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Research Article
Fungi Associated with Peanut Seeds and Their Extracellular Enzyme Activities under the Influence of Physical Factors

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Abstract

Background and Objective: Peanut is one of the most important crops all over the world. Global annual production of peeled peanuts reached 44 million t in 2016 and China produces 38% of total production. Peanut attacked by many of fungi which causes lot of quality or quantity losses The present investigation concerned with study of peanut seed-borne fungi and their extracellular enzyme activities under Egyptian condition. **Materials and Methods:** Commercial seed samples were collected from different four governorates with different conditions such as; Beheira, Ismailia, Giza and Sohag. All samples were subjected to seed healthy test using blotter method. Therefore, the most frequent fungi subjected to study their extra cellular enzyme activity. **Results:** Seventeen species of fungi belonging to 14 genera were isolated. Six fungi were recorded in all locations such as; *Aspergillus flavus*, *Fusarium moniliforme*, *Fusarium oxysporum*, *Penicillium* sp., *Rhizoctonia solani* and *Aspergillus niger*. The best activity for most enzymes was at 30°C. **Conclusion:** Tested fungi were varied in their enzyme activities between three evaluated enzymes such as; amylase, cellulase and lipase. *Aspergillus flavus* showed a remarkable activity of amylase and lipase while *Rhizoctonia solani* distinctive in cellulase.

Key words: Peanut, *Arachis hypogaea* L., associated fungi, enzyme activity, *Aspergillus flavus, Rhizoctonia solani*

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Peanut (*Arachis hypogaea* L.) is an important crop grown in the worldwide especially in sandy or yellow soil. Peanut originated in South American and spreads beyond China, Africa, Indian, Japan and United States of America, etc. This crop achieves cash yield more meaningful when the farmers follows bilateral or trilateral agricultural with planting early maturing varieties such as; a Giza 5 and 6. Peanut is one of legume crop which plays an important role of soil fertilization. Treating cultivated legume seeds with co-inoculation with rhizobacteria (PGPR) and rhizobia lead to enhancement and stabilize nitrogen which releases from the remaining nodules on plants roots. This amount of nitrogen improves soil fertility for next crop[1,2]. Peanut is also one of the important crops either as an economic crop as a result of export or because it is an important oil crop.

Peanut attacked by many economically important pathogenic fungi. These fungi invade all parts of the plant (root, stem, leaf and pod) directly or through wounds. Several researchers isolated many fungi such as; *Aspergillus niger*, *Aspergillus flavus*, *Alternaria dianthicola*, *Curvularia lunata*, *Curvularia pellescens*, *Fusarium oxysporum*, *Fusarium equiseti*, *Macrophominapha seolina*, *Rhizopus stolonifer*, *Penicillium digitatum* and *Penicillium chrysogenum*, etc[3]. These fungi cause different symptoms such as; discoloration, rotting, shrinking, seed necrosis, loss in germination capacity and toxification or change properties to oil seeds[3,4]. Janardhan *et al.*[5] noted that *Aspergillus flavus* is a common mold in tropical and subtropical environments and secret aflatoxin as a result of molding after badly stored of peanut seeds.

Amylases are a group of enzymes which plays an important role in degrading starch links and glycogen molecules. These enzymes refer to glycoside hydrolase enzymes group which have enzyme commission number (EC: 3.2.1). These enzymes are widely distributed in the microbial plant (bacteria and fungi) and animal kingdoms and varying in action patterns according to the source. Amylases have significant applications in food fermentation, textile, paper and pharmaceutical industries[6]. Generally, amylase group represents about 30% of the world enzyme production[7].

Bio-degradation of cellulose is a very important process which reduces the environmental pollution by converting the cellulosic biomass to fermentable sugars through biocatalyst cellulases obtained from cellulolytic organisms. Also, there is much importance of the cellulase in the industry that used for many years in textile production, food processing, waste-water treatment and detergent[8]. Other practical uses of cellulase are for beer production of wine, production of juice and also for production of ethanol and biofuels.

Microbial lipases are biocatalysts with distinct properties, as action under reasonable conditions, stability in organic solvents and specific substrate[9]. Lipases have achieved a notable position in the world market of the enzymes, clearly demonstrated by the amount of information reported in the literature, which comprises over 1,000 publications per year[10]. The present study was aimed to isolate and detect seed-borne fungi of peanut seeds and focus on enzymes produced by the most important frequently fungi as well as study the effect of temperature on these enzymes activities.

MATERIALS AND METHODS

Seed health test: Commercial samples of peanut seeds were collected from different four locations Beheira, Ismailia, Giza and Sohag governorates during season of 2017. Four hundred seed of each sample were used to seed a healthy test according to ISTA[11]. Seeds were plated in pre-sterilized Petri dishes containing 3 layers of wet blotters with rate 10 seeds in each Petri dish. All Petri dishes were incubated at 25°C for 7 days. Seeds were examined under stereomicroscope and microscope. The purification of fungi were made by transferring hyphal tips to Petri dishes containing PDA by using fine glass capillary tubes. All cultures were identified according to description sheets of Common Wealth Mycological Institute Kew[12-19].

Inoculum preparation: The most frequently and most prevalent fungi which recorded in all location were selected for the further experiments. Selected fungi were grown on PDA medium for 7 days at 25°C. Then equal disks 0.5 mm were made by Cork Borer.

Preparation of media: The medium used in these experiments was mineral media containing (KH_2PO_4 0.14 g, NH_4NO_3 1 g, KCl 0.5 g, $MgSO_4.7H_2O$ 0.01 g and $FeSO_4.7H_2O$ 0.001 g) supplemented by the tested source of carbon as a sole source[20]. All these components dissolved in 1000 mL distilled water at pH 6.5 and autoclaved at 121°C for 15 min.

Amylase activity: In the present study, previous mentioned mineral media was supplemented by polysaccharides substrate (soluble starch) with rate 2 g/1000 mL of the medium. Erlenmeyer flasks 250 mL were filled with 100 mL of medium and autoclaved at 121°C for 15 min. Flasks were

inoculated by one disk of each fungus separately with 3 replicates. All flasks were incubated in a shaking incubator for 48 h at 120 rpm and 30°C.

Extraction of crude amylase: The broth cultures were centrifuged at 7000 rpm for 30 min. Supernatants cell-free were used for estimation of the amylase activity.

Amylase assay: One milliliter of each extracted supernatant was transferred by pipette into the test tube and 1.0 mL of 1% soluble starch in citrate phosphate buffer pH 6.5 was added. All test tubes incubated at 30°C for 30 min. About 2 mL Dinitrosalicylic Acid (DNS) was added as a reagent to the reaction and the reaction was stopped by boiling the mixture for 5 min in water bath and cooled to room temperature and 10 mL of distilled water was added and color intensity was measured by using spectrophotometer[20] at 540 nm.

Effect of temperature on amylase activity: Sterilized flasks filled with 100 mL medium, mentioned in the previous experiment were divided into 6 groups each 1 including all tested fungi with 3 replicates. All treatments incubated at different temperature 5, 10, 20, 30, 40 and 50°C for 48 h. Activity of amylase enzyme was evaluated in each treatment.

Cellulase activity: In this experiment, mineral medium mentioned before supplemented by cellulose carboxymethyl cellulose (CMC) with rate 2 g/1000 mL of medium as a solo source of carbon. Flasks (250 mL) were filled with 100 mL of medium and autoclaved at 121°C for 15 min. Flasks were inoculated by 1 fungal disk (0.5 cm) of each one of the tested fungi with three replicates. All treatments were incubated in a shaking incubator for 48 h at 120 rpm and 30°C temperatures.

All cultures were centrifuged at 7000 rpm for 30 min. Supernatants cell-free were used for the cellulase estimation.

Cellulase assay: One milliliter of each filtrated supernatants was pipetted into a test tube and 1.0 mL of 1% CMC in citrate phosphate buffer pH 6.5 was added. All test tubes incubated at 30°C for 30 min. Then treated with 2 mL DNS and the reaction was stopped by boiling for 5 min, in water bath and cooled to room temperature then 10 mL of distilled water was added and color intensity[21] was measured using spectrophotometer at 540 nm.

Effect of temperature on cellulase activity: Sterilized flasks with medium mentioned in the previous experiment were inoculated with tested fungi each one separately. All treatments incubated at different temperature 5, 10, 20, 30, 40 and 50°C with 3 replicates for 2 days. Activity of cellulase enzyme was determined by using spectrophotometer at 540 nm as mentioned before[21].

Lipase activity: In the present work, medium mentioned before was supplemented by olive oil with rate 10 mL L^{-1} as a solo source of carbon. About 250 mL flasks filled with medium sterilized and inoculated by one of the equal disks of tested fungi. All flasks incubated at 30°C for 48 h.

Lipase assay: The activity of lipase was determined by using the titrimetric method by using pipette, 3.5 mL of tris buffer and 3 mL of olive oil were transferred into 50 mL Erlenmeyer flasks. All flasks placed in checker water bath at 60 rpm and 37.5°C for 10 min. About 1 mL of crude enzyme was added to previous reaction substrate then incubated at 30°C for 30 min at 60 rpm with keeping the blank tube at -4°C. The reaction was inhibited by adding 3 mL of 95% ethanol solution buffer and 4 drops of phenolphthalein solution were then added.

The amount of fatty acids released during the reaction is determined by direct titration with NaOH 0.05 Mm and the pink color indicate the end point[22]:

$$\text{Lipase activity (U mL}^{-1}) = \frac{(V - V_0)}{t \times n} \times M_0$$

where, V is the volume of NaOH solution consumed in the sample solution (mL), V_0 is the volume of NaOH solution consumed in blank solution (mL), t is the reaction time (min), n is the volume of enzyme solution (mL) and M is the concentration of NaOH solution.

Effect of temperature on lipase activity: Flasks (250 mL) containing 100 mL medium mentioned in the previous experiment were inoculated with tested fungi with three replicates for each tested fungus. All treatments incubated at different temperature 5, 10, 20, 30, 40 and 50°C for 2 days. Activity of lipase enzyme was evaluated in each treatment.

RESULTS AND DISCUSSION

Seed health test: Seventeen fungal species belonging to 14 genera were isolated from samples of peanut seeds collected from different four locations i.e., Beheira, Ismailia, Giza and Sohag governorates by using blotter method. In Table 1, *Fusarium moniliforme* and *Aspergillus flavus* were the most incidence at Beheira governorate followed by *Rhizoctonia solani*, *Aspergillus niger* and *Penicillium* sp. At

Table 1: Incidence and frequency of seed-borne fungi of peanut seeds collected from different 4 Egyptian governorates

	Incidence at each location (%)					
Isolates	Beheira	Ismailia	Giza	Sohag	Total Incidence	Frequency (%)
Alternaria alternate	6	4	4	9	23	5.3
Aspergillus flavus	14	22	13	11	60	13.7
Aspergillus niger	11	9	11	15	46	10.5
Curvularia sp.	4	-	3	-	7	1.6
Chaetomium sp.	6	5	-	-	11	2.5
Cephalosporium sp.	2	-	3	2	7	1.6
Cladosporium sp.	4	2	4	1	11	2.5
Fusarium solani	5	7	2	2	16	3.7
Fusarium moniliforme	15	10	11	14	50	11.4
Fusarium oxysporum	11	16	12	9	48	11
Mucor sp.	-	-	2	-	2	0.5
Penicillium sp.	11	17	7	13	48	11
Rhizoctonia solani	13	10	9	14	46	10.5
Rhizopus nigricans	9	5	3	12	29	6.6
Sclerotium rolifsii	4	5	-	5	14	3.2
Stemphylium sp.	3	-	-	1	4	0.9
Trichoderma spp.	5	8	3	-	16	3.7

Ismailia *Aspergillus flavus* recorded the highest incidence followed by *Penicillium* sp. and *Fusarium oxysporum*. *Aspergillus flavus*, *Fusarium oxysporum*, *Fusarium moniliforme* and *Aspergillus niger* appeared as the most incidence at Giza governorate. *Aspergillus niger*, *Fusarium moniliforme* and *Rhizoctonia solani* recorded the highest incidence at Sohag governorate.

From Table 1, it can be noticed that 10 isolates were recorded in all tested locations such as; *Alternaria alternate*, *Aspergillus flavus*, *Aspergillus niger cladosporium* sp., *Fusarium solani*, *Fusarium moniliforme*, *Fusarium oxysporum*, *Penicillium* sp., *Rhizoctonia solani* and *Rhizopus nigricance*. While, *Cephalosporium* sp., *Sclerotium rolifsii* and *Trichoderma* spp. recorded in 3 locations. Three fungal isolates were isolated from 2 locations *Curvularia* sp., *Chaetomium* sp. and *Stemphylium* sp., while *Moucor* sp. was recorded only in Giza. Generally, *Aspergillus flavus, Fusarium Moniliforme*, *Fusarium oxysporum*, *Penicillium* sp., *Aspergillus niger* and *Rhizoctonia solani* were the most frequently and most prevalent in all tested locations. Elwakil and El-Metwally[3] isolated 27 fungal species from the 50 commercial markets samples of Egypt.

Amylase activity: In the present study mineral media supplemented by polysaccharides substrate (soluble starch) with rate 2 g/1000 mL of medium was used to determine the ability of most prevalent and the most frequent fungi for secretion amylase enzyme. The 6 tested isolates such as; *Aspergillus flavus*, *Fusarium moniliforme*, *Fusarium oxysporum, Penicillium* sp., *Aspergillus niger* and *Rhizoctonia solani* showed different activity of amylase. Results presented in Fig. 1 which indicated that activity of amylase secreted by *Aspergillus flavus*, *Aspergillus niger* and *Penicillium* sp. was active far enough comparing with that secreted by the other three isolates. While, amylase secreted by *Fusarium oxysporum* was appeared with the less activity followed by *Fusarium Moniliforme*. Medium amylase activity was recorded by *Rhizoctonia solani*.

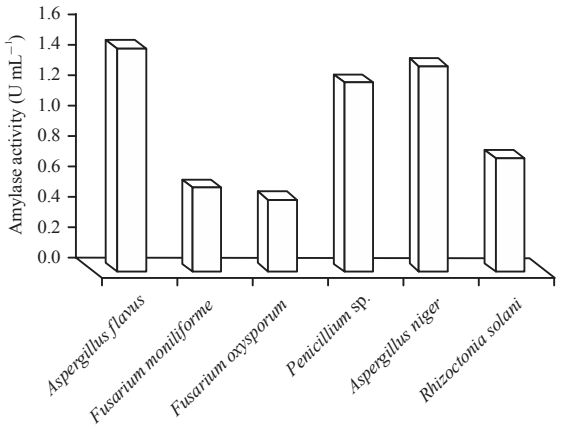

Fig. 1: Amylase activity of most prevalent fungal isolates from peanut seeds collected from different 4 governorates in Egypt

The optimum enzyme production by the all *Aspergillus* strain[23] was found at 30°C.

Effect of temperature on amylase activity: Data present in Fig. 2 showed that all tested fungi recorded maximum activity at 30°C and this activity decrease as temperature decreased or increased. Both *Aspergillus flavus*, *Aspergillus niger* and

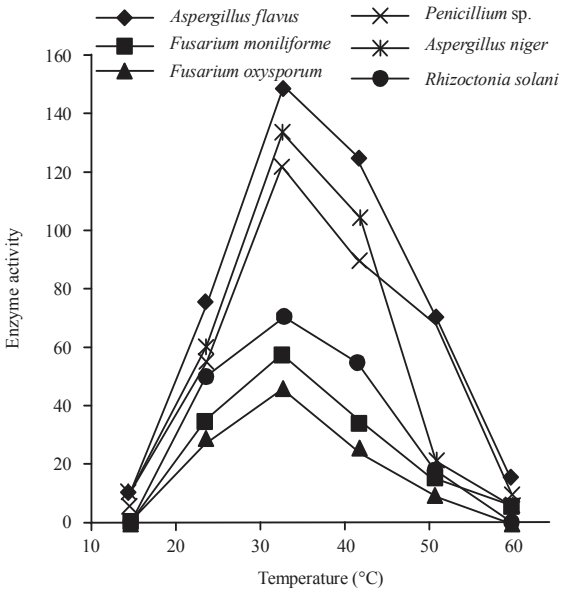

Fig. 2: Effect of different temperature on activity of amylase secreted by different 6 isolated fungi from the peanut seeds

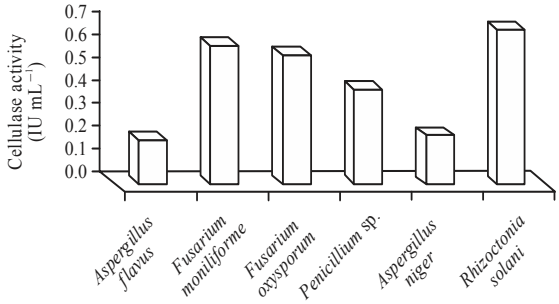

Fig. 3: Cellulase activity of most prevalent fungi isolated from peanut seeds collected from different 4 locations in Egypt

Penicillium sp. are characterized by thermal stability in range of temperature between 30-40°C, while *Rhizoctonia solani* characterized by thermal stability between 20 and 40°C. *Fusarium moniliforme* and *Fusarium oxysporum* showed maximum activity at 30°C with a fast reduction in activity with increasing or decreasing temperatures. Enzymatic activity corresponds directly proportional to the proper temperature for growth[24].

Cellulase activity: In this experiment mineral medium supplemented by sources of cellulose (CMC) with rate 2 g/1000 mL of medium as a solo source of carbon. Obtained data recorded in Fig. 3 showed that *Rhizoctonia solani* recorded maximum activity of cellulase enzyme,

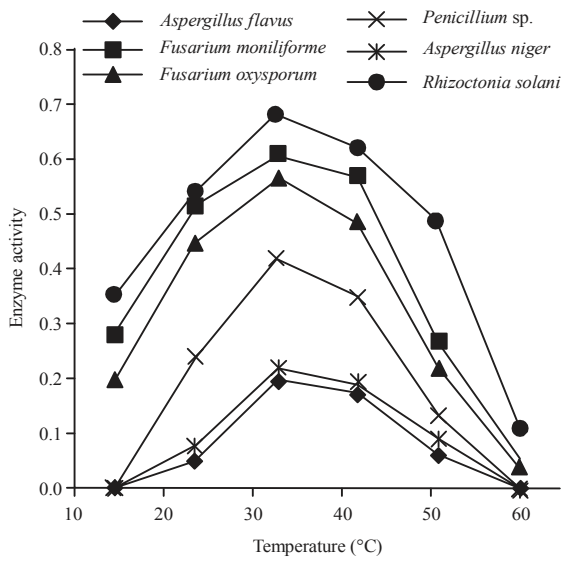

Fig. 4: Effect of different temperature on activity of cellulase secreted by different 6 fungi isolated from peanut

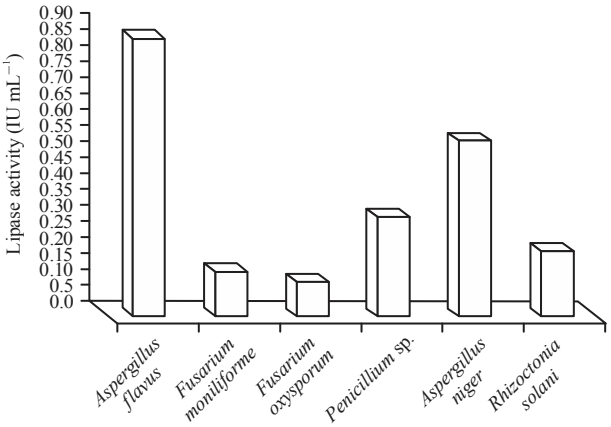

Fig. 5: Lipase activity of the most prevalent fungal isolates on peanut seeds collected from different four governorates in Egypt

F. moniliforme and *F. oxysporum* occupied the second and third rank, respectively. Cellulase enzyme secreted by *A. flavus* showed the lowest activity while *Penicillium* sp. and *Aspergillus niger* recorded medium level of cellulase enzyme activity. Cellulase activity produced by 5 isolated fungi was tested[21]. The result showed that the enzyme activity was superior of 2 isolates more than the other 3.

Effect of temperature on cellulase activity: Temperature is one of important factors which effect on enzyme activity. Results indicated that maximum cellulase activity of all tested fungi was recorded at 30°C with a difference in thermal stability between the tested fungi. *Rhizoctonia solani*

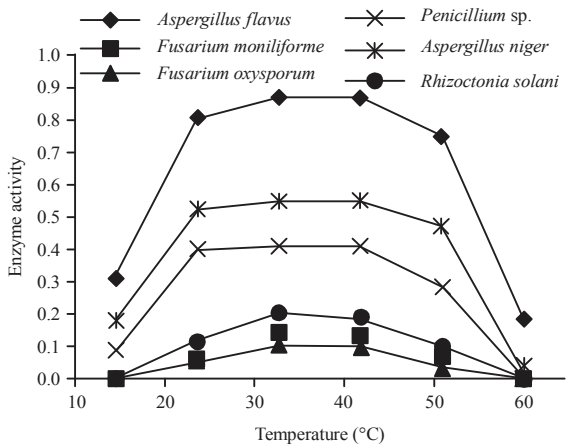

Fig. 6: Effect of different temperature on the lipase activity secreted by different 6 isolated fungi from peanut

characterized with a wide range of thermal stability ranged between 20-50°C. Also, thermal stability of *Fusarium moniliforme* and *Fusarium oxysporum* were between 20-40°C. Cellulase activity of *Penicillium* sp., *Aspergillus niger* and *Aspergillus flavus* recorded a noticeable reduction less than 30°C and more than 40°C. Effect of temperatures on enzymatic activity due to the impact of the effect of changes of temperature on the growth and cellular activity for each fungus. During the test of the effect of temperature on the cellulase activity, it was found that it reached maximum[21] at a temperature of 30°C.

Lipase activity: The results of evaluation for the activity of crude lipase enzyme extracted by the 6 tested fungi illustrated in Fig. 4. Data showed that lipase activity secreted by *Aspergillus flavus* was in highest activity followed by that secreted by *Aspergillus niger*. Medium activity was recorded by *Penicillium* sp. and *R. solani*. Very low activity of lipase enzyme was obtained by *F. monilifofrme* and *F. oxysporum*. Lipase activity of all isolated fungi from peanut seeds varied in lipase activity[25].

Effect of temperature on lipase activity: Effects of different temperatures on lipase activity of the 6 isolated fungi from peanut were tested. Data obtained in Fig. 6 showed lipolytic activity of all tested fungi were almost at the maximum between 30 and 40°C and differed in its stability. Lipolytic activity of *Aspergillus flavus* and *Aspergillus niger* were ranged from 20-50°C with the optimum between 30-40°C. Maximum of lipolytic activity of *Penicillium* sp. was between 20-40°C with sharp decrease at more or less than this range. While, the stability of the other 3 fungi was between 30-40°C with gradually decreasing at above or under previous range. *Penicillium notatum* showed significant maximum lipase activity at 20°C and followed by *Fusarium* sp. while, *Penicillium chrysogenum* showed maximum lipase production at 30°C as compared to other fungi[25].

Lower and higher temperatures appear falling in the activities values. Lower temperature decreases the activity because it is unsuitable for fungal growth. Consequently, decreased enzyme production while higher temperature lead to minimize media water content by vaporization, as a result, it effects on cells growth. In addition, the higher temperature limit oxygen concentration which effect on fungal activity[26].

All obtained data helped to give us some information about how these fungi cause infection and also the value of quality losses. Data proved that *Aspergelus flavus* has great activity of lipase which effect on quality and quantity of lipid contents, but has a neglected activity of cellulase which indicated that it has no ability of causing infection by itself while *Aspergillus flavus*, *Aspergillus niger* and *Penicillium* sp. have high activity of amylase which cause loss in carbohydrates contain. Thakare *et al.*[27] mentioned that amylase, protease and lipase are responsible for solubilizing reserve food material in form of starch, protein and lipid, respectively in the seed. In contrast, cellulose activity obtained from *R. solani* sufficient enough to penetrate the host plant and causing infection by itself while has very low lipase activity to change the properties of lipids in seeds. Heiler *et al.*[28] found that cellulase enzyme activity increased during appressorium development and reached a maximum when infection hyphae and haustorial mother cells were formed. Seven cellulolytic enzymes were separated by chromate focusing. While, the fungus caused the infection, the enzymatic activity reached to 3.3% during appressorium formation and increased to 36.6% when infection hyphae were formed and to 45.4% in haustorial mother cells.

CONCLUSION

Seventeen fungal species belonging to 14 genera were isolated from tested peanut seeds samples collected from 4 locations i.e., Beheira, Ismailia, Giza and Sohag governorates. *Aspergillus flavus, Fusarium moniliforme, Fusarium oxysporum, Penicillium* sp., *Aspergillus niger* and *Rhizoctonia solani* were the most frequently and most prevalent in all tested locations. *Rhizoctonia solani* characterized with maximum activity of cellulase enzyme while *Aspergillus flavus* recorded high activity of lipase enzyme. Temperature has different effect on enzymes stability along the 6 tested isolates. All enzymes secreted from all tested and *Aspergillus niger* has stability along wide range of temperature 20-50°C.

SIGNIFICANCE STATEMENT

This study gives researchers an idea about geographical distribution of seed-borne fungi for peanut crop in 4 governorates represent to the various climatic conditions in Egypt. Also, this study found link between development of infection and ability to secrete more efficient enzymes for example the fungus that has the ability to penetrate cellular walls rich in cellulose has the ability to secret cellulase enzyme like *Rhizoctonia solani, F. moniliforme* and *F. oxysporum* this information can be used in the control programs. This study will help the researcher to concentrate their research to restrict these genera that responsible for secreting this enzyme.

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