ISOLATION, IDENTIFICATION OF SOME FUGAL ISOLATES AND TESTING THEIR ABILITY FOR LIPOXYGENASE PRODUCTION

Nora T.Y. AL-Temime ,Amal K. G. AL-Asady ,

Alaa G.E. AL-Hashimi

Department of Food Science, Faculty of Agriculture, University of Basrah ,Basrh, Iraq.

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ABSTRACT

Eighty three local isolates of fungi were isolated from different resources (Peanuts , maize, rice, wheat, bread , domestic cheese of sheep, Milk local Cream, Iranian cream, Roquefort cheese and soil). These isolates were purified and identified, it include 14 isolates of *Aspergillus flavus*, 13 *Aspergillus niger*,8 *Aspergillus terreus*,3*Aspergillus parasaticus,3Alternaria spp.,15Penicilliumspp.*,7*Fusarium* spp.,5 *Trichoderma* spp., 11 *Rhizopus* spp. and 7*Mucor* spp. The ability of isolates for producing aflatoxin were tested, the toxic isolates(*Aspergillus flavus*,*Aspergillus terreus* ,and *Aspergillus parasaticus*) were removed.*Aspergillus niger* which was isolated from maize was choosing as the best lipoxygenase producer after Primary and secondary screening. The growth of the selected isolate colonies had the largest proportion than the Colonies of *Penicillium* sp. and *Trichoderma* sp..all so the same isolate had high enzymatic activity 801.4units/ml, while *Penicillium* sp. and *Trichoderma* sp. had (559.2 and 120) units/ml respectively.

INTRODUCTION

Filamentous fungi are eukaryotes that digest food extremely and absorb nutrients directly through its sell wall. Most of fungi reproduce by spores and have a body (thallus) composed of microscopic tubular cells called hyphae. *Aspergillus niger* is one of the filamentous fungus with black colonies so that it usually known as black mold[(5)]. It exist in all over the world in extend, although it causes the food

as well as the rest of fungi However, it was used in many fermentation in biotechnology such as organic acids production like citric acid, gluconic acid and enzymes production such as lipase and amylase[38,1].In spite of its usability exhaustion many of organic material which is necessary to produce enzymes[4]. *A.niger* is one of the mold using inbio-technology processing because it is safe in food , while some of *A.niger* strains may be distinguish itself via production of mycotoxins fungal[36]. Lipoxy genase (LOX) (linoleate: oxygen oxido reductase; EC:1.13.11.12) discovered first time in 1928 by Haas and Bohn when they observe the loss of yellow color from wheat flour sample after adding small amount of soybean flour. Lipoxy genase is a group of oxidized enzyme including non-hemiiron atom in its effective atom as well as it is stimulates to molecular of oxygen[33,23]. These enzymes are link the oxygen with unsaturated fatty acid such as arachidonic and linolenic and linoleic to produced peroxides and hydroperoxides[28], these free radicals are able to oxidase the sulpha hydreal groups of wheat flour proteins and form bi-sulphide bonds which strengthen the gluten, they found that the LOX extracted from soybeans for bleaching and increase the stability of wheat flour and improvement the rheological properties. LOX plays an important role in developing the flavor compounds such as the carbonate and alcohol compounds through produced of different type of peroxides isomers[19], which contain one of flavor compounds. LOX has special importance to produce aromatic compounds because it gives unique properties because it range of hydroperoxide isomers [11,9].LOX it exist widely in plant and animal kingdom and Micro-organisms[13,17]. LOX differs in substrate so essential fatty acids exist naturally such as linoleic acid (18: 2) and acid linolenic (18: 3) and arachidonic acid works as enzymes substrate [34].

Due to the lack of studies in LOX production from Micro-organisms in Iraq and because of the importance of this enzyme, and its role in food products, So this study aims to obtain some local fungal isolates for LOX production.

MATERIALS AND METHODS

Source of isolation: Many natural sources were collected for isolation of fungi (peanut, maize , local gamer, Iranian cream, local sheep cheese, blue cheese mold(Italian-made), bread , wheat , rice and soil Faculty of Agriculture) which were transferred to plastic containers sterile by UV at 254 nm for 15 minutes then the sample left at room temperature and air to enhance fungal growth before isolation.

Isolation of fungi: Each sample was transferred directly to sterile petri dishes contained Potato dextrose agar (PDA) and incubated for (28-30)°C \ (3-5) days[15]. after that a Part of colony for each fungi was transferred by a needle to another sterile petri dishes with new PDA[31,25]. This step was repeated three times till the colonies in each petri dishes became pure,then these fungal isolates were kept on slant of PDA at 4°C[3].

Detection of Aflatoxins: ammonia vapor method were adopted according to[32] to choose the viability of fungal isolates which was isolated from various sources to produce aflatoxin, through using coconut extract agar (CEA) which was prepared by[14].

Identification of isolated fungi: The isolates identified according to their morphological properties when growth on the PDA, The identification was at the level of genus and species according to classification keys mentioned in[29,20,27],the isolates were examined by using the morphological test for fungal colonies and microscopic test by using lacto phenol solution with the blue cotton dye[24].

Preparing of spore suspension: The new growing fungal colonies with thes pores on the slant of PDA were suspended with dist. water [12].then the spores were calculated by using Haemocytometer slide and light microscope. The spore suspension was diluted by distill water to the number of spores (1×10^6)spore / ml [35, 26].

Screening of fungal isolates for lipoxy genase production:

Primary screening (growth on PDA): The non-toxic isolates were testing by using the growth on PDA at 28°C\ 5 day. The chosen isolates were with big diameters of colonies [16, 22].

Secondary screening (growth in PD medium) :

Sterile PD medium (50)ml was used in a conical flask (100)ml capacity and inoculated with 1 ml of spores suspension(1×10^6) spore / ml and incubated in shaking incubator at 85 rpm \ min , 30 °C\ 7 days according to [30].

Extraction of lipoxy genase from biomass:

After incubation, the broth was filtered by what man filter paper no.1. The biomass was washed with deionized dist. water, then smashed by mortar and glass beads. After that, centrifugation at 10000 rpm \ min for 10 min, then the precipitate was removed and used the supernatant as extracted enzyme [30].

Estimation of lipoxy genase activity:

the activity of the enzyme was estimated according to [21]. 0.5 ml of enzyme solution added to 10ml of substrate (1gm of linoleic acid dissolved in 100 ml ethanol 95%) and mixed then incubated for 3minutes. at 30^0C.The reaction was stopped by addition 5ml of acidified ethanol solution (5ml of ethanol 95% and 0.1 ml of hydrochloric acid) to 1ml enzyme-substrate reaction solution (enzyme solution and substrate solution) and left for 2min. To this solution 0.015µl of ammonium ferrous sulfate 5% (5gm from ammonium ferrous sulfate dissolving in solution 3% hydrochloric acid and completed the volume to 100ml from HCl) and 0.5ml of ammonium thiocyanate solution(20gm from ammonium thiocyanate dissolved in deionized water and completed the volume to 100ml from deionized water)were added mixed, and the absorbance were measured at 480nm using spectrophotometer. The calibration solution(blank) solution prepared from deionized water instead of enzyme solution and substrate solution and completed the previous steps, Lipoxy genase activity was assayed by definition unit enzymatic activity.

$$\text{Activity} = \frac{\text{The change in the absorption}}{\text{The volume of the enzyme (ml)} \times \text{time (minutes)}} \times 60$$

Secondary screening (fungal capability of LOX production growing on liquid solution-PD by measuring the LOX activity)

The isolated fungi were growth on liquid PD in order to produce LOX from selected using shaking incubator ,inoculation glass flasks 100 ml contain50 ml of media inoculated by1ml of spore suspension the flask incubated at a temperature of 30°C for 7 days and incubated at 85 cycles/min [(30)].After incubation the control media filtering by paper Whitman No.1 to get the biomass washed by ions distilled water then crushed by mortar using glass balls then centrifuge10,000 rev/min then the separated of sludge stuck taking which represents the enzyme to assess the activities[(30)].

RESULTS AND DISCUSSION

Eighty three pure local isolates belong to 7 species of fungus was obtained as in this study Table 1 and Figure (1) indicated the fungus isolates which was obtained and purified on PDA after growing in incubator at (28 -30)°C for (3-5) days.

Table (1) the sources used for isolation of fungi

Sources	Number of isolates
Peanuts	**11**
Maize	**10**
Rice	**7**
Wheat	**9**
Bread	**15**
local cheese of sheep	**9**
Milk local gamer	**5**
Iranian cream	**4**
Roquefort cheese	**4**
Soil	**9**
Total isolates	**83**

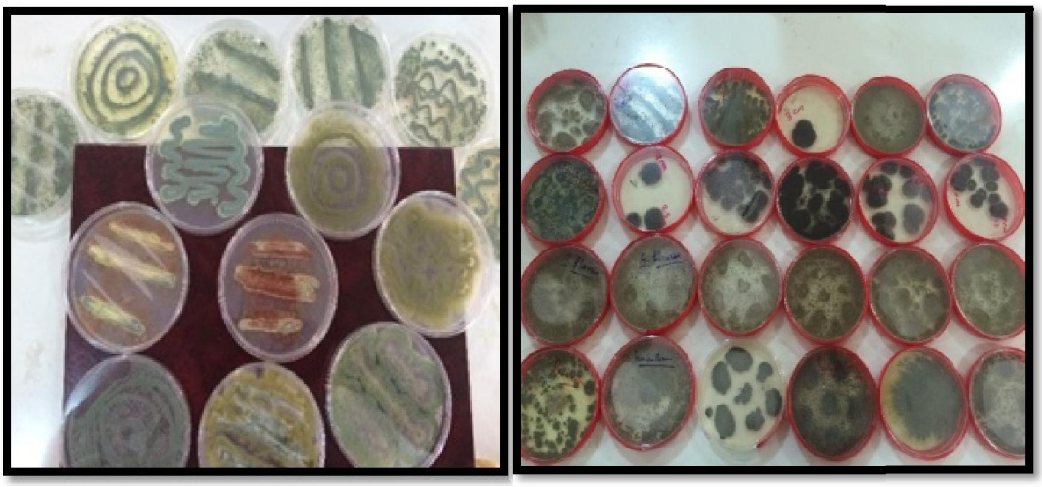


Figure (1) same of the pure fungal isolates which were grown on PDA

Detection of fungal isolates producing aflatoxin:

The results showed that there were 22 fungal isolates which produce aflatoxin, 11 isolates belong to *A.flavus*,8 isolates belonging to *A.terreus* and 3 isolates belonging to *A. parasaticus* (according to physical appearance). The results indicated that these isolates have the ability to change the color to coconut extract agar as in Figure 2 from white to pink after one hour of incubation also after 24 hours of incubation with ammonia steam at 5°C[21]. This result indicate a confirmative detection for production of aflatoxins[8]. While the other isolates given negative results which were *A.niger*, *Alternaria* sp.,*Fusarium* sp.,*Trichoderma* sp.,*Penicillium* sp.,*Mucor* sp. and *Rhizopus*sp. This agree with[38,6,40].

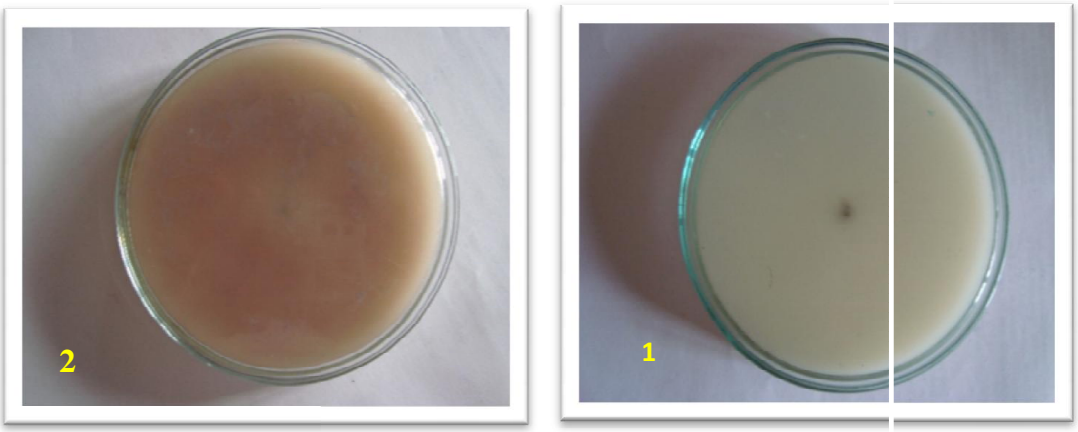
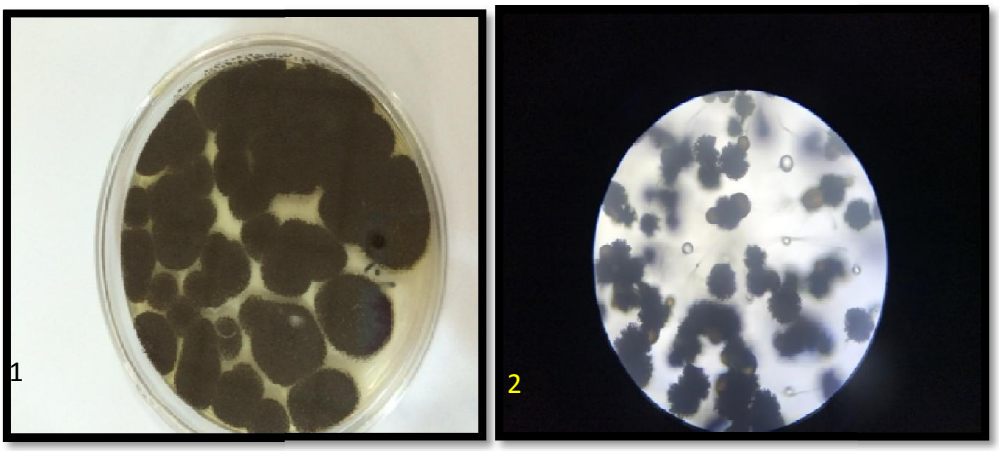


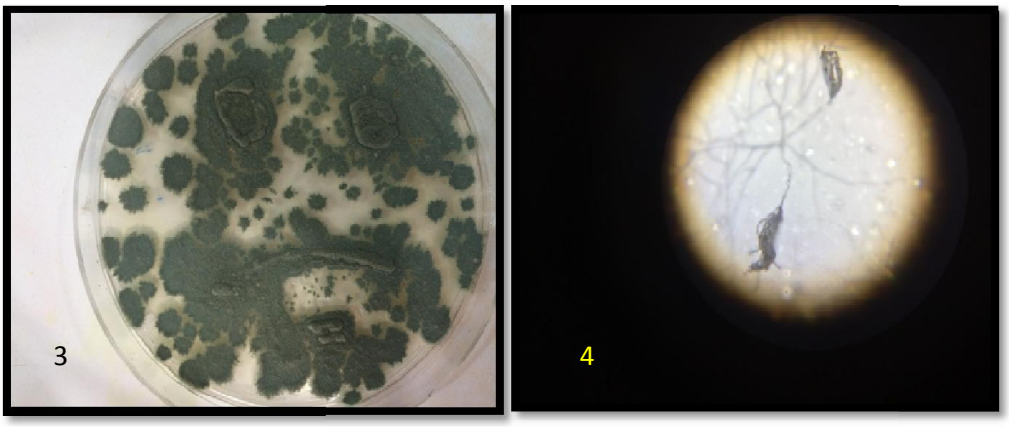
Figure (2) Tow samples of fungal isolates which were grown on CEA
1: *A.niger* (non-aflatoxins producer)2: *A. flavus* (aflatoxin producer)

fungal Identification:

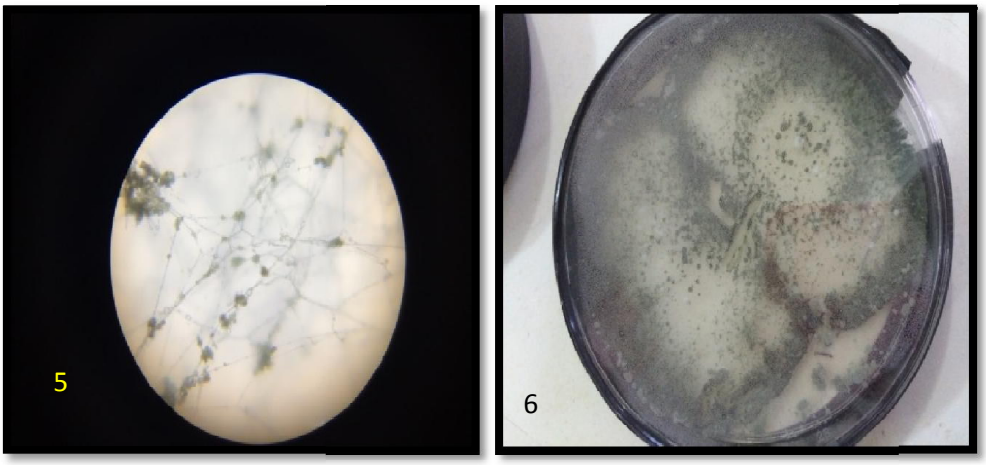
The fungal isolate identified according to the appearance (color, shape and nature of the growth) as well as microscopic test, according to dependable references[29,20,27] and 7 isolates of fungal species were isolated as in table (2), and *Aspergillus* was appeared to be predominant in different types *A.niger* and *A.flavus*, while the other species Figure 3were *Penicillium* sp., *Alternaria* sp., *Fusarium* sp., *Trichoderma* sp., *Rhizopus* sp.and *Mucor* sp.



1: *A.niger*pure colonies growing on PDA.2: sporseof *A.niger* under the microscope



3: *Penicillium*sp.purecolonies growing on PAD. 4:sporse of *Penicillium*sp. under the microscope



5: *Trichoderma* sp.purecolonies growing on PAD.6: sporseof *Trichoderma*sp.under the microscope

Figure (3) The morphological and microscopic tests of the selected fungal isolates for LOX production.

Table (2) numbers of identified fungal isolates which were isolated from different sources

sources / fungal isolates	Peanut	Maize	Roquefort cheese	local cheese of sheep	Milk local gamer	Iranian cream	Bread	Wheat	Rice	Soil	Total
Aspergillus flavus	2	2	–	–	1	1	2	1	1	1	11
Aspergillus niger	4	3	–	1	–	–	1	1	1	2	13
Aspergillus terreus	1	1	–	–	1	1	1	1	1	1	8
Aspergillus parasaticus	1	1	–	–	–	–	–	1	–	–	3
Alternaria sp.	–	1	–	–	1	–	–	–	1	–	3
Penicillium sp.	1	–	4	2	1	1	2	3	–	1	15
Fusarium sp.	1	1	–	–	–	–	2	1	1	1	7
Trichoderma sp.	–	–	–	2	–	–	1	–	–	2	5
Rhizopus sp.	1	1	–	1	1	–	4	1	2	–	11
Mucor sp.	–	1	–	2	–	–	2	1	1	–	7
Total	11	11	4	8	5	3	15	10	8	8	83

Fungal isolates frequency:

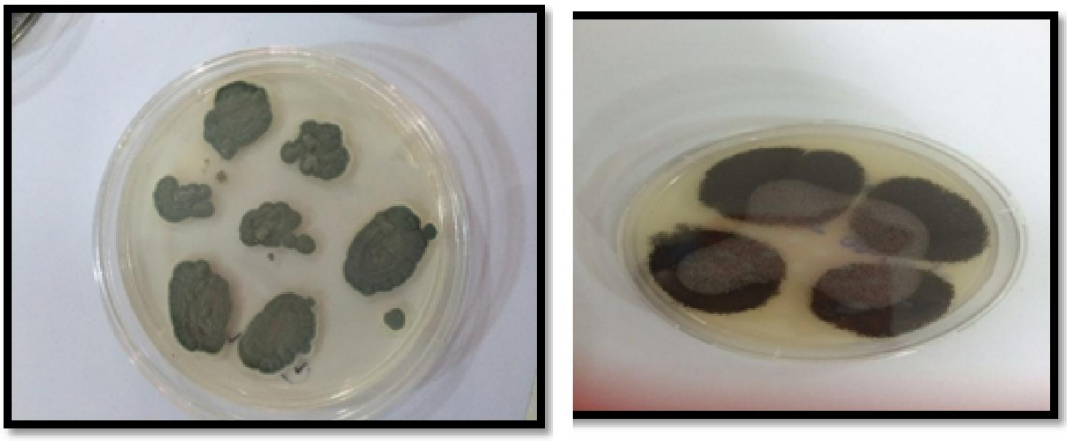
Table (3) illustrated that the highest frequency was 18.07 for *Penicillium sp.* Followed by *Aspergillus niger*15.66, while *Aspergillus parasaticus* and *Alternaria* sp.showed the lowest frequency

Table (3) fungal isolates from various sources and their frequencies

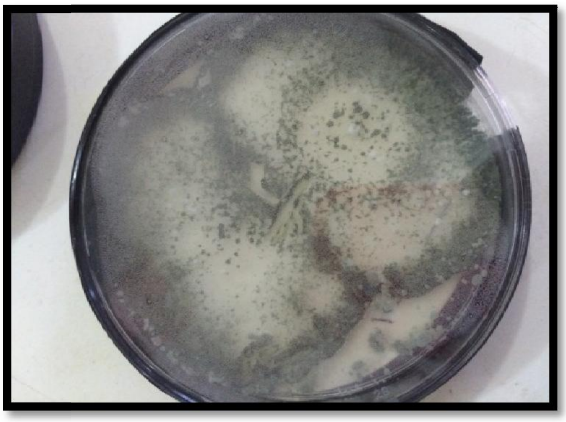
S	fungal isolates	Number	Frequency%
1	*Aspergillus flavus*	11	14.45
2	*Aspergillus niger*	13	15.66
3	*Aspergillus terreus*	8	9.63
4	*Aspergillusp arasaticus*	3	3.61
5	*Alternaria* sp.	3	3.61
6	*Penicillium* sp.	15	18.07
7	*Fusarium* sp.	7	8.43
8	*Trichoderma* sp.	5	6.02
9	*Rhizopus* sp.	11	13.25
10	*Mucor* sp.	7	8.43

PDA was used for primary screening of fungal isolates which was non- toxic[16, 22]. The numbers of these isolates 61 which were isolated from various sources.The results proved that *A.niger* which was isolated from

the maize, *Trichoderma sp.*from local sheep cheese and *Penicillium sp.*from the blue cheese were which given large diameter when growing on PDA comparing with other isolates sources Figure (4)



Penicillium sp *A.niger*



***Trichoderma* sp.**

Figure (4) the selected fungal isolate for LOX production by growing on PDA

It has been depend on the colonies diameter when selecting the isolate which produce enzyme. The fungal isolates compared depending on enzymatic activity for the crude enzymatic extracted for each one after growing on liquid media to make the secondary screening[37]. 24 fungal isolates were selected with big biomass and high enzymatic activity, these isolates belong to *A.niger, Penicillum* sp. And *Trichoderma sp*. After that only 3 isolates were selected were belong to the same three genus for production of LOX. A.niger was the best and this was agreed with [18]but disagreed with[7] he used *Fusarium proliferatum*as fungal isolates for

production of LOX .The isolates belong to *A.niger* showed enzymatic activity ranged from(790.5 - 801.4) followed by *Penicillum* sp. Isolates (545.8-559.2) while the Trichoderma sp. Isolates showed less enzymatic effectiveness.

Table (4) screening of isolates growing on Modified Shown liquid medium (MSM)

S	Fungal isolates	number	Rang activity (unit\ml)	The biomass weight rang(gm\100ml MSM)
1	***Aspergillus niger***	11	790.5-801.4	0.3 – 0.9
2	***Penicillum* sp.**	10	545.8-559.2	0.26 – 0.45
3	***Trichoderma* sp**	3	115–120	0.24 – 0.35

عزل وتشخيص بعض العزلات الفطرية وأختيار العزلات المنتجة لأنزيم اللايبوكسيجينيز

نورا طه ياسين التميمي ، آمال كاظم غضبان الاسدي ، آلاء غازي عيدان الهاشمي
قسم علوم الاغذية، كلية الزراعة، جامعة البصرة، البصرة ، العراق .

الخلاصة

تم الحصول على83عزلة محلية للاعفان من مصادر مختلفة(الفول السوداني والذرة الصفراء والرز والحنطة والخبز وجبن الغنم المحلي والقيمر المحلي والقشطة الإيرانية وجبن الروكفورت والتربة) إذ اجريت لها عمليات العزل والتنقية والتشخيص وتبين انها تضم14عزلة لعفن *Aspergillus flavus* و13عزلة لعفن *nige* *rAspergillus* و 8 عزلات لعفن *Aspergillus terreus* و 3 عزلات لعفن*parasaticusA spergillus* و 3 عزلات لعفن *Alternaria* spp. و15 عزلة لعفن *Penicillium* spp. و7 عزلات لعفن *Fusarium* spp. و 5 عزلات لعفن *Trichoderma* spp. و 11 عزلة لعفن *Rhizopus* spp. و7 عزلات لعفن *Mucor* spp. ثم اختبرت قابلية العزلات الفطرية على انتاج سموم الافلاتوكسين. استبعدت العزلات السمية وهي *Aspergillus flavus* و*Aspergillus terreus* و*Aspergillu sp arasaticus*، تم أختيار العزلة الأكفأ أنتاجاً لأنزيم اللايبوكسيجينيز *Aspergillus niger* المعزولة من الذرة الصفراء بعد أجراء عمليات الغربلة(الاولية والثانوية) للعزلات الفطرية غير السمية. و كان نمو مستعمرات العزلة المنتخبة ذو أبعاد أكبر من مستعمرات جميع الأعفان بما فيها عفن *Penicillium sp.* و *Trichoderma sp.* ، كما أعطت العزلة ذاتها فعالية أنزيمية عالية مقدارها 801.4 وحدة / مل، في حين كانت (559.2 و 120) وحدة / مل لعفن *Penicillium sp.* و *Trichoderma sp.*على التوالي.

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