.3.3. Effect of different corn oil concentrations in medium

Both isolates AUMC 16212 and AUMC 16213 exhibited their highest enzyme activity at 3 % corn oil (41.467 U/l and 32.022 U/l, respectively) as shown in Fig. (4). Our results indicated that the highest total protein concentration for isolate AUMC 16212 was reached at 2 % corn oil (25.41 mg/ml). However, in the other isolate, different corn oil concentrations had a neutral effect on total protein concentration.

A study suggested that *A. niger* grown on medium containing oil as the sole carbon source achieved higher lipolytic activity than that grown on carbohydrate-supported media [29]

Our study results are near to a study conducted by Alabdalall*et al*. (2020) [25] who added different oils to *A. niger*in medium andfound that adding 2.5% corn oil induced optimal lipase production while other oils as olive oil, sunflower oil, castor oil and palm oil showed optimal lipase production when adding only 2% to medium and the productivity decreased when their concentrations increased to 2.5%.

To our knowledge, there is not much studies that mention adding corn oil as the sole carbon source to induce the production of *A. niger* lipase. However, a study conducted on *A. niger* Lipase showed that the highest enzyme activity was achieved when combining corn oil and peptone with the concentrations 2% and 1% respectively [30]. Another study carried on *A. terreus* has optimized lipase production when cultured in medium containing 2 % corn oil [31].

Others reported that adding 10 g/l (0.92 %) olive or soybean oil to medium exhibits maximal *A. niger* lipase enzyme activity [32] and the addition of 3% soybean oil to medium exhibits maximal *A. oryzae*lipase enzyme activity[33].

Thus, oil is considered the main inducer of the production of fungal lipase in medium. Moreover, it was observed that the amount of unsaturated fatty acids in medium is directly proportional to enzyme activity more than the amount of saturated fatty acids added to medium [7].

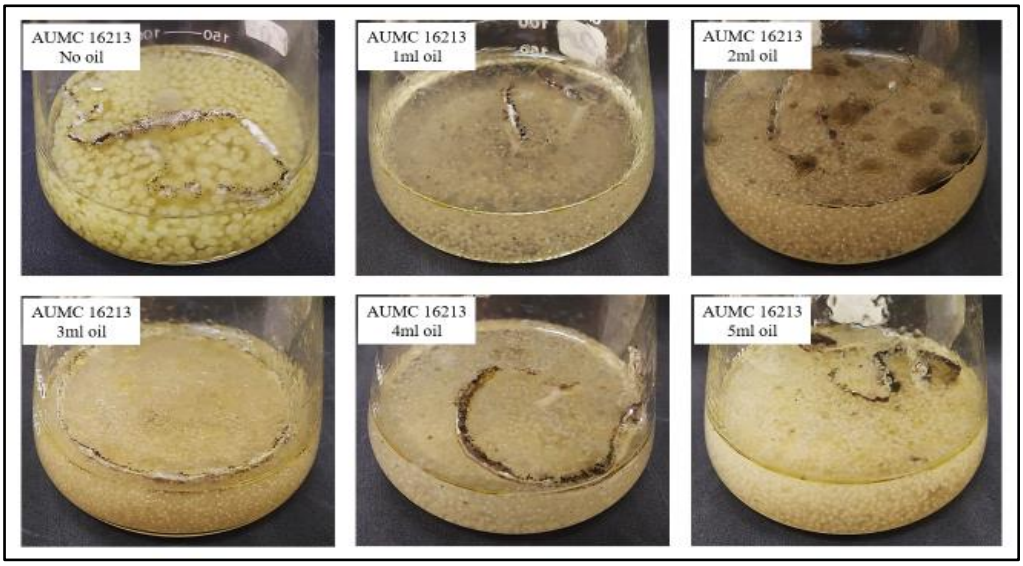

Figure 3: Effect of different concentrations of corn oil on lipase production

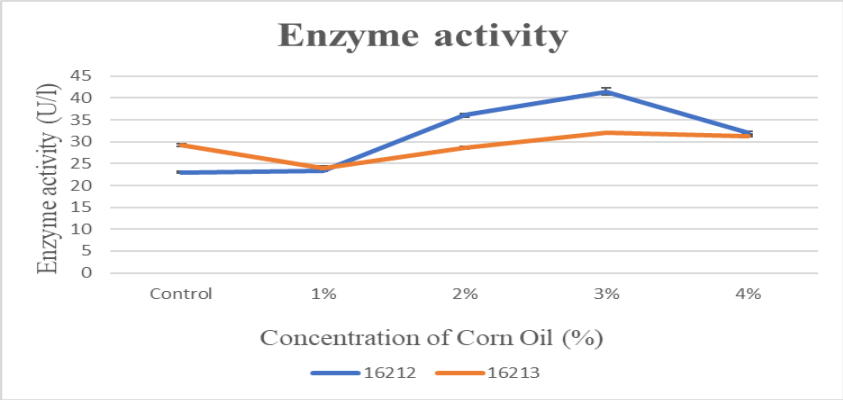

Figure 4: Effect of different corn oil concentrations on lipase activity

3.3.4. Effect of metal ions concentration

3.3.4.1. Manganous ions

Isolate AUMC 16212 exhibited its highest lipase activity at 10 mM Mn^{2+} (i.e.,154.511 U/l), and the other isolate at 8 mM Mn^{2+} (100.6 U/l), as shown in Fig. (6). While different manganous ion concentrations decreased total protein concentration of AUMC 16212 and had a neutral effect on the total protein concentration of the other isolate. The heaviest dry weight was recorded at 12 mM Mn^{2+} (0.897 g) for isolate AUMC 16212 while increasing manganous ion concentration caused a decrease in the dry weight of the other isolate.

Our results suggest that Mn^{2+} is a good inducer of lipase activity which is compatible with the findings of [34] who stated that Mn^{2+} has a positive effect on the activity of the Wild (LPF-5) and Mutant (HN1) Strains of *A. niger*. Another study worked on *A. niger* MH078571.1 found that combining 0.1% of manganese sulfate and zinc sulfate together in medium achieved the highest lipase activity (102%) [2].

Besides, Mn^{2+} enhanced the activity of lipases from *Halobacillus* sp. AP-MSU 8 [35] and *B. licheniformis*[36]. And gave maximum production of lipase by *Rhodotorula* sp. Y-23 when implementing $MnCl_2$ in medium for 96 h of incubation [37].

On the other hand, Shu *et al*. (2007) [38] observed that 2 mM Mn^{2+} ions inhibited lipase enzyme secretion from *A. niger*. And in terms of lipase production, Research has consistently shown that heavy metal ions can lead to growth inhibition and cell death in fungal species. Various *Aspergillusspp.* and *Sterigmatomyceshalophillus* have been observed to be inhibited by heavy metals such as Cu, Cd, Mn, Pb, and Zn [39], [40].

However, other studies mentioned that Mn^{2+} did not affect at all the enzyme activity of *Aspergillus japonicus* lipase nor *Penicillium roqueforti* IAM7268 lipase [41], [42].

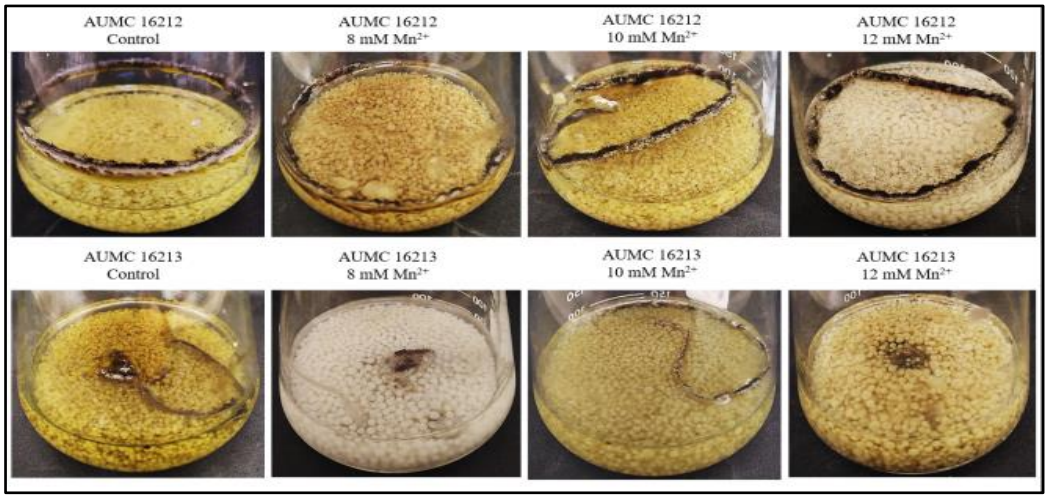

Figure 5: Effect of different concentrations of Mn^{2+} on lipase production

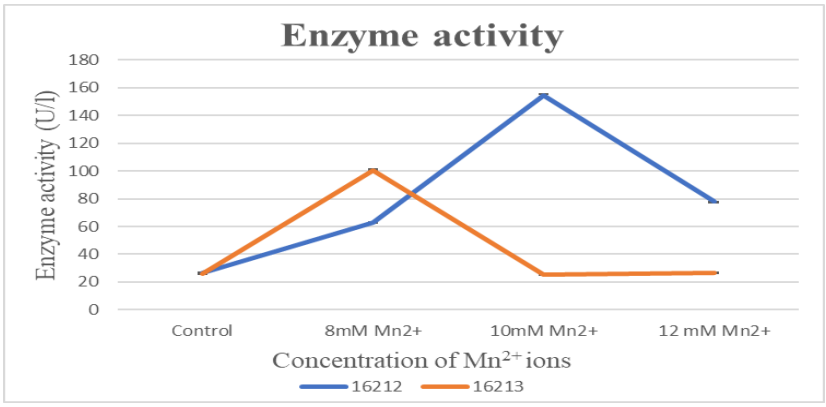

Figure 6: Effect of different concentrations of Mn^{2+} ions on lipase activity

3.3.4.2. Cuprous ions

Both isolates, AUMC 16212 and AUMC 16213exhibited the highest lipase activity at 10 mM Cu^{2+} (76.689 U/l & 99.311 respectively) as shown in Fig. (8). While our results recorded a decrease in total protein concentration and dry weight for both isolates when adding different concentrations of cuprous ions in medium.

Our findings are consistent with the studies on lipase enzyme produced by *A. niger* MH079049.1 [2], wild (LPF-5) and mutant (HN1) strains of *A. niger*[34] and on *Candida rugosa*[43] which found that Cu^{2+} was a good inducer of the enzyme activity.

However, Shu *et al*. (2007) [38] observed that 2 mM Cu^{2+} ions had a neutral effect on lipase activity produced by *A. niger*F044 while Yadav *et al.* (1998) [31] found that Cu^{2+} served as inhibitor for lipase enzyme secreted by *A. terreus*.

Regarding fungal growth, Akhtar *et al*. (2013) [44] reported that the tolerance limit of *A. niger* (SF-5) to Cu^{2+} was 1716 mg/l (27mM) and complete inhibition occurred at 1780 mg/l (28mM). Thippeswamy*et al*. (2012) [45] mentioned a tolerance limit of 500 mg/l (7.9 mM) for Cu^{2+}.

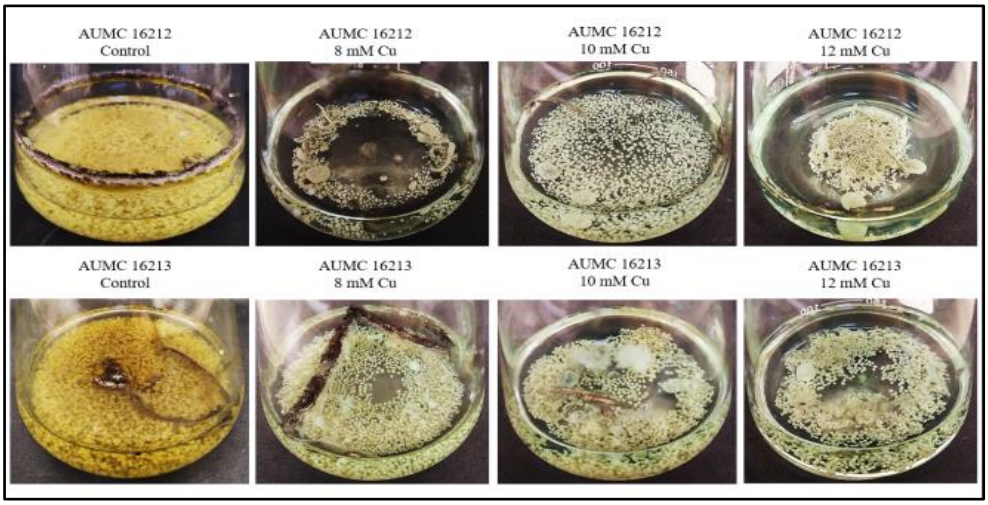

Figure 7: Effect of different concentrations of Cu^{2+} ions on lipase production

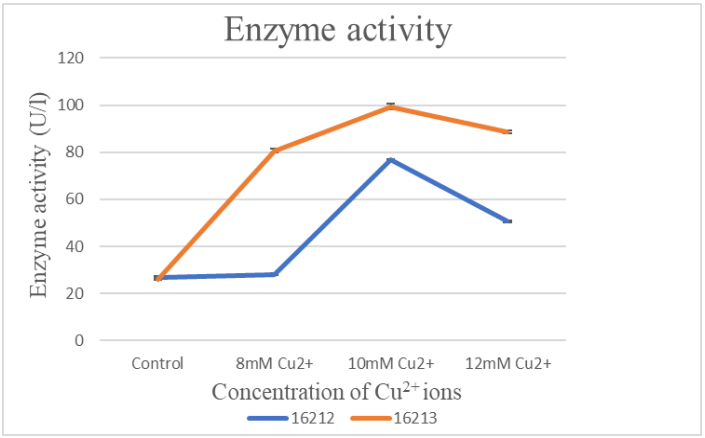

Figure 8: Effect of different concentrations of Cu^{2+} ions on lipase activity

3.3.4.3. Zinc ions

Both isolates AUMC 16212 and AUMC 16213 exhibited their highest enzyme activity at 2 mM Zn^{2+} (50.533 U/l and 53.8 U/l, respectively), as shown in Fig. (10). The highest total protein concentration was recorded for AUMC 16212 at 2 mM Zn^{2+} (20.066 mg/ml) and for AUMC 16213 at 4 mM Zn^{2+} (16.799 mg/ml). While the heaviest dry weight for AUMC 16212 was at 4 mM Zn^{2+} (0.913 g). On the other hand, adding different concentrations of zinc ions to medium showed negative effect on dry weight for AUMC 16213.

According to Alabdalall*et al.* (2021) [2] Zn^{2+} and Mg^{2+} ions are found to enhance the lipase activity of *A. niger* MH078571.1. In terms of lipase production, a study on *A. carbonarius* (Bainer) IMI 366159 found that zinc sulphate (0.05%) was the best trace element added to medium and achieved highest lipase production, followed by magnesium sulphate and manganese sulphate [46].

On the other hand, a study observed that 2 mM Zn^{2+} ions inhibited lipase enzyme secretion from *A. niger*[38] and another reported that *A. niger* can tolerate up to 250 mg/l (3.8mM) of Zn^{2+} in medium [45] while most research have consistently shown that heavy metal ions can lead to growth inhibition and cell death in fungal species. Various *Aspergillusspp.* and *Sterigmatomyceshalophillus* have been observed to be inhibited by heavy metals such as Cu, Cd, Mn, Pb, and Zn [39], [40]. However, it is worth noting that one of the factors affecting halophilic lipase stability to metal ions is the lipase-producing organism. For example, lipase activity of *Halobacillus* sp. AP-MSU 8 was completely suppressed when adding 5 mM Zn^{2+} to medium [35] but it had a neutral effect on *C. japonicus* [47].

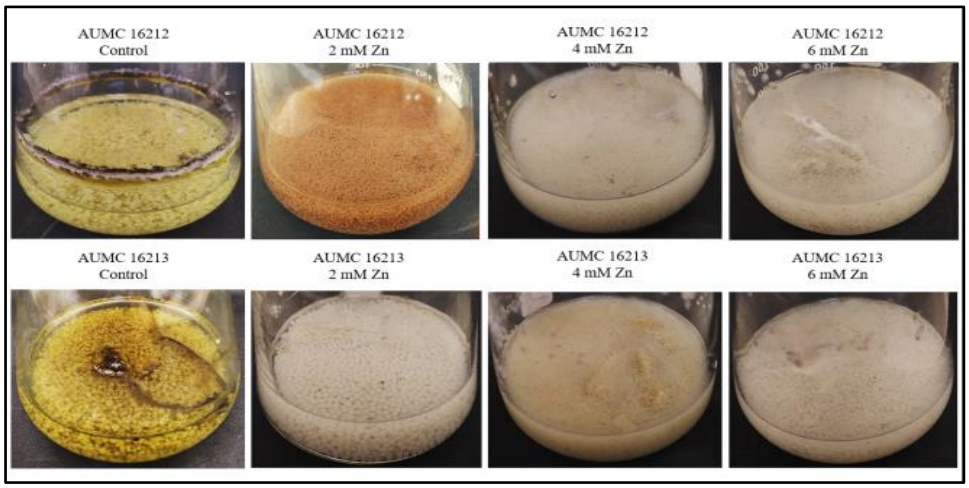

Figure 9: Effect of different concentrations of Zn^{2+} ions on lipase production

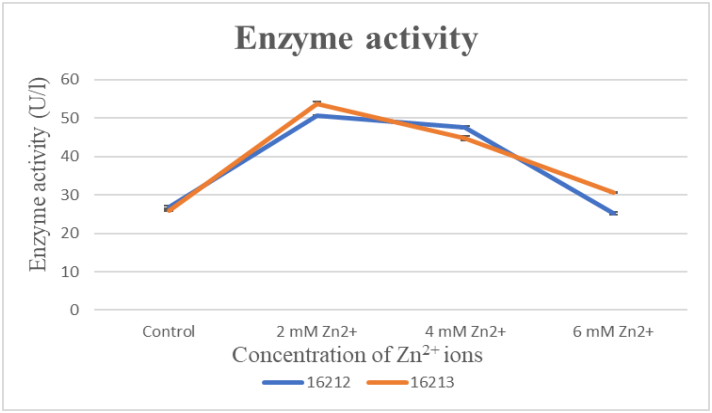

Figure 10: Effect of different concentrations of Zn^{2+} ions on lipase activity

3.3.4.4. Magnesium ions

Both isolates AUMC 16212 and AUMC 16213 exhibited their highest enzyme activity at 22 mM Mg^{2+} (27.667 U/l and 34.933 U/l, respectively), as shown in Fig. (12). The highest total protein concentration recorded for the first isolate was at 20 mM Mg^{2+} (19.626 mg/ml) and the other isolate at 22 mM Mg^{2} (18.68 mg/ml). Adding high concentrations of magnesium ions to medium had a negative effect on the dry weight of the first isolate and a neutral effect on the dry weight of the other.

Most studies on lipase producing organisms such as, the wild (LPF-5) and mutant (HN1) strains of *A. niger*[34], *A. niger* F044 [38], *A. terreus*[31], A. japonicus [41], *B. subtilis* [24], *Bacillus* sp. VITL8 [48], *Chromohalobacter canadensis*[49]*Haloarcula* sp. G41 [50] have found that Mg^{2+} ions served as activators for lipase enzyme activity and production. However, a study that worked on *Penicillium roqueforti* IAM7268 [42] showed that Mg^{2+} has a neutral effect on lipase activity.

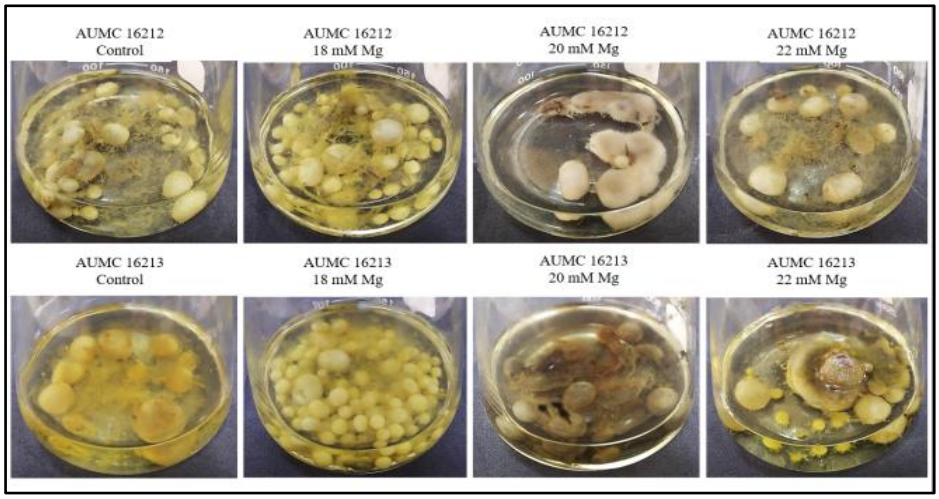

Figure 11: Effect of different concentrations of Mg^{2+} ions on lipase production

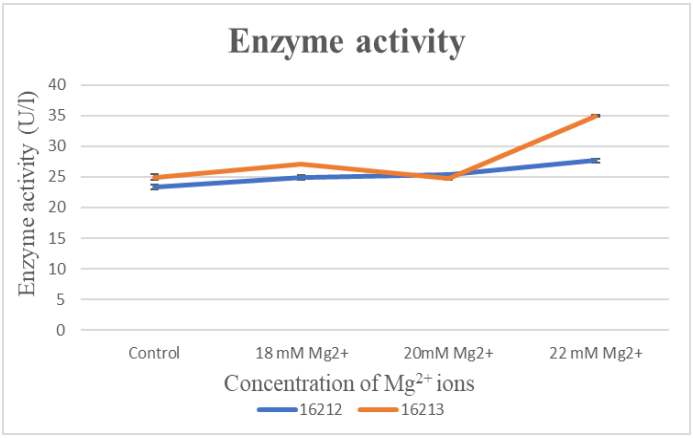

Figure 12: Effect of different concentrations of Mg^{2+} ions on lipase activity

In general, metal ions play an important role in enzymology, therefore, the effect of different metal ions with different concentrations on the enzyme production and activity was estimated in this study. some metal ions improve lipase activity and acts as a cofactor that facilitates the binding of the substrate to the enzyme's active site, holding them together.

They bind the structural domains of lipase to the substrate to form fatty acids in the interface between oil and water. However, other metal ions have inhibitory effect on the enzyme activity due to protein denaturation, changes in the behavior and solubility of the ion ionized fatty acids at water-oil interface or change in the catalytic properties of the enzyme [24], [51].

4. Conclusion

The production of lipase enzyme by *A. niger* is of considerable industrial and economic significance. This study serves to optimize the conditions for production of lipase enzyme from two Egyptian *A. niger*isolates. The most favorable conditions for both isolates were (pH 5 / 37°C / 3% corn oil / 10 mM Cu^{2+}/ 2 mM Zn^{2+} / 22 mM Mg^{2+}), besides 10 mM Mn^{2+} for AUMC16212 and 8 mM Mn^{2+} for AUMC 16213.

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