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# Submerged Production, Partial Purification and Characterization of Extracellular Chitinase from Local Endophytic Fungus for *Culex pipiens* Biocontrol: Strategy for Protein Stabilization

**Eman W. Elgammal[1], Eman F. Ahmed[1], Omnia M.H.M Kamel[2], Heba Yehia[1*]**

[1] *Chemistry of Natural and Microbial Products Department, Pharmaceutical and Drug Industries Research Institute, National Research Centre, Dokki, Giza 12622, Egypt*
[2] *Applied Organic Chemistry Department, National Research Centre, Dokki, Giza 12622, Egypt*

**Abstract**

The major barrier which restricts the enzymatic industrial application is their insufficient stability during processing. In this study, a native strain *Aspergillus niger* M-8, identified and deposited in GenBank under accession number MW940924, showed high chitinase production via submerged fermentation. The optimized operational conditions increased the enzyme production about 13 fold (up to 572.0 U/mg) using medium containing 0.1% untreated chitin, 0.1% starch and 0.3% tryptone at 35°C and pH 5. To increase the enzyme purification efficiency and stability, two co-solvent protein stabilizers (tryptone and yeast extract) were applied in the production medium and fractionation buffers. The results revealed that medium constituents, stabilizer type and pH greatly affected the yield and stability. Tryptone-optimized medium potentiated the enzyme specific activity (8885.0 U/mg), purification fold (16.3) and recovery (10.2%) more than the same medium supplemented with yeast extract (0.4%), under the same fractionation conditions. Furthermore, the fractionation buffer (acetate buffer, pH 5) containing 2 mM tryptone as stabilizer resulted in double the recovery (18.8%) in comparison to the yeast-containing buffer. The purified chitinase fraction using 40% (v/v) ethanol ($F_{40}$) reached its optimum catalytic activity at a temperature of 30°C and pH 5. Yet, it was also stable and retained most of its initial activity at a wide range of temperature (25-70°C) and pH values (4-7). Interestingly, the more stabilized $F_{40}$ chitinase had more virulence effect against mosquito larvae ($LC_{95}$= 222.9 ppm) after 48 h, suggesting that it can be applied for biological control of *Culex pipiens*.

**Keywords:** extracellular chitinase, enzyme production, enzyme purification, enzyme stability, *Aspergillus niger*, insecticides, *Culex pipiens*

## 1. Introduction

Industrial processes are usually carried out at quasi harsh conditions (temperature, pH, pressure, organic solvents, etc). Conventionally, this hurdled the employment of functional enzymes to avoid the protein denaturation via quaternary structure changes and hence, the loss of bioactivity. Thus, different strategies were investigated and applied over the years to enhance the enzymes' stability and retain the active structure and function [1]. For example, addition of polyols, inorganic salts and sugars in the production medium is regarded as an efficient method to protect enzymes against aggregation in the presence of organic solvents [1, 2].

Chitinase or poly-beta-glucosaminidase enzymes (E.C. 3.2.1.14) are ubiquitous and were reported from several sources. They are classified into endo- and exo-chitinases according to how they digest their substrate; insoluble polymer of N-acetylglucosamine linked by β-1,4-glycosidic bonds [3]. Endo-chitinases cleave internal linkages of chitin into diacetyl chitobiose and soluble low molecular mass β-1,4-N-acetylglucosamine polymers e.g.: chitotriose, and chitotetraose. Whereas, exo-chitinases generate diacetylchitobiose from the non-reducing terminal of chitin microfibril and N-acetyl-D-glucosamine monomers [4, 5]. Chitin substrate has three structural isoforms (α, β and γ); the α-form is the most stable and dominant and is considered the primary component of fungal cell wall, crustacean shells and insects. The β-form occurs only in marine organisms while the γ-form remains under study [6, 7].

*Corresponding author e-mail: hebayehya@hotmail.com (Heba Yehia)
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Most filamentous fungi are able to utilize chitin and were described to produce up to 20 different chitinolytic enzyme variants depending on mechanistic and structural properties [3]. This is especially evident among endophytic fungi which grow intercellularly among plant tissue without causing disease however, producing effective secondary metabolites stimulating the plant resistance against pathogenesis and pest infection [8]. For example *Aspergillus*, *Penicillium* and *Trichoderma* spp. produce chitinase, protease, glucanase and other hydrolyzing enzymes for biological control [9]. In addition to the aforementioned pest control value, chitinases have uses in different industrial, clinical and pharmaceutical purposes including biocontrol of pathogenic fungi and insects, isolation of protoplasts, single-cell protein and waste treatment [3, 5].

The role of chitinase as a pesticide in the biocontrol of mosquitoes is well reported since it is an extensively studied problem with serious public health hazards and high mortality rate [3, 7, 10–12]. In Egypt, *Culex pipiens* Linnaeus (Diptera: Culicidae) has been documented as the major filarial vector [10] in addition to many other diseases such as Rift Valley fever virus [11], Japanese encephalitis [12], and West Nile virus [12]. The application of chemical pesticides and insecticides to combat mosquito-borne arboviruses and diseases has resulted in many hazardous problems related to human and environmental safety. Also, the development of insecticide resistance among the vectors has been reported [13, 14]. Alternatively, bio-pesticides are appealing to control insect vectors on account of their larvicidal, adulticidal and repellent properties without endangering the environment and livestock [13, 15].

To produce chitinase for pesticide use, the principles of production cost-effectiveness apply ie: maximizing the enzymatic yield while reducing the production cost. Thus, the aim of this study was to optimize the operational production conditions (e.g.: medium composition, pH, temperature) of chitinase from the endophytic fungus *Aspergillus niger* M-8 and explore its potential application as a biocontrol agent against mosquito larvae. Moreover, the application of co-solvent strategy during production and fractionation was investigated to prolong the enzyme stability and improve the recovery.

## 2. Experimental

All chemicals were purchased from Sigma Aldrich and used as received, unless otherwise stated. The solvents were of HPLC grade and purchased from the same supplier.

### *2.1. Microorganisms, growth conditions and inoculum preparation*

Some endophytic fungi previously isolated from medicinal plants collected from El-Orman garden, Cairo, Egypt, were screened for chitinase production using medium consisting of yeast extract, 4; tryptone, 2; $MgSO_4.7H_2O$, 4; $KH_2PO_4$, 1.2; $K_2HPO_4$, 2.8; powder chitin, 2 g/L: adapted from [16, 17]. The enzyme production was detected after 6 days of incubation at 28°C, pH 6 and 200 rpm.

The fungi were cultured on potato dextrose agar (PDA) slants and incubated at 28ºC for 7 days and kept at 4°C until use. A spore suspension (equivalent to $10^5$ CFU/mL) was prepared from the 7 days old cultures, 2 mL thereof were inoculated into 50 mL of the designed production medium. The cultures were incubated in a shaker incubator adjusted at 200 rpm at 28ºC for 6 days. After cultivation, the total extracellular protein and chitinase activity were measured in cell free supernatant, adapted from [17].

### *2.2. Identification of the most active fungus isolate by sequencing the internal transcribed spacer of ribosomal DNA method*

#### *PCR amplification and Sequencing*

The fungus candidate was genetically identified as *Aspergillus niger* M-8, under accession number of MW940924, according to Sigma Scientific Services Company via the following method: DNA extraction was made by Quick-DNA™ Fungal/Bacterial Microprep Kit (Zymo research #D6007). Then PCR product clean-up was done using Gene JET™ PCR Purification Kit (Thermo K0701). Finally, the sequencing was made to the PCR product on GATC Company with ABI 3730xl DNA sequencer using PCR of ITS region - Fragments of the ITS1-5.8S-ITS2 which were amplified by the use of the primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS2 (GCTGCGTTCTTCATCGATGC) as mentioned by [19]. Comparisons with sequences in the GenBank database were achieved in BlastN searches at the National Center for Biotechnology Information (NCBI) site (https://www.ncbi.nlm.nih.gov/ ).

### *2.3. Colloidal chitin preparation*

10 g of chitin were dissolved in 60 mL concentrated HCl and the resulting solution was added to 400 mL deionized water with rapid stirring. The formed precipitate of colloidal chitin was collected by centrifugation and washed several times by distilled water, neutralized by 1 M NaOH then re-centrifuged and further washed with distilled water. The resulting pellet was kept at 4°C for further applications [16].

### *2.4. Chitinase assay*

The exo-chitinase N-acetylhexosaminidase activity was determined as follows [20]: p-Nitrophenyl-β-N-acetylglucosaminide (1 mg/mL in

0.05 M acetate buffer, pH 5) was equally mixed with enzyme filtrate and incubated in shaking water bath for 1 h at 30°C. The reaction was stopped by adding 2.5 mL of 0.125 M sodium borate buffer (pH 10). The amount of the released p-nitrophenol was measured spectroscopically at 410 nm. One unit of the enzyme activity was defined as the amount of enzyme that releases 1 µmol of p-nitrophenol per min under the specified assay conditions.

### *2.5. Estimation of protein*

Protein concentration was determined according to the Lowry method [21] using bovine serum albumin (BSA) as standard.

### *2.6. Optimization of fermentation conditions using one-variable-at-a-time technique*

#### *2.6.1. Effect of different media*

Four different media were screened for the enzyme production by *Aspergillus niger* M-8. The tested media were (g/L):

M1: $NaNO_3$, 3; $KH_2PO_4$, 1; sucrose, 10; $MgSO_4$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.0001: adapted from [18].

M2: $KH_2PO_4$, 0.5; $K_2HPO_4$, 0.5; $MgSO_4$, 0.3; yeast extract, 1.5: adapted from [18].

M3: Yeast extract, 4; tryptone,2; $MgSO_4.7H_2O$, 4; $KH_2PO_4$, 1.2; $K_2HPO_4$, 2.8: adapted from [16, 17].

M4: Sucrose, 10; $NaNO_3$, 3; $K_2HPO_4$, 1; $MgSO_4$, 0.5; KCl, 0.5 and $FeSO_4$, 0.01: adapted from [22].

Pure powder chitin (2 g/L) was added to all the previous media as an inducer and pH was adjusted at 6 then autoclaved. The sterilized media were inoculated by the spore suspension and the cultures were incubated at 28ºC in a shaking incubator adjusted at 200 rpm. The enzyme activity was determined after 6 days of incubation.

#### *2.6.2. Effect of different chitin inducer substrates*

To reach the maximum production of chitinase, different inducer substrates (colloidal chitin, pure powder chitin, and untreated chitin) were added in a concentration of 0.2% to the selected production medium obtained from the previous step (M3).

Different concentrations ranging from 0.05 to 0.8% of the selected low cost substrate (untreated chitin) were added to medium (M3). The medium was inoculated and cultivated as previously mentioned and finally checked for protein concentration and chitinase activity.

#### *2.6.3. Effect of different carbon sources*

Six different carbon sources were tested at a concentration of 0.1% namely; lactose, dextrin, sorbose, glucose, starch and sucrose. Subsequently, different concentrations of the best carbon source (starch) were applied in the range (0.05 to 0.8%).

#### *2.6.4. Investigation of different nitrogen sources*

Different nitrogen sources were investigated in the form of mixture of organic and inorganic sources compared to the control (yeast extract and tryptone). The organic (casein, corn steep liquor) and inorganic (sodium nitrate, ammonium molybdate) supplements were added one time instead of yeast extract and another instead of tryptone at the same concentration. All trials were compared to yeast extract alone (0.4%) and tryptone alone (0.2%). The favourable source (tryptone) was selected for the following experiments after verifying the optimal concentration.

#### *2.6.5. Effect of initial medium pH*

To study the effect of initial pH on chitinase production, different pH values ranging from 4 to 10 were adjusted using 1 N HCl/NaOH before sterilization. The prepared media were inoculated with the spore suspension and incubated for 6 days at 28ºC in a rotary shaker at 200 rpm. The cell free extract was checked for enzyme activity.

#### *2.6.6. Effect of temperature*

The effect of different temperatures was evaluated to test chitinase production. The temperatures applied ranged from 30°C to 45°C.

### *2.7. Partial purification of chitinase*

#### *2.7.1. Effect of medium composition on purification efficiency using ethanol fractionation at different pH values*

The crude enzyme obtained from the final optimized medium was compared to that obtained upon supplementing the same medium with yeast extract (0.4%) as protein stabilizer. The concentrated enzyme was dissolved in two different buffers (phosphate buffer at pH 7 and acetate buffer at pH 5) then precipitated by cold ethanol at concentrations of 0-40%, 40-60% and 60-80%. The precipitates were re-suspended in the same buffer and assayed for protein concentration and chitinase activity.

#### *2.7.2. Effect of stabilizers on purified enzyme*

Two different protein stabilizers (yeast extract and tryptone) were added to the crude enzyme at 2 mM concentration, dissolved in acetate buffer (pH 5) then precipitated by cold ethanol (0-40% concentration). Both stabilizers were also added to the partially purified enzyme to test their effect on the enzymatic solubility. Then the activity was determined.

### *2.8. Characterization of the partially purified exo-chitinase enzyme*

#### *2.8.1. Effect of pH on enzyme activity and stability*

A wide range of pH values (4 to 10) was examined for assessing the enzyme activity and stability. Acetate buffer was used for pH 4 and 5; phosphate buffer for pH 6 and 7; Tris-HCl buffer for pH 8 and glycine-NaOH buffer for pH 9 and 10 at concentration of 100 mM.

#### *2.8.2. Effect of temperature on enzyme activity and stability*

To detect the maximum activity and stability of chitinase, different degrees of temperature from 25 to 90ºC were applied. In case of enzyme stability, the reaction mixture was pre-incubated for 1 h at the test temperature and the residual activity was determined.

### *2.9. Insecticidal potential of chitinase*

#### *2.9.1. Tested mosquitoes*

Larvae of *Culex pipiens* provided from Medical Entomology Institute and self-perpetuating colonies were established and maintained in the laboratory of Entomology Department, Faculty of Science, Ain Shams University. Mosquitoes were reared under controlled conditions of temperature (27 ± 2ºC), relative humidity, R.H. (70% – 80%) and light – dark period (16: 8 h). Late third larval instars were used for toxicological studies.

#### *2.9.2. Larvicidal activity*

Different concentrations of the two partially purified enzymes which were produced from the two tested media as mentioned above were applied at the concentrations of 0-40%. The mortality data were recorded after 24 and 48 h and analysed by the probit analysis to calculate $LC_{50}$ and $LC_{95}$ which are defined as the lethal concentrations causing 50% and 95% mortality of the treated larvae, respectively [23].

### *2.10. Statistical analysis*

All data are presented as means ± standard deviation of means. The experiments were replicated 3 times (n=3). The given value for each result is the mean of the repeated measurements.

## 3. *Results*

### *3.1. Screening of endophytic fungal isolates for chitinase production*

In this study, ten endophytic fungi previously isolated from local medicinal plants were tested for chitinase production using medium containing powder chitin. All of them showed different degrees of chitinase activity. On the basis of specific activity, isolate no. eight (M-8) was the most potent one (44.3 U/mg) and thus, selected for further study (data not shown).

### *3.2. Identification of the most potent fungus using sequencing the internal transcribed spacer of ribosomal DNA method*

The internal transcribed spacer of ribosomal DNA sequencing technique was applied for the identification of the most potent fungus on the molecular level. Fig. 1 shows the phylogenetic tree of the fungus which is closely related to *Aspergillus niger* strain with identity reaching 98%, indicated by the similarity of the sequencing and multiple alignments. The fungus was submitted in the GenBank and registered under the accession number MW940924.

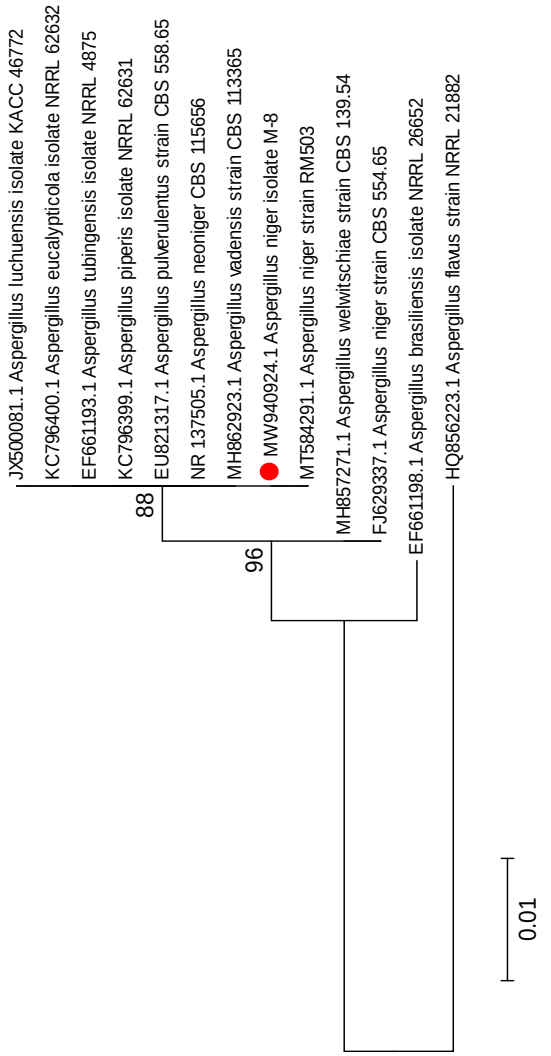


**Fig. 1. Molecular phylogenetic tree of *Aspergillus niger* isolate M-8 with the most related cultures of *Aspergillus* section *Nigri* according to [25]**

The phylogenetic analysis was performed in MEGA6 software [24] using Maximum Likelihood method and basing on internal transcribed spacer (ITS) gene. Accession number is indicated adjacent to each strain. The tree is constructed to scale with branch lengths measured in the number of substitutions per site. Bootstrap values above 50% (based on 500 replications) are indicated at nodes. *Aspergillus flavus* NRRL 21882 was used as an out-group in the analysis.

### *3.3. Optimization of fermentation conditions*

#### *3.3.1. Effect of different media on chitinase production*

The medium composition and cost are very important parameters to propagate the production process.

In the current study, four different media were examined to optimize chitinase production by *Aspergillus niger* M-8 (Fig. 2). Medium (M3) gave the maximum activity (50.2 U/mL) and specific activity (45.5 U/mg) followed by medium number 2 which gave enzyme activity 28.5 U/mL and approximately the same specific activity, whereas media number 1 and 4 showed very low production.

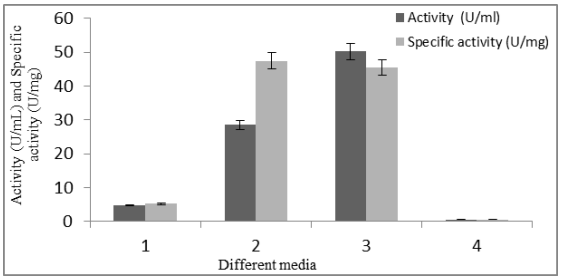


**Fig. 2. Effect of different media on chitinase production from *Aspergillus niger* M-8**

#### *3.3.2. Investigation of the role of different chitin substrates on the production*

Various substrate inducers such as colloidal chitin, pure powder chitin and untreated chitin were used for the enzyme production (Fig. 3a). All of them gave high chitinase activities and specific activities yet, colloidal chitin was the best (56.3 U/mg).

However, the untreated chitin also produced enzyme with high specific activity (43.6 U/mg). Therefore, further experiments were carried out using untreated chitin because of its lower cost. To optimize the concentration of untreated chitin, different amounts were tested (0.05-0.8%). Fig. 3b shows that 0.1% induced the maximum activity (64.7 U/mg) by *Aspergillus niger* M-8 while higher concentrations suppressed the production.

The rest of the chitinase production conditions were later improved by one-variable-at-a-time approach (OVAT), in shake flasks.

#### *3.3.3. Investigation of different carbon additives*

Different sources of carbon namely; glucose, sorbose, sucrose, lactose, starch and dextrin were added to the optimized medium (Fig. 3c). Supplementing the medium with 0.1% of each increased the enzyme productivity, compared to the control (containing only 0.1% chitin). The specific activity reached its maximum value when the medium was supplemented with starch or dextrin (115.5 and 114.3 U/mg, respectively) compared to the control (64.1 U/mg). Also, the best starch concentration was 0.1%, in equal ratio to chitin, while the higher concentrations repressed the enzyme specific activity (Fig. 3d).

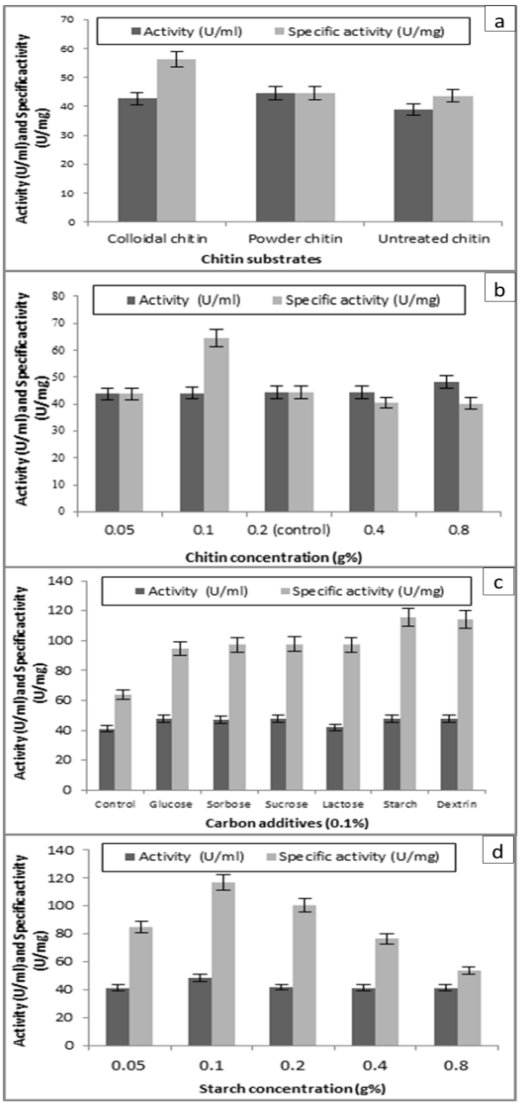


**Fig. 3. Effect of (a) chitin substrates, (b) chitin concentrations, (c) carbon additives and (d) starch concentrations on chitinase production from *Aspergillus niger* M-8**

#### *3.3.4. Investigation of different nitrogen additives*

From the previous experiments of medium optimization, it was observed that the sole employment of inorganic nitrogen had a suppressive effect on chitinase production as indicated upon using M1 and M4. Therefore, mixtures of different organic and inorganic nitrogen sources along with single tryptone or yeast extract (included together in the control of M3) were tested to optimize chitinase production (Table 1). Different sources were added one time instead of tryptone and another time instead of yeast extract at the same concentration of each. The data indicate that the single organic nitrogen source is

better than the mixed sources. Tryptone alone (0.2%) enhanced the yield to reach 348.3 U/mg followed by tryptone + corn steep (262.0 U/mg). Whereas yeast extract + $NaNO_3$ had a detrimental effect on chitinase production (56.4 U/mg). Next, tryptone optimal level was adjusted at 0.3% to achieve a specific activity of 400.7 U/mg (Table 2).

**Table 1. Effect of different nitrogen sources on chitinase production by *Aspergillus niger* M-8**

| Nitrogen sources | Activity (U/mL) | Specific activity (U/mg) |
|---|---|---|
| **Yeast extract+ tryptone (control)** | 40.0 ± 2.1 | 108.1± 2.2 |
| **Yeast extract** | 52.1 ± 3.0 | 153.2± 3.0 |
| **Tryptone** | 41.8 ± 2.7 | 348.3± 3.8 |
| **Yeast extract+ casein** | 47.2 ± 2.9 | 109.7± 2.6 |
| **Yeast extract+ corn steep** | 49.6 ± 2.8 | 141.7± 2.9 |
| **Yeast extract+ $NaNO_3$** | 20.9 ± 1.1 | 56.4± 2.7 |
| **Yeast extract+ amm. molybdate** | 37.9 ± 1.4 | 102.4± 1.9 |
| **Tryptone + casein** | 53.5 ± 2.3 | 157.4± 2.9 |
| **Tryptone + corn steep** | 41.9 ± 2.5 | 262.0± 4.1 |
| **Tryptone + $NaNO_3$** | 20.9 ± 1.1 | 110.0± 1.9 |
| **Tryptone + amm. molybdate** | 54.5 ± 2.7 | 259.5± 4.8 |
| **LSD at P ≤** | 0.83 | 1.10 |

### *3.3.5. Effect of different pH and temperature values*

Different pH values of the best medium composition (4-10) were studied for chitinase production. The results depicted in Fig. 4a indicated that there was a strong correlation between pH and the chitinolytic activity where, pH 5 was the optimum giving specific activity of 433.9 U/mg. The enzyme retained more than 70% of its activity up to pH 7 after that the specific activity declined steeply.

Fig. 4b shows a maximum value of 572.0 U/mg at 35°C, after which the specific activity decreased ie: the higher cultivation temperatures had adverse effect on the activity of the produced enzyme.

**Table 2. Effect of tryptone concentrations**

| Tryptone conc (g%) | Activity (U/mL) | Specific activity (U/mg) |
|---|---|---|
| **0.05** | 10.2 ± 1.2 | 204.0± 3.7 |
| **0.1** | 23.4 ± 1.9 | 260.0± 4.0 |
| **0.2 (control)** | 44.8 ± 2.2 | 344.6± 2.6 |
| **0.3** | 52.1 ± 2.1 | 400.7± 5.4 |
| **0.4** | 52.1 ± 1.8 | 372.1± 4.2 |
| **0.5** | 52.1 ± 2.1 | 306.4± 2.9 |
| **LSD at P ≤** | 1.72 | 1.85 |

### *3.4. Enzyme partial purification*

#### *3.4.1. Investigation medium composition on purification efficiency at different pH values*

It was noticed that the chitinase enzyme produced in this study tended to aggregate during precipitation by ethanol. Therefore, the following experiment aimed to correlate the production medium composition to the purification efficiency and enzymatic yield. The crude enzyme was collected from two different media (the optimized medium +/- 0.4% yeast extract) and precipitated by ethanol at two different pH values (5 and 7) (Table 3).The optimized medium at pH 5 gave the maximum specific activity (8885 U/mg), purification fold (16.3) and recovery (10.2) at lower ethanol concentration (0-40%) while the higher concentrations accelerated the enzyme deactivation. On the other hand, addition of yeast extract to the production medium increased the protein solubility during fractionation but it led to low enzymatic yield (5467.3 U/mg).

#### *3.4.2. Effect of protein stabilizer on purification efficiency during ethanol precipitation*

Protein aggregation means the disruption of its quaternary structure causing partial unfolding hence, herein, two stabilizers at 2 mM concentration were added separately to the crude enzyme before its precipitation at 0-40% concentration, designated as $F_{40}$ (Table 4). The results indicate that both stabilizers afforded additional boost to the purification fold but tryptone doubled the enzyme recovery.

**Table 3. Ethanol fractionation profile under the effect of two different media at two different pH values**

| Purification steps | | Total activity (U) | | Total protein (mg) | | Specific activity (U/mg) | | Purification fold | | Recovery (%) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | pH 5 | pH 7 | pH 5 | pH 7 | pH 5 | pH 7 | pH 5 | pH 7 | pH 5 | pH 7 |
| Crude extract | A | 52175.0 ± 7 | 52060.0 ± 8 | 95.5 ± 1 | 105.0 ± 2 | 546.3 ± 2 | 495.8 ± 2 | 1 | 1 | 100 | 100 |
| | B | 50485.0 ± 4 | 50450.0 ± 7 | 105.0 ± 2 | 105.0 ± 1 | 480.8 ± 4 | 480.4 ± 1 | 1 | 1 | 100 | 100 |
| Fraction 40% | A | 5331.0 ± 3 | 4982.4 ± 4 | 0.6 ± 0.1 | 1.5 ± 0.2 | 8885.0 ± 7 | 3321.6 ± 5 | 16.3 | 6.7 | 10.2 | 9.6 |
| | B | 4926.6 ± 2 | 5144.6 ± 3 | 0.9 ± 0.2 | 1.86 ± 0.2 | 5467.3 ± 5 | 2819.6 ± 3 | 10.0 | 5.9 | 9.8 | 10.2 |
| Fraction 60% | A | 5141.5 ± 3 | 5022.5 ± 4 | 2.75 ± 0.3 | 2.95 ± 0.3 | 1869.6 ± 4 | 1702.5 ± 3 | 3.4 | 3.4 | 9.5 | 9.5 |
| | B | 4807.5 ± 4 | 4972.0 ± 6 | 3.25 ± 0.4 | 3.8 ± 0.4 | 1479.2 ± 5 | 1307.9 ± 1 | 3.1 | 2.7 | 9.5 | 9.5 |
| Fraction 80% | A | 3848.0 ± 5 | 1304.5 ± 3 | 3.0 ± 0.2 | 1.2 ± 0.1 | 1282.7 ± 3 | 1087.1 ± 3 | 2.4 | 2.2 | 7.5 | 2.5 |
| | B | 4978.5 ± 2 | 2087.5 ± 5 | 4.3 ± 0.1 | 1.9 ± 0.2 | 1157.8 ± 4 | 1098.7 ± 2 | 2.4 | 2.3 | 9.5 | 4 |
| LSD at P ≤ | | 1.95 | 1.38 | 0.34 | 0.24 | 1.54 | 1.09 | - | - | - | - |

**A.** Optimized medium and **B.** Optimized medium + yeast extract (YE)

Furthermore, the addition of 2 mM of yeast extract and tryptone enhanced the enzyme activity by 220 and 300%, respectively (Data not shown).

### *3.5. Enzyme characterization*

#### *3.5.1. Effect of temperature on partially purified chitinase activity and stability*

Various temperature degrees (25-90°C) were applied to determine maximum chitinolytic activity and stability (Fig. 5 a,b). The enzyme activity was preserved when the reaction was carried out at (25-70°C) and depleted at higher temperatures losing almost 80% of the initial chitinolysis potential. The same goes for enzyme stability where the enzyme was incubated for 1 h at the corresponding temperature before assessing the chitinase activity.

#### *3.5.2. Effect of pH values on partially purified chitinase activity and stability*

Chitinase activity and stability were evaluated at different pH values ranging from 3 to 10 (Fig. 6 a,b).

The optimum activity was found at pH 4-5 with minimal decrease up to 7 and the enzyme maintained 50% of its original activity at the end of the tested range at pH 10. The same results were obtained as per the enzyme stability.

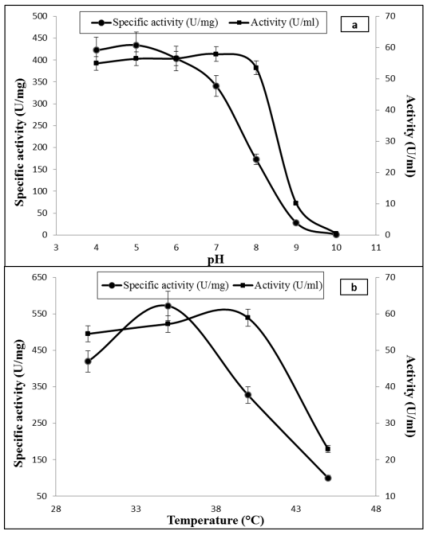


**Fig. 4. Effect of (a) pH and (b) temperature values on chitinase production from *Aspergillus niger* M-8**

### *3.6. Insecticidal effect of partially purified enzyme*

The larvicidal potency of the partially purified chitinase ($F_{40}$) produced from *Aspergillus niger* M-8

**Table 4. Effect of stabilizer addition during ethanol precipitation at 40% concentration**

| | Total activity (U) | Total protein (mg) | Specific activity(U/mg) | Purification fold | Recovery (%) |
|---|---|---|---|---|---|
| **Crude extract** | 51788.0± 6 | 94.2± 1 | 549.8± 3 | 1 | 100 |
| **Crude extract + YE** | 52205.3± 8 | 96.8± 2 | 539.4± 2 | 1 | 100 |
| **Crude extract +T** | 54318.6± 5 | 98.5± 1 | 551.2± 2 | 1 | 100 |
| **$F_{40}$ Crude extract** | 5320.8± 3 | 0.6± 0.1 | 8868.0± 5 | 16.1 | 10.3 |
| **$F_{40}$ Crude extract +YE** | 7405.3± 1 | 0.9± 0.2 | 8228.1± 3 | 15.3 | 14.2 |
| **$F_{40}$ Crude extract + T** | 10213.2± 2 | 1.0± 0.2 | 10213.2± 4 | 18.5 | 18.8 |
| **LSD at P ≤** | 2.28 | 0.48 | 1.58 | - | - |

YE (yeast extract) and T (tryptone)

using the two tested media (tryptone medium +/- yeast extract) was evaluated against the freshly molted 3rd instar larvae of *Culex pipiens* (Table5). The toxicity values depended on the enzyme concentrations and exposure time where $F_{40}$ of the tryptone-optimized medium was more effective against the mosquito larvae with $LC_{50}$ and $LC_{95}$ of 54 and 222.9 ppm, respectively, after 48 h. The microscopic observation, as well, shows some deformations of exo- and endo-skeletons of the treated larvae compared with normal ones where, there was a compressed darkness of abdomen segments and big vacuole beside the alimentary canal with rupture of segment cells, respectively (Fig. 7 a,b,c).

### *4. Discussion*

The endophytic fungi are known to produce various bioactive secondary metabolites and enzymes [4, 8]. Therefore, they have a great effect on their host plants such as promoting plant growth and resistance against biotic and abiotic stress [26]. Furthermore, it is thought that some medicinal plants produce bioactive compounds on account of the secondary metabolites produced by endogenous endophytes [26, 27]. In this context, different research groups have reported the isolation of various hydrolytic enzymes and bioactive compounds from many endophytic fungi genera, which were shown to influence their hosts' promiscuity.

To efficiently produce hydrolytic enzymes from the most potent fungus, first the medium type should be carefully selected. It was observed here that M2 and M3 greatly enhanced the productivity compared to M1 and M4, this is accounted to the presence of organic nitrogen in their composition which is deficient in M1 and M4.

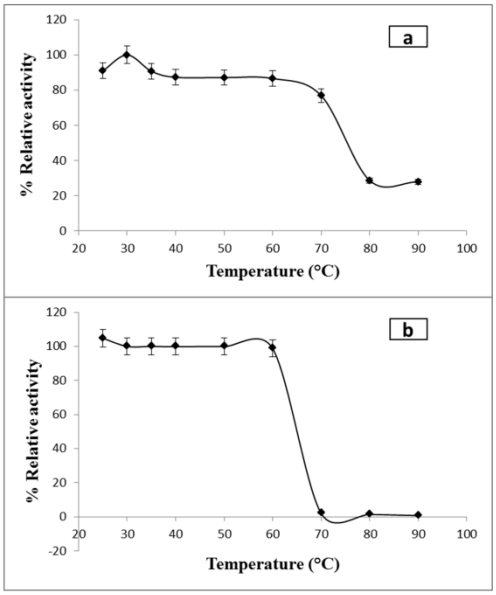


**Fig. 5. Effect of temperature variation on chitinolytic (a) activity and (b) stability**

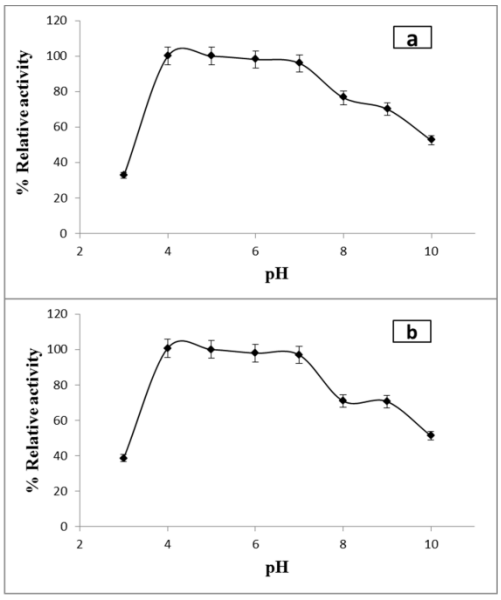


**Fig. 6. Effect of pH variation on chitinolytic (a) activity and (b) stability**

**Table 5. Susceptibility of 3rd instars larvae of *Cx. pipiens* to partially purified chitinase at different time intervals and coefficient limits**

| Concentration (ppm) | Mortality percent % | | | |
|---|---|---|---|---|
| | $F_{40}$ of optimized medium | | $F_{40}$ of mixed medium | |
| | 24 h | 48 h | 24 h | 48 h |
| 50 | 20 | 51.7 | 20 | 36.7 |
| 100 | 43.3 | 66.7 | 40 | 58.3 |
| 150 | 53.3 | 90 | 48.3 | 70 |
| 250 | 73.3 | 98.3 | 60 | 88.3 |
| $LC_{50}$ (ppm) (co. limit) | 127.47 (105.58-153.93) | 54 (42.59-68.25) | 162.05 (123.27-213.34) | 76.48 (61.29-95.32) |
| $LC_{95}$ (ppm) (co. limit) | 811.32 (419-1580.23) | 222.87 (163.2-305.06) | 1888.04 (569.42-6349) | 465.43 (278.71-780.84) |
| Slope ± SE | 2.05±0.1 | 2.7±0.2 | 1.54±0.1 | 2.1±0.1 |

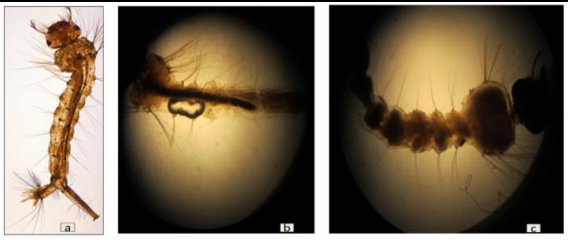

**Fig.7. Deformation in 3rd instars larvae of *Culex pipiens* treated by partially purified chitinase a: Normal larva of *Cx. Pipiens*, b and c: Larva treated with $F_{40}$ of partially purified chitinase**

Similar observation was reported as the chitinases produced from *Aspergillus niger* LOCK 62, *Aspergillus* sp. S1–13, *Beauveria bassiana* reached their maximum productivity using yeast extract-containing medium [18, 28, 29]. This finding has not only been reported in the cases of fungal production, but also in some cases of some *Streptomyces* spp. and bacterial species [30–32]. The exact explanation might not be quite conclusive but sound postulations attribute it to the better mycelial growth leading to better enzyme production or to the presence of N-acetylglucosamine within the yeast extract that, in turn, further induces the enzyme production [29]. Herein, the sole presence of tryptone enhanced the production by 3.2 fold, compared to the control. This was documented as well upon investigating the production of chitinase from *Alcaligenes xylosoxydans* IMI 385022 and *Aeromonas punctata* HS6 [16, 33].

On the other hand, the presence of sucrose (1%) in M1 and M4 led to the suppression of chitinase production, mostly due to catabolite repression [29].

Considering the presence of enzyme inducers (ie: the different chitin substrates), it was observed that colloidal chitin gave the highest chitinase production (56.3 U/mg). But out of economic prespective in this report, the further optimization of the fermentation medium was carried out using untreated chitin due to its lower cost and fairly high production of chitinase (43.6 U/mg). Similarly, colloidal chitin was previously reported as sole and good cultivation medium ingredient for producing chitinase in both bacteria and fungi [18, 31, 32, 34]. Furthermore, the optimal untreated chitin concentration was found at 0.1% giving 64.7 U/mg. Whereas higher concentration of colloidal chitin (0.3%) was needed to achieve the maximum chitinase production by *Aeromonas hydrophila* HS4 and *Aeromonas punctata* HS6 [16] and up to 1.5% for production from *Streptomyces* spp. [31].

The combination of chitin and different sugars was tested and the results indicated a high synergistic effect. These findings are in agreement with previous articles that described the synergistic effect of starch and chitin [16, 31]. While other researches stimulated chitinase production by the addition of glucose, glucosamine, N-acetylglucosamine [29], maltose [35] and pectin [36]. Moreover, the effective starch concentration was at 1% with *Aeromonas hydrophila HS4* and *Aeromonas punctata HS6*, which was higher than the obtained result here (0.1%) [16].

The pH value of the cultivation medium is an important physical factor that can affect the enzyme production and stability by influencing its structure integrity; either maintaining the proper folding or denaturing it leading to loss of activity, in addition to affecting the availability of nutrients for the growing organism through their ionization state [17, 37]. The optimum pH value here was found at 5 and the enzyme was adequately stable till pH 7. Same results were obtained for *Aspergillus terrus*, *Beauveria bassiana* and *Metarhizium anisopliae* [29, 38, 39].

Similarly, the microbial metabolic processes, including active enzymes production, are influenced by the incubation temperature [40, 41]. The obtained results support the previous convention that the optimum temperature for active enzyme production was at 30-35°C and beyond that, most of the activity was lost [18, 31, 34, 38].

The following section was carried out to study the influence of the production medium constituents, pH and ethanol concentration on the purification efficiency. The results illustrated that the highest specific activity (8885.0 U/mg), purification fold (16.3) and recovery (10.2%) were obtained using

tryptone-containing medium at lower ethanol concentration (0-40%) using acetate buffer at pH 5. And it was observed that by increasing the ethanol concentrations, the enzyme deactivation accelerated which could be attributed to damaging the protein structure, rigid conformation and dehydrating the enzyme [42, 43].

Furthermore, the addition of yeast extract as a stabilizing agent to the tryptone medium indeed increased the protein solubility during fractionation, but it afforded lower active enzyme yield. One plausible reason might be the reduction in aggregation degree of the inactive protein [44, 45]. In other words, yeast extract solubilized and increased the total protein content in the filtrate without a similar positive effect on the enzyme under test ie: it could be good stabilizer for other proteins in the cell-free supernatant as well. These results indicated that the presence of amino acids alone, as found in tryptone stabilizer, was preferred than the presence of amino acids combined with sugar stabilizers, as found in yeast extract which is opposite to previous data obtained upon utilizing yeast extract [46]. Hence, it can be concluded that the production step strongly impacted the purification of active enzyme yield. Appropriately to design a suitable solvent system, safe compounds as osmolytes or polyols (sugars, polymers and amino acids) can be added to act as protein stabilizers to reduce aggregation and maintain the structure [1, 2, 47, 48] by preserving the water molecules around the protein which reduces the dehydration process during solvent precipitation [2]. From these results, it might be deduced that certain protein stabilizers may be applied to reduce the *in vivo* protein aggregation that causes diseases due to the protein misfolding e.g.: Alzheimer's, cystic fibrosis, Parkinson's, etc. [49, 50].

As shown in the results section, the stabilizers (yeast extract and tryptone) at a 2 mM concentration were added separately to the crude enzyme at fractionation to increase the insufficient stability and recovery. The results revealed that tryptone doubled the enzyme recovery. Previously, it was shown that the stabilizer concentration is best kept between 1-3 mM depending on the inherent protein solubility and other properties [46]. Moreover, the two stabilizers at the same concentration were added to the partially purified chitinase to study their effect on activity, and the results indicated that both of them enhanced the enzyme activity by 220 and 300%, respectively. This may arise from the increased hydration around the protein molecules which plays a crucial role in the biological reactions by dissolving of polar residues of the enzyme facilitating the protein conformational changes during the catalytic process and speeding up the catalytic reaction [2].

To characterize the partially purified chitinase, some properties were studied such as the effect of temperature on enzyme activity and stability. The obtained results proved that chitinase from *A. niger* M-8 retained most of its activity and stability between 25-70°C. Previous studies have reported a narrower activity range for chitinases from *Aspergillus niger* and *Penicillium aculeatum* NRRL 2129 at 40°C and 50°C, respectively [18, 51]. While, a more thermostable chitinase has been produced and purified from *Humicola grisea* and exhibited maximum activity at 70°C [52].

Also, the pH stability potential of chitinase was tested and the enzyme was active and stable under a wide span between pH (4-7) retaining about 50% of its original activity until pH 10. The range of 5-9 was also convenient for the activity of *Bacillus circulans* chitinase [53]. The durable temperature and pH stability profile imply that the partially purified chitinase can be applied in different industrial sectors.

The insecticidal activity results of the partially purified enzyme ($F_{40}$ of tryptone medium +/- yeast extract) indicated that the yeast fortified sample was highly effective against mosquito larvae with $LC_{95}$ of 222.9 ppm after 48 h. Additionally, the presence of some deformations in both exo- and endo-skeletons was observed under the microscope. The present results show higher activity when compared to the chitinase isolated from *Penicillium chrysogenum* which had $LC_{90}$ of 443.004 and 5046.8 ppm after 48 h exposure to the free and immobilized enzyme, respectively [54]. Previous studies, similarly, confirm the biocidal effect of chitinases from *Bacillus* spp. against the white fly *Bemisia tabaci*, aphids *Myzus persicae* and *Aphis gossypii* [55, 56] and also the larvicidal activity of *B. subtilis* chitinase against the tomato leaf miner insect *Tuta absoluta* [57] indicating the degradation of the exoskeleton chitin of the tested insects' larvae.

## *5. Conclusion*

Endophytic fungi are still acting as a rich mine for secondary metabolites with a more exploitable potential in many applications. Therefore, the endophyte *Aspergillus niger* M-8 was isolated, identified and its operational production conditions of extracellular chitinase were successfully optimized to reach high enzymatic yield. The critical factor here was the folding and aggregation of the enzyme during ethanol precipitation which led to applying a co-solvent strategy using tryptone and yeast extract which clearly proved to have protective capabilities as they shift the dynamic equilibrium from the aggregate denatured form to a more stabilized one, which led to obtain a good turnout. *A. niger* chitinase ($F_{40}$) had a great effect against the mosquito *Culex pipiens*. This could help understanding how the potential of enzyme

application is dependent on its production and the detailed multistep processing.

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The authors do not have any conflict of interest.

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### *9. References*

[1] Beaufils C., Man H.M., de Poulpiquet A., Mazurenko I., and Lojou E., From enzyme stability to enzymatic bioelectrode stabilization processes, *Catalysts*, **11**, 497 (2021). doi:10.3390/catal11040497.

[2] Silva C., Martins M., Jing S., Fu J., and Cavaco-Paulo A., Practical insights on enzyme stabilization, *Critical Reviews in Biotechnology*, **38**, 335–350 (2018). doi:10.1080/07388551.2017.1355294.

[3] Oyeleye A., and Normi Y.M., Chitinase: diversity, limitations, and trends in engineering for suitable applications, *Bioscience Reports*, **38**, BSR2018032300 (2018). doi:10.1042/BSR20180323.

[4] Kidibule P.E., Santos-Moriano P., Plou F.J., and Fernández-Lobato M., Endo-chitinase Chit33 specificity on different chitinolytic materials allows the production of unexplored chitooligosaccharides with antioxidant activity, *Biotechnology Reports*, **27**, e00500 (2020). doi:10.1016/j.btre.2020.e00500.

[5] Churklam W., and Aunpad R., Enzymatic characterization and structure-function relationship of two chitinases, LmChiA and LmChiB, from *Listeria monocytogenes*, *Heliyon*, **6**, e04252 (2020). doi:10.1016/j.heliyon.2020.e04252.

[6] Sabab A., Liu S., Javadiyan S., McAdam C.J., Hanton L.R., Jukes A., Vreugde S., and Wormald P.-J., The effect of chemical and structural modifiers on the haemostatic process and cytotoxicity of the beta-chitin patch, *Scientific Reports*, **11**, 18577 (2021). doi:10.1038/s41598-021-97781-8.

[7] Hahn T., Tafi E., Paul A., Salvia R., Falabella P., and Zibek S., Current state of chitin purification and chitosan production from insects, *Journal of Chemical Technology & Biotechnology*, **95**, 2775–2795 (2020). doi:10.1002/jctb.6533.

[8] Alam B., Lǐ J., Gě Q., Khan M.A., Gōng J., Mehmood S., Yuán Y., and Gǒng W., Endophytic fungi: From symbiosis to secondary metabolite communications or vice versa?, *Frontiers in Plant Science*, **12**, 791033 (2021). doi:10.3389/fpls.2021.791033.

[9] Poveda J., Abril-Urias P., and Escobar C., Biological control of plant-parasitic nematodes by filamentous fungi inducers of resistance: *Trichoderma*, Mycorrhizal and endophytic Fungi, *Frontiers in Microbiology*, **11**, 992 (2020). doi:10.3389/fmicb.2020.00992.

[10] Sayed R.M., Abdalla R.S., Rizk S.A., and El sayed T.S., Control of *Culex pipiens* (Diptera: Culicidae), the vector of lymphatic filariasis, using irradiated and non-irradiated entomopathogenic nematode, *Steinernema scapterisci* (Rhabditida: Steinernematidae), *Egyptian Journal of Biological Pest Control*, **28**, 67 (2018). doi:10.1186/s41938-018-0070-z.

[11] Eifan S., Hanif A., Nour I., Alqahtani S., Eisa Z.M., Dafalla O., and Kohl A., Distribution and molecular identification of *Culex pipiens* and *Culex tritaeniorhynchus* as potential vectors of Rift Valley fever virus in Jazan, Saudi Arabia, *Pathogens*, **10**, 1334 (2021). doi:10.3390/pathogens10101334.

[12] Folly A.J., Dorey-Robinson D., Hernández-Triana L.M., Ackroyd S., Vidana B., Lean F.Z.X., Hicks D., Nuñez A., and Johnson N., Temperate conditions restrict Japanese encephalitis virus infection to the mid-gut and prevents systemic dissemination in *Culex pipiens* mosquitoes, *Scientific Reports*, **11**, 6133 (2021). doi:10.1038/s41598-021-85411-2.

[13] Richards S.L., Byrd B.D., Reiskind M.H., and White A. V, Assessing insecticide resistance in adult mosquitoes: Perspectives on current methods, *Environmental Health Insights*, **14**, 1178630220952790 (2020). doi:10.1177/1178630220952790.

[14] Correy G.J., Zaidman D., Harmelin A., Carvalho S., Mabbitt P.D., Calaora V., James P.J., Kotze A.C., Jackson C.J., and London N., Overcoming insecticide resistance through computational inhibitor design, *Proceedings of the National Academy of Sciences*, **116**, 21012–21021 (2019). doi:10.1073/pnas.1909130116.

[15] Baz M.M., Selim A., Radwan I.T., Alkhaibari A.M., and Khater H.F., Larvicidal and adulticidal effects of some Egyptian oils against *Culex pipiens*, *Scientific Reports*, **12**, 4406 (2022). doi:10.1038/s41598-022-08223-y.

[16] Saima S., Kuddus M., Roohi R., and Ahmad I., Isolation of novel chitinolytic bacteria and production optimization of extracellular chitinase, *Journal of Genetic Engineering and Biotechnology*, **11**, 39–46 (2013). doi:10.1016/j.jgeb.2013.03.001.

[17] Asif T., Javed U., Zafar S.B., Ansari A., Ul Qader S.A., and Aman A., Bioconversion of colloidal chitin using novel chitinase from *Glutamicibacter uratoxydans* exhibiting anti-fungal potential by hydrolyzing chitin within fungal cell wall, *Waste and Biomass Valorization*, **11**, 4129–4143 (2020). doi:10.1007/s12649-019-00746-2.

[18] Brzezinska M.S., and Jankiewicz U., Production of antifungal chitinase by *Aspergillus niger* LOCK 62 and its potential role in the biological control, *Current Microbiology*, **65**, 666–672 (2012). doi:10.1007/s00284-012-0208-2.

[19] White T.J., Bruns T., Lee S., and Taylor J., Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in: M.A.

Innis, D.H. Gelfand, J.J. Sninsky, T.J. White (Eds.), PCR Protocols: A guide to methods and applications, Academic Press, New York, 1990: pp. 315–322. doi:10.1016/b978-0-12-372180-8.50042-1.

[20] Coudron T.A., Kroha M.J., and Ignoffo C.M., Levels of chitinolytic activity during development of three entomopathogenic fungi, *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, **79**, 339–348 (1984). doi:10.1016/0305-0491(84)90386-9.

[21] Lowry O.H., Rosebrough N.J., Farr A., and Randall R.J., Protein measurement with the folin Phenol Reagent, *Journal of Biological Chemistry*, **193**, 256–275 (1951). doi:10.1016/S0021-9258(19)52451-6.

[22] Kaur N., and Sharma A.D., Production, optimization and characterization of extracellular invertase by an actinomycete strain, *Journal of Scientific and Industrial Research*, **64**, 515–519 (2005).

[23] Finney D.J., Probit analysis, 3rd ed., Cambridge University Press, New York, 1971. doi:10.1002/jps.2600600940.

[24] Tamura K., Stecher G., Peterson D., Filipski A., and Kumar S., MEGA6: Molecular evolutionary genetics analysis version 6.0., *Molecular Biology and Evolution*, **30**, 2725–2729 (2013). doi:10.1093/molbev/mst197.

[25] Samson R.A., Visagie C.M., Houbraken J., Hong S.-B., Hubka V., Klaassen C.H.W., Perrone G., Seifert K.A., Susca A., Tanney J.B., Varga J., Kocsubé S., Szigeti G., Yaguchi T., and Frisvad J.C., Phylogeny, identification and nomenclature of the genus *Aspergillus*., *Studies in Mycology*, **78**, 141–173 (2014). doi:10.1016/j.simyco.2014.07.004.

[26] Jia M., Chen L., Xin H.L., Zheng C.J., Rahman K., Han T., and Qin L.-P., A friendly relationship between endophytic fungi and medicinal plants: A systematic review, *Frontiers in Microbiology*, **7**, 906 (2016). doi:10.3389/fmicb.2016.00906.

[27] Zheng R., Li S., Zhang X., and Zhao C., Biological activities of some new secondary metabolites isolated from endophytic fungi: A review study, *International Journal of Molecular Sciences*, **22**, 959 (2021). doi:10.3390/ijms22020959.

[28] Rattanakit N., Yano S., Plikomol A., Wakayama M., and Tachiki T., Purification of *Aspergillus* sp. S1-13 chitinases and their role in saccharification of chitin in mash of solid-state culture with shellfish waste, *Journal of Bioscience and Bioengineering*, **103**, 535–541 (2007). doi:10.1263/jbb.103.535.

[29] Dhar P., and Kaur G., Effects of carbon and nitrogen sources on the induction and repression of chitinase enzyme from *Beauveria bassiana* isolates, *African Journal of Biotechnology*, **9**, 8092–8099 (2010). doi:10.5897/ajb09.1161.

[30] Sridevi M., and Mallaiah K. V., Factors effecting chitinase activity of *Rhizobium* sp. from Sesbania sesban, *Biologia*, **63**, 307–312 (2008). doi:10.2478/s11756-008-0070-7.

[31] Narayana K.J.P., and Vijayalakshmi M., Chitinase production by *Streptomyces* sp. ANU 6277, *Brazilian Journal of Microbiology*, **40**, 725–733 (2009). doi:10.1590/S1517-83822009000400002.

[32] Akeed Y., Atrash F., and Naffaa W., Partial purification and characterization of chitinase produced by *Bacillus licheniformis* B307, *Heliyon*, **6**, e03858 (2020). doi:10.1016/j.heliyon.2020.e03858.

[33] Vaidya R.J., Shah I.M., Vyas P.R., and Chhatpar H.S., Production of chitinase and its optimization from a novel isolate *Alcaligenes xylosoxydans*: potential in antifungal biocontrol, *World Journal of Microbiology and Biotechnology*, **17**, 691–696 (2001). doi:10.1023/A:1012927116756.

[34] Cheba B.A., Zaghloul T.I., El-Mahdy A.R., and El-Massry M.H., Effect of nitrogen sources and fermentation conditions on *Bacillus* sp. R2 chitinase production, *Procedia Manufacturing*, **22**, 280–287 (2018). doi:10.1016/j.promfg.2018.03.043.

[35] Liu B.-L., Kao P.-M., Tzeng Y.M., and Feng K.C., Production of chitinase from *Verticillium lecanii* F091 using submerged fermentation, *Enzyme and Microbial Technology*, **33**, 410–415 (2003). doi:10.1016/S0141-0229(03)00138-8.

[36] Chen H.-C., Hsu M.-F., and Jiang S.T., Purification and characterization of an exo-N,N′-diacetylchitobiohydrolase-like enzyme from *Cellulomonas flavigena* NTOU 1, *Enzyme and Microbial Technology*, **20**, 191–197 (1997). doi:10.1016/S0141-0229(96)00111-1.

[37] Deb P., Talukdar S.A., Mohsina K., Sarker P.K., and Sayem S.M.A., Production and partial characterization of extracellular amylase enzyme from *Bacillus amyloliquefaciens* P-001, *SpringerPlus*, **2**, 154 (2013). doi:10.1186/2193-1801-2-154.

[38] Farag A., and Alnusaire T., Production, optimization, characterization and antifungal activity of chitinase produced by *Aspergillus terrus*, *African Journal of Biotechnology*, **13**, 1567–1578 (2014). doi:10.5897/AJB2014.13628.

[39] Dhar P., and Kaur G., Effects of carbon and nitrogen sources on the induction and repression of chitinase enzyme from *Metarhizium anisopliae* isolates, *Annals of Microbiology*, **59**, 545 (2009). doi:10.1007/BF03175144.

[40] Chandrasekaran S., Kumaresan S.S.P., and Manavalan M., Production and optimization of protease by filamentous fungus isolated from paddy soil in Thiruvarur District Tamilnadu, *Journal of Applied Biology and Biotechnology*, **3**, 66–69 (2015). doi:10.7324/jabb.2015.3610.

[41] Sharma O., Sharma K., and Nath K., Production of proteolytic enzyme by fungi, *Revue Roumaine de Biochimie*, **17**, 209–215 (1980).

[42] Podkaminer K.K., Shao X., Hogsett D.A., and Lynd L.R., Enzyme inactivation by ethanol and development of a kinetic model for thermophilic simultaneous saccharification and fermentation at 50°C with

*Thermoanaerobacterium saccharolyticum* ALK2, *Biotechnology and Bioengineering*, **108**, 1268–1278 (2011). doi:10.1002/bit.23050.

[43] Stepankova V., Bidmanova S., Koudelakova T., Prokop Z., Chaloupkova R., and Damborsky J., Strategies for stabilization of enzymes in organic solvents, *ACS Catalysis*, **3**, 2823–2836 (2013). doi:10.1021/cs400684x.

[44] Okada J., Maruyama T., Motomura K., Kuroki K., Maenaka K., Sakono M., and Goto M., Enzyme-mediated protein refolding, *Chemical Communications*, 7197–7199 (2009). doi:10.1039/b916225a.

[45] Mirhosseini S.A., Latifi A.M., Hosseini H.M., Seidmoradi R., Aghamollaei H., and Farnoosh G., The efficient solubilization and refolding of recombinant organophosphorus hydrolyses inclusion bodies produced in *Escherichia coli*, *Journal of Applied Biotechnology Reports*, **6**, 20–25 (2019). doi:10.29252/JABR.06.01.04.

[46] Ralph K., and Peterson J., Method of stabilizing proteins at low pH, US 2004/0022792 A1, 2004. https://lens.org/158-858-333-375-431.

[47] Murphy E.L., Collier A.C., Kalish L.A., Assmann S.F., Para M.F., Flanigan T.P., Kumar P.N., Mintz L., Wallach F.R., Nemo G.J., and Study V.A.T., Highly active antiretroviral therapy decreases mortality and morbidity in patients with advanced HIV disease, *Annals of Internal Medicine*, **135**, 17–26 (2001).

[48] Canchi D.R., and García A.E., Cosolvent effects on protein stability, *Annual Review of Physical Chemistry*, **64**, 273–293 (2013). doi:10.1146/annurev-physchem-040412-110156.

[49] Denny R.A., Gavrin L.K., and Saiah E., Recent developments in targeting protein misfolding diseases, *Bioorganic & Medicinal Chemistry Letters*, **23**, 1935–1944 (2013). doi:10.1016/j.bmcl.2013.01.089.

[50] Nevone A., Merlini G., and Nuvolone M., Treating protein misfolding diseases: Therapeutic successes against systemic amyloidoses, *Frontiers in Pharmacology*, **11**, 1024 (2020). doi:10.3389/fphar.2020.01024.

[51] Adrangi S., Faramarzi M.A., Shahverdi A.R., and Sepehrizadeh Z., Purification and characterization of two extracellular endochitinases from *Massilia timonae*, *Carbohydrate Research*, **345**, 402–407 (2010). doi:10.1016/j.biotechadv.2013.09.012.

[52] Kumar M., Brar A., Vivekanand V., and Pareek N., Process optimization, purification and characterization of a novel acidic, thermostable chitinase from *Humicola grisea*, *International Journal of Biological Macromolecules*, **116**, 931–938 (2018). doi:10.1016/j.ijbiomac.2018.05.125.

[53] Siwayaprahm P., Audtho M., Ohmiya K., and Wiwat C., Purification and characterization of a *Bacillus circulans* No. 4.1 chitinase expressed in *Escherichia coli*, *World Journal of Microbiology and Biotechnology*, **22**, 331 (2006). doi:10.1007/s11274-005-9038-8.

[54] Mansour S.H., Abdel-Fattah A.M., Esawy M.A., Ahmed E.F., Haroun A.A., Hussein M.A., Hassanien N.M., and Khater H.M., Immobilization, thermodynamic studies and application of chitinase enzyme from *Penicillium chrysogenum*, *Egyptian Journal of Aquatic Biology & Fisheries*, **23**, 527–544 (2019).

[55] Mubarik N.R., Mahagiani I., Anindyaputri A., Santoso S., and Rusmana I., Chitinolytic bacteria isolated from Chili rhizosphere: Chitinase characterization and its application as biocontrol for Whitefly (*Bemisia tabaci* Genn.), *American Journal of Agricultural and Biological Sciences*, **5**, 430–435 (2010). doi:10.3844/ajabssp.2010.430.435.

[56] Nurdebyandaru N., Mubarik N., and Prawasti T., Chitinolytic bacteria isolated from Chili Rhizosphere: Chitinase characterization and application as biocontrol for *Aphis gossypii*, *Microbiology Indonesia*, **4**, 103–107 (2010). doi:10.5454/mi.4.3.1.

[57] Abdullah R., The efficacy of chitinolytic bacteria as a bioinsecticide against *Tuta absoluta* (Lepidoptera: Gelechiidae), *International Journal of Entomology Research*, **5**, 107–110 (2020).

## Arabic abstract

يعد عدم ثبات الانزيمات عند تنقيتها العائق الرئيسي الذي يحول دون التوسع في تطبيقاتها الصناعية. في هذه الدراسة وجد ان السلالة المحلية M-8 *Aspergillus niger* والمدرجة في الجين بنك تحت رقمMW940924، قد أعطت إنتاجًا عاليًا من الكيتيناز أثناءالتخميرالمغمور. حيث أدت ظروف التشغيل المثلى إلى زيادة إنتاج الإنزيم بحوالي 13 ضعفًا (572.0 وحدة / مجم) باستخدام وسط يحتوي على 0.1٪ كيتين غير معالج ، 0.1٪ نشا و 0.3٪ تريبتون عند 35 درجة مئوية ودرجة حموضة 5. ولزيادة كفاءة تنقية الإنزيم وثباته، تم تجربة اثنين من المواد المحافظة علي ثبات البروتين وبالتالي النشاط الانزيمي و هم التربتون وخلاصة الخميرة. وقد أظهرت النتائج أن مكونات الوسط الإنتاجي ونوع المثبت ودرجة الحموضة قد أثروا بشكل كبير. وقد سبق الوسط المُحسَّن باستخدام التربتون قرينة المحتوي علي 0.4٪ من مستخلص الخميرة حيث أدي إلى زيادة نسب كل من النشاط النوعي للإنزيم (8885.0 وحدة / مجم) ودرجة النقاوة (16.3) والإنتاجية (10.2٪) وذلك تحت نفس ظروف التنقية. علاوة على ذلك، أدى المحلول المحتوي على 2 mM من التربتون عند الرقم الهيدروجيني 5 إلى مضاعفة نسبة الانتاجية (18.8 ٪) مقارنةً بالمحلول الذي يحتوي على الخميرة.وقد وصل الكيتيناز المنقى باستخدام 40٪ إيثانول إلى نشاطه التحفيزي الأمثل عند درجة حرارة 30 درجة مئوية ودرجة حموضة 5. وأيضًا كان الانزيم مستقرًا واحتفظ بمعظم نشاطه الأولي في نطاق واسع من درجات الحرارة (25-70 درجة مئوية) وقيم الأس الهيدروجيني (4-7). ومن المثير للاهتمام أن الإنزيم المنقي والاكثر ثباتا كان له تأثير أكثر ضراوة ضد يرقات البعوض بعد 48 ساعة $LC_{95}$= 222.9 ppm ، مما يشير إلى إمكانية استخدامه للتحكم البيولوجي في بعوضة الكوليكس أو البعوضة المنزلية الشمالية.