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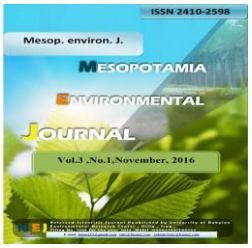

Molecular diagnosis of patulin toxic produced from *Penicillium expansum* in apples fruits

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Abstract

The present study aimed to test the ability of *P.expansum* to producing patulin toxic in some varieties of apples imported which founded in the local markets of the province of Babylon, and studying the molecular characteristics of toxic by using PCR technique and TLC. Results showed that *P.expansum* was the highest appearance in the red apples imported, which amounted to 80% and has the highest frequency in red apples imported (68.75)% and the lowest frequency in the apple golden yellow imported (17.3)% . There are four isolates of the *P.expansum* was ability to produce patulin (B, D, E and F) as the production rate of patulin 90% of the isolates were studied by TLC method, as well as the detection capability isolates fungus to produce patulin by Polymerase chain reaction (PCR) using primers (P450, IDHI, G3PDH, MSAS) .The technic of PCR was proved the presence of the gene(IDH) responsible for the production of patulin in four isolates of the *P.expansum* of the total 13 isolates where it was noted that the gene site has appeared at the base of nitrogen 500 bp also proved the existence of a gene MSAS in 2 isolates at the base of nitrogen 300 bp. As for the G3PDH gene it has been obtained 4 isolates a carrier of the gene, while not prove possession of the fungus isolates the gene P450. It was also investigating the viability of *P.expansum* isolates to produced three types of enzymes which Amylase , Protease and Lipase.

Key words: Penicillium expansum , patulin , apple fruits, PCR technics , enzymes .

Introduction :

Postharvest diseases cause losses that are greater than generally realized because the value of fresh fruits and vegetables increases several-fold with the passing from fields to consumers [1]. One of the economically most important problems worldwide is blue mold decay. It is caused by *Penicillium* spp., among which *P. expansum* (Link) Thom. is prevalent. Blue mold decay leads to significant economic losses during storage that also impacts fruit destined for processing by its production of the carcinogenic mycotoxin patulin [2]. The mycotoxin patulin is produced by several fungi, mostly species of *Aspergillus* and *Penicillium*. Included is *Penicillium expansum,* the blue-mold fungus of deciduous fruits. Its ability to grow at 0 C makes it a major cause of loss of apples and pears in storage, Other common fruit hosts include most Prunus sp.,grapes, and persimmons[5] . Although

patulin is acutely toxic to humans, its acute toxicity is believed unimportant because rotted flesh is easily avoided in fresh fruits and rotted fruits are eliminated before processing, thereby limiting ingestion. Patulin has been found in apple fruits [3, 4] and in apple juice [5, 6].

The best ways that reduce crop yields and food mycotoxin contamination by preventing the conditions for fungal growth and production of toxins in addition to the need to examine all the food processed for human consumption to make sure they do not contain these mycotoxins harmful to human health [7] .Depend on the data referred to above and for the detection of fungal isolates produced toxin patulin from apples this study was conducted which included isolate and diagnose fungus associated with the fruits of infected apples imported and local, in some markets the province of Babylon and detection capability isolates fungus *P.expansum* to produce cm patulin by (TLC) Thin layer chromatograph and Polymerase chain reaction (PCR) and study the effectiveness of some isolates fungus enzymes under study .

Material and method

Isolate and Diagnose Fungi Associated with the Apples Under Study

Detection of microorganisms was performed using a moist chamber method. The transfer of apple samples to a laboratory to isolate and diagnose fungi then cut each apple into five small pieces and put in petri- dishes (diameter 9 cm) containing the PDA for each class separately and repeated the process three times (replicates) for each class of the apples then incubated all dishes under a temperature of 25 ± 2°C for 7 days[8]. Developed fungi were identified by their morphological features according to the previously described methods[9] and all ratios were calculated according to the following equation: dishes under a temperature of 25 ± 2°C for 7 days[8]. Developed fungi were identified by their morphological features according to the previously described methods[9] and all ratios were calculated according to the following equation:

$$\text{The percentage of appearance (\%)} = \frac{\text{the number of samples that have fungus}}{\text{total number of isolates}} \times 100$$

Detection ability a number of isolates *P.expansum* to produce patulin

A-Thin layer chromatography plates (TLC)

Patulin-producing ability of *Penicillium* isolates were determined according to [10]. The isolates were grown on yeast extract sucrose agar for 7 days at 25C. Colony centers were transferred to vials containing the extraction solvent (chloroform : methanol, 3:1, v/v) and mixed in blander for at least 15 min. Thirty-microliter aliquots of extracts and patulin standard (10 mg/mL) were spotted on thin-layer chromatography (TLC) plates (SIL 20 × 20 cm G-25HR, Machery-Nagel and Co., Düren,Germany) where the active plate in the oven electric degree 120 º and for an hour before use the separation system. The spots were dried and the plates put in solvent system toluene : ethylacetate : formic acid (10:30:60, v/v/v) and heated at 130 C for 15 min. Patulin appeared as a yellow spot under visible light for reflection and transparency at the same time and a yellow orange fluorescence spot under long wavelength ultraviolet (UV) light (266 nm)[11].

B-DNA extraction and sequence analyses: After 7 days, growth of fungal colonies on PDA was counted and recorded in a colony forming unit per millilitre (cfu/ml). DNA was extracted from isolates using the genomic DNA purification Kit (Promega, Madison, WI, USA). Small subunit ribosomal RNA (mtSSU rRNA) and β-tubulin were then amplified by PCR using 4 primer pairs that primers (IDH1R/ TGG GAC AAT TCC TGA ACA TGC and IDH2F/ GCC CAT GTG CTC ATT ACA GG) and (G3PDH-R/ TGG AGG AGGGGA TGA TGT T and G3PDH-F/ CGG CTT CGG TCG TAT TGG) and (P450-2R1/ CCC GGA TTT GTA AAG ACT GGAC and P450-2F1/ AGC GGC CAA ACT CAT GAC TAA CTG) and (MSAS R2/ GAC CAT GTT GCC GGC CCA GTA TTC and MSAS F2/ CGA AAT CGC GGC CAG TGT TGT G) the conditions described[12,13] here are primers for patulin produced diagnosis of *P. expansum*. For detection of DNA, gel electrophoresis method was used by using Agarose gel (1% concentration). This method consisted of three steps: preparation of agarose gel, preparation of casting agarose and the addition of samples.

PCR amplification condition: This method is used to amplify DNA by using the specific primer. These methods are dependent on the volumes that are found in Master Mix provided from Bioneer (Table 1).

The time and vol. which is used for electrophoresis methods: To obtain clear band after PCR running , that used different time and volume during electrophoresis.

Table 1. Programme of PCR technique for IDH, G3PDH, P450 and MSAS primers

No. of cycles	Time	Temperature	steps	No .steps
1	30 min	94°C	Initial denaturation	1
24	1 min	94°C	Denaturation	2
1	15 min	50°C	Annealing	3
1	4 min	60°C	Elongation	4
1	10 min	72°C	Final extension	5

Detection of some isolates from *P.expansum* some apple varieties on the secretion of enzymes Protease and Amylase and Lipase

1- **Protease enzym**e

It was detected viability of some isolates *P.expansum* which isolated from some apple varieties under study on the production of the protease enzyme inoculating the petri-dish containing Nutrient Gelatin Agar by transfer a disk(5mm) grow in the PDA media and the age of a week from fungi and with three replicates leave the other plate without vaccination as control, and the plates were incubated at a temperature 28 º C for 5 days [14, 15] then add reagent Frazier to the plates and left for 5 minutes, the emergence of a transparent halo around the colony fungal proof that isolation producing the enzyme protease.

2- Amylase enzyme

Vaccinated plates containing yeast extract ribbed starch ability the isolated to production the enzyme amylase by taking a drop of 5 mm disc of fungal colonies developing on PDA by piercing cork and by three replicate to isolates and left with a plate without vaccination to control, and the plates were incubated at a temperature 28 º C for 5 days [14, 15]then add reagent potassium iodide to the plates and leave for five minutes and then poured into the detector, which was added to the plates , not that change the color of the media is proof that isolation wasn't producing amylase.

3- Lipase enzyme

After 7 days, growth of fungal colonies on PDA was counted and taken the disk (5mm) put it in petri- dishes which containing the peptone ribbed twin 80 by three replicate for fungal isolates and left other without vaccination to control, and the petri-dishes were incubated at a temperature of 25 m º for a week after the results recorded by the appearance of white around precipitation the colonies or by observing the halo transparent about the colony of fungus [16]

Results and discussion

1-Isolate and Diagnose Fungi Associated with the Apples

Table (2) showed that there are many types of fungi in apple varieties, pathogenic fungus *P. expansum* was recorded high in percentage with *P. italicum* appearance in most apple varieties. The highest rate of the apparance of *P. expansum* amounted to 90% in the red apples imported, and the lowest rate of 30% in the appearance of golden yellow apples imported. *A. terreus* was in highest percentage imported. *P. digitatium* was the highest rate imported (20%), but did not appear in green apple imported. *P. digitatium* was the highest rate in green apple imported (60%) and the lowest rate appearance was in the golden yellow apples imported at a rate of 30%. The percentage of the apparance of *Altrenaria* spp. in red apple local and golden yellow apple imported as 30% did not appear in green apples either. *A. niger* was the highest rates of its appearance in the golden yellow apple imported and that 60% did not appear in green apple imported. *A. flavus* was at high percentage in the golden yellow apples imported did not appear in green apple imported.

Table2: Number fungal species isolated from apple varieties

No.	Fungi types	No. of isolates
1	*Penicillium expansum*	65
2	*P.italicum*	80
3	*P.digitatium*	40
4	*Aspergillus terreus*	20
5	*A. flavus*	73
6	*A. niger*	41
7	*stolonifer Rhizopus*	25
8	*Alternaria* spp.	59
9	Yeasts	40
	total	443

. *P.expansum* and *P.italicum* the rate of high-appearance in most apple varieties and a higher rate of the *P.expansum* amounted to 80% in the red apples imported, and less of the appearance 30% in apples imported golden yellow as in Figure (1) and *A .terreus* was the highest rate was the golden yellow apples imported 20% did not appear in green apple imported, while the *P.digitatium* was the highest rate in the appearance of a green apple imported by 60% and the lowest rate was in the appearance of golden yellow apples imported by 30% as the rate of the apparance of fungi spp. reached *Altrenaria* in the local red apples and apple golden yellow importing 30% did not appear in green apples either *A.niger* was the highest appearance in the apple golden yellow importer and that 60% did not appear in green apple imported the *A.flavus* high rate of its appearance in the golden yellow apples were imported did not appear in green apple imported as in Figure (4) as for the yeasts were highest appearance in the golden yellow apples by 70% the lowest percentage of appearance in the green apple importing 10%. these results are similar to those reached by the [17] in which he suggested that *P.expansum* has recorded the highest percentage displayed on apples was 100%, while *A.terreus*, fungi and other record isolated from apples lowest rate emergence reached 25%, while not consistent with the findings of the [17], which is that *Penicillium* spp. comes in second place after *Rhizopus stolinifer* injury in the apples, where the latter record the highest in the appearance of apples. as well as the results of this study is similar to those reached by [18] *Aspergillius* spp. founded the highest frequency ratio followed by the *Penicillium* spp.

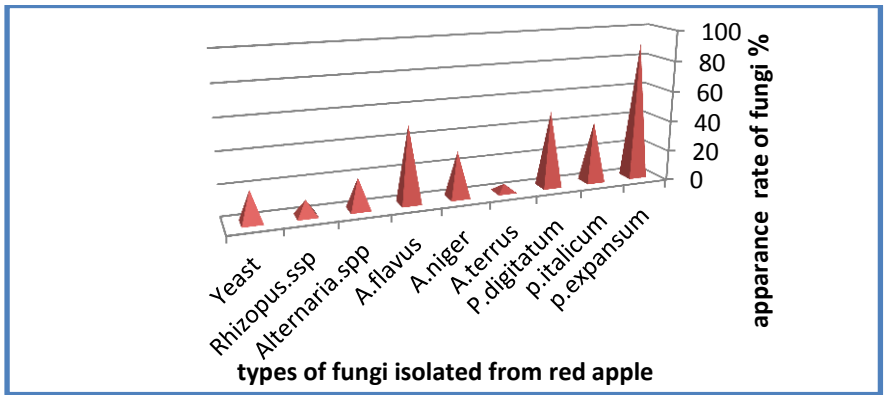

Figure1:The percentage appearance of fungi isolated from red apples imported.

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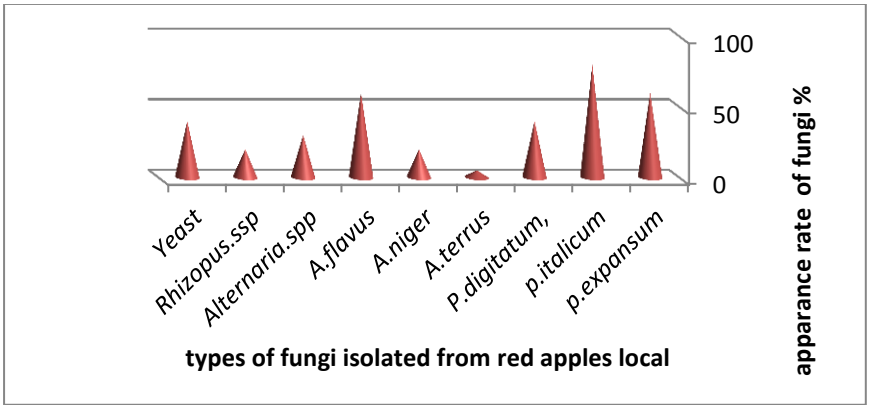


Figure 2: The percentage appearance of fungi isolated from red apples local.

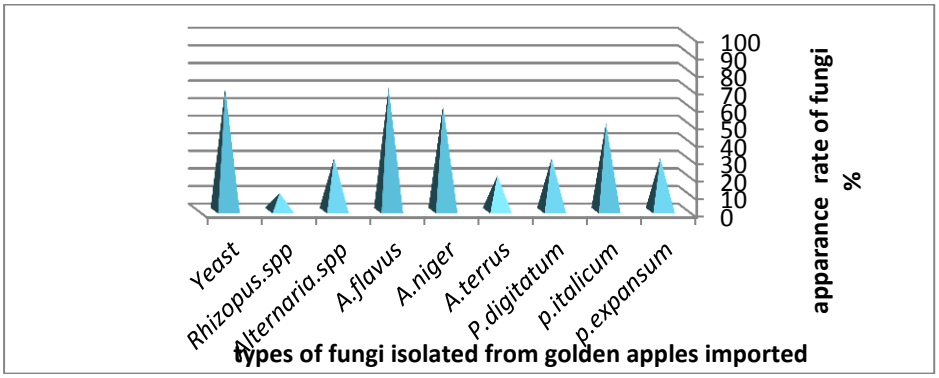


Figure 3: The percentage appearance of fungi isolated from golden apples imported

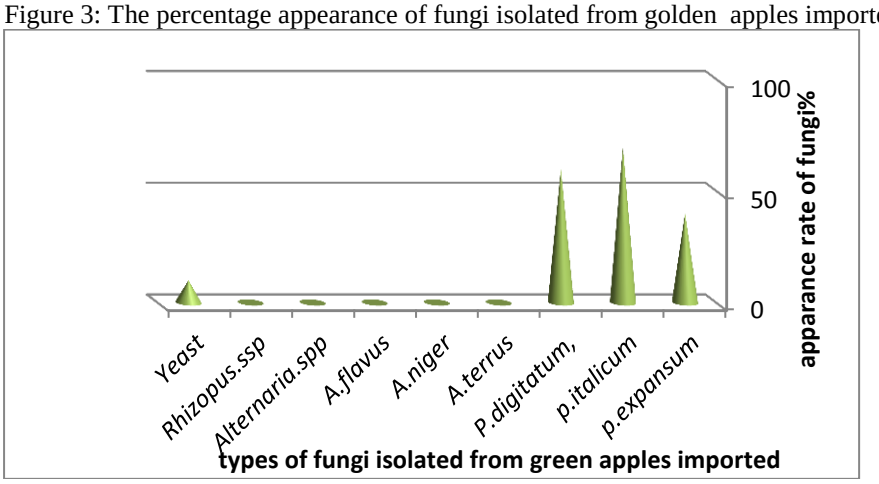


Figure 4: The percentage appearance of fungi isolated from green apples imported

2-Test the ability *P.expansum* to produce patulin toxic

A - TLC

The results of the detection by using plates thin layer chromotograph (TLC) that the four isolates which isolated from *P.expansum* producing patulin isolated from different types of apples, represented by red apples imported , Red apples local , apple golden apple and green apples imported, as the percentage production of patulin 90% through improved the visualization of the yellow-orange fluorescence spots against the background and calculated values of the relative movement of the spots separated Relative Flow (Rf). Was the relative flow values of the record to the toxic was (88.23 cm) either isolates produced toxin patulin values reached for isolate B was 8.23%, while the isolation D amounted relative flow of 87.90%, while the value of relative flow F and E (87.35%) that the biosynthesis of mycotoxins is influenced by many factors affecting significantly the production of toxins, especially the environmental conditions and the conditions of the implant where the genes responsible for the production of toxins [19] or may be the toxin contain large amounts of starch and fat[20], and this does not mean that isolates non-producing patulin toxin the fungus is able to produce these toxins may even due to the nature of the media components or temperature degree for the media [21] as well as the reason may be due to the presence of biogenic competition between fungi .

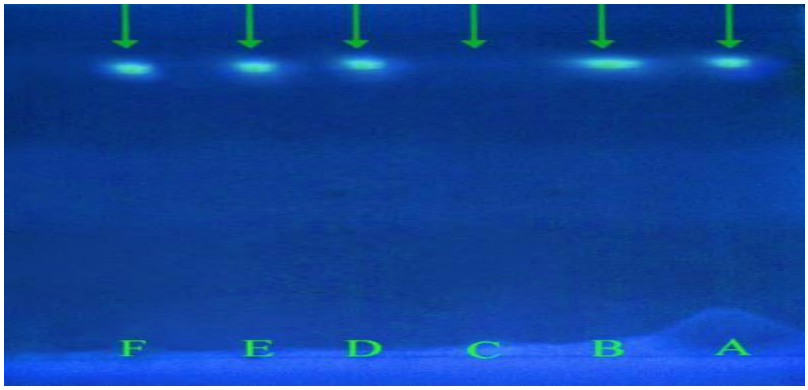


Figure 5: isolates the ability to produce *P.expansum* patulin using plate) Thin chromatography (TLC) where: (A) represents the standard toxic (F, E, D, B represents isolates Produced patulin toxic , (C) represents the isolate was not producing patulin

B- Detection the patulin produced by PCR technic

The results proved of PCR and the presence of the gene(IDH) responsible for the production of patulin in four isolates of the *P.expansum* of the total 13 isolates where it was noted that the gene site has appeared at the base of nitrogen 500 bp figure (6) and the result was agree with [13], which he used three isolates have found that all of these insulation having the same sequences (D1 / D2), and at the base of nitrogen, 500 bp site also proved the existence of a gene MSAS in 2 isolates at the base nitrogen 300bp figure (7). The gene G3PDH was obtained 4 isolates a carrier of these gene, while the other isolates gave a negative resul**t** as shown in figure (8), the isolates of the fungus not carrier the gene P450 figure (9) and this result does not agree with that founded [22], which that all isolates were carriers of the gene**.**

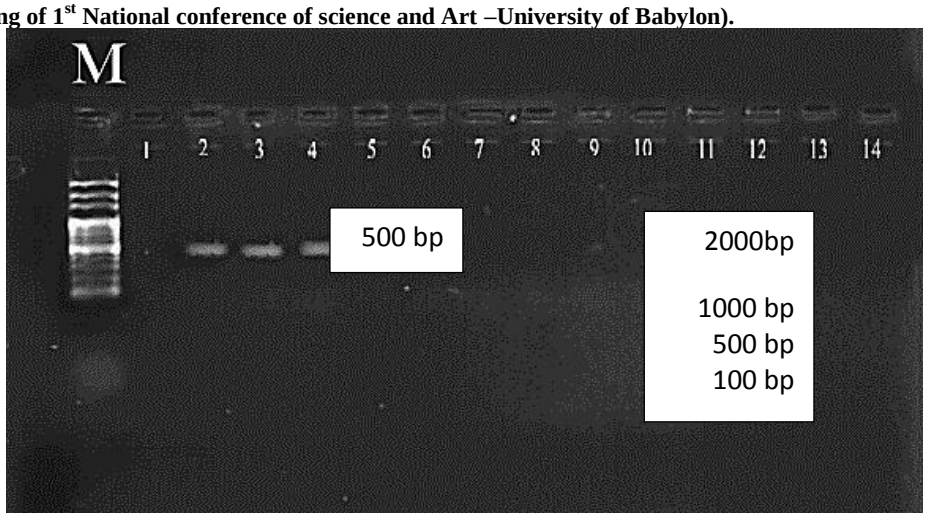


Fig 6: Gel electrophoresis of amplified PCR product from different *P.expansum* isolates, by using IDH primer (in Vol. = 100, agarose = 0.4%).

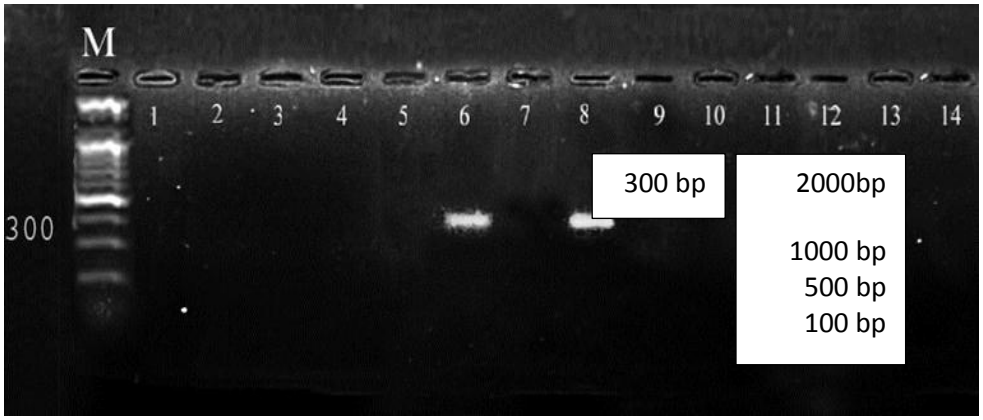


Fig. 7 Gel electrophoresis of amplified PCR product from different P.expansum isolates, by using MSAS primer (in Vol. = 100, agarose = 0.4%).

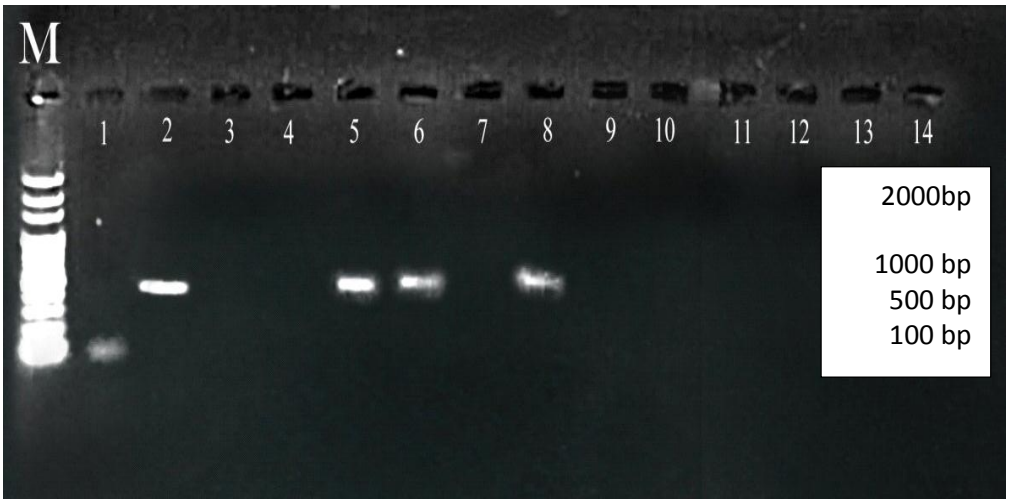
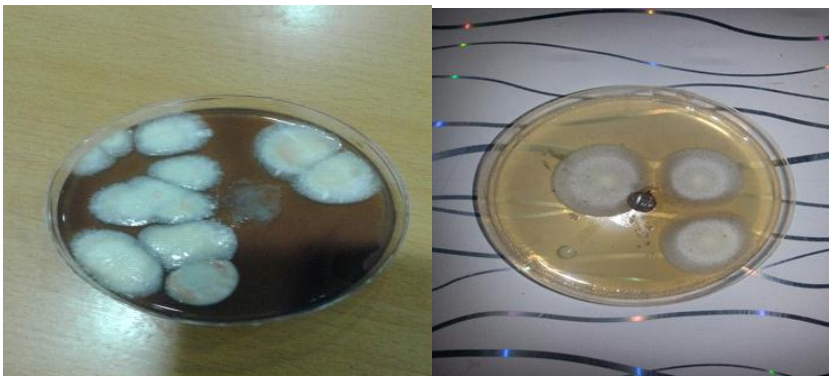


Fig. 8 Gel electrophoresis of amplified PCR product from different *P.expansum* isolates, by using G3PDH primer (in Vol. = 100, agarose = 0.4%).

Fig. 9 Gel electrophoresis of amplified PCR product from different *P.expansum* isolates, by using P450 primer (in Vol. = 100, agarose = 0.4%).

3- Detection of some isolates from *P.expansum* some apple varieties on the secretion of enzymes Protease and Amylase and Lipase

The result showed the test production of enzymes Amylase and Lipase and Protease founded in nine isolates belonging to *P.expansum* . The most of the *P.expansum* isolates have the ability to produce enzyme amylase and protease varying quantities table (3) and the result is similar to those reached by [23] , which was that the *P.expansum* ability to produce the enzyme amylase in large quantities and the result was positive media color change figure (10), also the *P.expansum* ability to secrete the protease enzyme high quantities and so the appearance of a halo transparent about the colony innate, that disparity in the production of the protease enzyme may be due to different rates and types of organic materials that are found in the source of organic nitrogen, particularly proteins, as casein nitrogen by 10% contains peptone nitrogen increased by 13.8% [21]. As well as most of these isolates were able to produce a Lipase enzyme as in figure (10) and the result was positive appearance of white deposits on the colony fungal and this study are similar to those reached by [22] which is that the *P.expansum* the ability to produce the Lipase enzyme at a temperature 35 C º.



A B

Figure 10 : Enzymatic effectiveness of *P.expansum* was A represents the form of the front of *P.expansum* on yeast extract and the result was a positive change the color of the media , while B represents the form of the front side of the *P .expansum* on Nutrient Gelatin Agar, the positive result was the apparent of a transparent halo around the fungal colony.

Table 3 : Test the ability of *P.expansum* to produce enzymes Amylase and Lipase and Protease.

No.	Source of isolate	Protease	Lipase	Amylase
1	Pe-1	++	+++	++
2	Pe-2	+++	-	-
3	Pe-3	-	++	++
4	Pe-4	++	++	+++
5	Pe-5	+++	++	++
6	Pe-6	+++	-	-
7	Pe-7	+	+++	+
8	Pe-8	++	++	++
9	Pe-9	-	-	++

+Represents the production of small amounts of the enzyme and enzyme production ++ represents the amount of medium and enzyme production +++ represents a large amount

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